**Brief UltraRapid Communication**

**AXL-Mediated Productive Infection of Human Endothelial Cells by Zika Virus**

Shufeng Liu, Leon J. DeLallo, Brant E. Isakson, Tony T. Wang

**Rationale:** The mosquito-borne Zika virus (ZIKV) is now recognized as a blood-borne pathogen, raising an important question about how the virus gets into human bloodstream. The imminent threat of the ZIKV epidemic to the global blood supply also demands novel therapeutics to stop virus transmission though transfusion.

**Objective:** We intend to characterize ZIKV tropism for human endothelial cells (ECs) and provide potential targets for intervention.

**Methods and Results:** We conducted immunostaining, plaque assay, and quantitative reverse transcription-polymerase chain reaction of ZIKV RNA to evaluate the possible infection of ECs by ZIKV. Both the African and the South American ZIKV strains readily infect human umbilical vein endothelial cells and human ECs derived from aortic and coronary artery, as well as the saphenous vein. Infected ECs released infectious progeny virus. Compared with the African strains, South American ZIKV isolates replicate faster in ECs and are partially cytopathic, suggesting enhanced virulence of these isolates. Flow cytometric analyses showed that the susceptibility of ECs positively correlated with the cell surface levels of tyrosine-protein kinase receptor UFO (AXL) receptor tyrosine kinase. Gain- and loss-of-function studies further revealed that AXL is required for ZIKV entry at a postbinding step. Finally, small-molecule inhibitors of the AXL kinase significantly reduced ZIKV infection of ECs.

**Conclusions:** We identified EC as a key cell type for ZIKV infection. These data support the view of hematogenous dissemination of ZIKV and implicate AXL as a new target for antiviral therapy. *(Circ Res. 2016;119:1183-1189. DOI: 10.1161/CIRCRESAHA.116.309866.)*

**Key Words:** axl receptor tyrosine kinase ■ blood ■ endothelial cells ■ infection ■ virus internalization ■ ZIka virus

**Editorial, see p 1149**

**In This Issue, see p 1145**

Conceptually, ZIKV may be carried into bloodstream by infected macrophages or dendritic cells after local mosquito bites. Here, we report that ZIKV directly infects human endothelial cells (ECs) and disseminates infectious progeny virus. Specifically, both the African and the South American ZIKV strains readily infect primary human ECs isolated from umbilical veins, the aorta, the coronary arteries, and the saphenous veins, leading to the release of infectious virus. The candidate receptor of entry seems to be the tyrosine-protein kinase receptor UFO (AXL) receptor tyrosine kinase (RTK) because specific small molecular inhibitors of AXL blocked ZIKV infection. Our results support a model that ZIKV spreads through infected ECs and bypasses the barriers that would otherwise restrict viral infection.

**Methods**

A detailed Methods section is provided in the Online Data Supplement.

Original received August 27, 2016; revision received September 10, 2016; accepted September 19, 2016. In August 2016, the average time from submission to first decision for all original research papers submitted to Circulation Research was 13.98 days.

Brief UltraRapid Communications are designed to be a format for manuscripts that are of outstanding interest to the readership, report definitive observations, but have a relatively narrow scope. Less comprehensive than Regular Articles but still scientifically rigorous, BURCs present seminal findings that have the potential to open up new avenues of research. A decision on BURCs is rendered within 7 days of submission.

From the Center for Infectious Diseases, Discovery Biology, SRI International, Harrisonburg, VA (S.L., T.T.W.); and Department of Molecular Physiology and Biophysics, Robert M. Berne Cardiovascular Research Center, University of Virginia School of Medicine, Charlottesville (L.J.D., B.E.I.).

The online-only Data Supplement is available with this article at http://circres.ahajournals.org/lookup/suppl/doi:10.1161/CIRCRESAHA.116.309866/-/DC1.

Correspondence to Tony Wang, PhD, 140 Research Dr, Harrisonburg, VA 22802. E-mail tony.wang@sri.com

© 2016 American Heart Association, Inc.

