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Short Communication

An investigation into the immune response of cultured neural rat cells following Zika virus infection

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ABSTRACT

The most notable effect of prenatal Zika virus (ZIKV) infection is severe microcephaly. ZIKV has a selective tropism for neural progenitor cells; however, it is not clear what role the immune cells of the brain, microglia, may have in mitigating or exacerbating neuronal cell death following ZIKV infection. We cultured hippocampal and cortical neural cells from neonatal rat pups and infected them with ZIKV at various multiplicities of infection (MOI). We found that the neuroimmune response to ZIKV infection is composed of both pro-inflammatory and type I interferon responses and is largely dependent upon the viral dose.

1. Introduction

Zika Virus (ZIKV) is thought to preferentially affect the developing brain because of the virus's specific neurotropism for radial glia coupled with the unique vulnerability of the developing immune system (Li et al., 2016a). The neural response to ZIKV infection is dynamic, with the neuroimmune response changing dependent on the time post-infection. ZIKV infection of embryonic mouse brain slices hinders apoptotic cell death within 24 h (Garcez et al., 2016; Qian et al., 2016), but then induces apoptosis in neural progenitor cells and stimulates associated pro-inflammatory cytokine expression within 72 h (Brault et al., 2016). Microglia are likely involved in this complex neural-immune activation profile, as microglia can inappropriately phagocytize stressed-but-viable neurons, and they are thought to be intimately linked to over-pruning of developing neurons resulting in abnormal brain development (Brown and Neher, 2014). Our experiments used a cell culture method to determine the neuroimmune and anti-viral response of developing rat neural cells to ZIKV infection. We found that neural cells up-regulate pro-inflammatory cytokines, as well as type I interferon and interferon-inducible anti-viral effectors in response to ZIKV infection; however, the profile of this response changed over time and was dependent on the dose of ZIKV.

2. Materials and methods

Whole hippocampi and cortex were collected from two-day old rat pups and dissociated into a single cell suspension using the Neural Tissue Dissociation Kit (Miltenyi Biotec). Two independent cell cultures, consisting of four sex-specific samples from at least two litters each, are represented within each experiment presented below. The neural cells were seeded at 50,000 cells per well and maintained in an incubator at 37 °C, 5% CO_2. They were treated with either (1) vehicle (MACS Buffer; 50 µL/50,000 cells), (2) ZIKV at a Multiplicity of Infection (MOI) of 0, 0.1, 1.0, 10 in 50 µL MACS Buffer/50,000 cells for 2 h followed by fresh media for 2 h, or (3) ZIKV at two doses (0.1 MOI or 1.0 MOI) for 4, 8, or 16 h. At the time of collection, the media was removed and 800 µL of Isol-RNA Lysis Reagent (Cat. No. 97064-950, VWR) was added to each well so that the cells could be collected for

