Zika virus-induced TNF-α signaling dysregulates expression of neurologic genes associated with psychiatric disorders

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Abstract

Background: Zika virus (ZIKV) is an emerging flavivirus of global concern. ZIKV infection of the central nervous system has been linked to a variety of clinical syndromes, including microcephaly in fetuses and rare but serious neurologic disease in adults. However, the potential for ZIKV to influence brain physiology and host behavior following apparently mild or subclinical infection is less well understood. Furthermore, though deficits in cognitive function are well-documented after recovery from neuroinvasive viral infection, the potential impact of ZIKV on other host behavioral domains has not been thoroughly explored.

Methods: We used transcriptomic profiling, including unbiased gene ontology enrichment analysis, to assess the impact of ZIKV infection on gene expression in primary cortical neuron cultures. These studies were extended with molecular biological analysis of gene expression and inflammatory cytokine signaling. In vitro observations were further confirmed using established in vivo models of ZIKV infection in immunocompetent hosts.

Results: Transcriptomic profiling of primary neuron cultures following ZIKV infection revealed altered expression of key genes associated with major psychiatric disorders, such as bipolar disorder and schizophrenia. Gene ontology enrichment analysis also revealed significant changes in gene expression associated with fundamental neurobiological processes, including neuronal development, neurotransmission, and others. These alterations to neurologic gene expression were also observed in the brain in vivo using several immunocompetent mouse models of ZIKV infection. Mechanistic studies identified TNF-α signaling via TNFR1 as a major regulatory mechanism controlling ZIKV-induced changes to neurologic gene expression.

Conclusions: Our studies reveal that cell-intrinsic innate immune responses to ZIKV infection profoundly shape neuronal transcriptional profiles, highlighting the need to further explore associations between ZIKV infection and disordered host behavioral states.

Introduction

Zika virus (ZIKV) is a mosquito-borne pathogen of global concern [1]. Like many other members of the genus Flaviviridae, ZIKV is both neuroinvasive and neurotropic [2]. Infection of the central nervous system has been linked to diverse clinical syndromes, including severe congenital neurodevelopmental abnormalities in infants following vertical infection in utero [3–5]. Severe neurologic disease is less frequent in adults, though cases of encephalitis, myelitis, and, more commonly, peripheral neuropathy have been reported [2, 6]. While research on ZIKV pathogenesis to date has heavily focused on severe neurologic disease, it remains unclear whether ZIKV accesses the central nervous system during mild and/or...
subclinical infection, though data from animal models suggest this is probable [7–9]. Even in the case of established neuroinvasive infection, the long-term neurologic consequences that follow viral clearance and recovery remain poorly understood. However, recent evidence suggests that a range of potential neurologic sequelae may occur in the postinfectious brain, including changes to host behavior [10–13].

In particular, recovery from neuroinvasive infection by flaviviruses, including ZIKV, has been associated with neurocognitive deficits [14–17]. These effects have been attributed in part to the activities of immune cells, including both T cells and microglia, which act in concert to aberrantly prune neuronal synapses following flavivirus recovery [18–20]. Flavivirus infection has also been shown to alter neurodevelopmental programs [21–24], including adult neurogenesis [25–27], a feature of flavivirus infection that contributes to altered learning and memory following recovery in rodent models [28]. Cognitive decline, including persistent memory loss, is also a common occurrence in human patients recovering from flavivirus encephalitis [29–31].

Despite these insights into the cognitive consequences of flavivirus infection, the potential impact of these viruses on other behavioral domains remains relatively unexplored. The multifaceted impact of neurotropic flaviviruses on a diverse array of neurologic functions suggests that such infections may also promote or exacerbate neuropsychiatric conditions, including mood and psychotic disorders. Indeed, depression is another common behavioral symptom reported in patients recovering from flavivirus infection [32–34]. Case reports have also documented the appearance of psychotic symptoms, including hallucinations, in adults infected with ZIKV [35, 36]. However, the cellular and molecular mechanisms that underlie these effects remain unknown. In particular, the potential for ZIKV infection to impact host behavior due to cell intrinsic effects on neuronal gene expression has been relatively unexplored.

In this study, we examined how ZIKV infection in neurons impacted the expression of gene pathways that promote homeostatic neural function, as well as genes associated with disordered behavioral states. Transcriptomic profiling of primary cortical neurons following ZIKV infection revealed altered expression of many genes associated with psychiatric disorders, including autism, depression, and schizophrenia. Moreover, unbiased gene ontology enrichment analysis revealed that ZIKV infection disproportionately impacted expression of genes associated with neurotransmission and neurodevelopment. These patterns of altered gene expression were also observed in vivo, using an established model of central nervous system (CNS) ZIKV infection in immunocompetent mice. Observed changes in gene expression were due, at least in part, to innate cytokine signaling via tumor necrosis factor receptor-1 (TNFR1). Our data describe a mechanism linking the cell intrinsic innate immune response to ZIKV with dysregulation of a diverse array of neurologic gene pathways, opening new avenues of inquiry into the effect of flavivirus infection on host behavior.

