Powassan Viruses Spread Cell to Cell During Direct Isolation from *Ixodes* Ticks and Persistently Infect Human Brain Endothelial Cells and Pericytes

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Running Title: POWVs Infect Brain Endothelial cells and Pericytes

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Abstract

Powassan viruses (POWVs) are neurovirulent tick-borne flaviviruses emerging in the Northeastern U.S., with a 2% prevalence in Long Island (LI) deer ticks (*Ixodes scapularis*). POWVs are transmitted in as little as 15 minutes of a tick bite, and enter the CNS to cause encephalitis (10% fatal) and long-term neuronal damage. POWV-LI9 and POWV-LI41 present in LI *Ixodes* ticks were isolated by directly inoculating VeroE6 cells with tick homogenates and detecting POWV infected cells by immunoperoxidase staining. Inoculated POWV-LI9 and LI41 were exclusively present in infected cell foci, indicative of spread cell to cell, despite growth in liquid culture without an overlay. Cloning and sequencing establish POWV-LI9 as a phylogenetically distinct lineage II POWV strain circulating in LI deer ticks. Primary human brain microvascular endothelial cells (hBMECs) and pericytes form a neurovascular complex that restricts entry into the CNS. We found that POWV-LI9, -LI41 and Lineage I POWV-LB, productively infect hBMECs and pericytes and that POWVs were basolaterally transmitted from hBMECs to lower chamber pericytes without permeabilizing polarized hBMECs. Synchronous POWV-LI9 infection of hBMECs and pericytes induced proinflammatory chemokines, interferon- β (IFN β) and IFN-stimulated genes, with delayed IFN β secretion by infected pericytes. IFN inhibited POWV infection, but despite IFN secretion a subset of POWV infected hBMECs and pericytes remained persistently infected. These findings suggest a potential mechanism for POWVs (LI9/LI41 and LB) to infect hBMECs, spread basolaterally to pericytes and enter the CNS. hBMEC and pericyte responses to POWV infection suggest a role for immunopathology in POWV neurovirulence and potential therapeutic targets for preventing POWV spread to neuronal compartments.

Importance

We isolated POWVs from LI deer ticks (*I. scapularis*) directly in VeroE6 cells and sequencing revealed POWV-LI9 as a distinct lineage II POWV strain. Remarkably, inoculating VeroE6 cells with POWV containing tick homogenates resulted in infected cell foci in liquid culture, consistent with cell to cell spread. POWV-LI9, -LI41, and Lineage I POWV-LB strains infected hBMECs and pericytes that comprise neurovascular complexes. POWVs were nonlytically transmitted basolaterally from infected hBMECs to lower chamber pericytes, suggesting a mechanism for POWV transmission across BBB. POWV-LI9 elicited inflammatory responses from infected hBMEC and pericytes that may contribute to immune cell recruitment and neuropathogenesis. This study reveals a potential mechanism for POWVs to enter the CNS by infecting hBMECs and spreading basolaterally to abluminal pericytes. Our findings reveal that POWV-LI9 persists in cells that form a neurovascular complex spanning the BBB, and suggest potential therapeutic targets for preventing POWV spread to neuronal compartments.

Introduction

Powassan virus (POWV) is a neurovirulent tick-borne flavivirus (FV) that was first isolated in 1958 from a fatal encephalitic case of human disease in Powassan, Ontario, Canada⁽¹⁾. POWV has recently emerged in ticks in the Northeastern U.S., with host reservoirs in white-footed mice, deer, skunks, woodchucks, and squirrels⁽²⁻⁵⁾. In humans, POWV enters the central nervous system (CNS), lytically infects neurons, and causes severe encephalitis with a ~10% mortality rate and long-term neurologic sequelae occurring in 50% of infected patients⁽⁶⁻⁹⁾. POWV awareness and diagnostic testing of patients has revealed an increasing number of human POWV encephalitis cases that suggest a high incidence of previously undiagnosed POWV disease, especially in areas where Lyme disease is endemic. Approximately 2% of deer ticks (*I. scapularis*), in the highly populated Long Island (LI), N.Y. area are POWV positive⁽⁶⁾. Currently there are no approved vaccines or therapeutics for POWV.

The presence of POWV in tick saliva accounts for POWV transmission to hosts or humans in as little as 15 minutes after a tick bite⁽⁸⁻¹⁰⁾. POWV transmission is followed by a 1-5 week incubation period, a 1 week of acute febrile illness and weeks to months of encephalitic manifestations of fever, headache and confusion that worsen into meningoencephalitis with seizures, impaired consciousness, coma and respiratory failure⁽⁹⁾. In autopsies of POWV patients, neurons in the central nervous system (CNS) were infected with POWV antigen and RNA within lesions in the cerebellum, brainstem, basal ganglia, and thalamus⁽¹¹⁾. The emergence of neurovirulent POWV in highly populated areas, rapid tick to human transmission and the severity of POWV disease provide urgency for understanding how POWVs spread to neuronal compartments, and defining potential therapeutic targets.

POWV is the only North American tick-borne flavivirus and POWVs are distantly related to Tick Borne Encephalitis virus (TBEV), the leading cause of arthropod borne encephalitis in Europe and Asia⁽¹²⁾. There are two genetic lineages of POWV, lineage I includes the LB strain derived from the 1958 human encephalitis case, and lineage II strains sequenced or isolated from North American deer ticks. POWV I and II strains share 94% amino acid identity and represent POWV genotypes carried by

discrete ticks and mammalian hosts⁽¹³⁾. Prototype Lineage I and II POWVs were first isolated following intracranial inoculation of mice and subsequent passage in Vero cells^(1, 8, 14, 15). However, there are no studies of POWV infection of cells that comprise the BBB and normally prevent viral access to neuronal compartments.

How POWV spreads from a tick bite site to the CNS, persists to cause a late acute febrile illness, or enters protected neuronal compartments remains to be determined. Reservoir cells where POWV persists following infection and mechanisms by which POWV crosses the BBB to enter the CNS remain enigmatic. There are no reports of POWV infection of hBMECs or pericytes, or cellular responses to POWV or TBEV infections. TBEV was found to infect only 2-5% of human brain microvascular ECs (hBMECs), was primarily apically released, and failed to lyse ECs, alter EC barrier functions or spread within EC monolayers⁽¹⁶⁾. In mice, TBEV reportedly enters the CNS without permeabilizing the BBB, and at late stages of infection induces proinflammatory cytokine and chemokine responses when high viral loads are already present in the brain⁽¹⁷⁾.

hBMECs have intimate basolateral contacts with pericytes, forming a neurovascular unit that plays a critical role in regulating BBB permeability and immune cell recruitment. POWV infection of hBMECs and brain pericytes have not been investigated. In this study, we isolated POWVs from Long Island deer ticks and evaluated their ability to replicate, persistently infect, and spread in VeroE6 cells, primary human BMECs and brain pericytes. We collected 438 deer ticks from Long Island, and from 44 pools of 10 ticks, we found 3 POWV positive pools by qRT-PCR. Tick homogenates inoculated into 44 wells of VeroE6 cells revealed 2 wells (9 and 41) with POWV antigen positive cells 7 dpi, both coinciding with PCR positivity. We observed that direct inoculation of VeroE6 cells with LI tick homogenates resulted in large POWV infected cell foci consistent with cell to cell spread despite the absence of restrictive overlays. POWV-LI9 and POWV-LI41 were propagated from supernatants and POWV-LI9 was cloned and sequenced, identifying it as a distinct Lineage II POWV strain.