*Circulation Research* is available at http://circres.ahajournals.org

DOI: 10.1161/CIRCRESAHA.116.309866

1183
## Results

The interior surfaces of vascular and lymphatic vessels are lined with endothelium, forming an interface between circulating blood or lymph in the lumen and the rest of the vessel wall. ECs are also major components of the blood–brain barrier and part of the placental blood barrier, preventing circulating virus from entering the brain and the fetal tissues, respectively. To investigate whether ZIKV directly infects ECs, we cultured the immortalized human blood–brain barrier EC line hCMEC/D3 (human cerebral microvascular endothelial cell line), which is a well-characterized blood–brain barrier cell system. The African MR766 and IbH 30656 (IbH) ZIKV strains (ZIKVAF), as well as the South American ZIKV isolates (ZIKVSA) PRVABC59 (PRV) and FLR, but not Dengue virus (DENV-2 strain Thailand 16681), readily infected hCMEC/D3 cells and produced infectious virus with titers comparable to that of the C6/36 cell line (Online Figure I).

To determine the extent of ZIKV EC infection, we used cultures of human ECs at low passages (passage numbers <7) isolated from umbilical veins (human umbilical vein endothelial cells [HUVECs]), aorta (human aortic endothelial cells), coronary artery (human coronary artery derived endothelial cells), and saphenous vein (human saphenous vein derived endothelial cells; Online Figure II). For comparison, we also obtained human lymphatic ECs (Online Figure III). Low-passage ECs were inoculated with the MR766 and IbH 30656 (IbH) ZIKV strains, and the PRV and FLR. Both ZIKVAF and ZIKVSA strains, but not DENV2, infected all vascular ECs from multiple donors, with HUVECs being significantly most susceptible (Figure 1A through 1E). Notably, ZIKV entry into vascular ECs also led to productive infection and the release of infectious progeny virus (Figure 1B and 1D). Although the ZIKVAF strains MR766 and IbH caused minimal morphological change, infection of HUVEC cells with the 2 ZIKVSA isolates induced significant cell death, suggesting enhanced virulence of these isolates (Figure 2A). The PRV isolate also formed noticeably larger plaques on plaque assays (Figure 2B). Indeed, when the kinetics of viral RNA replication was measured, the ZIKVSA isolates displayed faster growth rates (Figure 2C and 2D). Although we cannot completely rule out that the African isolates have been cultured for decades in the laboratory and hence adapted to be less cytopathic, our observation is consistent with a recent report that pups born from a Brazilian ZIKV (ZIKVBR) isolate-infected SJL pregnant mice displayed abnormalities resembling the microcephaly seen in humans.2 These findings highlight the differences between the original ZIKVAF strain, which causes no or very mild symptoms, and the circulating ZIKVSA strains that seem to be more pathogenic. The fact that HUVECs are more susceptible and the partial cytopathic effect of ZIKVSA isolates on these cells also suggest the potential involvement of ECs during vertical transmission of ZIKV.

Because vascular ECs form physical barriers with tight junctions between cells, we conducted confocal microscopy and Western blotting to evaluate the effects of ZIKV infection on endothelium integrity. Transendothelial electric resistance was measured for the assessment of endothelial barrier function. Infection by ZIKV did not directly disrupt the tight junctions or the barrier function of HUVEC or hCMEC/D3 cells (Online Figure IV). Therefore, we reason that the loss of endothelium integrity probably only occurs when significant cell death is caused by ZIKV infection.

