

Rubella virus tropism and single cell responses in human primary tissue and microglia-containing organoids

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Abstract

Rubella virus is an important human pathogen that can cause neurologic deficits in a developing fetus when contracted during pregnancy. Despite successful vaccination programs in the Americas and many developed countries, rubella remains endemic in many regions worldwide and outbreaks occur wherever population immunity is insufficient. Intense interest since rubella virus was first isolated in 1962 has advanced our understanding of clinical outcomes after infection disrupts key processes of fetal neurodevelopment. Yet it is still largely unknown which cell types in the developing brain are targeted. We show that in human brain slices, rubella virus predominantly infects microglia. This infection occurs in a heterogeneous population but not in a highly microglia-enriched monoculture in the absence of other cell types. By using an organoid-microglia model, we further demonstrate that rubella virus infection leads to a profound interferon response in non-microglial cells, including neurons and neural progenitor cells, and this response is attenuated by the presence of microglia.

eLife assessment

The manuscript represents an **important** study on the pathogenesis of rubella virus tropism and neuropathology in human microglia-containing human stem cell derived organoids and human fetal brain slices. The strength of evidence is **compelling**, employing two different human-relevant models. The findings will be of broad interest to virologists and infectious disease experts, as well as neurodevelopmental biologists. The findings could also be of interest to pediatrics and obstetrics clinical colleagues.

Introduction

Neurotropic viruses contracted during pregnancy can have grave consequences for the fetus. These comprise both viruses of longstanding concern like Human cytomegalovirus and Herpes simplex virus as well as emerging viruses like Zika virus. Yet our understanding of how direct viral infection and indirect inflammatory consequences affect fetal brain development is limited. This is true even for well-studied pathogens like rubella virus (RV), which is an enveloped, single-stranded RNA virus of the family *Matonaviridae* restricted to human transmission. Infection with RV typically causes a mild, self-limiting illness with a characteristic rash during childhood, often referred to as “German measles.” However, infection during pregnancy can cause miscarriage, stillbirth, or a range of birth defects including congenital rubella syndrome (CRS). The sequelae of congenital RV infection were first recognized in 1941 and although the first RV vaccines were licensed in 1969, an estimated 105,000 infants with CRS were born each year worldwide as of 2010 (Vynnycky et al., 2016). As of 2019, RV-containing vaccine coverage remains incomplete and inconsistent, with ongoing endemic transmission and reporting gaps primarily in the African, Eastern Mediterranean, and South-East Asian World Health Organization Regions (World Health Organization, 2020). Countries with RV-containing vaccine programs also remain susceptible to outbreaks, such as Japan and China where outbreaks in 2013-14 and 2018-19 caused a two-fold increase in reported rubella cases worldwide (26,033 total cases in 2018 vs 49,179 cases in 2019) (Plotkin, 2021) and included CRS the following year (423 total cases worldwide in 2019 vs 1,252 cases in 2020) (World Health Organization, 2022).

The most common features of CRS are congenital cataracts, sensorineural deafness, and cardiac defects (Banatvala & Brown, 2004). In addition, microcephaly (Munro, Sheppard, Smithells, Holzel, & Jones, 1987), developmental delay and autism (Chess, 1977), and schizophrenia spectrum disorders (Brown et al., 2001) are associated with the syndrome, but the pathophysiology of these neurological complications is not well described. To gain mechanistic insight into the pathophysiology of CRS it is essential to understand the tropism of the virus. Initial infection in the lymphoid tissues of the nasopharynx and upper respiratory tract leads to systemic viremia, with virus spread across the placenta and into nearly all fetal organs on post-mortem examination, primarily via infected mononuclear cells (Nguyen, Pham, & Abe, 2015). As for the fetal nervous system, RV was isolated from cerebrospinal fluid and brain tissue of fetuses and infants with CRS in studies from the 1960s (Bellanti et al., 1965; Esterly & Oppenheimer, 1967; Korones, 1965; Monif, Avery, Korones, & Sever, 1965). However, further details of where RV might replicate in the brain are lacking. Autopsies in that early era revealed nonspecific gliosis and cerebral vessel degeneration (Rorke & Spiro, 1967). In limited pathology specimens from more recent outbreaks, RV RNA and antigens were identified in rare cells in the cortex and cerebellum presumed to be “nerve cells” and neural progenitor cells (Lazar et al., 2016; Nguyen et al., 2015). Experimental infections of cells that might not accurately represent the primary cells in the developing brain have yielded little further insight. To complicate the matter, myelin oligodendrocyte glycoprotein (MOG) has been proposed as a cellular receptor for RV (Cong, Jiang, & Tien, 2011), but it is exclusively expressed in oligodendrocytes in the human brain and therefore cannot explain infection in other cell populations. Thus, there is clear evidence for the presence of RV in the central nervous system in infants with CRS, but the identity of infected cell type(s) remains elusive.

Here we address RV tropism in the human developing brain and other poorly understood molecular aspects of CRS. By combining primary human brain tissue with a variety of cell culture techniques, we show that microglia are the predominant cell type infected by RV. Furthermore, we show that diffusible factors from non-microglia cells are necessary to render microglia susceptible to RV. By using brain organoids supplemented with primary

microglia, we demonstrate that RV infection leads to a robust interferon response and leads to dysregulation of multiple genes implicated in human brain development. Finally, we compared transcriptomic changes between microglia-transplanted and non-transplanted organoids and found that in the presence of microglia, interferon pathway upregulation following RV exposure is reduced.

Results

RV infects microglia in the human developing brain

To investigate RV tropism in the human brain, cultured cortical slices from mid-gestation samples were infected with M33 RV, representing a laboratory strain originally derived from a clinical isolate (Figure 1A). At 72 hours post-infection, immunostaining for the RV capsid protein revealed numerous cells positive for the RV antigen, of which >90% were co-labeled with the microglia marker Iba1 (Figure 1B-D). To confirm functional transcription and translation of the viral genome, a new reporter construct of RV designed to express GFP within the non-structural P150 gene was generated (RV-GFP, GenBank Accession OM816675, Figure 1E) and validated by GFP expression in Vero cells (Figure 1 – figure supplement 1). In human primary brain slices infected with RV-GFP, GFP expression was detected predominantly in microglia, confirming the production of RV proteins inside this cell type (Figure 1F) consistent with the wildtype M33 RV.

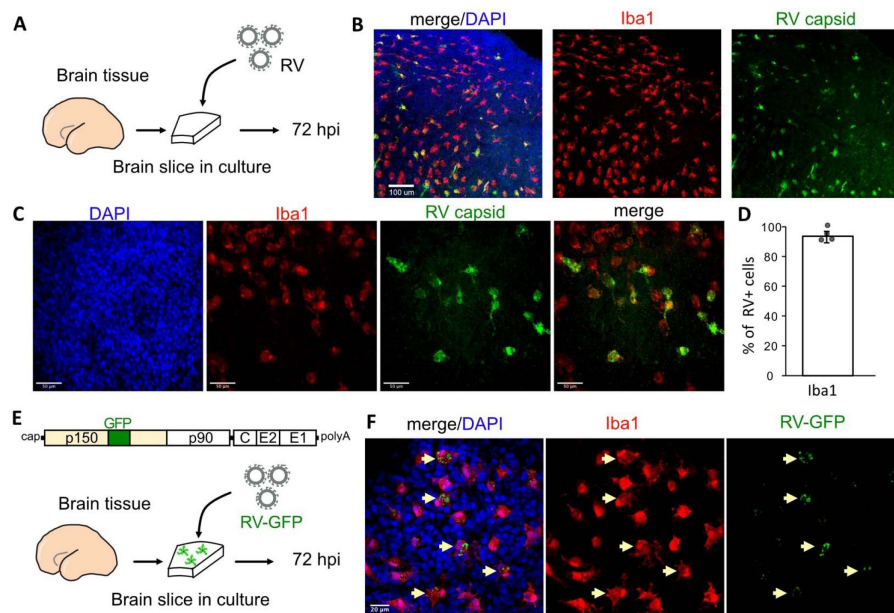


Figure 1.

Rubella virus infects primary human microglia in cultured brain slices.

A. Schematic for brain slice infection. Mid-gestation (GW18-23) human brain slices were infected with RV for 72 hours. **B,C.** Immunostaining for RV capsid and Iba1 on cultured cortical slices at 72 hpi, at 10x with scale bar 100 μm (B) and at 40x objective with scale bar 50 μm (C). **D.** Quantification of RV capsid-positive cells co-labeled with microglial marker Iba1: 764/819 (93.3%) of RV+ cells were microglia based on Iba1 staining across four biological replicates.

Error bars denote standard deviation. **E.** Diagram of viral genome of GFP-expressing RV (RV-GFP). Cortical brain slices were infected with RV-GFP for 72 hours. **F.** Examples of GFP fluorescence and Iba1 immunostaining at 72 hpi of cultured cortical slices with GFP-RV, at 62x with scale bar 20 μm. GFP expression of modified rubella virus is localized to Iba1-positive microglia cells (arrows).

Cell microenvironment influences RV infectivity

Such specificity of RV for microglia in this model is striking given that microglia represent only 1-5% of the cells of the human developing brain (Menassa et al., 2022). Moreover, the previously published viral entry factor MOG is not specific to microglia according to analysis of publicly available RNA and protein expression profiles of the human developing brain (Nowakowski et al., 2017) (<https://cells.ucsc.edu/?ds=cortex-dev&gene=MOG>) or human radial glial cells (Eze, Bhaduri, Haeussler, Nowakowski, & Kriegstein, 2021) (<https://cells.ucsc.edu/?ds=early-brain&gene=MOG>). Further, common components of the host cell membrane, such as sphingomyelin and cholesterol that appear to be essential for RV entry (Otsuki et al., 2018), cannot explain viral tropism for microglia.

Thus, to identify factors contributing to the relatively specific infection of microglia, RV infectivity was tested in monocultures of primary human microglia. Microglia from mid-gestation cortical brain samples were purified using magnetic-activated cell sorting (MACS) and then subsequently infected with RV (Figure 2A). Surprisingly, RV infection of the microglia monoculture was negligible (Figure 2B). To resolve this apparent paradox, we investigated whether microglia infectivity could be restored by the presence of other cell types, such as neurons or progenitor cells. Microglia were co-cultured with either neuronally-enriched cultures (sorted with PSA-NCAM magnetic beads), or the glial component (flow-through that was depleted of both the CD11b-positive microglia cells and the PSA-NCAM-positive population). Both conditions, together with mixed brain cells (flow-through from CD11b-depleted fraction; FT) successfully restored infection (Figure 2C-E). In the pure microglial cultures, less than 2% of microglia were positive for RV capsid by immunostaining, but when different cell fractions were added to the culture (neuronal; glial; or mixed cultures), up to 60% of microglia had RV capsid immunopositivity (Figure 2F). Similar to the cortical brain slices, microglia represented the main cell type infected with RV in the mixed co-cultures (Figure 2G). Furthermore, mixed cultures inoculated with lower viral titers had fewer cells with RV capsid immunopositivity overall, but retained a high proportion of infected microglia demonstrating specificity for microglia (Figure 2 – figure supplement 2A-C). Despite RV capsid co-localizing with microglia cells and GFP protein being produced from RV-GFP in microglia, RV titrating experiments failed to detect significant production of newly released virions in microglia co-cultures (Figure 2 – figure supplement 2D). No statistically significant difference was detected in RV infectivity in cells cultured with or without microglia (Figure 2 – figure supplement 3).

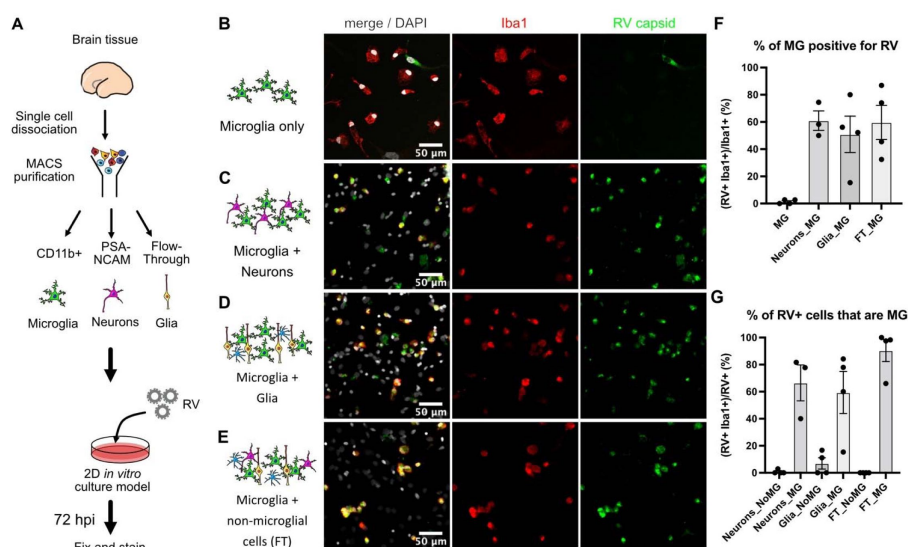


Figure 2.

Rubella virus infection of microglia is dependent on the presence of other cells.

