Zika Virus Infection Modulates Expression of Regulatory Complement Factors in SH-SY5Y Cells

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Abstract

Viruses that use the blood as a route of systemic spread frequently encode proteins allowing evasion of the complement system. Although complement neutralizes pathogens in the blood, the complement system has non-immune functions in the central nervous system (CNS) and mediates non-viral neuropathologies when triggered by damaging stimuli.

As viruses capable of both hematogenous spread and neuroinvasion, flaviviruses such as Zika virus (ZIKV) are a significant public health issue as the most common cause of arboviral disease. Outside of the brain, flaviviruses can modulate levels of complement proteins and surface bound complement regulators. We hypothesized that similar alterations occur in CNS cells, contributing to viral neuropathology.

In this study, we establish the relevance of viral complement modulation in infection of CNS cells. Using various CNS culture systems, endogenous expression of complement proteins and complement-related surface markers was investigated. In a co-culture system of cell lines SH-SY5Y (neuroblastoma) and HMC3 (microglia), endogenous complement protein production was confirmed in absence of Zika infection and stimulation. Furthermore, Zika infection modulated expression of surface bound inhibitors CD46 and CD55. Compared to mock, CD46 was upregulated in both infected and non-infected, bystander cells with stronger expression in infected

cells. CD55 was equally downregulated in infected and bystander cells. Finally, affinity between ZIKV non-structural protein 1 (NS1) and complement proteins was demonstrated.

These results support previous studies implicating complement modulation as a source of pathology in neuro-invasive flavivirus infection, providing insight on the precise biochemical changes behind this process.

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Preface

Firstly, I would like to thank Dr. Ernesto Marques for his mentorship and for the trust and creative freedom afforded to me on this project. I thank Dr. Priscila Castanha and future doctor Chen Yang who have made coming to the lab a daily joy. I also thank my committee members Drs. Velpandi Ayyavoo and Leonardo D'Aiuto for their instruction and roles in my scientific training. I could not have asked for better mentors or colleagues. I am indebted to the countless individuals (teachers, friends, and family) who have potentiated my science career and appreciate that practicing and advancing science is a privilege. During this time of pandemic, the importance of infectious disease research is brought to the forefront of the public conscience and I endeavor to continue as one of many scientists making incremental gains in combatting these diseases.

1.0 Introduction

Circulation of Zika virus (a flavivirus) is concerning in light of its association with severe neurologic and neuro-developmental disease (1, 2). Currently, there are no approved treatments or vaccines available to address disease caused by Zika virus. In the context of central nervous system (CNS) infection, identifying sources of pathogenesis could inform treatment strategies as well as vaccine design.

The complement system, a key part of the innate immune system, achieves a dynamically regulated balance which is perturbed in response to pathological or pathogenic insult. Accordingly, previous studies have implicated complement overactivation and dysregulation in severe flaviviral disease in and outside of the CNS (3, 4). However, the complement system has also been revealed to have key roles in normal functioning and development of the human brain (5-7). While finely controlled complement processes serve to maintain cellular health, overactivation of complement has been associated with disease-causing processes such as neurodegeneration (8-10).

These results lead us to hypothesize that dysregulation of complement plays an important role in Zika pathology of the brain. The connections between flaviviruses, complement, and the specific context of the CNS will be explored (Figure 1).

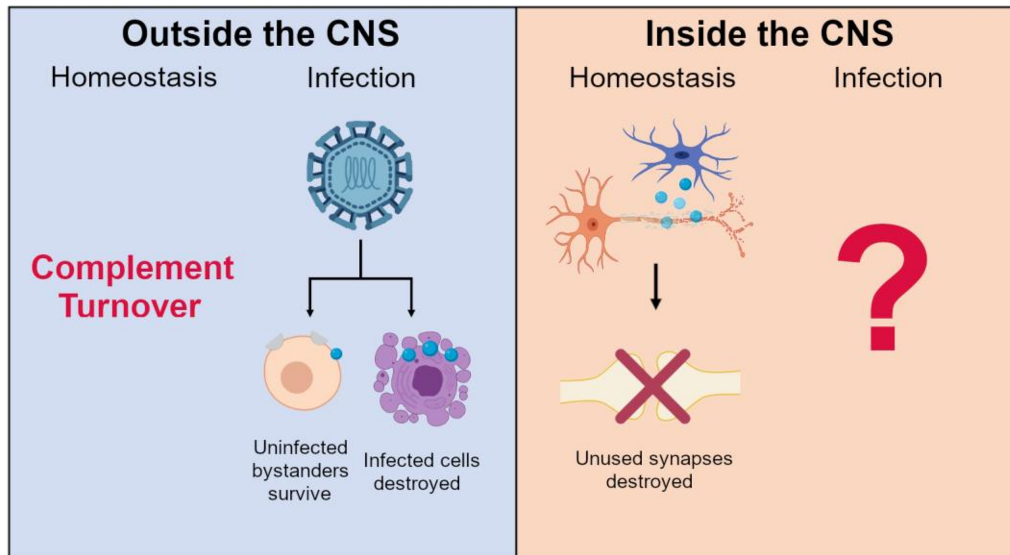

Figure 1 Distinct Roles for Complement in Immunity and Neurodevelopment Interact in the CNS

Complement has immune and neurodevelopmental functions which share common regulatory programs. During infection, these functions are hypothesized to intersect, potentially leading to poor outcomes.

1.1 Complement: Multiple Functions in the Immune System and CNS

1.1.1 Complement in the Periphery

Complement proteins have traditionally been regarded as soluble, serum factors circulating throughout the body (Figure 1). Comprised of over 60 factors and receptors, new functions for complement are being discovered despite early recognition of its role in the blood. Such functions span immunity, wound healing, cell homeostasis, and differentiation (11).

Principles of the complement system echo those of intracellular signaling pathways. Complement proteins function as proteases, amplifying localized production of active components

through cascades. There are three main pathways initiating the complement cascade—the classical, lectin, and alternative pathways.

• The classical pathway is initiated by binding of the C1 complex to a variety of ligands including pathogen associated molecules, C-reactive protein, and antibodies (primarily IgG and IgM).

• The lectin pathway recognizes carbohydrate groups on cells through mannose binding lectin (MBL) or ficolin.

• The alternative pathway primarily serves as an amplification loop for the other pathways although spontaneous activation of the alternative pathway can occur through hydrolysis of C3.

These three processes of initiation feed into a common pathway, typically leading to either formation of the terminal complement complex (TCC) or complement-enhanced phagocytosis upon successful activation. C3 and C5 cleavage also results in the production of anaphylotoxins C3a and C5a, both potent inducers of inflammation.

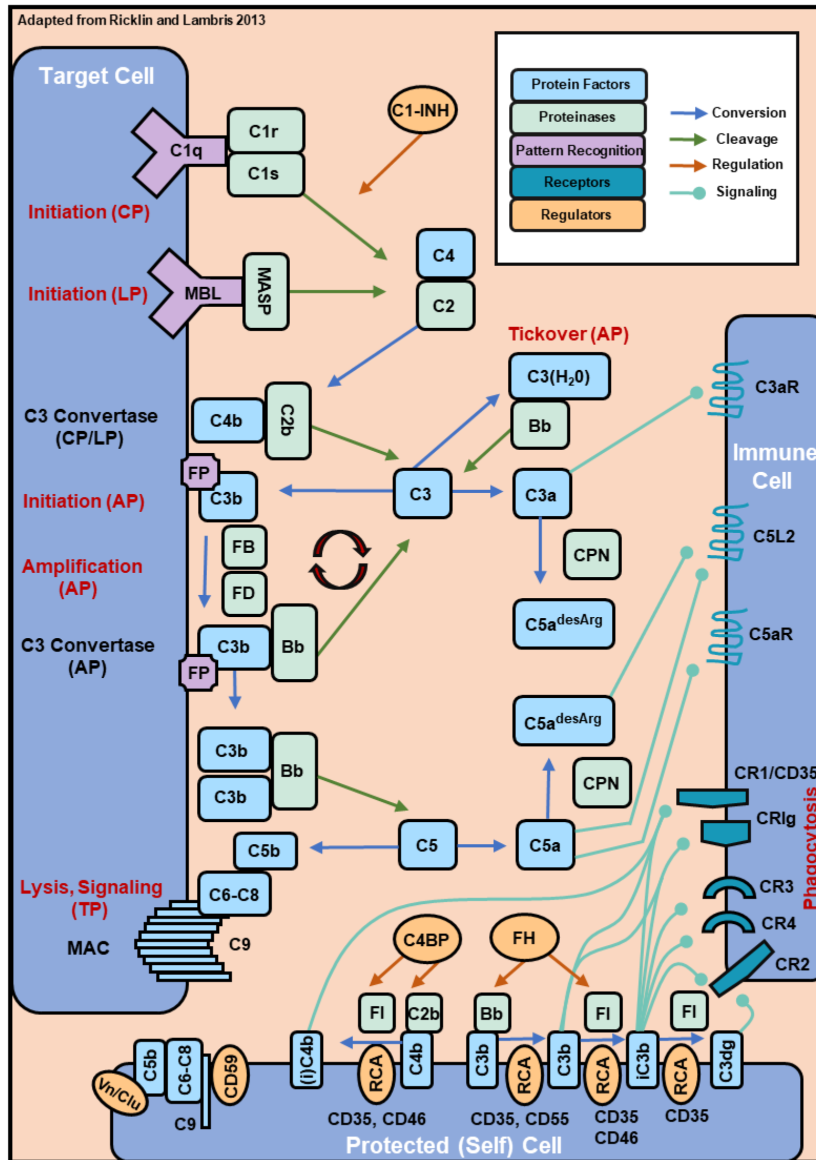

Figure 2 Overview of the Complement System

The three complement pathways differ in initiation but link to a common pathway downstream of C3. Various byproducts produced during these pathways have corresponding receptors, initiating cellular programs. Cells under homeostatic conditions retain surface-bound regulators of complement. Abbreviations: AP, alternative pathway; C1-INH, C1 inhibitor; C3aR, C3a receptor; C4BP, C4b-binding protein; C5aR, C5a receptor; C5L2, C5a receptor-like 2; Clu, clusterin; CP, classical pathway; CPN, carboxypeptidase-N; CR, complement receptor; FB, factor B; Fcn, ficolins; FD, factor D; FH, factor H; FI, factor I; LP, lectin pathway; MAC, membrane attack complex; MASP, MBL-associated protease; MBL, mannose-binding lectin; RCA, regulator of complement activation; Vn, vitronectin (12)

Recently, an intracellular activation pathway termed the "complosome" has been described. Active components of complement—cleaved C3 and C5—have been identified inside T and B cells (13). Furthermore, intracellular localization of C3b has been found in isolated monocytes, neutrophils, epithelial cells, endothelial cells, and fibroblasts (14). In lymphoid-derived cells, this activation promotes effector functioning and cell homeostasis. However, the broad consequences of complosome activation in other cells have yet to be determined.

Rather than inert, C3 is continuously cycled and cleaved during homeostatic conditions. Defined as C3 "tick-over", this occurrence permits rapid responsiveness of the alternative pathway (15, 16). An apt metaphor is that of a sonar system which detects objects by constantly surveilling the environment (17). Deposition and turnover of soluble complement occurs at a steady rate, decoupled from effector functions by local complement regulatory factors. Only when cleaved products accumulate past a threshold do these functions trigger. Reasonably, the balance between pro-activation members and regulatory proteins must be carefully maintained to prevent non-specific complement engagement.

Naturally, there are several proteins involved in regulation of the different pathways (Table 1). Regulation of the complement system largely occurs through inhibition of cleavage, cleavage to inactive products, dissociation of active complexes, or context-specific expression. Regulatory factors act at different stages of activation, allowing for tight control under homeostatic conditions. A key regulatory axis involving CFI occurs on the surface of C3b-bound cells. Complement factor I (CFI) converts C3b to iC3b with the assistance of complement factor H (CFH) and CD46, steering the cascade away from TCC formation but towards phagocytosis mediated by iC3b recognizing receptors such as complement receptor 3 (CR3; composed of CD11b and CD18) (18). Another axis involves depolymerization of C3 convertases. C4b-binding protein (C4BP), CFH,

and CD55 act to prevent further deposition of activated complement fragments, leading to resolution of the cascade. Finally, regulators CD59, vitronectin, and clusterin directly prevent TCC formation by competing with active components. Of these diverse regulators, surface-bound CD46, CD55, and CD59 act to limit off-target complement activation on bystanders (19).

1.1.2 Complement in the Brain

Besides providing protection against pathogens, complement is involved in regulated processes of neural development and homeostasis—specifically neuronal differentiation, proliferation, migration of progenitors, and synaptic pruning. However, complement also contributes to neurodegenerative diseases with the complement system playing a role in a wide array of CNS-related conditions including Alzheimer's, multiple sclerosis, and ischemic stroke (20-23). Consequently, perturbation of complement's natural balance could lead to some of the neurological complications found in adult Zika infection and congenital Zika syndrome (CZS).

In the periphery, a variety of cell types produce complement proteins although the liver is primarily involved in complement biogenesis and is the main source of serum C3 (24). As serum proteins experience difficulty crossing the intact BBB, complement factors of the brain are regarded as being produced within the CNS (25).

Throughout the brain, complement factors and corresponding receptors are expressed by discrete cell populations. As immune-responsive cells, neurons and astrocytes both produce select complement components as well as factors modulating complement expression (26-28). Microglia additionally display surface receptors for activated complement products and dynamically respond to the complement milieu. Based on preliminary *in vitro* studies conducted in the Marques and Nimgaonkar lab, neurons and microglia are likely sources of C3, C1Q, and C4 in the CNS

(Unpublished). Additionally, co-cultures of pluripotent stem cell-derived neurons, astrocytes, and microglia demonstrate minimal to no levels of terminal complement proteins. Consequently, the CNS may limit terminal complement formation by selective expression of components and experimental findings indicate a lack of polymerized terminal complement components (C5b9) in the brain when BBB integrity is maintained. Although not definitive, BBB integrity may influence levels of complement within the CNS, confounding claims of endogenous production (29). Finally, it is unclear if complement levels in the brain and cerebrospinal fluid (CSF) are high enough to meaningfully impact viral titers via neutralization; whereas incubation of virus with complement-containing serum clearly reduces infectivity, similar experiments using CSF have not been conducted or are obscure.

Complement patterns the developing brain, promoting cell-specific migration and differentiation. Early evidence for a complement developmental axis was derived from phenotyping of knockout mice. Knockout mice for various complement factors of the classical and lectin pathways exhibit impaired neuronal development with behavioral abnormalities. Involvement of the alternative pathway appears limited; knockout mice of Factor B, an essential component of the alternative pathway, develop normally with no reproductive or behavioral abnormalities (30).