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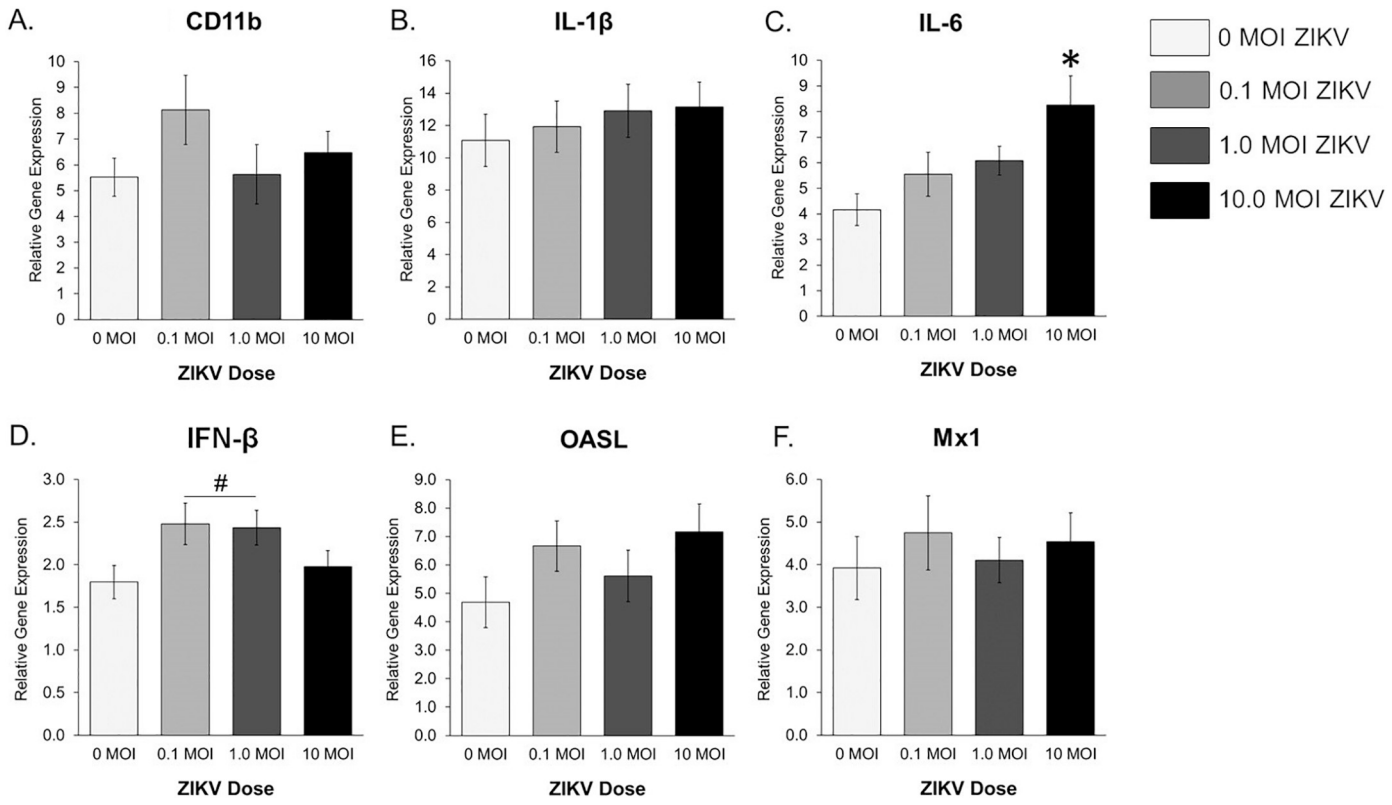

Fig. 1. Dose Response Curve to ZIKV Infection in Neonatal Rat Hippocampal and Cortical Cells. Hippocampal/cortical cells were cultured with increasing doses of ZIKV, including 0 MOI, 0.1 MOI, 1.0 MOI, and 10 MOI for 2 h, followed by fresh, uninfected media for two additional hours before being collected for the analysis of gene expression. (A) There were no significant main effects or interactions in the relative gene expression of CD11b. (B) There were no significant main effects or interactions in the relative gene expression of IL-1β. (C) There was a significant effect of ZIKV infection on IL-6 expression, such that there was significantly more IL-6 expressed in the cultured cells following infection with 10 MOI ZIKV compared uninfected cells or 0.1 MOI ZIKV (*$p < .05$). (D) There was a trending increase in IFN-β expression at the 0.1 and 1 MOI (#$p = .06$), such that at the two lower doses of ZIKV infection, there was significantly more IFN-β expression relative to uninfected cells. (E) There were no significant main effects or interactions in the relative gene expression of OASL. (F) There were no significant main effects or interactions in the relative gene expression of Mx1. Data were analyzed using a one-way ANOVA (1–4 statistical outliers per analysis were removed) and represent the mean ± SEM.

subsequent qPCR analysis. The human Puerto Rico (Dec 2015) Zika Virus (ZIKV, strain PRVABC59, ATCC® VR-1843, GenBank Accession: KU501215), propagated in Vero cells, was used for these studies.

3. Results

See Figs. 1–3 and Table 1 for all results. There was a significant main effect of ZIKV infection on the expression of IL-6 ($F_{3,67} = 4.08$, $p = .010$; Fig. 1C). There was significantly more IL-6 expressed in cells treated with 10 MOI ZIKV than uninfected cells ($p < .001$) or cells treated with 0.1 MOI ZIKV ($p < .002$). A pairwise comparison of IFN-β revealed an increase in relative gene expression of at the 0.1 ($p = .02$; $d = 0.683$) and 1.0 MOI ZIKV ($p = .03$; $d = 0.612$) doses relative to the uninfected cells (Fig. 1D).