A. Schematic of rubella infection. Primary prenatal brain tissue was dissociated and different cell types were purified using MACS. Microglia cells were cultured alone or in combination with neurons, glial cells, or all cell types. 2D cultures were infected with RV for 72 hours and processed for immunostaining. **B-E.**

Representative images of microglia cultured with different cell types. Cell cultures were stained for microglia marker Iba1 (red), RV capsid (green) and DAPI (grey; on the overlay Merge channel). **B.** Purified microglia only. **C.** Microglia and neurons (purified with PSA-NCAM magnetic beads) co-cultured at 1:5 ratio. **D.** Microglia and glial cell types (flow through fraction after PSA-NCAM magnetic beads) cultured together at 1:5 ratio. **E.** Microglia cultured with non-microglial cells (flow-through after CD11b magnetic beads; mixed cell populations) at 1:5 ratio. **F.** Quantification of RV capsid immunopositivity among microglia (Iba1+) for conditions in B-E. FT: flow through after microglia MACS purification. Error bars denote SEM. Each data point represents a field of view from the same experimental batch. **G.** Quantification of microglia (Iba1+) among RV capsid-positive cells.

We then tested whether RV capsid immunopositivity in microglia could be due to phagocytic activity by this macrophage population. To exclude microglia engulfing other infected cells, a transwell system was employed where microglia and other cell types are grown in compartments separated by a semi-permeable membrane that allows media exchange without direct cell-cell contacts (Figure 3A). Both the presence of other cell types in the same well (co-culture) and the media exchange between the two chambers (transwell) restored infection in microglia (Figure 3B-C). Consistent with our previous experiments, microglia represented the main cell type infected with RV (Figure 3D). Together, these results suggest that RV capsid immunostaining cannot be explained by phagocytosis of other infected cells, but it is possible that infection of microglia is influenced by diffusible factors from other cell populations found in the tissue microenvironment.

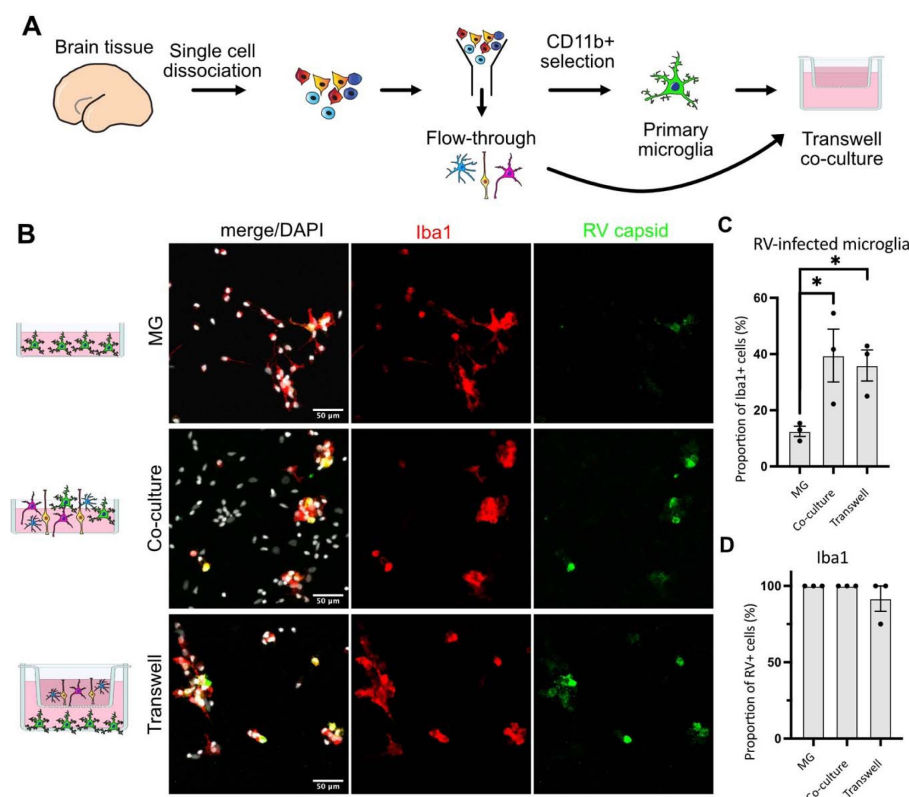


Figure 3.

Direct cell-cell contact is not required for microglia infection by rubella virus.

A. Schematic for experimental set up. Primary human brain tissue was dissociated, and microglia were cultured with or without microglia-depleted flow through portion. Cells were co-cultured in direct contact or in solution-permeable chambered transwells (TW). **B.** Representative images of microglia-enriched cultures (top row), microglia cultured with other cell types in the same well (middle row), and microglia cultured in the bottom compartment with other cell types cultured in a permeable transwell chamber (bottom row) infected with RV for 72 hours. **C.** Quantification of RV capsid immunopositivity among microglia

(Iba1+). Three fields of view across the same experiment were quantified for each condition. Error bars represent SEM. p-value between microglia and co-culture condition is 0.0479. p-value between microglia and trans well condition is 0.0159.

D. Quantification of microglia (Iba1+) among RV capsid-positive cells.

Rubella infection elicits an interferon response in brain organoids

Given the striking difference in infection rates in different cell environments, we next investigated how the presence of microglia modulates response to the viral infection in other cell populations. We used brain organoids as a model of early brain development that, unlike primary brain slices, can be cultured for prolonged period of time to investigate long-term consequences of RV exposure. Under standard protocols, brain organoids do not robustly develop any cells of myeloid origin, making them a useful reductionist model for investigating the role of immune cells in brain homeostasis and development (Nowakowski & Salama, 2022). Brain organoids were generated following previously established protocols (Pasca et al., 2015), and at five weeks of differentiation, when the majority of cell types are present in the organoids, mid-gestation primary human microglia were introduced as previously described (Popova et al., 2021). After allowing microglia to engraft into the organoids, we exposed neuroimmune organoids to RV or heat-inactivated control and cultured them for 72 hours or two weeks to identify short- and long-term consequences of the viral exposure (Figure 4A). In organoids with engrafted primary microglia subsequently exposed to RV, immunostaining revealed RV capsid in microglia, similar to primary tissue and co-culture experiments. We detected RV capsid in microglia, but not in other cell types, in both timepoints, suggesting that microglia remain the main cell population that harbors viral infection (Figure 4 B-C).

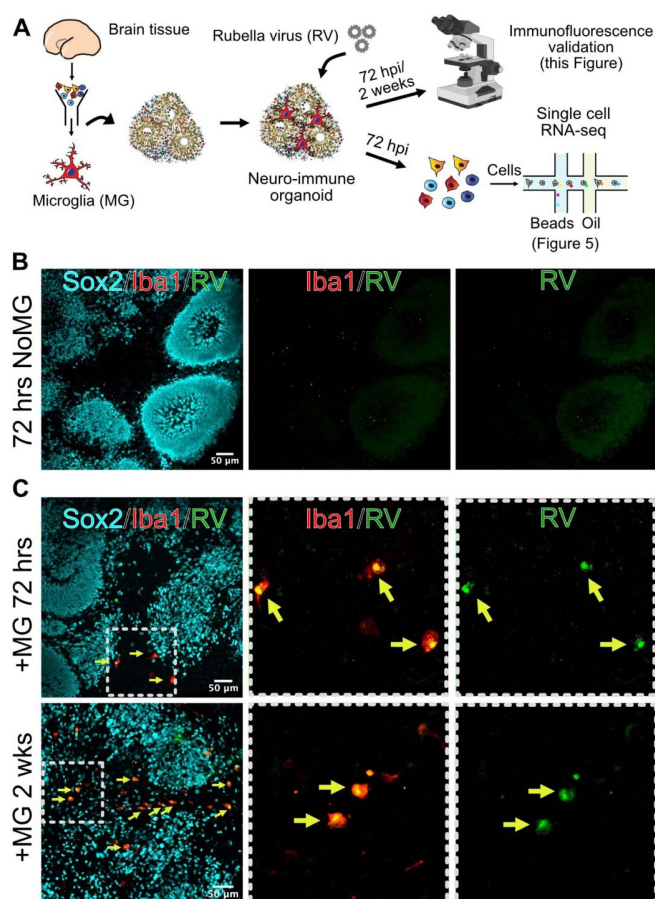


Figure 4.

Microglia in neuroimmune organoids are infected with rubella virus.

A. Primary human microglia were transplanted into brain organoids and resulting neuroimmune organoids were exposed to RV. After 72 hours or 2 weeks organoids were processed for immunofluorescence validation (this figure) or scRNAseq analysis (Figure 5). **B.** Representative immunofluorescence images of brain organoids without microglia subjected to RV exposure for 72 hrs. Radial glial cells are labeled with Sox2 (cyan), microglia are labeled with Iba1 (red) and RV is labeled with anti-RV capsid antibody (green). Scale bar is 50 μm. **C.** Representative immunofluorescence images of brain organoids with microglia at 72 hrs (top panel) or 2 weeks (bottom panel) after RV exposure. Radial glial cells are labeled with Sox2 (cyan), microglia are labeled with Iba1 (red) and RV is labeled with anti-RV capsid antibody (green). Dashed boxes represent zoomed-in examples of microglia cells. Scale bar is 50 μm.

To determine brain-wide consequences of the RV infection across different cell types, at 72 hours after RV exposure we processed neuroimmune organoids for single-cell RNA

sequencing with 10X Genomics and downstream analysis. After processing for single-cell RNA sequencing, cells with fewer than 500 detected genes and/or more than 20% mitochondrial genes were removed from the analysis. Ribosomal transcripts and pseudogenes were excluded. Approximately 11,000 cells passed filtering criteria (Figure 5A, Figure 5 – figure supplement 4A-C), revealing the expected major cell populations of the human developing brain (Nowakowski et al., 2017), including radial glia cells, immature and mature neurons, and astrocytes (Figure 5A-B). Cell cluster annotations were assigned based on combinations of co-expressed cluster marker genes, such as *FGFBP2* and *SOX2* for radial glial cells (clusters 5 and 10), *TAGLN3*, *HES6*, *NEUROD4* for neural progenitor cells (cluster 7), *TUBB2A*, *TUBB2B*, *STMN2* for neurons (cluster 2), *CLU*, *PTN* and *SPARCL1* for astrocytes (cluster 6), and *MKI67*, *UBE2C* and *CENPF* for dividing cells (clusters 3 and 4) (Figure 5B, Figure 5 – figure supplement 4 D-E for individual cluster marker genes, Figure 5 – source data file 1 for the full list of markers). Cells derived from organoids with and without microglia were present in all clusters (Figure 5C). A separate microglia cluster was not identified. Rare cells expressing the microglia marker *AIF1* (encoding the Iba1 protein) were present, but such cells have been previously reported to develop spontaneously in organoids (He et al., 2022) and the canonical microglia marker *P2RY12* was not detected in those cells (Figure 5 – figure supplement 4F). We attribute the apparent lack of microglia to both the small starting population and loss due to cell dissociation during processing for scRNAseq. Consistent with the lack of microglia cells in our scRNAseq data, we did not recover appreciable numbers of the viral transcripts. However, exposure of organoids to RV resulted in significant transcriptomic differences including genes involved in the interferon signaling pathway and its response (*IFI27*, *IFI6*, *IFITM3*)(*HLA-A* (Campbell, Bizilj, Colman, Tuch, & Harrison, 1986; Keskinen, Ronni, Matikainen, Lehtonen, & Julkunen, 1997) and *BST2* (Holmgren, Miller, Cavanaugh, & Rall, 2015)) (cluster 1, Figure 5E, Figure 5 – figure supplement 4 – source data file 1). The majority of cells in cluster 1 came from RV-exposed organoids (Figure 5D). While genes involved in the interferon response showed increased expression in organoids both with and without microglia, the magnitude of their upregulation was lower among cells in microglia-containing organoids (Figure 5E-F). We confirmed higher expression levels of IFITM3 protein in organoids with microglia exposed to RV in comparison to organoids with microglia exposed to heat-inactivated-RV at 72 hours post-exposure (Figure 5 G-H), while the overall cell numbers were not changed in either condition (Figure 5I).

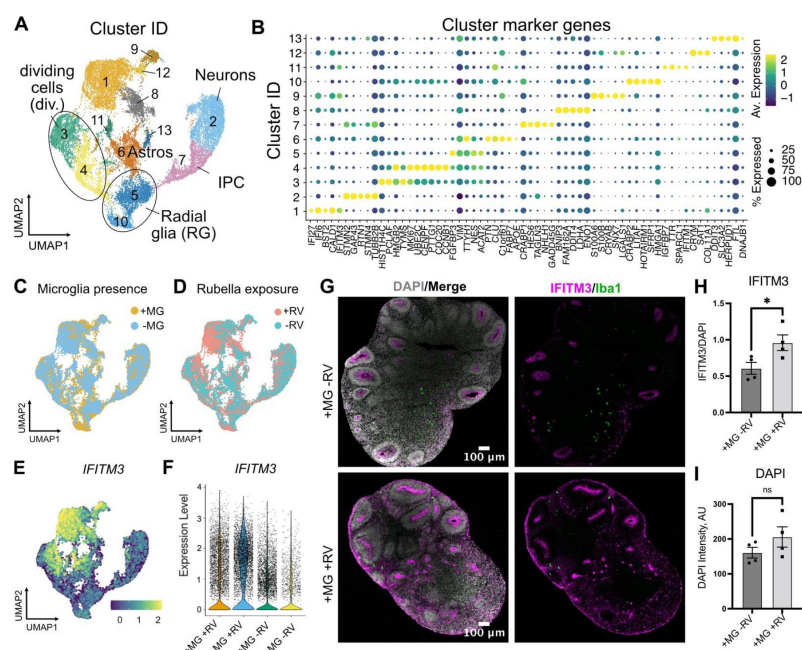


Figure 5.

