CHARACTERIZING THE CYTOPATHOGENICITY OF ZIKA VIRUS IN HUMAN SCHWANN CELLS

By
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ABSTRACT

Zika virus (ZIKV) is a newly reemerged RNA virus that belongs to the *flavivirus* genus of the family Flaviviridae. It is a mosquito-borne virus, transmitted mainly by the Aedes spp and was considered a relatively unimportant pathogen until a large outbreak in the Pacific Island of Yap in 2007, followed by outbreaks in the South Pacific, Caribbean and Americas in 2015. A systematic review of the Zika outbreaks found that ZIKV is a cause of congenital abnormalities and has been associated with an increase in cases of Guillain-Barré syndrome (GBS), a potentially life-threatening peripheral nerve disease characterized by rapidly progressive, symmetrical weakness of the extremities. The pathogenesis of GBS associated with ZIKV infection remains unclear, and therefore our study focused on human Schwann cells (hSCs) which play a central role in peripheral nerve function, maintenance and repair. Schwann cells ensheathe and myelinate axons of peripheral nerves by wrapping around the shaft of an individual peripheral axon.

Another major characteristic of the Zika outbreak in the 21st century was that it followed a similar geographic distribution as another flavivirus, Dengue (DENV). The cross-talk and epidemiological similarity between the two viruses leads to the possible role of antibody dependent enhancement (ADE) as a mechanism driving enhanced replication and more severe illness seen during the ZIKV emergency.

Our study revealed that three different strains of Zika virus (1968 Nigeria, 2014 Thailand and 2015 Brazil) can infect, induce cytopathic effect and replicate well in hSCs. The comparative infection with other flaviviruses like Dengue virus (NGC) and Yellow Fever virus (17D) showed that hSCs are more susceptible to ZIKV. ZIKV infection in hSCs led to the upregulation of proinflammatory cytokines, mRNAs for IL-6, IFNβ1, IFIT-1,
TNFα, and IL-23A were observed to be upregulated in ZIKV (Fortaleza) infected hSCs. As compared to Yellow Fever virus infected hSCs, ZIKV infected hSCs showed a robust type III interferon response (IL-29) with an increase in its receptor levels (IL-10RB and IFNLR1) and negative regulators (SOCS1/SOCS3). We found no evidence of ADE between DENV and ZIKV infection of hSCs. This study demonstrates the cytopathogenicity of ZIKV infection in peripheral nerve cells and helps to understand the biological properties potentially contributing to the development of Guillain-Barré syndrome.

**Primary Reader:** Dr. Diane E. Griffin  
**Secondary Reader:** Dr. Anna P. Durbin
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ABBREVIATIONS

ZIKV – Zika Virus
YFV – Yellow Fever Virus
DENV – Dengue Virus
WHO - World Health Organization
GBS - Guillan-Barré Syndrome
PHIEC - Public Health Emergency of International Concern
AIDP - Acute Inflammatory Demyelinating Polyneuropathy
AMAN - Acute Motor Axonal Neuropathy
ADE – Antibody Dependent Enhancement
hSC - Human Schwann cell
PBS - Phosphate Buffered Saline
Ab - Antibody
mAb – Monoclonal antibody
hpi – Hours post-infection
RT – Room Temperature
moi – Multiplicity of infection
INTRODUCTION

Zika Virus

Zika Virus (ZIKV) is a single stranded enveloped RNA virus belonging to the family Flaviviridae and the genus Flavivirus. It is closely related to other members of this genus, including Dengue, Yellow Fever, West Nile, Japanese encephalitis viruses and most closely, Spondweni virus [1]. ZIKV is a positive-sense RNA virus with a 10.7-kb genome encoding a single polyprotein that is cleaved into three structural proteins (C, PrM/M, and E) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) translated as a single polyprotein [2]. Like other flaviviruses, ZIKV can use multiple receptors for host cell attachment, clathrin-mediated endocytosis, pH-dependent membrane fusion and entry [3]. ZIKV is an arbovirus spread by Aedes spp. mosquitoes such as A. aegypti and A. albopictus. Additionally, ZIKV can also be transmitted sexually through infected semen and from mother to fetus [4].

History of Zika Virus

ZIKV was first isolated in 1947 while scientists were conducting routine surveillance for yellow fever in the Zika forest of Uganda. The isolate came from the vertebrate host of ZIKV, a sentinel rhesus macaque monkey [5]. It was not until 1952 that the first human cases of ZIKV were detected in Uganda and Tanzania [6] and the first evidence that ZIKV can cause human disease was not determined until 1964 when a researcher fell ill and experienced a mild illness [7]. For the next four decades sporadic ZIKV cases in humans were reported but the disease was regarded as benign and characterized as self-limiting acute febrile illness with fever, headache, myalgia and rash.
During this time the geographical distribution of ZIKV spread from Uganda to western Africa and equatorially through Asia [8]. The conventional phylogenetic analysis of the ZIKV genome indicates two distinct viral lineages, African and Asian, with the Asian lineage diverging from the African lineage (Figure 1) [9] [10].