**Materials and methods**

**Viruses**

ZIKV strain MR766 was originally provided by Dr. Andrew Oberst (University of Washington, Seattle, WA, USA). ZIKV-DAKAR-MA was first generated [7] and generously provided by Dr. Michael Diamond (Washington University, St. Louis, MO, USA). Viral stocks were generated by infecting Vero cells (MOI 0.01) and harvesting supernatants at 72hpi. Viral titers of stocks were determined via plaque assay on Vero cells (ATCC, #CCL-81). Cells were maintained in DMEM (Corning #10-013-CV) supplemented with 10% Heat Inactivated FBS (Gemini Biosciences #100-106), 1% Penicillin–Streptomycin–Glutamine (Gemini Biosciences #400-110), 1% Amphotericin B (Gemini Biosciences #400–104), 1% Non-Essential Amino Acids (Cytiva #SH30238.01), and 1% HEPES (Cytiva SH30237.01). Plaque assay basal media was 10X EMEM (Lonza # 12-684F) adjusted to 1X and supplemented with 2% Heat Inactivated FBS (Gemini Biosciences #100-106), 1% Penicillin–Streptomycin–Glutamine (Gemini Biosciences #400-110), 1% Amphotericin B (Gemini Biosciences #400-104), 1% Non-Essential Amino Acids (Cytiva #SH30238.01), and 1% HEPES (Cytiva SH30237.01), 0.75% Sodium Bicarbonate (VWR #BDH9280) and 0.5% Methyl Cellulose (VWR #K390). Plaque assays were developed 4dpi by removal of overlay media and staining/fixation using 10% neutral buffered formalin (VWR #89370) and 0.25% crystal violet (VWR #0528).

**Cell culture experiments**

Primary cerebral cortical neurons were generated using E15 C57BL/6 J embryos as described [37]. Cells were maintained on cell culture treated multiwell dishes supplemented by coating with 20 μg/mL Poly-L-Lysine (Sigma-Aldrich, #9155). Neurobasal B-27 supplemented medium was used for all experiments (Thermo-Fisher Scientific, #A3582901). All primary mouse cells were generated using pooled tissues derived from both male and female animals. For ZIKV infection experiments, primary neuron cultures were infected at an MOI of 0.1.
**Quantitative real-time PCR**

Total RNA from cultured cells was isolated with Qiagen RNeasy mini extraction kit (Qiagen, #74106) following the manufacturer’s protocol. RNA concentration was measured with a Quick Drop device (Molecular Devices). cDNA was subsequently synthesized with qScript cDNA Synthesis Kit (Quantabio, #95048). qPCR was performed with SYBR Green Master Mix (Applied Biosystems, #A25742) using a QuantStudio5 instrument (Applied Biosystems). Cycle threshold (CT) values for analyzed genes were normalized to CT values of the housekeeping gene 18S (ΔCT = CT_{Target} − CT_{18S}). Data were further normalized to baseline control values (ΔΔCT = ΔCT_{experimental} − ΔCT_{control}). Primers were designed using Primer3 (https://bioinfo.ut.ee/primer3/) against murine genomic sequences. A list of primer sequences used in the study appears in Additional file 1: Table S1.

**Murine models of ZIKV infection**

C57BL/6 J mice were bred in-house for all experiments. hSTAT2 knockin mice were originally obtained from Jackson Laboratories (strain 031630) and subsequently bred in-house. All animals were housed under pathogen-free conditions in the animal facilities in Nelson Biological Laboratories at Rutgers University. Both male and female mice were inoculated intracranially (10 µl) with 10⁴ PFU of ZIKV-MR766, intracranially with 10¹ PFU ZIKV-DAK-MA, or subcutaneously (50 µl) in a rear footpad with 10³ PFU ZIKV-DAK-MA, as described previously [7, 8].

**Tissue preparation**

All tissues harvested from mice were extracted following extensive cardiac perfusion with 30 mL of sterile PBS. Extracted tissues were weighed and homogenized using 1.0 mm diameter zirconia/silica beads (Biospec Products, #11079110z) in sterile PBS for ELISA (VWR #L01190) or TRI Reagent (Zymo, #R2050-1) for gene expression analysis. Homogenization was performed in an Omni Beadracter Elite for 2 sequential cycles of 20 s at a speed of 4 m/s. Total RNA was extracted using Zymo Direct-zol RNA Miniprep kit, as per manufacturer instructions (Zymo, #R2051).

**Flow cytometric analysis**

Leukocytes were isolated from whole brains after digestion in 0.05% collagenase A (Sigma-Aldrich, #C0130) and 10 mg/ml DNase I (Sigma-Aldrich, #D4527), then purified via centrifugation in 37% isotonic Percoll (VWR, #89428-524) as described [38]. Isolated leukocytes from brains were stained as described [39] with fluorescently conjugated antibodies to CD3e (Biolegend, clone 17A2), CD44 (Biolegend, clone IM7), CD19 (Biolegend, clone 6D5), CD107a (Biolegend, clone 1D4B), CD8a (Biolegend, clone 53-6.7), CD4 (Biolegend, clone RM4-5), CD45.2 (Biolegend, clone 104), MHC-II (Biolegend, clone M5/114.15.2), NK1.1 (Biolegend, clone PK136), CD11c (Biolegend, clone N418), F4/80 (Biolegend, clone BM8), CD11b (Biolegend, clone M1/70), Ly6G (Biolegend, clone 1A8), Ly6C (Biolegend, clone HK1.4), CD80 (Biolegend, clone 16-10A1), and Zombie NIR (Biolegend, 423105). Data collection and analysis were performed using an Cytek Northern Lights Cytometer and FlowJo software (Treestar).

