ZIKA VIRUS INFECTS HUMAN SERTOLI CELLS AND TRESPASSES THE BLOOD-TESTES BARRIER TO GAIN ENTRY INTO THE SEMINIFEROUS EPITHELIUM

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ABSTRACT

Over the past decade, Zika virus (ZIKV) has re-emerged as a pathogen of major health concern in the Western Hemisphere. Specifically, since 2007, 76 countries have announced new outbreaks of ZIKV, 29 of which also report increased incidence of CNS malformations such as microcephaly and also Guillain-Barre Syndrome [1]. These alarming statistics combined with the unique ability of ZIKV to be transmitted both sexually and *in utero* highlight the urgent need to study the mechanisms of ZIKV pathogenesis in order to ultimately develop vaccines, effective anti-viral therapies, and policies to control the spread of ZIKV disease.

While ZIKV can be detected in human seminal fluid for months after the clearance of viremia, the cellular targets and mechanisms associated with persistent infection in the testes remains unclear. Mouse and NHP studies have recently shown that ZIKV can infect and damage the seminiferous tubules within the testes [2-5]. The seminiferous tubules are an immune privileged organ with a tight blood-testes barrier also known as the Sertoli-cell barrier (SCB), which protects developing spermatozoa from peripheral pathogens and environmental toxins. However, increased inflammatory mediators such as TNF-α and IL-1β, matrix metalloproteinases, and cell-adhesion molecules (CAM) can disrupt the integrity of the SCB leading to pathologic outcomes [6, 7]. Therefore, the objective of this study was to characterize ZIKV replication kinetics and immune responses in primary human Sertoli cells (SC) and develop an in vitro SCB model to understand mechanisms of ZIKV transmigration across the barrier.

We demonstrate that primary human SC are highly susceptible to ZIKV as compared to the closely related dengue virus and induced expression of IFN-α, key cytokines and cell-adhesion molecules (VCAM-1 and ICAM-1). Further, using an in vitro SCB model, we show that ZIKV was released on the adluminal side of the SCB model with higher efficiency when compared to the blood-brain barrier model. ZIKV-infected SC also exhibited enhanced adhesion of leukocytes that correlated with decrease in the SCB integrity. While ZIKV infection did not affect the expression of tight and adherens junction proteins such as ZO-1, claudin and JAM-A, exposure of SC to inflammatory mediators derived from ZIKV-infected macrophages led to the degradation of ZO-1 protein that correlated with increased SCB permeability. Collectively, our data suggest that infection of SC may be one of the crucial steps by which ZIKV gains access to the site of spermatozoa development and identifies SC as a therapeutic target. Finally, the SCB model opens up opportunities to assess interactions of SC with other testicular cells and lays the platform for future studies to test the ability of anti-ZIKV drugs to cross the barrier and clear testicular infection.

TABLE OF CONTENTS

Acknowledgements………………………………………………………… **ii**

Abstract…………………………………………………………………….. **iii**

List of figures……………………………………………………………… **vii**

List of abbreviations……………………………………………………… **ix**

Chapter 1. Zika virus and human disease **1**

Discovery, history, and epidemiology of Zika virus……………….. **2**

Virology……………………………………………........................ **3**

Phylogeny………………………………………………..................... **5**

Life cycle of ZIKV………………………………......................... **7**

Mosquito and blood-borne transmission of ZIKV…………………. **9**

Sexual transmission of ZIKV…………………………………………… **10**

Vertical transmission of ZIKV…………………………………………… **15**

Clinical symptoms of ZIKV diseases…………………….................. **17**

Complications of ZIKV infection………………………..................... **18**

Pathogenesis of ZIKV…………………………………..................... **20**

Diagnosis, treatment, and prevention…...…………………………….. **24**

Vaccine and antiviral development.…….. **26**

Host response to ZIKV…………………...................................... **29**

Immune evasion of ZIKV…………………………………………….. **32**

Overview of the male reproductive tract.................................. 33

Mammalian testicular anatomy and spermatogenesis.............. 34

Literature on testicular infection of ZIKV................................ 37

Chapter 2: Thesis scope **39**

ZIKV a re-emerging flavivirus with increased disease severity.... 40

An update of ZIKV sexual transmission................................. 40

Gap, rationale, and hypothesis.. 41

Specific aims.. 42

Specific aim 1.. 42

Specific aim 2.. 43

Significance.. 45

Chapter 3: Zika virus infects human sertoli cells and modulates the integrity of the in vitro blood-testes barrier model **46**

Abstract.. 48

Importance and introduction... 49

Results... 52

Discussion.. 67

Materials and methods... 74

Supplemental figures... 80

Chapter 4: Summary and future directions................................ **81**

Bibliography.. **89**

LIST OF FIGURES

Chapter 1

Figure 1: Spread of ZIKV across the globe to date…………………. **3**

Figure 2: ZIKV genome and it's encoded proteins………………….. **4**

Figure 3: Organization of a mature flavivirus particle………………. **5**

Figure 4: Phylogenetic tree of ZIKV African and Asian lineages….. **6**

Figure 5: The replication cycle of flaviviruses……………………….. **8**

Figure 6: Presence of ZIKV in seminal fluid and sexually transmitted cases………………………………………………………….. **12**

Figure 7: Time until clearance of ZIKV RNA in serum, urine, and semen………………………………………………………………. **13**

Figure 8: Confirmed mother-to-child ZIKV transmission…………… **16**

Figure 9: Overview of ZIKV transmission cycles……..................... **17**

Figure 10: Infants with microcephaly as compared to a typical newborn……………………………………………………………….. **20**

Figure 11: Schematic of the course of human and mosquito infection……………………………………………………………….. **21**

Figure 12: ZIKV tissue and cell tropism……………………………… **23**

Figure 13: Algorithm for the laboratory diagnosis of ZIKV infection **25**

Figure 14: Proposed ZIKV vaccine candidates and platforms…….. **27**

Figure 15: Anatomy of the male reproductive system……………… **34**

Figure 16: Overview of spermatogenesis……………………………. **35**

Figure 17: Cross section of the mammalian testes…………………. **36**

Figure 18: ZIKV infection and pathogenesis in the male reproductive tract………………………………………………………….. **38**

Chapter 3

Figure 1: ZIKV infection in human sertoli cells is productive……….. 54

Figure 2: ZIKV infection induces secretion of antiviral and pro-inflammatory mediators in HSEC……………………………………… 56

Figure 3: Effect of ZIKV infection on cytokines, cell adhesion molecules and MMP…………………………………………………… 58

Figure 4: ZIKV infection does not alter the expression of tight and adherens junction proteins in HSEC……………………………… 59

Figure 5: Cell free ZIKV is efficiently released on the adluminal side of an in vitro SCB model without affecting barrier integrity…………… 62

Figure 6: ZIKV infection in human macrophages induces mediators that alter SCB permeability……………………………………………… 65

Figure 7: Anti-flavivirus drug, NITD008, blocks ZIKV infection in HSEC in a dose-dependent manner…………………………………… 67

Figure 8: Putative interactions between ZIKV and the SCB………… 72

Supplemental Figure 1: Infection with ZIKV, DENV, and WNV does not result in cell death in HSEC…………………………………………… 80

Supplemental Figure 2: ZIKV, DENV, and WNV cause productive infection in HSEC…………………………………………………………… 80

Chapter 4

Figure 1: Hypothetical model for flavivirus recognition by TIM/TAM receptors…………………………………………………………………….. 86

ABBREVIATIONS

ADE	Antibody-dependent enhancement
APC	Antigen presenting cells
BBB	Blood-brain barrier
BRB	Blood-retinal barrier
BTB	Blood-testes barrier
CAM	Cell-adhesion molecules
CDC	Centers for Disease Control and Prevention
CHIKV	Chikungunya virus
CNS	Central nervous system
CSF	Cerebral spinal fluid
DC	Dendritic cells
DENV	Dengue virus
FDA	Federal Drug Administration
GBS	Guillain-Barré syndrome
HBMVEC	Human brain microvascular endothelial cell
HSEC	Human sertoli cells
ICAM-1	Intracellular cell adhesion molecule 1
IFN	Interferon
ISG	Interferon stimulated gene
IL	Interleukin
JAM-A	Junctional adhesion molecule alpha

JEV	Japanese encephalitis virus
MDA5	Melanoma differentiation-associated gene 5
MOI	Multiplicity of infection
NHP	Non-human primate
NIH	National Institutes of Health
OFC	Occipitofrontal head circumference
PBMC	Peripheral blood mononuclear cells
PFU	Plaque forming units
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RLR	RIG like receptor
SC	Sertoli cells
SCB	Sertoli-cell barrier
TBEV	Tick-borne encephalitis virus
TEER	Trans-endothelial electrical resistance
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
ZIKV	Zika virus
VCAM-1	Vascular cell adhesion molecule 1
WHO	World Health Organization
WNV	West Nile virus
YF	Yellow fever virus
ZO-1	Zona occludens 1

Chapter 1

Zika virus and human disease

Discovery, history, and epidemiology of Zika virus:

Zika virus (ZIKV) was first isolated from a sentinel Rhesus monkey in 1947 and subsequently in 1948 from a pool of *A. africanus* mosquitos found in the Zika forest of Uganda [8]. The first human case, however, was not described until 1954 from three patients in Nigeria [9]. Thereafter, serologic and entomologic surveys began to suggest a wide geographic distribution in the band of equatorial African countries (Sierra Leone, Gabon, Central African Republic, Senegal) and in parts of Asia (Pakistan, Malaysia, Indonesia) [10]. For over half a century, ZIKV was repeatedly isolated in many of these countries, however only 14 mild-febrile human infections were documented [11].

The first large outbreak occurred in 2007 on Yap island in the Federated States of Micronesia, where an estimated 5,005 (72.6%) of the 6,892 residents were infected [12]. The next major outbreak happened in 2013 in French Polynesia, where an estimated 30,000 (11.5%) of the 270,000 inhabitants were exposed [13]. As seen in Figure 1, during and after this time, ZIKV spread rapidly to other Pacific islands including New Caledonia, Cook Islands, Easter Island, Vanuatu, Soloman Islands, Samoa, and Fiji [14]. However, it wasn't until ZIKV made landfall in Brazil in early 2015 that the virus truly emerged on the global stage where it caused between 440,000 to 1,300,000 infections by December 2015 [15]. Futhermore, the association of ZIKV with more severe complications including Guillain-Barré syndrome (GBS) and severe fetal abnormalities such as microcephaly was a cause for serious alarm.

Soon after, nearby countries in South America and the Caribbean began reporting autochthonous ZIKV circulation, attributed to the widespread distribution of *Ae. aegypti*

and *Ae. albopictus* in these regions [16]. Since then, 61 new countries worldwide have reported an outbreak of mosquito-borne Zika virus transmission, 29 of which have also reported increased incidence of microcephaly, other CNS malformations, and GBS potentially associated with ZIKV infection [1]. Currently in the U.S. and US territories there have been 5,285 and 36,583 symptomatic ZIKV disease cases reported, respectively [17].

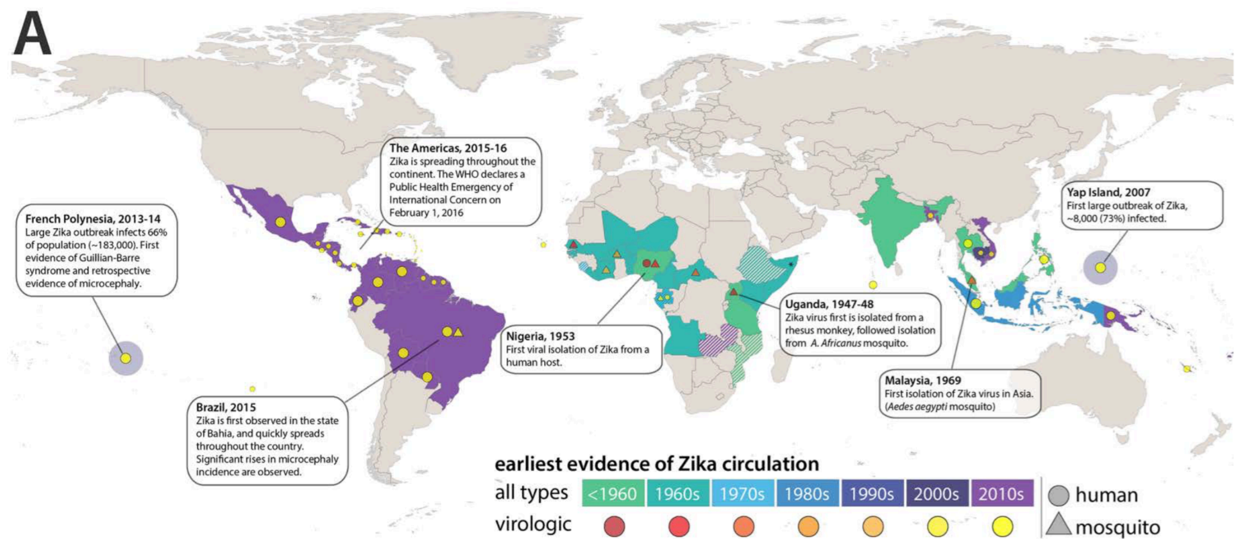

Figure 1: Spread of ZIKV across the globe to date [14]

Virology

ZIKV belongs to the flavivirus genus of the *Flaviviridae* family, which includes other globally relevant arthropod-transmitted human pathogens such as dengue (DENV), West Nile (WNV), and Japanese encephalitis (JEV) viruses. Similar to these related viruses, ZIKV has an enveloped, spherical particle with an estimated diameter of ~50 nm. The viral genome is a single stand of linear, positive sense RNA roughly 10.8 kilo-bases in length. At the 5' end is a type I cap structure ($m^7GpppAmG$), which plays an important role in evasion of host restriction. Following the cap structure is a 5' non-coding region

(NCR) that is required for modulating viral translation and RNA replication and is comprised of cis-acting RNA sequences and structures. Next is the open reading frame (ORF), which is translated into a polyprotein of 3,423 amino acids that is cleaved post translationally into three structural proteins (capsid, pre-membrane/membrane, and envelope) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5), as shown in **Figure 2** [18]. In general, the capsid (C) protein is involved with packaging of the viral genome. The pre-membrane (PrM) protein chaperones the folding and assembly of the envelope (E), which is the major protein involved in receptor binding and fusion [19, 20]. The non-structural proteins are responsible to form the viral replicase complex, which functions as a protease, helicase, methyltransferase, and RNA-dependent RNA polymerase [21]. At the 3' end of the genome, there is another NCR, which works together through long distance RNA-RNA interaction with the 5' NCR and is also involved in counteracting the host cell's response by serving as a substrate for host cell exoribonucleases to generate short non-coding sub-genomic flaviviral RNAs [10].

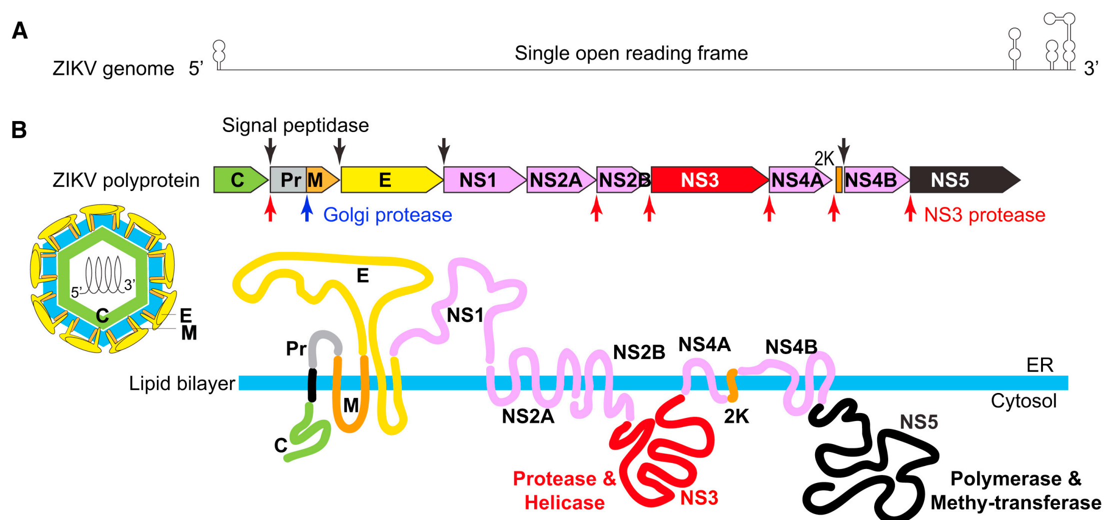

Figure 2. ZIKV genome and it's encoded proteins [18]

A mature ZIKV particle consists of one copy of genomic RNA surrounded by multiple copies of the viral capsid protein, which form the nucleocapsid. This nucleocapsid is enclosed in a lipid bilayer derived from the modified membrane of the endoplasmic reticulum, with two surface proteins, membrane (M) and envelope (E) anchored through their two C-terminal transmembrane domains [11]. The M protein is a small proteolytic fragment of its precursor form prM, which acts as a transmembrane protein under the E-protein shell of the mature virion. The smooth outer layer of the mature particle is constituted by 180 copies of the E protein, arranged as 90 antiparallel homodimers with an icosahedral symmetry [16] as depicted in **Figure 3.**

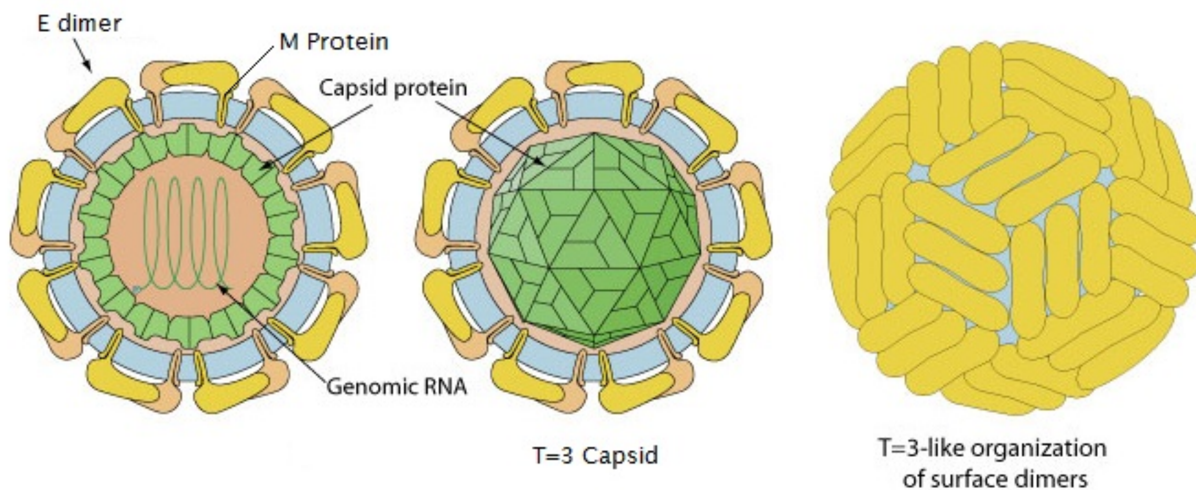

Figure 3: Organization of a mature flavivirus particle [22]

Phylogeny of ZIKV

ZIKV is most closely related to the Spondweni virus, and these two viruses form the Spondweni serocomplex within the mosquito borne-clade of flaviviruses as shown in **Figure 4**. The next nearest relatives include the Illheus, Roico, and St. Louis encephalitis viruses [23]. ZIKV is also closely related to the four serotypes of DENV with approximately

43% amino acid identity across the viral polyprotein as well as the ectodomain of the envelope protein [24]. As described in the previous section (Discovery, History, and Epidemiology), it is assumed that the virus originated in East Africa and then spread to both West Africa and Asia about 50-100 years ago [25]. Following this historical pattern and based on the comparison of full and partial genome sequences of ZIKV, there are three distinct genotypes of Zika including the West African (eg. Nigerian and Senegal cluster), East African (eg. MR766 prototype cluster), and Asian (eg. Malaysian, Yap, and French Polynesian) strains. The Zika strain responsible for the current epidemic in Brazil and Central America is derived from the Asian lineage.

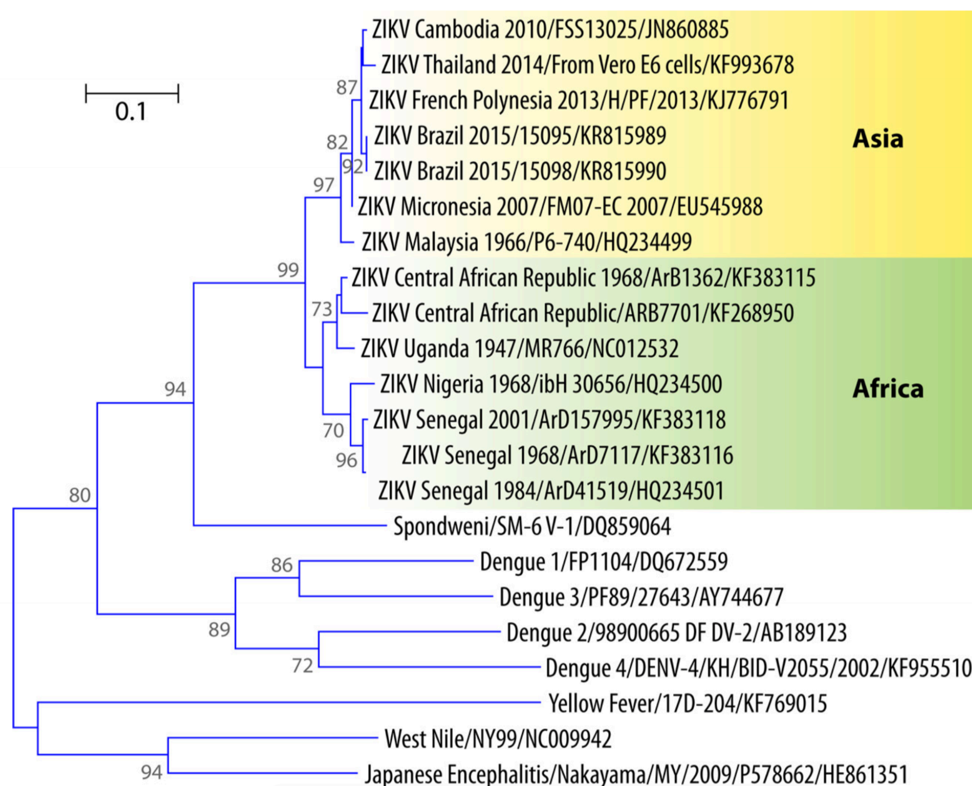

Figure 4: Phylogenic tree of ZIKV African and Asian lineages [16]

It is estimated that the virus mutates about 10 bases a year or 0.01% of the genome [26]. Taking a closer look at the genomes between ZIKV strains, the percent identity of the ZIKV 2007 Yap strain with that of the prototype ZIKV MR 766 prototype strain isolated in 1947 is 88.9% at the nucleic acid level (96.5% at the amino acid level) [16]. Interestingly, the 2007 Yap strain, the French Polynesian H/PF/2013 epidemic strain, and three strains of ZIKV from Senegal have a glycosylation motif at position 154 of the envelope, which is associated with an increase in virulence and is missing in the ZIKV MR 766 prototype strain [16]. Sequences from Brazil, Colombia, Puerto Rico, and Guatemala were all more than 99% identical with the French Polynesian strain (H/PF/2013), which provides further evidence that the virus was derived from Asia and the Pacific [16].