The first recent large outbreak of ZIKV occurred in 2007 on the Pacific island of Yap, in the Federated States of Micronesia. Of the island’s small population of 11,250 people, 185 suspected cases of ZIKV were identified during the outbreak. Of these, 49 were confirmed (RNA identified by PCR) and 59 were classified as probable (patients with IgM antibody against Zika virus who had a potentially cross-reactive neutralizing-antibody response) [11]. This was followed by outbreaks beginning in October 2013 and continuing throughout 2014 across Oceania in four groups of Pacific islands: French Polynesia, Easter Island, the Cook Islands, and New Caledonia. These outbreaks saw approximately 8,750 reported cases of ZIKV, the largest to date [12]. Most startling are the results of a retrospective study of the French Polynesian outbreak which provided evidence that suggested Zika virus infection was causing Guillain-Barré syndrome [13]. Continuing its equatorial spread, the first report of locally acquired Zika disease in the Americas occurred on May 5, 2015 in Brazil. Throughout 2015 ZIKV spread to other countries in South America, Central America, North America, and the Caribbean. Throughout this epidemic the symptoms of ZIKV infection were not only its historically mild symptoms but also included reports of infection with neurological complications and congenital malformations [14].

On February 1, 2016 the World Health Organization (WHO) declared that the recent association of Zika virus infection with clusters of microcephaly, other congenital
malformations, and neurological disorders constituted a public health emergency of international concern. On March 31, 2016 the WHO announced, “Based on observational, cohort and case-control studies there is strong scientific consensus that Zika virus is a cause of Guillan-Barré Syndrome, microcephaly and other neurological disorders [15].” This statement was updated on September 7, 2016 after a systematic review of the scientific literature to include the following: “The most likely explanation of available evidence from outbreaks of Zika virus infection and Guillain-Barré syndrome is that Zika virus infection is a trigger of GBS [16].”

On November 18, 2016 the WHO ended the status of “Public Health Emergency of International Concern (PHIEC)” for the ZIKV epidemic but did note that “Zika virus and associated consequences remain a significant enduring public health challenge requiring intense action but no longer represent a PHEIC [17].” Throughout 2017 ZIKV cases dramatically dropped with no resumption to outbreak levels, however ZIKV can still thrive in susceptible populations that have yet to be infected leading to potential future flare-ups [18]. Figure 2 shows the rise and fall of suspected and confirmed Zika case in three regions of the Americas: Central, South, and Caribbean (2015-2017).
**Figure 1** - Maximum clade credibility tree of Zika Virus constructed based on shared amino acid replacements showing the two distinct lineages of ZIKV, Asian and African. The tips correspond to the isolate years [10].
Figure 2 - Distribution of suspected and confirmed Zika cases by sub-region. Region of the Americas, 2015 – 2017. Credit: (Graphic) J. You/Science [19]; (Data) Pan American Health Organization/World Health Organization [18].
Guillain–Barré Syndrome

Guillain-Barré syndrome (GBS) is a potentially life-threatening peripheral nerve disease characterized by a rapid onset of bilateral weakness progressing to paralysis that may be accompanied by sensory symptoms. GBS typically occurs 1-3 weeks after an infectious disease which triggers an immune response producing antibodies that cross react against the nerve cells of the peripheral nervous system and their support structures. Symptoms peak within 4 weeks, followed by a recovery period that can last months or years, as the immune response decays and the peripheral nerve undergoes an endogenous repair process (Figure 3) [20].

The most common subtypes of GBS are acute inflammatory demyelinating polyneuropathy (AIDP) and acute motor axonal neuropathy (AMAN), differentiated by the target location of the crossreactive antibody on the neuronal axon (Figure 4) [21]. In AMAN membranes on the nerve axon are the primary target for immune-related injury. Individuals suffering from the AMAN phenotype frequently have serum antibodies against gangliosides (a result of molecular mimicry) on the peripheral nerve at or near the node of Ranvier, binding to the nodal axolemma, leading to complement activation and the disappearance of voltage-gated sodium channels resulting in nerve conduction failure. In AIDP the immune injury specifically takes place on the myelin sheath produced by Schwann cells which wrap around segments of the axon. Once the antibodies target the myelin sheath they activate complement, which leads to the formation of the membrane attack complex on the outer surface of the Schwann cell, initiation of vesicular degeneration, and invasion of myelin by macrophages. The main clinical features of AIDP are characterized as sensorimotor symptoms combined with cranial nerve deficits and
frequent autonomic dysfunction, while the main features of AMAN are described as pure motor symptoms with cranial nerves rarely affected [20] [21].

The epidemiology of GBS can be described as rare, with an incidence of 0.81–1.89 (median 1.11) per 100,000 people per year and is more common in men than in women (ratio 3:2). The pathogenesis of GBS occurs as a post-infectious disorder, two-thirds of patients report symptoms of a respiratory or gastrointestinal tract infection before the onset of GBS. *Campylobacter jejuni* is the predominant preceding infection, found in 25–50% of the adult patients. Other pathogens that cause antecedent infections related to GBS are cytomegalovirus, Epstein–Barr virus, *Mycoplasma pneumonia, Haemophilus influenzae*, and influenza A virus. Despite this strong association, GBS is still a sporadic disorder with only one in 1,000–5,000 patients with Campylobacter enteritis developing GBS in the subsequent 2 months. The major driving force behind the development of GBS is molecular mimicry between microbial and nerve antigens but the factors that dictate if and how the immune response shifts towards autoreactivity is still not well understood. Treatment of GBS usually combines multidisciplinary supportive medical care and immunotherapy, specifically intravenous immunoglobulin therapy (IVIg) and plasma exchange [21].
Figure 3 - Guillain-Barré syndrome time course [20].