**ELISA**

A TNF-α sandwich ELISA kit (EBioscience, #MTA00B) was used for detection of cytokine levels in cell culture supernatants and brain tissue homogenates. Colorimetric reading of ELISA plates was performed with a microplate reader and Gen5 software (BioTek Instruments, Inc.).

**Neutralizing-antibody studies**

Neutralizing-antibody studies were performed after 30 min of pretreatment with purified anti-mouse TNFR1 (Invitrogen, #16-1202-85) and anti-mouse IFNAR1 (Leinco, # 1-400) antibodies. IgG isotype antibodies (EBioscience, # eBio299Arm; Leinco, # 1-443) were used as controls.

**Curation of psychiatric disorder-associated gene list**

Genes associated with autism spectrum disorder (ASD) for our bioinformatics study were identified using the Sfari Gene database (https://gene.sfari.org) [40]. The Sfari database includes a ranked list of genes with known associations to ASD. We included genes within the top 3 levels of evidential strength of association (syndromic, category 1, and category 2). All genes within this curation have at least two reported de novo likely-gene-disrupting mutations. We were unable to identify similar database resources for other psychiatric disorders. We thus assembled gene lists for additional disorders by consultation of recent and/or highly cited literature in these areas, including metanalyses and systematic reviews. More information about our gene list can be found in Additional file 1: Table S2.

**Microarray analysis**

Microarray studies were performed on primary cortical neurons derived from C57BL/6 J mouse embryos following 24 h exposure to PBS or ZIKV-MR766 (MOI 0.1), as described [8]. Microarray analysis was conducted using Agilent Whole Mouse Genome arrays (Agilent Technologies) according to manufacturer’s instructions.
Expression values and additional sample metadata are publicly available through the NCBI Gene Expression Omnibus Database (accession: GSE122121).

Bioinformatics and statistical analysis
Secondary analysis of microarray data was performed in GEO2R and the GO Enrichment Analysis tool (geneontology.org). Biological pathways were defined using the PANTHER (Protein Analysis Through Evolutionary Relationships) classification system. Corrected \( p \) values (false discovery rate) were determined using the Benjamini & Hochberg procedure. For molecular biology assays, two-way analysis of variance (ANOVA) with Sidak’s correction for multiple comparisons was performed using GraphPad Prism Software v8 (GraphPad Software, San Diego, CA). \( P < 0.05 \) was considered statistically significant. Data points in all experiments represent biological replicates unless otherwise noted.

Results
ZIKV infection in neurons dysregulates expression of genes associated with abnormal or pathologic behavioral states
We first assessed whether ZIKV infection in neurons resulted in altered expression of genes associated with abnormal and/or disordered behavioral states. To do so, we first generated a list of 676 genes that have been linked in previous studies to psychiatric disorders, including autism, attention deficit hyperactivity disorder (ADHD), bipolar disorder, major depressive disorder, and schizophrenia [40–87] (Additional file 1: Table S2). This list of genes includes a combination of known risk genes as well as genes associated with behavioral abnormalities in each of the above human disorders and related animal models. While we stress that the list is not designed to be comprehensive or definitive, it serves as a starting point for probing the behavioral consequences of neuronal ZIKV infection. We assessed the impact of ZIKV infection on the expression of these genes using a data set previously published by our group and others in which primary cortical neurons derived from C57BL/6 J mice were infected with 0.01 MOI ZIKV-MR766 [8]. Gene expression in this study was profiled via microarray analysis at 24 h following infection.

Cross-comparison of the differentially expressed genes (DEGs) from the microarray analysis with our curated gene list revealed that 181 out of 676 (26.8%) genes exhibited significant differential expression following ZIKV infection (Fig. 1A). Of these significant DEGs, 85 (46.96%) were downregulated by ZIKV infection, while 96 (53.04%) were upregulated by ZIKV infection. The 13 genes with the lowest \( p \) value in this analysis included \( Nr4a2 \), \( Ltg3b \), and \( Slc7a5 \), which were downregulated by ZIKV infection, along with \( Nlgn1 \), \( Myt1l \), \( Dpp6 \), \( Thsd7a \), \( Hepcam \), \( Syn3 \), \( Cacna1e \), \( Met \), \( Pyhin1 \), and \( Tor3a \), which were upregulated by ZIKV infection (Fig. 1B). The functions of these genes are summarized in Fig. 1C, and generally include synaptic function, ion channel physiology, and neurodevelopmental processes. None of the specific psychiatric disorder gene lists were significantly over-represented among the list of significant DEGs (Fig. 1D). Together, these data suggest that ZIKV infection in neurons dysregulates expression of a broad set of genes associated with abnormal or pathologic behavioral states.