The developmental functions of complement are linked to generation of anaphylotoxins, C3a and C5a. C3a signaling in the neural crest was found to promote cell migration of neural crest cells (7). Additionally, the lectin pathway (initiated by MASP-1 and -2) is integral for C3a-dependent neuronal migration. Expression of complement proteins is highly regulated temporally and spatially in these processes. Gorelik et al. interrogated transcriptional dynamics of Masp-1, Masp-2, C3, C3aR and C5aR using fetal tissues and found expression levels to be dependent on

developmental stage (31). MASP-1, MASP-2, and C3 were expressed in a radiating pattern throughout the fetal brain with low levels of expression in the ventricular zone and higher levels near the cortical plate (31).

Unlike C3a which plays a role in cell migration, C5a is involved in proliferation and differentiation. It is suspected that production of C5 occurs in an autocrine manner from hNPCs in neural rosettes. Mice studies have demonstrated C5a signaling through C5aR1 promotes hNPC proliferation and maintains cell polarity (32). These events are spatially limited to the apical (facing the lumen) surface of the ventricular zone and temporally limited to early stages of neural development as expression of both C5 and C5aR1 decrease after hNPCs differentiate. Finally, C5a appears at higher levels within CSF of developing embryos than adult CSF, mirroring its role in early development (32).

Synaptic pruning describes the coordinated removal of a synapse between two neuronal cells. This process contributes to key cognitive phenomena such as learning and forgetting but cognitive impairment when dysregulated (33). Physiologically, synaptic pruning matures neuronal circuitry by removing "excess" connections (Figure 3).

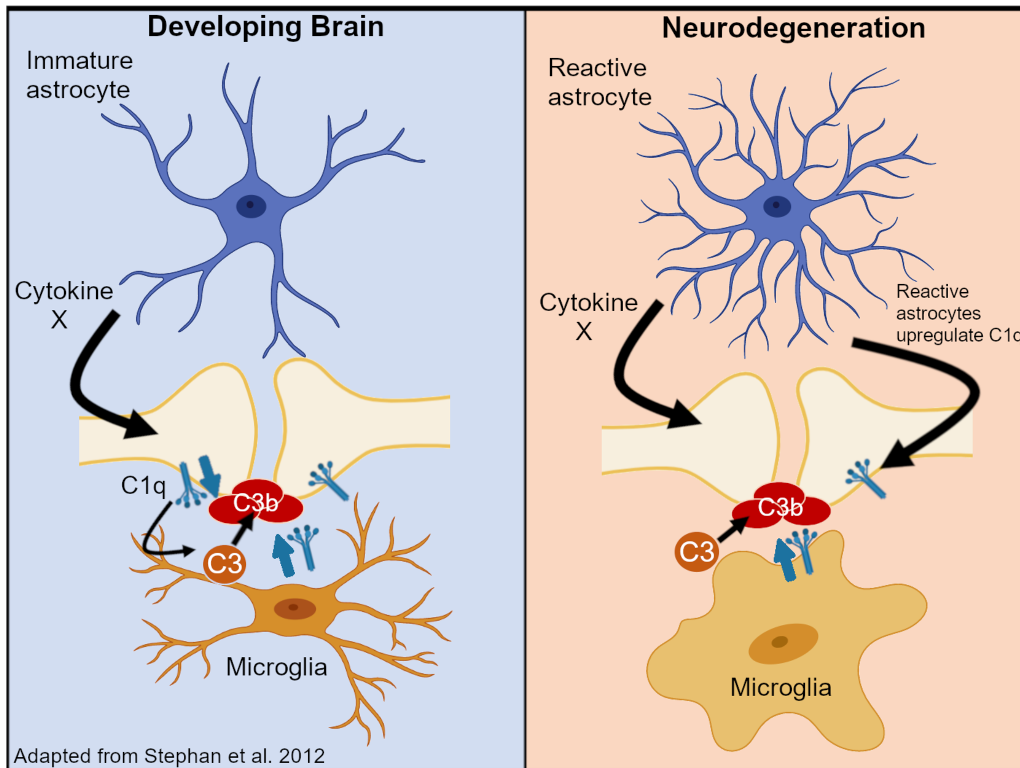

Figure 3 Synaptic Pruning Relies on Elements of the Complement System

Left: In the absence of activated complement, synapses remain stable. In the developing retina, astrocytes can induce expression of C1q in retinal ganglion neurons through TGF-β. Neuron and/or microglial C1q will initiate the classical cascade on synapses to be pruned. Synapses covered in C3b products are subject to phagocytosis. Right: Neurodegenerative pruning involves similar effectors. Reactive phenotypes for microglia and astrocytes are highly inflammatory and lead to the upregulation of cytokines ("X") and complement factors. Secretion of these factors instigates complement activation and loss of synapses in pathological conditions (27).

Under homeostatic conditions, less active synapses trigger the process of pruning. Although details remain unclear, current models assert under-utilized synapses initiate crosstalk with adjoining astrocytes and microglia (34). Crosstalk—entailing the exchange of soluble factors—enables surface expression of pro-phagocytic proteins ("Eat me" signals), reduction of anti-phagocytic markers ("Don't eat me"), increased soluble complement, and chemoattraction of microglia to the synapse (35). Following migration, synaptic pruning is believed to function

through microglial phagocytosis enhanced by the classical pathway (6, 9). Consequently, C1q and C3 have been implicated as mediators.

Microglia are the primary source of C1q as microglial-specific deletion in mice results in near complete ablation of C1q production in the brain (36). Retinal ganglion neurons are also capable of C1q expression in presence of astrocyte-derived TGF-β (26). While C1q deposition on synapses initiates the classical pathway and subsequent C3 deposition, C1q binding elicits other direct effects. C1q deposition leads to local activation of apoptosis-like processes in axons and the corresponding receptor C1qR enhances phagocytosis upon ligation, both potentially promoting engulfment independent of downstream C3 cleavage (37, 38). However, C3 clearly plays a role in many pruning or pruning-like mechanisms. Blockage of CR3 (which recognizes surface iC3b) on microglia abrogated synaptic pruning (6). It is unclear if the alternative pathway can effectively initiate synaptic pruning during physiologic pruning. However, such initiation may be feasible in multiple sclerosis as evidenced by mice demonstrating C3 but not C1q on actively pruned synapses and synapse loss in C1qa knockout mice (23, 39).

1.2 Flaviviruses

1.2.1 Clinical Spectrum of Zika CNS Infection

While the existence of ZIKV was known since 1947, research was minimal until the World Health Organization declared ZIKV an international health emergency. During the 2015 ZIKV outbreak, incidence of severe neurological symptoms alarmed health professionals around the world. Coupled with a propensity for mild or asymptomatic acute infection, diagnosis of ZIKV

infection in early stages can be challenging. More recently, clinical and experimental evidence suggests ZIKV can induce pathology within the brain (with associated neurodevelopmental or cognitive delay) in the absence of other overt symptoms (40-42). The possibility of such "silent pathologies" have drawn attention in both children and adults (Figure 4).

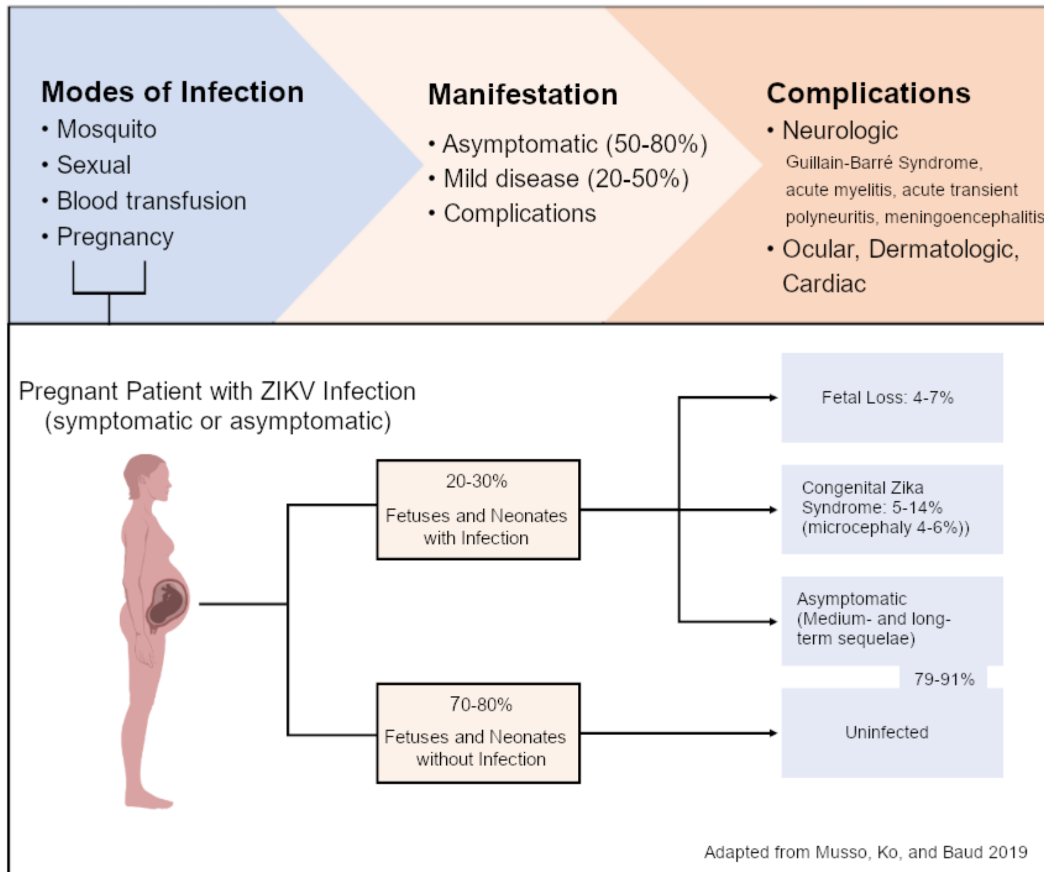

Figure 4 Clinical Manifestations of Zika Infection

The vast majority of Zika infections are asymptomatic. In rare instances, patients can manifest severe neurologic or neurodevelopmental symptoms (43).

Recognition of an association between ZIKV infection and microcephaly fueled global concern during the Brazilian outbreak. However, the consequences of fetal ZIKV infection

(termed congenital Zika syndrome [CZS]) represent a broad spectrum of neurological presentations. This heterogeneity is associated with time of maternal infection due to the uneven timing of human neurogenesis, the bulk of which occurs before gestational week 25 (44). One of the earliest post-mortem studies of a ZIKV-infected neonate, Mlakar et al. presented findings of complete agyria, regions of calcification, ventriculomegaly, diffuse microglial and macrophage infiltration, yet a general lack of inflammation (45). Driggers et al. described a single case exhibiting smaller brain size, calcification, diffuse infiltration of macrophages, and partially differentiated neuronal cells in the neocortex (1). Through pathological examination of ten ZIKV-infected neonates, Chimelli et al. categorized cases into three groups: neonates with severe ventriculomegaly; neonates with low total brain mass and mild to moderate ventriculomegaly; and neonates with localized calcification yet little overall morphological change (41). This third group was associated with maternal exposure in late-term pregnancy. In aggregate, these diverse pathologies most commonly manifest as neurodevelopmental delay and ophthalmic abnormalities (43). Although no analogous evidence exists in humans, infection of animal models in early life also leads to poor outcomes with both mice and rhesus macaque models demonstrating behavioral abnormalities and persistent anatomical changes in the brain (46, 47).

Adult CNS infections can lead to equally diverse outcomes with findings such as encephalitis, meningoencephalitis, and myelitis (48, 49). An atypical Guillain-Barre syndrome demonstrating CNS involvement is also possible leading to functional changes detectable by neuroimaging in the acute period (50). While neurological symptoms in adult infection are relatively rare, it is possible silent pathologies exist in adult infection as well. Adult mice models of CNS infection demonstrate memory decline in absence of mortality (42, 51).

1.2.2 Zika virus tropism in the brain

Neuroinvasion is exhibited by many flaviviruses. West Nile, Zika, Japanese encephalitis virus, and tick-borne encephalitis virus clearly demonstrate neuro-invasiveness although Dengue and Yellow Fever viruses are capable of CNS infection (52, 53).

In maternal infection, the mechanism ZIKV uses to gain access to the fetal brain is still undescribed. The route from site of maternal exposure to the fetal brain is a circuitous one. ZIKV infection presumably spreads through systemic maternal circulation, bypasses the placental interface, enters fetal systemic circulation, bypasses the fetal blood brain barrier (BBB), and penetrates deeper into the CNS. This path necessitates circumvention of two cellular barriers: the placenta and fetal blood brain barrier. Accordingly, flaviviruses are highly capable of circumventing immunity at cellular barriers with such bypass linked to flaviviral nonstructural protein 1 (NS1) (54, 55).

Bypass of the BBB could be destructive or non-destructive. BBB breakdown was demonstrated in adult mice models, allowing infiltration of leukocytes into the CNS (56). However, degradation could be secondary to ZIKV bypass of the BBB. Infection of human brain microvascular endothelial cells simulating the BBB *in vitro* allowed passage of competent ZIKV into the CNS with minimal disruption (57, 58). Correspondingly, variable levels of BBB permeabilization or damage were demonstrated in animal studies (56, 59, 60).

Neurotropism of ZIKV has been demonstrated *in vivo* and *in vitro*. Several experimental models including mice, non-human primates, explants, and human organoids exhibit active replication in CNS cells with maternal or direct inoculation (61-65). Neurons, neuroprogenitor cells, neural stem cells, astrocytes, and microglia are all susceptible to ZIKV infection. Once infection is established within the brain, virus can persist despite clearance in the periphery (66).

Tropism across models is relatively consistent. Myelinating cultures showed wildtype and *Ifnar1*-deficient mice CNS but not PNS cells were ZIKV permissive (67). In adult *Ifnar* $^{-/-}$ mice, astrocytes and neurons could support viral growth, a pattern seen throughout the brain (56). Isolated human fetal brain phagocytes including microglia demonstrated susceptibility to viral infection (65). ZIKV can also stably infect human neuroprogenitor cells (hNPC) *in vitro*, affecting their differentiation and growth (68, 69). Lin et al. included comprehensive immunostaining of fetal tissues from second trimester donors, presenting a more nuanced view of hNPC tropism. In accordance with *in vitro* models, intermediate progenitor cells (a type of hNPC) appeared highly permissive to ZIKV. However, the sparse co-localization of ZIKV envelope protein and either SOX2 or nestin found in donor brains suggest neuroepithelial cells and radial glial cells (also hNPCs) were largely un-infected. Lin et al. confirmed that while hNPCs were permissive in the context of neural monolayers, hNPC infection was rarer in donor samples. The absence of infected hNPCs has been interpreted as rapid depletion of this population, perhaps providing a basis for cortical thinning observed in CZS (70). Adult infection is believed to similarly deplete hNPCs, a cell population which contributes to plasticity and regeneration in the mature brain (62).

