Differential Neuronal Susceptibility and Apoptosis in Congenital Zika Virus Infection

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To characterize the mechanism of Zika virus (ZIKV)-associated microcephaly, we performed immunolabeling on brain tissue from a 20-week fetus with intrauterine ZIKV infection. Although ZIKV demonstrated a wide range of neuronal and non-neuronal tropism, the infection rate was highest in intermediate progenitor cells and immature neurons. Apoptosis was observed in both infected and uninfected bystander cortical neurons, suggesting a role for paracrine factors in induction of neuronal apoptosis. Our results highlight differential neuronal susceptibility and neuronal apoptosis as potential mechanisms in the development of ZIKV-associated microcephaly, and may provide insights into the design and best timing of future therapy.

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Zika virus (ZIKV) is a flavivirus in the family Flaviviridae. It is transmitted mainly by Aedes species mosquitoes A. aegypti and A. albopictus. After initial discovery in 1947, human infections were only sporadically reported in Africa and Asia, typically accompanied by mild illness. In February 2016, as infection spread rapidly from episodic large clusters of disease in the Pacific Islands to the Americas, the World Health Organization declared ZIKV infection a Public Health Emergency of International Concern due to its association with microcephaly in the setting of congenital infection and other neurological disorders.1,2 There is now scientific consensus that ZIKV causes microcephaly.3 The mechanism of how congenital ZIKV infection leads to microcephaly, however, remains to be elucidated.

Recent studies in human induced pluripotent stem cell–derived neural progenitor and organoid culture systems have provided novel insight into the pathogenesis of ZIKV-related microcephaly. These studies show that ZIKV preferentially infects neural progenitor cells (NPCs), attenuates cell growth, and induces cell death.4-6 These findings have also been observed in mouse models, which further demonstrated the development of microcephaly in infected fetal mice.7-9 Although cell culture and animal models are valuable resources for ZIKV studies, they may not fully recapitulate human disease, due to the inherently artificial components of the experimental setup, including required manipulation of murine host immune responses for infection. Therefore, human studies remain critical for investigating virus–host interactions and phenotypic variability of ZIKV-induced neurologic injury.

Several postmortem studies of ZIKV-infected fetuses and infants have been published since the inception of the ZIKV outbreak. They have demonstrated a spectrum of gross and microscopic brain anomalies including microcephaly, lissencephaly, hydrocephalus, cerebellar hypoplasia, intracranial calcifications, brain parenchyma necrosis, and inflammatory infiltrates.10-14 Of note, the majority of the cases were fetuses examined in the third trimester of pregnancy (after antecedent infection earlier in the pregnancy), at a point when neurogenesis has been largely completed. This may explain why attenuated growth or apoptosis of NPCs, the key finding...
of cell culture and animal studies to date, has not been observed in infected human fetuses. To investigate whether findings in cell culture and animal model systems are representative of pathogenesis in human fetal brain tissue, we performed immunofluorescence analysis on brain tissue from a 20-week gestation fetus with confirmed ZIKV infection and retarded brain growth. Compared with other postmortem studies, our case is unique in that neurogenesis remains ongoing and robust at this gestational age.

**Materials and Methods**

**Postmortem Tissue Study**
The study utilized solely autopsy tissue and therefore does not qualify as human subjects research, as per institutional review board guidelines.

**Immunofluorescent Staining of Tissue Sections**
The brain was fixed in formalin for 2 weeks before processing. Formalin-fixed paraffin-embedded brain tissue was then sectioned at 4 μm. The sections on slides were deparaffinized and underwent antigen retrieval in Tris-HCl, pH 10 buffer (Sigma-Aldrich, St Louis, MO) at boiling temperature for 10 minutes. After cooling down for 30 minutes, tissue sections were permeabilized in phosphate-buffered saline (PBS) containing 0.3% Triton X-100 (PBST) for 20 minutes and washed in PBS for 5 minutes × 3. Sections were then blocked with PBS containing 5% normal goat or donkey serum (Sigma-Aldrich) for 30 minutes at room temperature. All sections were incubated overnight at 4°C with primary antibodies diluted in blocking solution. The primary antibodies used were mouse anti–flavivirus group antigen antibody (MAB10216; Millipore, Billerica, MA; 1:500), rabbit anti-SOX2 (ab92494; Abcam, Cambridge, UK; 1:250), chicken anti-nestin (Aves Labs, Tigard, OR; 1:500), rabbit anti-Tbr2 (ab23345, Abcam; 1:250), rabbit anti-DCX (ab18723, Abcam; 1:500), rabbit anti-NeuN (ABN78, Millipore; 1:250), rabbit anti–cleaved caspase-3 (9664; Cell Signaling Technology, Danvers, MA; 1:1,000), chicken anti-GFAP (ab4674, Abcam; 1:2,000), rabbit anti-Olig2 (AB9610, Millipore; 1:250), rabbit anti-CD68 (SAB555070, Sigma-Aldrich; 1:250), rabbit anti-CD3 (SAB555057, Sigma-Aldrich; 1:250), and rabbit anti-CD20 (SAB555049, Sigma-Aldrich; 1:250). Sections were washed with PBST, and incubated with Alexa Fluor–conjugated secondary antibodies at room temperature for 1 hour. The secondary antibodies were all purchased from Thermo Fisher (Waltham, MA) and used at 1:400. Nuclear counterstain was performed with 4,6-diamidino-2-phenylindole (DAPI; Thermo Fisher; 1:5,000) after incubation of secondary antibodies. Images were obtained using Zeiss (Oberkochen, Germany) LSM510 confocal microscope systems.