ZIKV infection results in age-dependent changes in gene pathways associated with fundamental neurobiological processes
To better understand the consequences of ZIKV infection on neurologic gene expression, we next took an unbiased approach by performing gene ontology (GO) enrichment analysis on the DEGs derived from our microarray data set. Similar to our results using the curated psychiatric disorder-associated gene list, this unbiased analysis revealed significant enrichment of several GO terms related to neurotransmission, neuronal stress responses, and neurodevelopment, a subset of which are highlighted in Fig. 2A, B. Notably, regulatory pathways influencing ion transport and ion homeostasis were particularly enriched in our data set. We next questioned whether there were clear patterns in the direction of differential expression among the significantly enriched GO terms. Heatmaps depicting the expression of genes associated with several representative GO terms are shown in Fig. 2C–E, each of which revealed a mixed set of both up- and down-regulated genes. These findings suggest that the impact of ZIKV infection on the transcriptional state of neurons likely involves complex alterations to a variety of fundamental neurobiological processes.

While our microarray data revealed profound alterations to neurologic gene expression in primary neuronal
Fig. 1 (See legend on previous page.)
culture following ZIKV infection, we next wanted to assess whether similar changes to gene expression occur in vivo. To do so, we inoculated male and female wildtype (C57BL/6 J) mice intracranially with $10^4$ PFU ZIKV-MR766. We performed these studies in separate cohorts of adolescent (3 weeks) and adult (8 weeks) animals to account for potential differences in the expression of neurologic genes across development (Fig. 3A). On days 2 and 4 following infection, we harvested brains and used qRT-PCR to assess the expression of a panel of genes derived from the top DEGs identified in our microarray analysis. This candidate gene panel included genes from both our curated psychiatric disorder-associated gene list, as well as DEGs from the highly enriched GO terms identified in Fig. 2.

These experiments revealed that a majority of genes in our panel did exhibit differential expression at the whole-brain level following intracranial ZIKV infection in vivo. However, the magnitude and direction of differential expression exhibited complex patterns that differed across time post-infection and between 3-week-old and 8-week-old animals. In particular, 3-week-old animals exhibited a mix of significantly downregulated and upregulated expression of selected neurologic genes 2 day post-infection (dpi), with a marked shift to primarily upregulated expression at 4dpi (Fig. 3B, C). In contrast, 8-week-old animals exhibited an essentially inverse pattern, with nearly uniform upregulation of significant DEGs at 2dpi, but a mixture of down- and up-regulated DEGs at 4dpi (Fig. 3D, E). These patterns of differential expression were evident across GO terms, including neurogenesis (Fig. 3F), neuropeptide signaling pathway (Fig. 3G), and regulation of behavior (Fig. 3H), as well as genes taken from our curated list of psychiatric disorder-associated genes (Fig. 3I). Together, these data confirm that gene expression associated with disease-relevant
neurologic pathways is significantly dysregulated following ZIKV infection of the brain in vivo, but the mechanisms that control these transcriptional responses are under complex regulation by factors that vary with host age.

To assess whether differences in virologic burden or inflammation might correspond to age-dependent changes in neurologic gene expression, we profiled viral burden and inflammatory infiltrates following intracranial ZIKV-MR766 infection. Mice of both ages exhibited similar levels of viral RNA in the brain following infection, with 8-week-old mice showing a modest decrease in brain viral burden at 4dpi compared to 3-week-old animals (Fig. 4A). We next performed flow cytometric analysis of infiltrating leukocytes in the brain at 4dpi. While numbers of CD45lo CD11b+ microglia were unchanged between groups, mice of both ages exhibited similarly robust recruitment of CD45hi CD11b+ peripheral immune cells (Fig. 4B–D), including myeloid subsets expressing Ly6C (Fig. 4E) and Ly6G (Fig. 4F). Numbers of CD11c+ MHC-II+ antigen presenting cells were also increased similarly in both age cohorts following infection (Fig. 4G). Mice of both ages also exhibited similar recruitment of lymphocytes to the infected CNS (Fig. 4H), including CD4 T cells (Fig. 4I), CD8 T cells (Fig. 4J), and NK cells (Fig. 4K). Together, these data suggest that differences in neurologic gene expression between adolescent and adult mice following ZIKV infection were not driven by overt differences in CNS viral burden or infiltrating immune cells, suggesting that other age-dependent factors underlie this phenotype.

Expression of candidate neurologic genes is significantly dysregulated in vivo models of mild/asymptomatic ZIKV infection

While our transcriptional findings to this point were promising, a limitation of the intracranial ZIKV-MR766 infection model is a high degree of virulence, including uniform mortality around 1 week following infection (data not shown). To better model the mild disease course experienced by most human hosts, we thus repeated key analyses using two separate models of infection. In the
first, we performed intracranial inoculation with a low dose (10 PFU) of a mouse adapted African lineage strain (DAK-41525) [7], here referred to as ZIKV-DAK-MA, in 8-week-old C57BL/6J mice. All mice survive this challenge with no observation of neurologic signs of disease. Infected mice exhibited substantial levels of viral RNA in the brain at 7dpi, with greatly diminished but still detectable levels of viral RNA at 28dpi (Fig. 5A). We next analyzed a panel of our candidate neurologic genes in whole brain homogenates using qRT-PCR (Fig. 5B–O). While the extent and magnitude of gene expression changes were not as robust as the virulent ZIKV-MR766 infection model, we nevertheless saw significant changes in the expression of 7 of 14 genes analyzed, including