1.2.3 Flavivirus immunity in the matured brain

Bypass of the BBB is accompanied by establishment of infection, activation of local immune responses, and infiltration of immune cells into the CNS. Once brain resident cells are infected, viral detection through pathogen recognition receptors such as TLR3 leads to local immune responses and apoptosis (71, 72). Consequently, several cytokines are produced and secreted during CNS infection.

Neurons, astrocytes, and microglia all express various receptors for cytokines (73). Cytokine production instigates both expression of anti-viral proteins as well as engagement of innate immune effector cells. Type I interferon signaling, a major player in early response to viral infection, potently restricts flaviviral replication but is efficiently antagonized by multiple flaviviral proteins (74-76). Additional cytokines orchestrate the host response within the brain. Elevated soluble IL-6, IL-10, and IFN-γ was found in CSF of Zika-positive pediatric encephalitis patients (77). However, elevated levels of IL1β and TNF-α were detected in ZIKV-infected neuronal cultures and brains of mice but not in CSF of rhesus macaques (42, 66, 78).

After the early stages of infection, cells infiltrate the brain and adopt an active phenotype, eliciting cellular immunity. For ZIKV, CD8+ cells can appear in large number within the brain 7 days post infection. The predominant infiltrating cell population in *Ifnar* $^{-/-}$ mice shifts with macrophages and polymorphonuclear leukocytes (PMNs) composing the majority earlier in infection (day 5) and CD8+ cells later (day 7) (56). However, type I interferon deficiency is a crucial caveat to these results and may limit generalization to human infection. In Manangeeswaran et al., the majority of infiltrating cells in one day old wildtype mice were CD4+ or CD8+ whereas PMNs predominated in *Ifnar* $^{-/-}$ mice 15 days post infection, supporting distinct immune responses (79).

Infiltrating cells have distinct functional niches during CNS flavivirus infection. Phagocytes from the periphery and microglia migrate to the site of infection, secrete antiviral cytokines, and serve as antigen presenting cells—linking innate responses to adaptive immunity. Dengue encephalitis models demonstrate the critical importance of microglia in controlling viral titers in the brain (80, 81). Microglia-mediated protection heavily relies on subsequent infiltration of IFN-γ+ CD8+ cells (51, 81). Activated microglia present antigen to infiltrating T cells,

upregulating molecules associated with antigen presentation such as CD80, CD86, and MHC class II (81). Activation of T cells can mediate cytolytic and non-cytolytic viral control, in turn producing cytokines acting on neurons, glia, and leukocytes. Despite their contribution to viral clearance, cytotoxic T cells may also contribute to certain ZIKV-associated neurological complications. Adult ZIKV-infected *Ifnar* $^{-/-}$ mice experience hindlimb paralysis upon infection, a symptom which does not develop in CD8+ depleting conditions (56).

1.2.4 Role of Complement in Flaviviral Pathogenicity

Involvement of the complement system in flaviviruses related to ZIKV such as West Nile Virus (WNV) and Dengue virus (DENV) has been experimentally confirmed with many foundational studies originating from the Diamond lab. Of the two related viruses, WNV is of particular interest due to its neuroinvasiveness and the well-established role of complement in the development of chronic neurologic symptoms. Additionally, flaviviral nonstructural protein 1 (NS1) has affinity for several complement proteins and can influence complement activation (Table 1).

Table 1 Flavivirus Non-structural Protein 1 Interactions with Complement

DENV	MBL (82), Vitronectin, C5, C6, C7, C9 (83), C1Q (84, 85), C4 (86), C4 + C1S (87), C4BP (82)
WNV	Factor H (88), C4 + C1S (87), C4BP (82)
YFV	C4 + C1S (87), C4BP (82)
ZIKV	C1Q (85)

All three complement pathways have been evaluated in mice models of WNV infection. In serum, the classical pathway primes T cell responses and limits viral pathogenicity (89, 90). In the brain, the classical pathway contributes to abnormal synaptic pruning in WNV-infected mice as evidenced by upregulation of C1QA during disease and neuroprotection in C3-deficient conditions (8). In the same study, mice with abnormally low numbers of microglia also exhibited protection from this synaptic pruning. On the other hand, the lectin pathway only minimally contributes to protection, demonstrated by modestly increased susceptibility in infected MBL-A$^{-/-}$ $\times$ MBL-C$^{-/-}$ and MASP-2$^{-/-}$ knockout mice versus wildtype controls (91). Comparable experiments designed to address the alternative pathway demonstrated faster systemic spread in knockout mice (89). WNV proteins also directly affect the complement cascade during infection. WNV NS1 protein interferes with full activation of complement by directly associating with Factor H (88). In summary, studies of WNV in the CNS reveal a core role for complement in disease progression with individual pathways contributing variably to the disease process.

As neurological complications of DENV infection are rare, the vast majority of research connecting complement and DENV are focused in other tissues. DENV can infect hepatocytes (major producers of complement) and phagocytes (cellular effectors of complement), altering expression of complement-related proteins (4, 92). Notably, complement is an integral component of several theories regarding DENV severity, stemming from its potent modulation of antibody responses, NS1-induced vascular permeability, and antibody-dependent enhancement. Complement activation has been extensively correlated with clinical severity and development of vasculopathy characteristic of Dengue hemorrhagic fever and increased levels of anaphylatoxins C3a and C5a have been detected in serum of patients with more severe clinical symptoms (3, 93). In terms of host genetics, polymorphisms in *MBL2* contribute to variation in DENV serum

neutralization and associate with the development of severe disease (82). DENV NS1 has also been demonstrated to bind complement proteins directly. Interactions with C1Q, C4 and C1S in complex, C4BP, C5, C6, C7, C9, MBL, and vitronectin have been reported using techniques ranging from immunoassay to affinity purification mass spectrometry (82-87). These interactions have functional consequences. For instance, binding of MBL by DENV NS1 abrogates activation of the lectin pathway *in vitro* (82). Similarly, NS1-vitronectin complexes were isolated from patient sera and reduce terminal complement complex formation (83).

Complement in ZIKV infection is modestly characterized in comparison. Complement-mediated neutralization of ZIKV was demonstrated in normal human serum (85). Intriguingly, antibodies binding complement protein C1Q have been identified in the sera of ZIKV-infected mice and monkeys, driven by homology between C1Q and ZIKV E protein (94). Furthermore, affinity between ZIKV NS1 and C1Q has been reported, mirroring interactions seen in other flaviviruses (85).

Due to complement's role in early development (differentiation and migration) and flaviviral NS1's capacity to bind complement proteins, it could be hypothesized that ZIKV NS1 contributes to the cortical disorganization observed in CZS. This possibility was addressed by Yoon et al. in supplemental experiments (95). Embryonic mouse cortexes transfected with ZIKV NS1 demonstrated no difference in neuroprogenitor cell proliferation (specifically $Pax6^+$ radial glial cells) compared to control mice. While this does not entirely preclude involvement of NS1 in CZS pathology, the lack of reported defects suggests other viral proteins have a greater contribution to neurodevelopmental sequelae.

The Klein lab has performed highly informative studies evaluating neuroinfection with ZIKV and WNV in adult mice (8, 32). Vasek et al. was the initial paper describing complement

activation as a molecular mechanism for memory decline post-WNV infection. The following study Garber et al. highlights key differences between WNV and ZIKV CNS pathology in their mouse model. While WNV was primarily associated with loss of synaptophysin$^+$ (Syp) presynaptic termini in the hippocampal CA3 region, ZIKV initiated apoptosis and loss of Homer1$^+$ post-synaptic termini in neurons. Another major paper evaluating synapse loss in ZIKV-infected adult mice, Figueiredo et al. demonstrated similar pathologic findings overall with decreased colocalization of pre-synaptic Syp and post-synaptic Homer1 in the CA3 region. Notably, these studies used ZIKV strains from different lineages indicating synaptic loss is a conserved feature of hippocampal infection.

As ZIKV and WNV infection lead to divergent synaptic pathologies, pruning-like mechanisms in other viral infections are perhaps even less similar. For example, Di Liberto et al. demonstrates synapse loss in experimental LCMV infection is not complement-mediated but rather a distinct process similarly involving microglia, T cells, and IFN-γ signaling (96). Viral-initiated synapse loss appears to generally rely on CD8-derived IFN-γ but not necessarily complement. Consequently, it is important not to conflate what happens during infection with what occurs during homeostatic pruning or other diseases. Although current understanding hints that pathologic pruning involves overactivation of an otherwise conserved complement cascade, there are likely multiple pathways leading to synaptic pathology with aspects distinct to each phenomenon.

This body of research leaves us with several unresolved questions. Is the induction of complement-mediated pruning-like mechanisms the “default” outcome in response to viral infection? Is initiation of the complement cascade due to binding of viral determinants or triggered by the neuron’s cellular state? Are differential outcomes between WNV and ZIKV infections related to disparate cytokine profiles, changes in soluble complement / surface-bound complement

regulators, or interactions involving complement and viral proteins? Is inhibition of complement activation detrimental to adaptive responses in the brain? Based on accumulated evidence, it is highly probable complement is involved with adult infection and CZS in some capacity, but the clinical relevance of such involvement remains to be determined.

1.3 Translational Prospects

Complement therapies are being investigated for efficacy in diverse disease etiologies (97). Complement's extensive, multi-component cascade provides numerous molecular targets for intervention. However, investigation into CNS complement could additionally provide novel tools for neuroscience research.

With a conserved role for complement in viral CNS pathogenesis, development of therapies applicable to a broad range of neuroinfectious viruses appears feasible. Memory decline and learning deficits are common, debilitating symptoms associated with viral CNS infections and complement inhibition could have therapeutic benefit across viral families.

With the current state of CNS complement research, considerably more is known about how to inhibit complement-mediated synaptic pruning than how such pruning is initiated (33, 39). Study of viral-initiated pruning-like mechanisms could provide insight into this process applicable to normal, physiologic conditions or non-viral neurodegenerative diseases. Viral heterogeneity could be an advantageous experimental feature. Diverse complement evasion mechanisms exist due to selective pressure exerted by serum complement, leading to conserved and non-conserved interactions among pathogens which spread via blood. Comparison of these strategies in viruses which do and do not induce synaptic damage provide an experimental framework for probing CNS

complement regulation. Ideally, such knowledge could be translated to experimental control of microglia-mediated pruning. Engineered viruses triggering circuit-specific pruning would clarify the contribution of specific synapses to complex cognitive functions or provide therapeutic benefit in conditions marked by over-connectivity or over-responsiveness (such as epilepsy or neuropathic pain).

These exciting (albeit distant) translational prospects highlight the potential value of studying complement-pathogen interactions in the brain.

2.0 Statement of the Project

The proposed study will clarify the nature of Zika-mediated complement dysregulation within the human brain. Experiments aim to validate the applicability of cell lines, characterize changes in brain complement during infection, and investigate how these mechanisms could contribute to pathology. Each of these aims addresses unknowns in the fields of CNS complement and infection.

Basic science research involving CNS complement predominantly uses mice as experimental models. However, complement differs substantially between mice and humans, especially in surface-bound inhibitory proteins. Advancements in CNS culturing methodologies provide greater representative human models at the cost of great investment (both time and money). While characterization of complement in CNS cell lines has been previously conducted (25, 98, 99), these results have not been independently validated and uptake of these systems has been poor. Consequently, we believe cell lines have utility (albeit limited) in such studies.

While some aspects of complement during ZIKV CNS infection have been explored (42, 51), observations only provide a macroscopic view of complement's involvement. The regulatory balance—the interplay between effectors and inhibitors—has not been evaluated. This balance is additionally influenced by viral proteins such as NS1. Experiments addressing these factors will offer a more granular view of complement-related processes.

Lastly, precise mechanisms for these processes remain elusive, partially due to difficulties associated with *in vivo* characterization. Our *in vitro* system and complement-related reagents will generate information regarding protein localization and experimental control not seen in previous

work. This aim will attempt to tie our observations seen in previous aims to pathogenesis observed *in vivo*.

3.0 Specific Aims

3.1 Specific Aim 1. Identify appropriate *in vitro* models for assessing complement-virus interactions.

Hypothesis. Development of an appropriate *in vitro* model will require co-culture of multiple cell types: neurons, astrocytes, and microglia.

Experimental Approach. Initial characterization and co-culture will focus on immortalized cell lines comprising neuroblastomas (SH SY5Y) and microglia (HMC3). Using established enzyme-linked immunosorbent assays (ELISAs) and flow cytometry, production of complement-related proteins will be assessed in single population cultures as well as co-cultures. Co-cultures will be established using standard ratios for each population (1:5 - microglia to neuroblastoma).

3.2 Specific Aim 2. Assess viral induced changes in the complement system due to Zika infection.

Hypothesis. Downregulation of complement regulatory molecules and upregulation of effectors will occur in Zika CNS infection, mirroring changes seen in the periphery.

Experimental Approach. ELISAs and flow cytometry will be used to observe complement changes in uninfected and infected cells. Existing ELISAs (C1Q, C1R, MBL, C3, C3a, C4, C5, C5a, CFH, CFI, C6, C8, and sC5b9) optimized in our lab have been previously

validated on reference samples and are highly sensitive. Analogous flow cytometry protocols have been established as well (CD11b, CD46, CD55, CD59). ZIKV infection will be performed on SH SY5Y/HMC3 co-cultures.

3.3 Specific Aim 3. Identify mechanisms linking changes in complement-associated proteins to pathology.

Hypothesis. Observed changes in the complement system lead to greater levels of neuroblastoma cell death and involves specific localization of complement and Zika NS1.

Experimental Approach. Expanding on the previous aim, flow cytometry will allow cell viability to be assessed across conditions. Confocal microscopy/immunostaining will be used to localize complement proteins (C3d, iC3b) to cellular compartments and surfaces. ELISAs will be developed to investigate interactions between ZIKV NS1 and complement proteins. Exposure to Zika NS1 at a physiologically relevant concentration will also be performed.