We found that POWV-LI9 and POWV-LI41 strains productively and persistently infected VeroE6

110 cells, hBMECs and human pericytes nonlytically, reaching respective POWV-LI9 titers of 8×10^5 , $1 \times$
 111 10^4 , and 5×10^4 . POWV-LI9 failed to permeabilize polarized hBMECs or VeroE6 cells, but was
 112 preferentially released basolaterally from cells (3-12-fold) to lower chamber pericytes in a BBB-like
 113 Transwell model. Prototypic POWV-LB acquired from the ATCC has been passaged extensively in
 114 Vero cells since 1958⁽¹⁾, and similar analysis of POWV-LB infection of VeroE6 cells resulted in a few
 115 small clusters of infected cells but primarily spread uniformly within the monolayer. However, like
 116 POWV-LI9, we found that POWV-LB also productively infected hBMECs and pericytes and POWV-
 117 LB was basolaterally released from polarized hBMECs (3-fold over apical titers). These findings
 118 suggest a potential mechanism for Lineage I and II POWVs to infect hBMECs and pericytes and cross
 119 the BBB.

120 Lectin pretreating hBMECs permitted POWV-LI9 to synchronously infect hBMECs (>90%) and we
 121 used this approach to evaluate hBMEC and pericyte responses to POWV infection. POWV induced
 122 IFN β , CCL5 (>3000-fold) an array of inflammatory chemokines, and IFN-stimulated genes (ISGs) 1-3
 123 dpi, with conserved and novel responses between POWV infected hBMECs and pericytes. IFN β
 124 induction and secretion were restricted in POWV infected pericytes, but not hBMECs, 1 dpi, but IFN β
 125 was highly secreted by both hBMECs and pericytes 2 dpi. Pretreating VeroE6 cells, hBMECs or
 126 pericytes with Type I IFN prevented POWV infection. Despite IFN β induction and sensitivity, a subset
 127 of hBMECs and pericytes were persistently infected with POWV-LI9, 12-30 dpi and through cell
 128 passage. These findings differ from ZIKV which spreads and replicates continuously in hBMECs in the
 129 absence of secreted IFN β ⁽¹⁸⁾. Our results reveal novel POWV-LI9 regulation of hBMEC and pericyte
 130 responses and suggest that hBMEC-pericyte neurovascular complexes may serve as POWV reservoirs
 131 and conduits for POWV to spread basolaterally across the BBB and into neuronal compartments.

Results

POWV-LI9 isolation from *Ixodes scapularis* ticks

In November 2020 we collected 438 *I. scapularis* adult ticks, in Suffolk County, Long Island, New York. Homogenates of 44 pools of 10 ticks were prepared in PBS by dounce homogenization, and following centrifugation, supernatants were inoculated into VeroE6 cells and hBMECs in 24-well plates and screened for POWV RNA by qRT-PCR. Three tick pool inocula were PCR positive for POWV RNA. One week after inoculation, infected cell supernatants were harvested and cell monolayers were immunoperoxidase stained using anti-POWV HMAF antibody (ATCC). Coincident with RNA positive tick pools, VeroE6 cell wells 9 and 41 had POWV antigen positive infected cells 7 dpi (Figure 1A).

Despite growth in liquid culture, POWV antigen positive VeroE6 cells were present in large infected cell foci, consistent with cell to cell spread, without apparent cell lysis (Figure 1A). Infection of VeroE6 cells with passage 1 POWV-LI9 or POWV-LI41 stocks resulted in smaller more dispersed infected cell foci (Figure 1A). A time course of POWV-LI9 spread in VeroE6 cells from 1-10 dpi shows that virus spread from initial infected cell foci 3-5 dpi to uniformly infect monolayers 6-10 dpi (Figure 1B). Similar initial foci and later spread was observed when POWV-LI41 infected VeroE6 cells (Supp. Fig. 1). There was no evidence of cytopathic effects (CPE) in POWV-LI9 infected VeroE6 cells with ~100% of VeroE6 cells still infected 10-13 dpi (Figure 1B). Supernatant titers of POWV-LI9 in VeroE6 cells reached 8×10^5 FFU/ml (Figure 1C) and VeroE6 cells remained highly infected following cellular passage ($>10^5$ FFU/mL). Infection of VeroE6 cells with extensively passaged Lineage I POWV-LB (ATCC-1958) resulted in some small clusters of 3-12 infected VeroE6 cells 1 dpi (Supp. Fig. 2A), and the uniform infection of VeroE6 cell monolayers with titers of 1×10^6 /ml (Supp. Fig. 2A-B). As POWV spread in tissue culture cells following direct tick inoculation has not been previously examined, it remains to be seen if initial cell to cell spread is unique to POWV-LI9/LI41 or a fundamental attribute of direct tick inoculation of POWVs in mammalian cells. Overall these findings demonstrate the direct isolation and productive replication of POWV-LI9 and POWV-LI41 from LI ticks in VeroE6 cells.

POWV-LI9 is a Discrete Lineage II Strain of POWV Present in LI Deer Ticks

The POWV-LI9 isolate was passaged in VeroE6 cells for 7-10 days with supernatants collected for viral stocks and we cloned and sequenced the POWV-LI9 genome from RNA isolated from passage 3 POWV-LI9 infected VeroE6 cell lysates. Random primed cDNAs were amplified into ~2 kb fragments, using conserved POWV primers, and cloned into pMiniT 2.0. Clones encompassing the complete POWV genome were sequenced with 5' RACE and a 3' end oligo used to amplify non-coding POWV-LI9 genome termini (GeneBank accession MZ576219)⁽¹⁹⁾.

Sequences of POWV-LI9 were phylogenetically compared to 15 full-length POWV lineage II sequences, 10 POWV lineage I sequences and TBEV^(20, 21). Phylogenetic tree analysis revealed that the POWV-LI9 isolate is a lineage II POWV strain that forms a distinct clade by comparison with other Northeastern, New York and Wisconsin POWV lineage II sequences (Figure 2A)⁽²⁰⁻²²⁾. Sequence alignment of POWV strains with POWV-LI9 revealed an average 99% nucleotide and amino acid identity with POWV genomes collected in the Northeastern region (Figure 2B, group A), 93.5% nucleotide and 97% amino acid identity with POWV collected in Wisconsin (Figure 2B, group B), and 85% nucleotide and 94.7% amino acid identity with POWV Lineage I (Figure 2B, group C). Novel POWV-LI9 residues include Envelope (Env) protein residues S119 and K151, and NS2B (F125), NS3 (D402) and NS5 (T282) residues. The POWV-LI41 isolate is derived from discrete tick homogenates but has yet to be sequenced to determine if it differs from POWV-LI9.

In initial experiments, POWV-LI9 (10³ FFUs) was inoculated into the footpad of C57BL/6 mice and sera collected 12 dpi were found to elicit antibody responses that detect envelope proteins from POWV-LI9 and POWV-LB infected VeroE6 cells by Western blot (Supp. Fig. 3A); and neutralize POWV-LI9 and POWV-LB viruses (Supp. Fig. 3B). Collectively, these findings establish that POWV-LI9 is a lineage II POWV strain newly discovered in *Ixodes s.* ticks on Long Island, that infects mice and elicits neutralizing antibody responses to Lineage I and II POWVs.

182 POWVs Productively Infect Primary Human BMECs and Pericytes

183 Mechanisms by which POWVs enter neuronal compartments in order to lytically infect neurons
184 have not been investigated. Here we assessed the ability of POWV-LI9 to infect, replicate and persist in
185 primary human BMECs and pericytes that form a neurovascular complex within the BBB⁽²³⁻²⁶⁾. We
186 found that POWV-LI9 productively infects hBMECs 1-8 dpi, with low viral titers and a subset of
187 hBMECs remaining persistently infected 6-8 dpi (Figure 3A,B). Infecting hBMECs with POWV-LI9 at
188 an MOI of 5 only marginally increased the number of infected hBMECs 1 dpi (10-15% max),
189 suggesting that entry was restricted to a subset of hBMECs.