![Fig. 4](image_url)

3-week-old and 8-week-old mice exhibit similar neuroinflammatory responses to intracranial ZIKV infection. A qRT-PCR analysis of ZIKV RNA in the brains of mice at indicated day post-infection (dpi) following intracranial infection with ZIKV-MR766 (10^4 PFU). Values are reported in plaque forming unit-equivalents (PFUe) per hemisphere of brain tissue. n = 5–7 mice/group. B–K Flow cytometric analysis of infiltrating immune cells in brains of mice at 4dpi following intracranial ZIKV-MR766 infection. B Representative flow cytometry plots showing percentages of infiltrating (CD45^hi) vs. resident (CD45^lo) immune cells. C–G Quantification of absolute numbers of myeloid cell populations expressing indicated markers.

H Representative flow cytometry plots showing CD45^hi CD3^+ cells stratified by expression of T cell markers CD4 and CD8. I–K Quantification of absolute numbers of CD45^hi CD3^+ cell populations expressing indicated markers. n = 3–4 mice/group. * p < 0.05, ** p < 0.01, *** p < 0.001. Bars represent group means.
Adora2 (Fig. 5B), Cacna1e (Fig. 5C), Ccl5 (Fig. 5D), Gpr84 (Fig. 5E), Nlgn1 (Fig. 5I), Pmch (Fig. 5L), and Tor3a (Fig. 5O). Remarkably, differential expression of 6 of these genes persisted out to day 28 following infection. These data suggest that differential expression of neurologic genes following intracranial ZIKV infection was not dependent on severe clinical disease or uncontrolled viral replication within the CNS.

We next assessed whether our target neurologic genes would be impacted using a model of subcutaneous ZIKV infection, which most faithfully models the route of infection experienced by human hosts following the bite of an infected mosquito. As WT mice strongly resist ZIKV infection in the periphery due to an inability of ZIKV to antagonize murine STAT2 signaling, we used a mouse line harboring a homozygous replacement of the murine Stat2 gene with human STAT2 (hSTAT2 knockin) [7]. These mice are normal and immunocompetent but are susceptible to ZIKV-mediated antagonism of STAT2-mediated antiviral signaling, more faithfully modeling...
The human innate immune response to ZIKV-infection. 5-week-old hSTAT2 mice were infected with $10^3$ PFU ZIKV-DAK-MA in a rear footpad. All mice exhibited detectable viral RNA in the brain at 7dpi, confirming that neuroinvasion occurs in this model of peripheral ZIKV inoculation (Fig. 6A). All mice survived this challenge with no clear exhibition of clinical signs of neurologic disease; moreover, most mice completely cleared infection in the brain by 14dpi (Fig. 6A). Remarkably, qRT-PCR analysis of our neurologic gene panel (Fig. 6B–O) revealed significant differential expression of 10 of 14 genes analyzed, including Ccl5 (Fig. 6D), Gpr84 (Fig. 6E), Itgb3 (Fig. 6F), Lhx6 (Fig. 6H), Nlgn1 (Fig. 6I), Nr4a2 (Fig. 6J), Pmch (Fig. 6L), Rarb (Fig. 6M), Tac1 (Fig. 6N), and Tor3a (Fig. 6O). Many of these genes exhibited differential expression at 14dpi, a timepoint when viral RNA was undetectable in the majority of animals, though all genes had returned to baseline expression by 28dpi. These data suggest that genes associated with neurologic function and neuropsychiatric disorders are impacted in

![Figure 6](image)

**Fig. 6** Expression of candidate neurologic genes is significantly dysregulated following subcutaneous inoculation with ZIKV-DAK-MA in hSTAT2 knockin mice. **A** qRT-PCR analysis of ZIKV RNA in the brains of 5-week-old hSTAT2 knockin mice at indicated day post-infection (dpi) following subcutaneous inoculation with ZIKV-DAK-MA ($10^3$ PFU). Values are reported in plaque forming unit-equivalents (PFUe) per hemisphere of brain tissue. $n = 5–7$ mice/group. **B–O** Expression profiles of 14 candidate genes assessed by qRT-PCR analysis on indicated days following intracranial ZIKV-DAK-MA infection. $n = 5–7$ mice/group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Bars represent group means.
the brain in a physiologic model of subcutaneous ZIKV infection, and that changes to neurologic gene expression persist for some time following the resolution of active CNS infection.