Analysis of CD11b revealed a significant interaction of ZIKV infection at 0.1 MOI and time post-infection ($F_{1,77} = 3.82$, $p = .0265$; Fig. 2A). After 4 h, ZIKV significantly increased the expression of CD11b relative to uninfected cells ($p = .049$; $d = 0.776$); but by 16 hours post-infection, ZIKV significantly decreased CD11b expression relative to uninfected cells ($p = .422$; $d = -0.695$). Analysis of IFN-β revealed a significant interaction of ZIKV infection and time ($F_{1,78} = 2.98$, $p = .05$; Fig. 2D). IFN-β expression was significantly increased at 8 hours post-infection ($p = .023$ compared to uninfected cells).

Treatment of neural cells with 1.0 MOI ZIKV produced a significant increase in the interferon-inducible genes OASL ($F_{1,80} = 3.23$, $p = .04$; Fig. 3E) and Mx1 ($F_{1,78} = 5.79$, $p = .004$; Fig. 3F) that varied across time. Both OASL and Mx1 expression were significantly increased at 8 hours post-infection compared to expression in uninfected cells ($p = .05$; $d = 0.237$ and $p = .03$; $d = 0.810$ respectively). However, by 16 hours post-infection, these levels of both these genes had decreased.

We analyzed ZIKV RNA in the neural cells, which revealed a significant main effect of treatment, specifically that ZIKV expression was detectable following either the 0.1 or 1.0 MOI ZIKV ($p < .001$ compared to uninfected cells), regardless of the time post-infection (Figs. 2G and 3G).