ZIKV is known to utilize multiple cell surface receptors, including DC-SIGN, AXL, Tyro3, and TIM-1, to gain entry with a major role for the RTK AXL. A recent single-cell expression analysis revealed that the candidate entry receptor AXL is highly expressed in neural stem cells and ECs in developing human cortex. To probe into the potential involvement of AXL, we first performed flow cytometric analysis and determined the AXL expression levels on low-passage cultures of human ECs. Notably, although most ECs express AXL, HUVECs seem to express AXL to the highest level, followed by human aortic endothelial cells and human saphenous vein–derived endothelial cells (Figure 3A). By contrast, human coronary artery–derived endothelial cells expressed little AXL. Although we cannot completely exclude potential donor variability, the cell surface expression levels of AXL positively correlate with cellular susceptibilities to ZIKV (Figures 1E and 3A). AXL is an RTK that transduces signals from the extracellular matrix into the cytoplasm by binding to the vitamin K–dependent protein growth arrest-specific 6 (Gas6) gene. To explore the role of AXL in the observed ZIKV infection, we conducted 3 sets of experiments. First, a polyclonal antibody that recognizes the extracellular portion of human AXL blocked the entry of virus into HUVECs and hCMEC/D3 cells (Figure 3B). Notably, addition of the antibody did not reduce the attachment of ZIKV to cells (Figure 3C) and was unable to block ZIKV infection if added 3 hours after the infection had been initiated (Figure 3D). These data suggest that AXL is a ZIKV entry factor required at a late stage during entry. Second, ectopic expression of AXL in 293T cells promoted ZIKV infection without enhancing virus binding (Figure 3E, 3F, and 3H). By contrast, the kinase-dead AXL, which carries a K567R mutation that destroys an ATP-binding site and inhibits Axl phosphorylation and signaling,20–22 is significantly impaired its ability to confer permissiveness to 293T cells (Figure 3F and 3H). These observations reinforced the idea that AXL promotes ZIKV entry at a postbinding step and also imply that AXL-mediated signaling is needed for ZIKV entry. Finally, 2 known inhibitors of AXL phosphorylation, Cabozantinib23 and R428,24 significantly impaired ZIKA infection of hCMEC/D3 and the HUVECs in a dose-dependent manner (Figure 4A through 4D). By contrast, RTK inhibitors Sunitinib malate and Sorafenib had none or marginal inhibition at one micromolar (Online Figure V). These results indicate that AXL RTK activity is potentially important to its function in ZIKV entry. The kinetics of R428-mediated inhibition, generated from a time-of-addition experiment, showed that the compound remained inhibitory even during vertical transmission of ZIKV.

### Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>ECs</th>
<th>endothelial cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>HUVECs</td>
<td>human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>AXL</td>
<td>tyrosine-protein kinase receptor UFO</td>
</tr>
<tr>
<td>ZIKVAF</td>
<td>African Zika virus stains</td>
</tr>
<tr>
<td>ZIKVSA</td>
<td>South American Zika virus strains</td>
</tr>
</tbody>
</table>

---

15. By guest on November 18, 2017 http://circres.ahajournals.org/ Downloaded from
Figure 1. Productive infection of primary human endothelial cells by Zika virus (ZIKV). A, human umbilical vein endothelial cells (HUVECs) from ATCC (passage 2) were infected by indicated ZIKV strains/isolates at multiplicity of infection of 1 for 48 h followed by immunostaining of ZIKV Env protein. B, Infectious titers of supernatants collected at 48 h post infection from cells in a (n=3). C, ECs were infected by ZIKV MR766 or PRVABC59 at multiplicity of infection of 1 and then imaged for the presence of viral Env protein at 48 h post infection. D, Infectious titers of supernatants collected at 48 h post infection from cells in B (n=3). E, Summary of infection efficiencies of ECs from different donors by MR766 or PRVABC59. Each solid shape represents 1 donor. Data are shown as the percent of ZIKV Env-positive cells relative to the total number of nuclei (as assessed by DAPI [4',6-diamidino-2-phenylindole]). HAoEC indicates human aortic ECs; HCoECs, human coronary artery–derived ECs; and HSaVECs, human saphenous vein–derived ECs.
Figure 2. Enhanced virulence of South American ZIKV isolates. A, Representative phase-contrast images of infected human umbilical vein endothelial cell (HUVEC) cells (ATCC, passage 2) from Figure 1A at 48 h post infection. The percentage of cell viability was quantified using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). n=3, *P<0.05. B, Representative photographs of plaques using supernatants from above cells. Plaque sizes were indicated in the brackets. C and D, HUVECs or hCMEC/D3 (human cerebral microvascular endothelial cell line) cells were infected by ZIKV MR766, PRVABC59, or FLR at multiplicity of infection of 0.1 for 1 h. After extensive wash in PBS, cellular RNA was extracted at indicated time points post infection for quantitative reverse transcription-polymerase chain reaction to determine the kinetics of virus RNA replication (n=4, each experiment contains 3 technical replicates; error bars, SD).
when added at 1 hour after the virus was added but drastically lost its effect if added at 2 hours after infection had been initiated (Figure 4E). Therefore, R428 interferes with a postbinding process during ZIKV entry. Of note, treatment of HUVECs with AXL inhibitors neither altered AXL cell surface expression (Figure 4F) nor reduced cell viability (Online Figure VC).
Discussion