Figure 4 - Major Guillain-Barré syndrome subtypes in which antibody-mediated effector pathways, including complement activation, cause glial or axonal membrane injury [20].
Zika Virus and Guillain–Barré Syndrome

The relationship between Zika virus and Guillain-Barré syndrome was first observed during the ZIKV outbreak in French Polynesia in 2013. Between November 2013 and February 2014, 42 patients presented at hospital with GBS of which 98% had anti-ZIKV IgM or IgG, and all had neutralizing antibodies against ZIKV. This translated to a 20-fold increase in GBS incidence over the previous four years. Most notable of this newfound relationship was that while electrophysiological findings were compatible with the acute motor axonal neuropathy (AMAN) type of GBS the typical AMAN-associated anti-ganglioside antibodies were rarely present [13]. However, it should be noted that the results of this study on the outbreak in French Polynesia were challenged due to concerns over restricting the control group, confirmation bias, non-specific application of the Brighton criteria, and using blood samples collected about 3 months after Guillain-Barré syndrome diagnosis [22] [23] [24].

During the outbreak of ZIKV in Colombia (Nov. 2015 - March 2016) 68 patients with GBS were evaluated when clusters of cases of GBS arose. Among the 68 patients, 66 had symptoms compatible with ZIKV infection before the onset of GBS. Among 46 patients in whom nerve-conduction studies and electromyography were performed, the results in 36 patients (78%) were consistent with the AIDP subtype of GBS. This further lent support to the idea that ZIKV infection can develop into the AIDP subtype of GBS [25]. Findings from a prospective study done in Martinique were consistent with the results in the Colombia study, determining that the incidence ratio of GBS during the ZIKV outbreak was 4.52 (95% confidence interval, 2.80 - 7.64; P = .0001) and that patients had findings consistent with the AIDP pattern of GBS [26].
In January 2017 The World Health Organization conducted a systematic review of 36 studies in several countries for the association of ZIKV and GBS and found sufficient evidence to say that Zika virus is a trigger of GBS [27]. Figure 5 illustrates the trends in ZIKV and GBS cases in the region of the Americas [18].

A noteworthy characteristic observed between ZIKV and GBS during the outbreak is the rapid presentation of symptoms. The time from viral prodrome to neurological symptom onset in most cases studied were a median of 5-10 days. This differs from the classical form of GBS in which almost two thirds of adult patients develop symptoms 14-30 days after infection. This suggests that GBS associated with ZIKV is parainfectious, in contrast to infection with other antigens in which GBS is postinfectious. However this rapid progression does not translate to increased morbidity or mortality [28].
Figure 5 - Distribution of suspected and confirmed cases of Zika and GBS by EW. Region of the Americas, 2015 - 2017 (as of EW 32) [18].
Antibody-Dependent Enhancement

Antibody-dependent enhancement (ADE) is a phenomenon in which crossreactive non-neutralizing antibodies are able to bind to the virus and enhance the entry of virus through interaction with Fc and/or complement receptors. In vitro, ADE has been shown for influenza A virus, Coxsackievirus B, respiratory syncytial virus, Ebola virus, human immunodeficiency virus (HIV), and many other viruses. In vivo ADE has been linked with the severity of HIV and most widely studied, dengue virus infection [29].

A major characteristic of the Zika outbreak in the 21st century was that it followed a similar geographic distribution as another flavivirus, dengue virus, and shares the same genus of mosquito for transmission, Aedes [30]. Several studies reviewed by the WHO during the ZIKV outbreak reported past dengue virus infection in ZIKV cases [27]. Recent papers have identified the structural details of a quaternary epitope that provides a previously unrecognized link of potent cross-neutralization between Zika and dengue viruses [30] [31]. This cross-talk and epidemiological similarity leads to the possible role of antibody dependent enhancement (ADE) as a mechanism driving enhanced replication and more severe illness seen during the ZIKV emergency. This mechanism of enhancement has been shown in a myeloid cell line, U937, in which DENV immune plasma was able to drive ADE of ZIKV infection [31].
Research Aims

The public health relevance of Zika virus and its trigger of Guillain–Barré Syndrome offers a unique opportunity to investigate the pathogenesis of GBS associated with a specific known infection. In that context this study will focus on the myelinating cells targeted in AIDP associated GBS, Schwann cells, and their relationship with ZIKV inducing GBS. Specifically, this study will test the hypothesis that human Schwann cells (hSCs) are susceptible to ZIKV infection, assess the cellular response of hSCs to ZIKV and determine whether antibody to DENV1, 2, 3 or 4 enhances ZIKV infection. Results of this study will provide a comprehensive picture of the potential role of hSC infection in ZIKV-induced peripheral neuropathies.
MATERIALS AND METHODS