**Quantification of ZIKV-Infected or Activated Caspase 3–Positive Cells**
Brain tissue sections containing different brain regions were coimmunostained with flavivirus envelope protein antibody and 1 or 2 lineage markers and counterstained with DAPI. For non-neuronal lineage cells, astrocytes and lymphocytes were imaged from the cortical plate, and oligodendrocytes and macrophages/microglia were imaged from the deep white matter. For quantification of activated caspase 3 (aCasp3)-positive cells, tissue sections were stained with aCasp3 antibody and counterstained with DAPI. Three to 5 random microscopic fields per brain region were photographed by C.-Y.H. and then quantified in an automated fashion using the ImageJ Cell Counter plug-in. Percentage of ZIKV⁺ cells in a given lineage was defined as the number of flavivirus envelope protein and lineage marker double-positive cells divided by lineage marker-positive cells. GraphPad (La Jolla, CA) Prism and Microsoft (Redmond, WA) Excel software were used to perform data analysis.

**Results**

**Variability in ZIKV Infection among Different Neuronal Cell Populations and Stages of Differentiation in Fetal Brain**
To identify the types of neural lineage cells that are most susceptible to ZIKV infection, we coimmunolabeled fetal brain sections for flavivirus envelope protein and various neuronal lineage markers including NPC markers SOX2 and nestin, intermediate progenitor cell (IPC) marker Tbr2, immature neuronal marker doublecortin (DCX), and mature neuronal marker NeuN. ZIKV was detected in a wide range of neural lineage cells (Fig 1), but susceptibility to viral infection was variable: Tbr2⁺ IPCs showed the highest frequency of ZIKV⁺ cells (81.4 ± 12.0%), followed by DCX⁺ immature neurons (51.5 ± 13.9%) and SOX2⁺ nestin⁺ NPCs from the dorsal ventricular zone (VZ; 26.6 ± 13.4%); NeuN⁺ mature neurons had the lowest frequency of ZIKV⁺ cells (10.0 ± 7.0%).

**Variable Tropism of ZIKV Infection among Different Anatomic Regions of the Fetal Brain**
As compared to the dorsal VZ, which showed severe cell loss in the germinal matrix, the ganglionic eminence (GE; ventral VZ) germinal matrix of this ZIKV-infected brain appeared normal on premortem fetal magnetic resonance imaging and postmortem immunohistochemical analysis (Fig 2). The volume, cellularity, and NPC morphology all appeared normal in this region. Strikingly, only rare cells in the GE were ZIKV-infected. The significant difference in the infection rate between NPCs in the dorsal VZ and GE suggests that there is differential cellular tropism of ZIKV even within the NPC population.

**Quantitation and Characterization of Apoptosis in ZIKV-Infected and Uninfected Fetal Brain Neuronal Cells**
Apoptosis as a mechanism of neuronal cell death has been demonstrated in the setting of other viral infections of the central nervous system in the fetus. Previously,
increased apoptosis and suppressed NPC proliferation have been proposed as preceding determinants of ZIKV-associated microcephaly. To determine whether cell death was universally present among the neural lineage cell population, apoptotic cells were quantified by activated (cleaved) caspase 3 labeling in different fetal brain regions. In concordance with the data from the animal model, apoptotic cell death was most abundant in the cortical plate, which contained immature and mature neurons (Fig 3). In comparison, only rare apoptotic cells were observed in the intermediate zone, despite a high frequency of ZIKV-positive IPCs in this region (see Fig 1C, F). The presence of apoptotic cells was also extremely low in the GE and dorsal VZ, despite the previously mentioned abundance of infected cells in the dorsal VZ. Interestingly, many noninfected cells were aCasp3-positive (percentage of ZIKV-negative cells among aCasp3-positive cells: 66.3 ± 5.9%), suggesting a potential paracrine mechanism of apoptosis induction in neighboring infected cells.