Lifecycle of ZIKV

Like most other flaviviruses, ZIKV follows a general replication cycle that begins with virions attaching to the surface of target cells. Although the specific cell entry receptor utilized by ZIKV to bind and enter host cells is not well characterized, it has been reported that ZIKV enters via adhesion factors such as Heparin sulphate, C-type lectin receptors (ex. DC-SIGN), the low-density lipoprotein receptor, and diverse members of the phosphatidylserine receptor family (ex. AXL, Tyro3, and TIM-1) [27, 28].

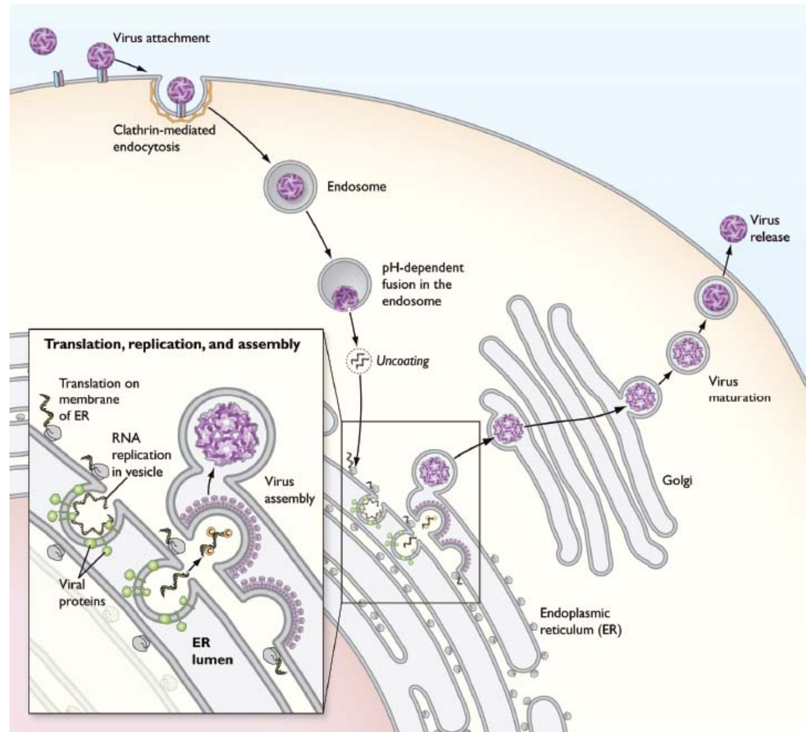

Figure 5. The replication cycle of flaviviruses [28]

Following receptor binding, ZIKV enters target cells via clatherin-mediated endocytosis and is transported to endosomes [10]. The low pH within the endosome induces a conformational change in the E glycoprotein that allows fusion of the virus with the endosomal membrane. Viral genomic RNA is then released form the virion into the cytoplasm and is translated into a single polypeptide at the endoplasmic reticulum (ER). The polyprotein is cleaved by host cellular signalase, furin, and viral serine protease (NS3) into the three structure and 7 non-structural (NS) viral proteins. These NS proteins form a replication complex inside the virus-induced ER-derived membranous compartments for the synthesis of complementary negative-sense RNA. This intermediate then serves as a template for production of additional (+)-sense genomic RNAs and further translation of viral proteins [10, 28]

Viral assembly takes place on the ER membrane and is facilitated by the prM and E proteins, which form a heterodimer and drive the budding of the viral genomic RNA and C proteins into the ER lumen to produce an immature non-infectious virion. These particles then must move through the cellular secretory pathway, during which furin and furin-like proteases cleave prM, generating the M structural protein. This processing leads to structural rearrangements of the M and E glycoproteins and generate mature virions that are then trafficked within vesicles to the cell surface where they are released by exocytosis [10, 28]. A general overview of ZIKV replication is shown in **Figure 5**.

Mosquito and blood-borne transmission of ZIKV

In Africa, ZIKV is maintained in an enzootic cycle between forest-dwelling species of *Aedes* mosquitos and nonhuman primates, however in urban and suburban environments, ZIKV is mainly transmitted in a human-mosquito-human cycle [29]. The *Aedes* genus of mosquito is specifically known to transmit ZIKV, however only a subset of species are competent vectors, (*A. aegypti, A. albopictus, A. hensilli,* and *A. polynesiensis),* with *A. aegypti* being the principal vector spreading the current outbreak in Latin America and the Caribbean. This is most likely because *Ae. aegypti* lives in close association to human habitats, feeds primarily on humans, and often bites multiple humans in a single blood meal [29]. There is currently no evidence that animals other than humans and non-human primates serve as amplifying hosts for ZIKV, suggesting a mode of transmission similar to dengue, Yellow Fever (YF), and Chikungunya viruses (CHIKV), but not like WNV that uses birds as amplification host [24].

Although the dose of ZIKV delivered by mosquitos is not available, previous studies with WNV and DENV help to speculate how much is required to cause infection. For example, in a mouse model it was demonstrated that WNV infected mosquitos inoculate 10^4-10^6 PFU of virus extravascularly while probing [30]. Another study estimated that DENV transmitted by *Ae. aegypti* ranges from 10^4-10^5 PFU in a mosquito infectious dose [31]. Finally, experimental DENV challenges in human subjects have injected 10^3 of DENV-1 and 10^5 DENV-3 subcutaneously to cause viremia and disease symptoms in humans [32]. Applying this data to ZIKV, it appears that the minimum dose to cause infection is between 10^3-10^6 PFU, however the exact range remains to be determined.

While *A. aegypti* and *A. albopictus* mosquitos are the main vectors responsible for ZIKV outbreaks, other modes of transmission have been reported, for example through blood transfusion in Brazil. Also during the French Polynesian outbreak in 2013, it was discovered that 2.8% of blood donors tested positive for Zika virus [33]. Although information about the possible risk of ZIKV infection via blood products is limited, Lustig et al detected ZIKV RNA in frozen whole blood samples up to two months after collection [34]. Fortunately, due to revised guidelines by the FDA to screen all donated blood samples for ZIKV, the U.S. has not reported any blood-transfusion related infections [33].

Sexual transmission of ZIKV

During acute infection, it has been documented that ZIKV can disseminate to the reproductive organs and be transmitted person to person through sexual intercourse. The first case of sexual transmission was reported in the United States in 2008 after a 36-

year-old man, who had been preforming a mosquito sampling project in Senegal, returned home with ZIKV consistent symptoms. He and his wife reported having sexual intercourse in the days after he returned home and, within a week, his wife developed symptoms nearly identical to his own. Interestingly, the man also reported signs of hematospermia during this time-period. ZIKV was confirmed in both individuals by serologic testing. His wife had never traveled to Africa or Asia and had not left the US since 2007 [35]. The next affirmation came from a 44-year-old man in Tahiti who was infected with ZIKV during a large outbreak in 2013. This patient also noted hematospermia, which prompted blood, urine, and semen sample collection. Molecular diagnostics confirmed ZIKV RNA in the semen and urine at the level of 10^7 and 10^3 copies/ml respectively, two weeks after resolution of his symptoms, inoculation of the patient's seminal fluid in Vero cells, produced replicative ZIKV particles, yet no viral replication was noted when cuturing a urine specimen [36]. Although hematospermia has been described in these initial patients, it is not a common symptom in ZIKV-infected men suggesting that sexual transmission is not the result of hematospermia- induced blood-borne transmission. Additional case reports from a recently published review by Grischott et al are described in **Figure 6** [37].

Lead author	Location	Type of paper	Patients	Presence of ZIKV disease and sexual transmission	Key findings
Foy et al.	Colorado, USA	Case report	US scientist returning from Bandafassi, Senegal, and his wife	Onset of symptoms after sexual intercourse	Female sexual partner developed symptoms 10 days after return of husband. Symptoms included prostatitis, hematospermia and aphthous ulcers.
Hearn et al.	England	Case report	Male traveller returning from Cook Island	No sexual intercourse	28 after rash onset: ZIKV positive semen sample (PCR), while blood and urine were negative.
Musso et al.	Tahiti	Case report	44-year-old man	No sexual intercourse	High ZIKV RNA load in semen and urine samples, replicative ZIKV in semen but not in urine or blood. Symptoms included hematospermia.
McCarthy	Texas, USA	Case report	Sexual partner of a person returning from Venezuela	Not specified	Patient who had not recently travelled outside of the U.S. developed Zika illness symptoms after sexual contact with the traveller.
Venturi et al.	Florence, Italy	Case report	Sexual partner of traveller returning from Thailand	Not specified	9 days after onset of symptoms in male patient, partner developed Zika-like symptoms.
Atkinson et al.	United Kingdom	Case report	68-year-old traveller returning from Cook Island	No sexual intercourse	Serum and urine were ZIKV rRT-PCR negative at 27 and 62 days after onset of febrile illness, while semen was positive for ZIKV and with stronger signals. No culture of infectious virus from semen was attempted.
Hills et al.	USA	Case series	2 confirmed and 4 probable cases of ZIKV disease in female sexual partners	Yes, or symptoms had only just resolved	Women developed symptoms 10—14 days after sexual contact. Several women were pregnant.
Armstrong et al.	USA	Case series	5 US sexual partners of travellers to areas with active ZIKV transmission	Yes	5 laboratory-confirmed sexually transmitted ZIKV disease cases (2 cases were previously reported as probable cases in Hills et al.).
Ministerio de Salud	Chile	Case report	48-old sexual partner of traveller returning from Haiti	Yes	First sexually transmitted ZIKV disease in Chile.
Mansuy et al.	Toulouse, France	Case report	32-year old man returning from Brazil and French Guyana	No sexual intercourse	Viral load in semen 2 weeks after symptom onset was roughly 100 000 times that of his blood or urine. ZIKV in semen could replicate in African green monkey cells.

Figure 6: Presence of ZIKV in seminal fluid and sexually transmitted cases [37]

These case studies raise many interesting questions including the length of time that the virus can be detected in semen and in what fractions of seminal fluid can the virus be found. Currently, the longest documented persistence of ZIKV RNA in seminal fluid for 181 days was described in a man in his early forties [38]. The shedding was sustained, persistent, and ranged from 10^3 and 10^4 copies/mL. ZIKV RNA was also detected in his plasma, urine, and saliva up to 9, 15, and 47 days after symptom onset, respectively [38]. Furthermore, ZIKV was found associated with the cellular component of his semen and remained undetectable in seminal plasma. While this patient may represent an extreme case, a recent cohort study with data from 150 acutely-infected human participants demonstrated that the median time until loss of ZIKV RNA in the serum is around 14 days compared to 34 days in seminal fluid **(Figure 7)** [39]. The prolonged detection of ZIKV

RNA in human seminal fluid, long after resolution of the virus in serum, strongly suggests that ZIKV has the ability to establish persistent testicular infection.

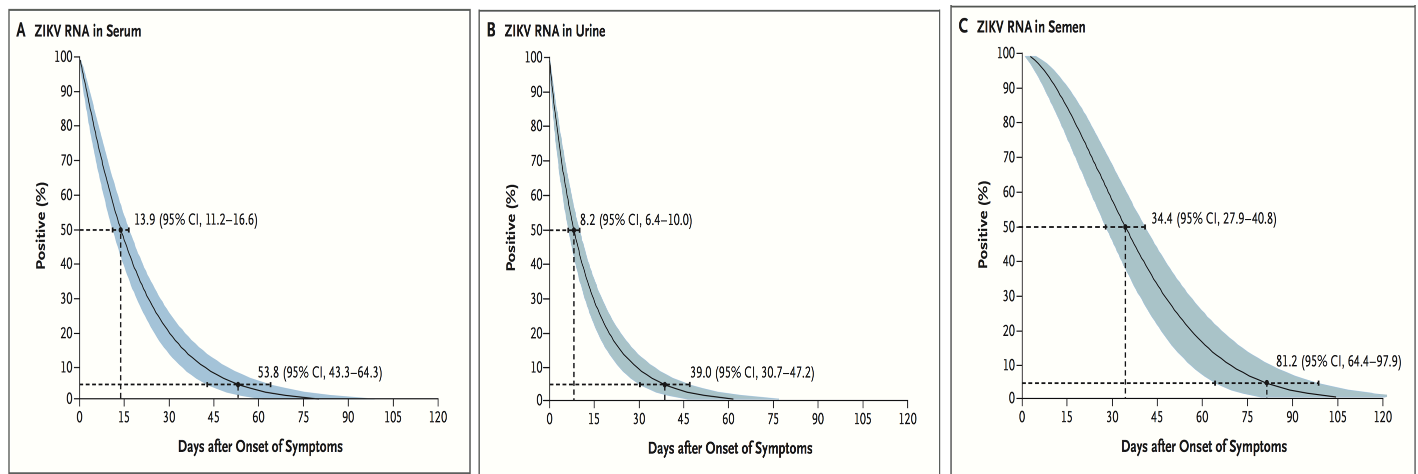

Figure 7: Time until clearance of ZIKV RNA in serum, urine, and semen [39]

Other questions that are important to the field are for how long can semen-positive males infect their partners? Do asymptomatic men also harbor virus in their seminal fluid? Can someone without symptoms sexually transmit the virus? In regard to the question of delayed sexual transmission, the longest documented case of sexual transmission is 44 days after onset [40]. Two reports also suggests that ZIKV can be present in the semen and can be sexually transmitted in asymptomatic males [41, 42], however larger cohort studies are needed for stronger evidence. Other unexpected findings in regards to ZIKV sexual transmission includes male-to-male transmission through anal sex [43], presence of ZIKV RNA in the genital tract of females [44], and even a report of ZIKV transmission from a female to a male [45]. Of note, these reports are isolated case studies and further validation of these findings are required with larger cohort studies.

Currently, there have been 46 confirmed cases of sexual transmission reported in the United States [17]. Additionally, 12 other countries have reported cases of sexual transmission including Argentina, Canada, Chile, Peru, France, Germany, Italy, Netherlands, Portugal, Spain, New Zealand, the United Kingdom [1]. Although there is no convincing data currently available to determine the contribution of sexual vs. mosquito borne transmission in ZIKV endemic areas, mathematical modeling has determined that sexual transmission increases the risk of infection and epidemic size, prolongs current outbreaks, and broadens the geographic potential of ZIKV infection to *Aedes* non-endemic regions [46]. Corroborating this model, recent data from Brazil demonstrates a higher incidence of ZIKV infection in women of reproductive age, suggesting the potential influence of sexual transmission [47].

Although, ZIKV is currently the only known arbovirus linked to sexual transmission in humans [47], other related viruses have been detected in the male reproductive tract and seminal fluids. For example, in a patient who suffered terminal WNV encephalitis, viral antigens were detected in the prostate and testes [48]. Although not phylogenetically related, Ebola virus RNA has also been detected in semen as long as 7 to 9 months after disease onset and sexually transmitted cases have been confirmed [49, 50].

Vertical Transmission of ZIKV

Another new route of ZIKV transmission, which has never been described in any other flavivirus before, is the vertical transmission from mother-to-fetus during pregnancy. Substantial clinical and epidemiologic evidence now indicates that ZIKV can be passed to the developing child via this route. For example, ZIKV has been detected in amniotic fluid of mothers whose fetuses had cerebral abnormalities [29]. Viral antigen and RNA has also been identified in the brain tissue and placentas of children who were born with microcephaly and died soon after birth as well as in tissues from miscarriages [29]. Additionally, there have been cases of possible ZIKV transmission through breast milk in mothers who developed ZIKV symptoms just before giving birth, which has also been previously suggested for dengue, West Nile, and yellow fever [37, 51].

The first case of congenital ZIKV infection in the United States was actually reported here on Oahu in December 2015 [52]. The mother was specifically a 32 year-old woman from Brazil who at 7 weeks gestation was clinically diagnosed with a ZIKV infection. At 39 weeks gestation, she delivered a male newborn that showed progessive neurologic deterioration in the first month of life [52]. Follow up studies examining archieved blood samples from mothers who gave birth to babies with microcephly in Hawaii between 2009 and 2012 further determined that ZIKV positive cases and associated microcephly occurred in the United States as early as 2009 [53]. Selected studies with confirmed ZIKV infection and fetal outcomes from a recent review by Grischott et al. are described in **Figure 8**.

Lead author	Location, country	Type of paper	Patients	Symptoms of ZIKV in mother during pregnancy (gestational weeks)	Key findings
Intrauterine transmission					
Sarno et al	Salvador, Brazil	Case report	32-week-old fetus	No	Microcephaly, hydranencephaly, intracranial calcifications, hydrops fetalis, arthrogryposis, fetal demise. ZIKV RNA in ZNS and amniotic fluid.
Calvet et al.	Paraíba, Brazil	Case series	2 28-week-old fetuses	18th and 10th week, respectively	Fetus 1: microcephaly with calcifications. Fetus 2: microcephaly, ventriculomegaly, microphthalmia, cataract, arthrogryposis. ZIKV RNA in amniotic fluid.
Mlakar et al.	Ljubljana, Slovenia	Case report	32-week-old fetus	13th week, while mother lived in Natal, Rio Grande do Norte, Brazil	Microcephaly, agyria, hydrocephalus, brain calcifications, cortical displacement, focal inflammation. Placenta: calcifications, low placental-fetal weight ratio. ZIKV RNA in brain.
Martines et al.	Rio Grande do Norte, Brazil	Case series	2 newborns (36 and 38 weeks); 2 miscarriages (11 and 13 weeks)	In all 4 mothers, in first trimester	Microcephalic newborns who died within 20 h of birth: ZIKV RNA in brain with calcification, microglial nodules, gliosis, cell degeneration, necrosis. Viral antigen in mononuclear cells and chorionic villi. Placenta: heterogeneous chorionic villi with calcification, fibrosis, fibrin deposition, intervillositis, villitis.
Oliveira et al.	Paraíba, Brazil	Case series	2 fetuses (30.1 and 29.2 weeks); 6 children	In all of the mothers	Microcephalic fetus 1: brain atrophy with calcifications, corpus callosal and vermian dysgenesis, enlarged cisterna magna. Microcephalic fetus 2: asymmetric hemispheres with ventriculomegaly, no visible corpus callosum and thalami, mass at the position of the basal ganglia, calcifications and cataracts in eyes. 6 children: 2 cases with cerebellar involvement and 3 with brain calcifications, 1 with arthrogryposis.
Faria et al.	Tejuçuoca, Ceará, Brazil; Sumaré, São Paulo, Brazil	Case series	Newborn; 52-year-old blood donor	Unknown	Microcephalic newborn who died after birth: microcephaly, arthrogryposis: ZIKV isolation from brain. Blood donor: RT-PCR positive blood.
Meaney-Delman et al.	USA	Case series	9 fetuses with microcephaly	All 9 mothers (6 in first trimester), all travellers from countries with ZIKV transmission	ZIKV infection in 1st trimester: 2 pregnancy losses, 2 elective terminations because of severe brain malformations, 1 live-born infant with microcephaly, brain calcifications, pale optic nerve, chorioretinitis), 1 pregnancy continuing. Infection in 2nd trimester: 1 healthy infant, 1 pregnancy continuing. Infection in 3rd trimester: 1 healthy infant.
Butler	Colombia	Case series	3 newborns	Unknown	Colombia's first case of a newborn with microcephaly, 2 others with congenital brain abnormalities.
Villamil-Gómez et al.	Sincelejo, Sucre, Colombia	Cohort study	28 pregnant women	In 21 (75%) women	Preliminary analysis of ongoing study: 2 cases with brain calcifications (1 of them with positive IgG serology for toxoplasmosis).
Brasil et al.	Rio de Janeiro, Brazil	Case series	72 pregnant women	In all	Deaths at 36 and 38 weeks of gestation (2 fetuses), in-utero growth restriction (5 fetuses), ventricular calcifications or other CNS lesions (7 fetuses), abnormal amniotic fluid volume, cerebral or umbilical artery flow (7 fetuses).
Driggers et al.	Washington D.C., USA	Case report	21-week old fetus	11th week, after returning from Mexico/ Guatemala/Belize	Brain abnormalities without microcephaly or calcifications. High viral loads in brain, membranes, umbilical cord and placenta, lower amounts in muscle, liver, lung, spleen, amniotic fluid. Replicative ZIKV from fetal brain. ZIKV RNA in serum of mother 4 and 10 weeks after clinical onset, not after delivery.
Youannic et al.	French Polynesia	Case series	4 fetuses	In 3 of 4 cases, in first trimenon	Ventricular dilatation, absent corpus callosum, abnormal gyration, cerebral calcifications, vermian hypoplasia, intrauterine growth restriction. ZIKV positive amniotic fluid.
Intrapartum/perinatal transmission					
Besnard et al.	French Polynesia	Case series	2 newborns at 38 weeks' gestation	Mother 1: 2 days prior delivery; mother 2: 3 days post delivery	Infant 1: healthy. Infant 2: hypotrophy, hypoglycaemia (mother with gestational diabetes), rash on day 4.

Figure 8: Confirmed mother-to-child ZIKV transmission [37]

In 2016, the WHO reported that of 6,480 suspected cases of ZIKV-associated microcephaly in Brazil, 2,212 were investigated, and of which 39% (863) infants were confirmed to have microcephaly [54]. Furthermore, applying Shepards's and Bradford Hill criteria to this phenomenon, vertical transmission of ZIKV satisfies the epidemiologic requirements to be identified as a teratogen [55]. Although many aspects of ZIKV transmission need further clarification, **Figure 9** depicts a general overview.