4.0 Methods

4.1 Complement Protein Elisas

High-binding, half-area 96-well plates (Corning, USA) were coated overnight with associated antibodies in carbonate-bicarbonate buffer at 4°C in a humid chamber. The next day, plates were washed with PBS-T buffer [PBS with 0.05% (v/v) Tween 20]. Plates were blocked with bovine serum albumin (Sigma) at 5% (w/v) in PBS for an hour at room temperature (RT; 21°C to 23°C). A standard curve was generated using purified protein 2-fold serially diluted in blocking buffer resulting in 10 points total. Normal human serum (Complement Technology) at a protein specific dilution and a dilution buffer blank were included. All dilutions were made in 1% (w/v) BSA in PBS Standards, controls, and samples (unaltered cell supernatants) were incubated for 2 hour at RT. Plates were washed and incubated for 1 hour at RT with a corresponding mouse anti-human protein antibody, purified or conjugated to biotin. For detection, plates were washed and incubated for 1 hour at RT with either horseradish peroxidase (HRP)-linked goat anti-mouse antibodies or HRP-linked streptavidin. Following additional washes, plates were incubated for 30 minutes at RT with TMB substrate (KPL, USA) and the reaction was stopped with 1N hydrochloric acid (HCl; Sigma). Optical densities at wavelength of 450 nm (OD450) were determined using SpectraMax Plus PC380 microplate spectrophotometer using the SoftMax Pro software version 6.4 (Molecular Devices). Protein concentrations were calculated using 4-parameter non-linear regression from the R package ‘drc’ (R version 3.5.3; package version 3.0-1). A list of reagents is provided in Appendix A.

4.2 ZIKV NS1 Complement Binding Elisas

High-binding 96-well plates (Corning, USA) were coated with recombinant purified ZIKV NS1 (Native Antigen Company) in carbonate-bicarbonate buffer overnight at 4°C. The following day, plates were aspirated and tapped on a hard surface to remove excess liquid. Controls with no coating step or coated with the corresponding complement protein (1 µg/mL) were included for each protein tested. Plates were blocked with bovine serum albumin (Sigma) at 5% (w/v) in PBS buffer for an hour at RT. Plates were again aspirated and tapped. Purified complement proteins were added to wells at 5 µg/mL. Controls with no protein added were also included, leading to four groups – NS1 / protein, protein / blank, blank / protein, and blank / blank (coated protein / added protein). All dilutions were in PBS. Samples were incubated for two hours at RT. Plates were washed with PBS-T buffer [PBS with 0.05% (v/v) Tween 20] and incubated for 1 hour at RT with a corresponding mouse anti-human protein antibody, purified or conjugated to biotin. For detection, plates were washed and incubated for 1 hour at RT with either horseradish peroxidase (HRP)-linked goat anti-mouse antibodies or HRP-linked streptavidin. Following additional washes, plates were incubated for 30 minutes at RT with TMB substrate (KPL, USA) and the reaction was stopped with 1N hydrochloric acid (HCl; Sigma). Optical densities at wavelength of 450 nm (OD450) were determined using SpectraMax Plus PC380 microplate spectrophotometer using the SoftMax Pro software version 6.4 (Molecular Devices). Values were represented as the ratio of mean OD450 of test samples (NS1 / protein) to experimental controls (blank / protein and blank / blank).

4.3 Cell Culture

Cortical organoids and stem cell-derived CNS cells were generously provided by the Vishwajit Nimgaonkar lab (Western Psychiatric Hospital, University of Pittsburgh Medical Center) (100). SH SY5Y (ATCC® CRL-2266™), HMC3 (ATCC® CRL-3304™), and PHA cell lines were generously provided by the Velpandi Ayyavoo lab (University of Pittsburgh Graduate School of Public Health).

For cell lines, bulk cells were grown in flasks and plated on 96 well flat-bottom plates (Falcon). In flasks, SH SY5Y cells were maintained in 10% FBS in DMEM:F12K (1:1) media while HMC3 cells were maintained in 10% FBS DMEM. When plated in 96 well format, co-cultures of SH SY5Y and HMC3 were maintained in 7% FBS DMEM. Co-cultures were seeded with $3x10^4$ cells in each well with a 1:5 ratio (HMC3:SH SY5Y). Media for 96 well plates were collected and changed every 3 days. ZIKV infection was performed at MOI 1 with strain PE243 (GenBank ref. number KX197192). Cell supernatants were collected and stored at -20°C until use.

HMC3 and co-culture stimulation with IFN-γ1b was performed with recombinant protein (Miltenyi Biotec) at 100 units/mL. After initial administration, subsequent media was also supplemented with IFN-γ1b.

4.4 Flow Cytometry for Complement Related Proteins

Permeabilization and fixation buffers for intracellular staining were from BD Biosciences. Antibodies are as follows: CD11b-PE-Cy7, C5aR-APC, C3aR-FITC, gC1qR-FITC, CD11c-PE,

C5L2-PE, CD21-APC, CD59-PE, CD55-PE-Cy7, CD46-APC, goat anti-mouse Alexa Fluor 488, and corresponding isotypes. The clone 4G2 anti-flavivirus envelope protein antibody was derived from unpurified hybridoma supernatant.

Isotype controls and unstained negative controls were included with every run. Flow acquisition was performed on a BD Biosciences LSR Fortessa Cell Analyzer. Data was acquired with FACS Diva software and analyzed using Flow Jo version 10.

4.5 Imaging Studies

Brightfield images were obtained with an EVOS XL Core Imaging System and were desaturated with an image processing program (GIMP). Millicell EZ chamber slides were seeded with $3x10^4$ cells. All washing steps were performed using Hank's balanced salt solution (HBSS). Cells were fixed using acetone and blocked with the appropriate host serum at 5% (v/v). Antibodies for immunofluorescence are as follows: C3d (BioLegend Cat. No. 846302), iC3b (Quidel Cat. No. A209), and ZIKV NS2B (GeneTex Cat. No. GTX133308).

4.6 Statistical Analysis

Statistical analysis was either performed in GraphPad Prism 8.2.1 or R (version 3.6.3) (101).

5.0 Results

5.1 Specific Aim 1. Identify appropriate *in vitro* models for assessing complement-virus interactions.

5.1.1 Complement protein levels in CNS cell models

ELISAs developed in-house were applied to culture media samples from 2D pluripotent stem cell-derived CNS cells, 3D cortical brain organoid cultures, and immortalized cell lines. Cortical brain organoid media had undetectable levels for all tested proteins (C1q, C3, C5, C8, SC5b9, CFH; data not shown). Other surveyed supernatants with detected complement were largely limited to detection of C3 although other complement proteins were detected (Figure 5). Later evaluations of soluble complement levels using media from 96 wells demonstrated improved detectable ranges compared to media from flasks.

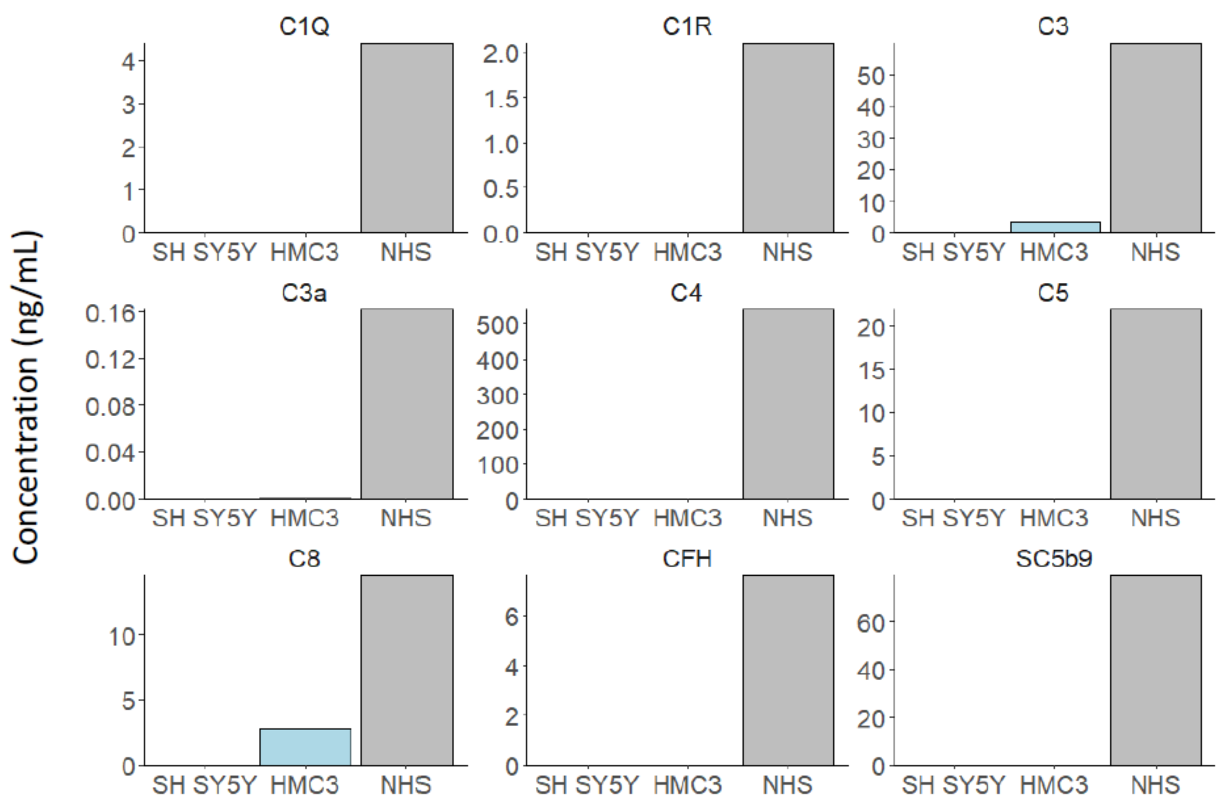

Figure 5 Detection of Complement Proteins in media of CNS Cell Lines

Culture media from cell culture flasks were subjected to various ELISAs. Values are derived from pooled experimental replicates (n = 2) with two technical replicates each. Normal human serum was included as a positive control. NHS – normal human serum.

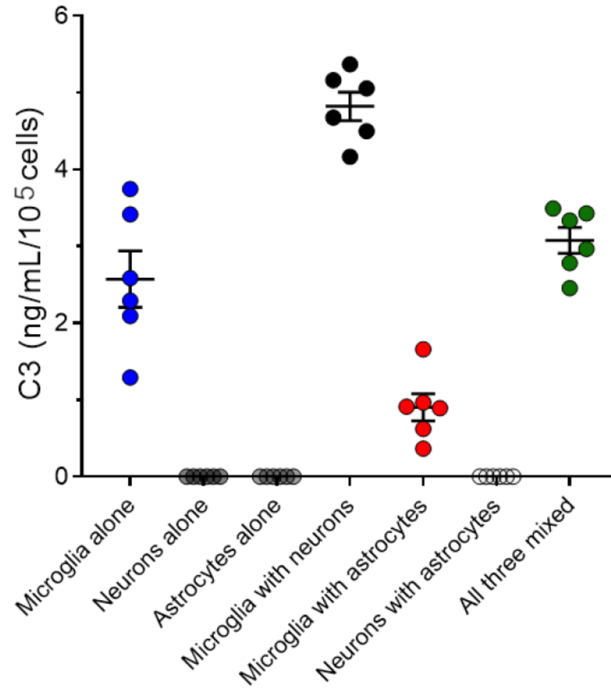

Figure 6 Co-culture of Stem Cell-derived CNS Cells Alters C3 Levels in Supernatant

Levels of C3 in Stem Cell-derived CNS cell supernatant were determined in monocultures and co-cultures. Values were adjusted for the number of microglia initially seeded. Bars represent the median and quartiles.

In differentiated cells and cell lines, microglia appeared to be the sole producers of C3 in culture (Figure 6). Notably, co-culture of differentiated microglia and neurons enhanced C3 production after adjustment for number of microglia. Additionally, complement detection in co-cultures of differentiated cells and of cell lines SH SY5Y and HMC3 were perfectly correlated.

HMC3 cells and SH SY5Y / HMC3 co-cultures were stimulated with IFN-γ1b at 100 Units / mL. No changes in complement levels (C1q, C3, C3a, C5, CFH, CFB) were observed at day 3 and day 5 timepoints (data not shown).

5.1.2 Complement receptors and regulators in SH SY5Y, HMC3, and hNPCs

Various antibody cocktails were developed to investigate complement-related surface proteins on SH SY5Y, HMC3, and hNPCs using flow cytometry. Levels of proteins were evaluated on live, singlet cells and compared to an isotype control.