Rubella virus exposure of brain organoids leads to interferon response.

A. Single cell RNA sequencing analysis identified 13 clusters, including neurons and glial cells (Div.: dividing cells, RG: radial glia, Astros: astrocytes, IPC: intermediate progenitor cells). **B.** Dot plot depicting cluster marker genes for each cluster. **C.** UMAP plots of organoids colored by the presence or absence of microglia. **D.** UMAP plots of organoids colored by the presence or absence of RV treatment. **E.** Feature plot for expression levels of *IFITM3*. **F.** *IFITM3* expression in all cells across different conditions. **G.** Representative images of *IFITM3*

immunofluorescence in brain organoids with microglia with wild type RV (bottom panel) or heat-inactivated control (top panel) 72 hours post-infection. IFITM3 is labeled in magenta, microglia are labeled with Iba1 (green), cell nuclei are labeled with DAPI (grey). **H.** Quantification of fluorescence intensity of IFITM3 normalized to DAPI intensity per organoid. Columns represent mean of four organoids. Dots represent averages across several section for each individual organoid. Error bars represent SEM. Unpaired parametric student t-test was used to compare the two groups in H-I. p-value=0.04 **I.** Quantification of fluorescence intensity of DAPI staining per organoid. p-value=0.22.

To investigate how the presence of microglia and RV exposure modulate gene expression profiles across different cell populations, we stratified gene expression differences based on the major cell types for each of the four conditions (absence and presence of microglia; absence or presence of RV exposure). Radial glia and dividing cells had a greater transcriptomic response to RV exposure as compared to neurons, both with and without microglia (Figure 6A). Cells captured from microglia-containing organoids showed fewer differentially expressed genes in response to RV in each of the major cell classes compared to organoids that did not contain microglia (Figure 6A, top vs bottom panel), with radial glia and neurons reaching statistically significant levels (p-values shown on the right side of the panel) and neural progenitor cells showing the overall trend without reaching statistical significance. One gene family that was specifically downregulated in the presence of RV in organoids without microglia included nuclear factor I – *NFIB* and *NFIA* (Figure 6A, Figure 5 – source data file 2) – two genes that form heterodimers *in vivo* and are associated with induction of gliogenesis (Tchieu et al., 2019) in embryonic brain development. Early disruption in the function of either gene is associated with neurodevelopmental deficits and perinatal mortality in mice (das Neves et al., 1999; Steele-Perkins et al., 2005) and with intellectual disability in humans (Schanze et al., 2018).

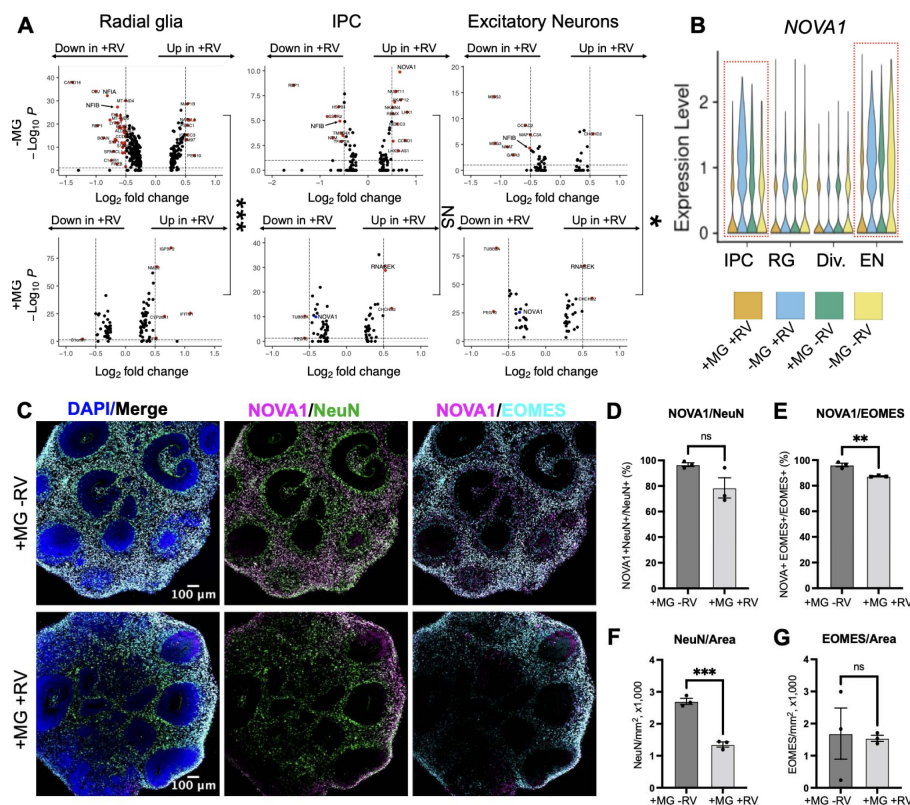


Figure 6.

NOVA1 expression is reduced in response to rubella virus exposure.

A. Differentially expressed genes in different cell types in response to RV treatment without (top panel) and with microglia (bottom panel). IPC – intermediate progenitor cells. In the presence of microglia, fewer differentially expressed genes in response to RV treatment were identified across all major cell types. In organoids with microglia, NOVA1 trended towards a decrease in IPC and neurons (labeled in blue in the panel). Kolmogorov-Smirnov test was used on DEGs with p-value<0.05. *** <0.001, NS – not significant, * <0.05. **B.** Violin plot for NOVA1 that is differentially expressed in response to RV and presence of microglia. IPC – intermediate progenitor cells, RG – radial glia, Div. – dividing cells, EN – excitatory neurons. **C.** Representative images of

RV-exposed organoids with microglia at 2 weeks post-exposure, stained with DAPI for cell nuclei (blue), NOVA1 (magenta), NeuN for neurons (green) and EOMES for intermediate progenitor cells (cyan). **D.** Cell number quantification for NeuN+ neurons that were also positive for NOVA1 in control (heat-inactivated RV) or RV condition. Unpaired parametric student t-test was used to compare the two groups in D-G. p-value=0.088. **E.** Cell number quantification for EOMES+ intermediate progenitors that were also positive for NOVA1 in control (heat-inactivated RV) or RV condition. p-value=0.0042. **F.** Cell number quantification for NeuN+ neurons per organoid area displayed in 1,000cells x mm² or organoid surface area in control (heat-inactivated RV) or RV condition. p-value=0.0004. **G.** Cell number quantification for EOMES+ intermediate progenitor cells per organoid area displayed in 1,000cells x mm² or organoid surface area in control (heat-inactivated RV) or RV condition. p-value=0.86.

Genes with expression levels affected both by the presence of microglia and by RV exposure included NOVA alternative splicing regulator 1 (*NOVA1*) (Figure 6B). *NOVA1* is a master regulator of alternative splicing (Zhang et al., 2010) in the central nervous system with potential links to neurologic diseases (Parikshak et al., 2016). Unlike primary brain slices, brain organoids can be cultured for extended periods of time, providing a human-specific model for studying long-term consequences of RV infection. To better mimic normal human brain development, we used neuroimmune organoids with microglia exposed to RV or heat-inactivated controls to determine how the presence of the viral infection influences *NOVA1* expression and neuronal cell differentiation. After two weeks, we used immunostaining to quantify numbers of neurons or intermediate progenitor cells (IPC) – two major cell types with the most robust predicted *NOVA1* level changes based on the scRNAseq experiment (Figure 6C). We detected a statistically significant decrease of *NOVA1*+ IPCs in response to RV exposure (Figure 6E). Numbers of *NOVA1*+ neurons also had a trend towards reduction (Figure 6D); however, it did not reach statistical significance. Concurrently with reduction of *NOVA1*+ cells, we detected lower numbers of neurons (Figure 6F), but not IPCs (Figure 6G), in organoids after RV exposure.

Discussion

Here we demonstrate that in the developing brain RV predominantly infects microglia, the resident macrophage population. This finding is consistent with RV tropism for monocytes in the periphery (Perelygina et al., 2021; van der Logt, van Loon, & van der Veen, 1980), and adds new information to the limited understanding of RV infection in the central nervous system. Supporting data from real-world infections including post-mortem specimens would be helpful to evaluate clinical strains. Tropism for microglia raises interesting questions about how and where RV persists in CRS, perhaps in brain tissue during the extended period of viral shedding, similar to other relatively immuno-privileged sites such as the eye (Doan et al., 2016; Sugishita et al., 2016). Notably, we did not detect significant production of newly released virions, suggesting potential limitations of the culture system. Our findings also help contextualize CRS in comparison to congenital infections by other neurotropic viruses: Human immunodeficiency virus type 1 and Zika virus, which target microglia directly; Herpes simplex virus, which replicates poorly in microglia with cytopathic effect; and Human cytomegalovirus, which causes microglia to produce antiviral cytokines without productive infection or cytopathic effect (Lum et al., 2017; Retallack et al., 2016; Rock et al., 2004).

Like some of these other viruses, we found that by establishing viral transcription and translation in microglia, RV elicits a strong interferon response in other cell types. It has been previously shown that the interferon response in neurons derived from induced pluripotent stem cells can induce molecular and morphological changes associated with

neurodevelopmental disorders, including neurite length and gene expression changes associated with schizophrenia and autism (Warre-Cornish et al., 2020). The interferon response is additionally associated with pathobiology in a range of congenital infections and interferonopathies (Crow & Manel, 2015). Furthermore, in our preliminary experiments in organoids, where microglia do not develop under standard protocols, the RV-induced interferon response was attenuated in the presence of microglia, suggesting a possible protective role of microglia on other cell types. One limitation of the current work is the lack of information on transcriptional differences in microglia in the context of RV-exposed organoids due to the low number of recovered microglia in scRNAseq experiments. However, our data on molecular changes in neural progenitor cells and neurons, which likely produce the bulk of neurological symptoms seen in CRS, provide a valuable resource for future investigation of congenital viral infections. Our finding that the presence of microglia may reduce RV-associated transcriptional differences across different cell populations may also shed light on neuroimmune consequences of other congenital infections that coincide temporally with phases of microglia population expansion and reduction (Menassa et al., 2022).

Interestingly, RV infection rates were largely influenced by the local cell environment, where proximity to non-microglia cells was necessary for RV infection of microglia. This requirement did not depend on cell-to-cell contact or cell type, thus eliminating phagocytosis of infected non-microglia cells or cell type-specific factors as the explanation for enhanced microglia infectivity. It is possible that the non-microglia supporting cells generate a reservoir of virus, though infection of non-microglia cells was limited and it is unclear how these virions would be different from virions in the viral stocks. More likely, diffusible factors contribute to RV infection of microglia, perhaps in conjunction with other ubiquitous cell surface elements. For instance, such factors could alter the activation state of microglia and thereby alter infectivity. Based on previous reports in 2D cell cultures and pathology examination of infected tissues, RV can establish infection in a variety of cell types, suggesting that the viral entry receptor is ubiquitously expressed, or that viral entry is facilitated by cell membrane components and their modifications. Indeed, membrane phospholipids and glycolipids have been shown to participate in viral entry (Mastromarino, Cioe, Rieti, & Orsi, 1990; Otsuki et al., 2018). While our study did not directly address the molecular mechanisms of entry, our findings motivate new directions to advance our limited understanding of host factors needed for RV entry and infection. Moreover, our study highlights the importance of considering tissue complexity when studying viral infection in brain organoids. Complex, multi-lineage organoids can now be designed by incorporating vascular or immune cells into differentiation protocols (Cakir et al., 2022; Cakir et al., 2019; Popova et al., 2021). We show that transcriptomic consequences of RV exposure are dependent on the presence of microglia in the organoid tissue environment, while future studies will be needed to determine the precise mechanisms that mediate this effect. One possibility is that microglia become the predominant cellular target of RV infection. Another possibility is microglia actively altering the microenvironment to modulate the antiviral response.

Clearly, efforts to eliminate RV worldwide through vaccination are a priority. However, our work on neuroimmune interactions in CRS may inform how early brain development goes awry in many contexts including prenatal infection with other neurotropic viruses, genetic conditions associated with dysregulated interferon responses such as Aicardi Goutières Syndrome, and a variety of perturbations that activate common inflammatory pathways. Understanding the specific role of microglia may be key to unlocking the pathophysiology and developing therapies to prevent or mitigate damage.

Material and data availability

Sequences of RV and RV-GFP have been deposited at Genbank under accessions OM816674 and OM816675 respectively. Single-cell RNA-seq data for iPSC-derived organoids are available from Gene Expression Omnibus (GEO) under the accession code GSE232462. Processed single-cell data, including dimensionality reduction object, is freely available at <https://cells.ucsc.edu/?ds=rubella-organoids>. Code associated with analysis of the single cell analysis can be accessed at Github: <https://github.com/cnk113/analysis-scripts/blob/main/rmd/rub.Rmd>.