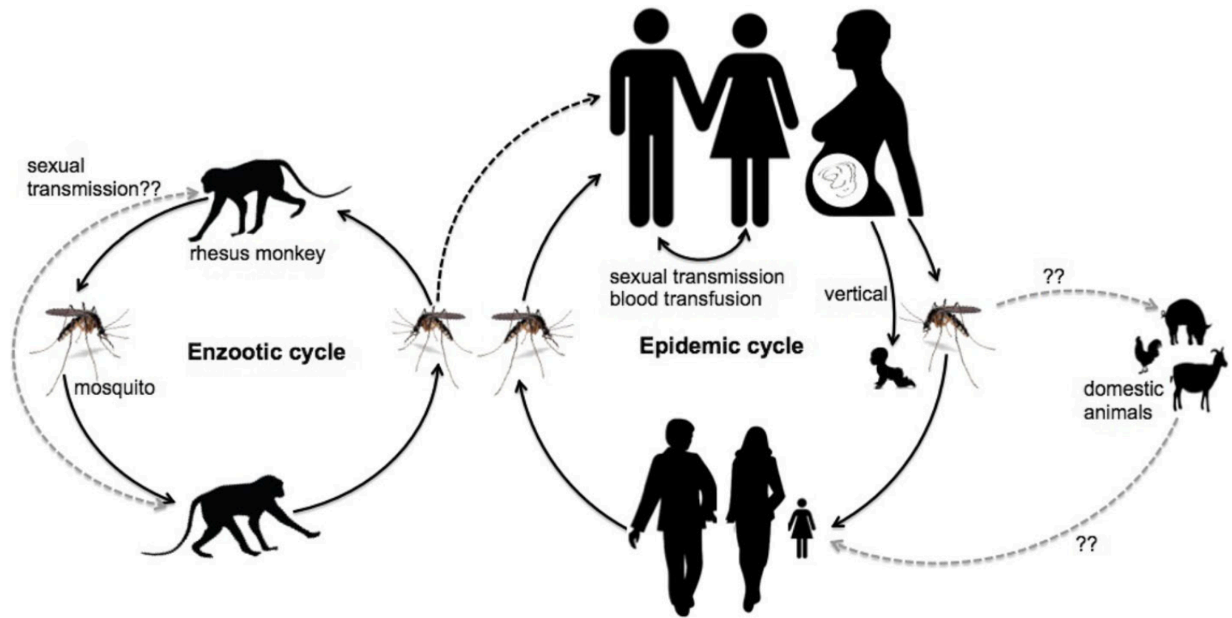

Figure 9: Overview of ZIKV transmission cycles [56]

Clinical symptoms of ZIKV disease

It is estimated that only 20% of patients infected with ZIKV will become symptomatic with clinical manifestations [57]. In most adult cases, symptoms only last a few days to a week and include a low-grade fever, maculopapular pruritic rash, arthralgia, or non-purulent conjunctivitis. Other symptoms include headache, edema, myalgia gastrointestinal disorders, and lymphadenopathy [57]. Ninety-five percent of patients who do develop symptoms will do so by day 11 post infection [58]. Furthermore, the median time of viral persistence in serum is 10-14 days after infection, however this range can extend weeks further [39, 58].

Laboratory tests are generally in the normal range, including blood cell and platelet counts as well as liver and kidney function tests [59]. However, leukopenia, mild thrombocytopenia, and increased transaminases have been described in some cases

[59]. There have also been some reports of mild hemorrhagic symptoms such as petechiae, minor mucosal bleeding, and hematospermia [59]. Generally, ZIKV is less neuroinvasive in adults than encephalitic flaviviruses such as WNV and Tick-borne-encephalitis virus (TBEV) and fatality is rare excluding fetal losses among women infected during pregnancy and newborns with severe congenital ZIKV disease [60].

Complications of ZIKV infection

More serious neurological effects associated with ZIKV infection in adults include Guillain-Barré syndrome. GBS is a rare condition in which a person’s immune system attacks peripheral nerves that control muscle movement as well as transmit pain, temperature, and touch sensations. This can result in muscle weakness and loss of sensation in the legs and/or arms [61]. Symptoms typically last a few weeks and most individuals recover without long term neurological complications, however 3-5% of GBS patients are known to die from complications, which can include paralysis of the muscles that control breathing, lung clots, or cardiac arrest [61]. Specifically, in the French Polynesian outbreak, 38 cases of GBS occurred among an estimated 28,000 persons who sought medical care [29]. Since then, 22 other countries have reported ZIKV associated GBS cases [1].

Vertical transmission of ZIKV is associated with several sequelae including congenital microcephaly, other neurologic disorders, and fetal loss. Microcephaly is defined as an occipitofrontal head circumference (OFC) below the third centile or more than two standard deviations (SD) below the mean for sex, age, and ethnicity [62]. This abnormality is associated with a reduction in brain volume and often intellectual and/or motor

disabilities [62]. The phenotype of microcephaly is variable and the spectrum of disorders is large. Microcephaly may be evident at birth (primary) or postnatally (secondary) when a child with a normal OFC drops to a value more than two SDs below the mean. Severe microcephaly is described as a child with an OFC below three SDs of the mean as shown in **Figure 10** [62]. Typical findings of microcephaly via neuroimaging consist of calcifications, cortical disorders, and ventriculomegaly [63]. Other clinical manifestations include craniofacial disproportion, spasticity, seizures, and irritability. Brainstem dysfunctions, feeding difficulties, and ocular abnormalities have also been described [63].

Microcephaly, however, is not a common condition. In the United States, birth defect tracking systems have estimated that microcephaly cases before ZIKV introduction ranged from 2-12 babies per 10,000 live births (0.02-0.12%) [64]. Furthermore, based on Hawaii Birth Defects Surveillance Report (1986–2005), there was a declining trend of incidence of microcephaly in Hawaii, with a rate of 13.6 per 10,000 total births in 1986 to 4.8 per 10,000 total births in 2005. Over the period (1986–2005), a total of 370 cases of microcephaly were reported in Hawaii, which is equivalent to 9.4 per 10,000 total births [53]. However, based on the University of Hawaii Biorepository data, over the period of 2007–2013, microcephaly rate was 14.7 per 10,000 total births. This increase in microcephaly rate coincides with ZIKV outbreaks in the Pacific starting in 2007 [53]

Additional reports from the 2013-2014 French Polynesia outbreak suggest that the risk of microcephaly due to ZIKV infection in the first trimester of pregnancy was 0.95% [65] and modeling from the 2015-2016 outbreak in Brazil estimated the risk of microcephaly to be between 0.88-13% in the first trimester [66]. In the United States, a recent study showed

that in pregnancies with evidence of ZIKV infection, birth defects were reported in 5% of completed pregnancies [66]. This number is alarmingly higher than the risk of microcephaly pre-ZIKV era. Further, recent studies also suggest that the risk of microcephaly is highest if ZIKV exposure is in the first trimester of pregnancy [67].

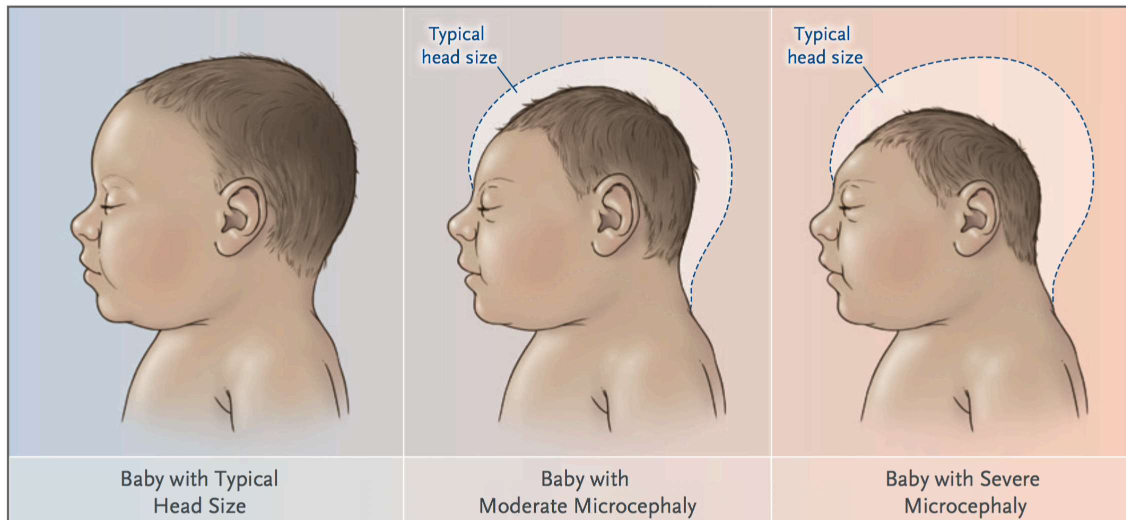

Figure 10: Infants with microcephaly as compared to a typical newborn [29]

Pathogenesis of ZIKV

ZIKV is primarily transmitted by the *Aedes* mosquito, which deposits the virus in the human epidermis and dermis while taking a blood meal. Once in the skin, the virus is thought to infect skin fibroblasts, epidermal keratinocytes, and dendritic cells (DC) based on in vitro studies [27]. Although it is yet to be shown with ZIKV, in vivo experiments using both WNV and DENV in mice have also demonstrated that skin Langerhans cells, macrophages, and infiltrating monocytes are also capable of sustaining productive infection of flaviviruses [68, 69]. Following peripheral inoculation, these skin antigen presenting cells (APC) subsequently migrate to the nearest draining lymph nodes, from

where the virus enters the bloodstream and establishes further sites of infection [68]. Human in vitro studies have also demonstrated that aortic, coronary artery, saphenous vein, and lymphatic endothelial cells are susceptible to infection, which could conceivably also facilitate hematogenous dissemination of the virus resulting in primary viremia [70].

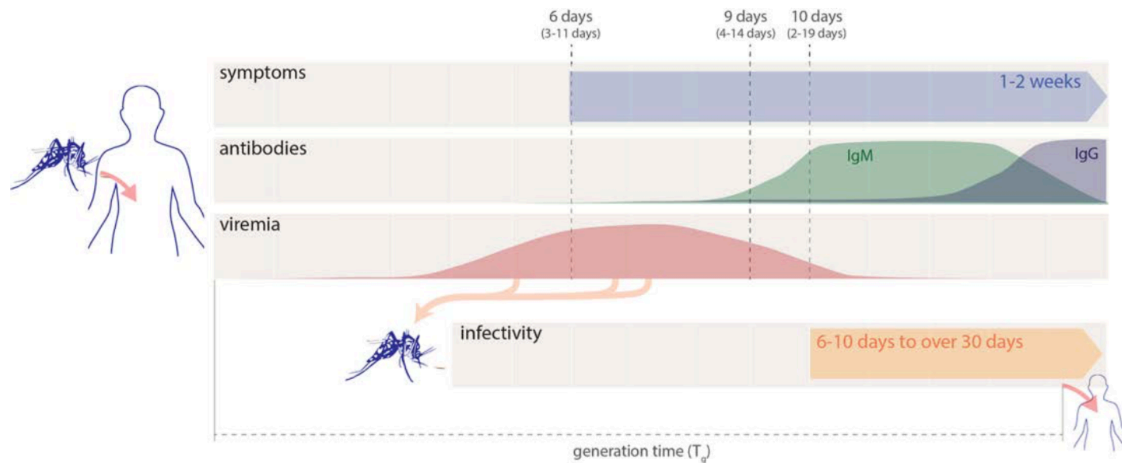

Figure 11: Schematic of the course of human and mosquito infection [14]

As shown in **Figure 11,** the estimated median incubation period of ZIKV infection in humans is six days and ZIKV RNA is usually detectable in blood within the first 10 days after infection [59] (Fig. 6). In the blood, peak viral load coincides with onset of symptoms (i.e. 5-7 days post infection) with titers typically ranging from 10^3-10^5 copies/ml, however high levels 10^7-10^9 copies/ml and prolonged viremia has been documented in some cases [59]. Non-human primate (NHP) studies have further validated the presence of viral RNA in peripheral tissue such as the spleen, kidney, bladder, joints, and peripheral nervous tissue [5, 71]. Interestingly, viral RNA was also found to be associated with the spinal cord, cerebrospinal fluid (CSF), and cerebellum [5, 71]. In the brain, ZIKV specifically infects neuronal cell types including neural progenitor cells, mature neurons, and

astrocytes [60]. Although there have been rare cases of ZIKV-associated meningoencephalitis in humans [72] healthy adults rarely experience such sequelae and most neurologic complications are associated with birth defects in infants such as microcephaly.

Several human studies now provide strong evidence of ZIKV RNA in both maternal and fetal tissues, including cord blood, several placental cell types (Hofbauer cells, trophoblasts, and endothelial cells), amniotic fluid, and the developing fetal brain [60]. ZIKV RNA has also been detected in the brain and placenta of spontaneously aborted human fetuses in the first and second trimesters [60]. Several studies now collectively suggest that ZIKV may have unique mechanisms to cross the placental barrier and cause fetal damage [59].

Ocular abnormalities related to ZIKV have also been reported in infants, which closely resembles those caused by other RNA viruses such as CHIKV, DENV, and WNV [73]. Some of the ocular findings include macular problems such as pigment mottling and chorioretinal atrophy, in addition to optic nerve disorders for example hypoplasia [57]. Conjunctivitis is also a common symptom in infected adults [24]. These clinical manifestations are further corroborated with the discovery that ZIKV RNA and infectious virus can be recovered from human conjunctival fluids and tears [60]. Mouse studies have specifically demonstrated that the cornea, optic nerve, and neurosensory retina are susceptible to infection [60]. Furthermore, it has been proposed that ZIKV directly infects cells lining the blood-retinal-barrier (BRB), may establish a reservoir in the eye, and facilitate viral dissemination to other organs including the brain [73].

The female reproductive tract is also susceptible to ZIKV. In humans, viral RNA has been detected in urine and vaginal secretions [44] and in NHPs ZIKV has been found in the uterus and ovaries [5]. In vitro studies have further demonstrated that the vaginal epithelium and human uterine fibroblasts are susceptible to both African and Asian strains of ZIKV [60]. These in vitro and in vivo findings confirm the potential for sexual transmission and that uterine infection may contribute to impaired fetal development [60].

Overall, there are two unique findings associated with ZIKV replication kinetics. First, ZIKV exhibits a broad tissue tropism and has the ability to access immune privileged sites, such as the placenta, brain, eye, and testes perhaps more readily than other flaviviruses, which may help to explain virus-induced congenital defects and spread through sex **(Figure 12)** [57]. Secondly, it has been noted that ZIKV preferentially infects and damages neural progenitor cells, which may explain its ability to impair development of the fetal brain, cause microcephaly, and other neurodevelopmental injuries [60].

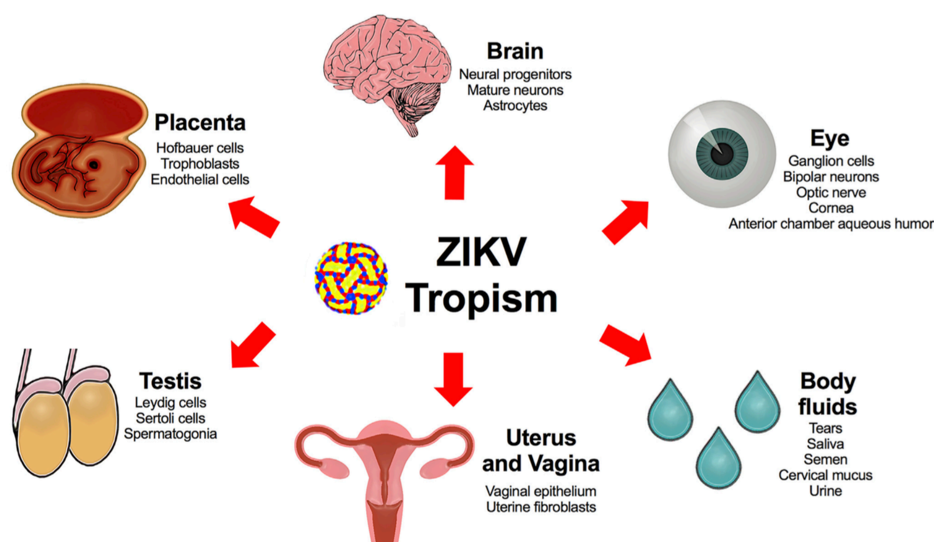

Figure 12: ZIKV tissue and cell tropism [60]

Diagnosis, treatment, and prevention of ZIKV

Laboratory diagnosis of ZIKV infection in humans is typically performed by detection of viral nucleic acid in the serum using qRT-PCR [29]. CDC currently recommends that for all symptomatic patients serum and urine samples should be obtained and tested via qRT-PCR [74]. However, as viremia is transient in most affected individuals, diagnosis by qRT-PCR is most successful only within one week after the onset of clinical illness [29]. As shown in **Figure 12,** while a positive result via qRT-PCR confirms ZIKV infection, a negative result does not exclude the possibility and requires other diagnostic methods to rule out infection [59]. Other patient specimens such as saliva and urine have also been studied for potential diagnostic value. While urine demonstrates the consistent presence of viral RNA for up to two weeks, ZIKV is infrequently detected in saliva [39].

Another well-established diagnostic method is the detection of IgM antibodies in serum or CSF by IgM-capture enzyme-linked immunosorbent assay (MAC-ELISA) [29]. Generally, IgM appears as viremia declines within the first week after symptom onset and persists for several months after infection [29]. Diagnosis by serology is the gold standard, however sometimes the results are complicated due to potential cross-reactivity to other flaviviruses. For example, a recent ZIKV infection may also evoke a positive MAC-ELISA result for DENV. Therefore, as shown in **Figure 13,** the plaque reduction neutralization test (PRNT) is used to differentiate between the two and verify MAC-ELISA results [29]. In general, a PRNT $\geq$10 for ZIKV and $\leq$10 for DENV indicates a recent ZIKV infection [75]. In ZIKV endemic areas, asymptomatic pregnant women should undergo IgM testing as part of routine obstetric care in the 1st and 2nd trimester [74].

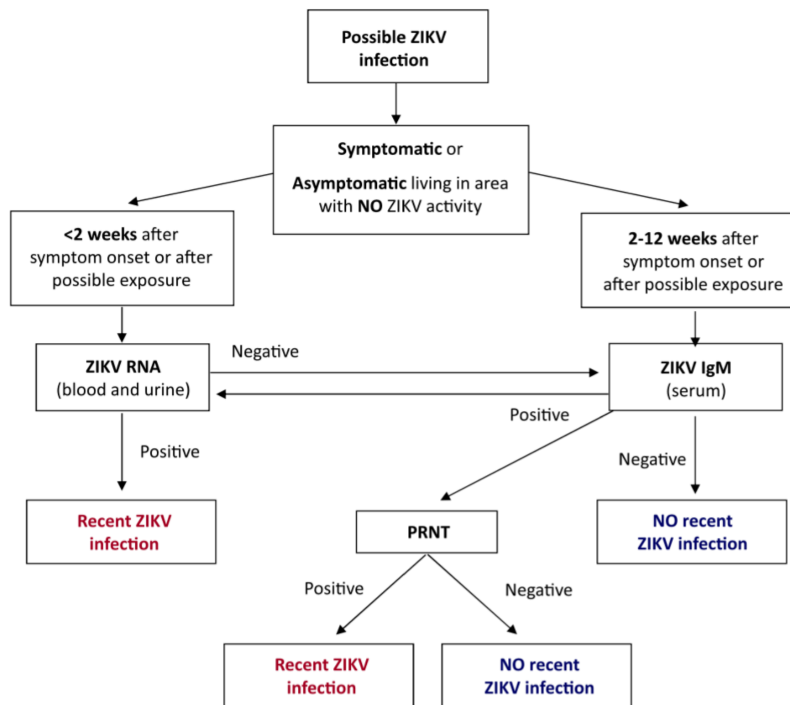

Figure 13: Algorithm for the laboratory diagnosis of Zika virus infection [59]

As with other mosquito-borne flaviviruses treatment for uncomplicated ZIKV infection is largely focused on symptoms as there is no specific antiviral available for use in humans [29]. Strategies of care include the use of analgesics such as acetaminophen (Tylenol) to reduce fever and combat pain, antiemetics, and rehydration for nausea and vomiting [76]. It is also suggested for patients to not take aspirin and other non-steroidal anti-inflammatory drugs (NSAIDS) until dengue can be ruled out to reduce the risk of bleeding [76]. It is also recommended that patients get plenty of rest. For patients experiencing GBS, patients should be hospitalized and closely monitored. Additional treatments such as plasma exchange to remove antibodies can help improve symptoms and shorten the duration of GBS [61].

There is no vaccine currently available for ZIKV, therefore prevention and control measures are centered on avoiding mosquito bites, reducing sexual transmission, and controlling the mosquito vector [29]. Current CDC recommendations suggest that women who have been exposed to ZIKV wait at least 8 weeks from symptom onset before attempting conception. Men with possible ZIKV exposure should use condoms or abstain from sex for at least 6 months [77]. Health care providers should counsel couples that correct and consistent use of condoms reduces the risk for sexually transmitted diseases and discuss the use of the most effective contraceptive methods that can be used correctly and consistently [42]. Other preventative measures include using mosquito repellent and permethrin treatment for clothing, bed nets, window screens, and air conditioning. However, the most effective *A. aegypti* vector control relies on elimination of mosquito breeding sites, in addition to application of larvicides, and insecticides to kill adult mosquitoes [29].

Vaccine and antiviral Development

Development of an effective vaccine to prevent ZIKV infection in high-risk populations is an area of intense investigation. Several companies, research organizations, and academic institutions, as listed in **Figure 14**, are actively working on ZIKV vaccine development using different methods [78]. While the platforms employed by these developers vary, majority of the candidates are based on strategies that have been developed for other related flaviviruses and include use of live attenuated virus, whole inactivated virus, and subunit recombinant proteins.