Neuronal TNF-α signaling downstream of ZIKV infection dysregulates expression of genes relevant to neuronal function

We next questioned whether innate immune activation following ZIKV infection in neurons may be linked to observed changes in neurologic gene expression. To answer this, we returned to our gene ontology enrichment analysis to identify innate immune signaling pathways that were most significantly impacted by neuronal ZIKV infection. Well-known antiviral cytokine responses were among the most enriched pathways in this analysis, particularly those related to innate cytokines, including type I interferon (IFN), interleukin (IL)-1 and IL-6, and tumor necrosis factor (TNF)-α (Fig. 7A, B). Pathways related to signaling by the inflammatory transcription factor nuclear factor kappa B (NF-κB) were also particularly enriched within the list of significant DEGs. We next

| Gene Ontology Term                        | Number of Associated Genes | Fold Enrichment | FDR Rate (adj. P-value) |
|------------------------------------------|----------------------------|-----------------|-------------------------|
| cellular response to interferon-beta     | 17                         | 25.41           | 1.03e-014               |
| positive regulation of cytokine production | 33                      | 5.17            | 2.84e-011               |
| cellular response to interferon-gamma    | 17                         | 12.06           | 2.64e-010               |
| positive regulation of NF-kappaB          | 14                         | 6.69            | 1.23e-005               |
| transcription factor activity             |                            |                 |                         |
| regulation of interleukin-1-beta production | 10                     | 8.57            | 9.69e-005               |
| regulation of interleukin-1 production    | 10                         | 7.02            | 4.23e-004               |
| regulation of interleukin-kappaB signaling | 13                      | 5.05            | 4.62e-004               |
| regulation of tumor necrosis factor       | 12                         | 4.99            | 1.08e-003               |
| superfamily cytokine production           |                            |                 |                         |
| regulation of toll-like receptor signaling pathway | 8                     | 8.39            | 1.09e-003               |
| chemokine-mediated signaling pathway      | 7                          | 9.11            | 2.16e-003               |
| cellular response to interferon-alpha     | 4                          | 23.42           | 4.8e-003                |
| positive regulation of toll-like receptor signaling pathway | 5                     | 12.11           | 7.52e-003               |
| cellular response to tumor necrosis factor | 9                       | 5.14            | 7.53e-003               |
| cellular response to interleukin-1        | 7                          | 6.47            | 1.09e-002               |
| positive regulation of interferon-gamma-mediated signaling pathway | 3                     | 30.11           | 1.72e-002               |
| regulation of interleukin-6 production    | 9                          | 4.05            | 2.8e-002                |

Fig. 7 Neurons mount a robust innate immune cytokine response to ZIKV infection. A, B Results of GO enrichment analysis of microarray data derived from primary cortical neurons following ZIKV-MR766 infection (MOI 0.1) compared to PBS-treated controls after 24 h. Selected GO terms focus on cytokine activation or inflammatory transcription factor responses. Tabular results (A) are graphically represented in a bubble plot (B) to demonstrate associations between fold enrichment, FDR, and number of associated genes for each GO term. C-H qRT-PCR analysis was performed measuring cytokine genes Ifna6 (C), Ifnb (D), Ifng (E), Il1b (F), Il6 (G), and Tnfa (H) at indicated timepoints in 8-week-old adult mouse brains following intracranial ZIKV-MR766 infection. n = 5. ns not significant, * p < 0.05, ** p < 0.01, *** p < 0.001. Bars represent group means.
confirmed that each of these major cytokine responses was induced in the brains of 8-week-old mice following intracranial infection with ZIKV. Expression analysis via qRT-PCR showed that ZIKV infection induced significant upregulation of each of the cytokines analyzed, including Ifna6, Ifnb, Ifng, Il1b, Il6, and Tnfa (Fig. 7C–H). Together, these data confirm that wildtype neurons mount a robust innate immune cytokine response to ZIKV infection, including several cytokines previously established to influence brain function and behavior.

To examine whether neuronal cytokines may be implicated in ZIKV-induced changes in neurologic gene expression, we generated primary cultures of cortical neurons and examined expression of targets in our candidate gene panel following treatment with exogenous cytokines, including IFN-β, IL-6, and TNF-α (each at 10 pg/ml). We then compared the direction of differential expression for each gene to that induced by ZIKV to see which, if any, cytokine most closely phenocopied the pattern of gene expression induced by ZIKV infection. While IFN-β and IL-6 did significantly alter the expression of some target genes, significant DEGs following these treatments did not closely follow the pattern of downregulation (Fig. 8A) or upregulation (Fig. 8B) induced by ZIKV infection. In contrast, TNF-α treatment induced a strikingly similar pattern of differential expression to that induced by ZIKV infection. Moreover, while IFN-β and IL-6 only induced significant changes in expression of a handful of neurologic genes in our analysis (Fig. 8C, D), TNF-α significantly altered 17 out of 18 genes in our panel (Fig. 8E), and of these, all but 2 matched the pattern of up- or downregulated expression observed in ZIKV-infected neuronal cultures. These data identified TNF-α signaling as a promising candidate mechanism for the altered neurologic gene expression observed in the setting of neuronal ZIKV infection.