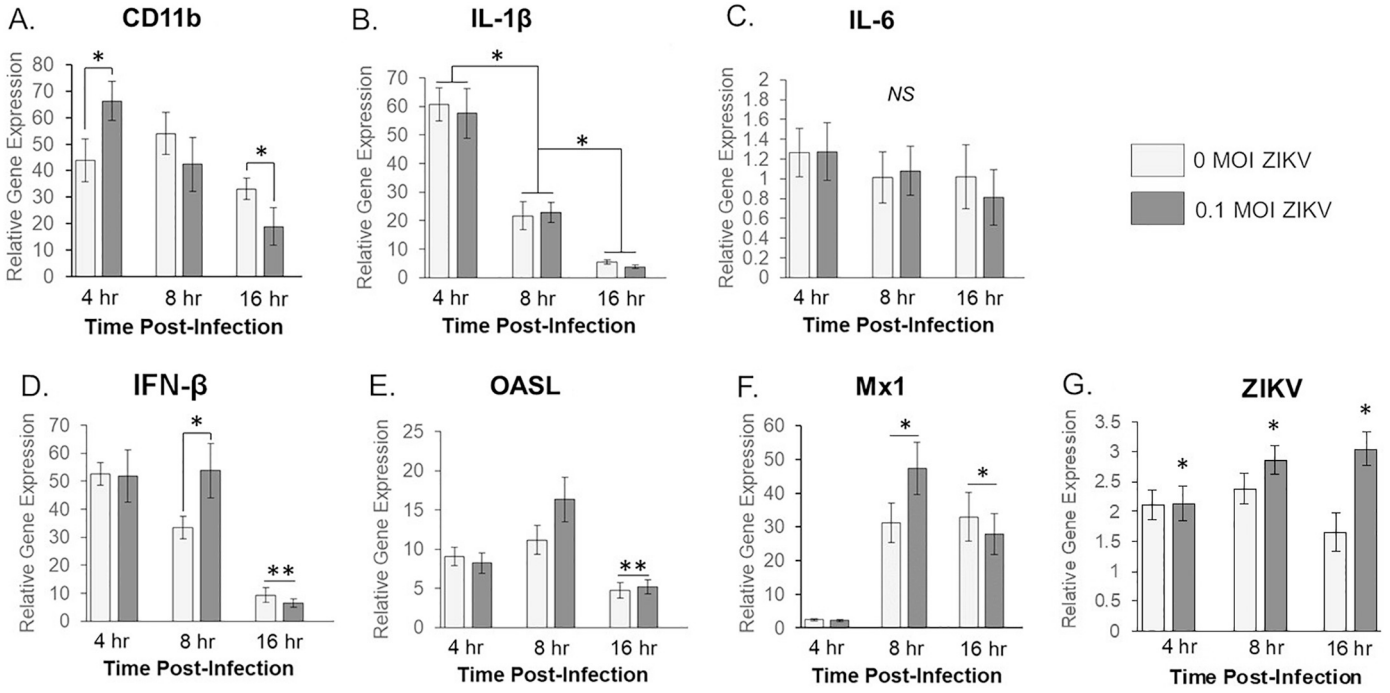

Fig. 2. Effect of 0.1 MOI ZIKV Infection on Developing Neural Cells. Hippocampal/cortical cells were cultured with ZIKV at either 0 MOI or 0.1 MOI for 4, 8, or 16 h before being collected for the analysis of gene expression. (A) There was a significant interaction of ZIKV infection and time on the expression of CD11b, such that ZIKV infection increased CD11b expression at 4 hours post-infection compared to uninfected cells (*$p < .05$), but significantly decreased at 16 hours post-infection compared to uninfected cells (*$p < .05$). (B) There was a significant main effect of time post treatment on the expression of IL- β, such that IL-β decreases significantly at 8 hours post-infection and 16 hours post-infection regardless of treatment (*$p < .001$). (C) There were no significant main effects or interactions in the relative gene expression of IL-6. (D) There was an increase in IFN-β expression at the 0.1 MOI as compared to saline at the 8 h time point only (*$p < .05$), while expression of IFN-β significantly decreased at 16 h relative to the other time points of culture. (E) There was a significant main effect of time on OASL expression. OASL expression was significantly decreased at 16 h into the culture compared to the earlier time points ($p < .05$). (F) Analysis of Mx1 expression revealed a significant main effect of time, such that Mx1 expression is significantly increased at 8 and 16 h into the culture compared to the 4 h time point ($p < .05$). (G) Analysis of ZIKV expression revealed a significant effect of ZIKV infection (*$p < .03$ compared to 0 MOI) with infected cells having a detectable amount of ZIKV regardless of the time point post-infection. Data were analyzed using a two-way ANOVA (1–4 statistical outliers per analysis were removed) and represent the mean ± SEM.

4. Discussion

The experiments presented here are the first of their kind to identify the immune response to various doses of ZIKV infection of developing rat neural cells. This approach allowed us the opportunity to; **(1)** Determine the time course of the acute immune response within the first 16 h after ZIKV infection, **(2)** Determine the sensitivity of the immune response to various titers of ZIKV infection, and **(3)** Identify the variety of immune molecules produced specifically by neural cells to this emergent virus in the absence of other factors that may be derived from the periphery or the maternal immune system during a prenatal infection. We were also able to determine that ZIKV is actively located and replicating within primary rat neural cells.

Our dose-response curve showed that following a high dose of ZIKV infection, there is a rapid, robust induction of IL-6 expression by the neural cells. Using an intermediate dose of 0.1 MOI ZIKV we were able to identify a distinct time-dependent response in the expression of the microglial activation marker CD11b, which was significantly increased at the 4-hour time point, then significantly decreased at the 16-hour time point. This suggests that the microglia had completed their initial phase of activation within 16 hours post-infection. While there was no significant effect of ZIKV on CD11b expression 8 hours post-infection, there was a significant increase in the expression of IFN-β. IFN-β is the first interferon induced upon the detection of a virus, triggering surrounding cells to express anti-viral proteins that "interfere" with the virus and its ability to replicate in the target cell. Notably, the intermediate dose of 1.0 MOI ZIKV did not trigger an increase in CD11b expression, however it did have a significant effect on the common anti-viral, interferon-inducible genes oligoadenylate synthetase-like (OASL), an enhancer of RIG-I activation and OAS/RNAse L activity, and myxovirus resistance protein Mx1 (Mx1), a GTP-binding protein.