Cell Cultures

In vitro studies of Schwann cells have generally used cell lines established from schwannomas (e.g. ST88-14) because primary SCs are difficult to maintain in culture [32]. A recently established line of human SCs from 60-80 day fetal sciatic nerves that were immortalized with SV40 large T antigen and human telomerase reverse transcriptase were produced by colleagues at the Johns Hopkins Neurology Department [33]. The cells express SC-characteristic proteins (e.g. S100B), transcription factors (e.g. Slug, TWIST), cell surface receptors (e.g. p75NTR), chemokines (e.g. CCL2) and neurotrophic factors (e.g. NGF, BDNF, NT-3) and can myelinate axons in vitro. This established line of hSCs are grown in DMEM supplemented with 0.2% glucose, 2mM L-glutamine, 10% heat-inactivated fetal bovine serum, 2µM forskolin and penicillin (100U/ml) and streptomycin (100µg/ml) at 37°C in a 5% CO₂ incubator.

For titration of viruses, African green monkey epithelial cells (Vero) obtained from the American Type Culture Collection and were grown in DMEM with 10% FBS at 37°C in a 5% CO₂ incubator. Virus stocks were grown in Aedes albopictus C6/36 cells which were obtained from the American Type Culture Collection and grown at 28°C in a 5% CO₂ incubator in DMEM + 10% heat-inactivated fetal bovine serum supplemented with 1x minimal essential amino acids in addition to 2mM L-glutamine, penicillin (100U/ml) and streptomycin (100µg/ml).

For antibody dependent enhancement experiments, K562 cells, a human erythroleukemia cell line, expressing DCSIGN were used as a positive control due to their
expression of FcγRII and DCSIGN, a receptor for flaviviruses [34]. The cells were kindly provided to us by Dr. Anna Durbin and grown at 37°C in a 5% CO₂ incubator with Roswell Park Memorial Institute medium (RPMI) + 10% heat-inactivated fetal bovine serum.

**Viral Stocks**

Virus strains used for this study were Yellow Fever 17D (a vaccine strain), Dengue virus serotype 2 (NGC) and three strains of Zika Virus: ZIKV 1968 Nigeria (IBH 30656), 2014 Thailand (SCV0127/14) and 2015 Brazil (Fortaleza) which were grown in C6/36 mosquito cells. Viral titers were determined by focus-forming assay on Vero cells for DENV and YFV and plaque assay for ZIKV strains 72 hours after infection [35]. The three distinct ZIKV strains were chosen because they represent the historical shifts ZIKV experienced as it moved from Africa to Asia and finally the Americas.

**Focus Forming Assay**

Supernatant fluids collected from infected hSCs were diluted in DMEM supplemented with 1% FBS and used to infect Vero cells for 1hr at 37°C. Cells and inoculum were overlaid with methylcellulose (OptiMEM [Corning], 2% FBS, 2mM glutamine, 50 μg/mL gentamicin and 1% methylcellulose [Sigma Aldrich]) and incubated for 96hr at 37°C. Cells were washed with PBS to remove methylcellulose and fixed with a 4:1 methanol:PBS mixture for 10min. Cells were blocked with 5% milk in PBS at RT for 10min. Cells were incubated with primary antibody (mouse 4G2 monoclonal antibody) diluted 1:2000 in 5% milk in PBS for 1hr at RT. Cells were incubated with secondary antibody, HRP-conjugated goat anti-mouse IgG (KPL Cat. #50-78-02), diluted 1:3000 in
5% milk in PBS for 1hr at RT. Foci were developed with TrueBlue Peroxidase Substrate (KPL Cat. # 71-00-68).

**Replication Kinetics and Cell Viability Assay**

Determining the replication kinetics of each virus in hSCs was performed by infecting hSCs at an moi of 5 and incubating for 24, 48, 72, 96 and 120 hours post infection. The supernatant fluids were collected at each time point for determining the viral titer using Vero cells by plaque assay [35] (for ZIKV) or focus forming assay (for YFV and DENV). Cell viability was determined using the same parameters (moi and timepoints) and measured using the trypan blue exclusion assay.

**Flow Cytometric Analysis**

Flow cytometric staining was performed for the detection of ZIKV in hSCs and the presence of Fc receptors on hSCs. hSCs were infected with the Fortaleza strain of ZIKV at an moi of 5 for the detection of ZIKV and an moi of 1 for Fc receptor detection. Table 1 describes the antibodies used for staining. For the detection of ZIKV, cells were fixed with 100µl of 2% formaldehyde in PBS and permeabilized with 0.2% Triton in FACS Buffer before staining with the primary antibody (concentration 0.5µg/µl) followed by the secondary antibody, PE conjugated goat anti-mouse IgG. Live dead staining used Thermo Fisher Scientific Violet L/D dye (Cat no. L34955). Compensation for PE and live/dead staining was done using BD compensation beads (Cat no. 552845) for the former and Thermo Fisher Scientific compensation beads (Cat no. A10346) for the latter. Analysis was performed using Flow Jo. Antibodies were graciously provided to us by Dr. Ted Pierson (NIH) and Dr. Anna Durbin (Johns Hopkins).
<table>
<thead>
<tr>
<th>Target</th>
<th>Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZIKV</td>
<td>4G2 (pan-flavivirus)</td>
</tr>
<tr>
<td>ZIKV</td>
<td>2H2 (pan-flavivirus)</td>
</tr>
<tr>
<td>ZIKV</td>
<td>Z13 (ZIKV specific)</td>
</tr>
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<td>ZIKV</td>
<td>Z67 (ZIKV specific)</td>
</tr>
<tr>
<td>Isotype Control</td>
<td>Mouse IgG2a κ</td>
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<tr>
<td>CD16 (FcγRIII)</td>
<td>FITC mouse antihuman CD16 (BD 555406)</td>
</tr>
<tr>
<td>Isotype Control</td>
<td>FITC mouse IgG1 κ (BD 551954)</td>
</tr>
</tbody>
</table>