While we previously confirmed that Tnfa was induced at the transcriptional level in the brain in vivo following ZIKV infection, we next wanted to confirm that a robust TNF-α-dependent signature could indeed be observed following infection. We thus performed enzyme-linked immunosorbent assay (ELISA) to confirm that TNF-α was upregulated at the protein level in both supernatants of primary neuronal cultures at 24 h following infection (Fig. 8F) and whole brain homogenates derived from 8-week-old animals on days 2 and 4 following intracranial ZIKV inoculation (Fig. 8G). We also confirmed upregulation of known TNF-α transcriptional targets, including Cd69, Ccl2, Ccl5, and Stat1, in the brains of infected 8-week-old mice (Fig. 8H–K). In contrast, transcript expression of the TNF-α receptors TNFR1 (Tnfrsf1a) and TNFR2 (Tnfrsf1b) were not altered in the brain following infection (Fig. 8L, M), suggesting that enhanced TNF-α signaling in this setting is mediated primarily through induction of cytokine expression. Together, these data confirm that TNF-α signaling is active in both cultured neurons and the brain in vivo following ZIKV infection.

To more carefully assess whether TNF-α signaling was required for ZIKV-mediated alterations to neurologic gene expression, we cultured primary cortical neurons and pretreated with neutralizing antibodies against cytokine receptors for 2 h prior to infection. After 24 h, we then performed qRT-PCR analysis of major DEGs from our microarray analysis to assess the impact of cytokine signaling on ZIKV-induced gene expression. These experiments revealed that blockade of type I IFN signaling via neutralization of the IFN α/β receptor (IFNAR) had no impact on ZIKV-induced changes in expression of the neurologic genes we analyzed (Fig. 8N), findings which mirrored our previous result showing that exogenous IFN-β treatment did not phenocopy ZIKV-induced patterns of expression in our target gene list (Fig. 8A, B). In contrast, blockade of TNFR1 rescued ZIKV-induced changes in each of the 6 genes we analyzed, including Chac1, Nr4a2, Lhx6, Dpp6, Cacna1e, and Tor3a (Fig. 8O). Taken together, these data suggest that the induction of TNF-α following neuronal ZIKV infection is a major regulatory mechanism that alters expression of genes relevant to neuronal function.

(See figure on next page.)

**Fig. 8** Induction of TNF-α following neuronal ZIKV infection is a major regulatory mechanism that alters expression of genes relevant to neuronal function. A, B Expression values of candidate genes previously shown to be either downregulated (A) or upregulated (B) by ZIKV infection were measured via qRT-PCR in neuronal cultures treated for 24 h with 10 pg/ml exogenous cytokines: IFN-β, IL-6, or TNF-α (n = 6). C–E Volcano plots depicting mean z-score and -log10FDR of representative DEGs in neurons treated with IFN-β (C), IL-6 (D), or TNF-α (E). Significant differences (p < 0.05) are noted in red (upregulated DEGs) or blue (downregulated DEGs). n = 6. F, G Concentrations of TNF-α in supernatants of in vitro neuronal cell cultures infected with ZIKV for 24 h (n = 4) (F) and brains harvested following in vivo intracranial infection (n = 5) (G). Cytokine concentrations were quantified via ELISA assay. H–M qRT-PCR analysis was performed for known TNF-α transcriptional target genes, Cd69 (H), Ccl2 (I), Ccl5 (J), Stat1 (K), and TNF-α receptor genes, Tnfrsf1a (L) and Tnfrsf1b (M), in 8-week-old adult mouse brains following ZIKV-MR766 infection at 2 or 4 dpi. n = 5. N, O qRT-PCR analysis of representative DEGs associated with neurologic functions (Chac1, Nr4a2, Lhx6, Dpp6, Cacna1e, and Tor3a) following pretreatment with neutralizing antibodies against IFN α/β receptor (IFNAR) (N) or TNFR1 (O) and subsequent 24 h infection with ZIKV-MR766. n = 3–6 biological replicates per group. Data in (A) and (B) represent normalized and z-transformed values of qRT-PCR expression data. ns not significant, * p < 0.05, ** p < 0.01, *** p < 0.001. Bars represent group means.
Discussion
Emerging flaviviruses represent a significant and growing challenge to global public health. While most famously associated with rare but severe clinical manifestations, including encephalitis, congenital abnormalities, etc., the consequences of apparently mild and/or asymptomatic infection by neuroinvasive flaviviruses remain poorly understood [2, 88]. The observation of behavioral sequelae following recovery from severe flavivirus infections raises the possibility that subclinical neuroinvasive infection may also impact brain function in ways that promote or exacerbate psychiatric disorders. This idea is
supported by some case reports [15, 35, 89–91], though, to our knowledge, this hypothesis has not been rigorously tested in the clinical literature. The prevalence of psychiatric sequelae following ZIKV infection, in particular, may be hard to discern due to the relatively low neurovirulence of ZIKV compared to other flaviviruses, resulting in symptoms that may not be severe enough to warrant clinical attention and the documentation of infection status. Our study highlights the need for increased attention to behavioral symptoms in patients who are seropositive for ZIKV and other neuroinvasive flaviviruses, as well as further mechanistic investigation into the cellular and molecular impacts of flavivirus infection on brain physiology and function.