Type	Candidate	Status
Inactivated	PaxVax, California	Preclinical
	NewLink Genetics, Massachusetts	Preclinical
	GSK, United States/Belgium	Preclinical
	Bharat Biotech, India	Preclinical
	WRAIR/Sanofi Pasteur, United States and France	Phase 1: 2016–2017
Subunit/peptide	Protein Sciences, Connecticut	Preclinical
	Hawaii Biotech, Hawaii	Preclinical
	Bharat Biotech, India	Preclinical
	Replikins, Massachusetts	Preclinical
Live	NIAID-LID/Instituto Butantan, United States/Brazil	Phase 1: Q4 2016
	UTMB/Instituto Evandro Chagas, United States/Brazil	Preclinical
	Sanofi Pasteur, France	Preclinical
Vectored	Jenner Institute (chimpanzee adenovirus), UK	Preclinical
	Harvard University (VSV), Massachusetts	Preclinical
	Themis Bioscience (measles), Austria	Preclinical
DNA/RNA	NIAID-VRC (Biojector needle-free), United States	Phase 1: Q3 2016
	Inovio Pharmaceuticals (electroporation), Pennsylvania	Phase 1: Q3 2016
	GSK (RNA), United States/Belgium	Preclinical

Abbreviation: VRC, Vaccine Research Center; VSV, vesicular stomatitis virus.

Figure 14: Proposed ZIKV vaccine candidates/platforms [78]

One example includes the National Institutes of Health (NIH) live-attenuated chimeric ZIKV vaccine, which is based on the components of their live-attenuated tetravalent dengue vaccine. Specifically, this vaccine candidate is comprised of the prM and E proteins of ZIKV combined with the nonstructural proteins of DENV-2 and is expected to begin Phase 1 clinical trials soon [78]. Should the vaccine prove to be immunogenic in Phase 1 clinical evaluation, NIH hopes to combine this ZIKV vaccine with their live attenuated tetravalent dengue vaccine to create a pentavalent vaccine that would be administered to children in endemic areas [78].

Another approach is a purified inactivated Zika vaccine under development by the Walter Reed Army Institute of Research (WRAIR) called ZPIV. This vaccine is based on a similar

approach in which WRAIR used to develop a vaccine against JEV and has entered phase 1 clinical trials [79]. This type of vaccine generally requires multiple doses and very high virus titers/quantity of protein to induce a protective immune response.

Newer strategies such as DNA and subunit vaccines are also under development [78]. For example, Inovio Pharmaceuticals is developing a DNA vaccine (GLS-5700), which has received approval from the FDA to begin Phase 1 clinical trials [78]. This vaccine specifically contains a single plasmid with DNA encoding for the ZIKV pre-membrane (prM) and envelope (E) proteins. NIAID has developed a similar DNA vaccine (entitled VRC 705), which just entered a Phase 2 clinical trial in seven countries [79]. Subunit vaccines strategies include expression of various portions of ZIKV E protein in both bacterial and insect cell lines. The benefit of subunit vaccines is the ability to produce robust immunity to specific antigenic epitopes [80]. Although these vaccine candidates are still in pre-clinical stages, they have demonstrated robust protection in mouse and NHP models.

There are also several mRNA vaccines under development at GlaxoSmithKline (GSK), University of Pennsylvania, and Moderna/Valera. These vaccines utilize lipid-nanoparticles to deliver nucleoside-modified mRNA encoding ZIKV prM and E proteins. The Moderna/Valera candidate is currently being evaluated in a Phase 1 trial [79].

In terms of antivirals, there is currently no clinically approved therapy for ZIKV available. However, two strategies are currently being pursued for antiviral development. These

include repurposing existing clinical compounds that have previously been developed for unrelated diseases, in addition to discovering new bonafide inhibitors of ZIKV replication. In particular, nucleoside/nucleotide inhibitors have shown efficacy against flaviviruses [81]. One drug in particular, NITD008, has been demonstrated to be effective against suppressing peak viremia, reducing cytokine elevation, and preventing death in vivo against DENV, WNV, and ZIKV [82-84]. Furthermore, NITD008 has good in vivo pharmacokinetic properties and is biologically available through oral administration [82]. While there are no current clinical studies ongoing with anti-flaviviral drugs, nucleoside/nucleotide inhibitors may prove to be beneficial and warrants further study.

Host response to ZIKV

Several recent studies have used in vitro and animal models to delineate specific host responses to ZIKV and its comparison to other flaviviruses. Upon introduction of ZIKV via an infected mosquito, skin fibroblasts, epidermal keratinocytes, and dendritic cells are some of the first cell types to encounter the virus. One of the characteristic features of early host response to ZIKV is the induction of robust innate immune pathways such as production of antiviral type I IFN and inflammatory cytokines. In primary human skin fibroblasts, ZIKV has been shown to induce the expression of pattern recognition receptors (PRRs) such as TLR3, RIG-I, and MDA5 that are involved in the recognition of ZIKV dsRNA intermediates. Recognition of ZIKV dsRNA by these PRRs activates downstream signaling, which results in the production of IFN-α, IFN-β, and transcription of several interferon stimulated genes (ISGs), including OAS2, ISG15, and MX1 [27]. Additionally, ZIKV-induces pro-inflammatory cytokines (IL-1 β and IL-6) and chemokines

(CCL5, MCP-1, and CXCL10) in many cell types [27, 85]. In vitro, several cell types are shown to elicit antiviral responses such as human fibroblasts, monocytes, dendritic cells, human placental macrophages, skin epithelial cells, retinal cells, and neural progenitor cells [27, 73, 85-88]. However, ZIKV-induced downstream effects vary in different cell types. On one hand it causes apoptotic cell death in neurons, neuronal progenitor cells and retinal epithelial cells, but on the other hand, specific endothelial cells are resistant to virus-associated cell death. Studies are still ongoing to determine the precise role of ZIKV-induced innate immunity in host protection vs. virus pathogenesis.

The role of type I IFN and ISGs is to control virus replication and spread to neighboring cells, and like other flaviviruses, ZIKV is sensitive to the antiviral effects of both type I and type II Interferons [27]. Mouse models have further confirmed this finding, as Ifnar1$^{-/-}$ mice and Irf3$^{-/-}$/Irf5$^{-/-}$/Irf7$^{-/-}$ triple knockout mice develop high viremia, neurological disease, and succumb to ZIKV infection, whereas wild-type (WT) mice do not [89]. IFN transmembrane proteins (IFITMs) specifically IFITM1 and IFITM3 have also been shown to inhibit ZIKV infection [59]. Although the exact mechanism of IFTM-mediated restriction is unknown, experimental approaches suggest that IFITMs can halt ZIKV early in the viral replication cycle and prevent cell death [90].

Lack of a reliable mouse model has hampered progress in our understanding of host response to ZIKV. Although ZIKV infection does cause disease in an age-dependent manner in WT C57BL/6 mice, the resulting viremia is short-lived from about 1-3 days [89]. Since these models have significant limitations in terms of biological relevance, guinea

pig and NHP models have been utilized to provide critical information about tissue tropism and host response to the virus [91, 92]. In rhesus macaques, ZIKV infection causes an increase in CD169+ monocytes, DCs, and CD16+ natural killer cells in the plasma during early infection, which is similar to the monocytosis observed early during DENV and WNV infection in humans [71, 93]. ZIKV infection also induced an upregulation of IL-1RA, MCP-1, CXCL-10, and CXCL-11 cytokines and chemokines as compared to the controls in the plasma of NHPs [71].

Other innate immune responses to ZIKV include inflammasome and complement activation. Specifically, in glial cells, ZIKV activates the NLRP3 inflammasome and subsequently releases mature Il- 1β after infection [94]. Previous studies have shown that complement demonstrates protective effects against flaviviruses through direct inactivation of virions, activating monocytes and other granulocytes by C3a/C5a, opsonization of viral particles by C3b, and lysis of enveloped viral particles and infected cells by the membrane attack complexes (MACs) [95]. This pathway for ZIKV however is yet to be studied.

Clearance of ZIKV is also mediated by both humoral and cellular immune responses. In both mouse and NHP models, proliferating CD4+ and CD8+ T-cells can be detected within the first week of infection [71, 96]. Additionally, the number of circulating plasmablasts also expands during this time. In NHPs, ZIKV IgM becomes detectable between 7-10 dpi with IgG following between days 8-21 dpi. These neutralizing antibodies are directed towards the ZIKV E protein, which is consistent with antibody responses

against other flaviviruses [71]. Interestingly, prior infection with ZIKV from an Asian-lineage seems to protect against heterologous infection with an African strain demonstrating similar cross-neutralizing epitopes between the two strains [60].

This antibody response, however, may be complicated in patients who live in both ZIKV and DENV endemic areas. For example, due to the high degree of structural and sequence similarity between ZIKV and DENV, antibodies produced against these two flavivurses can interact with each other. This event is known as antibody-dependent enhancement (ADE) and is believed to promote infection of myeloid cells, leading to increased disease severity [60]. Cross-reactive anti-DENV antibodies can enhance ZIKV infection in cell culture and reciprocally, cross-reactive human anti-ZIKV antibodies can promote DENV infection in vitro and in mice [97, 98]. It however remains unknown whether ADE of ZIKV by anti-DENV antibodies occurs in humans and further studies are warranted to improve our understanding of cross-reactivity between ZIKV and DENV antibodies.

Immune evasion of ZIKV

Flaviviruses, including DENV, have evolved several mechanisms to counteract the host antiviral response. In ZIKV, the studies are limited, however they show that ZIKV NS5 has the ability to modulate the type I interferon signaling pathway through antagonism of STAT1 and STAT2 phosphorylation [85]. Additionally, ZIKV NS1 and NS4B have been shown to interact with TBK1 to inhibit production of IFN and NS2B-NS3 can inhibit the JAK-STAT pathway by degradation of Jak1 [99]. NS2B3 is also reported to attenuate RIG

like receptor (RLR)-induced apoptotic death [99]. Recently, it has been shown that ZIKV-infected human DCs, could not induce expression of CD80/86, which was coupled with decreased production of inflammatory cytokines/chemokines suggesting that ZIKV can block the innate-adaptive interface [85]. Other immunomodulatory capabilities of ZIKV included the downregulation of NF-kB activation through TLR3 and IL-1 receptor pathways [71] and inhibition of complement mediated MAC formation on cell membranes through ZIKV NS1 protein [100]. Finally, also like DENV, ZIKV is known to hijack autophagy to support viral replication, which has been demonstrated in human skin fibroblasts and fetal neural stem cells [27, 101].

Overview of the male reproductive tract

Since ZIKV is associated with sexual transmission and persistence in seminal fluid, understanding the normal functioning of the male reproductive tract is important for this study. As shown in **Figure 15**, the human male reproductive tract is composed of several different organs that interact to produce sperm cells and transfer them to the female reproductive tract. The paired testes are a crucial component to this process as they produce both sperm and androgens, such as testosterone. In close association with the testes is the epididymis, which is where sperm cells mature, develop the motility, and are stored until ejaculation. To be released, sperm must next pass through a long tube from the testes to the prostate gland called the Vas Deferens. Although the evidence of ZIKV infection in the male human reproductive tract is limited, ZIKV RNA has been detected in the testes, prostate, and seminal vesicles of NHPs [5].

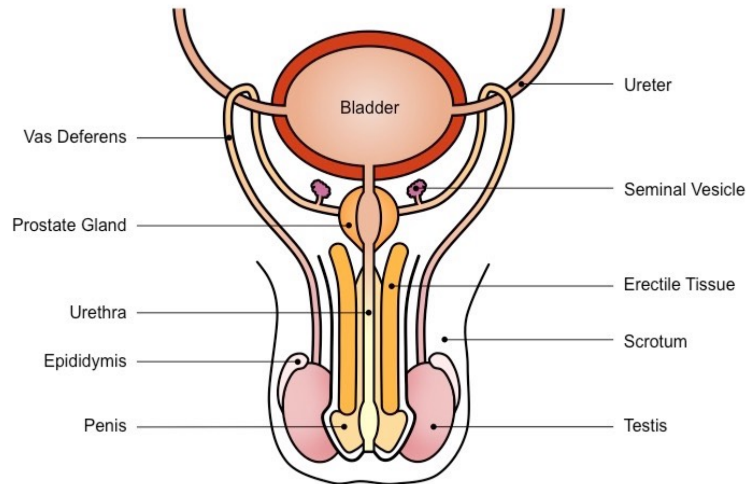

Figure 15: Anatomy of the male reproductive system [102]

Mammalian testicular anatomy and spermatogenesis

Based on the epidemiologic evidence that ZIKV RNA persists in seminal fluid even after the virus has been cleared from the blood suggests that it is able to establish a persistent infection in the male reproductive tract. As the testes are the site of spermatogenesis and are sheltered from peripheral immune responses, they may represent a likely reservoir. As shown in **Figures 16** and **17**, the mammalian testes are specifically divided into two compartments, the peritubular compartment (aka interstitial space) and the seminiferous tubules. The peritubular compartment specifically consists of blood vessels, testosterone producing Leydig cells, and testicular macrophages, while seminiferous tubules contains Sertoli cells (SC) and is the site of spermatogenesis [103]. The process of spermatogenesis is further depicted in **Figure 16.**

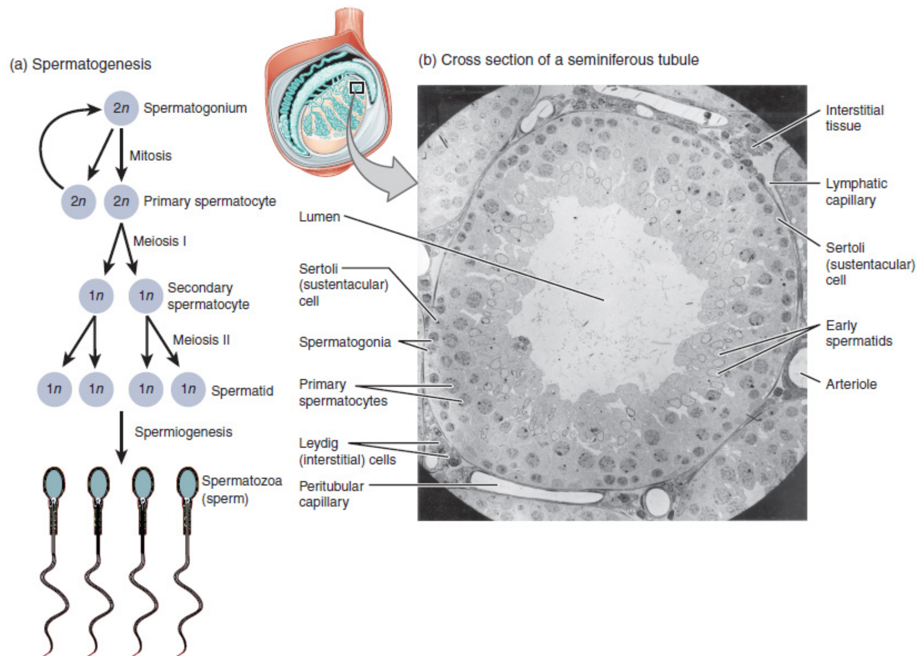

Figure 16: Overview of spermatogenesis [102]

Spermatogenesis begins with the mitosis of the diploid spermatogonia, resulting in two identical diploid cells. While one of these cells remains a spermatogonium, the other becomes a primary spermatocyte (PSc), which must undergo meiosis to produce haploid secondary spermatocytes (SSc). Another round of meiotic division in SSc results in a total of four cells with half the number of chromosomes called spermatids. Finally, a process called spermiogenesis then reduces the cytoplasm and begins the development of structures found in formed sperm also known as spermatozoa. One production cycle from spermatogonia through mature sperm, takes approximately 64 days [102].

As shown in **Figure 17**, spermatogenesis occurs within seminiferous tubules, which are separated from the peritubular compartment by *the blood-testes barrier, also known as the Sertoli-cell barrier (SCB)* [103]. The SCB mainly functions to protect developing germ

cells from systemic attack by adaptive immune cells and cytotoxic molecules, while simultaneously providing nutritional and structural support [104]. It represents one of the tightest blood-tissue barriers in the human body and is constituted by tight junction proteins (TJP) (eg. ZO-1, occludin, claudins) and adherens junction proteins (β-catenin and JAM- A) between connecting SC as shown in **Figure 17** [103]. These junctions are unique compared to other epithelial tight junctions in that they are located basally rather than apically [104].

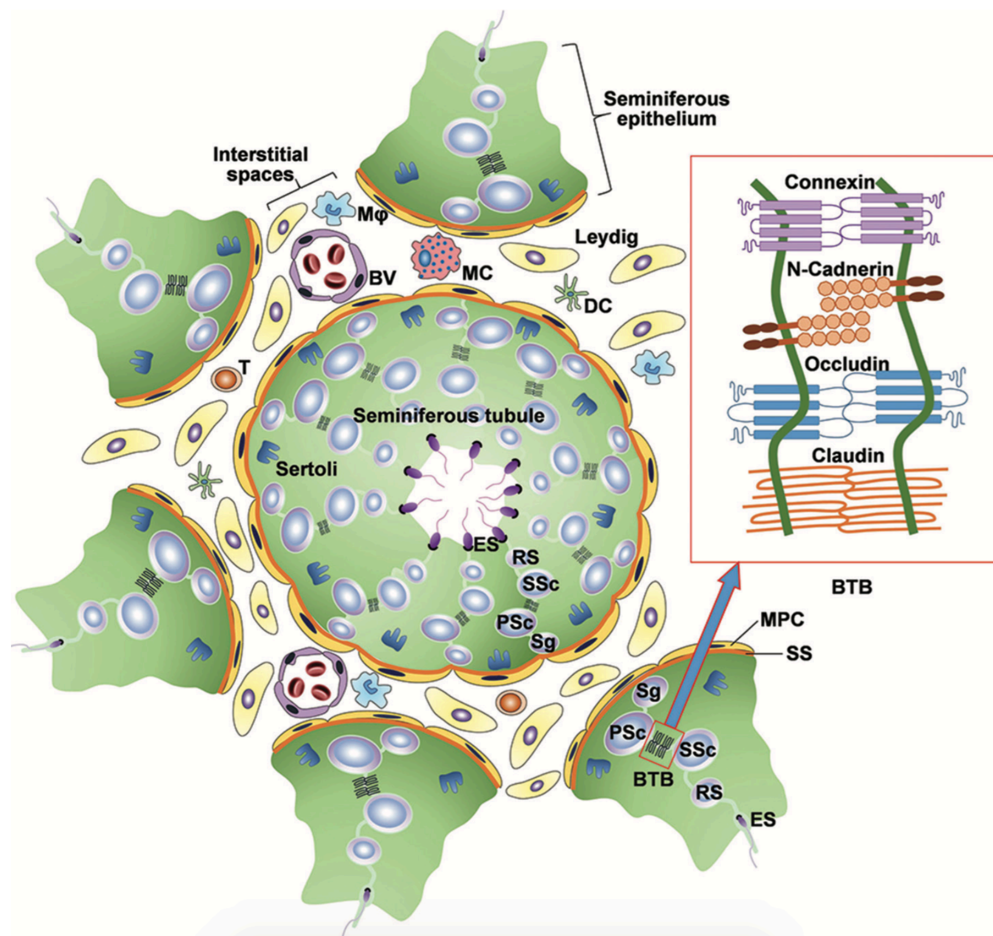

Figure 17: Cross section of the mammalian testes [105]. **MPC=** myoid peritubular cells, **Sg=** spermatogonia, **PSc=** primary spermatocytes, **SSc=** secondary spermatocytes, **SS=** secretion substances, **RS=** round spermatids, **ES=** elongating spermatids, **BTB=** blood-testes barrier, **DC=** dendritic cells, **MC=** mast cells, **BV=** blood vessels, **MΦ=** macrophage

In addition to forming an anatomical barrier, SC also contribute to the overall immune-privileged nature of the testes by participating in local production of anti-inflammatory cytokines and immunosuppressive factors, further aided by Leydig cells, testicular macrophages, and regulatory T cells [104]. However, upon exposure to viral infections, SC have also been shown to induce both innate immune and pro-inflammatory responses [3, 106, 107]. The subsequent production of inflammatory mediators associated with the disruption of blood-tissue barriers, such as TNF-α, matrix metalloproteinases (MMP), and cell- adhesion molecules (CAM) by SC and other testes resident cells are proposed to mediate degradation of tight junctions between the SCB allowing pathogens to enter the protected adluminal compartment of the seminiferous tubules. Our objective, described in chapter two is therefore based on these features of the SCB.

Literature on testicular infection of ZIKV

Although the evidence of ZIKV infection in the male human reproductive tract is limited, mouse and NHP models provide strong evidence that ZIKV is able to target the male reproductive tract. Specifically, in NHPs, ZIKV RNA has been detected in the testes eight days after infection and in seminal fluid (plasma) for up to three weeks [4, 5]. ZIKV RNA has also been detected in the prostate, seminal vesicles, and testes of NHPs via *in situ* hybridization [5]. Additional data has been obtained utilizing an immunocompromised mouse model [89]. While wild type mice do not develop disease symptoms, persistent testicular infection for up to 42 days has been documented in mice lacking functional IFN-α/β receptors (IFNAR1 deficient mice) [2]. These mouse studies have further demonstrated presence of ZIKV in the Leydig cells, Sertoli cells, and spermatogonia [2]. Infection of murine Sertoli and Leydig cells in vitro also lead to an upregulation of major

pro-inflammatory cytokines such as TNF-α, IL-6. IFN-β, and CXCL10 suggesting that they play an essential role ZIKV pathogenesis within the testes [3]. Further, infection in immunocompromised mice is associated with a destruction of testes architecture, decrease in sex hormones, reduction of motile sperm count, and even leads to male infertility [2, 3]. Although these in vivo findings are derived from severely immunocompromised mouse models and such severe testicular injury is yet to be documented in humans or NHPs, they do form the basis of this study to understand human testicular ZIKV infection.