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Author contributions

These authors contributed equally: G.P., H.R..

G.P. and H.R. performed the experiments and analyzed the data. C.N.K. analyzed single cell RNA sequencing data. D.S. provided brain organoids and A.W. performed immunofluorescence and image analysis. G.P. and H.R. prepared figures and wrote the manuscript with input from all authors. T.J.N. and J.D. provided oversight of the project. All authors reviewed the manuscript and agreed on its content.

Materials and Methods

Cell lines

Vero cells were obtained from ATCC (CRL-1587) and maintained in DMEM (ThermoFisher, 11965-118) with 10% (vol/vol) fetal bovine serum (ThermoFisher, 10438026), 10mM HEPES (ThermoFisher, 15630-080), and 1X penicillin/streptomycin (ThermoFisher, 10378016). Cell cultures were routinely checked to be free from mycoplasma.

Rubella Virus

To generate viral stocks, a plasmid containing a full infectious clone of RV-M33 was linearized then added to an *in vitro* transcription reaction with Sp6 (New England Biolabs,

M0207L). The resulting RNA was purified then polyadenylated (New England Biolabs, M0276S) and capped using Vaccinia Capping System (New England Biolabs, M2080S). This RNA was then introduced to Vero cells using TransIT-mRNA transfection (Mirus Bio, MIR 2250). At 72 hours post-transfection, culture media was collected and passaged onto fresh Vero cells. To generate viral stocks, Vero cells were inoculated with low passage number RV (P2-P3) and cultured at 37°C. Culture media was collected at 72 hours post-inoculation, clarified, and stored at -80°C. Immunofluorescent titering assays were performed on Vero cells using anti-RV capsid antibody (ab34749), yielding titers of 10⁵-10⁶ focus-forming units/mL (ffu/mL) for RV stocks. RV-GFP stocks were prepared in the same manner, from a plasmid that had been modified through an *in vitro* reaction with nCas9 and custom guides to cut the RV-M33 plasmid midway through the p150 gene at residues 717-718 (dgRNA system with DNA oligos for RNA in vitro transcription as follows: tracrRNA sequence: AAA AAG CAC CGA CTC GGT GCC ACT TTT TCA AGT TGA TAA CGG ACT AGC CTT ATT TTA ACT TGC TAT GCT GTC CTA TAG TGA GTC GTA TTA, crRNA oRV012 sequence: CAA AAC AGC ATA GCT CTA AAA CGC TCG CGG CCA CGT CAC CGC CTA TAG TGA GTC GTA TTA). After cutting the plasmid, an sfGFP sequence flanked by Gly-Gly-Ser-Gly-Gly linkers (PCR-amplified using primers oRV010: CTG GCC CCG GCC AGC TCG GAG GAT CGG GCG GAA TGA GCA AGG GCG AGG AG and oRV011: GTG ACG TGG CCG CGA GTC CTC CTG ATC CGC CAG TGA TCC CGG CGG CG) was inserted using InFusion (TakaraBio, 638916). GFP expression of the resulting virus was validated through co-labeling of RV-GFP infected Vero cells with anti-RV capsid antibody. All viral stocks were tested to be free from mycoplasma.

Consent statement UCSF

De-identified tissue samples were collected with previous patient consent in strict observance of the legal and institutional ethical regulations. Protocols related to human iPSC cells were approved by the Human Gamete, Embryo, and Stem Cell Research Committee (institutional review board) at the University of California, San Francisco.

Primary prenatal brain slices

Deidentified primary tissue samples were collected with previous patient consent in strict observance of the legal and institutional ethical regulations. Cortical brain tissue was immediately placed in a sterile conical tube filled with oxygenated artificial cerebrospinal fluid (aCSF) containing 125 mM NaCl, 2.5 mM KCl, 1mM MgCl₂, 1 mM CaCl₂, and 1.25 mM NaH₂PO₄ bubbled with carbogen (95% O₂/5% CO₂). Blood vessels and meninges were removed from the cortical tissue, and then the tissue block was embedded in 3.5% low-melting-point agarose (Thermo Fisher, BP165-25) and sectioned perpendicular to the ventricle to 300 μm using a Leica VT1200S vibrating blade microtome in a sucrose protective aCSF containing 185 mM sucrose, 2.5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 1.25 mM NaH₂PO₄, 25 mM NaHCO₃, 25 mM d-(+)-glucose. Slices were transferred to slice culture inserts (Millicell, PICM03050) on six-well culture plates (Corning) and cultured in prenatal brain slice culture medium containing 66% (vol/vol) Eagle's basal medium, 25% (vol/vol) HBSS, 2% (vol/vol) B27, 1% N2 supplement, 1% penicillin/streptomycin and GlutaMax (Thermo Fisher). Slices were cultured in a 37 °C incubator at 5% CO₂, 8% O₂ at the air-liquid interface created by the cell culture insert.

Primary human microglia purification

Deidentified primary tissue samples were collected with previous patient consent in strict observance of the legal and institutional ethical regulations. Brain tissue was immediately placed in a sterile conical tube filled with oxygenated artificial spinal fluid (aSCF) containing

125 mM NaCl, 2.5 mM KCl, 1mM MgCl₂, 1 mM CaCl₂, and 1.25 mM NaH₂PO₄ bubbled with carbogen (95% O₂/5% CO₂). Prenatal human microglia were purified from primary brain tissue from mid-gestation (gestational week 18-23) samples using magnetic-activated cell sorting (MACS) kit with CD11b magnetic beads (Miltenyi Biotec, 130-049-601) following manufacturer's instructions. Briefly, primary brain tissue was minced to 1mm³ pieces and enzymatically digested in 10 ml of 0.25% trypsin reconstituted from 2.5% trypsin (Gibco, 15090046) in DPBS (Gibco, 14190250) for 30 mins at 37 °C. 0.5 ml of 10 mg/ml of Dnase (Sigma Aldrich, DN25) was added in the last 5 minutes of dissociation. After the enzymatic digestion, tissue was mechanically triturated using a 10 ml pipette, filtered through a 40 µm cell strainer (Corning 352340), pelleted at 300xg for 5 minutes and washed twice with DBPS. Dissociated cells were resuspended in MACS buffer (DPBS with 1 mM EGTA and 0.5% BSA) with addition of 0.5 mg/ml DNase and incubated with CD11b antibody for 15 minutes on ice. After the incubation, cells were washed with 10 ml of MACS buffer and loaded on LS columns (Miltenyi Biotec, 130-042-401) on the magnetic stand. Cells were washed 3 times with 3 ml of MACS buffer, then the column was removed from the magnetic field and microglia cells were eluted in 5 ml of MACS buffer. Cells were pelleted at 300xg, re-suspended in 1 ml of culture media, counted, and used for downstream analysis. We routinely obtained 1×10⁶ of microglia cells from a single MACS purification.

For experiments requiring microglia co-culture with different cell types, the flow through eluent from microglia selection served either as a cell type fraction depleted of microglia (denoted as “flow through”) or was used for an additional separation between neuronal and glial fractions by using PSA-NCAM antibody (Miltenyi Biotec, 130-092-966) following the same procedure described for microglia purification.

2D microglia cultures

Microglia were cultured on glass-bottom 24 well plates (Cellvis, P24-1.5H-N) pre-coated with 0.1 mg/ml of poly-d-lysine (Sigma Aldrich, P7280) for 1 hr and 1:200 laminin (Thermo Fisher, 23017015) and 1:1,000 fibronectin (Corning, 354008) for 2 hrs. Microglia were plated at 1×10⁵ cells/well and maintained in culture media containing 66% (vol/vol) Eagle's basal medium, 25% (vol/vol) HBSS, 2% (vol/vol) B27 (Thermo Fisher, 17504001), 1% N2 supplement (Thermo Fisher, 17502001), 1% penicillin/streptomycin, and GlutaMax (Thermo Fisher) additionally supplemented with 100 ng/ml IL34 (Peprotech, 200-34), 2 ng/ml TGFβ2 (Peprotech, 100-35B), and 1x CD lipid concentrate (Thermo Fisher, 11905031) for 5-8 days. For co-culture experiments, other cell types were cultured with microglia at 5:1 ratio (1×10⁵ microglia cells for each 5×10⁵ non-microglial cells).

iPSC lines

All work related to human iPS cells has been approved by the UCSF Committee on Human Research and the UCSF GESCR (Gamete, Embryo, and Stem Cell Research) Committee.

Human iPS cell line “WTC-10” derived from healthy 30-year-old Japanese male fibroblasts was from the Conklin Lab, UCSF (Bershteyn et al., 2017; Kreitzer et al., 2013). Human iPS line “13325” was derived from 9-year-old female fibroblasts originally obtained from Coriell cell repository.

Human iPS line “1323-4” derived from healthy 48-year-old Caucasian female fibroblasts (gift from the Conklin Lab, UCSF) was used for immunofluorescence validation analysis as we found that this line generates more reproducible brain organoids.

Organoid generation

Cerebral organoids were generated based on a previously published method (Pasca et al., 2015) with several modifications. Briefly, hiPSCs cultured on Matrigel were dissociated into clumps using 0.5 mM EDTA in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free DPBS and transferred into ultra-low attachment 6-well plates in neural induction media (GMEM containing 20% (v/v) KSR, 1% (v/v) penicillin-streptomycin, 1% (v/v) non-essential amino acids, 1% (v/v) sodium pyruvate, and 0.1mM 2-mercaptoethanol). For the first nine days, neural induction media was supplemented with the SMAD inhibitors SB431542 (5 μM) and dorsomorphin (2 μM), and the Wnt inhibitor IWR1-endo (3 μM). Additionally, the Rho Kinase Inhibitor Y-27632 (20 μM) was added during the first four days of neural induction to promote survival. Neural induction media was replaced every two days for eight days, and Y-27632 was removed from the media on the fourth day. After neural induction, plates containing cortical organoids were transferred to a plate shaker rotating at 80 rpm. Between days 9-25, organoids were transferred to an expansion media (1:1 mixture of Neurobasal and DMEM/F12 containing 2% (v/v) B27 without vitamin A, 1% N2, 1% (v/v) non-essential amino acids, 1% (v/v) Glutamax, 1% (v/v) antibiotic/antimycotic, 0.1mM 2-mercaptoethanol) supplemented with FGF β (10 ng/mL) and EGF (10 ng/mL). Between days 25-35, organoids were maintained in neural differentiation media without FGF or EGF. From Day 35 onward, organoids were maintained in neural differentiation media containing B27 with vitamin A with full media exchanges every 2-3 days.

Microglia-organoid engraftment and co-culture

Microglia from mid-gestation cortical tissue were MACS-purified and immediately added to organoids between week 5 and 6 in 6-well plates at 1×10^5 microglia cells/organoid and kept off the shaker overnight. The following day, the plates were returned to the shaker and maintained following a usual organoid maintenance protocol.

Rubella virus infection

Cells cultured in 2D were inoculated by adding RV stock virus to culture media in 1:1 dilution (250 μl of media to the equal volume of viral stock, 1.75×10^5 total ffu/well) to achieve a multiplicity of infection (MOI) of 2. After four hours, media was exchanged with fresh cell culture media. Cortical brain slices were treated with 500 μl of RV viral stock (3.5×10^5 total ffu/slice) applied over the slice culture filter for four hours, and then the viral culture media was removed and replaced with fresh slice culture media. Organoids were treated in 6-well plates with 2ml of 1:1 dilution of viral stock:organoid maintenance media (7×10^5 total ffu) for four hours, and then viral media was exchanged for fresh media. For all experimental conditions, cells were fixed and processed for downstream analysis at 72 hours post infection. Supernatant from non-infected Vero cells (mock) or heat-inactivated RV (65 $^{\circ}\text{C}$, 30 mins) was used as control.

For titering experiments, microglia co-cultures or Vero cells (as controls) were infected at the indicated MOI. Cells were inoculated for 4 hours, then fresh media was replaced, and sampled at the indicated timepoints. Media samples were clarified and flash frozen. Viral titer was then quantified in the media samples using immunofluorescence titering assay.

Immunofluorescence

Cells cultured on glass-bottom well plates were fixed in 4% PFA at the room temperature for 10 minutes and washed with PBS three times for five minutes each wash. Blocking and

permeabilization were performed in a blocking solution consisting of 10% normal donkey serum, 1% Triton X-100, and 0.2% gelatin for 1 hour. Primary and secondary antibodies were diluted and incubated in the blocking solution. Cell cultures were incubated with primary antibodies at the room temperature for 1 hour, washed 3x with washing buffer (0.1% Triton X-100 in PBS), and incubated with secondary antibodies for 1 hour at the room temperature.

Organoid samples were fixed in 4% PFA at the room temperature for 1 hour. Whole organoids were incubated in 30% sucrose (w/v) at 4°C overnight, cryopreserved in OCT/30% sucrose (1:1) and then cryosectioned at 20 µm thickness. Blocking and permeabilization were performed in a blocking solution consisting of 10% normal donkey serum, 1% Triton X-100, and 0.2% gelatin for 1 hour. Primary and secondary antibodies were diluted and incubated in the blocking solution. Cryosections were incubated with primary antibodies at 4°C overnight, washed 3x for 10 minutes each with washing buffer (0.1% Triton X-100 in PBS). Slides were incubated with species-specific AlexaFluor secondary antibodies (1:2,000) overnight at 4°C and then washed with washing buffer for at least 3x for 10 minutes each. Finally, slices were mounted with glass coverslips using DAPI Fluoromount-G (Southern Biotech, 0100-20) mounting media.