In our study, we show that neurons mount a robust innate cytokine response to ZIKV infection, including a number of cytokines with previously established effects on behavior. A large body of evidence has established that neuroinflammation and inflammatory cytokine signaling is associated with psychiatric disorders, including major depressive disorder [92–95] and schizophrenia [96–98]. TNF-α is a major pleiotropic cytokine induced strongly in the CNS by ZIKV and other flaviviruses [99–102]. Notably, recent work has described complex neuromodulatory effects of TNF-α signaling, including direct effects on glutamatergic neurotransmission [103–105], neuronal differentiation [106–108], and other fundamental neurologic processes [109–111]. In our study, ZIKV-mediated changes to neurologic gene expression greatly overlapped those induced by TNF-α, and gene expression changes induced by ZIKV could be rescued in part by blockade of TNFR1 signaling. These data identify TNF-α as a candidate for further mechanistic investigation of the potential impacts of flavivirus infection on neuronal function.

To date, the most well-described behavioral outcomes of neuroinvasive flavivirus infection in animal models are changes to learning and memory [18, 19, 27, 112–114]. While it is clear that a variety of pathogenic processes related both to viral infection and neuroinflammation can impact cognition, comparatively less attention has been devoted to how flavivirus infection impacts other behavioral domains, including mood, affect, and emotional regulation. This discrepancy is likely due, in part, to technical limitations, including difficulty modeling these behavioral domains in rodents and containment issues related to using ABL2 and ABL3 pathogens within behavioral laboratories. Nevertheless, our data identify a need for more robust assessment of behavioral changes in models of flavivirus infection, particularly measures of anxiety, fear/avoidance, and other paradigms with relevance to human psychiatric disorders.

Finally, our data add to a growing body of evidence suggesting that the impact of flavivirus infection varies across the lifespan. In our study, intracranial ZIKV infection resulted in very different impacts on neurologic gene expression in adolescent compared to adult animals, suggesting that developmental factors may significantly influence the neurologic outcomes of flavivirus infection. Recent work has shown that neuroimmune responses to other flaviviruses are impacted by aging [115, 116], and thus differential engagement of cytokine signaling, adaptive immune priming, and blood–brain barrier function may all be relevant variables in determining how flavivirus infection might impact behavior differentially across life stages. Moreover, while the potential for ZIKV to induce severe congenital abnormalities following vertical transmission in utero has now been well established, it remains less clear what the impact of ZIKV infection is on apparently developmentally normal fetuses, including those who are exposed to ZIKV late in gestation, when rates of microcephaly and severe birth defects are exceedingly rare [114, 117, 118]. Further work will be needed to assess whether ZIKV infection in this context may result in changes to neurodevelopment and brain function that impact behavior postnatally and beyond.

Conclusions
Our study describes the impact of ZIKV infection on both immunologic and neurologic transcriptional responses in neurons, adding new insight into how neuronal physiology and function may be altered in the setting of neuroinvasive flavivirus infection. The apparent dysregulation of numerous genes with known associations with human psychiatric disorders, in particular, suggests that more robust analyses of the impact of flavivirus infection on host behavior are warranted.

Abbreviations
ZIKV: Zika virus; TNF-α: Tumor necrosis factor-alpha; TNFR1: Tumor necrosis factor-alpha receptor 1; CNS: Central nervous system; DMEM: Dulbecco’s modified eagle medium; FBS: Fetal bovine serum; HepES: 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid; dpi: Day post-infection; M0: Multiplicity of infection; PPU: Plaque forming unit; PBS: Phosphate buffer saline; ELISA: Enzyme-linked immunosorbent assay; IFNAR1: Interferon-alpha receptor 1; ASD: Autism spectrum disorder; GO: Gene ontology; PANTHER: Protein analysis through evolutionary relationships; ANOVA: Analysis of variance; ADHD: Attention deficit hyperactivity disorder; DEG: Differentially expressed gene; IFN: Interferon; IL: Interleukin; NF-kB: Nuclear factor kappa B; ABL: Animal biosafety level; FDR: False discovery rate.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12974-022-02460-8.

Additional file 1: Table S1. Primer sequences for qRT-PCR. Table S2. Curated psychiatric disorder-associated gene list.
Acknowledgements
Not applicable.

Author contributions
Conceptualization: PLK and BPD; Investigation and analysis: PLK, TWC, ML, NPC, IE, BOB, CA, and BPD; Writing—original draft: PLK and BPD; Writing—review and editing: PLK, TWC, NPC, CA, and BPD; Supervision: CA and BPD; Funding acquisition: BPD. All authors read and approved the final manuscript.

Funding
This work was supported by NIH Grant R21 MH125034 and startup funds from Rutgers University (to BPD). PLK was supported in part by a Division of Life Sciences Summer Undergraduate Research Fellowship from Rutgers University. IE was supported in part by NIH Grant R25 GM055145 and an NIH Research Supplement to Promote Diversity (R01 NS120895-02).

Availability of data and materials
All data are available upon reasonable request to the corresponding author. Microarray data used in this study is deposited in NCBI’s Gene Expression Omnibus and can be accessed under accession number GSE122121.

Declarations
Ethics approval and consent to participate
All animal experiments were performed with approval of the Rutgers University Institutional Animal Care and Use Committee (IACUC).

Consent for publication
Not applicable.

Competing interests
The authors declare they have no competing interests.

Received: 27 November 2021   Accepted: 7 April 2022

Published online: 24 April 2022

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