With the imminent threat of the ZIKV epidemic to pregnant women and to the global blood supply, this timely study provides mechanistic understanding of ZIKV tropism and pathogenicity. The significance of our findings is 3-fold: (1) human ECs are likely one of the principal cell types of ZIKV infection. In vivo, the release of infectious ZIKV by ECs would conceivably allow the virus to rapidly enter or leave the bloodstream and potentially contributes to the intrauterine and transfusion-mediated ZIKV transmission (Online Figure VI). Although several human placental cell types, including cytotrophoblasts, epithelial cells, fibroblasts, and Hofbauer macrophages, were reportedly permissive to ZIKV,25,26 Miner et al5 recently found that ZIKV-infected fibroblasts, and Hofbauer macrophages, were reportedly permissive to ZIKVPRV was added to HUVECs cells at 4°C and incubated for an additional 24 h before RT-qPCR assay of ZIKV RNA. Inhibition was calculated as 100% =−2 h where R428 was added back after removal of the virus and incubated for additional 2 h before removal (exception is t=−2 h where R428 was added back after removal of the virus and incubated for additional 2 h before removal). Unbound virus was washed off with cold media, and the cells were shifted to 37°C (set as a 0-h time point) to initiate synchronous infection. At the indicated time points, 1 μmol/L R428 or DMSO was added into the media and incubated for 2 h before removal. Infected cells were incubated at 37°C for an additional 24 h before RT-qPCR assay of ZIKV RNA. Inhibition was calculated as 100% infection relative to infections containing no inhibitors. Fitted lines represent sigmoidal time-dependent curves (mean of n=2; error bars, SD). F, Cell surface levels of AXL on compound-treated HUVECs were quantified by flow cytometry using the anti-AXL antibody.

Discourse

Development of antiviral therapies that isocancer activities, may serve as unique antiviral therapeutics that facilitate hematogenous dissemination of the virus and bypass the barriers that would otherwise restrict viral infection and thus reach tissues where viruses typically cannot reach. Such a route allows the virus to rapidly enter or leave the bloodstream and potentially contributes to the intrauterine and transfusion-mediated ZIKV transmission (Online Figure VI). Although several human placental cell types, including cytotrophoblasts, epithelial cells, fibroblasts, and Hofbauer macrophages, were reportedly permissive to ZIKV,25,26 Miner et al5 recently found that ZIKV-infected Ifnar–/– pregnant mice show signs of vascular damage in the placenta and fewer fetal blood vessels, evidencing ZIKV infection of fetal ECs in vivo. (2) The ZIKVSA isolates replicate faster in ECs than the ZIKVSF strains. Although the ZIKVSF strains MR766 and IbH have been around for many years and might have adapted more through continuous passages in cultures, faster replication kinetics could contribute to the enhanced virulence of ZIKVSA isolates. We speculate that the partial cytopathic effect of the ZIKVSA isolates may trigger vascular changes in vivo, from severe placental vascular damage and a reduction in fetal blood vessels early in pregnancy to hemorrhagic retinopathy and torpedo maculopathy.27 In this regard, it would be highly interesting to assess the prevalence of vascular complications among ZIKV-infected individuals. (3) ZIKV tropism for ECs positively correlates with cell surface levels of AXL. Despite previous studies have implicated a role of AXL in ZIKV entry, our results clearly demonstrated that AXL functions at a postbinding step, where its catalytic activity is required. Several AXL inhibitors, including Cabozantinib and R428 that are currently in clinical trials for anticancer activities, may serve as unique antiviral therapeutics that suppress ZIKV infection of ECs.