4.1. Discussion of cell culture paradigm and possible limitations

This study examined the neonatal *immune* and *anti-viral* response to ZIKV through quantitative PCR. A future goal of these experiments would be to measure inflammatory cytokine secretion (e.g. IL-6 and IFN-β) into the culture media, or the expression of key anti-viral proteins in the cells. Both peripheral macrophages and perivascular immune molecules could pose a threat to the purity of our neural cell

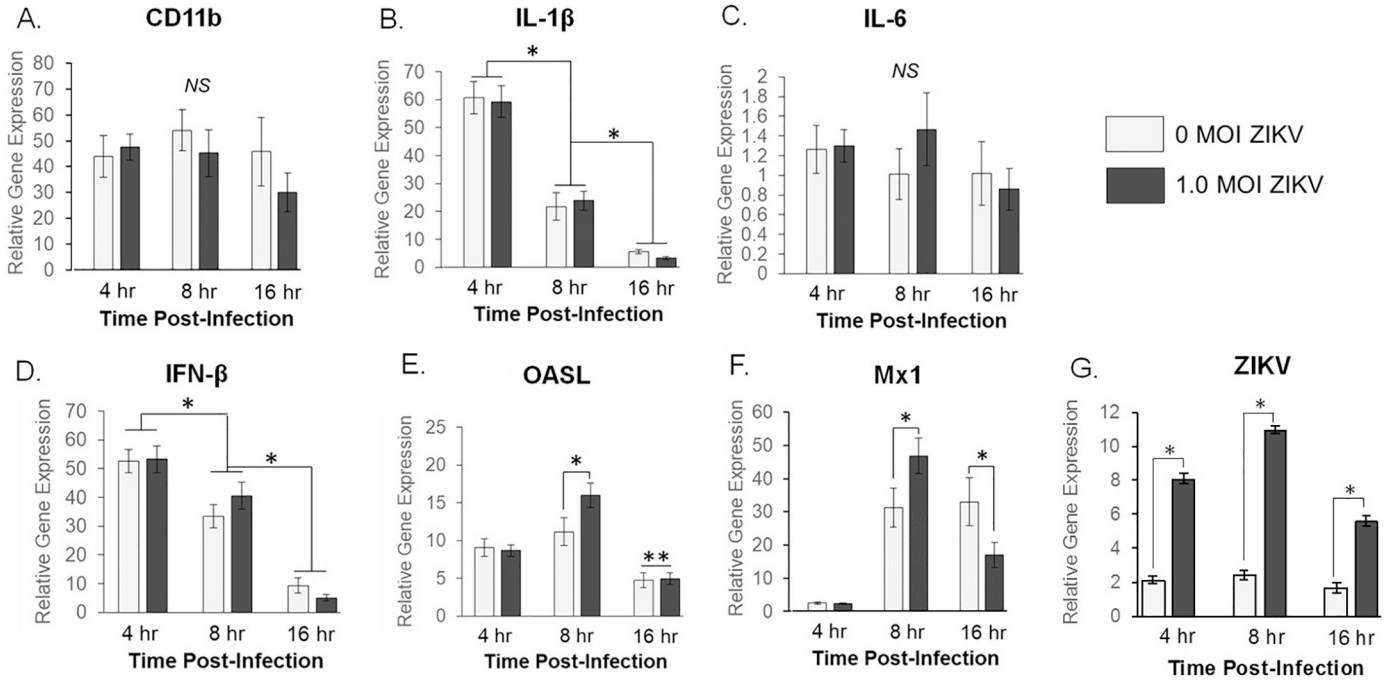

Fig. 3. Effect of 1.0 MOI ZIKV Infection on Developing Neural Cells. Hippocampal/cortical cells were cultured with ZIKV at either 0 MOI or 0.1 MOI for 4, 8, or 16 h before being collected for the analysis of gene expression. (A) Analysis of CD11b revealed a significant effect of time post-infection; however, post hoc analysis revealed no significant pair-wise comparisons across time. (B) IL-1β expression significantly decreased during the time since the cells were initially dissociated and plated. (C) Analysis of IL-6 revealed no significant main effects or interactions of ZIKV infection and time post-infection. (D) Analysis of IFN-β revealed significant effect of time post-infection (*$p < .001$ compared to other time points). (E) Analysis of OASL expression revealed a significant interaction of ZIKV infection at 1.0 MOI and time, such that OASL expression is significantly increased at 8 hours post-infection compared to expression in uninfected cells ($p < .05$). By 16 hours post-infection, these levels had decreased such that there was no longer a significant effect of ZIKV infection on OASL expression. (F) Analysis of Mx1 expression revealed a significant interaction of ZIKV infection at 1.0 MOI and time, such that Mx1 expression is significantly increased at 8 hours post-infection compared to expression in uninfected cells ($p < .05$), but by 16 hours post-infection, these levels decreased such that they were significantly lower than the expression of Mx1 in uninfected cells at this time ($p < .05$). (G) Analysis of ZIKV expression revealed a significant effect of ZIKV infection (*$p < .03$) with infected cells having a significantly greater amount of ZIKV. Data were analyzed using a two-way ANOVA (1–4 statistical outliers per analysis were removed) and represent the mean ± SEM.