190 To determine if attachment limited hBMEC infection, we treated POWV-LI9 with wheat germ
191 agglutinin (WGA, 1 µg/ml, 5 min) prior to infecting hBMECs, a method previously used to direct
192 efficient HIV infection of ECs⁽²⁷⁻²⁹⁾. Pretreating POWV with WGA prior to adsorbing POWV-LI9 to
193 cells, resulted in a synchronous ~95% infection of hBMECs (1 dpi) (Figure 3C). The ability of WGA
194 treated POWV to efficiently direct the infection of hBMECs suggests that POWV attachment and entry,
195 rather than post-attachment replication, is restricted to a subset of hBMECs and permits analysis of
196 hBMEC responses to a synchronous POWV infection.

197 In BBB capillaries, human brain pericytes are attached to the basolateral side of hBMECs and
198 form abluminal contacts with astrocytes and neurons^(23-25, 30, 31). Pericyte responses and interactions with
199 hBMECs control the integrity and immune cell recruiting functions of the BBB⁽³¹⁾. We found that
200 POWV-LI9 infected primary human brain pericytes spreading from 1 to 3 dpi to infect ~80% of
201 pericytes (Figure 3D) and produce titers of 5×10^4 /ml (Figure 3E) without apparent cytopathology.
202 From 3-8 dpi POWV titers were reduced in pericyte supernatants, but a high percentage of pericytes
203 remained POWV infected 8-30 dpi and after pericyte passage. Unlike POWV-LI9 infection of VeroE6
204 cells, we found that POWV-LI9 infection of pericytes failed to form infected cell foci and instead
205 infected single cells that spread ubiquitously. Similar to POWV-LI9 findings, we found that POWV-
206 LI41 and POWV-LB also productively infect hBMECs and pericytes (Supp. Fig. 4). Collectively, these

findings demonstrate that Lineage I and II POWVs infect hBMECs and pericytes which form neurovascular complexes within the BBB, and thereby suggest potential mechanisms for POWVs to enter neuronal compartments.

Preferential Basolateral Release of POWVs from hBMECs Directs Pericyte Infection

hBMECs form polarized monolayers with apical and basolateral surfaces that mimic luminal and abluminal capillary surfaces⁽²⁴⁾. The ability of POWV-LI9 to infect hBMECs prompted us to determine whether POWV spreads basolaterally across polarized hBMECs. Neither POWV infected hBMECs, pericytes, or VeroE6 cells showed signs of cytopathic effects and 3 dpi POWV-LI9 infected cells were nearly all viable by Calcein-AM staining (green-live) with little propidium iodide uptake (red-dead) (Figure 4A). Consistent with this, we found no apparent disruption of tight junction protein ZO-1 in cell-to-cell contacts of POWV-LI9 infected hBMECs (Figure 4B).

To determine whether POWVs permeabilize hBMECs or VeroE6 cells we analyzed polarized cells grown on Transwell inserts for changes in Trans endothelial electrical resistance (TEER). We found no significant change in TEER of POWV infected VeroE6 or hBMECs 1-3 dpi versus uninfected cells, indicating that POWV infection did not permeabilize infected hBMEC monolayers (Figure 4C). Infection of hBMECs and VeroE6 cells on polarized Transwell inserts permitted analysis of apical and basolateral titers following POWV infection. We found that POWV-LI9 titers in basolateral supernatants were enhanced >3-fold in VeroE6 and >12-fold in hBMECs over apically released virus (Figure 4D, E). Analysis of POWV-LB found that POWV titers were enhanced 3 fold in basolateral versus apical supernatants (Supp. Fig. 4D).

POWV basolateral release from hBMECs suggested the potential for POWV to directly infect pericytes. We POWV-LI9 infected polarized hBMECs on Transwell inserts, transferred inserts to wells containing lower chamber pericytes (1dpi) and found that pericytes were infected by basolaterally released POWV (Figure 4F). Preferential basolateral release of POWV from hBMECs suggests a

potential mechanism for POWVs to spread across the BBB to abluminal pericytes that reside within neuronal compartments.

Interferon Pretreatment blocks POWV Infection and Spread

To determine if POWV infections are IFN sensitive, we pretreated VeroE6 cells, hBMECs and pericytes with Type I IFN (IFN α) 2-18 hours prior to POWV-LI9 infection and quantitated infected cells 1 dpi. We found that pretreating cells with IFN α nearly completely abolished POWV infection (Figure 5A), indicating that preexisting or induced Type I IFN prevents POWV infection and spread in hBMECs and pericytes. We analyzed POWV infected hBMEC and pericyte supernatants for IFN β by ELISA and found that POWV infected hBMECs highly secreted IFN β 1 dpi, while IFN β secretion by POWV infected pericytes was not apparent until 2 dpi (Figure 5B,C). Secreted IFN β responses are consistent with the early restriction of POWV-LI9 spread in hBMECs, and continued POWV spread in pericytes 1-3 dpi (Figure 5D). In both cell types IFN β secretion is dramatically reduced 3 dpi suggesting a potential mechanism for POWVs to inhibit late IFN expression and persist in infected hBMECs and pericytes.

POWV Infected hBMECs and Pericytes Elicit Immune-Enhancing Responses

hBMECs and basolateral pericytes form a functional unit that determine the integrity of the BBB through synergistic responses that regulate vascular permeability, cell proliferation, and immune cell transit across the endothelium^(30, 31). To evaluate transcriptional changes of POWV infected hBMECs, we WGA treated POWV to synchronously infect 85-90% of cells 1 dpi and compared transcriptional responses to WGA treated, mock infected, hBMECs. RNA from control and POWV infected hBMECs were analyzed by Affymetrix arrays 1-3 dpi. POWV infection of hBMECs resulted in the dramatic induction of chemokines (CCL5, CXCL10, CCL20, CXCL11), IFN β , IFN stimulated genes (ISGs: RSAD2, OASL, IFIT2/3, MX2, ISG15), IFN response factors (IRFs-1/2/7), Transcription factors (ATF3 and EGR1), and coagulation factor B over mock infected hBMEC controls (Figure 6A). Selected POWV directed hBMEC transcriptional changes are presented in Figure 5A, and complete data is available in

the NCBI GEO database (GSE176251). POWV induction of high levels of IFN β , CCL5, and ISG15 in hBMECs was verified by qRT-PCR (Figure 6B).

We also analyzed human brain pericyte transcriptional responses following synchronous POWV-LI9 infection (MOI 5) compared to mock-infected pericytes (NCBI GEO accession GSE176252). ISGs were the most highly induced responses observed in POWV infected pericytes 1 dpi, with 100-1700 fold increases over a wide array of ISGs (Figure 6C). Despite high ISG responses, IFN β was only induced 9 fold 1 dpi, but increased dramatically to 463-fold by 2 dpi and 281-fold 3 dpi. Chemokines CCL5 and CXCL10 were moderately induced 93-156 fold 1 dpi but were induced 1800-3300 fold 2-3 dpi (Figure 6C). Complement-activating factors (CFB, C3, C1S, C1R, C7), IL6 (111-256-fold 2-3 dpi) and IL1 β (10-19-fold) were highly induced by POWV infection of pericytes 2-3 dpi. Despite high level IFN β and ISG responses, POWV productively replicated and spread in pericytes 1-3 dpi, with persistent infection apparent >8 dpi (Figure 3D,E). The induction of selected chemokines, cytokines and ISGs induced by POWV infection was validated by qRT-PCR (Figure 6D). Our findings indicate that POWV infection of human pericytes transcriptionally induces chemotactic cytokine and complement responses that are known to enhance inflammation and immune cell recruitment to the endothelium. How POWV spreads and persists in pericytes in the presence of high level IFN and ISG responses at later times after infection remains to be investigated.