Acknowledgments

We thank Dr Matthew Kappes for critical reading of this article. Zika Virus, MR766, NR-50065 and IbH 30656, NR-50066 were obtained through BEI Resources, the National Institute of Allergy and Infectious Diseases (NIAID), and the National Institutes of Health (NIH) as part of the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA) program. The following reagents were obtained through BEI Resources, NIAID, NIH: Zika virus, PRVABC59, NR-50240 and FLR, NR-50183.

T. Wang designed the overall experiments. L. DeLalio prepared the primary human endothelial cells. S. Liu and T. Wang performed the experiments and analyzed the data. S. Liu, B. Isakson, and T. Wang wrote the article. All authors read and approved the final article. All authors have provided the corresponding author with written permission to be named in the article.

Sources of Funding

This study was sponsored by National Institutes of Health grant R01DK088787 (to T. Wang), R01HL088554 (B.E. Isakson). The funders had no role in study design, data collection, and interpretation, or the decision to submit the work for publication.
Endothelial cells are major components of the blood–brain barrier and are partially cytopathic. Besides infecting the developing fetal brain, Zika virus has also been recognized as a blood-borne pathogen. Zika virus infection during pregnancy in mice causes placental damage and fetal demise. Cell. 2016;2016:26:645–654. doi: 10.1016/j.cell.2016.05.008.


AXL-Mediated Productive Infection of Human Endothelial Cells by Zika Virus
Shufeng Liu, Leon J. DeLalio, Brant E. Isakson and Tony T. Wang

Circ Res. 2016;119:1183-1189; originally published online September 20, 2016;
doi: 10.1161/CIRCRESAHA.116.309866
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2016 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circres.ahajournals.org/content/119/11/1183

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2016/09/20/CIRCRESAHA.116.309866.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published
in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the
Editorial Office. Once the online version of the published article for which permission is being requested is
located, click Request Permissions in the middle column of the Web page under Services. Further information
about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
Material and Methods

Cells

293T, BHK-21, Vero, and C6/36 cell lines were purchased from ATCC (American type culture collection) and maintained as instructed by the manufacturer (Cat# CRL-11268, CCL-10, CCL-81, and CCL-1660). Cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% Penicillin and streptomycin, 1% non-essential amino acids and 10% fetal bovine serum (Invitrogen). The immortalized BBB cell line hCMEC/D3 (Millipore #SCC066) was maintained in EndoGRO™-MV Complete Media Kit (Millipore # SCME004) supplemented with 1 ng/mL FGF-2 (Millipore #GF003). Cell lines were tested negative for mycoplasma contamination using a homemade PCR detection kit. Primary HUVECs were purchased from ATCC (PCS-100-010, Lot# 62245802, passage #2) or Cell Applications (200K-05, Donor #1 & #2, passages 2 and 3 respectively). Primary HSaVECs and HAoECs were from PromoCell (C-12231 and C-12271, passage 3, 5, and 6.) and HCoAECs were purchased from Lonza (CC-2585, passage 3 and 6 respectively) All ECs were maintained under standard cell culture conditions in endothelial growth medium (EGM-2MV) from Lonza as we have published\(^1\). The purity of ECs was validated by the presence of CD31/PECAM1 on cell surface (Online Figure 2A).

Viruses

Zika virus strains/isolates MR766 (Rhesus/1947/Uganda), IbH 30656 (Human/1968/Nigeria), PRVABC59 (Human/2015/Puerto Rico), and FLR (Human/2015/Colombia) were initially obtained from Biodefense and Emerging Infections Research Resources Repository (BEI Resources, Manassas, VA) and subsequently propagated in C6/36 cells. Approximately 2 x 10^6
cells were seeded 24 hours prior to infection in a T75 cell culture flask. When the cells had reached ~60-80% confluency they were infected with virus at a multiplicity of infection (MOI) of 0.01. The cells were incubated for 7 days or until they began to die from the cytopathic effect caused by the virus. After propagation, the virus containing media was collected and centrifuged at 1500g for 5 minutes to remove extracellular debris. The supernatant was then passed through a 0.22 µm filter syringe and aliquoted before it was frozen at -80 °C for long term storage. The infectious titers of stock virus were determined by plaque assays and summarized in Online Table I. The production of DENV-2 strain Thailand 16681 has been previously published². Experiments involving infectious virus were approved by SRI Biosafety Committee and conducted in a Biosafety level 2 laboratory.