Cortical slices were fixed in 4% PFA at room temperature for 1 hour. Antibody staining was performed as for organoid samples above, with the exceptions that no cryosectioning was performed.

Images were collected using Leica SP8 confocal system with 20x air lens (0.75 NA) and 63x oil lens (1.40 NA). Images were processed using ImageJ/Fiji and Affinity Designer software.

Antibodies

Primary antibodies used in this study included: rabbit Iba1 (1:500, Wako, 019-19741), guinea pig Iba1 (1:500, Synaptic Systems, 234 004), mouse rubella virus capsid (1:500, Abcam, ab34749), rat Sox2 (1:500, Invitrogen, 14-9811-82), chicken GFP (1:1,000, Aves labs, GFP-1020), mouse NOVA1 (1:500, Santa Cruz, sc100334), rabbit EOMES (1:200, Sigma Aldrich, HPA028896), chicken NeuN (1:1,000, Millipore, ABN91), rabbit IFITM3 (1:500, Proteintech, 11714-1-AP).

Organoid single-cell capture for single-cell RNA sequencing

Two organoids per experimental condition were washed with Ca²⁺/Mg²⁺-free DPBS and cut into 1 mm² pieces and enzymatically digested with papain digestion kit (Worthington, LK003163) with the addition of DNase for 1 hr at 37°C. Following enzymatic digestion, organoids were mechanically triturated using a P1000 pipette, filtered through a 40 µm cell strainer test tube (Corning 352235), pelleted at 300xg for 5 minutes, washed twice with DPBS and re-suspended in 180 µl of DPBS on ice for barcoding with MULTI-seq indices (McGinnis et al., 2019) for multiplexing. Anchor and barcoded strands unique for each sample were mixed in 1:1 molar ratio in DPBS (without BSA or FBS to avoid sequestering labeling oligonucleotides) and 20 µl of 10x Anchor:Barcode mixture was added to 180 µl of cell suspension. Cells were incubated on ice for 5 minutes, and then 20 µl of co-anchor was added to each tube. Cells were incubated on ice for additional 5 minutes and washed with ice-cold 1% BSA in DPBS. Cells were counted and kept on ice to prevent barcode loss. Two organoid lines with and without microglia that were treated with RV or uninfected Vero cell supernatant were combined and captured across seven lanes of 10x Genomics using Chromium single cell 3' reagent kit (v2 Chemistry) following the manufacturer's protocol.

Single cell RNA-seq libraries were generated using the 10x Genomics Chromium 3' Gene Expression Kit. Briefly, barcoded single cell mixtures from different conditions ranging from 2-3 individual conditions per lane were loaded onto chromium chips with a capture target of 10,000 cells per sample. The 10x protocol was modified for collection of MULTI-seq barcodes. During SPRI clean up immediately following cDNA amplification, supernatant was saved to recover the barcode fraction. Endogenous transcript cDNA remained bound to the SPRI beads and the protocol was continued for endogenous transcripts without change. Libraries were prepared following the manufacturer's protocol and sequenced on an Illumina NovaSeq with a targeted sequencing depth of 50,000 reads per cell. BCL files from sequencing were then used as inputs to the 10X Genomics Cell Ranger pipeline.

MULTI-seq barcode amplification

Supernatant collected after cDNA amplification clean up step was transferred to fresh 1.5 mL Eppendorf tubes, and 260 μ L SPRI (for a final ratio of 3.2X) and 180 μ L 100% isopropanol (for a final ratio of 1.8X) were added. After pipette mixing 10 times, the solution was incubated at room temperature for 5 minutes, placed on magnetic rack for solution to clear. The supernatant was removed, and the beads were washed with 500 μ L of 80% ethanol twice. Air-dry beads were removed from magnet, resuspended in 50 μ L buffer EB. After clearing the solution on the magnet, supernatant was transferred to a new PCR strip. Libraries were prepared with KAPA HiFi master mix with universal IS primers and RPI primers unique for each 10x lane. PCR was performed with the following protocol: 95 °C for 5 min, (98 °C for 15 sec, 60 °C for 30 sec, 72 °C for 30 sec) repeated for 10 times, 72 °C for 1 min, 4 °C hold.

PCR product was cleaned with 1.6X SPRI beads and resuspended in 25 μ L buffer EB. Barcode libraries were quantified at 1:5 concentration using Bioanalyzer High Sensitivity DNA analysis. Barcodes were sequenced as fraction of endogenous cDNA library with a target of 3000-5000 barcode reads per cell.

Single cell RNA-seq analysis

CellRanger 3.0 was used to create a cell by gene matrix which was then processed using Solo (Fleming, Marioni, & Babadi, 2019) for doublet detection and removal. A minimum of 1000 genes, 500 UMI counts, and 20% mitochondrial cutoff were used to remove low quality cells from all datasets. MAST (Finak et al., 2015) was used on log normalized raw counts for all differential expression tests. The gene marker lists were filtered after testing by specifically removing unannotated genes from HGNC. Organoid demultiplexing and doublet filtering was done through deMULTiplex (McGinnis et al., 2019) (<https://github.com/chris-mcginnis-ucsf/MULTI-seq>). Uniform manifold approximation and projection (UMAP) (Leland McInnes, John Healy, Nathaniel Saul, & Großberger, 2018) embeddings and neighbors for Leiden clustering (Traag, Waltman, & van Eck, 2019) were used for clustering and visualization. Nebulosa was used to generate density plots and (Bunis, Andrews, Fragiadakis, Burt, & Sirota, 2020) for color-blind friendly plotting of clusters. Pearson correlation was calculated on the intersection of the shared genes between datasets which averaged Pearson residuals for each cluster. Organoid cells were batch corrected using default parameters of the SCTransform (Hafemeister & Satija, 2019) integration workflow.

Image analysis and statistical tests

2D microglia co-cultures

Cell co-localization with the RV capsid was quantified using the CellProfiler 3.0 software (McQuin et al., 2018). First, individual cells were identified by using IdentifyPrimaryObjects module with threshold strategy “Global”, threshold method “Otsu” and a two-class thresholding for each individual channel for DAPI, Iba1 and RV capsid fluorescence images. Then resulting cell objects were paired by using RelateObjects module to identify Iba1-positive, RV-positive and double-positive DAPI objects. Finally, CalculateMath was used to quantify proportions for each cell population, including RV-positive and RV-negative Iba1 microglia cells and non-microglia cells, depending on the analysis.

Organoid immunofluorescence quantifications

For cytoplasmic IFITM3 staining, whole organoid sections were treated as regions of interest and average fluorescence intensity for IFITM3 was normalized to DAPI fluorescence using QuPath 0.3.2. To conduct IFITM3 intensity quantifications, the entire organoid section was defined as region of interest by using the wand tool. Intensity calculation features with the analyze function for the DAPI and IFITM3 channels were then used to determine ROI fluorescence intensities. To set up the calculation, preferred pixel size was set to 0.76 μm according to image resolution. Relevant measurements including intensity mean, standard deviation, min & max, and organoid area were calculated and retrieved from QuPath detection measurements.

For analyzing nuclear signal for NOVA1/EOMES/NeuN experiments, QuPath 0.3.2 was used to identify and quantify individual cell nuclei. First, each organoid was selected as region of interest by using the wand tool. To establish total EOMES count in each organoid, the cell detection function was applied to the appropriate channel where adjustments were made, including thresholding and deselecting. The positive cell detection function with the EOMES channel thresholding value were then applied on NOVA1 channel to identify cells that are NOVA1+/EOMES+ double positive. Thresholding adjustments were made to account for imaging variations in EOMES channel. To quantify NOVA1+/NeuN+ nuclei, the same 2-step detection procedure described above was utilized with NOVA1 and NeuN channels. All relevant quantification values were then collected from QuPath detection measurements. Three to five organoid sections per each organoid were imaged and analyzed, with each data point representing an average of several sections per individual organoid.

Prism 9.3.1 was used for statistical analysis and data plotting. Unpaired t-test with assumed Gaussian distribution of the variants and the same standard deviations were used to calculate statistical significance for cell counts. Unpaired nonparametric Kolmogorov-Smirnov test was used to compare differentially expressed genes that reached significance value of $p=0.05$ between conditions in organoids.

Parts of figure schematics were done using Biorender.com.

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Reviewer #1 (Public Review):

The authors sought to address the longstanding question of which cell types are infected during congenital or perinatal rubella virus infection. They used brain slice and organoid-microglia experimental models to demonstrate that the main cell types targeted by rubella virus are microglia. The authors further show that infection results in augmented interferon responses in neighboring neuronal cells but not in the microglia themselves. The data convincingly support the conclusions, with major strengths being the sophisticated primary cell models and single-cell RNA-Seq used to pinpoint microglia as the main cellular targets of rubella virus, and neurons as the bystander targets of immune signaling. This study reveals a new cellular target that will have important implications for basic studies on rubella virus-host interactions and for the potential development of therapies or improved vaccines targeting this virus. As rubella virus is a pathogen of high concern during human pregnancy, this study is also relevant in the field of neonatal infectious diseases.

Reviewer #2 (Public Review):

Maternal infection by Rubella virus (RV) early during pregnancy is a serious threat to the health of the fetus. It can cause brain malformation and later expose the newborn to a constellation of symptoms collectively named Congenital Rubella Syndrome (CRS). In this manuscript, the authors provide novel exciting findings on the pathophysiological mechanisms of RV infection during human brain development. By combining analyses of human fetal brain material and cerebral organoids, Popova and colleagues uncovered a selective tropism of RV for microglial cells. Their results suggest that the infection of microglia by RV relies on the presence of diffusible factors secreted by neighboring brain cells. Moreover, the authors showed that RV infection of human cerebral organoids leads to a strong interferon response and dysregulation of neurodevelopmental genes, which both may contribute to brain malformation. Altogether, these data shed some new light on the cellular tropism and the pathophysiological mechanisms triggered by RV infection in the developing brain. This study also raises the importance of using human cell-based models to further understand the pathophysiological mechanisms of CRS. Identifying the cellular and molecular targets of Rubella virus will also pave the way to develop therapies against CRS.

Author Response

The following is the authors' response to the original reviews.

We thank the reviewers for their time in evaluating the strengths and weaknesses of our manuscript.

We are pleased to see that all reviewers recognized the high significance of our work, noting that the manuscript addresses “longstanding question of which cell types are infected during congenital or perinatal rubella virus infection”. As noted by reviewer 1, “This study reveals a new cellular target that will have important implications for basic studies on rubella virus-host interactions and for the potential development of therapies or improved vaccines targeting this virus. As the rubella virus is a pathogen of high concern during human pregnancy, this study also has important implications in the field of neonatal infectious diseases”.

Below, we provide responses (in blue) to specific critiques:

Reviewer #1 (Public Review):

A weakness is that the current data do not provide information on the full replicative potential of the rubella virus in microglia, or whether the virus persists in this system.

See our response below. Briefly, we include new experimental evidence from primary tissue, microglia-transplanted organoids, and Vero cells to further characterize the dynamics of viral infection.

Reviewer #1 (Recommendations for the authors):

Most of the viral assays in the brain slices and organoids examine viral protein synthesis, which is a surrogate for genome replication. However, basic virological characterization is lacking and would improve the robustness of the model and its potential utility to understand better rubella virus-microglia interactions. Questions the authors should consider with new experiments include:

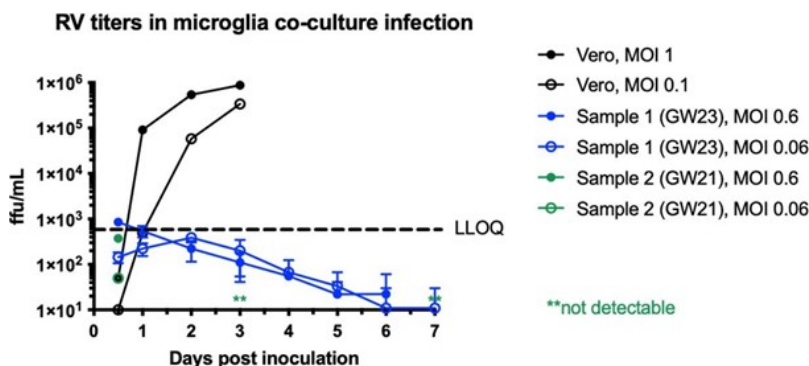
Are new virions produced? Can viruses be detected in the media?

Or, are the infections abortive, with viral protein synthesis occurring, but no virus production?

We performed RV titering experiments in dissociated microglia co-cultured with other cell types, as well as Vero cells as a control. While we can detect a robust increase in viral titer from Vero cells, it fell below detection levels in microglia co-cultures. See Author response image 1. We now include these data in Supplementary Figure 2D.