Species	Sample	Time	Findings	Ref.
Human	Seminal Fluid Spermatozoa	10-141 dpi 56 dpi	-Viral load via qRT-PCR ranged from $10^{8.6}$ to $10^{3.5}$ copies/mL -ZIKV identified in head of spermatozoa. 3.52% of spermatozoa positive for ZIKV	[108]
Human	Seminal Fluid	69 dpi	-Infectious ZIKV isolated from semen 69 dpi determined by Plaque Assay	[109]
Human	Seminal Fluid	181 dpi	-Viral load via qRT-PCR ranged from 10^3 to 10^4 copies/mL -Viral RNA associated with the cellular component of semen, undetectable in seminal plasma	[38]
Cynomolgous Macaques	Testis	4-8 dpi	-ZIKV strains PRVABC59 (Puerto Rico) and FSS13025 (Cambodia) detected between 10^3 - 10^5 copies/g tissue	[4]
Cynomolgous Macaques	Testis, Prostate, and Seminal Vesicles	28 dpi	-ZIKV RNA detected by RNA scope *in situ* hybridization	[5]
Cynomolgous Macaques	Seminal Fluid	7-28 dpi	-Viral load ranged from 10^6 to 10^2 copies/mL between this time period	[5]
C57BL/6 mice treated with IFNAR-1 Ab	Testis and Epididymis Seminal fluid	7-42 dpi 7-21 dpi	-Viral load ranged from 10^7 to 10^6 copies/g tissue -ZIKV detected in spermatogonia, 1^0 spermatocytes, and Sertoli cells -Infection associated with a noticeable decrease in testis weight/size, sex hormones, and fertility -Leukocyte infiltration and damaged seminiferous architecture observed with ZIKV infection -Viral load via plaque assay ranged from 10^6-10^5 FFU/mL	[2]
IFNAR $^{-/-}$ C57BL/6 mice	Testis and epididymis	8 dpi	-ZIKV detected via immunofluorescence -Infiltrating macrophages and leukocytes prominent in the interstitum -Necrotic Leydig cells and numerous degenerating germ cells observed in seminiferous tubules	[3]

Figure 18: ZIKV infection and pathogenesis in the male reproductive tract

Chapter 2

Thesis scope

Recap of background: Zika virus is a re-emerging flavivirus with increased disease severity

Zika virus (ZIKV), a largely neglected mosquito-borne virus belongs to the flavivirus genus of the *Flaviviridae* family, which includes other globally relevant arthropod-transmitted human pathogens such as dengue (DENV), West Nile (WNV) and Japanese encephalitis (JEV) viruses [110]. Similar to these related viruses, ZIKV is an enveloped, spherical particle with a linear, positive sense RNA genome roughly 10.7 kilobases in length [110]. Originally identified as an isolate from rhesus monkeys in the Zika forest in Uganda in 1947, for half a century, fewer than 20 mild-febrile human infections were documented [16]. The first large reported outbreak of ZIKV occurred on the Western Pacific island of Yap in 2007 followed by an even larger epidemic in French Polynesia in 2013-2014. However, it wasn't until it made landfall in South America in 2015 that the virus truly began to emerge on the global stage, especially due to its associations with more severe complications including Guillain-Barré syndrome (GBS) and severe fetal abnormalities such as microcephaly [15, 16, 111, 112]. Since 2015, fifty-nine countries and territories have reported an outbreak of mosquito-borne ZIKV transmission. Furthermore, 29 countries have reported microcephaly, other CNS malformations, and incresed incidence of GBS potentially associated with ZIKV infection [17].

An update of ZIKV sexual transmission:

Similar to DENV, WNV, and JEV, ZIKV is primarily transmitted by mosquitos. However unlike other related viruses, ZIKV has caught the world's attention because of two unexpected disease transmission routes. First, *in utero* transmission suspected to be associated with dramatic surge in microcephaly cases and second, sexual transmission

mainly from infected males to their partners. In the US alone, there have been 1,883 pregnancies with laboratory evidence of possible ZIKV infection and 46 confirmed cases of sexual ZIKV disease transmission [17]. Furthermore, 12 other countries have also reported male-to-female sexual transmission [1].

These unanticipated routes have led the Center for Disease Control and Prevention (CDC) to issue an advisory to pregnant women to consider all possible options to protect their pregnancy [33, 113]. Based on reports of ZIKV in the seminal fluid and sperm of several male patients [36, 108, 114], it appears that the virus can be spread by males before disease symptoms start, when disease symptoms are present, and after the symptoms end [36, 115]. There is also mounting evidence that the virus is able to establish a persistent infection within the male reproductive tract, specifically within the seminiferous tubules of the testes, where sheltered sperm is produced.

Although the contribution of sex in disease transmission may be difficult to predict in ZIKV endemic regions, it certainly complicates the virus epidemiology in regions where the mosquito vector is absent. Given the strong evidence of ZIKV in human seminal fluid even after the clearance of viremia, and lack of any approved treatment option, it has become critically important to understand the mechanisms associated with testicular infection.

Gap, rationale, and hypothesis:

Despite the clinical observations and animal findings described in the previous chapter little is known about ZIKV infection in the male reproductive tract, which has left many questions unanswered. For example, what is the association of the level of viremia with testicular invasion of ZIKV? What are some of the host factors that affect persistence of

the virus in seminal fluid? Does the viral load in the semen differ between symptomatic and asymptomatic men? Efforts to address these questions, however, are hindered because of a large **gap** in our understanding of the specific cell types that support ZIKV infection in the human testes and the mechanisms by which the virus establishes persistent infection in the immune-privileged seminiferous tubules. Further, absence of appropriate tools limits studies to understand virus transmigration kinetics across the SCB. Therefore, the *objective of this study is to characterize ZIKV replication kinetics and immune response in primary Human Sertoli cells (HSEC) and develop an in vitro SCB model to understand mechanisms of ZIKV transmigration across the SCB.*

Hypothesis: We hypothesize that ZIKV can productively infect HSEC and macrophages and induce robust immune response that can collectively affect the integrity of the blood-testes barrier model.

Specific Aim 1: To determine virus replication kinetics and immune response in HSEC and macrophages following ZIKV infection

Rationale: To be able to sexually transmit for weeks after clearance of viremia, it is important for ZIKV to establish a persistent infection locally in the compartment where spermatozoa development occurs. A recent study with data from 150 newly-infected human participants has shown the median time until the loss of ZIKV RNA in the serum is around 14 days compared to 34 days in seminal fluid [39]. The rationale for this aim is based on mouse studies that show presence of ZIKV RNA in the testes peritubular myoid cells, SC, and germ cells [2, 3]. However, the cell tropism of ZIKV in humans has yet to be characterized. Although ZIKV has been shown to infect human placental macrophages

(Hofbauer cells) [86], ZIKV infection in human peripheral macrophages is not yet described. Therefore, we ***hypothesize*** that ZIKV can sustain a robust infection in human SC and macrophages, induce production of pro-inflammatory mediators including type I IFN and cytokines, and alter markers of SCB integrity.

Objective:

- Determine the replication kinetics of ZIKV, DENV-2, and WNV in HSEC at different multiplicity of infections (MOIs).
- Define the ZIKV infection kinetics and inflammatory response in human monocytes-derived macrophages (MDM)
- Characterize the anti-viral innate immune response and expression of multiple CAM and TJP in HSEC following ZIKV infection
- Test the ability of a known flavivirus inhibitor (NITD008) in preventing ZIKV infection in HSEC

Specific Aim 2: To test the hypothesis that ZIKV infection of SC and macrophages can lead to disruption of in vitro SCB model

Rationale: To further test the consequence of ZIKV infection on virus-transmigration across the barrier, we propose to utilize a human in vitro SCB model. In vitro SCB models comprising of human and mouse SC have been routinely used to study mechanisms of SC function in spermatogenesis and effects of environmental toxins on barrier integrity leading to male infertility [116-119], but have so far not been used to examine virus transmigration kinetics. This study, for the first time, will use the in vitro SCB model to

characterize virus transmigration and to further understand interactions of peripheral immune cells with infected SC. Additionally, since macrophages make up the majority of immune cells in the mammalian testes and are able to release inflammatory molecules capable of affecting the integrity of the SCB, it is imperative to also characterize their role in SCB integrity.

Objective:

- Optimize an in vitro SCB model and determine ZIKV transmigration by measuring viral titers in the upper (UCS) vs. lower chamber supernatants (LCS)
- Quantify SCB integrity following ZIKV infection by measuring Trans-endothelial electrical resistance (TEER) and through FITC-labeled dextran transmigration assays
- Establish whether ZIKV induced CAM affect interaction of HSEC with leukocytes
- Determine the effect of MDM-derived inflammatory mediators on SCB integrity

Significance: Since January 1, 2015 there have been 46 confirmed cases of ZIKV sexual transmission cases, 72 cases of live born infants with birth defects, and 8 pregnancy losses with birth defects in the US alone [17]. These disconcerting statistics combined with the fact that ZIKV is now associated with severe clinical diseases such as microcephaly and GBS highlights the utmost importance of understanding the pathogenesis of ZIKV disease and sexual transmission. Furthermore, this route may contribute significantly to the disease spread in non-endemic regions such as the United States. As seen in animal models and with other testes-tropic pathogens, the possibility of ZIKV causing testicular pathology and male infertility also exists. So far there are no antiviral drugs approved to specifically target ZIKV infection, therefore NIH has identified sexual transmission of ZIKV as a high priority research area. The data obtained from this study will be significant because it will identify one of the potential routes ZIKV may utilize to establish persistence in to the testes. Additionally, given the recent evidence of Ebola virus RNA in seminal fluid for 6 to 9 months after clearance of acute infection [49], it has become critically important to understand the mechanisms by which these pathogens hide in the immune privileged site of testes and infect developing spermatozoa for months after clearance of viremia. Novel insights into these mechanisms will properly inform CDC and WHO health policies. Furthermore, use of in vitro SCB models to test the ability of new antiviral drugs to cross the SCB may also impact the development of novel therapeutic strategies to clear testicular infection of ZIKV and other viruses.

Chapter 3

Zika virus infects human sertoli cells and modulates the integrity of the in vitro blood-testes barrier model

Zika virus infects human Sertoli cells and modulates the integrity of the in vitro blood-testes barrier model

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Running title: ZIKV alters integrity of the Sertoli-cell barrier

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Abstract

Confirmed reports of ZIKV in human seminal fluid for months after the clearance of viremia suggest the ability of ZIKV to establish persistent infection in the seminiferous tubules, an immune privileged site in the testes protected by the blood-testes barrier, also called the Sertoli cell barrier (SCB). However, cellular targets of ZIKV in human testes and mechanisms by which the virus enters seminiferous tubules remain unclear. We demonstrate that primary human SC are highly susceptible to ZIKV as compared to the closely related dengue virus and induced expression of IFN-α, key cytokines and cell-adhesion molecules (VCAM-1 and ICAM-1). Further, using an in vitro SCB model, we demonstrate that ZIKV was released on the adluminal side of the SCB model with higher efficiency as compared to the blood-brain barrier. ZIKV-infected SC exhibited enhanced adhesion of leukocytes that correlated with decrease in the SCB integrity. ZIKV infection did not affect the expression of tight and adherens junction proteins such as ZO-1, claudin and JAM-A, however exposure of SC to inflammatory mediators derived from ZIKV-infected macrophages led to the degradation of ZO-1 protein that correlated with increased SCB permeability. Taken together, our data suggest that infection of SC may be one of the crucial steps by which ZIKV gains access to the site of spermatozoa development and identify SC as a therapeutic target. Further, the SCB model opens up opportunities to assess interactions of SC with other testicular cells and test the ability of anti-ZIKV drugs to cross the barrier and clear testicular infection.

Importance

Recent outbreaks of ZIKV, a neglected mosquito-borne flavivirus, have identified sexual transmission as a new route of disease spread, not reported for other flaviviruses. To be able to sexually transmit for months after clearance of the viremia, ZIKV must establish infection in the seminiferous tubules, a site for spermatozoa development. However, little is known about the cell types that support ZIKV infection in the human testes. Currently there are no models to study mechanisms of virus persistence in the seminiferous tubules. We provide evidence that ZIKV infection of human Sertoli cells, important component of the seminiferous tubules, is robust and induce strong antiviral response. The use in vitro Sertoli cell barrier to describe how ZIKV or inflammatory mediators derived from ZIKV-infected macrophages compromise the barrier integrity will enable studies to explore interaction of other testicular cells with Sertoli cells and test novel antivirals for clearing testicular ZIKV infection

Introduction:

Zika virus (ZIKV), a largely neglected arbovirus belongs to the flavivirus genus of the *Flaviviridae* family, which includes other globally relevant arthropod-transmitted human pathogens such as dengue, West Nile (WNV) and Japanese encephalitis (JEV) viruses. Recent re-emergence of ZIKV in the South Pacific and Latin America in 2015-2016 has been associated with more severe complications including Guillain-Barré syndrome and severe fetal abnormalities [16]. So far, 38,527 locally acquired cases have been reported in the U.S including American Samoa, U.S. Virgin Islands and Puerto Rico [17]. However, what caught the world's attention during the recent ZIKV outbreak was the two unexpected

disease transmission routes: first, *in utero* transmission associated with a dramatic surge in microcephaly cases and second, the sexual transmission from infected males to their partners. In the US alone, 46 cases of ZIKV disease transmission via the sexual route has been confirmed so far [17] and at least 12 other countries have also reported male-to-male and male-to-female transmission, leading to an urgent advisory to pregnant woman to consider all possible options to protect their pregnancy [1]. Based on reports of the duration of the presence of ZIKV in the seminal fluid, it appears that the virus can be spread by males before disease symptoms start, when disease symptoms are present, and after the symptoms end [36, 115]. Further, it is unclear if infected individuals who remain asymptomatic can also sexually transmit ZIKV, and if there is any association of the level of viremia with testicular invasion of ZIKV. Although the contribution of the sexual route in disease transmission may be difficult to predict in ZIKV endemic regions, it certainly complicates the virus epidemiology in non-endemic regions where the mosquito vector is absent. A recent cohort study reported that 56% of the ZIKV serum-positive males were also positive for the virus in the semen and the median time until the loss of ZIKV RNA in the semen was 34 days as compared to 14 days in the serum thus suggesting a much longer infectious phase of ZIKV as compared to other flaviviruses traditionally transmitted via mosquitoes [39]. Considering the lack of any measures approved to clear ZIKV infection and the detection of RNA of other re-emerging pathogens such as Ebola virus in the semen [36, 49], it has become critical to understand the mechanisms associated with testicular infection of ZIKV.

The mammalian testes are divided into two compartments, the peritubular compartment, which consists of Leydig cells and testicular macrophages, and the seminiferous tubules compartment with germ cells protected by Sertoli cells (SC). These SC form the blood-testes barrier, also known as the Sertoli-cell barrier (SCB) that mainly functions to protect developing germ cells from systemic attack by adaptive immune cells, while simultaneously providing nutritional and structural support [103, 104]. As one of the tightest blood-tissue barriers, the SCB is formed by tight junction proteins (TJP) complexes such as ZO-1, occludin, and claudins, as well as adherens junction proteins between connecting Sertoli cells [103]. Similar to other blood-tissue barriers such as blood-brain barrier (BBB), in addition to providing a physiologic barrier, SCB also participates in the immune response to invading pathogens. Several viruses including mumps virus have been shown to infect human testes and induce inflammatory mediators associated with the disruption of blood-tissue barriers including TNF-α, type I IFN, matrix metalloproteinases (MMP) and cell-adhesion molecules (CAM) [107].

The data on testicular infection of ZIKV in humans is limited, however mouse and nonhuman primate models of ZIKV infection provide strong evidence of persistent ZIKV replication in the testes. Immunocompetent mice do not develop disease symptoms, but persistent testicular infection for up to 45 days in IFNAR1 deficient mice has been associated with pro-inflammatory responses, infiltration of leukocytes into the testes, and damaged architecture of the seminiferous epithelium [2, 3]. Similarly, nonhuman primate studies have also reported presence of ZIKV RNA in the testes at 7-8 days after infection [5]. In humans, not all, but some ZIKV-infected men present with hematospermia, however

presence of virus in the semen even after the clearance of the viremia suggests that sexual transmission is not the result of hematospermia-induced blood-borne transmission and that ZIKV has the ability to establish persistent testicular infection. However, little is known about the cell types that support ZIKV infection in testes and currently there are no models that can be used to study mechanisms by which the virus establishes persistence in the seminiferous tubules. Here, we show that primary human SC are highly susceptible to ZIKV infection as compared to dengue virus and are capable of inducing robust antiviral immune and inflammatory responses. We further developed an in vitro SCB model to systematically investigate whether ZIKV can be released on the adluminal side of the SCB and test how ZIKV infection of SC and macrophages affects barrier integrity. We demonstrate that ZIKV can cross the in vitro SCB more efficiently as compared to the BBB model without altering barrier permeability and expression of junction proteins. However, inflammatory mediators secreted from ZIKV-infected macrophages compromised the barrier integrity. Our data suggest that infection of SC may be one of the crucial steps by which ZIKV gains access to immune privileged seminiferous tubules and may serve as a reservoir for infection of other resident testicular cells including developing spermatozoa.

Results

ZIKV can infect and replicate in human Sertoli cells

Since SC are the primary component of the SCB that protects entry of pathogens into the seminiferous tubules, we first determined if primary Human Sertoli cells (HSEC) were susceptible to ZIKV. Low passage HSEC were infected with ZIKV strain PRVABC59 at a multiplicity of infection (MOI) of 1 and 5, and virus replication kinetics was analyzed using

multiple virological assays. As seen in Fig. 1, while no ZIKV titers were detected at 6 hrs after infection, titers reached about log 4.5 PFU/mL in supernatant at 24 hrs after infection and further peaked at 48 and 72 hrs after infection. Intracellular ZIKV replication also followed a similar trend wherein peak RNA levels were observed at 48 hrs after infection. However, we did not observe any cytopathic effect or cell death (Supplemental Figure 1). Further confirmation of the HSEC susceptibility to ZIKV by immunostaining using flavivirus specific 4G2 antibody demonstrated a robust signal of ZIKV-bound antibody in HSEC at 48 hrs after infection (Fig. 1C). Since ZIKV is also a neurotropic virus [89], we next compared the infectivity of ZIKV in HSEC vs. human brain microvascular endothelial cells (HBMVEC), a major component of the BBB. ZIKV titers in the supernatants from primary HBMVEC demonstrated similar replication kinetics as observed in HSEC. However, peak viral titers were almost half a log lower as compared to HSEC at 72 and 96 hrs after infection and this difference was statistically significant ($p<0.05$, Fig. 1D). We also compared the relative infection of ZIKV, West Nile virus (WNV), and dengue virus 2 (DENV2) in HSEC at different MOIs. As seen in Fig. 1E and Supplemental Fig. 2, DENV replication was not detected at 24 hrs and the titers were 3 logs lower than ZIKV titers at 48 and 72 hrs after infection ($p<0.05$). However, interestingly SC supported robust infection of WNV and the virus titers were comparable to ZIKV at all time points (Fig. 1E). These data suggest that while ZIKV can infect both HSEC and HBMVEC, virus replication is lower in HBMVEC and that HSEC are not a good target of dengue virus.

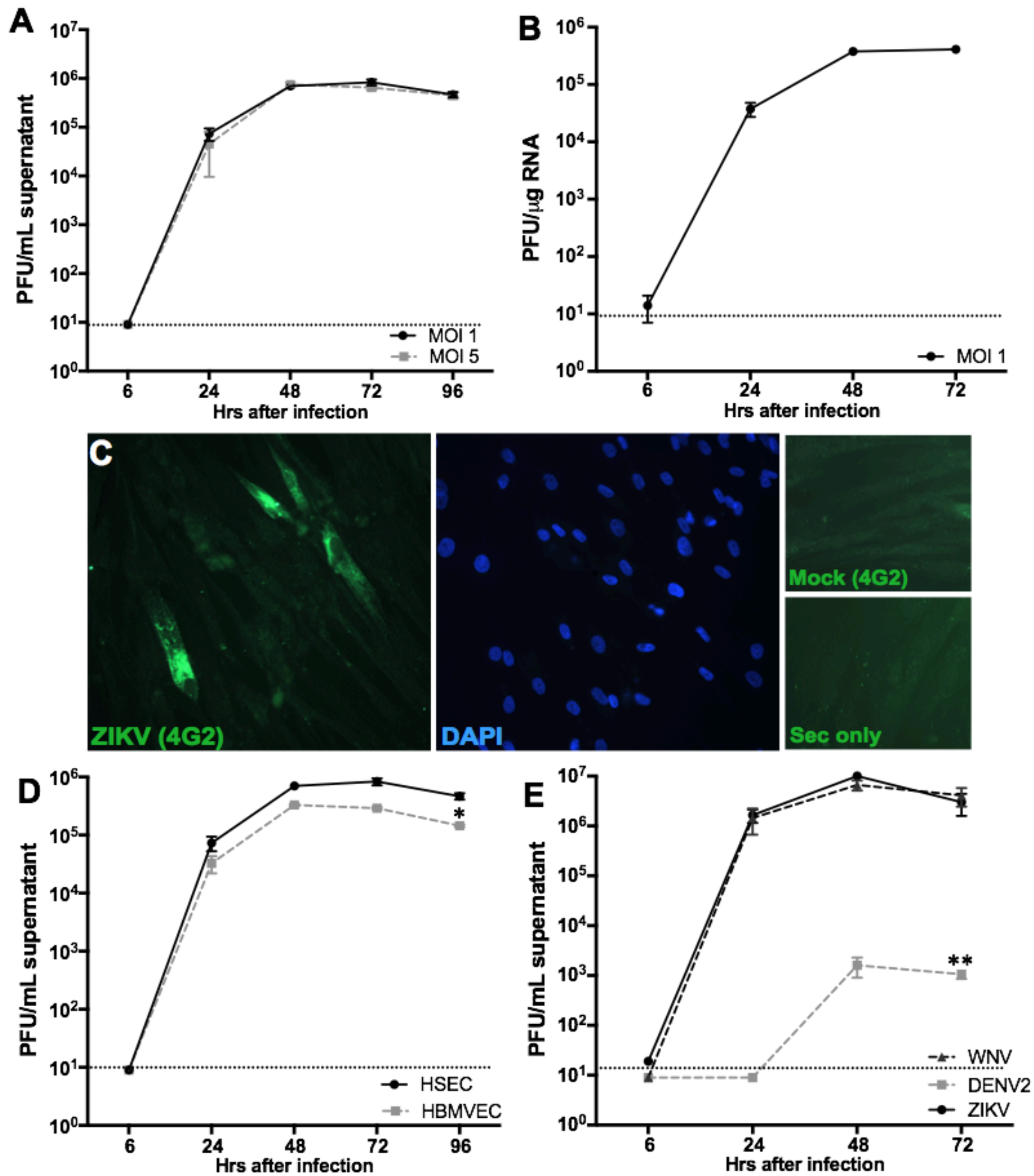

Fig. 1. ZIKV infection in human Sertoli cells is productive. HSEC were infected with ZIKV and **(A)** Virus titers were quantified by plaque assay using Vero cells and expressed as PFU/mL supernatant. **(B)** ZIKV mRNA determined using qRT-PCR and expressed as PFU equivalent/μg RNA **(C)** Representative image of ZIKV immunostaining in infected HSEC at 48 hrs after infection at MOI1. Cells were immunostained for envelope protein (green) using the 4G2 anti-flavivirus group antibody, secondary antibody alone and nuclei using DAPI (blue). **(D)** HSEC and HBMVEC were infected with ZIKV at MOI1 and titers were measured by plaque assay. **(E)** HSEC were infected with ZIKV, WNV, and DENV 2 (MOI1) and viral titers at different time points were measured using plaque assay. Error bars represent SEM of at least 3-5 independent infections. *, $p<0.05$ and **, $p<0.01$

ZIKV infection induces type I IFN signaling and inflammatory cytokines in HSEC

Robust production of type I IFN and inflammatory cytokines is required to restrict virus dissemination into immune privileged tissues such as the testes. Therefore, to test whether HSEC were capable of initiating antiviral responses to ZIKV, we next measured the levels of IFN-α and key antiviral cytokines. As seen in Fig. 2A, the mRNA transcripts of the key IFN-stimulated gene IFIT1 and cytokines (TNF-α and IL-6) were found to be elevated at 48 and 72 hrs after infection. ZIKV infection did not induce IFN-α secretion during early time points, however a robust induction was observed in the supernatant at 48 and 72 hrs after infection (Fig. 2B). Furthermore, levels of multiple inflammatory cytokines such as IFN-γ, IL-1α, IL-1β, IL-6, IL-8 and TNF-α were significantly elevated in the supernatant at 48 and/or 72 hrs after infection that correlated well with peak virus titers. The secretion of chemokines involved in recruiting leukocytes to inflammatory sites, such as RANTES (CCL5), fractalkine (CX3CL1), and IP-10 (CXCL10) were also significantly increased at 72 hrs after infection. Interestingly, GRO (growth-related oncogene CXCL1, which is structurally related to IL-8) chemokine shown to attract neutrophils and T lymphocytes in the testes during orchitis of various origins [120], was also found to be significantly elevated at both 48 and 72 hrs after infection (Fig. 2B). Collectively, these results demonstrate that HSEC are capable of generating a strong innate immune response to ZIKV infection.