cultures; however, these cell types were likely removed during the process of neural dissociation and plating. As a result, we are confident that microglia are the primary producers of the immune molecules measured here, though it is possible that neurons or astrocytes may respond to ZIKV and induce the expression of certain immune molecules (e.g. IL-6), and this possibility cannot be ruled out (Erta et al., 2012).

5. Conclusions

ZIKV can infect developing neural cells where it replicates and causes widespread neurological damage (Li et al., 2016b). As a positive-sense, single-stranded RNA virus, ZIKV can initiate cellular machinery to translate its genetic material directly into proteins (Duarte et al., 2017). As such, viruses like ZIKV hijack the cell's genetic control of immune genes, modulating apoptosis while simultaneously replicating viral proteins within the host cell (Santoro et al., 2003). Our dose response curve revealed that neonatal rat hippocampal and cortical cells upregulated IL-6 at the highest dose of ZIKV (10 MOI), but upregulated IFN-β at the lower ZIKV doses (0.1 and 1 MOI). The upregulation of IL-6 expression suggests that a robust pro-inflammatory response was triggered by the microglial innate immune response being overwhelmed by the high MOI of 10. This is supported by past literature that states there is increased IL-6 expression in the CNS during both acute and immunocompromising viral infections (Erta et al., 2012). Conversely, at the lower 0.1 and 1.0 MOI doses, there was an increase in IFN-β gene expression which indicates a more specific anti-viral response.

Our results suggest that cultured rat neural cells are susceptible to ZIKV infection and furthermore indicate that microglial cells in culture actively mount an immune response early in the ZIKV infection that is completed or even reversed 12 h later. Our findings reveal that the expression of IFN-β-inducible genes OASL and Mx1 is significantly upregulated at the intermediate dose of 1.0 MOI, and occurs in a time-dependent manner. This aligns with our previous CD11b and IFN-β results, suggesting that early on in the infection (the 4-hour time point) microglia are activated, followed by a robust anti-viral response of IFN-β (at the lower dose) and OASL and Mx1 (at the higher dose) all within the 8-hour time point. At 16-hours post-infection, the mRNA expression levels of IFN-β, OASL, and Mx1 decrease, regardless of viral titer. This data supports a complex microglial-anti-viral relationship. Depending on the dose of the ZIKV exposure, microglia may produce either a specific anti-viral response (IFN-β, OASL and Mx1) or a general, robust cytokine response (e.g. IL-6), which may be either protective or cytopathic for the surrounding neural cells. Taken together, these findings

Table 1
Gene expression statistical analysis. All statistics from the initial dose response curve as well as the time response curve post treatment with intermediate titers of ZIKV are shown. (A) There were significant effects of ZIKV in IL-6 and IFN-β. (B) There were significant effects of ZIKV in CD11b; Time in IL-1β, OASL, and Mx1; and ZIKV x Time in IFN-β. (C) There were significant effects of Time in CD11b, IL-1β, IFN-β; and ZIKV x Time in OASL and Mx1.

A) Dose response curve – ZIKV infection (0, 0.1, 1.0 and 10.0 MOI)

One-way ANOVA [factor: ZIKV dose]

Gene	Effect	$F_{,df}$	*p* value	n =
CD11b	–	$F_{3,72} = 1.28$	0.287	17–19/group
IL-1β	–	$F_{3,72} = 0.79$	0.796	17–19/group
IL-6	ZIKV	$F_{3,67} = 4.08$	0.010	16–19/group
IFN-β	ZIKV	$F_{3,69} = 2.50$	0.06	16–18/group
OASL	–	$F_{3,69} = 1.23$	0.31	17–18/group
Mx1	–	$F_{3,67} = 0.27$	0.842	16–18/group