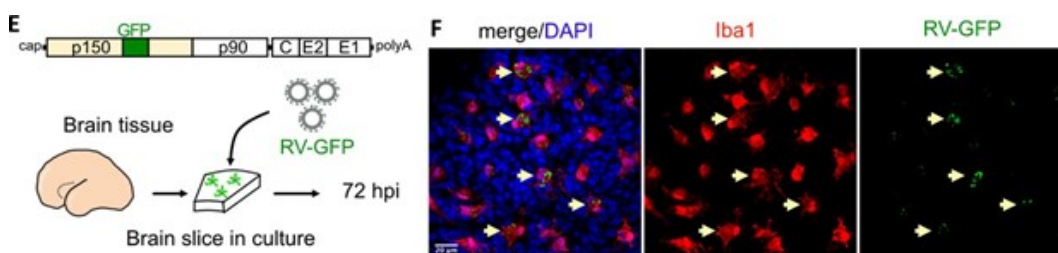
Author response image 1.

Rubella virus titering experiment performed in Vero cells (positive control) or dissociated microglia co-cultures. In primary microglia co-cultures, viral titer falls below detection levels after several days of infection.



While we could not detect an increase in the viral particles from microglia mixed cultures, we confirmed the presence of GFP from the RV-GFP reporter construct, and we believe it serves as a proof that the virus can infect microglia cells and lead to production of functional viral protein (Author response image 2, Figure 1E-F of the current manuscript):

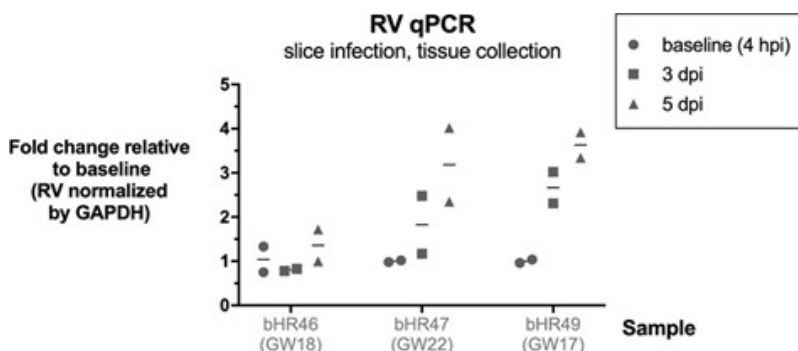
Author response image 2.



We also observed an increase in RV RNA over time in tissue slice infections, using qPCR (Author response image 3, not included in the manuscript).

Author response image 3.

Modest increase in RV RNA over time in brain slice infections. Rubella virus RNA measured by qPCR relative to GAPDH gene, in n=3 samples (2 technical replicates each condition). Brain slices were exposed to RV, then collected at end of inoculation (4 hours post infection), or at 3 or 5 days post infection, and processed for RNA extraction and RT-qPCR.

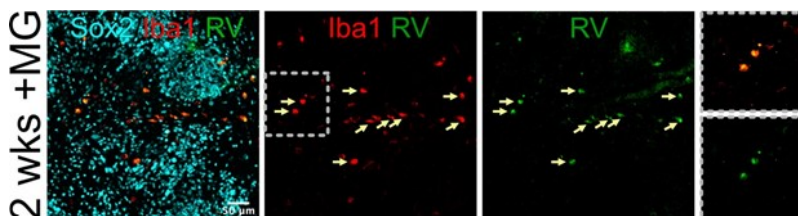


How long do the infections persist in the model? What is the fate of infected microglia over time? Time courses to monitor infection and cell health would be useful.

We performed a longer infection with RV in organoids transplanted with microglia, and after two weeks of infection, we can detect multiple microglia cells positive for the RV capsid. These data are now included in Figure 4 of the current manuscript.

Author response image 4.

After 2 weeks post infection, microglia remain positive for RV capsid.



Reviewer #2 (Public Review):

Weaknesses

The set of data is rather descriptive. It suggests that microglia are the predominant brain target of RV in vivo, without identifying the targeting mechanism that provides cell type specificity. Moreover, what are the diffusible cues released from the brain environment that increase microglia infection and RV replication?

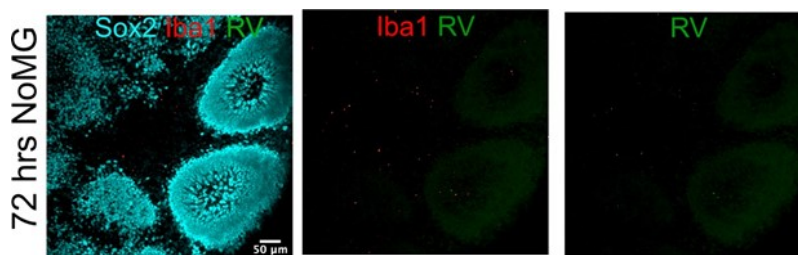
We agree with the reviewer that identifying molecular mechanisms that underlie this phenotype will be very interesting to explore in future research, and we acknowledge the limitation of the study in the Discussion.

It is unclear why brain organoids not supplemented by microglia are susceptible to RV inoculation.

We could not detect RV capsid in organoids without microglia after 72 hours of inoculation. We attribute any changes seen at the level of single cell transcriptomics in the absence of microglia transplantation to exposure to virus-associated particles, including but not limited to viral RNA species, viral proteins, or even other components of the viral stocks made in Vero cells. These factors may induce transcriptomic differences even in the absence of RV infection. In the text, we take care to refer to these condition as “Rubella virus-exposed” rather than “Rubella virus- infected”. We now include the following panel from Author response image 5 in Figure 4B of the current manuscript.

Author response image 5.

Organoids without microglia do not show positive RV immunofluorescence.



Reviewer #2 (Recommendations for the authors):

Several points could be further addressed to improve the data set and shed more light on some aspects of this manuscript:

- *Figure 1. Additional microglia markers should be used to reinforce the evidence that microglia cells are the principal RV targets. Since Iba1 is a marker of activated microglia, does RV have a selective tropism to all microglia or only to activated ones in human fetal brain slices?*

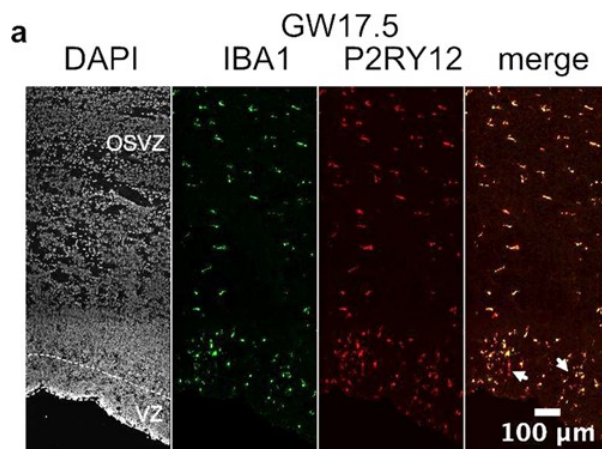
The reviewer brings up an interesting point that, in our mind, can be separated into two independent questions:

1. Are Iba1-positive cells bona fide microglia, or are there other cell populations of macrophage/monocyte origin that are labeled with Iba1? Therefore, additional markers should be used for immunolabeling;
2. Is RV infection selective for microglia “activation” status, when only 5 immune-primed cells can be infected?

For the first point, we have previously shown that in the developing human brain, virtually all Iba1-positive cells are also P2RY12-positive (unpublished; Author response image 6). Therefore, in primary human brain slices, there is a negligible amount of non-microglia macrophages. However, in culture microglia quickly lose their “homeostatic” identity, including P2RY12 expression, as quickly as six hours after ex vivo extraction (Gosselin et al., 2017; DOI: 10.1126/science.aal3222).

Author response image 6.

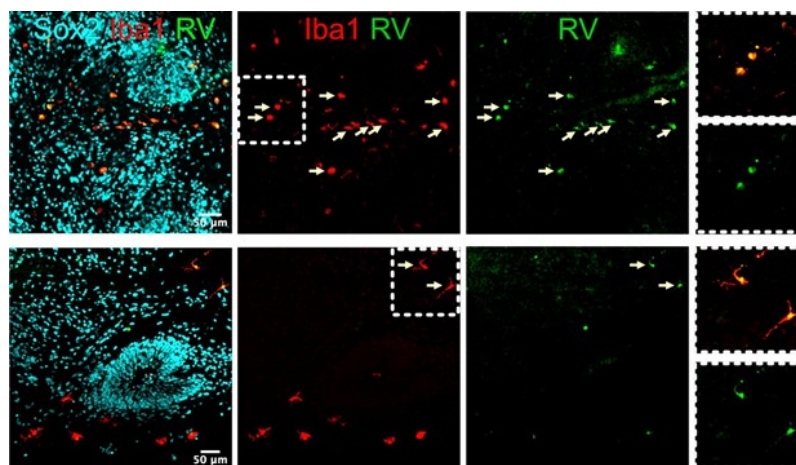
P2RY12 co-localizes with Iba1 in primary brain tissue from gestational week 17.5, including cells with more amoeboid morphology (arrows)



However, in organoids at 2 weeks post-RV exposure, we found microglia with both ameboid and more ramified morphology (Author response image 7). It would be challenging and beyond the scope of this manuscript to use morphology or Iba1 intensity levels to determine cause and effect as microglia activation state relates to RV infectivity (i.e. do activated microglia preferentially get infected with the virus, or do infected microglia become activated and upregulate Iba1 levels and change morphology).

Author response image 7.

Examples of microglia with round (top) and ramified (bottom) morphology that co-localize with RV capsid staining.

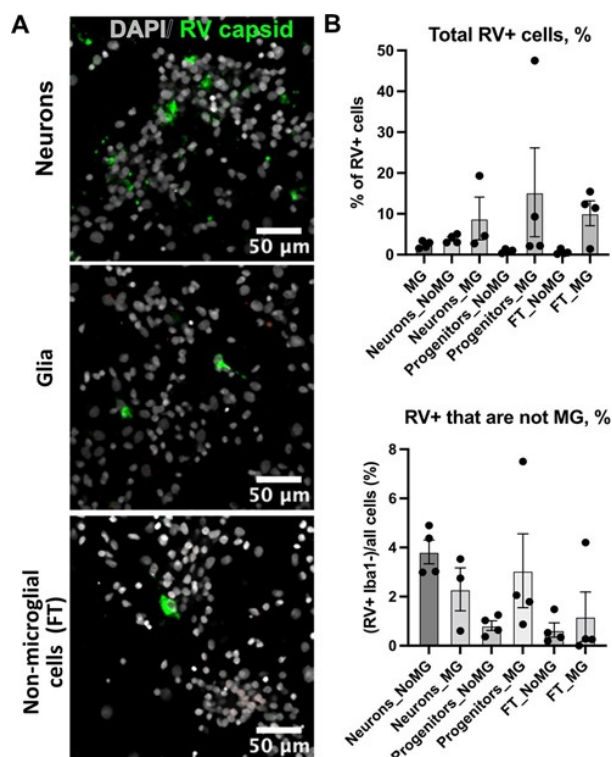


Regarding RV tropism in the 2D culture of microglia, some Iba⁺ cells are infected by RV as they show capsid staining. What are these cells? Are neurons and/or glia also susceptible to RV in vitro infection? Are non-microglial cells getting RV infected in the absence of microglia?

In the absence of microglia cells, a small proportion of non-microglia cells get infected with RV. There is no statistically significant difference in the number of cells that get infected with RV in the presence or absence of microglia across different cell types. We add these data as Supplement Figure 3.

Author response image 8.

Rubella infection in non-microglia cells. A. Representative images of different cell types depleted of microglia. Cell cultures were stained RV capsid (green) and DAPI. B. Quantification of total cells that are positive for RV capsid across conditions. C. Quantification of RV+ cells that are not microglia across different cell populations. No statistically significant difference was detected in RV infectivity in cells c-cultured with or without microglia.



• Figure 3. The low rate of Rubella virus infection in homogenous CD11b⁺ cell culture raises the question of whether the Rubella virus can infect microglia at a specific activation stage. It is also surprising that there is no infection of such cell population (also CD11b⁺) alone while cultured in 2D, as reported in figure 2. Why such a difference?

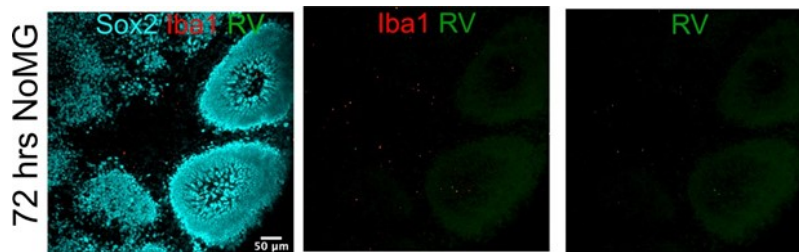
It is well established that culture of microglial cells isolated from brain tissue alters their molecular properties, which likely alters the cell surface protein composition. In the revised discussion, we include activation as a possible mechanism that will require further investigation.

• Fig 4A-B, it is unclear whether organoids that are not engrafted with microglia get infected upon RV (with active viral replication) inoculation. If non-microglia-supplemented organoids are indeed infected and allow RV replication, this suggests that organoids might not be the ideal system to model human fetal brain RV infection at GW18-23.

We could not detect RV capsid in organoids without microglia after 72 hours of inoculation. We include the following panel from Author response image 9 in Figure 4 now.

Author response image 9.

Organoids without microglia do not show positive RV immunofluorescence.