Table 1 - List of primary antibodies used in flow cytometric experiments
**Immunofluorescence**

Human Schwann cells were grown on poly-L-lysine coated cover slips and infected with ZIKV Fortaleza and DENV (NGC) at an moi of 5. At 24 hpi, cells were fixed with 4% formaldehyde, permeabilized with 0.2% triton X 100 in PBS, and blocked in 5% normal goat serum in PBS. The cells were incubated with primary antibody (4G2 fusion loop; cross reactive) at a concentration of 1:1000 in PBS for an hour followed by staining with anti-mouse Alexafluor594 (Invitrogen) as the secondary antibody. DAPI was used for staining the nuclei. Images were captured on Zeiss Axio Imager light microscope.

**Quantitative real-time PCR (qPCR)**

hSCs were grown as described above and seeded in 6 well flat bottom cell culture plates, then infected with ZIKV Fortaleza and YFV (17D) at an moi of 5 in triplicates. Cells were collected in RLT buffer at 0, 12, 24, 48, and 72 hpi. Qiagen RNeasy Plus Mini Kit (Cat No. 74136) was used for RNA isolation and quantified. cDNA was synthesized using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Ref 4368814) with an RNA concentration of 500ng/µl in a 20µl total reaction volume. qPCR was performed with the Promega GoTaq® qPCR Master Mix for Dye-Based Detection (Ref A6001) using gene-specific oligonucleotide primers for SYBR Green-based measurements or the Applied Biosystems TaqMan probes (Part No. 4331182) listed in Table 2. Applied Biosystems 7500 Real Time PCR System was used under the following conditions: initial hold for 2 min at 50°C and hold for 10 min at 95°C followed by 40 cycles consisting of denature 15 s at 95°C; anneal 60 s at 60°C. All gene expression data were normalized against GAPDH levels and fold change was calculated as $2^{-\Delta\Delta ct}$. 
### SYBR Green Primers (IDT-DNA Primer Quest)

<table>
<thead>
<tr>
<th>Gene</th>
<th>RefSeq.</th>
<th>Forward Primer (5' - 3')</th>
<th>Reverse Primer (5' - 3')</th>
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<td>GAGACCAGGCCATCCACC</td>
<td>AATTTCCCTATCTCCTTCGCA</td>
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<tr>
<td>FCGR1C</td>
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### TaqMan Probes (Applied Biosystems)

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*Table 2 – Primers and probes used for RT-PCR*
Antibody-Dependent Enhancement (ADE) Assay

Plasma samples of DENV (serotypes 1-4) and control sera were heat inactivated at 56°C for 30 minutes before being serially diluted ten-fold for one set of experiments and four-fold for the subsequent experiments. Viruses (NGC, 17D, ZIKV Fortaleza, ZIKV Nigeria, and ZIKV Thailand) were added to diluted sera for an moi of 0.1 for one set of experiments and 1 for subsequent experiments. The virus/sera mixtures were incubated for 1 hour at 37°C with 5% CO₂. Following incubation, the virus/sera mixture was added to hSCs or the K562 positive control cell line seeded into 12 well plates and incubated at 37°C with 5% CO₂ for 1 hour. Following incubation hSC media or RPMI media (described above) with 1% heat-inactivated fetal bovine serum was added to each well bringing the total well volume to 400µl and plates were incubated for 48 hours at 37°C with 5% CO₂. Supernatant fluids were collected after incubation and stored at -80°C before analysis was done by either flow cytometry or focus forming assay (as described above).

Statistics

Data was compared using a two-way ANOVA test and presented as mean +/- standard error of the mean (SEM). A value of P < 0.05 was used to establish significant difference between samples. All statistical analyses were performed on GraphPad Prism 5. Statistical significance was reported as follows, *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.
RESULTS

Human Schwann Cells Are Susceptible to Zika Virus Infection and Exhibit Strain Specific Characteristics