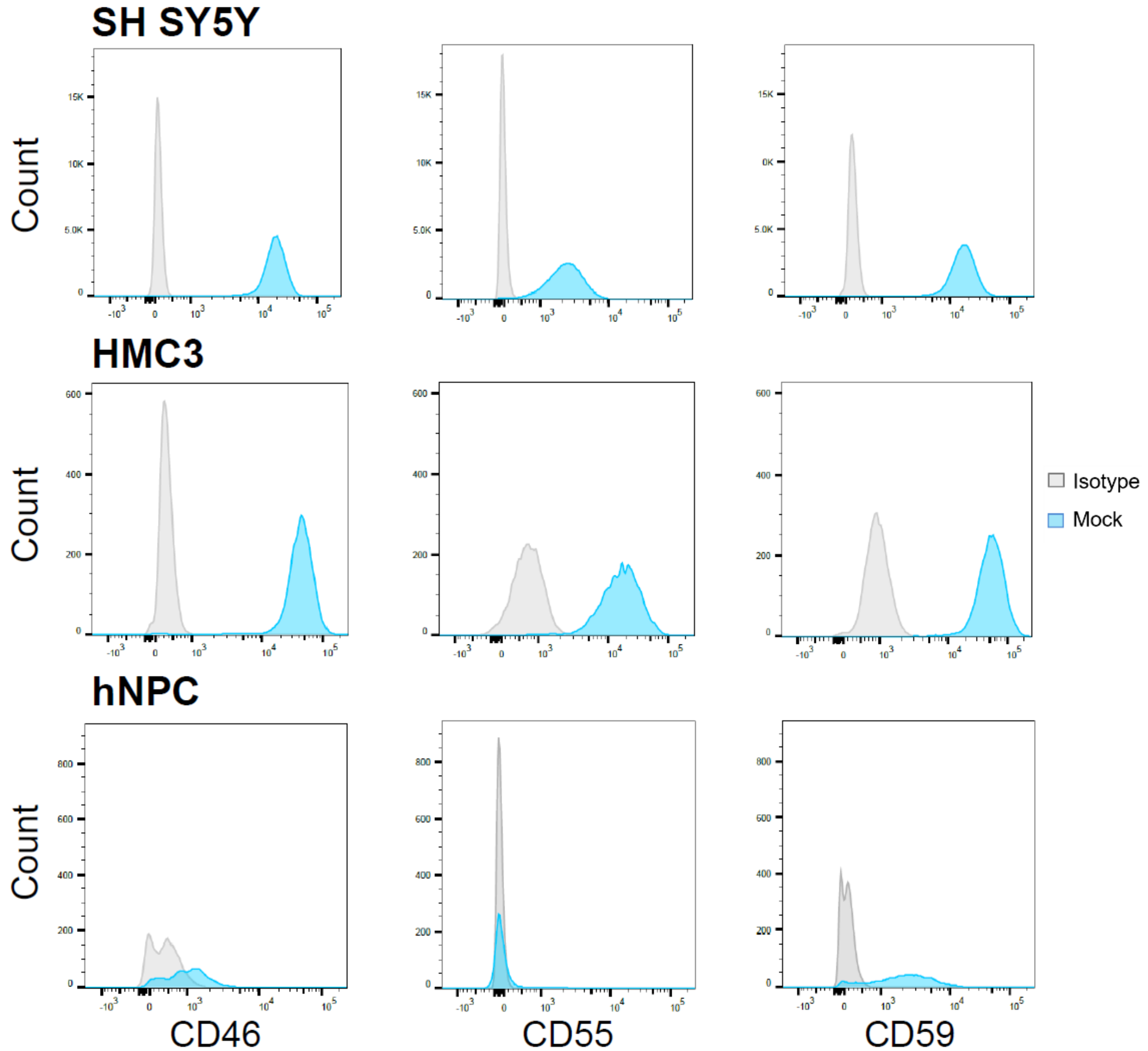

Figure 7 Cell Surface Complement Regulators on SH SY5Y, HMC3, and hNPCs

Flow cytometry plots depicting fluorescence intensity for CD46, CD55, and CD59 on various cell types. Expression was evaluated on live, singlet cells.

SH-SY5Y and HMC3 demonstrated all three complement regulators on cell surfaces (Figure 7). In comparison, hNPCs expressed lower levels of CD46 and CD59 and did not exhibit CD55. The presence of these regulators under typical culture conditions allows for the detection of changes in infected cultures.

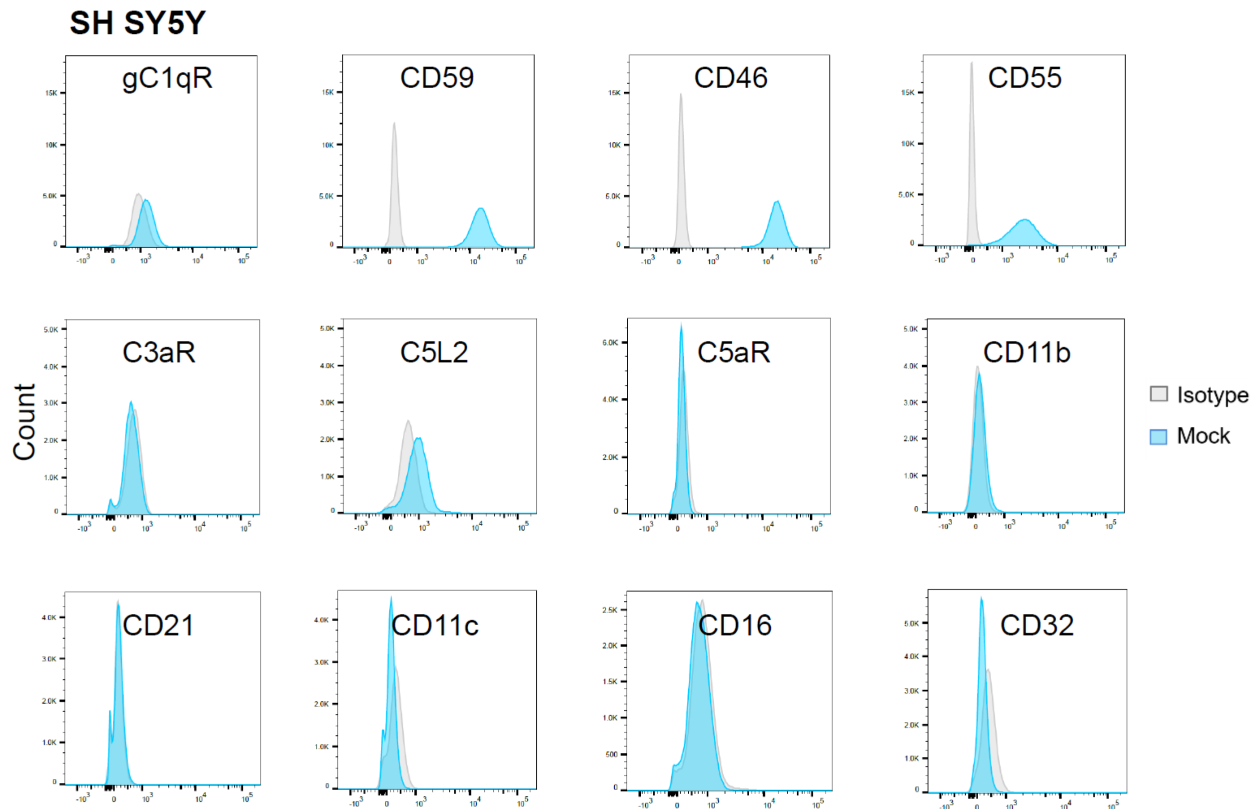

Figure 8 Complement-related Proteins on SH SY5Y Cells

Flow cytometry plots depicting fluorescence intensity for gC1qR, CD46, CD55, CD59, C3aR, C5L2, C5aR, CD11b, CD21, CD11c, CD16, and CD32 on unstimulated SH SY5Y cells. Expression was evaluated on live, singlet cells.

Additional complement-related surface proteins were assessed on SH SY5Y cells (Figure 8). Of these additional proteins, modest expression of gC1qR and C5L2 was detected. In summary, surface complement-related proteins are largely limited to regulatory molecules which are present on both SH SY5Y and HMC3 cells.

5.1.3 Infection of HMC3 with ZIKV

While ZIKV infection of SH SY5Y cells has been reported in previous studies, HMC3 infection has not been studied (102). ZIKV infection of unstimulated HMC3 was assessed at MOI 1 by immunostaining (Figure 9).

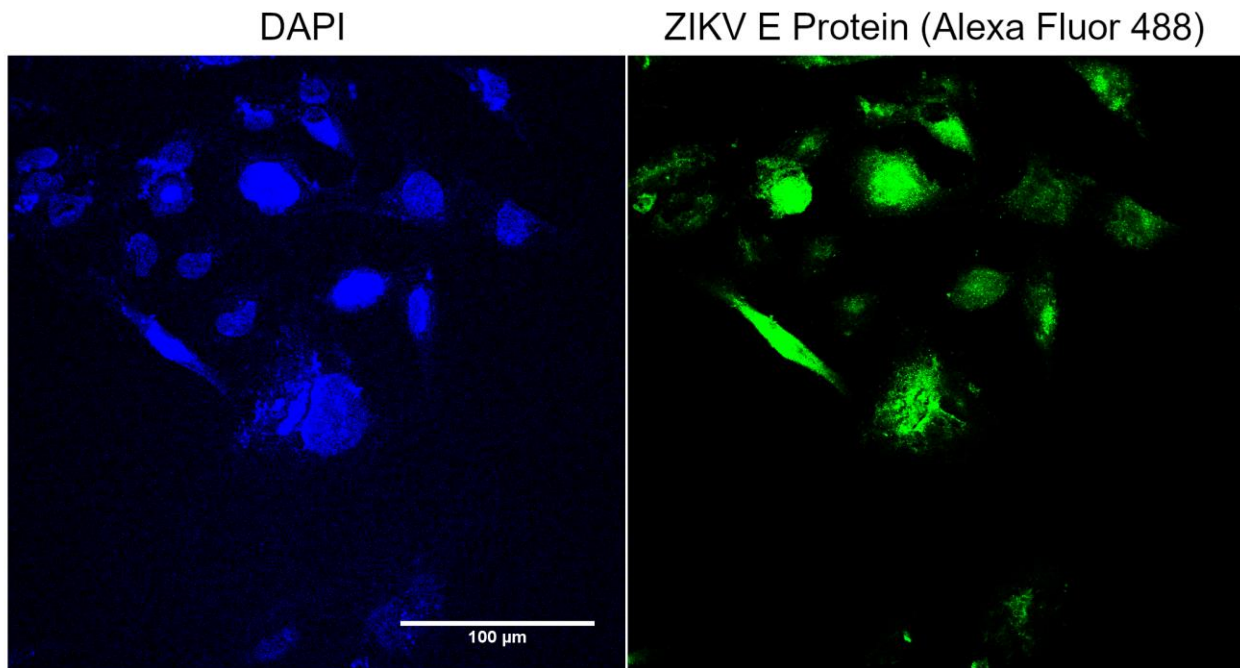

Figure 9 ZIKV Infection of HMC3

Immunostaining for ZIKV Envelope protein (Alexa Fluor 488) taken with a 40x objective.

Although HMC3 cells demonstrated extensive infection with immunostaining, infection was difficult to detect in live HMC3 cells using flow cytometry (data not shown). At 5 DPI, HMC3 cells demonstrated cytopathic effect with brightfield microscopy. Compared to mock infected cells, ZIKV exposed HMC3 were greatly depleted in culture (Figure 10).

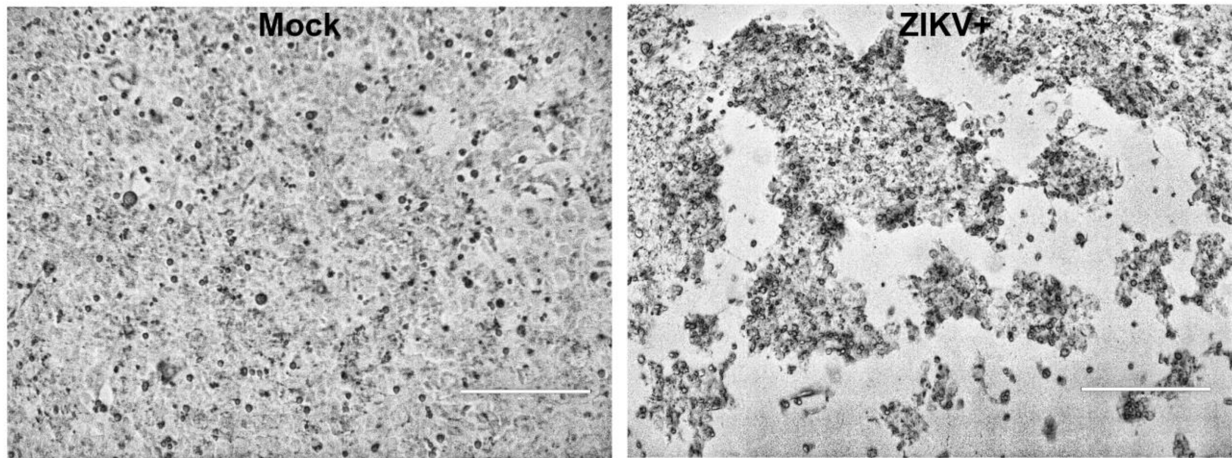

Figure 10 ZIKV Cytopathic Effect in HMC3 Cells

Brightfield microscopy images of ZIKV-infected HMC3 cells taken with a 10x objective. Scale bar is 400 µm.

5.1.4 Establishment of an SH SY5Y / HMC3 co-culture

Initially, co-cultures with SH SY5Y, HMC3, and an astrocytic cell line were evaluated. However, the inclusion of immortalized astrocytes PHA lead to dense cellular clumps in culture flasks centered around astrocytes (Figure 11). As such clumps may compromise cellular viability and impede downstream flow analysis, astrocytic cell lines were excluded from subsequent experiments. SH SY5Y / HMC3 co-cultures (5:1 ratio) were established with both cell types capable of growth in a common medium (7 or 10% FBS in DMEM).

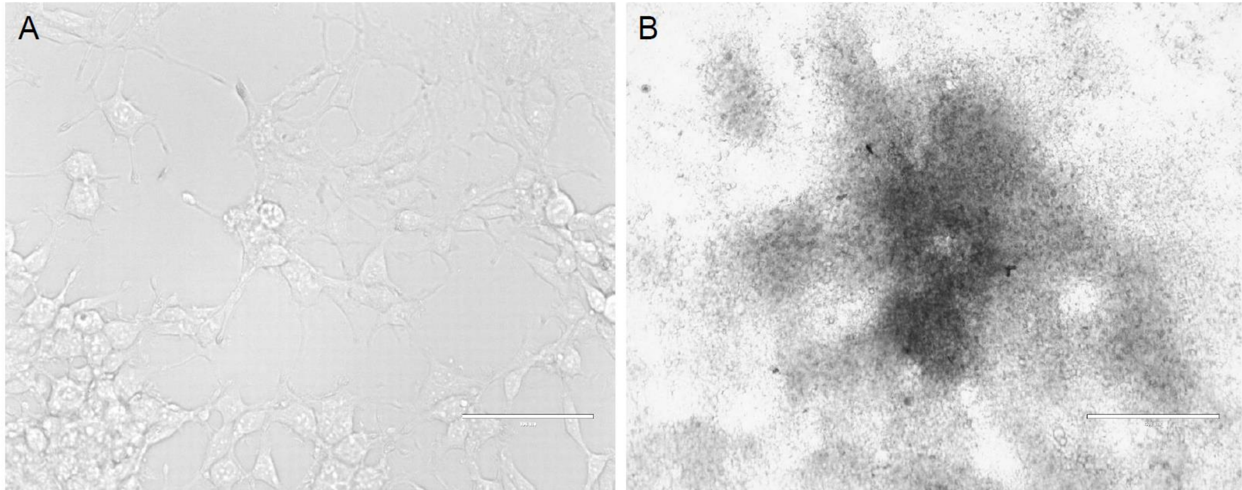

Figure 11 Inclusion of an Astrocytic Cell Line Leads to Cellular Aggregates

Brightfield microscopy of SH SY5Y, HMC3, and PHA co-cultures A) In regions without astrocytes, co-cultures develop in a flat monolayer. B) Other regions have dense cellular aggregates centered around astrocytes. A was taken with a 40x objective while B was taken with a 10x objective.