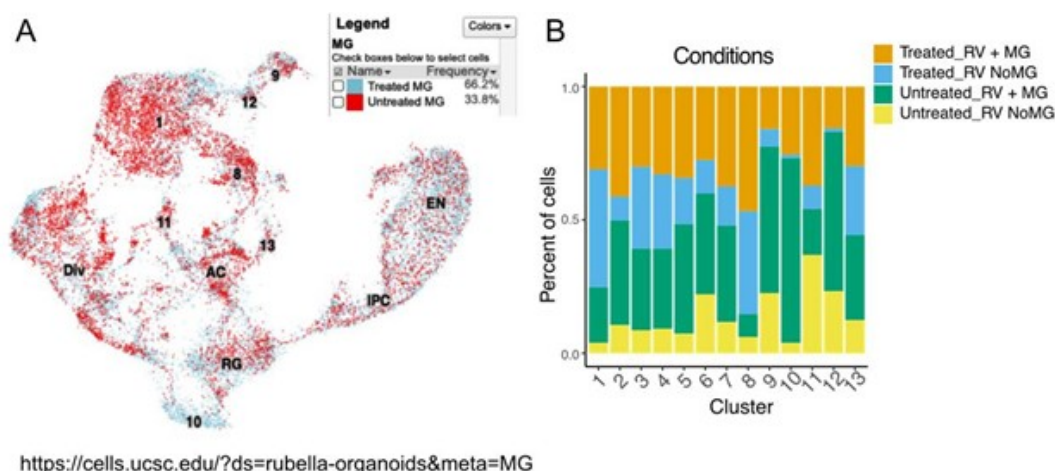
• Figure 4E, why are cells derived from microglia-free organoids so much enriched in the UMAP plots as compared to the other organoid condition? Is RV impacting cell fitness, proliferation, or neurodifferentiation?

This perceived difference is due to data presentation. Based on cell proportions, cells from organoids that were treated with microglia are more represented in the scRNAseq data, and this difference most likely comes from user-introduced imbalance in cell loading and possible cell losses during demultiplexing (Author response image 10, panel A). Cell number composition across different conditions and cell types, including RV and MG treatment, are shown in Supplement Figure 4 of the current manuscript (Author response image 10, panel B).

Contribution of each condition can be visualized via UCSC single cell data browser: <https://cells.ucsc.edu/?ds=rubella-organoids>

Author response image 10.

Data composition depending on condition. A. Cell number contribution from organoids with and without microglia. B. Contribution of each condition to each cluster composition.



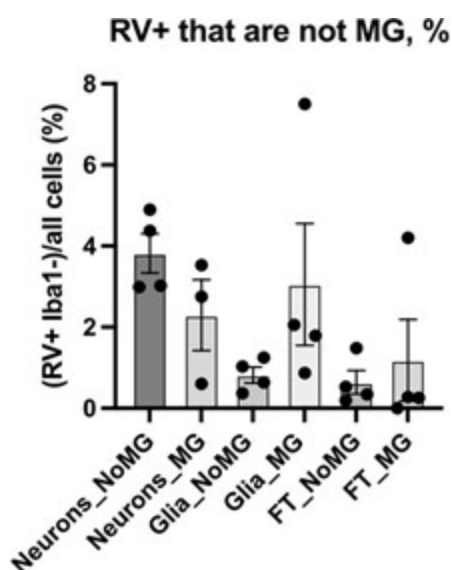
• Figure 4F-H. If microglia is the predominant target for RV in the brain, why are microglia-free organoids susceptible to RV and who are the other cellular targets, whose infection leads to activation of interleukin pathway genes and dysregulation of brain developmental markers in selected subpopulations (RGCs, ENs..).

Thank you for bringing this point. We did not detect any appreciable RV genomic RNA in our published single cell data, nor did we identify RV capsid in the RV-exposed organoids without microglia. Our experiments on dissociated cell cultures show that a small population (~1-4%) of other cell types was positive for the RV capsid, including neuron-enriched and glial-enriched fractions (Author response image 11; Supplementary Figure 3C in current

manuscript). We expect a similar proportion of non-microglia cells to be infected in the brain organoids. One possible explanation for the robust interferon response even in the absence of productive infection in other cell types is exposure to virions and virus-associated particles, including but not limited to viral RNA species, viral proteins, or even other components of the viral stocks made in Vero cells (which is a cell line that should not produce interferons, but may produce other unmeasured cytokines as a virally infected cell culture).

Author response image 11.

Quantification of RV+ cells that are not microglia across different cell populations. No statistically significant difference was detected in RV infectivity in cells cultured with or without microglia.



- QRT-PCR validations of some of these key brain targets should be performed.

We agree with the reviewer that further validation of the predicted molecular changes downstream of Rubella exposure would be valuable. We have opted to validate IFITM3 and NOVA1 expression differences using immunostaining, and the results are consistent with our predictions from scRNAseq, and the data is presented in revised Figure 5 and 6 of the current manuscript.

Reviewer #3 (Public Review):

Weaknesses of the paper: Overall, additional control experiments are needed to support the stated conclusions. Affinity chromatography is used to purify microglia and other cell types, but the overall cell enrichment is not quantified.

We appreciate the reviewer concern. However, affinity based enrichments rarely guarantee purity of the enrichment, and we do not believe accurate estimation of the purification purity would alter the biological interpretation of the data.

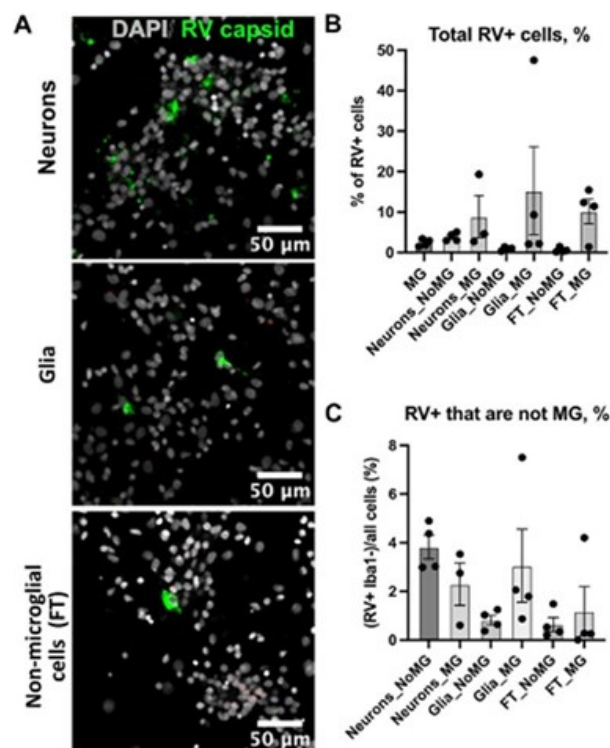
In cell mixing experiments, the authors do not rule out the possibility that the added non-microglia cells also become infected, releasing additional infectious viruses. The finding that a diffusible factor is required for RV infection would be unusual if not unprecedented; therefore, additional data are required to support this claim and rule out other interpretations.

We provide quantification of non-microglia cells that are positive for RV capsid in the presence and absence of microglia. Small (~1-4%) of non-microglia cells get infected with the virus and can potentially release more of the virus (see Author response image 12), but we do not know how this newly produced virus would be different from the one that was applied to the cells directly. To follow up our co-culture experiments, we wanted to exclude a possibility of microglia engulfing RV- infected cells in co-cultures, therefore we separated the two cell fractions by a liquid-permeable membrane (Figure 3 of the current manuscript). It is possible that factors secreted by other cell populations in the transwell assay experiments act on microglia cells to upregulate a yet unidentified receptor on microglia surface or other infection-dependent molecule rendering them infectable by the virus.

We re-phrase the text by de-emphasizing “soluble factors” and focusing on excluding phagocytosis of infected cells as a possible mechanism of RV capsid immunoreactivity in microglia cells.

Author response image 12.

Rubella infection in non-microglia cells. A. Representative images of different cell types depleted of microglia. Cell cultures were stained RV capsid (green) and DAPI. B. Quantification of total cells that are positive for RV capsid across conditions. C. Quantification of RV+ cells that are not microglia across different cell populations. No statistically significant difference was detected in RV infectivity in cells c-cultured with or without microglia.



The methods section would be improved by including details about the iPSC line that was used.

We include the following section in Materials and Methods:

iPSC lines.

All work related to human iPS cells has been approved by the UCSF Committee on Human Research and the UCSF GESCR (Gamete, Embryo, and Stem Cell Research) Committee. Human iPS cell line “WTC-10” derived from healthy 30-year-old Japanese male fibroblasts was from the Conklin Lab, UCSF (Bershteyn et al., 2017; Kreitzer et al., 2013). Human iPSC line “13325” was derived from 9-year-old female fibroblasts originally obtained from Coriell cell repository. Human iPSC line “1323-4” derived from healthy 48-year-old Caucasian female fibroblasts (gift from the Conklin Lab, UCSF) was used for immunofluorescence validation analysis as we found that this line generates more reproducible brain organoid differentiations.

and by a more thorough description of virus-specific details, including the numbers of infectious particles added per volume of incubation media.

We now include the following data in the Materials and Methods section:

Rubella virus infection

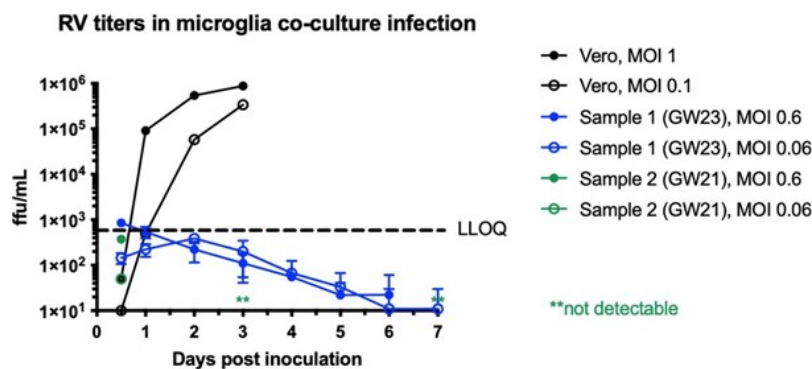
Cells cultured in 2D were inoculated by adding RV stock virus to culture media in 1:1 dilution (250 ul of media to the equal volume of viral stock, 1.75×10^5 total ffu/well) to achieve a multiplicity of infection (MOI) of 2. After four hours, media was exchanged with fresh cell culture media. Cortical brain slices were treated with 500 ul of RV viral stock (3.5×10^5 total ffu/slice) applied over the slice culture filter for four hours, and then the viral culture media was removed and replaced with fresh slice culture media. Organoids were treated in 6-well plates with 2ml of 1:1 dilution of viral stock:organoid maintenance media (7×10^5 total ffu) for four hours, and then viral media was exchanged for fresh media. For all experimental conditions, cells were fixed and processed for downstream analysis at 72 hours post infection. Supernatant from non-infected Vero cells (mock) or heat-inactivated RV (650C, 30 mins) was used as control.

In addition to immunofluorescence, adding additional data to demonstrate and quantify virus infection (PCR and plaque assays, or immunofluorescence using an anti-double-stranded RNA antibody such as J2) from the infected brain slices and organoids would provide greater assurance that the virus is indeed replicating under the experimental conditions.

We performed RV titering experiment in dissociated microglia co-cultured with other cell types, as well as Vero cells control. While we can detect a robust increase in viral titer from Vero cells, it fell below detection levels in microglia co-cultures. We now include these data in Supplementary Figure 2D.

Author response image 13.

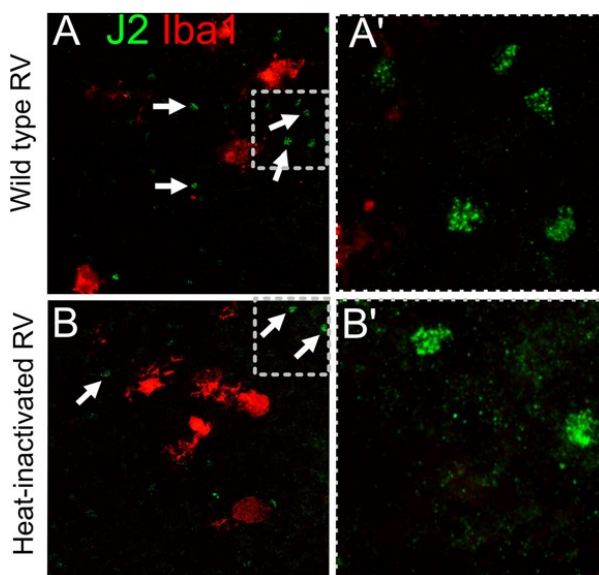
Rubella virus titering experiment performed in Vero cells (positive control) or dissociated microglia co-cultures. In primary microglia co-cultures, viral titer falls below detection levels after several days of infection.



Unfortunately, we did not find J2 staining informative because we could detect signal in both wild type RV infection conditions and in heat-inactivated RV, presumably due to native dsRNA species present in cells. We did not detect any increase or difference in the pattern of staining between RV and heat-inactivated virus-exposed conditions (Author response image 14; not included in the manuscript).

Author response image 14.

J2 antibody labels dsRNA in both RV-exposed and control heat-inactivated virus conditions, presumably due to native dsRNA that is not unique to the viral replication.