Using an immortalized human Schwann cell line [33] we assessed the ability of Yellow Fever virus (17D), Dengue virus serotype 2 (NGC) and three strains of Zika Virus: ZIKV 1968 Nigeria (IBH 30656), 2014 Thailand (SCV0127/14) and 2015 Brazil (Fortaleza) to infect and replicate in hSCs. hSCs were infected at an moi of 5 for each virus and collected at varying time points of 0, 24, 48, 72, 96, and 120 hours post infection for analysis by plaque assay for ZIKV strains and focus forming assay for YFV and DENV (Figure 6). While all viruses successfully infected hScs, replication of ZIKV strains was highest. ZIKV 2015 Brazil (Fortaleza) exhibited the highest titer levels of virus at 120 hours post infection suggesting the best ability to replicate in hSCs. When comparing replication kinetics of similar flaviviruses, YFV (17D) and DENV (NGC), YFV had similar replication in hSCs. But, DENV replication in hSCs was minimal.
Figure 6 - Replication kinetics of Yellow Fever virus (17D), Dengue virus serotype 2 (NGC) and three strains of Zika Virus: ZIKV 1968 Nigeria (IBH 30656), 2014 Thailand (SCV0127/14) and 2015 Brazil (Fortaleza) in human Schwann cells. Moi = 5.
To further confirm the susceptibility of hSCs to ZIKV infection, detection of ZIKV by monoclonal antibodies and through immunofluorescence were conducted. Using ZIKV 2015 Brazil (Fortaleza) hSCs were infected at an moi of 5 and incubated for 24 hours before analysis by immunofluorescence and 96 hours before analysis by flow cytometry using Zika-specific mAbs, Z13 and Z67. Figure 7 shows the immunofluorescence image of ZIKV infected hSCs and of mock treated hSCs. Blue DAPI stained the cell nuclei while red Alexafluor594 stained ZIKV. Figure 8 displays the flow cytometry histogram analysis of ZIKV infected hSCs. Blue identifies the mock treated cells while red identifies the ZIKV infected cells, the shift as detected by ZIKV specific mAbs indicates infection of hSCs with ZIKV.
**Figure 7**– Immunofluorescence image of mock infected (top) and ZIKV Fortaleza infected (bottom) HSCs at 24hpi with an moi of 5. Cells were stained with pan flavivirus 4G2, Alexafluor594, and DAPI.
Figure 8 - ZIKV Fortaleza detection in hSCs by mAbs, 96 hours post infection at an moi of 5.
To explore further the strain specific differences amongst the three ZIKV strains, a cell viability assay was carried out (Figure 9). The Thailand strain of ZIKV induced cell death significantly faster than the other two strains of ZIKV. However, by 72 hours the Nigeria strain has induced the most cell death. By 120 hours post infection all strains caused similar levels of cell death. The ZIKV Fortaleza strain induced cytopathogenicity at the slowest rate creating a longer lasting infection. Together, our data suggest that evolutionarily distinct ZIKV strains can infect hSCs and exhibit varying cell death capacities during infection of hSCs.
Figure 9 - Human Schwann cell viability after infection with three strains of Zika virus at moi 5 as determined by focus forming assay.
Zika Virus Fortaleza Infection of Human Schwann Cells Induces Cytokine Expression

We examined the consequences of infection of hSCs at an moi of 5 at 0, 12, 24, 48, and 72 hours post infection by Zika Virus Fortaleza. hSCs were also infected at an moi of 5 with Yellow Fever virus (17D), which had similar replication kinetics in hSCs (Figure 6). Figures 10-12 display quantitative real time PCR results of the transcript level expression of inflammatory and regulatory cytokines after infection with ZIKV Fortaleza and YFV. Figure 10 displays mRNA levels for innate immune antiviral response cytokines and proinflammatory cytokines. IFNβ1 and IFN response gene IFIT-1 are both significantly upregulated 24 hours after infection with both YFV and ZIKV, with highest levels induced by infection with YFV. NLRP3 inflammasome gene is induced by YFV, but only minimally at 48 hours post infection by ZIKV infection. IL23a which stimulates the induction of other cytokines such as IL6 [36] peaked at 48 hpi only in ZIKV and IL6 mRNA expression was increased at 72 hpi only with ZIKV infection consistent with prior induction of IL23a mRNA. Expression of TNFα mRNA was induced in both ZIKV and YFV infected hSCs, however ZIKV infected hSCs saw a continual exponential rise of TNFα expression while YFV infected hSCs leveled off after 48hpi. The results presented in Figure 10 demonstrates that ZIKV Fortaleza infection induces an innate immune response in hSCs that is somewhat distinct from that induced by YFV.
Figure 10 - qPCR results of innate immune response cytokine mRNA levels in hSCs infected with ZIKV Fortaleza and Yellow Fever virus (17D) at an moi of 5.
The greatest cytokine mRNA expression observed in infected hSCs was the expression of IL-29 (IFNλ1) a type III interferon which exhibits antiviral and anti-proliferative activities produced through a different receptor but the same downstream signaling pathways as IFNα and IFNβ [37]. A 22,000-fold increase in mRNA expression was seen in ZIKV Fortaleza infected hSCs at 48 hours post infection (Figure 11), nearly seven times the expression observed in YFV infected hSCs. To further explore the role of IL-29, expression of mRNAs for the heterodimeric receptor complex for IL-29, INFLR1 and IL10RB, were examined. Expression of both receptors was increased only in ZIKV infected hSCs (Figure 11). In addition, the negative regulators of IL-29, SOCS1/3 [38], were prominently expressed in ZIKV infected hSCs at 72hpi that corresponds with the lack of expression of IL-29 at 72hpi. We also looked at MX1 expression, a GTPase that is part of the antiviral response induced by type I and type III interferons [39], and observed significant upregulation with YFV infection beginning at 24 hpi and significant upregulations with ZIKV at 72 hpi. These results suggest that ZIKV Fortaleza infection in hSCs induces a more robust expression of the type III interferon, IL-29, than YFV 17D.
Figure 11 - qPCR results of cytokine mRNA levels of IL-29 (IFNλ1), interferon induced GTPase MX1, IL29 negative regulators (SOCS1/3) and its heterodimeric receptor complex (IFNLR1 and IL10RB) in hSCs infected with ZIKV Fortaleza and Yellow Fever virus (17D) at an moi of 5.
ZIKV replication in human retinal pigment epithelial cells disturbs function of tight junction proteins like claudin-1 and occludin, that could influence hSC function [40] [41]. Therefore, we examined mRNA expression of the following tight junction proteins: TJPI, Occludin (OCLN), and Claudin 1/6 (CLDN). However, no significant change in expression of these genes at the transcript level was observed (Figure 12)
Figure 12 - qPCR results of cytokine mRNA levels of tight junction proteins in hSCs infected with ZIKV Fortaleza and Yellow Fever virus (17D) at an moi of 5.
Human Schwann Cells Have Limited Expression of Fc Receptors