**Antibodies and Reagents**

The pan-flavivirus anti-E protein murine monoclonal antibody D1-4G2-4-15 was produced from hybridoma cells purchased from ATCC (HB-112) and purified as instructed by the manufacturer. The Alexa Fluor 568 goat-anti-mouse and Alexa Fluor 488 goat-anti-rabbit secondary antibodies were purchased from molecular probes (Invitrogen cat # A11019 and #A-11008). Goat anti-human AXL polyclonal antibody (AF-154), mouse anti-human AXL antibody (MAB 154), and CD31/PECAM-1 antibody (BBA7) were purchased from R&D systems. Polyclonal ZO-1 antibody (Cat No. 61-7300) was from ThermoFisher Scientific. Cabozantinib (XL-184, VEGFR2/Met/Ret/Kit/FLT//AXL inhibitor), Sorafenib (Raf kinases and tyrosine kinases inhibitor), Sunitinib malate (VEGFR/PDGFRβ/ KIT/ FLT3/RET/CSF-1R inhibitor), and R428 (Selective Axl inhibitor) were purchased from APExBIO Technology (Houston, TX). Notably, Cabozantinib potently inhibits several RTKs including AXL phosphorylation³. R428
has been well characterized in its ability to inhibit AXL phosphorylation and downstream signaling\textsuperscript{4}. pWZL-Neo-Myr-Flag-AXL\textsuperscript{5} and the AXL kinase dead AXL (K567R)\textsuperscript{6} were obtained from Addgene. Mutating the K567 residue of AXL is known to destroy an ATP-binding site and to inhibit Axl phosphorylation\textsuperscript{6-8}.

**ZIKV plaque assay**

Vero cells were plated the day before infection into 12 well plates at 5 x 10\textsuperscript{5} cells/well. On the day of the experiment, serial dilutions of virus were made in 0.4 ml media. Five different 10-fold dilutions of purified virus were spread onto monolayers of Vero cells at 37°C for 1 h to initiate binding to cells. Then, medium containing virus particles was replaced with 1 ml overlay containing a 1:1 mixture of 1% SeaPlaque\textsuperscript{TM} agarose(Lonza) and 2X Modified Eagle Medium (Gibco) with 10% (vol/vol) FBS. Cells were maintained at 37°C in 5% CO\textsubscript{2}. On day 5, cells were stained with 1 ml secondary overlay containing a 1:1 mixture of SeaPlaque\textsuperscript{TM} agarose (Lonza) and 2X Modified Eagle Medium and 0.33% neutral red (Sigma). Cells were incubated at 37°C and plaques were counted after 48 hours. Resultant plaques were photographed using a Canon scanner. For each ZIKV strain, averages of 10 plaques from two plates were analyzed using the ImageJ software (NIH). The size of a plaque was determined by comparing the measured area of a plaque to that of a well in the 12-well (380 mm\textsuperscript{2}) using the formula: Size of a plaque = \( \frac{\text{AREA}_{\text{plaque}}}{\text{AREA}_{\text{well}}} \times 380\text{mm}^2 \).

**Immunofluorescence assay**

Cells were plated on gelatin-coated glass coverslips the day before infection into 24 well plates at 5 x 10\textsuperscript{4} cells/well. The cells were washed for 5 minutes 3 times with 1X phosphate.
buffered saline. After wash, cells were fixed in 4% paraformaldehyde for 15 minutes at room temperature and then permeabilized with 0.2% triton-X 100 for 10 minutes. The ZIKV envelope protein was detected using a 1:300 dilution of the 4G2 primary antibody isolated and 1:500 of the Alexa Fluor 568 goat-anti-mouse antibody. Images were captured by a Zeiss LSM 700 laser scanning confocal microscope.