Characterization of ZIKV Infection in Non-Neuronal Cell Populations in Fetal Brain

To investigate whether non-neuronal cells in fetal brain were also ZIKV-infected, we performed double immunofluorescent staining for flavivirus envelope protein and various lineage markers for astrocytes, oligodendrocytes, macrophages, and lymphocytes. As demonstrated in Figure 4, the frequency of ZIKV+ cells was remarkably high in cerebral cortical astrocytes (44.5 ± 7.7%), white matter oligodendrocytes (66.1 ± 8.8%), and white matter macrophages/microglia (84.2 ± 10.8%). The frequency of ZIKV infection appeared low in T and B lymphocytes, but we were unable to obtain meaningful quantitative data due to the rarity of lymphocytes present in this fetal brain. Our data suggest that in addition to direct cell damage by viral infection, inflammation caused by astrocytes, macrophages, and microglia may play an important role in the pathogenesis of ZIKV infection.

**FIGURE 1:** Variable percentage of Zika virus (ZIKV)-infected neuronal lineage cells in different stages of neurogenesis within fetal brain. (A) Human fetal brain structures at 20-week gestation. CP = cortical plate; LGE = lateral ganglionic eminence; MGE = medial ganglionic eminence; VZ = ventricular zone of the dorsal forebrain. (B) VZ neural progenitor cells from a ZIKV-infected 20-week gestation fetus coimmunostained for flavivirus envelope protein (ZIKV; red), nestin (green), and SOX2 (blue). LV = lateral ventricle. (C) Intermediate progenitor cells (IPCs) from the subventricular zone and intermediate zone (IZ) coimmunostained for ZIKV (red) and Tbr2 (green). (D) Mature neurons from the CP coimmunostained for ZIKV (red) and doublecortin (DCX; green). (E) Mature neurons from the CP coimmunostained for ZIKV (red) and NeuN (green). (F) Quantification of ZIKV+ neuronal lineage cells. Note that the percentage of ZIKV+ cells is the highest in IPCs and the lowest in mature neurons. Values represent means ± standard deviation; *p < 0.05, ***p < 0.001, n.s. = nonsignificant; Student t test. Scale bars = 20 μm.
role in ZIKV-associated neural injury and the subsequent development of microcephaly.

Discussion

By analyzing ZIKV-infected fetal brain tissue, our study provides valuable insights into ZIKV disease pathogenesis in vivo, identifying the importance of neuronal lineage and cell type in susceptibility to infection, as well as establishing apoptosis as a mechanism of both direct and indirect ZIKV-induced injury to neuronal cell populations. Our study also suggests that non-neuronal cell populations are susceptible to ZIKV infection in vivo and that resultant inflammatory responses may play an additional pathologic role in brain injury.

Our study is the first to demonstrate differential infectivity and susceptibility of neuronal lineage cells to ZIKV infection. The surprisingly high frequency of ZIKV+ IPCs and immature neurons implies either that these more differentiated neural lineage cells remain susceptible to ZIKV infection, or that infection can be supported until the immature neuron stage of differentiation. The resistance of mature neurons to ZIKV infection may also explain why ZIKV infection in the third trimester poses less risk of neurologic insult and microcephaly in infants.17 Gene expression studies to identify differentially expressed surface molecules in IPCs and immature neurons may shed light on the identification of key ZIKV entry factors. These studies may yield an explanation for the propensity of ZIKV infection to result in the most severe fetal brain injury at discrete phases of gestation/neuronal development.

Our study also revealed not only that neuronal lineage a factor in susceptibility, but that neuronal cell types appear to be differentially susceptible to ZIKV infection. The significant difference in infection rate between NPCs in the dorsal VZ and GE suggests that there is differential cellular tropism of ZIKV even within the NPC population. The GE (ventral VZ) consists predominantly of inhibitory γ-aminobutyric acid-ergic (GABAergic) interneuron precursors, whereas the dorsal VZ consists mainly of excitatory

FIGURE 2: Resistance of neural progenitor cells (NPCs) in ganglionic eminence (GE) to ZIKV infection. (A) Axial diffusion image at the level of the atria of the lateral ventricles from a normal 20-week fetus. Three distinct layers are evident: the cortical plate (CP; upper, shortest arrow), the subplate (middle arrow), and the ventricular zone (VZ; lower longest arrow). Bright diffusion signal of the CP and VZ reflects high cellular density (high concentration of neuronal cells). (B) Axial diffusion image of the Zika virus (ZIKV)-infected fetus in this study at a similar level similar to that shown in A demonstrates abnormally enlarged lateral ventricles (asterisks). There is no bright diffusion signal in the expected locations of the CP and VZ, reflecting severe cell loss. (C, D) Axial T2-weighted magnetic resonance images of a normal 20-week fetal brain (C) and ZIKV-infected fetal brain in this study (D) at the level of the basal ganglia and GE. The region of the basal ganglia/GE (denoted by arrows) appears relatively normal in the ZIKV-infected fetus. Note markedly enlarged frontal horns (asterisks). (E) NPCs of GE coimmunostained for ZIKV (red), nestin (green), and SOX2 (blue). Arrowheads indicate nonspecific staining of red blood cells in the vasculature. Scale bar = 20 μm. (F) Quantification of ZIKV+ NPCs in the VZ of the dorsal forebrain (see Fig 1B) and GE. Values represent means ± standard deviation; *p<0.05; Student t test.
Our findings raise the possibility that ZIKV specifically targets glutamatergic neuronal precursors, which later differentiate into principal neurons of the cerebral cortex. The differential tropism within the neural stem cell populations appears to be a novel phenomenon that has not been reported in any other neurotropic viral infections. Further studies of cell surface molecule expression in glutamatergic and GABAergic neuronal precursors will be crucial for uncovering key players in ZIKV entry.