Pathway gene ontology (GO) analysis⁽³²⁾ of hBMECs and pericyte transcriptomes showed similar GO enriched responses related to cytokine production, leukocyte migration, type I IFN signaling, ISG15-protein conjugation, cell death and apoptosis regulation, angiogenesis, and EC migration (Figure 6E). Regulation of prosurvival ERK1/2 signaling, that was recently shown to be required for ZIKV persistence in hBMECs⁽³³⁾, was specifically associated with POWV induced hBMEC transcriptomes 2-3 dpi (Figure 6E, left panel). ERK1/2 pathway activation is consistent with CCR3/CCR5 activation by high levels of CCL5 induced by POWV-infected hBMECs and pericytes⁽³³⁾. Pericyte specific responses

279 included cell cycle, NF- κ B signaling, antigen processing and presentation, and complement activation 2-
280 3 dpi (Figure 5E, right panel).

281 Collectively, chemokine responses elicited by POWV infected hBMECs appear to be synergistic
282 with robust chemokine and IFN responses induced by POWV-LI9 infected pericytes. These findings
283 indicate that POWV infection of pericytes is likely to enhance immune cell recruitment to hBMECs that
284 may contribute to immune enhanced pathogenesis. In contrast to IFN secretion 1 dpi by hBMECs,
285 POWV infected pericytes inhibit IFN secretion 1 dpi, yet both cell types induce late IFN and ISG
286 responses that fail to prevent POWV persistence. These findings suggest that POWV differentially
287 regulates early IFN responses in pericytes and hBMECs, and suggest that at later times a subset of
288 POWV infected hBMECs and pericytes may become resistant to IFN and ISG responses.

Discussion

POWV is transmitted to humans in tick saliva during a short ~15 minute tick bite⁽³⁴⁾. POWVs cause a severe neurovirulent disease that is 10% fatal and results in long term neurologic deficits in 50% of patients⁽⁸⁾. The rapid transmission of POWV is unlike Lyme disease, which requires long term tick attachment and where antibiotics are available to clear the infection. For POWV, there are no therapeutic treatments or approved vaccines to prevent neurovirulent infection and disease. POWV carrying ticks and human POWV infections have increased in the Northeastern (N.E.) U.S.^(22, 35, 36). Climate change is expected to exacerbate future POWV incidence by expanding the range and density of *I. scapularis* and mammalian disease reservoirs in addition to increasing tick survival and seasonal activity⁽³⁷⁾. A recent study found a 2% prevalence of POWV in *Ixodes scapularis* ticks on Long Island, NY⁽⁶⁾, and the spread of POWV and the severity of disease rationalized evaluating POWVs circulating in this densely populated area. In this report, we isolated POWVs from *I. scapularis* ticks on LI and demonstrate that POWVs (LI9, LI41 and LB) infect human brain microvascular endothelial cells and pericytes that form a neurovascular complex that spans the BBB. Our findings suggest the potential for persistently infected hBMECs and pericytes to be reservoirs of POWV spread, and suggest a mechanism for POWVs to cross the BBB and enter neuronal compartments.

We isolated POWVs (LI9 and LI41) from 3/44 PCR positive LI tick pools by directly inoculating tick homogenates into VeroE6 cells. Cloning and sequencing of the POWV-LI9 tissue culture isolate revealed it to be a phylogenetically distinct lineage II POWV strain present in LI *Ixodes scapularis* ticks. The POWV-LI9 isolate is 99% identical to POWV genomes collected in Rhode Island and Maine (Figure 2A)⁽²⁰⁻²²⁾, and shares 95% amino acid identity with the prototypic 1958 lineage I POWV-LB strain (Figure 2B)⁽²⁰⁻²²⁾. However, other than sequencing, the isolation and tissue culture analysis of NE POWV strains has yet to be reported.

We isolated POWV- LI9 and POWV-LI41 by directly infecting VeroE6 cells with tick homogenates and observed large POWV infected cell foci in liquid media 7 dpi. In this setting, viruses are normally

dispersed in cell supernatants and spread to single cells throughout the monolayer. The observation that POWV-LI9 and -LI4 form foci indicates that these POWVs spread cell to cell. This is novel and not previously evaluated or reported for any POWV⁽³⁸⁾. In subsequent POWV-LI9 passages, focal spread observed in passage 1 and 2, changed to a more uniform infection of VeroE6 cells. Whether cell to cell spread is a fundamental feature of POWVs directly inoculated into mammalian cells has yet to be considered and remains to be determined as there are no studies of POWV spread following direct tissue culture isolation from ticks. Prototypic POWV-LB and POWV-SP strains were isolated by intracranial inoculation of mice prior to extensive passage in Vero cells^(1, 8, 14, 15). Prior studies of POWV-LB infected VeroE6 cells did not report infected cell foci, but also lack analysis of POWV spread in infected cells. We found that POWV-LB uniformly infected VeroE6 cells with a few grouped infected cells (Supp Fig 2), but no large infected cell foci indicative of cell to cell spread.

Initial cell to cell spread of POWV strains remains an enigma that requires analysis of more POWVs by direct isolation in tissue culture, and the analysis of potential POWV genetic changes during isolation and early passage. These findings also raise the question of whether POWVs are initially restricted to cell to cell spread following a tick bite, and whether this contributes to the long prodrome of POWV disease. These findings are fundamental to initial POWV spread and may need to be considered in studies of POWV spread and neuropathogenesis in murine models that are uniformly based on POWVs that were first passaged in mouse brain.

The ability of POWV-LI9 and -LI41 strains to form infected foci are new and novel observations, as there are no reports of cell to cell spread for other mosquito or tick-borne flaviviruses⁽³⁸⁾. Focal cell to cell spread has been shown for several viruses including Herpesvirus, rhabdovirus, vaccinia virus, HIV-1 and the Flavivirus HCV⁽³⁸⁻⁴⁴⁾. Neurovirulent measles virus forms infectious foci in primary human airway epithelial cells, with viral spread dependent on intercellular adherens junction localized pores⁽⁴²⁾. Similar to POWV, measles virus foci form without cytopathology, cell to cell fusion or syncytia formation⁽⁴²⁾. Additional experiments that differentiate POWV cell to cell spread, basolateral release or

spread via intercellular membrane pores are required to understand how POWV-LI9 forms foci, and to define mechanisms of POWV spread from infected hBMECs to the CNS.

How POWVs enter the CNS remains unknown and there is no information on cellular targets of POWV infection, or the ability of POWVs to infect and replicate in cells of the BBB. The BBB is a highly selective semipermeable barrier that separates the circulating blood from the CNS and prevents pathogens from entering the brain⁽⁴⁵⁾. The BBB is composed of brain microvascular endothelial cells, pericytes, astrocytes and microglia^(31, 45, 46). Pericytes form intimate basolateral contacts with both BMECs and astrocytes, forming a neurovascular unit that regulates functions of luminal endothelial cells and abluminal pericytes and astrocytes within the neuronal compartment^(25, 47).