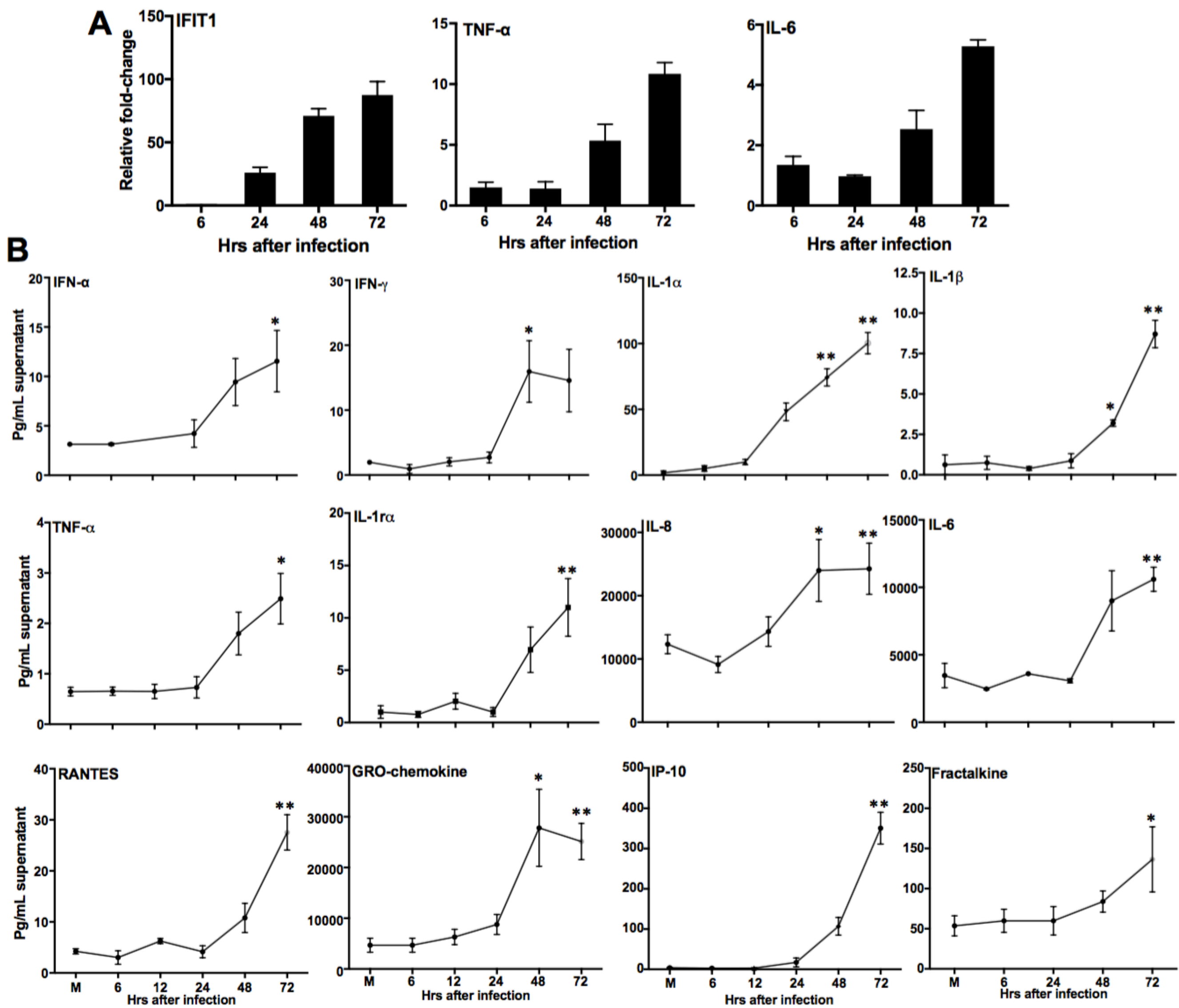

Fig. 2. ZIKV infection induces secretion of antiviral and pro-inflammatory mediators in HSEC. HSEC were infected with ZIKV at MOI1 and at different time points **(A)** Total RNA extracted from lysates was used to measure relative mRNA fold-change of IFIT1, TNF-α and IL-6 as compared to controls by normalizing to GAPDH. **(B)** IFN-α levels in the supernatant were measured by Luminex assay. Secretion of pro-inflammatory cytokines (IFN-γ, IL-1β, IL-1rα, IL-1α, TNF-α, IL-8, IL-6) and chemokines (RANTES, GRO-chemokine, and IP-10) in the supernatant at different time points after infection was analyzed using multiplex bead-based assay. Error bars represent SEM of at least 3-6 independent infections for each time point. *, $p<0.05$ and **, $p<0.01$ as compared to mock.

Effect of ZIKV infection on CAM, MMP and tight junction proteins

Under healthy conditions SC express very low levels of CAM, however inflammatory triggers or infection can induce the expression of multiple CAM and MMP associated with degradation of TJP [6, 121]. Therefore, we next determined if ZIKV infection could alter the expression of CAM and MMP in HSEC. As seen in Fig. 3A, we observed an increase in the mRNA transcripts of vascular cell adhesion molecule 1 (VCAM-1) and intracellular adhesion molecules (ICAM-1) but not of E-selectin (data not shown) at 72 hrs after infection. Further analysis of CAM using western blotting demonstrated a significant increase in the protein levels of VCAM-1, while expression of E-selectin did not change significantly thus supporting the qRT-PCR data (Fig. 3B and C). Among key MMP, ZIKV infection did not affect the mRNA expression of MMP-1 and MMP-3 (data not shown). Transcription of MMP-9, however, was modestly upregulated (2-3 fold) at 72 hrs after infection, but this increase could not be observed at the protein level (Fig. 3A and B).

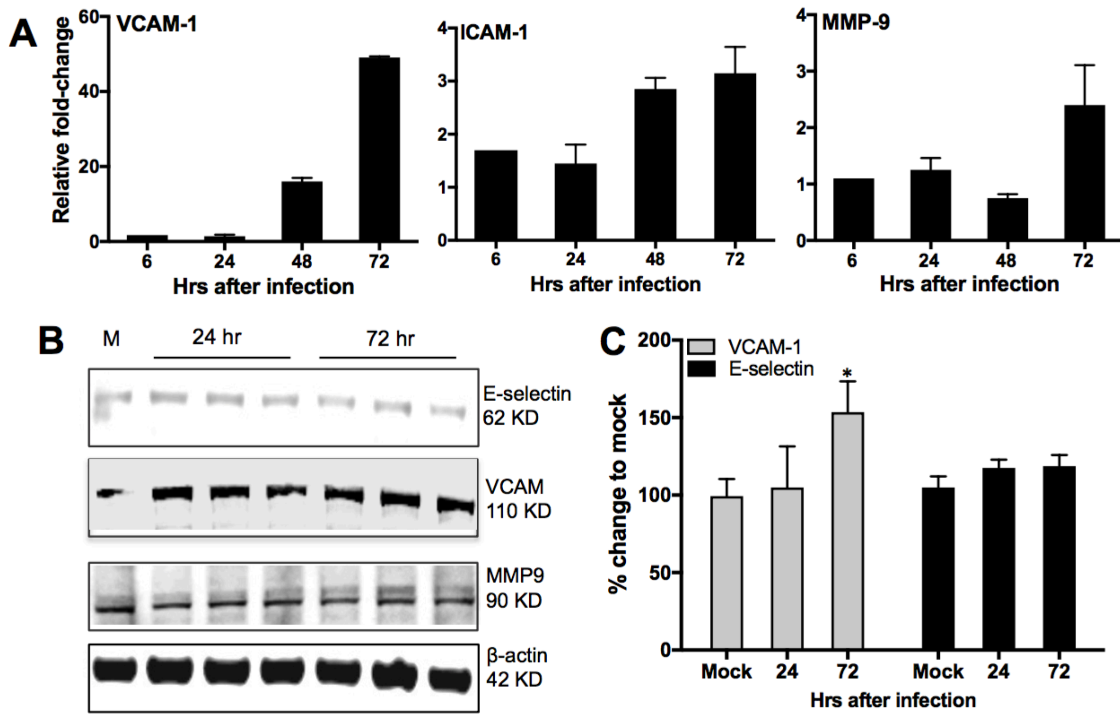

Fig. 3. Effect of ZIKV infection on cytokines, CAM, and MMP (A) Total RNA extracted from ZIKV-infected HSEC lysates (MOI-1) was used to measure relative mRNA fold-change of VCAM-1, ICAM-1 and MMP-9 as compared to controls by normalizing to GAPDH. **(B)** Whole cell extracts were subjected to Western blotting and stained for VCAM-1, E-selectin, MMP-9 and β-actin. The Western blot is representative of at least three independent infections. **(C)** Percent change in the protein levels as compared to controls measured using densitometric analysis and normalized to β-actin. *, $p<0.05$ as compared to mock

Altered gene expression or degradation of tight and adherens junction proteins is one of the critical events associated with leakiness of SCB and other tissue-barriers. Therefore, we next investigated if ZIKV modulates the expression of key TJP, such as ZO-1, occludin, and claudin-1, in HSEC. The mRNA transcripts of ZO-1, claudin-1 and occludin were comparable to controls at all time points after infection (data not shown). At the protein level also, we observed no change in ZO-1, occludin and claudin-1 expression in HSEC at any time points after infection (Fig. 4A). Furthermore, immunocytochemical analysis also

exhibited comparable signals of ZO-1 immunostaining in mock- and ZIKV-infected HSEC monolayers (Fig. 4C). Similarly, the expression of key adherens junction proteins, β-catenin and JAM-A, also did not change at any time points following ZIKV infection (Fig. 4B), thus suggesting that direct ZIKV infection of HSEC does not result in degradation of junction proteins.

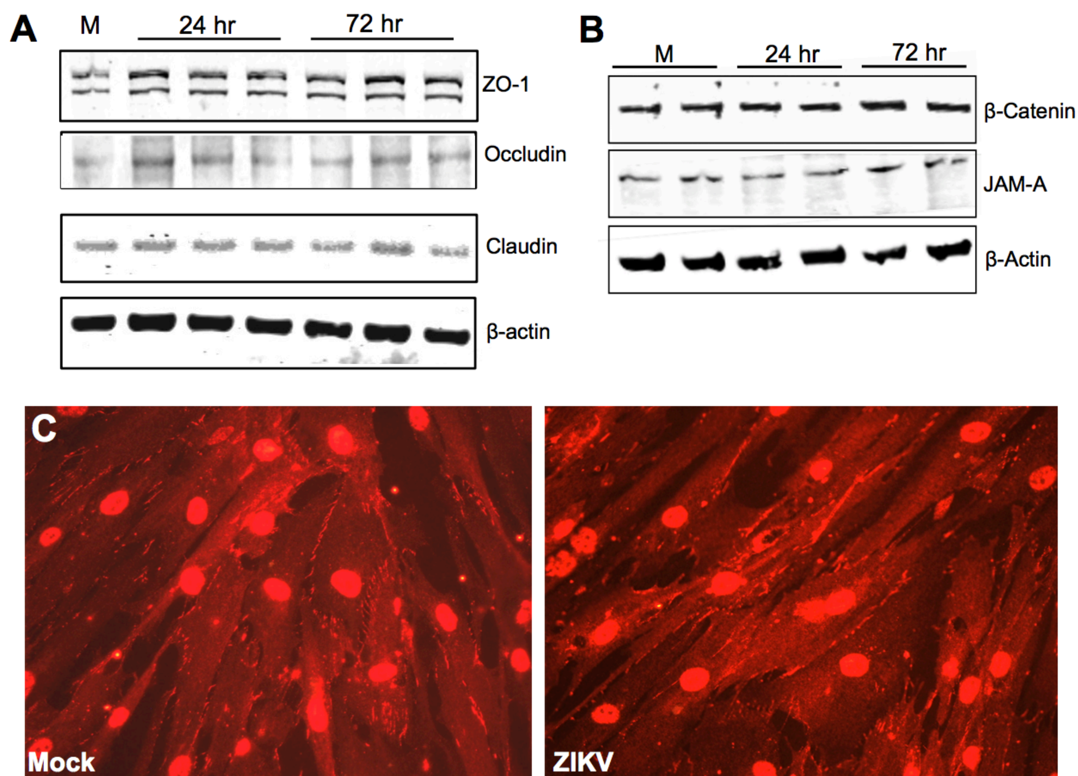

Fig. 4. ZIKV infection does not alter the expression of tight junction and adherens junction proteins in HSEC. Total protein extracted from ZIKV-infected HSEC (MOI-1) was used to measure levels of **(A)** TJP (ZO-1, occludin and claudin) and **(B)** adherens junction proteins (β-catenin and JAM-A) and β-actin by Western blotting using specific antibodies. The Western blot is representative of at least three independent infections. **(C)** Immunostaining of ZO-1 in mock- and ZIKV-infected HSEC at 48 hrs after infection showed comparable ZO-1 signal at the cell borders and cell-to-cell contact sites.

Cell free ZIKV is efficiently released on the adluminal side of the in vitro SCB model

To determine if ZIKV infection of SC represents a route for the virus to enter the seminiferous tubules, we utilized an in vitro SCB model using trans-well cell culture PET inserts and quantitated the transmigration of ZIKV across the inserts. Since SCB restricts the movement of ions, the well-documented methods to assess the tightness of the tissue-barrier are transendothelial electrical resistance (TEER) and FITC-labeled dextran transmigration assays [122, 123]. The SCB permeability was assessed starting at day 4 after seeding and demonstrated a gradual increase of resistance from 25 to 110-130 Ω/cm^2, which was comparable to other SCB studies using similar inserts with 0.3 cm^2 surface area [124]. At day 8 or 10 after seeding, after ensuring TEER >100 Ω/cm^2, the SCB model was infected with ZIKV or UV-ZIKV at MOIs 1 and 5 and virus titers in the upper chamber supernatant (UCS) representing the peritubular side of the SCB and lower chamber supernatant (LCS) corresponding to the lumen of seminiferous tubules were determined at different time points. Very low levels of ZIKV were detected at 6 hrs after infection, which represented the residual inoculum after gentle washing of the inserts (Fig. 5A). The replication kinetics of ZIKV observed in the UCS was similar to that observed in ZIKV-infected HSEC monolayers in culture plates (Fig. 1A) and showed a marked increase at 24 hrs and peaked at 48 hrs after infection. ZIKV titers in the LCS were very low at 24 hrs (in the same range as at 6 hrs after infection), but a sharp increase to log 4-5 PFU/mL was observed at 48 and 72 hrs after infection. However, virus titers in the LCS were approximately 1.5 to 2 logs lower than the UCS at all time points ($p<0.01$), and as expected, no plaques were detected in the SCB models infected with UV-ZIKV. We further compared ZIKV transmigration across the BBB model prepared as described in our previous studies

[122, 123]. As seen in Fig. 5A, ZIKV was detected both in the UCS and LCS at 48 and 72 hrs infection, but the transmigrated virus in the LCS was significantly lower in the BBB (less than log 4 PFU/mL) as compared to SCB models (log 6 PFU/mL).

To assess if ZIKV infection and release in the LCS has any effect on the integrity of the SCB model, we next measured the permeability before and at day 3 after infection. The TEER values were above 100 Ω/cm^2 in all inserts before infection and remained comparable at day 3 after infection in both the controls and UV-ZIKV-infected models as compared to their respective TEER values before infection. While ZIKV infection at MOI-1 did not affect the TEER values, we observed a slight reduction in the TEER values in inserts infected at MOI-5 as compared to controls (Fig. 5B). Analysis of SCB permeability using the FITC-dextran assay also demonstrated that approximately 4.5% of total input FITC-dextran on the UCS transmigrated to the LCS in control inserts, which increased modestly to (5.5) in infected inserts at day 3 of infection, but this difference was not statistically significant (Fig. 5C). Similarly, FITC-dextran transmigration (Fig. 5C) and TEER values (data not shown) remained comparable in mock and ZIKV-infected (MOI-1) BBB models. Collectively, these results demonstrate that while cell-free ZIKV is released on the adluminal side of both SCB and BBB models, it does not significantly affect barrier integrity. Furthermore, the ability of the virus to transmigrate across the SCB is much higher as compared to the BBB.

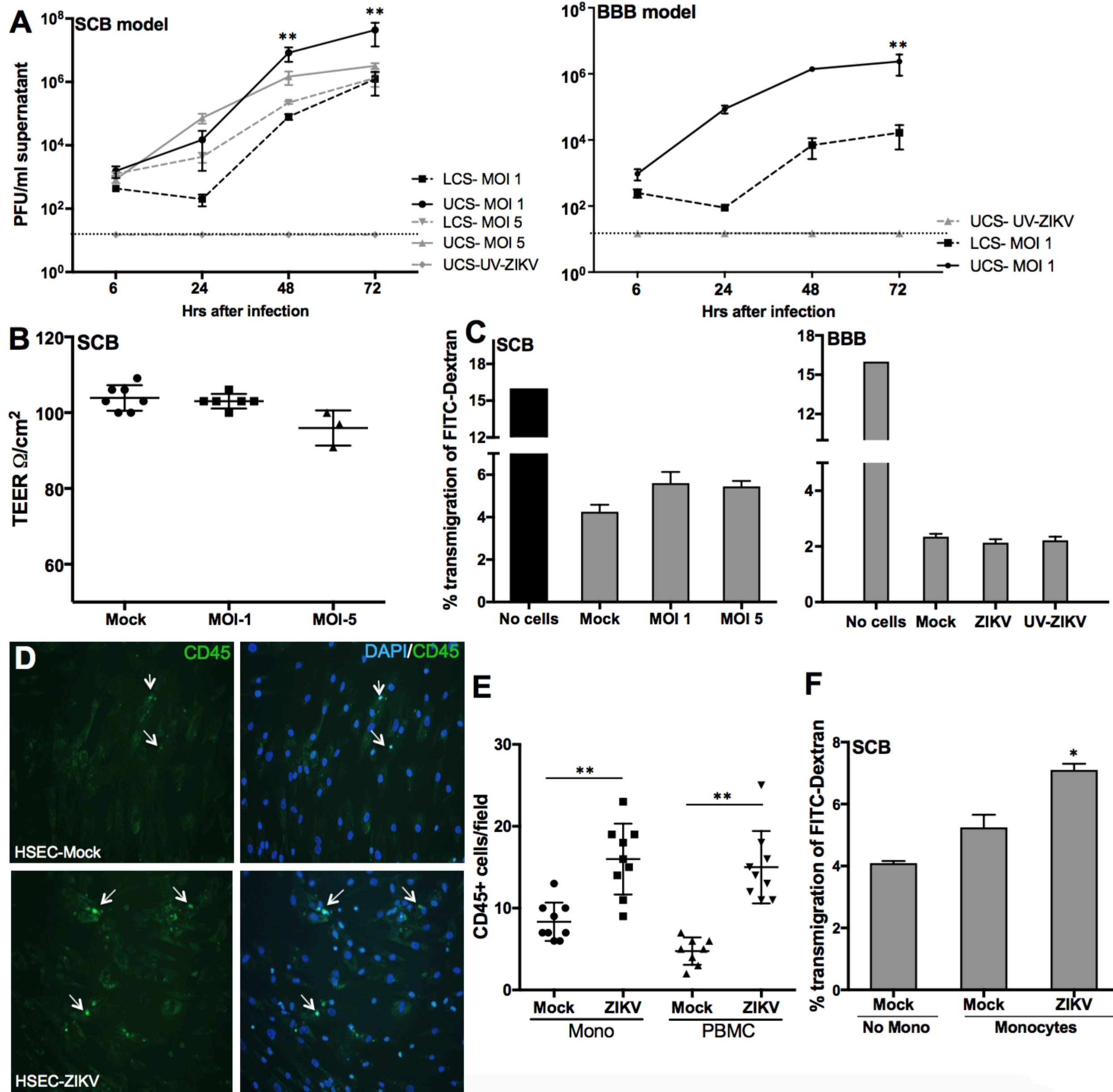

Fig. 5. Cell free ZIKV is efficiently released on the adluminal side of an in vitro SCB model without affecting barrier integrity. At day eight after seeding, the upper chamber of the **(A)** in vitro SCB and BBB models were infected with mock, UV-ZIKV and ZIKV, and washed gently to remove unattached virus. The virus titers determined by plaque assay demonstrated a significant increase in both, UCS and LCS from days 1 to 3 after infection. Data represent mean ± SEM of two independent infection experiments in triplicate. **(B)** The integrity of the SCB model as determined by measuring the TEER at 72 hrs after infection. TEER values are presented as Ω/cm^2 and did not change significantly after infection as compared to controls. The data is representative of at least three independent experiments. **$p<0.01$, compared to LCS **(C)** FITC-dextran permeability assay of the SCB and BBB models at 72 hrs after infection. The percentage of FITC-dextran that crossed the BBB model was determined based on the total input FITC-dextran and did not vary significantly

in ZIKV or UV-ZIKV infected inserts as compared to un-infected controls. **(D)** HSEC monolayers on the coverslips were infected with ZIKV for 72 hrs and then 2 x 10^5 monocytes or PBMCs were added to the cells. After two hrs of incubation, the non-adhered leukocytes were vigorously washed off and adherent cells were stained with CD45 (white arrows). Immunofluorescence micrograph of CD45-stained monocytes. **(E)** Quantitative representation of $CD45^+$ monocytes and PBMCs from six different areas per coverslip from at least three independent infections. **p<0.01, compared to mock-infected inserts **(F)** The integrity of the in vitro SCB model was determined by measuring the FITC-dextran transmigration after four hrs of incubation with monocytes. *p<0.05, compared to control SCB without monocytes incubation.