**Real-Time PCR**

Quantification of RNA was conducted using iTaq™ Universal SYBR® Green One-Step Kit (Bio-Rad) with an in-house developed protocol on a Step One Real-Time PCR system (Applied Biosystems). The copy number of Zika RNA was calculated by comparing to a standard curve obtained with serial dilutions of a full-length Zika genome encoding plasmid. Primers for qPCR were as follows: ZIKV MR766 forward: 5′- CTTGAAATCCGGTTTGAGGA-3′ and reverse: 5′-CTTTCCCTGGGCCTTATCTCC-3′; ZIKV IbH 30656 forward: 5′-CACAAAGGGAGGTCCTGGTC-3′ and reverse: 5′-CACAGCAAAAGTGTCACACGG-3; ZIKV PRVABC59 and ZIKV FLR forward: 5′- CACTGTGAGAGGTGCCAAGA-3′ and reverse: 5′-TTTGTGTTCAGACCCAACCA-3′; human GAPDH RNA forward: 5′-GAATTTGCCATGGGTGGAAT-3′ and reverse: 5′-ATGTCCAGCCTCAAACTTC-3′.

**Flow cytometry**

4x10^5 cells were trypsinized and resuspended in PBS. Control mouse IgG or mouse anti-human AXL antibody (MAB 154) was added to the cells at 1:100 and then incubated on ice for 30 minutes. After three washes in PBS, cells were incubated with Goat-anti-Mouse-FITC antibody (1:100) for 30 minutes on ice. Expression of AXL was quantified by BD FACSCalibur (BD Biosciences). Data was analyzed by flowing software.
Transendothelial Electric Resistance Measurement

2000 HUVECs cells were seeded on a 24-well transwell plate (Corning, 6.5-mm membrane diameter, 0.4-μm pore size). Cells that were resuspended in 0.2 ml of medium were added to the upper chamber, and 0.5 ml of medium was added to the lower chamber. After reaching full confluence, cells were infected with ZIKV (MOI 1). Transendothelial electric resistance (TEER) was measured daily for 2 days using an epithelial voltohmmeter (EVOM, World Precision Instruments, Sarasota, FL). A blank well with no cells was maintained, and the average resistance from the blank was subtracted from the sample. Resistance ($R$) per unit area was calculated as $(R_{\text{sample}} - R_{\text{blank}}) \times 0.33 \text{ cm}^2$. The data represent means of two independent experiments ± SD, each done in duplicate.

Time-of-addition assays

ZIKV was added to HUVECs at 4°C and incubated for 2 hrs. Unbound virus was washed off with cold media, and the cells were shifted to 37°C (set as 0 hr time point) to initiate synchronous infection. At the indicated time points, R428 or DMSO was added into the media and incubated for two hours prior to removal (exception is t= -2hr where inhibitors were added back after removal of the virus and incubated for additional two hours prior to removal). Infected cells were incubated at 37° for an additional 24 hours prior to quantification of ZIKV RNA by RT-qPCR. Inhibition was calculated as $100 - \%$ infection relative to infections containing no inhibitors.

Immunoblotting
Cells were grown in 6-well plates and lysates were prepared with RIPA buffer (50 mM Tris-HCl [pH 7.4]; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; protease inhibitor cocktail (Sigma); 1 mM sodium orthovanadate), and insoluble material was precipitated by brief centrifugation. Protein concentration of lysates was determined by BCA protein assay (Thermo Scientific). Lysates containing equal amounts of protein were loaded onto 4-20% SDS-PAGE gels and transferred to a nitrocellulose membrane (LI-COR, Lincoln, NE), blocked with 10% milk for 1 h, and incubated with the primary antibody overnight at 4 °C. Membranes were blocked with Odyssey Blocking buffer (LI-COR, Lincoln, NE), followed by incubation with primary antibodies at 1:1000 dilutions. Membranes were washed three times with 1X PBS containing 0.05% Tween20 (v/v), incubated with IRDye secondary antibodies (LI-COR, Lincoln, NE) for 1 h, and washed again to remove unbound antibody. Odyssey CLx (LI-COR Biosystems, Lincoln, NE) was used to detect bound antibody complexes.

**Statistical analysis**

All experiments were independently performed at least twice times as indicated in the Figure legends. Except were specified, bar graphs were plotted to show mean ± standard deviation (SD). Statistical analyses were performed using Prism 6. A p value of <0.05 in the Wilcoxon and Kruskal-Wallis tests was considered statistically significant.