5.2 Specific Aim 2. Assess viral induced changes in the complement system due to Zika infection.

5.2.1 ZIKV-induced changes in soluble C3 levels

Levels of soluble complement factors were assessed after infection of cultures. C1q, C5, CFB, CFH, and CFI remained undetectable post-infection (data not shown). However, C3 retained detectable levels (Figure 12).

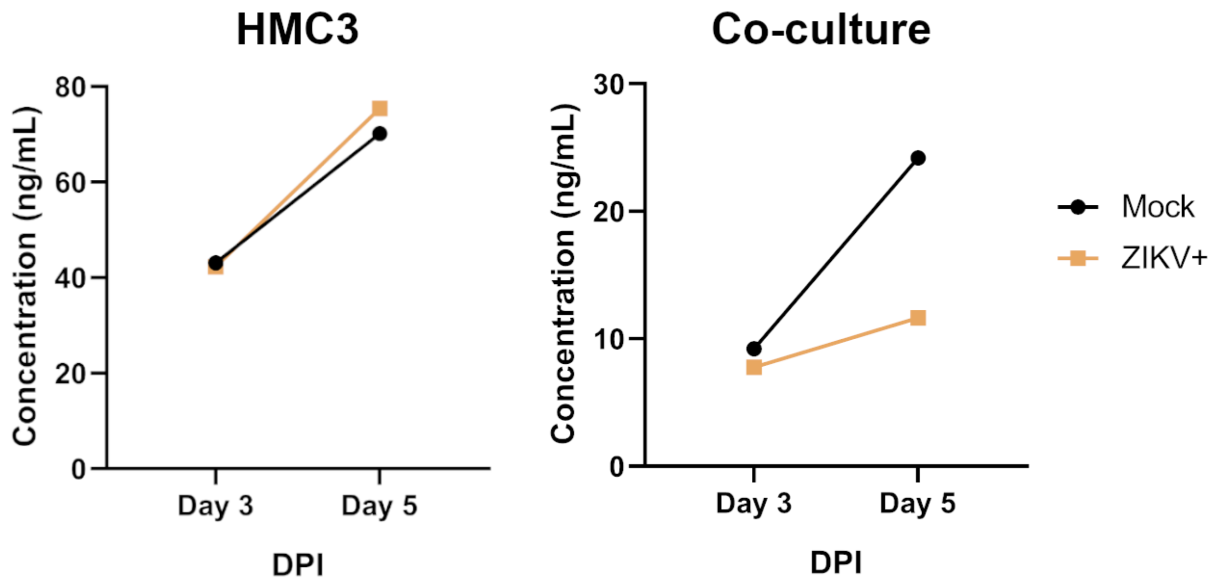

Figure 12 C3 Levels during ZIKV Infection of HMC3 and Co-cultures

C3 levels in infected and mock cultures were assessed by ELISA at 3 and 5 days post infection (DPI). Values are derived from pooled experimental replicates (n = 2) with two technical replicates each.

The dynamics of C3 during infection differ between HMC3 monocultures and SH SY5Y / HMC3 co-cultures. While both HMC3 and co-cultures demonstrated increases in C3 from day 3 to 5, infected co-cultures had reduced soluble C3 in comparison to matched mock controls, an observation absent in infected HMC3 monocultures. This reduction could represent greater cleavage of C3 or a decline in C3 production in co-cultures.

5.2.2 ZIKV-induced changes in surface-bound complement-related proteins

Unstimulated HMC3 monocultures were infected with ZIKV and analyzed 5 days post infection (DPI) for surface expression of CD11b, CD46, and CD59 (Figure 13). For CD11b, two distinct populations emerge: CD11b+ and CD11b- groups. ZIKV infection appears to induce the

expression of CD11b in some HMC3 while downregulating it in others. In contrast, reduction only of CD46 and CD59 was observed in exposed cultures.

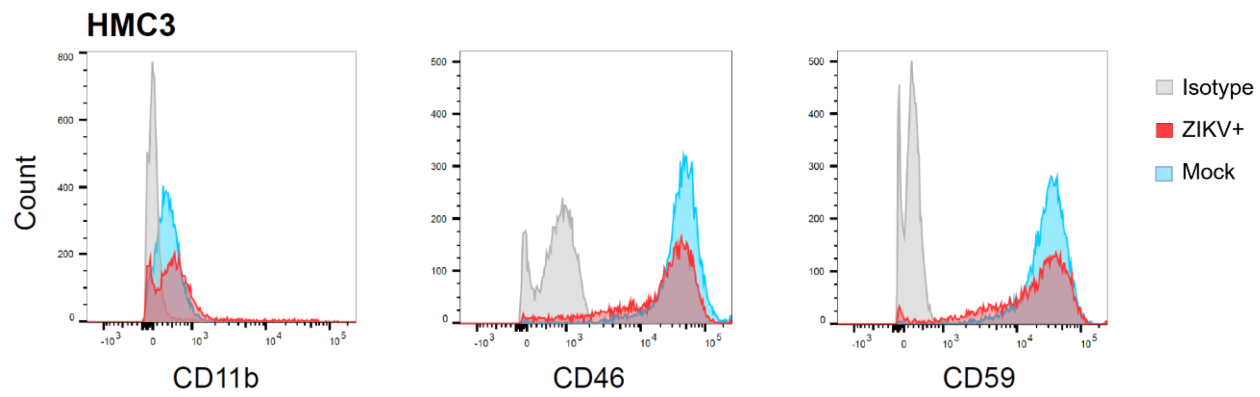

Figure 13 Changes in CD11b, CD46, and CD59 in Infected HMC3 Cells

Flow cytometry plots depicting fluorescence intensity for CD46, CD59, and CD11b on ZIKV-infected HMC3 and Mock cells. Expression was evaluated on live, singlet cells.

Unstimulated SH SY5Y monocultures and SH SY5Y / HMC3 co-cultures were infected with ZIKV and analyzed at 5 DPI for changes in surface-bound complement regulators CD46, CD55, and CD59 (Figure 15). Staining for ZIKV envelope protein allowed for differentiation of actively infected or non-infected cells within the ZIKV-exposed culture. A representative plot showing the gating strategy of an exposed co-culture is depicted in Figure 14.

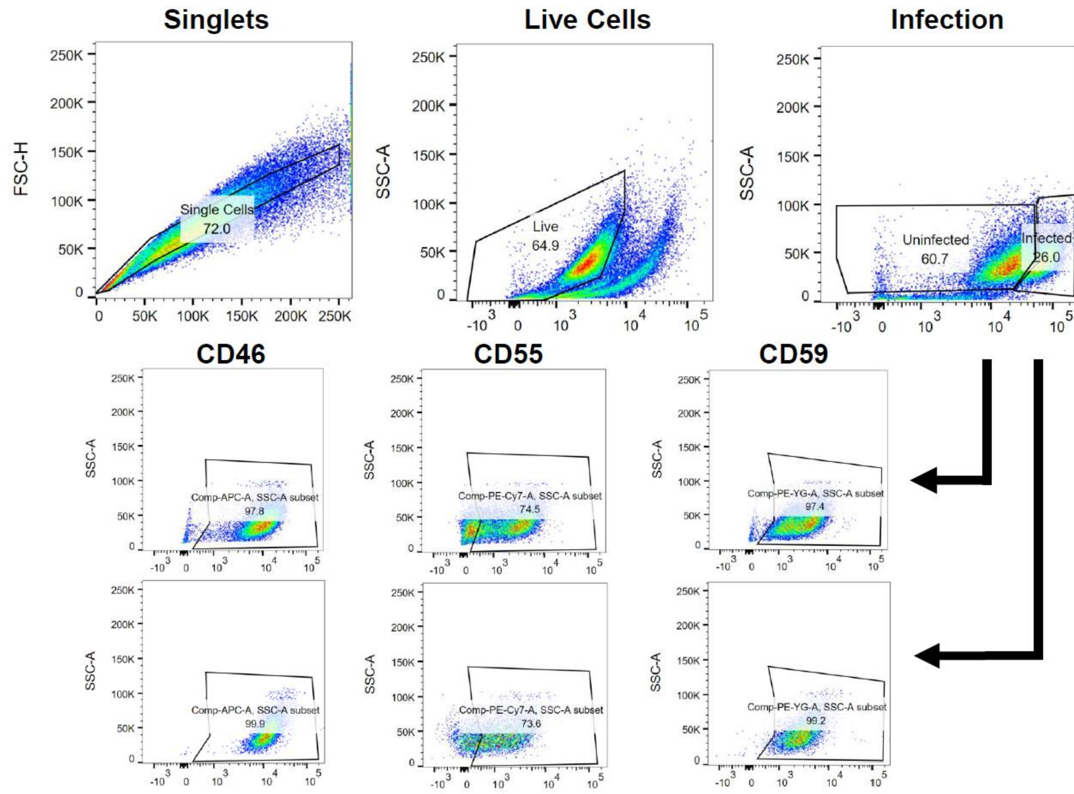

Figure 14 Gating Strategy for Analysis of ZIKV-Infected Co-cultures

A representative plot demonstrating the gating strategy for ZIKV-infected co-cultures of SH SY5Y and HMC3.

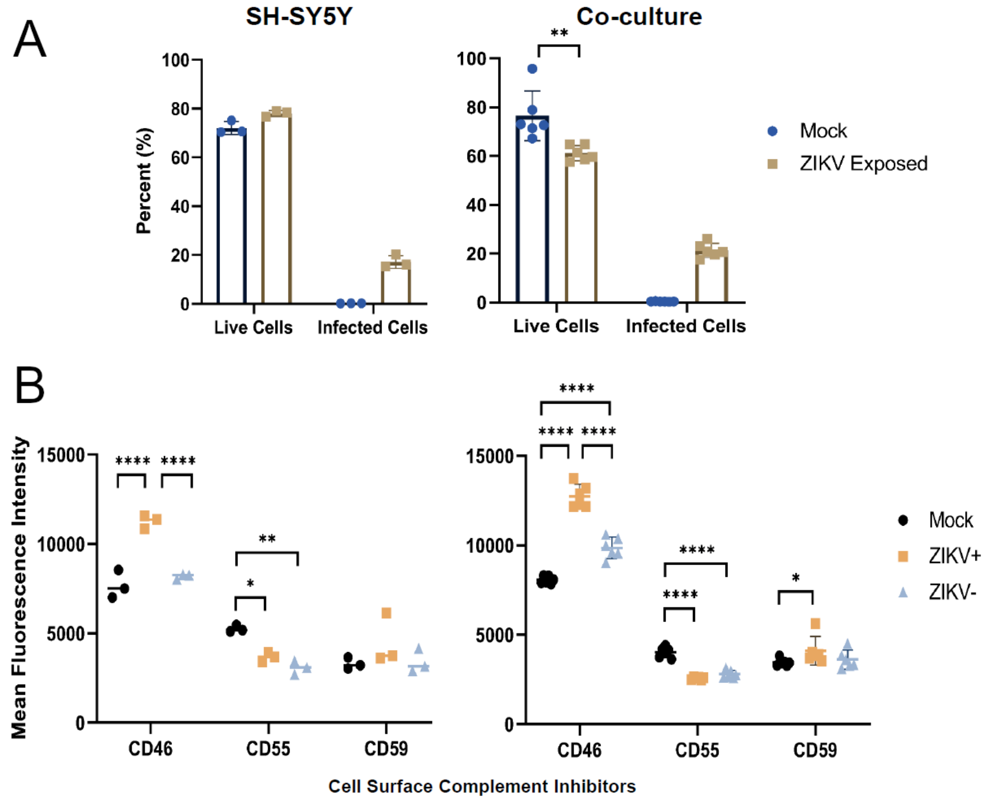

Figure 15 Changes in Cell Surface Complement Regulators CD46, CD55, and CD59 During ZIKV Infection

A) Co-culture of ZIKV Infected SH-SY5Y with HMC3 reduces overall cell viability. While viability did not significantly differ for SH-SY5Y mono-culture, differences in mean viability between mock and infected co-cultures were observed (76.5% vs 61.2%). At an MOI of 1, ZIKV infection resulted in an average of 17.1% (SH-SY5Y; n = 3) and 21.3% (SH-SY5Y + HMC3 [5:1 ratio]; n = 6) infected cells. B) CD46 is upregulated on ZIKV infected cells while CD55 is downregulated in both infected and bystander cells. ANOVA was performed followed by Tukey's multiple comparisons test. Multiplicity adjusted p-values are reported. * denotes $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p \leq 0.0001$.

In co-cultures, CD46 is upregulated on ZIKV infected cells while CD55 is downregulated in both infected and bystander cells. The upregulation of CD46 could preferentially generate iC3b on infected cells, leading to CR3-mediated synaptic pruning but protection from further complement activation. The downregulation of CD55 could allow destruction of infected and bystander cells if terminal complement components make it past the blood brain barrier.

5.3 Specific Aim 3. Identify mechanisms linking changes in complement-associated proteins to pathology.

5.3.1 Visualization of C3d and iC3b deposition on co-cultures

To visualize the impact of altered surface complement regulators, immunostaining with antibodies for C3d (which recognizes an epitope present on C3, C3b, iC3b, and C3d) and iC3b.

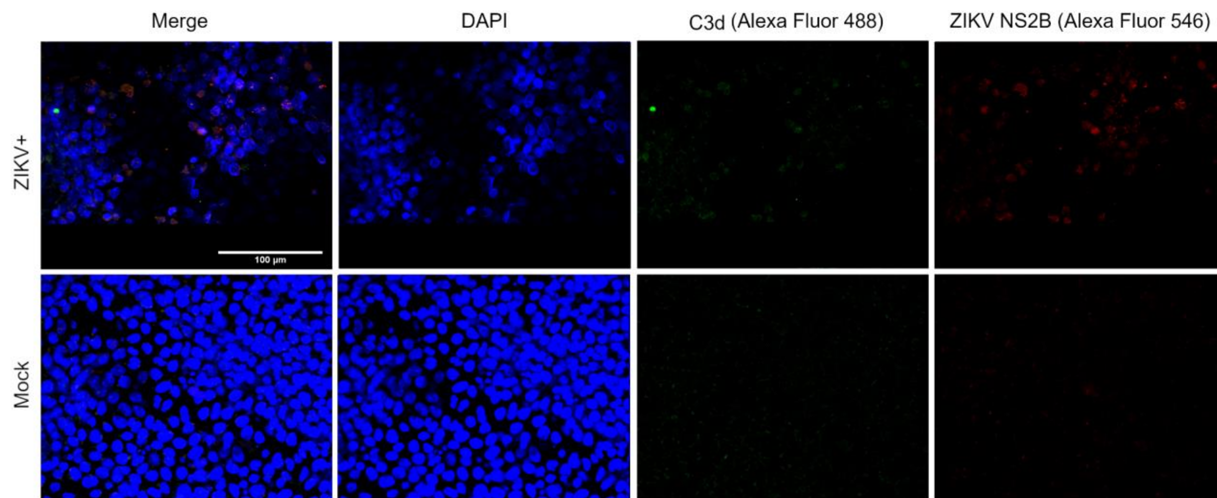

Figure 16 Immunostaining for C3d on ZIKV-infected Co-cultures

A representative field depicting immunostaining of acetone-fixed co-cultures cultures in chamber slides. Mock and infected cultures demonstrated similar levels of C3d. Images were captured with a 40x objective.