Fc receptors are a family of surface proteins that specifically bind the Fc region of antibodies and are involved in activation and regulation of the immune response. Fc receptors can facilitate viral entry into cells and play a key role in the phenomenon of antibody dependent enhancement [42]. Three classes of Fcγ receptors exist in humans: FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16). FcγRI and FcγRIIa facilitate ADE in a human monocytic cell line [43]. Using flow cytometry, we analyzed the expression of these three classes of Fc receptors on hSCs (Figure 13). Only minimal expression of FcγRI (CD64) was observed, corroborating with other studies [44]. To determine the effect of ZIKV infection on the expression of Fcγ receptors, hSCs were infected with ZIKV Fortaleza at an moi of 1 and analyzed at 0, 2, 48, and 72 hours post-infection. Cells were stained for both FcγRI and FcγRIIa expression. Neither expression of FcγRI (CD64) or expression of FcγRII (CD32) detected by flow cytometry was changed by infection with ZIKV Fortaleza (Figure 14).
Figure 13 - FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16) expression in hSCs at moi 1
Figure 14 - FcγRI (CD64) and FcγRII (CD32) expression in hSCs upon Zika Fortaleza Infection (moi 1) at 0 hours, 2 hours, 24 hours, 48 hours, 72 hours
To further validate the findings from our flow cytometry results, we also examined FcγR expression at the transcript level with qPCR (Figure 15). hSCs were infected with both ZIKV Fortaleza and YFV (17D) at an moi of 5 and assessed using custom primers (Table 2) at 0, 12, 24, 48, and 72 hours post-infection. No significant expression of any of the three classes of Fcγ receptors were found.
Figure 15 - qPCR results of cytokine levels of the three classes of Fcγ receptors which exist in humans: FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16). FcγRI and FcγRIIa. Results from hSCs infected with ZIKV Fortaleza and Yellow Fever (17D) at an moi of 5.
**Antibody Dependent Enhancement by Antibodies against Dengue Virus is not Observed with Zika Virus Infection**

To determine if plasma from DENV infected subjects could promote antibody-dependent enhancement (ADE) in human Schwann cells we infected hSCs at an moi of 0.1 with all three strains of ZIKV (Fortaleza, Nigeria, and Thailand), DENV (NGC), and YFV (17D) in the presence of antibodies against each serotype of DENV (1-4) and measured the effect on infection by focus forming assay. None of the three ZIKV strains showed enhancement of viral entry into hSCs with 12 sera dilutions for each DENV serotype antibody (Figure 16). Infection with YFV (17D) showed relatively similar results to the control sera and infection with DENV (NGC) showed a slight enhancement in the presence of DENV-1 sera at dilutions of $10^{10}$ and $10^{11}$ (Figure 16).
Figure 16 - Results of focus forming assay looking at virus levels in the presence of serially diluted control, DENV1, DENV2, DENV3, DENV4 sera.
We also assessed the promotion of ADE using a positive control cell line, K562 cells expressing DCSIGN. These cells were infected with ZIKV Fortaleza, ZIKV Thailand, and DENV (NGC) at an moi of 1 and measured by flow cytometry using 4G2 (pan-flavivirus) as the primary antibody. This assay showed enhancement of ZIKV infection of K562 cells in the presence of antibodies against DENV serotype 2 (Figure 17). Enhancement of ZIKV infection occurred at plasma dilutions of 1:5 to 1:80. As expected, DENV (NGC) infection was enhanced at higher dilutions of antibody against DENV-2. When hSCs were infected, under the same conditions as the positive cell line, no enhancement was observed (Figure 18). To confirm these negative results, we replaced the control sera with another control sera and performed the assay once again with the ZIKV strains of Fortaleza and Thailand. The results confirmed previous ADE assays, showing no enhancement in hSCs in the presence of DENV-2 serum compared to the control serum (Figure 19).
Figure 17 - Flow Cytometry results of the percent of positive cells for two strains of ZIKV in a positive control cell line, K562 cells expressing DCSIGN. Viruses infected cells at an moi of 1 in the presence of serially diluted control, DENV1, DENV2, DENV3, DENV4 anti-sera.
Figure 18 - Flow Cytometry results of the percent of positive cells for three strains of ZIKV in human Schwann cells. Viruses infected cells at an moi of 1 in the presence of serially diluted control and DENV2 sera.
**Figure 19** - Flow Cytometry results of the percent of positive cells for two strains of ZIKV (Fortaleza and Thailand) in human Schwann cells. Viruses infected cells at an moi of 1 in the presence of serially diluted control and DENV2 sera.
DISCUSSION