Our study identified apoptosis as a mechanism of cell death in ZIKV-infected neurons, as well as in an even greater number of adjacent neurons without evidence of

**FIGURE 3:** Differential apoptosis in different brain regions and bystander apoptosis in the cortical plate. (A–E) Quantification of apoptotic cells in different brain regions by activated caspase 3 (aCasp3) immunolabeling. CP = cortical plate; DAPI = 4,6-diamidino-2-phenylindole; GE = ganglionic eminence; IZ = intermediate zone; LV = lateral ventricle; VZ = ventricular zone of the dorsal forebrain; ZIKV = Zika virus. Values represent means ± standard deviation; **p < 0.01; Student t test. (F) Cells in the CP costained with flavivirus envelope antibody (red), aCasp3 (green), and DAPI (blue). Scale bars = 20 μm.

**FIGURE 4:** Zika virus (ZIKV) infection in non-neuronal cells of the fetal brain. (A–E) Astrocytes in the cortical plate (CP; A), oligodendrocytes in the white matter (WM; B), macrophages in the WM (C), and T lymphocytes (D) and B lymphocytes (E) in the CP coimmunostained with ZIKV (red) and respective lineage markers GFAP, Olig2, CD68, CD3, and CD20 (green). Arrowhead indicates nonspecific staining of red blood cells in the vasculature. Scale bars = 20 μm. (F) Quantification of ZIKV+ astrocytes in the CP (GFAP+, see A), oligodendrocytes in the WM (Olig2+, see B), and macrophages/microglia (CD68+, see C) in the WM. Values represent means ± standard deviation; *p < 0.05, **p < 0.01, n.s. = nonsignificant; Student t test.
infection. One potential interpretation of these results is that ZIKV-negative apoptotic cells may have been infected by ZIKV earlier and underwent delayed apoptosis after intracellular viral load had decreased below detection limits. This seems unlikely, because we used expression of envelope protein, a gene that is expressed relatively late after infection of host cells, as an indicator of ZIKV infection. An alternate interpretation of these results is that an infection-independent mechanism of cell death may be important for ZIKV-induced neuronal injury. The phenomenon of bystander apoptosis has precedents in the setting of other viral infections such as HIV and flaviviral infections including Dengue virus and West Nile virus infection. The proposed mechanism of bystander apoptosis is that cytotoxic factors released by infected neurons may induce apoptosis in noninfected neurons. Additionally, non-neuronal cells in the brain, such as astrocytes, microglia, macrophages, and lymphocytes, may also become activated and release excitotoxins, reactive oxygen species, and proinflammatory cytokines that may contribute to bystander injuries to neurons.

Finally, our study demonstrated that ZIKV infection occurred in a substantial proportion of non-neuronal cells (e.g., astrocytes, macrophages, and microglia), suggesting that in addition to direct ZIKV-induced apoptosis of neuronal cell populations, infection-induced inflammatory responses may also contribute to the development of microcephaly in the setting of congenital ZIKV infection. Although neurons within the ganglionic eminence appeared resistant to ZIKV infection in our study, secondary changes of ZIKV infection, such as microglial aggregates and macrophage infiltrates, may occur as a more generalized response to infection, with resultant injury to this brain region. This is one potential explanation for why calcifications have been detected in basal ganglia in many other published congenital ZIKV infection cases.

Characterization of differentiation-stage-dependent expression of ZIKV entry factors, delineation of ZIKV-induced apoptotic signaling cascades, and identification of ZIKV-induced activation of inflammatory cytokines and signaling factors may potentially facilitate the design of novel treatment strategies for ZIKV-related brain injury in the setting of congenital infection.

Author Contributions
C.-Y.H., J.S.L., J.S., M.T., F.J.R., A.d.P, and R.L.D. conceptualized and designed the study. C.-Y.H., H.M.A., A.T., and G.V. acquired data and conducted data analysis. C.-Y.H. and H.M.A. drafted the text and prepared the figures.

Potential Conflicts of Interest
Nothing to report.

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