ZIKV infection increases the adhesion of leukocytes to SC

Since VCAM-1 and ICAM-1 are the key CAM involved in cell-cell interactions, the first step during leukocytes transmigration across epithelial and endothelial cells, we next utilized a cell adhesion assay to determine whether ZIKV-infected HSEC exhibited any difference in their ability to mediate leukocyte adhesion. As seen in Fig. 5D, while co-culture of naïve monocytes with mock-infected HSEC monolayers on coverslips demonstrated only a small number of monocytes adhered to the mock-infected HSEC, they increased significantly in the infected HSEC (day 3 after infection, p<0.01). We also observed similar results of increased adherence of $CD45^+$ PBMCs to the ZIKV-infected HSEC (Fig. 5E). To further determine the consequence of leukocyte interaction with the SCB, 250,000 naïve monocytes were incubated with the inserts when the induction of CAM by ZIKV was at its peak (day 3 after infection). SCB permeability was assessed using FITC-dextran transmigration and as seen in Fig. 5F, a significant increase in the percent transmigration of FITC-dextran was observed only in the ZIKV-infected inserts incubated with monocytes (p<0.05).

ZIKV infection in human macrophages induces changes in SCB integrity

Testicular macrophages are important cell types in the peritubular compartment of the testes and are shown to contribute to testicular inflammation [125]. Therefore, we first characterized replication kinetics of ZIKV in human macrophages. As seen in Fig. 6A, peak ZIKV replication was observed at 24 hrs, however the infection was not as robust as HSEC. Peak virus titers were coupled with increased mRNA transcripts of IFN-α and IFIT1 genes (Fig. 6B), as well as secretion of multiple inflammatory cytokines including TNF-α, IL-1α and IL-8, at 24 and/or 48 hrs after infection. Additionally, ZIKV also induced important chemotactic chemokines, such as GRO, IP-10 and MCP-1, at 48 hrs after infection (Fig. 6C). To determine whether ZIKV-induced inflammatory mediators derived from macrophages could influence SCB integrity, we conducted experiments using the UV-inactivated supernatant from either ZIKV- or mock-infected macrophages. At day 9 after seeding, when the TEER readings of the inserts were above 120 Ω/cm^2, the SCB models were incubated with ZIKV-infected macrophage supernatant collected at 48 hrs after infection. While the treatment of inserts with supernatant from mock-infected macrophages did not change TEER values or FITC-dextran transmigration as compared to control SCB models (only HSEC media), decreased TEER readings were observed in the inserts treated with supernatant from ZIKV-infected macrophages that correlated with a significant increase in the percentage of FITC-dextran transmigrated to the LCS (Fig. 6 D). In parallel, we also incubated naïve HSEC monolayers on coverslips with supernatant from infected macrophages for 3 hrs. As seen in Fig. 6E, the intensity of ZO-1 immunostaining reduced markedly only in the coverslips treated with TNF-α (positive control) and supernatant from ZIKV-infected macrophages at 48 hrs after infection. These results collectively suggest that

inflammatory mediators released from ZIKV-infected cells in the peritubular compartment possess the potential of disrupting the SCB.

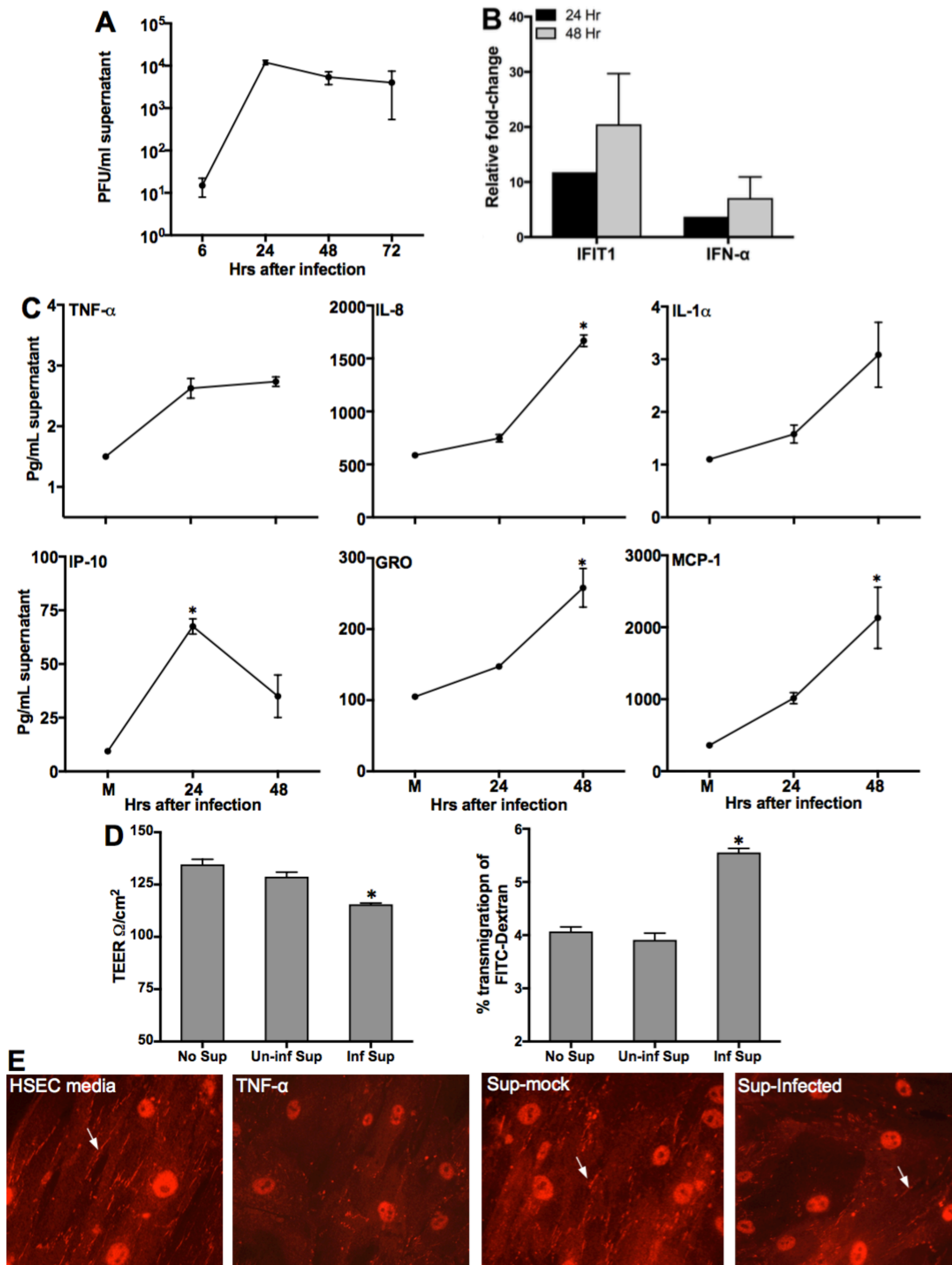

Fig. 6. ZIKV infection in human macrophages induces mediators that alter SCB permeability. Human macrophages were infected with ZIKV at MOI1. (**A**) Culture supernatants collected at different time points after infection were used to determine titers

using plaque assay on Vero cells. (**B**) qRT-PCR analysis of IFIT1 and IFN-α in infected macrophages. The data are normalized to the values of GAPDH and expressed as relative fold increase compared to uninfected controls. **(C)** Secretion of pro-inflammatory cytokines and chemokines in the supernatant was analyzed using multiplex bead-based assay. Data represent the mean ± SEM from three independent experiments. *, $p<0.05$ as compared to mock. **(D)** At day 9 after seeding, HSEC and SCB models were incubated for 2 hrs with supernatant from mock- and ZIKV-infected macrophages at 48 hrs time point and TEER values, presented as Ω/cm^2, demonstrated a decrease only in the inserts incubated with either supernatant from infected macrophages. FITC-dextran permeability assay demonstrated an increase in transmigration across the inserts incubated with supernatant from infected macrophages. *, $p<0.05$ as compared to inserts with no treatment **(E)** HSEC monolayers on coverslips were incubated with supernatant from mock- or ZIKV-infected macrophages (48 hrs time point) and TNF-α as positive control, and immunostained with anti-ZO-1 (white arrows).

Anti-flavivirus drug NITD008 blocks ZIKV infection in HSEC in a dose-dependent manner.

Current small molecule drugs against flaviviruses include NITD008, a nucleoside analog targeting viral polymerases, that has been shown to exhibit potent antiviral activity against DENV, WNV and hepatitis C virus, and more recently against ZIKV in Vero cells [83, 84]. Therefore, we next evaluated if this drug can block ZIKV replication in HSEC. The HSEC monolayers were treated with three different concentrations of NITD008 following ZIKV infection at MOI-1 and we observed a dose-dependent inhibition of ZIKV replication as demonstrated by plaque assay. In HSEC monolayers treated with 5 μM concentration, NITD008 completely blocked ZIKV replication (Fig. 7).

Fig. 7. Anti-flavivirus drug NITD008 blocks ZIKV infection in HSEC in a dose-dependent manner. HSECs were infected with ZIKV at a MOI 1 followed by the treatment with vehicle or indicated concentrations of NITD008. Supernatants collected at different time points were used to measure viral titers by plaque assay using Vero cells. The error bars represent data from three independent experiments.

Discussion

Considering a sudden increase in microcephaly cases in the recent ZIKV outbreak, there is a significant interest in understanding the pathogenesis of ZIKV in the testes, specifically to identify potential targets for intervention. Based on our results using human HSEC, macrophages and a relevant in vitro SCB model, we propose that ZIKV infects SC with higher efficiency than dengue virus and induces a robust yet balanced antiviral defense response with minimal cell death. ZIKV is released on the lumen side of the in vitro SCB model with higher efficiency than the blood-brain barrier model without affecting the barrier permeability. However, ZIKV-infected HSECs exhibit enhanced adhesion of monocytes, most likely via increased expression of CAM, leading to the disruption of SCB. Our results

also highlight the contribution of macrophages in testicular infection of ZIKV and show that inflammatory mediators derived from infected macrophages have the potential to compromise integrity of the in vitro SCB. Lastly, our study shows that SC can be one of the potential targets of anti-flavivirus compound NITD008 and provides an experimental SCB model to test future antiviral drugs to clear testicular infections.

The SCB, comprised of SC with unique tight and adherens junctions, is critical to preserve the integrity of immune privileged seminiferous tubules by restricting entry of pathogens and systemic immune cells, while simultaneously providing support to differentiating germ cells. Human SC are shown to be the target of other testes-tropic viruses including mumps and HSV [106], but not of HIV and SIV, which are mostly detected in testicular macrophages and T cells [126, 127]. However, Govero and colleagues recently reported presence of ZIKV RNA in mouse testicular cells including SC, spermatogonia and Leydig cells at day 7, which persisted until day 21 after infection in an immunocompromised mouse model [2]. Our in vitro data using human SC is in agreement with the mouse data, and suggests that human SC are also one of the targets of ZIKV infection. Further, a direct comparison of cell-tropism demonstrating significantly reduced replication of DENV in HSEC leads us to speculate that striking differences in the susceptibility of SC to ZIKV and DENV may be one of the reasons why only ZIKV and not DENV is able to establish persistent infection in the testes. Moreover, similar infection kinetics of WNV and ZIKV in SC is not surprising, as WNV is known for wide range of cell- and tissue-tropism as compared to DENV. Previous studies have reported shedding of WNV in the urine in human patients for months after recovery from disease symptoms, however presence of WNV in the seminal fluid of infected

humans is not shown so far. Since WNV requires an avian host for amplification of the virus, the possibility of human-to-human transmission of WNV via sexual route is relatively low.

Our data showing a robust antiviral defense response to ZIKV including production of IFN-α and several inflammatory cytokines agree with a recent in vitro study that reported production of TNF-α, IL-6 and IFN-β in mouse SC, but not in germ cells or peritubular myeloid cells, thus suggesting that SC are one of the major sources of antiviral cytokines in human testes [3]. Similarly, Singh and colleagues recently demonstrated that ZIKV infection in the cells of blood-retinal barrier is also associated with an antiviral defense response [73] suggesting that different blood-tissue barrier cells are able to mount a protective response to ZIKV. Given that many pathogen-recognition receptors including TLR3 and RIG- I are expressed in SC and that the synthetic dsRNA analogue poly I:C can induce cytokine production [128], it is highly likely that detection of ZIKV-derived dsRNA by TLR3 and/or RIG-I is one of the upstream pathways associated with the innate immune response elicited following infection.

In addition to restricting entry of pathogens and immune cells into the seminiferous tubules, another important function of the mammalian SCB is to facilitate movement of developing germ cells into the adluminal compartment of the seminiferous tubules. This unique mechanism of transit of spermatocytes requires restructuring of the SCB via modulating TJP throughout spermatogenesis without compromising the homeostasis of the seminiferous tubules [104]. However, cell adhesion molecules such as ICAM-1 and VCAM-1 and MMP induced by inflammatory triggers including TNF-α and IL-1α play a critical role

in junction disruption and cell movement [6]. The pattern of ZIKV-induced response in the SCB is very similar to WNV-BBB interaction. Our previous studies showed that while CAM such as VCAM-1 and ICAM-1 were induced by WNV in BBB-endothelial cells and mediated leukocyte infiltration in the CNS, the source of MMP involved in the degradation of TJP of the BBB were resident astrocytes [122, 123]. Similarly, severity of vascular leakage induced by DENV is associated with increased levels of VCAM-1 [129], suggesting a common trend of all flaviviruses to induce CAM to facilitate transmigration of peripheral immune cells across the barrier.

Histological analysis has revealed intensive damage of mouse seminiferous tubules accompanied with diminished populations of SC and infiltration of $CD45^+$ leukocytes in the testes at days 14 and 21 after ZIKV infection of mice [2]. A major question that remains is what are the mechanisms by which ZIKV and leukocytes gain access to the seminiferous tubules and cause infection of developing spermatozoa and/or testicular damage? One possible explanation based on our data is that direct infection of SC may represent one of the routes of ZIKV entry into the immune privileged compartment of the testes, but this process may not directly affect the barrier integrity or cause cell death (Fig. 8). The direct consequence of the ZIKV infection of the SC would be to avoid immune detection and be a reservoir to infect developing spermatozoa or elongated spermatids in the lumen, thereby contributing to the persistence of infection even after clearance of viremia. However, ZIKV infection may also increase the susceptibility of the SC to interact with other peritubular immune cells most likely via CAM leading to increased infiltration across the barrier and

contribute to barrier disruption as one of the mechanisms underlying ZIKV-associated damage to the seminiferous tubules.

Circulating monocytes or testicular macrophages in the peritubular compartment regulate the testicular immune environment in a manner that provides protection for the developing male germ cells, while permitting balanced inflammatory responses and protection against infections [125, 130]. However, in experimental autoimmune orchitis and LPS-induced murine models of testicular inflammation, activated macrophages are shown to contribute to the inflammation, ultimately causing massive infiltration in the seminiferous tubules, disruption of barrier junctions, and atrophy of SC [130]. Limited data exists on the infection kinetics of ZIKV in human macrophages, although Quicke and colleagues have recently documented productive ZIKV infection coupled with inflammatory responses in human Hofbauer cells, placental macrophages [86]. Therefore, the demonstration that ZIKV infection in human macrophages follows the same trend of production of type I IFN and pro-inflammatory cytokines as seen in Hofbauer cells, leads to the conclusion that macrophages are capable of mounting a strong antiviral defense to ZIKV.

Since testicular macrophages are in close proximity to the seminiferous tubules, we hypothesize that they contribute to the testicular infection of ZIKV in two ways. First, they could serve as a primary source for ZIKV dissemination to other resident testicular cells including SC and Leydig cells during the viremic phase, and secondly, inflammatory mediators released from infected macrophages may alter the testicular immune environment, thus favoring loss of TJP and leakiness of the SCB as shown in Fig. 8. Our

data demonstrating loss of ZO-1 and decreased barrier integrity following treatment with supernatant from infected macrophages support this notion that SC are susceptible to changes in the local inflammatory mediators. In an in vivo scenario, a direct consequence of ZIKV infection of both SC and testicular macrophages could be an altered local inflammation status leading to an enhanced infiltration of macrophages and adaptive immune cells into the lumen, thereby damaging the developing spermatocytes. However, the role of other cell types, e.g., Leydig cells and peritubular cells, in modulating the local inflammatory milieu and acting as a reservoir of ZIKV cannot be ruled out.

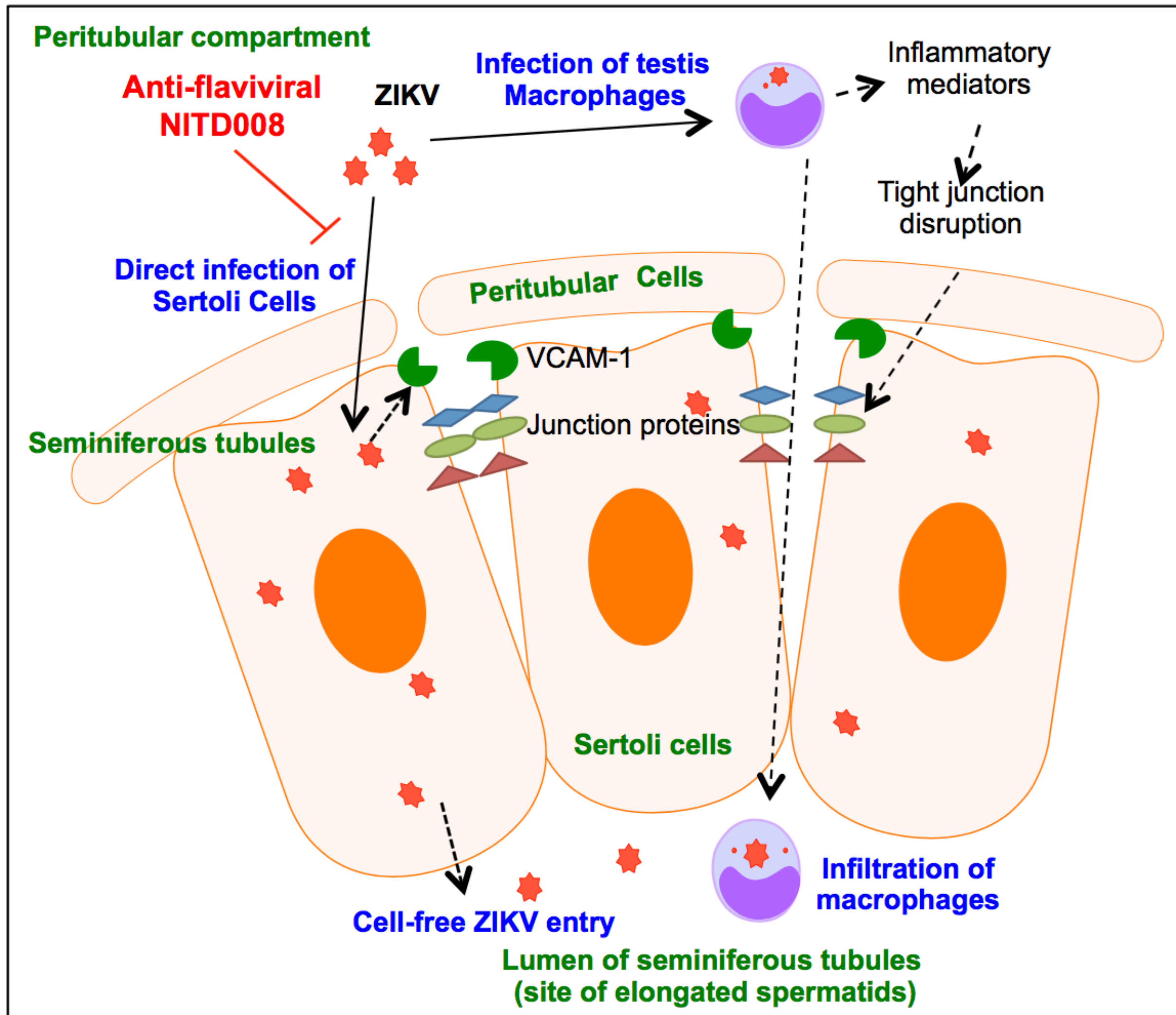

Fig. 8. Putative interactions between ZIKV and the SCB. During viremic stage, ZIKV reaches the peritubular compartment of the testes via peripheral immune cells and infects SC. Infection of SC induces robust innate immune response with no cytopathic effects. The virus replication does not affect levels of TJP or SCB integrity, but is efficiently released on the adluminal side of seminiferous tubules, where elongated spermatids are present. ZIKV infection also induces expression of cell-adhesion molecules like VCAM-1 and ICAM-1 that facilitate adhesion of naïve immune cells to SC and compromise SCB permeability. ZIKV infection in macrophages results in the production of inflammatory cytokines and chemokines that can degrade TJP ZO-1 and directly affect the integrity of the SCB. Further, anti-viral NITD008 is able to completely block ZIKV infection in SC. A direct consequence of ZIKV infection in these cell types may include establishment of persistence in the seminiferous tubules and infiltration of macrophages in the lumen.