C3d staining was minimal (likely due to a sub-optimal antibody concentration) on both mock and ZIKV-exposed co-cultures (Figure 16). On the other hand, iC3b staining visibly appeared greater in exposed vs mock cultures (Figure 17). Qualitatively, iC3b staining appeared brighter in association with infected cells, an expected result based on CD59 downregulation and CD46 upregulation.

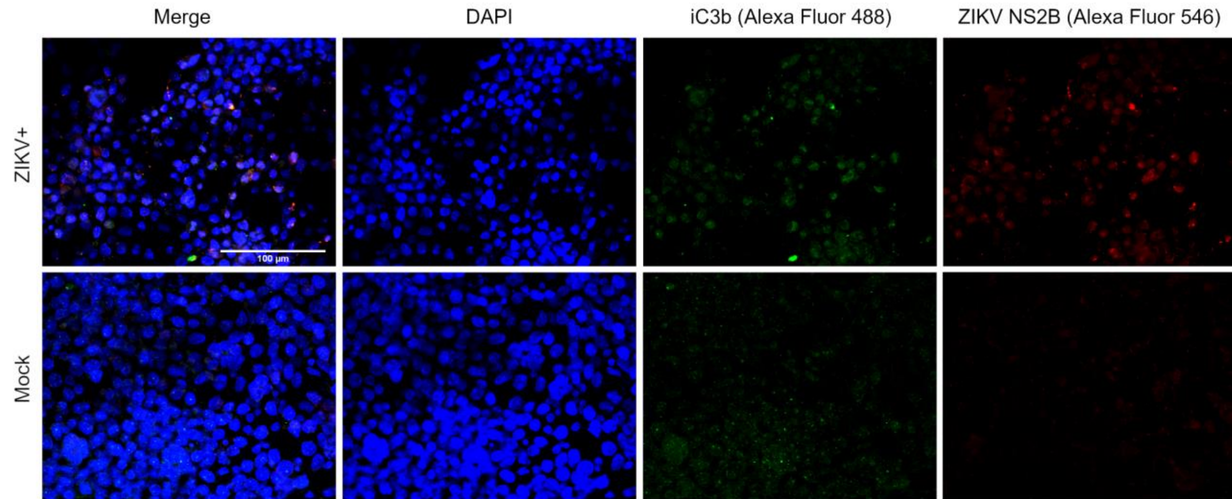

Figure 17 Immunostaining for iC3b on ZIKV-infected Co-cultures

A representative field depicting immunostaining of acetone-fixed co-cultures cultures in chamber slides. Mock and infected cultures demonstrated similar levels of C3d. Images were captured with a 40x objective.

5.3.2 Evaluation of binding between ZIKV NS1 and complement proteins

As several interactions between flaviviral NS1 and complement components have been reported, we sought to investigate ZIKV NS1-complement associations using an ELISA.

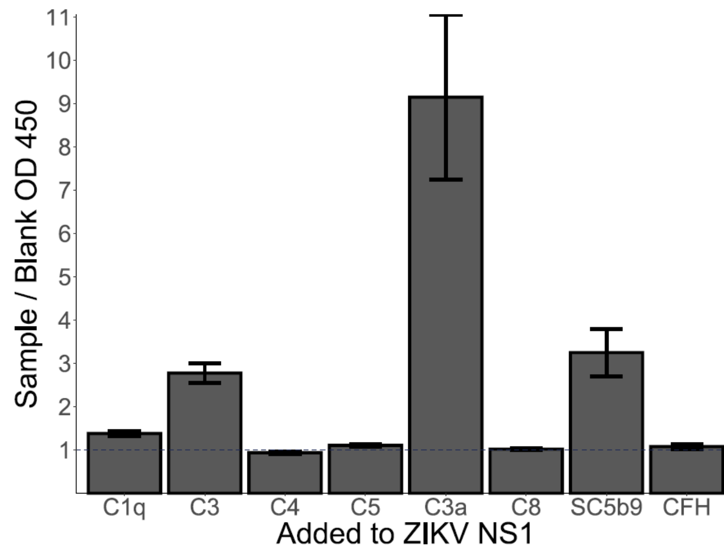

Figure 18 ZIKV NS1 Binding of Complement Proteins

ZIKV NS1 binds complement components. Binding was detected using an ELISA with ZIKV NS1 coated wells exposed to various complement components. Each condition was run with three technical replicates. The y axis is the ratio of mean OD450 of test samples (NS1 coated / protein added) to experimental controls (blank coated / protein added and blank coated / blank added).

C3, C3a, and SC5b9 (soluble polymerized complement factors 5 through 9) demonstrated some degree of affinity with NS1 (Figure 18). The greater levels of bound C3a suggests cleavage of C3 may reveal a binding site with high affinity for NS1. Additionally, binding of multimeric SC5b9 complexes could be due to a specific component of the terminal complement complex. As flaviviral NS1-complement interactions have functional consequences, further experiments examining these interactions in the context of complement activation are warranted.

5.3.3 Exposure of SH SY5Y and HMC3 to purified recombinant ZIKV NS1

Previous studies indicated that macrophage exposure to DENV NS1 in co-culture could elicit inflammatory transcriptional changes (103). We sought to test if exposure to ZIKV NS1 was sufficient to induce the changes in surface complement regulators seen during infection (Figure 19).

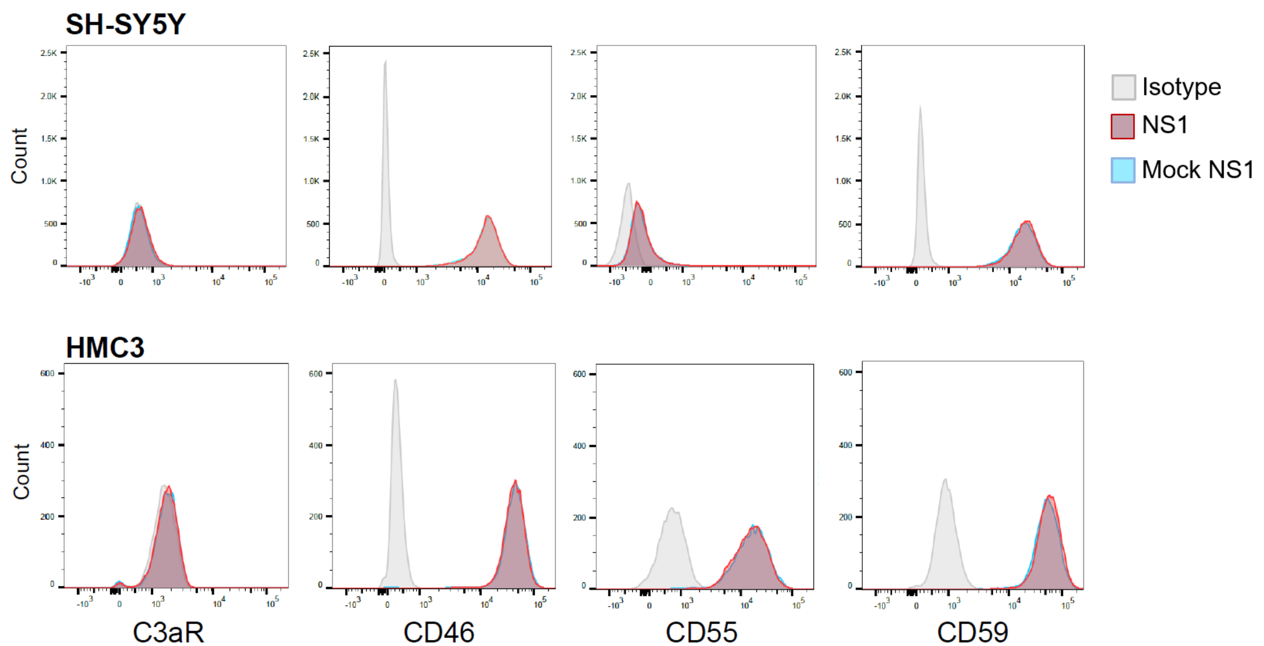

Figure 19 ZIKV NS1 Treatment of SH SY5Y and HMC3 Cells

ZIKV NS1 treatment does not affect the expression of C3aR, CD46, CD55, and CD59. Flow cytometry plots depicting fluorescence intensity for C3aR, CD46, CD55, and CD59 on ZIKV NS1-exposed SH SY5Y and HMC3 cells. Expression was evaluated on live, singlet cells.

ZIKV NS1 treatment does not affect the expression of C3aR, CD46, CD55, and CD59. SH-SY5Y and HMC3 cells exposed to 6 µg/mL of purified, recombinant ZIKV NS1 demonstrated no change in viability (not shown) or expression of select complement-related proteins.

6.0 Discussion

Aim 1 involved the establishment of an *in vitro* model of CNS complement production. Cell lines were capable of modeling infection and expressed the central complement protein, C3.

In line with previous research, infection was detected in both SH SY5Y and HMC3 cell lines at an MOI of 1 (102, 104, 105). While ZIKV-exposed HMC3 cells were greatly depleted in culture, SH SY5Y did not demonstrate a cytopathic effect with comparable viabilities between mock and exposed cultures. Accordingly, percentages of infected cells increased and overall viability decreased when HMC3 were introduced into infected co-cultures. Depletion of HMC3 cells may complicate interpretation of results, especially at later time points. As HMC3 are the main producers of several complement proteins, declines in detectable levels may be due to depletion of HMC3 rather than increased degradation through activation. Overall, the co-culture of SH SY5Y and HMC3 represents a viable system to assess viral-induced changes in the complement system.

Many complement proteins expressed in the periphery were undetectable in various CNS co-culture models. C3 was the only key complement protein detected, limiting activation to C3 tickover. The Fontaine lab previously validated complement production in several CNS cell lines in monoculture (18, 77-79). However, our results are discordant. While many of the Fontaine studies demonstrate production of several complement proteins at the protein and mRNA level, we detected only select complement factors in the various systems surveyed. Particularly, our ELISAs were unable to detect terminal components C6, C8, C9 despite high sensitivity. Based on such drastic discordance, we speculate that production of complement by cell lines is potentially subject to culture conditions. Additionally, cellular populations capable of directly producing or

stimulating production of these missing proteins may have been excluded from the existing co-culture. As such, CNS complement production remains ambiguous. Further *in vitro* studies are needed to clarify soluble factors and cell populations responsible for complement production.

Based on results, it appears that the use of cell lines in lieu of more advanced cell models is feasible. Production of complement proteins in cell line co-cultures and iPSC-derived co-cultures were perfectly correlated. While cell lines are generally less representative, the substantial effort required to maintain iPSC-derived cultures can be mitigated with usage of both systems in tandem. Validation with more advanced culture systems is still warranted as cancerous cells can co-opt complement signaling to promote survival, potentially confounding experiments (106).

Finally, the use of astrocytic cell lines was abandoned. The inclusion of immortalized astrocytes in co-culture lead to cell aggregates in culture. As astrocytes likely play an important role in ZIKV infection and in complement production, alternative culture techniques should be explored.

Aim 2 assessed the impact of infection on complement production and regulation. Although ZIKV infection upregulated complement components in immortalized hepatocytes and placental trophoblasts (not shown), similar findings were not seen in CNS cell lines. C3 levels were decreased in infected co-cultures but not HMC3 cultures compared to mock exposed controls at 5 DPI. This reduction in soluble C3 could be tied to greater activation or decreased production by HMC3 in co-culture. As iC3b appears greater in infected co-cultures and infected HMC3 monocultures did not exhibit the same pattern, this is likely due to increased cleavage of C3 via C3 tickover and not decreased production through HMC3 depletion. Despite this, measurement of soluble C3a levels would be an appropriate follow-up experiment to confirm greater activation in co-culture.

The complement regulators CD46, CD55, and CD59 were altered by ZIKV infection. The dynamics of CD46 and CD55 imply CD46 upregulation is a direct consequence of viral infection while CD55 downregulation is mediated by soluble factors released into culture. These changes could lead to different functional fates for bystander and infected neuroblastoma cells.

CD55 downregulation alone was observed in neuroblastoma bystander cells. CD55 downregulation allows for the accumulation of C3 convertase on cell surfaces and subsequent deposition of C3b fragments. If terminal complement components are present, deposition will trigger the formation of the terminal complement complex, lysing the C3b tagged cell. Moreover, C3b and downstream iC3b have been implicated in synaptic pathology (6). Consequently, decreased CD55 *in vivo* might contribute to loss of viable, un-infected neurons or their synapses.

Both an increase in CD46 and decrease in CD55 surface protein was detected on ZIKV-infected SH SY5Y cells. Coupled with increased deposition of C3b through CD55 downregulation, CD46 upregulation is hypothesized to increase generation of iC3b as CD46 is a cofactor for proteolytic CFI. This is supported by the qualitative association of iC3b and infection detected by immunostaining. The functional relevance of this finding is difficult to determine in culture. In the periphery, iC3b recognition by complement receptors, primarily CR3 (composed of CD11b and CD18), induces phagocytosis. Phagocytosis of infected CNS cells could contribute to patterning of downstream adaptive responses as well as pruning of synapses on infected cells. iC3b-induced phagocytosis by antigen presenting cells leads to antigenic tolerance via anti-inflammatory cytokine production outside the CNS (107). Analogous roles in the brain could influence T cell activation status in the CNS antiviral response, delaying clearance. While phagocytosis of infected cells was not evaluated in this study, functional *in vitro* assays exist and could address this possibility. Additionally, C3b and possibly iC3b are believed to tag synapses for removal through

CR3 (6). Increased production of iC3b through CD46 could provide a mechanism for pruning of infected cell synapses observed in mice models (Figure 20).