B) ZIKV infection (0.1 MOI) collected 4, 8, or 16 hours post-infection

Two-way ANOVA [factors: ZIKV × time post-infection]

Gene	Effect	$F_{,df}$	p value	n =
CD11b	ZIKV	$F_{1,77} = 3.82$	0.0265	12–14/group
IL-1β	Time	$F_{1,79} = 67.98$	< 0.001	12–14/group
IL-6	–	–	0.887	12–14/group
IFN-β	ZIKV × Time	$F_{1,78} = 2.98$	0.05	12–14/group
OASL	Time	$F_{1,77} = 14.98$	< 0.001	11–15/group
Mx1	Time	$F_{1,76} = 26.11$	< 0.001	11–14/group

C) ZIKV infection (1.0 MOI) collected 4, 8, or 16 hours post-infection

Two-way ANOVA [factors: ZIKV × time post-infection]

Gene	Effect	$F_{,df}$	p value	n =
CD11b	Time	$F_{1,82} = 3.51$	0.034	12–14/group
IL-1β	Time	–	< 0.001	12–14/group
IL-6	–	–	0.625	12–14/group
IFN-β	Time	$F_{1,82} = 75.7$	< 0.001	12–14/group
OASL	ZIKV × Time	$F_{1,80} = 3.23$	0.04	11–15/group
Mx1	ZIKV × Time	$F_{1,78} = 5.79$	0.004	12–14/group

may help to explain the wide variety of developing neural defects caused by prenatal ZIKV infection, which are likely dependent upon the amount of ZIKV titers to which the developing neural cells are exposed and the subsequent immune response to these ZIKV titers.

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Declarations of interest

None.

References

Brault, J.-B., Khou, C., Basset, J., Coquand, L., Fraisier, V., Frenkiel, M.-P., et al., Baffet, A.D., 2016. Comparative Analysis Between Flaviviruses Reveals Specific Neural Stem Cell Tropism for Zika Virus in the Mouse Developing Neocortex. https://doi.org/10.1016/j.ebiom.2016.07.018.

Brown, G.C., Neher, J.J., 2014. Microglial phagocytosis of live neurons. Nat. Publ. Group 15. https://doi.org/10.1038/nrn3710.

Duarte, G., Moron, A., Timerman, A., Fernandes, C., Mariani Neto, C., Almeida Filho, G., ... Francisco, R., 2017. Zika virus infection in pregnant women and microcephaly. Revista Brasileira de Ginecologia E Obstetrícia / RBGO Gynecology and Obstetrics 39 (5), 235–248. https://doi.org/10.1055/s-0037-1603450.

Erta, M., Quintana, A., Hidalgo, J., 2012. Interleukin-6, a major cytokine in the central nervous system. Int. J. Biol. Sci. 8 (9), 1254–1266. https://doi.org/10.7150/ijbs.4679.

Garcez, P.P., Loiola, E.C., Madeiro da Costa, R., Higa, L.M., Trindade, P., Delvecchio, R., ... Rehen, S.K., 2016. Zika virus impairs growth in human neurospheres and brain organoids. Science (New York, N.Y.) 352 (6287), 816–818. https://doi.org/10.1126/science.aaf6116.

Li, C., Xu, D., Ye, Q., Shi, L., Qin, C.-F., Xu Correspondence, Z., ... Xu, Z., 2016a. Zika virus disrupts neural progenitor development and leads to microcephaly in mice. Cell 19, 120–126. https://doi.org/10.1016/j.stem.2016.04.017.

Li, H., Saucedo-Vuevas, L., Shresta, S., Gleeson, J.G., 2016b. The neurobiology of Zika virus. Neuron Rev. 92 (5), 949–958. https://doi.org/10.1-1016/j.neuron.2016.11.031.

Qian, X., Nguyen, H.N., Song, M.M., Hadiono, C., Ogden, S.C., Hammack, C., ... Ming, G.-L., 2016. Brain-region-specific organoids using mini-bioreactors for modeling ZIKV exposure. Cell 165 (5), 1238–1254. https://doi.org/10.1016/j.cell.2016.04.032.

Santoro, M.G., Rossi, A., Amici, C., 2003. NF-kappaB and virus infection: who controls whom. EMBO J. 22 (11), 2552–2560. https://doi.org/10.1093/emboj/cdg267.