There are no prior studies of POWV infection of hBMECs and pericytes. We found that POWV-LI9, -LI41 and POWV-LB infect hBMECs resulting in single infected cells with little cell to cell spread after initial infection. This differs from POWV-LI9 infection of VeroE6 cells and it remains to be determined if these differences are the result of hBMEC receptors or IFN restricted spread that is absent in VeroE6 cells. POWVs productively infected hBMECs without apparent cell lysis or permeabilizing effects (Figure 4C). However, a comparison of POWV titers from apical and basolateral sides of polarized VeroE6 or hBMECs in Transwell plates demonstrated that POWV-LI9 is preferentially released from the basolateral side (3-12-fold) (Figure 4D, E). Similar analysis of POWV-LB found both apical and basolateral spread from infected hBMECs with a 3-fold increase in basolateral spread (Supp Fig 4B). Only a few studies demonstrate preferential basolateral viral spread⁽⁴⁸⁻⁵⁰⁾. Vesicular stomatitis virus (VSV) and Marburg virus (MARV) bud from the basolateral side of polarized MDCK cells⁽⁴⁸⁻⁵⁰⁾, and arenaviruses are basolaterally released from airway epithelial cells⁽⁵¹⁾. Basolateral spread of POWVs suggests a mechanism for POWVs to cross the BBB and spread abluminally to neuronal compartments.

In the brain, pericytes form a neurovascular complex with hBMECs spanning the BBB and contacting astrocyte end feet, microglia, and neurons^(30, 31, 52). POWV infection of hBMECs prompted us to analyze whether pericytes are infected and whether hBMECs convey POWV infection to pericytes.

364 We found that POWV-LI9, LI41 and POWV-LB highly infect primary human brain pericytes (Figure
365 3D,E; Supp Fig. 4A,C,E). In a BBB-like in vitro model POWV-LI9 infected hBMECs also basolaterally
366 transmitted POWV to pericytes in the lower chamber (Figure 4F). Our findings demonstrate that
367 POWVs productively and persistently infect hBMECs and pericytes, and suggest hBMEC-pericyte
368 complexes as potential POWV reservoirs and conduits for viral spread to neuronal compartments.

369 Neurotropic flaviviruses including ZIKV, JEV, WNV and TBEV are reportedly transmitted across
370 polarized ECs without permeabilizing or lysing cells^(16, 18, 53, 54). In TBEV infected mice, BBB
371 permeability was not required for viral entry into the CNS, and permeability was only observed at late
372 stages of infection, and after high viral loads and inflammatory damage was evident in the brain⁽¹⁷⁾. In
373 innate immune compromised mice, neurotropic flaviviruses invade the CNS during early viremic
374 stages⁽⁵⁵⁾ with infection of the BBB one of the most likely routes of neuronal invasion⁽⁴⁵⁾.

375 Many murine POWV pathogenesis models use POWV-LB and -SP strains that were first passaged in
376 mouse brains^(14, 56), and IFNAR antibody blockades to promote lethal outcomes^(57, 58). Initial inoculation
377 of POWV-LI9 strain in WT C57BL/6 mice resulted in murine seroconversion and antibodies that
378 neutralized both POWV-LI9 and POWV-LB (Supp. Fig. 3A,B). It remains to be determined if POWV-
379 LI9 and -41 strains are neurovirulent in mice. However, analysis of POWV-LI9 viremia, CNS spread,
380 pathology and neurovirulence in mice, at early or late passage, is beyond the scope of this initial study of
381 newly isolated POWV strains circulating on LI.

382 POWV-LI9 persistence in hBMECs and pericytes is consistent with the delayed onset of
383 neurologic disease in POWV patients and the long-term pathology of POWV infection^(4, 8, 59). However,
384 POWV persistence is very different from the persistent infection of hBMECs by ZIKV^(18, 33). ZIKV
385 replicates and spreads persistently in hBMECs without permeabilizing or lysing cells, and persistence
386 continues through serial cellular passage⁽¹⁸⁾. Recent findings indicate that ZIKV persistence requires the
387 suppression of IFN secretion and the high level secretion of CCL5 that acts as an autocrine activator of
388 ERK1/2 survival signaling responses⁽³³⁾.

389 In contrast to ZIKV, POWV infection of hBMECs does not result in the constitutive spread of
 390 POWV throughout the monolayer. ZIKV, DENV and POWV are inhibited by pretreating cells with
 391 Type I IFN^(18, 60). However, ZIKV bypasses this restriction by blocking IFN β secretion from infected
 392 hBMECs, while DENV is cleared from infected ECs by IFN β and fails to establish a persistent
 393 infection^(18, 60). Although, POWV spread is restricted by secreted IFN β , a subset of infected hBMECs
 394 remain persistently infected, viable and POWV antigen positive. Curiously, POWV inhibits pericyte
 395 secretion of IFN β 1 dpi, permitting early viral spread within pericytes. This suggests novel POWV
 396 regulation of early IFN responses in pericytes and suggests that POWV resistance to IFN secreted 2-3
 397 dpi permits POWV to persistently infect cells. It remains unknown whether POWV proteins, generated
 398 early in infection, permit POWV persistence or resistance to IFN/ISG responses hBMECs or pericytes
 399 or how secreted IFN β levels 3 dpi are restricted, despite highly induced IFN β transcripts. Collectively
 400 these findings indicate that POWV persists in hBMECs and pericytes without a total blockade of IFN β
 401 secretion observed during ZIKV infection⁽¹⁸⁾.

402 BMECs basolaterally secrete PDGF which recruits and stabilizes pericyte binding to BMECs and
 403 reciprocally, pericytes secrete factors that act on BMEC receptors which regulate barrier integrity,
 404 angiogenesis, and immune cell recruitment^(25, 47). Pericytes regulate the BBB, and targeting PDGFs or
 405 pericyte-BMEC interactions using imatinib or nintedanib has been suggested as a potential therapeutic
 406 approach to control damage of this neurovascular complex^(25, 47, 61). Our analysis of POWV-LI9 directed
 407 changes in hBMEC and pericyte transcriptional responses included finding the induction of several pro-
 408 survival genes including EGR1, ATF3 and BIRC3⁽⁶²⁻⁶⁴⁾, and chemokine CCL5, a cell survival response
 409 required for ZIKV persistence in hBMECs⁽³³⁾. Highly induced CCL5 activates ERK1/2 cell survival
 410 pathways in ECs⁽³³⁾ that may regulate anti-apoptotic and proliferative responses⁽⁶⁵⁾ and contribute to the
 411 viability of POWV infected hBMECs and pericytes.

412 Several chemokines were upregulated in both cell types, however, CCL5 and CXCL10 were the
 413 most highly induced, especially by POWV infected pericytes (Figure 6). CXCL10 is prominently

upregulated in the CSF of human patients and mouse models during TBEV and WNV infections⁽⁶⁶⁻⁷⁰⁾ and higher CXCL10 expression in the brain is correlated with more severe TBEV disease in mice⁽⁷¹⁾. CCL5 and CXCL10 also play an important role in recruiting activated T cells into sites of tissue inflammation⁽⁷²⁾ and these chemokines are implicated in both viral clearance and the neuropathology of neurotropic flavivirus infections⁽⁷³⁾. CCL5 is produced by several cell types that impact inflammation in the CNS, including hBMECs, pericytes, astrocytes, microglia, and neurons^(73, 74). Increased CCL5 has been detected in the CSF, but not in the serum, of TBEV patients and TBEV-infected mice^(68, 75). During TBEV infections, CCL5-directed neuroinflammation may contribute to brain pathology as treatment of mice with CCL5 antagonists prolonged survival and reduced brain inflammation after TBEV infection⁽⁷⁶⁾. Similarly, the CCL5 receptor antagonist, Maraviroc, reduced JEV-induced CNS inflammation and increased the survival of JEV infected mice⁽⁷⁷⁾. CCR5 knockout mice were also protected from fatal WNV or JEV encephalitis^(70, 78). ZIKV persistence in hBMECs was recently shown to require high level CCL5 secretion to support prosurvival responses, and CCL5 receptor inhibitors, that prevent ZIKV persistence in hBMECs, are suggested as therapeutics that clear ZIKV infections⁽³³⁾.