**Online Table I. Infectious titers of ZIKV stocks used in the study**

<table>
<thead>
<tr>
<th>ZIKV Strain/Isolate</th>
<th>Viral Titer(pfu/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRVABC59</td>
<td>8.6 X10⁶</td>
</tr>
<tr>
<td>MR766</td>
<td>3.6 X10⁶</td>
</tr>
<tr>
<td>IbH30656</td>
<td>2.1X10⁶</td>
</tr>
<tr>
<td>FLR</td>
<td>2.0 X10⁶</td>
</tr>
</tbody>
</table>
References


Online Figure I. Productive infection of hCMEC/D3 cells by ZIKV
(A) The human BBB cell line hCMEC/D3 were infected by indicated ZIKV strains/isolates at MOI of 1 for 48 hrs followed by immunostaining of ZIKV Env protein. (B) Infectious titers of supernatants collected at 48 hrs post-infection from cells in a. Data presented are averages of three independent experiments. N=3, Error bars, s.d.
Online Figure II. Primary human ECs are much less permissive to DENV2 infection. (A) Expression of CD31/PECAM-1 on primary ECs. ECs were immunostained by anti-CD31 antibody (1:100) and visualized on cell surface (Green). Nearly all cells are positive for CD31. (B) Primary human ECs from the same donor were infected with DENV2 (top) or PRVABC59 at MOI of 1. Forty-eight hours post-infection, cells were immunostained for viral Env protein (red). The percentage of infection (shown at the lower left corner of each image) was calculated by enumerating the number of Env+ cells over the number of nuclei. BHK-21 cells were infected a positive control for infection.
Online Figure III. Infection of lymphatic endothelial cells (LECs) by ZKIV. (A) LECs (passage 6) were infected by indicated ZIKV MR766 or PRV strain/isolate or DENV1 (Thailand 16681) at MOI of 1 for 48 hrs followed by immunostaining of ZIKV Env protein. (B) Representative phase-contrast images of infected cells at 48 hrs post-infection. Significant cell death was seen in PRV and FLR infected cells.
Online Figure IV. ZIKV infection does not directly disrupt tight junctions (TJs).
Representative confocal images of ZIKV infected HUVEC cells from Fig. 1A (A) or hCMEC/D3 cells (B) at 48 hrs post-infection. Notably, only those viable cells were left on the glass cover slips for imaging. TJ protein ZO-1 (green) and ZIKV Env protein (red) were immunostained. Right inset within b is the western blotting image of cells by ZIKV MR766 or IbH strains after 48 hours post-infection. OCLN, occludin; CLDN1, claudin-1. (C) Transendothelial resistance (TEER) was measured on HUVECs or hCMEC/D3 (day 2 after PRV infection, MOI=0.1). (n=2, each done in duplicate; error bars, s.d.). As a positive control, EDTA (2 µM) was added to HUVECs at Day 2 for 40 minutes to disrupt TEER.
Online Figure V. AXL inhibitors. (A) Chemical structures of four compounds used in the study. (B) hCMEC/D3 cells were pre-treated with Sorafenib or Sunitinib malate (1 µM) for 1 hour followed by ZIKV infection (MOI 1) of 1 hour, after which a medium change occurred. Twenty-four hours post-infection cellular RNA was extracted for real-time RT-PCR quantification of ZIKV RNA. The obtained results were normalized against levels of GAPDH and the ZIKV RNA levels from DMSO-treated cells were set to 1. (n=2; error bars, s.d.; *P<0.05.) (C) The cell viability of compound-treated HUVECs was quantified using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). The assay measures the cellular ATP levels using a luciferase based assay (in RLU). (n=3; error bars, s.d.)
Online Figure VI. Implications of ZIKV infection of ECs. (A) Productive of infection of vascular endothelium leads to shedding of virus to surroundings. (B) Productive infection of microvascular endothelial cells in the brain results in breaching the blood-brain barrier. (C) ZIKV virus carried from the infected mother feeds trophoblasts for potential breach of the placental-fetal barrier.