In vitro SCB models comprised of human and mouse SC have been used to study mechanisms of SC function in spermatogenesis and effects of environmental toxins on barrier integrity leading to male infertility [116, 117]. However, this study for the first time uses an in vitro model of SCB to characterize virus transmigration kinetics and to understand interactions of infected peritubular leukocytes with SC. Further, our data demonstrating the dose-dependent effect of a well-tested anti-flavivirus compound, NITD008, in blocking ZIKV replication in SC is encouraging and warrants future in vivo studies to test whether this drug can cross the SCB and clear testicular ZIKV infection. A recent study has reported inhibition of ZIKV in Vero cells and in a mouse model by NITD008, however this study did not examine ability of this drug to clear testicular infections [84]. Our in vitro model of SCB can not only be used as a tool to characterize testicular infections of other viruses including Ebola virus, but will also allow rapid testing of new antiviral drugs to cross the SCB and mitigate infection in the seminiferous tubules. In summary, this study provides the much-needed evidence that ZIKV infection of human SC is one of the mechanisms by which this virus might enter the intratubular compartment of the seminiferous tubules to establish persistent infection in testes. The data further provide

insights that disruption of the SCB may be one of the mechanisms by which ZIKV-infected macrophages cause testicular damage and lays a platform for future studies on how ZIKV infection may affect the interaction between SC and other testes resident cells including Leydig cells. Once the specific pathways and cell types involved in the dissemination of virus to developing spermatids are known, the system can be manipulated pharmacologically to test innovative approaches for the treatment of testicular ZIKV infection in humans.

Materials and methods:

Ethics statement: Human monocytes and PBMCs were isolated from fresh blood obtained from healthy donors under a protocol (CHS#24091) approved by the University of Hawaii Institutional Review Board for Human Subjects. All subjects were adult and gave informed written consent.

Cells and virus: Low-passage primary human Sertoli cells (HSEC) purchased from Lonza (MM-HSE-2305) were propagated in DMEM and F-12 media in the ratio of 1:1 with HEPES, L-Gln, 100 units/mL Pen-Strep and 5% untreated FBS according to manufactures protocol. Early-passage HBMVEC purchased from Cell Systems Cooperation (CSC 2M1) were grown as previously described [131]. All experiments of primary HSEC and HBMVEC cells were conducted using passage 6-9 at 80-90% confluency. ZIKV strain PRVABC59 (Human/2015/Puerto Rico) acquired from American Type Culture Collection (ATCC) was propagated once in Vero E6 cells (ATCC). DENV-2 New Guinea C strain and WNV NY99 strains were also propagated once in Vero cells. Stock virus was used for infection at MOI

1 or 5 for 1 hr at 37°C followed by 2 washes and replaced with fresh media. UV-inactivated ZIKV (UV-ZIKV) was generated by exposing the virus stock to UV radiation for 10 min (Stratagene, Stratalinker 2400) as described previously for WNV [131]. In some experiments, HSEC were infected with DENV2 (New Guinea C strain) or ZIKV in the presence of NITD008, an anti-flavivirus compound at different concentrations prepared as described previously [84].

PBMC isolation and monocyte separation: The PBMCs were isolated from 20-40 mL of anti-coagulated blood of healthy donors using Ficoll density gradient centrifugation and monocytes were differentiated into macrophages by culturing adhered monocytes in 24-well plates in X-vivo media for 6 days. For some experiments, monocytes were separated using the EasyStep Negative Selection Human Monocyte Enrichment Kit (StemCell Technologies) as described previously [122] and used immediately for incubation with HSEC monolayers or inserts. By this method, the purity of monocytes was 99% and the viability was 97-99%.

ZIKV quantitation using plaque assay and qRT-PCR: ZIKV titers in the cell culture supernatants were analyzed by a standard plaque assay using Vero cells [131]. Intracellular virus replication was measured by qRT-PCR using primer and probe specific for ZIKV Env region as described previously [132]. The data are expressed as ZIKV PFU per mL supernatant or PFU equivalents per µg RNA.

Analysis of host response: Total RNA extracted from HSEC and macrophages collected at different time points after infection was used to measure changes in the transcripts of multiple innate immune genes, TJP and CAM by qRT-PCR using specific primers as described previously [122, 131]. The housekeeping gene GAPDH was utilized to normalize each gene and fold change as compared to un-infected control was calculated as described previously [131]. The protein levels of TJP (ZO-1, claudin, occludin and JAM-A) and CAM (VCAM-1 and E-selectin) in HSEC were also determined by Western blotting using specific antibodies as described previously [131]. For further confirmation by immunostaining, mock- and ZIKV-infected HSEC grown on coverslips were fixed in 4% paraformaldehyde (PFA) at 48 hrs after infection and immunostained using biotinylated monoclonal mouse or anti-ZO-1 (1:100 dilution) followed by streptavidin 488 conjugated anti-mouse secondary antibodies and examined using an Axiocam MR camera mounted on a Zeiss Axiovert 200 microscope as described previously [131]. Multiple cytokines and chemokines including IFN-α secreted in the cell culture supernatant from ZIKV-infected HSEC and human macrophages were quantitated by Luminex assay using a Milliplex Map Human Cytokine/Chemokine kit (Millipore) and read on a Luminex200 LiquiChip analyzer as described previously [133].

Development of Sertoli-cell barrier (SCB) model, ZIKV infection and permeability assay: An in vitro SCB model was constructed using Corning® Biocoat™ Human Fibronectin PET (polyethylene terephthalate) inserts with a 3.0 μm pore size and 0.33 cm^2 membrane surface area in a 24-well plate. Briefly, after rehydrating the inserts following manufacturer's protocol, they were seeded with 6 x 10^4 HSEC or HBMVEC on the upper surface and

incubated with 500 µl media in both upper and lower chambers at 37°C, 5% CO_2, and 100% humidity. The integrity of the in-vitro SCB model was first determined four days after seeding, then subsequently every 2-3 days by measuring Trans-Endothelial Electrical Resistance (TEER) using an Epithelial Volt/Ohm Meter (EVOMX, World Precision Instrument) and Endohm-6 chamber and expressed as (Ω/cm^2) as described previously [131]. The media was replaced from both the upper and lower chambers during each TEER measurement. Ten days after seeding when the TEER readings crossed 110 Ω/cm^2, the inserts were infected with ZIKV or UV-ZIKV for 1 hr following which the inserts were gently washed twice with HSEC media to remove unbound virus. TEER was measured at 24, 48, and 72 hrs after infection and the upper and lower chamber supernatants (UCS and LCS, respectively) were collected for quantitative virus assays. For some experiments the upper chambers of ZIKV-infected SCB inserts were incubated with either 2.5 x 10^4 naïve PBMCs and monocytes or 350ul of supernatant from ZIKV-infected human macrophages collected at 48 hrs after infection mixed with 150 µl of HSEC media and TEER was measured after 2 hrs of treatment. The integrity of inserts following PBMC/monocytes transmigration and supernatant treatment was also assessed using the FITC-dextran (4-kDA MW, Sigma) transmigration assay as described in our previous studies [131]. The transmigration of FITC-dextran across the inserts was calculated as percentage of the total amount added in the upper chamber.

Adhesion assay: Confluent monolayers of HSEC cells grown on coverslips in 24 well plates were infected with ZIKV at MOI 1 and 5. At day 3 of infection, 2.5 x 10^4 naïve monocytes or PBMCs were added on the coverslips and co-cultured with HSEC for 2 hrs

at 37°C. At the end of the incubation period, the non-adherent monocytes or PBMCs were removed by washing four times to remove loosely attached cells. The co-cultured monolayers were fixed in 4% PFA for 10 minutes and were stained with FITC conjugated anti-CD45 (a surface leukocyte marker) diluted 1:100 and DAPI for nuclear staining as described previously [122]. Controls included uninfected HSEC cells co-cultured with leukocytes. The number of leukocytes bound to the monolayers was determined by counting the number of adherent $CD45^{+}$ cells in two central and two peripheral randomly selected fields of at least 3 coverslips from two independent infections.

Cell viability assay:

Cell viability was measured in HSEC 72 hours after infection with ZIKV, DENV, and WNV using the Promega CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (Catalog # G3582). Briefly 20µL of One Solution Reagent was added to both mock- and viral-infected wells containing 100µL of HSEC media. Wells were incubated for 2 hours before reading the absorbance at 490nm on a Victor X Plate Reader.

Statistical analysis: ZIKV virus titer, mRNA fold-change and Luminex data are reported as mean ± SEM of at least triplicates from two independent experiments. Unpaired Student t-test was used to compare the values of permeability and transmigration assays using GraphPad Prism 5.0 0 (GraphPad software, San Diego, CA). $p<0.05$ was considered as statistically significant for all analyses.

Acknowledgments:

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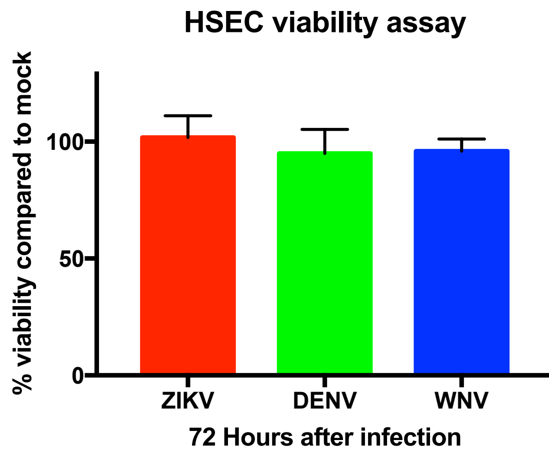
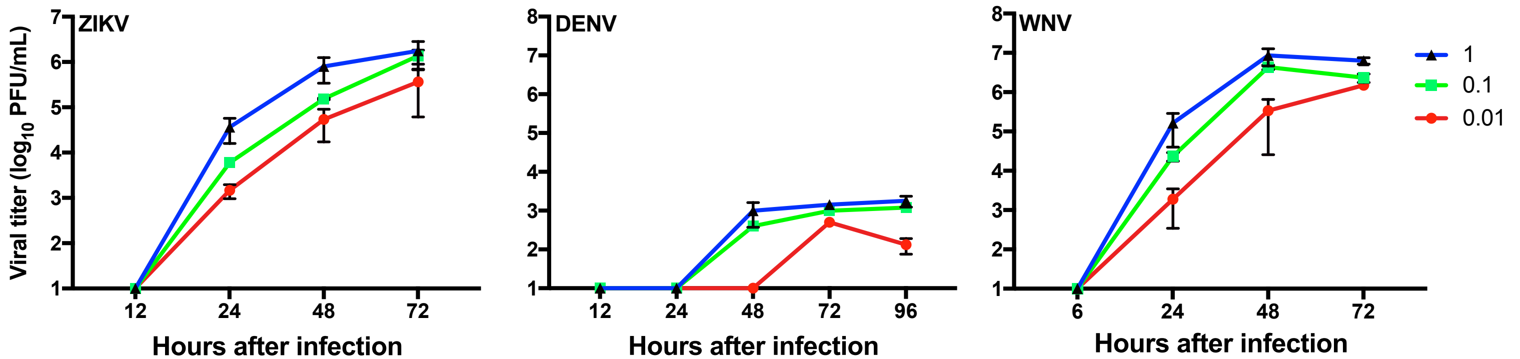
Supplemental figures:



Supplemental Fig. 1: Infection with ZIKV, DENV, and WNV does not result in CPE or cell death in HSEC Cell viability was measured in HSEC 72 hours after infection with ZIKV, DENV, and WNV at MOI 1 using the CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay.



Supplemental Fig. 2: ZIKV, DENV, and WNV cause productive infection in HSEC HSEC were infected with ZIKV, DENV, and WNV at MOIs 0.01, 0.1, and 1.0. Virus titers were quantified by plaque assay using Vero cells and expressed as PFU/mL supernatant.

Chapter 4

Summary and future directions

Summary:

Re-emerging ZIKV is a major health concern as it continues to expand to new regions of the world with increased cases and disease severity. Contributing to this current outbreak are unique features of the virus such as its ability to infect a wide range of tissues/organs, invade immune privileged organs such as the brain, eye, and testes, and to be transmitted both sexually and *in utero.* Shockingly, these findings have never been described with old world ZIKV and it is not entirely clear whether these disease characteristics were undiagnosed in previous outbreaks or are because of mutations in the viral genome and/or ADE like phenomenon as suggested by few experts in the field [24]. We believe that as sexual transmission increases the risk of infection, prolongs current outbreaks, and represents a serious threat in women of reproductive age, it is of the utmost importance to study the cellular and molecular mechanisms responsible for both prevention and disease management.

When we initiated this study, case reports on the newly documented phenomenon of ZIKV sexual transmission and virus presence in human seminal fluid were the only available literature [35, 36, 114]. Later, few in vivo NHP and mouse models provided the first evidence that ZIKV was able to target the testes in the male reproductive tract and provided the strongest scientific premise to take up this study [2-5]. Based on the immune privileged nature of the testes and association of ZIKV with the cellular portion of seminal fluid and sperm, we formulated the hypothesis that ZIKV will infect human Sertoli cells and cross the Sertoli-cell barrier to establish a persistent infection in the seminiferous tubules. Our overall objective, therefore, was to characterize ZIKV replication kinetics and

immune responses in primary human Sertoli cells (HSEC) and develop an in vitro SCB model to understand mechanisms of ZIKV transmigration across the SCB.

For specific aim 1, we utilized HSEC to compare infection of the recently isolated Puerto Rican strain of ZIKV (PRVABC59) with related flaviruses such as DENV-2 (New Guinea C strain) and WNV (NY99) at three different MOIs (0.01, 0.1, and 1.0). We found that HSEC were highly susceptible to ZIKV with viral titers peaking at 48 and 72 hours after infection suggesting that they may be one of the targets of the virus in the testes. HSEC also supported robust infection of WNV and this finding was at first surprising since WNV has so far not been labeled as a sexually transmitted pathogen. However, it is well established that WNV has far more wide range of tissue tropism as compared to DENV, which may explain our data. Also, due to the fact that WNV requires birds as an amplifying host in nature to achieve high virus titers to induce viremia and human disease, we speculate that even if in rare cases WNV infects testes, it may not be able to sexually transmit as the virus may not achieve titers high enough in the semen to induce viremia in their partners. Another interesting finding was that DENV produced much lower viral progeny than ZIKV and WNV thus indicating that flaviviruses exhibit different infection kinetics in SC.

The first aim also sought to characterize innate immune responses and barrier integrity markers in HSEC and MDM following ZIKV infection. Our results support our hypothesis and demonstrate robust antiviral and inflammatory responses in both HSEC and MDM. These data also support previous studies that indicated that SC could participate in

immune responses to other testes-tropic viruses [106]. Another novel finding of this aim is that direct infection of ZIKV does not affect the expression of TJP, which are critical for maintain the SCB integrity. As the data on the replication kinetics of ZIKV in MDM is relatively limited, our results from human MDM are also important in understanding peripheral immunity to the ZIKV. Finally, our data demonstrating complete blockage of ZIKV infection by NITD008 in HSEC highlights the potential for in vivo testing of this drug to treat testicular infection.

The objective of the second aim was to test the hypothesis that ZIKV infection of HSEC and macrophages lead to disruption of the in vitro SCB model. This model had only been used to study the mechanisms of SC function in spermatogenesis and the effects of environmental toxins on barrier integrity, therefore our study was the first to utilize the model to examine virus transmigration kinetics. We found that ZIKV was able to successfully transmigrate across the in vitro SCB barrier to the adluminal side of the membrane. Interestingly, infection also increased the ability of peripheral immune cells to adhere to the HSEC monolayer, which correlated with an increase in barrier permeability. Lastly, inflammatory mediators secreted from ZIKV infected macrophages were also found to compromise the barrier integrity and suggest that inflammatory mediators released from ZIKV-infected cells in the peritubular compartment possess the potential of disrupting the SCB. Our results collectively supported the overall hypothesis of this aim and provided strong support for the use of this in vitro SCB model to delineate interaction between different testicular cells.

Overall, our data suggest that infection of SC may be one of the crucial steps by which ZIKV gains access to immune privileged seminiferous tubules. We propose that SC may serve as a reservoir for infection of other resident testicular cells including spermatogonia and developing spermatozoa. Furthermore, since testicular macrophages are in close proximity to the seminiferous tubules, we strongly believe that they could serve as a primary source of ZIKV dissemination to resident testicular cells and release inflammatory mediators that may alter and affect the structure of the SCB. Although this study provides useful insight into the mechanisms of ZIKV pathogenesis in the human testes and serves as a proof of concept, additional follow up studies both in vitro and in vivo will be needed to further delineate the role of other cell types in ZIKV testicular infection.

Future directions:

The data obtained from this study opens up many new avenues of investigation into the mechanisms of testicular ZIKV infection. Since the specific cell receptor/receptors that ZIKV utilizes to enter host cells is currently under intense investigation, the most interesting future direction would be to identify the cell receptors involved in ZIKV entry into Sertoli cells. Previous studies have demonstrated that certain TAM proteins enhance infection of DENV, WNV, and YF in astrocytes and epithelial cells [134]. TAM proteins specifically belong to a family of receptor tyrosine kinases (RTKs), which includes Tyro3, Axl, and Mertk. These RTKs have two common ligands, namely, growth arrest specific gene 6 (Gas6) and protein S (ProS) **(Figure 1)** and the TAM-Gas6/ProS complex plays critical roles in both the phagocytic removal of apoptotic cells and regulating immune responses [105, 134].

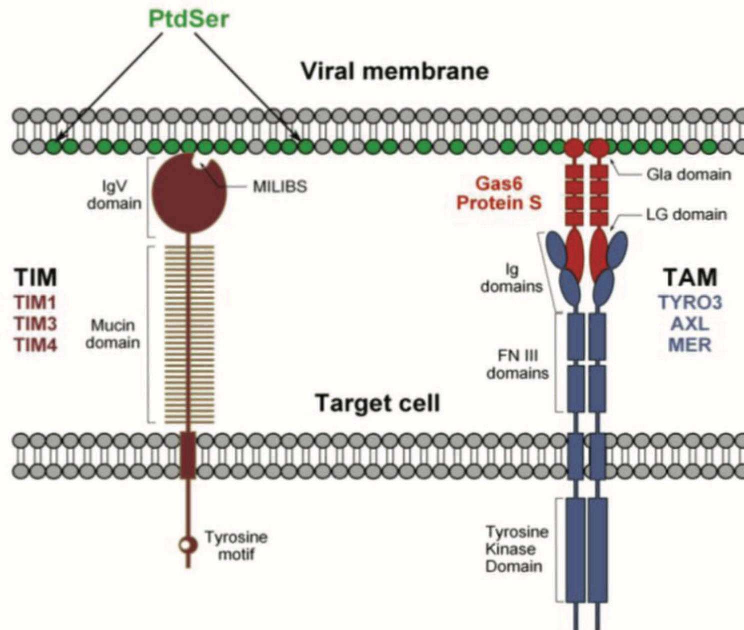


Figure 1: Hypothetical model for flavivirus recognition by TAM receptors [134]

Recent studies have shown that TAM receptors, Axl in particular, are involved in ZIKV entry in skin fibroblasts, glial cells, and neural stem cells [27, 135, 136] [137]. However, it appears that there is some cell-specificity in dependency of Axl as an entry receptor as Wells et al. demonstrated that blocking Axl receptor did not limit ZIKV entry and infection in human neural progenitor cells and cerebral organoids [138]. This suggests that ZIKV may use more than one receptor to enter and that may define higher replication of the virus in one cell type versus another.

Interestingly, the testes are one of the few organs that expresses high basal levels of TAM receptors. SC are specifically known to highly express Axl on their surface that is thought to help maintain homeostasis [105]. Since our data suggests that HSEC are one of the targets of ZIKV in human testes, the next logical extension would be to determine

if Axl plays an essential role in mediating ZIKV entry into these cells. Furthermore, I would like to investigate whether ZIKV infection in HSEC increases the secretion of Gas6, Axl ligand. If the Axl-Gas6 complex can bind to ZIKV and facilitate its entry, it could be taken advantage for the development of therapeutic strategies to prevent testicular infection.

Although our data indicated that SC induced robust innate immunity to ZIKV, the specific PRRs associated with detection or ZIKV and downstream pathways of innate immunity are yet to be defined. Therefore, another important follow up direction would be to investigate what are the upstream PPR pathways activated by ZIKV in SC. This could be accomplished by utilizing high throughput next generation RNA-sequencing (RNA-seq) technology, which would allow comprehensive analysis of global host response to ZIKV and help us identify if PRRs such as TLR3 or RIG-I and downstream pathways are activated by ZIKV. Additionally, RNA-seq will also potentially identify other important pathways/networks involved in SC-germ cell signaling as well as apoptotic and cell cycle arrest pathways. Activation of these pathways may further suggest that ZIKV can affect the function of the seminiferous epithelium and spermatogenesis. Collectively, this information will provide mechanistic clues to ZIKV testicular infection and lay platform for future studies to assess function of key host pathways in ZIKV persistence in the testes.

Finally, while this study supports the strength of SCB model in understanding human testicular infection of ZIKV, it also opens up opportunities to test other in vitro models that more closely represent human testes. A major limitation of the monolayer cell cultures is that they do not allow assessment of the interaction of multiple testicular cells, specifically

between SC and developing spermatozoa. Given that the existing immunocompromised animal models also have their limitations regarding applicability to human disease, there is a need to develop novel tools to study human testicular virus infections. One available model is the three dimensional (3D) human testicular organoid (HTO) culture system, which was recently developed for drug-screening and human testicular function [139]. Specifically, this system is composed of spermatogonial stem cells (SSC), Sertoli, Leydig, and peritubular cells isolated from human testicular tissue, which grow together to form organoids and closely mimics the human testicular microenvironment [139]. Furthermore, in vitro studies have demonstrated that this model produces testosterone continuously, maintains more than 85% viability throughout long-term culture of 23 days, and provides evidence of differentiation of SSC into meiotic spermatogonia [139]. This 3D HTO culture system could be utilized as a novel tool to address specific questions that cannot be assessed in a monolayer culture system. Because the 3D HTO culture system allows differentiation of germ cells, it could be used to evaluate the effect of ZIKV infection on different stages of spermatogenesis.

Collectively, the information acquired from our study and the proposed future experiments may provide mechanistic insight into the testicular pathology and persistence of ZIKV and may identify therapeutic targets to combat the virus. In conclusion, active research is warranted in delineating pathogenesis of ZIKV in human testes as it will ultimately help inform public health guidelines, impact the development of novel therapeutic strategies, and the ultimate goal of decreasing the disease burden and ZIKV-associated birth defects worldwide.

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