We were surprised to find that complement related factors CFB (149-fold), C3 (27-fold), C1r (30-fold), and C1s (14-fold) were highly induced by POWV infected pericytes. Complement proteins in the brain can be induced by astrocytes and microglia in response to injury⁽⁷⁹⁾, but little is known about brain pericytes actively producing complement proteins. WNV and HIV-1 induce complement activation within the CNS^(80, 81), and elevated C1q levels were found in the CSF of WNV and TBEV infected patients⁽⁸²⁾, and suggested to contribute to neurocognitive impairment in recovering patients⁽⁸³⁾. Our findings suggest that POWV-infected pericytes might enhance complement activation and C3a and C5a production, which contribute to immune cell chemotaxis, and T cell priming responses involved in both viral clearance and POWV neuropathology.

In this study, we demonstrate that POWVs productively infect and are basolaterally released from brain endothelial cells, suggesting a potential mechanism by which POWVs infect pericytes and

439 cross neurovascular complexes within the BBB. A subset of hBMECs and pericytes are persistently
440 infected by POWV for 8-30 days providing a novel location for the replication and spread of a
441 neurovirulent virus to the CNS. The abluminal location of pericytes in association with BBB hBMEC
442 makes them ideally suited to convey POWV to the CNS and recruit immune cells to the BBB that
443 contribute to neuronal immunopathogenesis. The ability of POWV to persist in hBMECs and pericytes
444 also suggests that these cellular niches are potential therapeutic targets for preventing POWV
445 neurovirulence.

Material and Methods

Cells. VeroE6 cells (ATCC CRL 1586) were grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with 5% fetal bovine serum (FBS) and penicillin (100 µg/ml), streptomycin sulfate (100 µg/ml), and amphotericin B (50 µg/ml; Mediatech) at 37°C and 5% CO₂. hBMECs (passages 4 to 10) were purchased from Cell Biologics (H-6023) and grown in EC basal medium-2 MV (EBM-2 MV; Lonza) supplemented with EGM-2 MV SingleQuots (Lonza) and incubated at 37°C and 5% CO₂. Human brain vascular pericytes (hPCs - passage 1) were purchased from ScienCell (#1200) and grown in pericyte medium (PM, #1201) supplemented with pericyte growth supplement (#1252) and incubated at 37°C and 5% CO₂.

POWV-LI9 Isolation from *Ixodes scapularis* Ticks. Ticks were collected during the fall season peak (October to November) throughout Suffolk County, New York, in 2020. Questing ticks were collected from vegetation along trails by flagging a 1-m² cotton flannel fabric attached on both ends to a wooden pole between 10:00 and 14:00 h during sunny days. 438 adults *Ixodes scapularis* ticks were identified morphologically on a dissecting microscope for taxonomic keys. Pools of 10 ticks were homogenized using an eppendorf tube mortar and pestle in 500 µL of phosphate-buffered saline (PBS) and debris removed by centrifuging for 5 min at 12,000 x g.

qRT-PCR for tick screening. Tick pool homogenates were tested for POWV RNA using a multiplex one-step reverse transcription PCR (qRT-PCR) using a forward primer (GTGATGTGGCAGCGCACC), a reverse primer (CTGCGTCGGGAGCGACCA) and a probe (CCTACTGCGGCAGCACACACAGTG) (6). The final reaction mixture contained 5 µl of template and 20 µl of master mixture. The master mix contained 0.2 µM of forward primer, 0.2 µM of reverse primer, 0.1 µM of probe, 1.25 µl of RNA UltraSense enzyme mix, and 5 µl RNA UltraSense 5× reaction mix. The reverse transcription step was performed at 55°C for 15 min, followed by incubation at 95°C for 10 min. The PCR consisted of 40 cycles at 95°C for 15 s and 60°C for 30 s. The qPCR was performed on a Bio-Rad C1000 Touch system

with a CFX96 optical module (Bio-Rad) using the RNA UltraSense one-step quantitative RT-PCR system protocol (Invitrogen).

Inoculation of Mammalian Cells. Tick pool homogenates were inoculated onto individual 24 well or 6-well plates of VeroE6 cells (150 μ l/well) in DMEM (supplemented with 2% FBS; penicillin 100 μ g/ml; streptomycin sulfate 100 μ g/ml; and amphotericin B 50 μ g/ml). Plates were transferred to the Stony Brook BSL3 facility for growth at 37°C and 5% CO₂. At 7-10 dpi cell supernatants were harvested and cell monolayers were 100% methanol fixed and POWV infected cells detected by immunoperoxidase staining with anti-POWV HMAF sera (ATCC). Supernatants positive for POWV RNA by qRT-PCR and POWV antigen staining in infected cells and were inoculated into T25 flasks containing VeroE6 cells and propagated for 7 days in DMEM with 2% FBS.

POWV-LI9 Infection and Immunoperoxidase staining. POWV-LI9 was adsorbed to ~60% confluent hBMEC or human pericyte monolayers for 1 h. Following adsorption, monolayers were washed with PBS and grown in supplemented EBM-2 MV or PM with 5% FBS, respectively. VeroE6 cells were similarly infected, washed, and supplemented with DMEM with 8% FBS. POWV-LI9 titers were determined by serial dilution and infection of VeroE6 cells, quantifying infected cell foci at 24 hpi by immunoperoxidase staining with anti-POWV hyperimmune mouse ascites fluid (HMAF; 1:5,000; ATCC), horseradish peroxidase (HRP)-labeled anti-mouse IgG (1:2,000; KPL-074-1806), and 3-amino-9-ethylcarbazole staining⁽⁶⁰⁾. IFN- α inhibition assay, medium was supplemented with 1,000 U/ml IFN- α (Sigma-Aldrich) at indicated times and cells incubated at 37°C and 5% CO₂.

POWV-LI9 Cloning and Sequencing. Total RNA was purified using RNeasy (Qiagen) from early passage POWV-infected Vero E6 cells (passage 3). cDNA synthesis was performed using a Transcriptor first-strand cDNA synthesis kit (Roche) using random hexamers as primers (25°C for 10 min, 50°C for 60 min, and 90°C for 5 min). POWV genome was amplified using primer pairs to regions located at near the junctions between capsid (forward: AGGAGAACAAGAGCTGGGAGTGGTC) and envelope (reverse: TGCTCCGACTCCCATTGTCATCAT); NS1 (forward: GACTATGGATGTGCAGTTGATCC) and NS2B

(reverse: TCTGCGTGCTGATGAGAAGA); NS3 (forward: ACTGACCTTGTATTCTCAGGG) and NS4B (reverse: GTGCATGAGTTCAACCGTTG); and NS5 (forward: CTAGAAGGGGTGGAGCAGAGG; reverse: TTAGATTATTGAGCTCTCTAGCTTG). 5'UTR was obtained using the Template Switching RT Enzyme Mix (NEB #M0466) following the manufacturer's protocol. 3' UTR was obtained using the reverse primer (GCGGGTGTTCCTCCGAGTCACACAC). PCR fragments were obtained using Phusion polymerase (NEB M0530L) with 37 cycles at 98°C for 30s, 68°C for 30s, and 72°C using 30s per kb. PCR fragments were gel purified (NEB T1020L) and cloned into pMiniT 2.0 vector (NEB E1202S). POWV-LI9 genome was sequenced using vector and internal primers, assembling both strands at least twice and submitted to GenBank (MZ576219).