Zika virus was declared a “Public Health Emergency of International Concern” by the World Health Organization on February 1, 2016 because of the emergence of neurological abnormalities [15]. One such neurological abnormality triggered by ZIKV was Guillain-Barré syndrome (GBS) [27]. A crucial detail of this new-found relationship was that the evidence pointed to ZIKV triggering the Acute Inflammatory Demyelinating Polyneuropathy (AIDP) subtype of GBS [25]. This suggested that ZIKV might target the myelin sheath produced by Schwann cells which wrap around segments of the neuronal axon in the peripheral nervous system and that the immune response might target a viral rather than a cellular antigen. This newly discovered relationship provided a unique opportunity for investigation of the pathogenesis of GBS associated with ZIKV. Therefore, our study focused on Schwann cells and understanding how ZIKV interacts with these cells which when infected in vivo might lead to AIDP type GBS.

Using a unique immortalized line of primary human SCs developed at Johns Hopkins [33] we observed the ability of three evolutionarily distinct strains of ZIKV (1968 Nigeria, 2014 Thailand, and 2015 Brazil) to infect and replicate in hSCs. Additionally, we tested the ability of two related flaviviruses (Yellow Fever 17D and Dengue) and found they also replicated in hSCs but not as well as the three ZIKV strains. The ability for ZIKV to infect hSCs supports a possible role for ZIKV in inducing GBS. We also found that when comparing strains of ZIKV in HSCs the strain responsible for the ZIKV epidemic, 2015 Brazil Fortaleza, induced cell death at a rate slower than the other two strains of ZIKV. It is possible that this strain causes a more persistent infection than the other two
strains before leading to cell death that might lead to clinical manifestations not seen historically with ZIKV infection.

Examination of the cellular response of hSCs upon ZIKV Fortaleza infection and YFV (17D) infection revealed an innate antiviral response when infected with both flaviviruses. However, infection by ZIKV 2015 Brazil induced greater IL-6 and IL-29 response than YFV in hSCs. This suggests a type III interferon response specific to ZIKV. The expression of IL29 negative regulators, SOCS1/3, and heterodimeric receptor complex were also observed during ZIKV. To further characterize the type III interferon response in hSCs future studies will have to examine the expression of IL28A and IL28B. Although the precise cellular and molecular mechanisms by which IL29 is regulated in hSCs upon viral and/or other cellular factors remain to be determined, its presence in ZIKV suggests a role which is not found amongst all flaviviruses.

This study also explored the phenomenon of antibody dependent enhancement (ADE) in hSCs upon infection with ZIKV and antibodies against DENV and found no evidence that enhanced viral replication was occurring. We also did not find significant expression of Fcγ receptors on hSCs, the receptor facilitating viral entry into cells when ADE occurs. While initially studies were published reporting ADE and immunological crosstalk occurring *in vitro* between plasma immune to DENV and ZIKV [30] [31] more recent studies have found no signs of ADE *in vivo* in patients with acute ZIKV infection who had prior exposure to DENV [45]. While several studies reviewed by the WHO during the ZIKV outbreak reported past dengue virus infection in ZIKV cases [27] our study suggests that past DENV infection does not promote ZIKV infection in hSCs.
The mechanisms between ZIKV and GBS are not well understood. Speculated mechanisms include: Antibody dependent enhancement with DENV sera, an immune mediated mechanism inducing damage through molecular mimicry, cellular mediated inflammation and demyelination induced by compliment and macrophage activation, or a direct viral pathogenic effect due to the parainfectious profile of ZIKV-GBS [28]. Of these four mechanisms our study of the relationship between ZIKV and GBS, specifically examining hSCs, suggests that GBS could be mediated by pathogen-specific antibodies/T cells or a direct viral effect rather than the traditionally held belief of cross-reactive antibodies to host proteins/lipids. The understanding that ZIKV can replicate within hSCs and induces a cellular response that this study demonstrates facilitates a better understanding of the role of ZIKV and GBS in the most recent epidemic.
REFERENCES


BIograPhical skEtCh

Gaurav Dhiman was born in Madison, Wisconsin before moving to Logan, Utah where he graduated from Logan High School in 2010. Gaurav entered George Washington University in Washington, DC where he received a Bachelor of Science with a major in biology and a secondary field in international affairs concentrating in global public health. Gaurav spent one year working at the American Association for the Advancement of Science before entering the ScM program in Molecular Microbiology and Immunology at the Johns Hopkins Bloomberg School of Public Health.