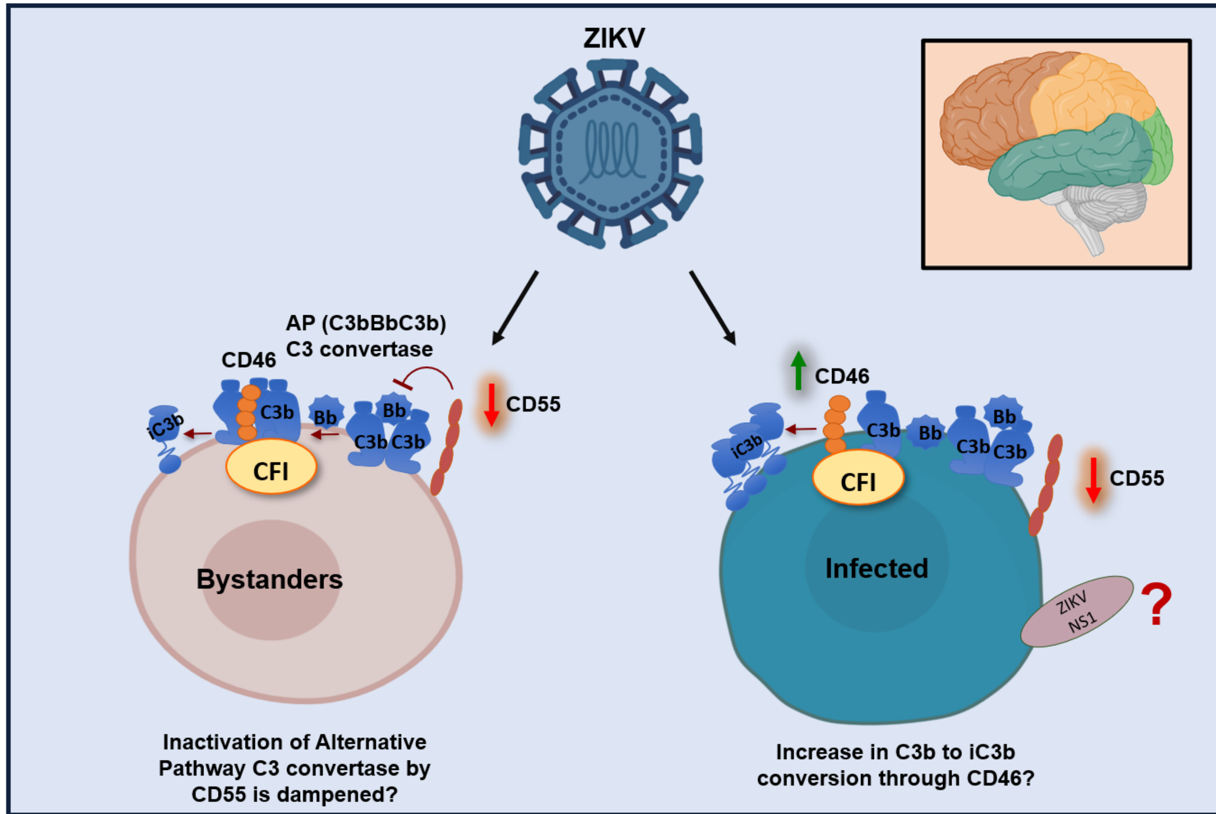

Figure 20 Changes in Surface-bound Complement Inhibitors During ZIKV Infection

Changes in surface regulators could lead to different functional fates for infected and bystander SH SY5Y cells. ZIKV NS1 could additionally impact cell fate through interactions with complement proteins. CD55 downregulation allows for deposition of C3b as CD55 antagonizes the alternative pathway (AP) convertase. CD46 upregulation leads to increased CD46 by serving as a cofactor for CFI.

It is unclear how these changes reconcile with loss of post-synaptic but not pre-synaptic termini observed in Garber et al. (51). Mice lack CD46 on most cells but express Crry, a functional analog of CD46 in rodents (107). Furthermore, the distribution of complement inhibitors varies considerably between mice and humans. As such, mice models are greatly limited in their

generalizability to human infection and investigation of similar phenomena in mice models will be restricted.

For HMC3 monocultures, decreases in CD46 and CD59 were detected in infected cultures, mirroring observations seen in DENV infected hepatocytes (Unpublished). Additionally, CD11b+ and CD11b- populations appeared during ZIKV infection. As reduction of CD59 and CD11b has been reported previously in DENV-infected peripheral monocytes (92), such downregulation appears to be conserved across flaviviral infection of professional phagocytes. In prior, unpublished studies with hepatocytes, these changes were restricted to bystander but not infected cells and we hypothesize that HMC3 infection is similar. Such changes (tied to phosphorylation of STAT3 in the unpublished work) may represent a more general response to flaviviral infection that does not occur in SH SY5Y cells.

As expected, ZIKV NS1 appears to bind complement components C3, C3a, and SC5b9. The greater affinity for C3a than C3 suggests cleavage reveals a binding site, leading to stronger interactions. Binding of SC5b9 could be due to one or several of the proteins in the multimeric complex. Finally, ZIKV NS1 treatment did not induce changes in complement regulators. NS1 in absence of infection is capable of triggering pathological changes. As NS1 binding perturbs complement activation, future studies could elucidate ZIKV NS1's involvement in CNS pathology.

In conclusion, the above study has established a working CNS co-culture model to assess flaviviral-induced changes in the complement system. Additionally, results describe previously unreported changes in several regulatory molecules which occur in a cell specific manner. Further investigation supporting these preliminary findings and linking observations to pathology are needed.

7.0 Public Health Significance

Zika virus is a positive-strand RNA virus of the family Flaviviridae typically causing mild to inapparent infection (43). While the existence of Zika was known since 1947, the virus remained obscure until the World Health Organization declared an international health emergency during the Brazilian outbreak. The explosive nature of the pandemic and rapid subsequent decline of cases has led to uncertainty regarding potential future outbreaks. With the continually expanding geographical range of Zika's primary vector (the *Aedes* species mosquito), threat of re-emergence persists (108).

Despite Zika's decline, ongoing circulation and spread have been demonstrated (109, 110). A recent report indicated a 2018 outbreak in Cuba went largely un-noticed and un-reported to local and global public health agencies (110). Such "hidden" epidemics are possible due to Zika virus' propensity for inapparent infection and current limitations in diagnostics and surveillance. Considering these factors, evaluation and development of vaccines and therapies are essential for future epidemic preparedness.

Outbreaks of Zika are particularly concerning with the increased incidence of severe neurological symptoms among both neonates and adults during the 2015 Zika outbreak (1, 2). For neonates, congenital Zika syndrome describes an amalgamation of potential findings associated with maternal infection, ranging from neurodevelopmental delay (in absence of overt physical defects) to microcephaly (111-113). Exposed neonates may appear un-affected at birth but require care later in life. For adults, neurological complications are typified by ZIKV-associated Guillain-Barré syndrome. Emerging evidence additionally associates adult CNS infection with memory decline, mirroring observations in West Nile virus CNS infection (8, 42, 51). Long-term care of

affected individuals imposes substantial financial and emotional burden on individuals, families, and health systems (114).

Death of neurons is observed in both adult and congenital infection, likely related to development of neurologic sequelae (67, 115). The complement system has recently been tied to severity of Zika CNS infection (42, 51). A series of serum proteases sequentially activated through enzymatic cleavage, complement's traditional functions comprise tagging pathogens for lysis, phagocytosis, and opsonization. In recent years, complement has demonstrated other emerging roles including regulation of cell metabolism, detection of intracellular pathogens, synaptic pruning, and neuronal differentiation. The link between complement and flaviviral CNS infection fits into a larger constellation of neurodegenerative diseases such as stroke, schizophrenia, Alzheimer's, Lyme disease, and HIV-associated neurocognitive disorders (10, 20, 116-118). While investigation of complement in Zika CNS infection is specific, investigation will likely produce general insights into how complement functions in the brain—insights which will improve our understanding of neurodegeneration.

8.0 Future Directions

Substantial gaps remain concerning interactions between ZIKV infection and complement. The proposed experiments would contextualize our existing findings.

1. Assess the influence of cytokines on complement secretion and correlation with microglial activation *in vitro*.
2. Intracellular staining of complement proteins in CNS culture systems.
3. Replication of phenomena (surface-bound complement modulation) in more robust CNS cell models such as differentiated pluripotent stem cells.
4. Validation of ZIKV NS1 interactions with complement proteins and characterization of functional consequences during infection.
5. Analogous experiments with DENV or attenuated WNV.
6. Treatment of cultures with complement therapeutics (compstatin, cobra venom factor) to identify changes in cell viability or viral load.

More broadly, investigation of virally induced synaptic pruning would benefit from comparing viruses restricted to hematogenous spread (such as ZIKV and WNV) to viruses capable of trans-synaptic transport mechanisms (rabies, pseudorabies, and herpes simplex virus 1 [HSV1]). Intuitively, viruses capable of retrograde or anterograde axonal transport may experience selection for synapse preservation rather than loss, particularly HSV1 which relies on latent reactivation for communicability. Comparing CNS infections would provide a framework for understanding molecular changes necessary for pruning-like mechanisms.

While C3 and C1q knockout mice demonstrate greater synaptic density compared to wildtype mice, reciprocal experiments evaluating knockout or knockdown of surface-bound

inhibitors (such as Crry) have yet to be performed. While increased levels of brain complement are likely contributors to pathology under the highly inflammatory conditions of viral infection, it is unclear if downregulation of complement regulatory molecules coupled with homeostatic complement levels is equally capable of triggering synapse loss. Performing these studies would clarify the contributions of both increased activation and decreased inhibition to pathology. Establishment of these models is arduous due to embryonic lethality; however, such mice have been previously generated but not had synaptic connectivity evaluated (119).

Appendix Complement ELISA Reagents

Appendix Table 1 Capture Antibodies

#	Target	Capture ab		
		Clonality/Host	**Cat# / Manufacturer**	**Dilution**
1	**C1q**	pab/Goat antisera	A301 / Quidel	1:5,000
2	**C3**	pab/Goat antisera	A213 / Complement technology	1:500
3	**CFH**	pab/Goat antisera	341276/ Calbiochem	1:500
4	**CFI**	pab/Goat antisera	A238 / Complement technology	1:500
5	**CFB**	mAB/Mouse (clone KT21)	ab110651 / Abcam	1:250
7	**C5**	pab/Goat antisera	A306 / Quidel	1:500
8	**C6**	pab/Goat antisera	A307 / Quidel	1:500
9	**C8**	pab/Goat antisera	A309 / Quidel	1:500
11	**SC5b-9**	pab/Rabbit antisera	A227 / Complement technology	1:500
12	**Oligomer MBL**	mAB/Mouse (clone 3B6)	BPD-HYB-131-01 / Enzo Life sciences	1:1,000
13	**C5a**	mAB/Mouse	Part 842755 / R&D systems	1:180
14	**C1r**	pab/Sheep IgG	LS-C22276/ LSBio	1:500

Appendix Table 2 Standard Curve

#	Target	Standard Curve				
		Range (ng/mL)	Fold dilution (# points)	Cat# / Manufacturer	Stock conc. (mg/mL)	Serum dilution
1	**C1q**	200-0.2	2 (11)	A400/Quidel	1	5,000-10,000X
2	**C3**	500-0.1	2 (11)	A401/Quidel	1	20,000X
3	**CFH**	1,000-1	2 (6)	A410/Quidel	1	2,500-5,000X
4	**CFI**	4,000-4	2 (11)	A138/Complement technology	1	250-500X
5	**CFB**	1,000-30	2 (6)	A408/Quidel	1	1,250X
7	**C5**	1000-2	2 (10)	A403/Quidel	1	500-1,000X
8	**C6**	10-0.01	2 (11)	A404/Quidel	1	5,000X
9	**C8**	100-0.1	2 (11)	A406/Quidel	1	1,000X
11	**SC5b-9**	1000-15	2 (7)	A127/Complement technology	1	25X-100X
12	**Oligomer MBL**	40-0.08	2 (10)	2307-MB/R&D systems	0.1	100X
13	**C5a**	0.2-0.03	2 (7)	Part 842757/R&D Systems	0.00013	1,000X
14	**C1r**	4,000-32	2 (8)	A102/Complement technology	1	125-250X

Appendix Table 3 Detection Antibody

#	Target	Detection ab			
		Clonality/Host	Cat# / Manufacturer	Format	Dilution
1	**C1q**	mab/Mouse IgG1	sc-53544/Santa Cruz	Purified	1:4000
2	**C3**	pab/Rabbit IgG	ab48342/Abcam	Biotin	1:1,000
3	**CFH**	mab/Mouse (clone OX23)	ab17928/Abcam	Purified	1:10,000
4	**CFI**	mab/Mouse IgG (clone 9E11)	NBP1-05071/Novus Biologicals	Purified	1:1,000
5	**CFB**	mab/Mouse IgG	A712/Quidel	Biotin	1:500
7	**C5**	mab/Mouse IgG1	ab17457/Abcam	Purified	1:5000
8	**C6**	mab/Mouse IgG	A219/Quidel	Purified	1:1,000
9	**C8**	mab/Mouse IgG	A249/Quidel	Purified	1:1,000
11	**SC5b-9**	mab/Mouse IgG	A711/Quidel	Biotin	1:500
12	**Oligomer MBL**	mab/Mouse IgG (clone 3B6)	BPD-HYB-131-01B/Enzo Life sciences	Biotin	1:2000
13	**C5a**	mab/Mouse IgG	Part 842756/R&D Systems	Biotin	1:180
14	**C1r**	mab/IgG2b	MAB1807/R&D Systems	Purified	1:250

Appendix Table 4 HRP Conjugated Antibody

#	Target	HRP-labeled protein		
		Host	Cat# / Manufacturer	Dilution
1	**C1q**	Goat	115-036-006/Jackson Imuno	1:2000
2	**C3**	Streptavidin	S000-03/Rockland	1:20,000
3	**CFH**	Goat	115-036-006/Jackson Imuno	1:2000
4	**CFI**	Goat	115-036-006/Jackson Imuno	1:2000
5	**CFB**	Streptavidin	S000-03/Rockland	1:20,000
7	**C5**	Goat	115-036-006/Jackson Imuno	1:2000
8	**C6**	Goat	115-036-006/Jackson Imuno	1:2000
9	**C8**	Goat	115-036-006/Jackson Imuno	1:2000
11	**SC5b-9**	Streptavidin	S000-03/Rockland	1:20,000
12	**Oligomer MBL**	Streptavidin	S000-03/Rockland	1:20,000
13	**C5a**	Streptavidin	S000-03/Rockland	1:20,000
14	**C1r**	Goat	115-036-006/Jackson Imuno	1:2000

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