Phylogenetic analysis. A phylogenetic tree of POWV sequences was generated using the neighbor-joining method⁽⁸⁴⁾ with a maximum composite likelihood model and bootstrap test of phylogeny with 100 replications⁽⁸⁵⁾ in MEGA version X (86). Phylogenetic analysis was performed with the POWV-LI9 sequence and reference full genome sequences representing POWV lineages I and II, and TBEV as outgroup, obtained from NCBI, as follows: Lineage I (LB L06436, LEIV-5530 KT224351, Ternay HQ231415, Pow-24 MG652438, Ulysses HQ231414, Spassk-9 EU770575, Nadezdinsk-1991 EU670438, LEIV-3070Prm KT224350, POWPa06 EU543649, POWANY64-7062 HM440563), Lineage II (RTS81 MG647779, RTS82 MG647780, RTS84 MG647783, RTS92 MG647781, RTS96 MG647782, NSF001 HM440559, MeW17-228 MK309362, P0375 KU886216, LI-1 KJ746872, DTVMeC17 MK104144, CTB30 AF311056, DTVWi99 HM440558, DTVWiA08 HM440560, DTVWiB08 HM440561, DTVWiC08 HM440562), and TBEV (NC_001672).

Viability assay. PI (Calbiochem) and calcein-AM (Invitrogen) uptake were used to evaluate Vero E6 and hBMEC viability as previously described⁽¹⁸⁾. Briefly, POWV-infected (MOI, 5) or mock-infected cells were seeded into 24-well plates and co-stained by the membrane-permeable dye calcein-AM (3 µM; green fluorescence in live cells), and 2.5 µM propidium iodide (red fluorescent DNA stain to detect

519 dead cells) 3 dpi. Images of calcein-AM-positive versus PI-positive cells were resolved using an
520 Olympus IX51 microscope and Olympus DP71 camera.

521 **Confocal Immunofluorescence.** POWV-LI9 (MOI, 5) was adsorbed to 80% confluent VeroE6
522 monolayers cultured in Lab-Tek II chambers (Nunc), washed with PBS, and grown in DMEM with 8%
523 FBS during 48h. Cells were washed with PBS, fixed for 10 min with 4% paraformaldehyde-PBS, and
524 permeabilized with 0.1% Triton X-100 in PBS for 10 min. Cells were blocked using 5% bovine serum
525 albumin in PBS for 2 h and incubated with anti-POWV HMAF (1:10,000) and anti-ZO1 rabbit
526 monoclonal antibody (1:100) in blocking solution for 18 h at 4°C. Cells were washed and incubated for
527 2 h with Alexa 488-conjugated goat anti-mouse IgG antibody (Invitrogen) and with Alexa 546-
528 conjugated goat anti-rabbit IgG antibody (Invitrogen) diluted 1:400 in blocking solution at room
529 temperature. hBMECs were subsequently incubated with 5 µM 4,6-diamidino-2-phenylindole (DAPI;
530 Sigma) for 5 min at room temperature. Slides were mounted using ProLong Antifade (Thermo Fisher)
531 solution and observed using a Zeiss LSM 510 META/NLO confocal microscope.

532 **Affymetrix gene array analysis.** Primary hBMECs or human pericytes (passages 4 to 10) were
533 synchronously infected with POWV-LI9 (MOI, 5) or mock infected. For hBMECs only, POWV-LI9 or
534 equivalent media were incubated with WGA (1 µg/ml) for 5 minutes prior to adsorbing POWV to cells
535 for 1 hour, or mock infecting cells with equivalent amounts of WGA as controls. Following adsorption,
536 cells were washed with PBS and media was replaced. Total RNA was purified 1-3 dpi from mock- or
537 POWV-infected hBMECs or hPCs using RLT lysis buffer and RNeasy columns (Qiagen). Purified RNA
538 was quantitated, and transcriptional responses detected on Affymetrix Clariom-S chip arrays in the
539 Stony Brook Genomics Core Facility. POWV-infected cell transcriptional responses were compared to
540 those in mock-infected cells harvested at each time point and fold changes in POWV versus control
541 transcripts were analyzed using Affymetrix TAC software. Data obtained from these studies were
542 submitted to the NCBI Gene Expression Omnibus database (hBMECs: GEO GSE176251; hPCs: GEO
543 GSE176252).

544 **qRT-PCR analysis.** Quantitative real-time PCR was performed on purified cellular RNAs derived from
 545 mock- or POWV-infected hBMECs/hPCs as described above. cDNA synthesis was performed using a
 546 Transcriptor first strand cDNA synthesis kit (Roche) using random hexamers as primers (25°C for 10
 547 min, 50°C for 60 min, and 90°C for 5 min). qRT-PCR primers for specific genes were designed
 548 according to the NCBI gene database with 60°C annealing profiles (provided by Operon). Genes were
 549 analyzed using PerfeCTa SYBR green SuperMix (Quanta Biosciences) on a Bio-Rad C1000 Touch
 550 system with a CFX96 optical module (Bio-Rad). Responses were normalized to internal glyceraldehyde-
 551 3-phosphate dehydrogenase (GAPDH) mRNA levels, and the fold induction was calculated using the $2^{-\Delta\Delta CT}$
 552 method for differences between mock- and POWV-infected RNA levels at each time point.

553 **Trans Endothelial/Epithelial Electrical Resistance (TEER).** hBMECs or VeroE6 were plated on
 554 Costar Transwell inserts (3- μ m pore size; Corning) at high density, and 7 days post seeding monolayers
 555 were analyzed for TEER (EVOM2; STX3; World Precision Instruments, Inc.). Confluent Transwell
 556 cultures were infected with POWV-LI9 (MOI, 5) or mock infected, and TEER values for POWV-
 557 infected versus mock-infected cells were compared at 1 to 3 dpi. For analysis of apical and basolateral
 558 release of POWV-LI9, hBMECs and VeroE6 were similarly infected 3 dpi prior to analyzing viral titers
 559 in apical and basolateral supernatants.

560 **POWV Transcytosis Assay.** hBMECs were plated on Costar Transwell inserts (3- μ m pore size;
 561 Corning) at high density, and 7 days post seeding hBMECs were mock- or POWV-infected in the upper
 562 chambers for 1 h, cells were washed with PBS, and the inserts were transferred to 24-well plates
 563 containing 70% confluent primary human brain vascular pericytes.

564 **ELISA.** IFN- β in the supernatants of mock- and POWV-infected hBMECs or hPCs at 1, 2, and 3 dpi
 565 were measured using a DuoSet ELISA (R&D Systems). ELISA plates (Immunolon 2, U-bottom;
 566 Dynatech Laboratories) were coated with anti-IFN- β capture antibody according to the manufacturer.
 567 Viral supernatants were incubated with antibody coated plates (2 h), washed with PBS (0.1% Tween 20),
 568 and bound protein was detected with IFN- β specific antibodies conjugated to streptavidin-HRP and

developed using tetramethylbenzidine. Secreted IFN- β in samples were compared to a purified IFN- β (R&D Systems) standard curve using a BioTek EL312e microplate reader (450 nm).

Statistical analysis. Results shown in each figure were derived from two to three independent experiments with comparable findings; the data presented are means standard errors of the means (SEM), with the indicated P values of <0.01 and <0.001 considered significant. Two-way comparisons were performed two-tailed analysis of variance and an unpaired Student's t test. All analyses were performed using GraphPad Prism software version 9.0.

Data availability. Sequencing data from POWV-LI9 was submitted to GenBank (MZ576219). Microarray data obtained from these studies were submitted to the NCBI Gene Expression Omnibus database (hBMECs: GSE176251; hPCs: GSE176252).

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Figure Legends

Figure 1. POWV-LI9 isolation from *Ixodes scapularis* ticks. (A) *I. scapularis* ticks were collected in Long Island, N.Y. Tick homogenates from groups of 10 ticks were added to wells of 24-well plates containing VeroE6 cells. After 7 days, cells were fixed and immunoperoxidase stained using a specific anti-POWV HMAF (1:10,000). Wells #9 and # 41 were positive for POWV antigen and formed distinct infected cell foci. First passage (P1) virus was inoculated into a well of 24-well plate, and cells were fixed and immunoperoxidase stained using a specific anti-POWV HMAF (1:10,000) after 7 days (left panels). Right panels show the magnification of the cell foci from initial viral isolation and P1 infection of VeroE6 cells. Bars represent 50 μ m. (B) VeroE6 were infected with POWV-LI9 (P3) at an MOI of 5 or mock-infected, cells were fixed at 1, 3, 5, 8, and 10 dpi, and immunoperoxidase stained using anti-POWV HMAF (1:10,000). Bars represent 50 μ m. (C) Viral titration from POWV-infected VeroE6 supernatants was determined by FFU assay at 1 dpi in Vero E6 cells.

Figure 2. Genetic Analysis of POWV-LI9 Isolates. (A) Maximum-likelihood tree analysis between POWV-LI9 and POWV-related strains using complete genome sequences⁽⁸⁴⁾. Branch numbers are bootstrap confidence estimates based on 100 replicates. Tick-borne encephalitis virus (TBEV) is shown as an outgroup. (B) Multiple-sequence analysis comparison using Clustal Omega⁽⁸⁷⁾ between POWV-LI9 and other POWV sequences deposited in the GenBank. POWV genomes were divided into three groups based on the genetic identity compared with POWV-LI9: sequences from the viruses collected in the Northeastern U.S. (group A), in Wisconsin (group B), and sequences from lineage I POWV (group C). Black bars within the sequences represent nucleotide differences compared to POWV-LI9. (C) Among 5 residue differences with Northeastern POWVs there are 2 not conserved residues in the Envelope (Env), including Domain II residues S119 and K151 in POWV-LI9 and additional unique residues in the NS2B (F125), NS3 (D402) and NS5 (T282) proteins of POWV-LI9.

Figure 3. POWV-LI9 Productively and Persistently Infect human BMECs and Pericytes. (A) hBMECs were infected with POWV-LI9 (P3) at an MOI of 5 or mock-infected, cells were fixed at 1, 2, 3, 6, and 8 dpi, and immunoperoxidase stained using anti-POWV HMAF (1:10,000). Bars represent 50 μ m. (B) Viral titration from POWV-infected hBMECs supernatants was determined by FFU assay at 1 dpi in Vero E6 cells. (C) POWV-LI9 was pretreated with WGA (1 μ g/mL) for 5 min. Control mock WGA treated media (1 μ g/mL), or WGA treated POWV was adsorbed to hBMECs for 1 hour, and subsequently cells were PBS washed and resupplemented with EBM2-2% FBS media. Cells were fixed and immunoperoxidase stained using anti-POWV HMAF (1:10,000) at 1 dpi. (D) Human brain pericytes (hPCs) were infected with POWV-LI9 (P3) at an MOI of 5 or mock-infected, cells were fixed at 1, 2, 3, 6, and 8 dpi, and immunoperoxidase stained using a specific anti-POWV HMAF (1:10,000). Bars represent 50 μ m. (E) Viral titers from POWV-infected pericyte supernatants were determined by FFU assay at 1 dpi in Vero E6 cells.

Figure 4. POWV-LI9 is Basolaterally Released from Cells Without Barrier Disruption. (A) Polarized hBMECs and VeroE6 cells grown for 5 days in Transwell inserts, were apically infected with POWV-LI9 (MOI 5). Cells were visualized by phase contrast microscopy for CPE and costained with Calcein-AM (green-live cells) and propidium iodide (red -dead cells) to visualize cell viability 3 dpi. (B) Confocal immunofluorescence. VeroE6 grown on microslides were infected with POWV-LI9 (MOI, 5). Cells were fixed 2 dpi, immunostained with anti-POWV HMAF and anti-ZO-1 antibodies, DAPI counterstained, and visualized by confocal microscopy. Experiments were done in triplicate, repeated at least three times, and representative data are presented. Bars represent 10 μ m. (C) Polarized hBMECs and VeroE6 cells grown for 5 days in Transwell inserts, were apically infected with POWV-LI9 (MOI, 5) in triplicate, and TEER was measured 1 to 3 dpi. (D) hBMECs and (E) VeroE6, grown for 5 days in Transwell inserts, were apically infected with POWV-LI9 (MOI, 5) in triplicate. Cells were washed and viral titers from both apical or basolateral compartments were titered 3 dpi. (F) Schematic representation

of hBMECs and human pericyte co-culture model (left); hBMECs were seeded on the apical side of Transwell inserts, grown for 7 days confluent by TEER resistance. hBMECs were mock or POWV infected from the apical side for 1 h, cells were washed, and 1 dpi Transwell inserts were transferred to 24-well plates containing monolayers of primary human pericytes. Pericytes were immunoperoxidase stained for POWV antigen 3 dpi using anti-POWV HMAF (1:10,000). Bars represent 50 μ m.

Figure 5. POWV-LI9 is susceptible to exogenous interferon addition. (A) Vero E6, hBMECs and human brain pericytes (hPCs) were pretreated with IFN- α (1,000 U/ml) for 2 or 18 h and before POWV infection (MOI, 5). One day post-infection cells were methanol fixed, immunoperoxidase stained using anti-POWV HMAF (1:10,000) and the number of infected cells quantitated and compared untreated controls. (B) hBMECs and (C) hPCs were mock or POWV-LI9 infected (MOI 5) and 1-3 dpi supernatants of cells were assayed for secreted IFN β by ELISA (R&D systems).

Figure 6. Global transcriptional changes in POWV-LI9-infected hBMECs and Pericytes. (A) POWV-LI9 was pretreated with WGA (1 μ g/mL) for 5 min. Control mock WGA treated media (1 μ g/mL), or WGA treated POWV was adsorbed to hBMECs for 1 hour, and subsequently cells were PBS washed resupplemented with EBM2-5% FBS media and grown for 1-3 days. Total cellular RNA from mock-WGA-treated and WGA-treated POWV infected hBMECs was harvested in RLT buffer, purified, DNase treated and subjected to Affymetrix array analysis. Log₂-induced genes from POWV-infected samples were compared to respective mock-infected time points, generating a heatmap with 0- to 100-fold induction scale, where induced genes above 100-fold were in black. Genes were separated into functional categories as chemokines, interferons (IFN), ISGs, IRFs, cytokines (Cytok.), transcriptional factors (TFs), apoptosis-related genes (Apop.), and ISGylation (ISGyl.) genes. (B) Selected genes from the Affymetrix array analysis were confirmed by qRT-PCR. (C) Total RNA from mock- and POWV-infected hPCs was isolated at 1-3 dpi and submitted to Affymetrix array analysis as in A. Log₂-induced

912 genes from POWV-infected samples were compared against its respective mock-infected timepoint,
 913 generating a heatmap with 0- to 500-fold (left graph) or 100-fold (right graph) induction scale, where
 914 induced genes above 500- or 100-fold were represented as black color. Genes were separated in
 915 functional categories as chemokines, interferons (IFN), ISGs, IRFs, cytokines (Cytok.), transcriptional
 916 factors (TFs), apoptosis-related (Apop.), ISGylation-related (ISGyl.), and complement related genes. (D)
 917 Selected genes induced by POWV infection of pericytes by Affymetrix array were confirmed by qRT-
 918 PCR. (E) Gene Ontology (GO) enrichment analysis was conducted by querying the upregulated genes
 919 from infected hBMECs or hPCs 1, 2, and 3 dpi using STRING v11⁽³²⁾. GO terms from selected
 920 biological processes were presented.

B

C

A

hBMECs

C

hPCs

B

D

E

hBMECs

hPCs