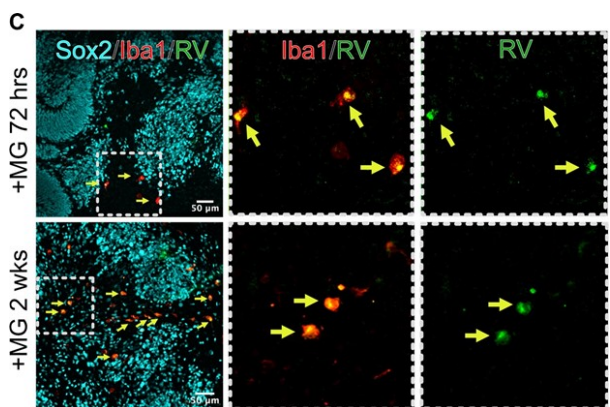


Organoid imaging with immunofluorescence would be very informative in demonstrating the presence of microglia and also in showing which cells are virus-infected in the context of organoid structures.

We provide images from 72hrs and 2 week RV infection, providing a zoomed-out view of organoids with microglia and RV capsid staining. We also provide images of 72hrs post-infection in organoids without microglia (Author response image 15, Figure 4C in current manuscript).

Author response image 15.

Microglia in organoids co-localize with RV capsid staining.



GenBank accession numbers are listed for the recombinant RV and GFP-RV reporter, but a search using those numbers did not locate the deposits--perhaps the deposits were very recent?

Both viral construct information is now available on GenBank:

M33 RV strain can be found here: <https://www.ncbi.nlm.nih.gov/nuccore/OM816674>

RV-GFP can be found here: <https://www.ncbi.nlm.nih.gov/nuccore/OM816675>

The authors incorrectly refer to the GFP virus as a new strain; it is not a viral strain and should be referred to as a reporter virus.

Thank you, we changed the description to

"To confirm functional transcription and translation of the viral genome, a new reporter construct of RV designed to express GFP within the non-structural P150 gene was generated (RV-GFP, GenBank Accession OM816675)"

Given that the authors show that Vero cell cultures are infected by the Rubella virus in the absence of other cells, additional evidence is needed to demonstrate that a diffusible factor from other cells enables microglia to be infected by the Rubella virus.

We have revised the manuscript to indicate that our data is consistent with the possibility that a diffusible factor is involved. Our experiment utilizing transwell assay argues against phagocytosis and physical interactions as primary drivers, but future studies will be needed to determine if soluble factors are involved.

The authors did not detect Rubella virus transcripts in the single-cell RNA sequencing experiment, nor was a microglia cluster found.

Indeed, microglia recovery using scRNAseq is very inefficient. We note this limitation in the discussion.

Innate immune responses can be activated in the presence of viral particles but without virus replication, as in inactivated viral vaccines; therefore changes in interferon responses do not necessarily prove virus replication.

We agree with the reviewer on this point, it is difficult, if at all possible, to entirely eliminate the possibility that some of the transcriptomic changes, particularly the interferon responses, are not induced by the exposure to viral particles. We have revised the manuscript to more rigorously described the conditions as "RV-exposed".

Figure 4: it would be helpful to define the abbreviations used in the figure legend (e.g. IPC, RG, EN). In the volcano plots, the gene names are blocked by the dots, and the figure becomes very pixelated when enlarged to read the text.

We have added abbreviations and replaced the figure files with higher resolution images (Figure 6 in current manuscript).

The value of including Supplemental Figure 2 (MOG) is not clear because it receives little mention in the text and also seems to be previously published data that could be cited.

We have removed the figure and replaced it with a citation and a link to the Cell Browser.

Supplemental Figure 4: In panel G, the legend shows "YH10" and "13325". These terms are not described in the Figure legend, nor did a search of the manuscript identify these terms. In its current form Supp. Fig. 4G is not interpretable. In addition, would be more clear to use the term "RV-infected" instead of "treated" to describe the addition of the virus.

We have expanded the Methods section to include the description of different organoid lines and added a revised legend for Supplementary Figure 4. We do not provide evidence of RV infecting organoids without microglia, therefore we have revised the claims that organoid cells become infected with the virus and replaced it with "RV-exposed" to better reflect the conditions studied.

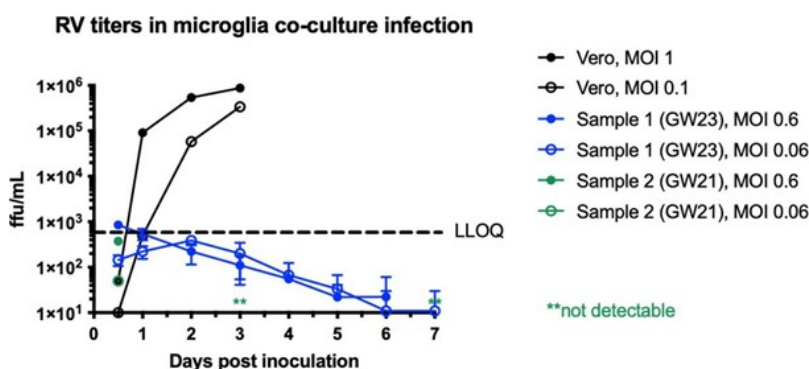
Reviewer #3 (Recommendations for the authors):

1. Demonstrate and quantify virus replication to provide data to complement the imaging. In order of data quality, plaque assays would be most convincing in demonstrating infection and release of infectious virus, while a time course of PCR on RV transcripts would support a conclusion of replicating virus. Further, staining with an anti-double-stranded RNA antibody (J2) would represent evidence of virus replication.

In response to the reviewer's comment, we performed an RV titering experiment in dissociated microglia co-cultured with other cell types, as well as Vero cells control. While we can detect a robust increase in viral titer from Vero cells, it fell below detection levels in microglia co-cultures. We now include these data in Supplementary Figure 2D.

Author response image 16.

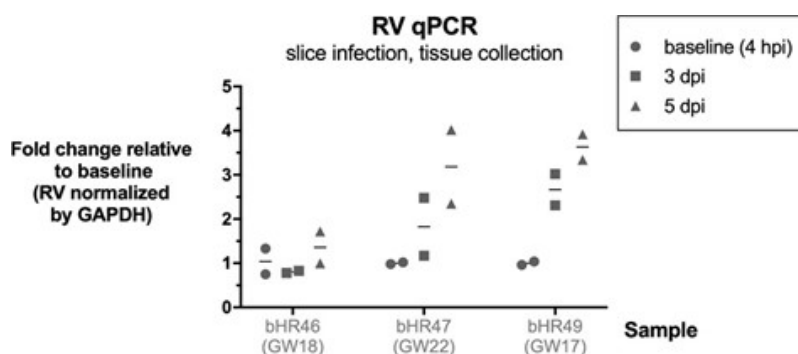
Rubella virus titering experiment performed in Vero cells (positive control) or dissociated microglia co-cultures. In primary microglia co-cultures, viral titer falls below detection levels after several days of infection.



We detected a very modest increase in RV RNA in infected brain slices over time using RT-qPCR (see Author response image 17, not included in current manuscript)

Author response image 17.

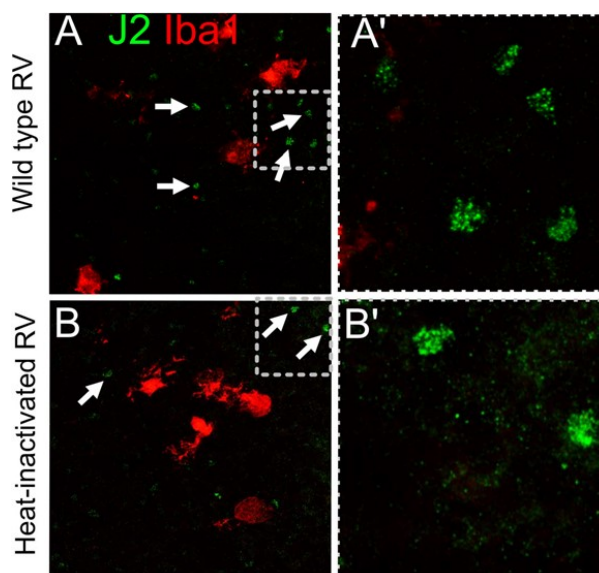
Modest increase in RV RNA over time in brain slice infections. Rubella virus RNA measured by qPCR relative to GAPDH gene, in n=3 samples (2 technical replicates each condition). Brain slices were exposed to RV, then collected at end of inoculation (4 hours post infection), or at 3 or 5 days post infection, and processed for RNA extraction and RT-qPCR.



Unfortunately, we did not find J2 staining informative because we could detect signal in both wild type RV infection conditions and in heat-inactivated RV, presumably due to native dsRNA species present in cells. We did not detect any increase of difference in the pattern of staining between RV and heat-inactivated virus-exposed conditions (Author response image 18; not included in the manuscript).

Author response image 18.

J2 antibody labels dsRNA in both RV-exposed and control heat-inactivated virus conditions, presumably due to native dsRNA that is not unique to the viral replication.

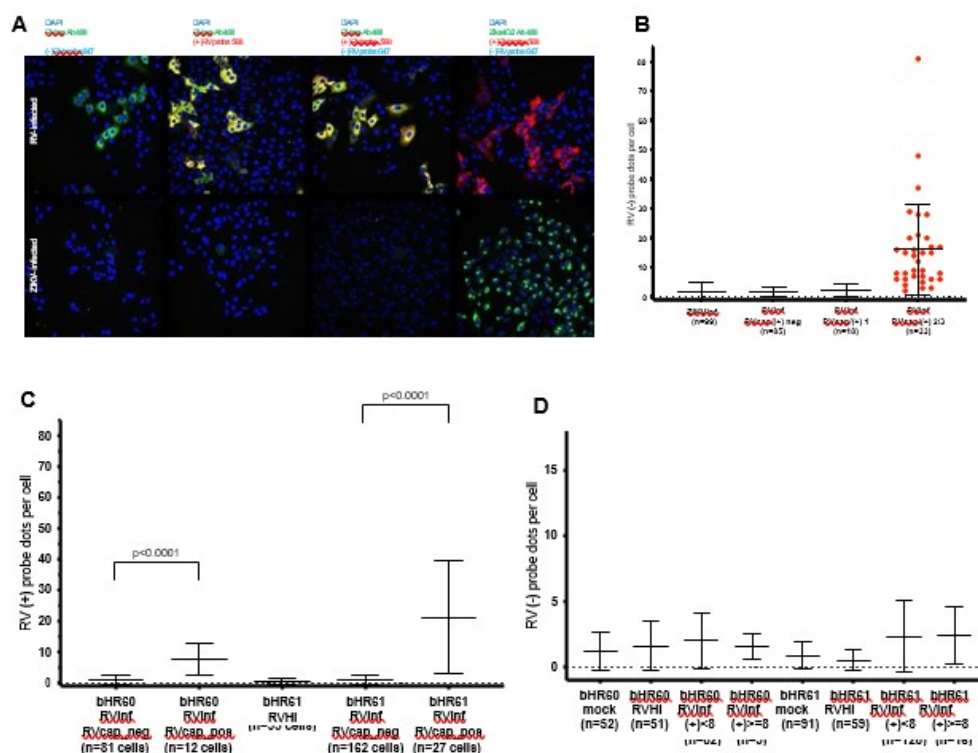


We utilized FISH to detect negative-stranded (non-genomic) RV RNA as an alternative to J2 to indicate RNA replication. However, it proved to be not very sensitive, as a small quantity of

negative-strand RV RNA could be detected in highly infected Vero cells, but negative-strand RV RNA was not detected in more modestly infected microglia (based on positive-strand RV RNA quantification), as in Author response image 19, not included in current manuscript.

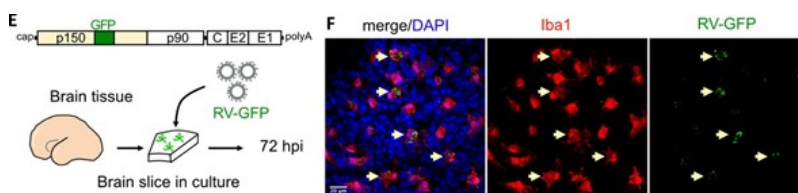
Author response image 19.

FISH probes to positive strand (genomic) and negative strand (replication template) RV RNA in Vero cells and microglia co-cultures. A: representative images of Vero cells infected with RV (top row) or Zika virus as control (bottom row). At 72hpi, cells were fixed and processed for immunofluorescence with anti-RV capsid antibody (RVcap) or Zika virus antibody (Zika4G2), and then FISH was performed using probes to positive strand (+) or negative strand (-) RV RNA. Negative strand RV RNA difficult to visualize at low-power magnification, and required quantification within cell borders defined by wheat germ agglutinin staining with results in panel B. B: In Vero cells, negative strand RV RNA is detected in strongly infected cells. Infection strength determined by intensity of RV capsid immunofluorescence staining and positive strand RV RNA (RVcap/(+) 2/3 indicates robust infection, RVcap/(+) 1 indicates weak infection). ZIKVinf = Zika virus infected control. C: In microglia co-cultures, positive strand RV RNA detected in cells with RV capsid immunopositivity (RVcap_pos). RVinf = RV infected. RVHI = heat-inactivated RV. D: In microglia co-cultures, negative strand RV RNA quantification not significantly different between mock, heat-inactivated RV (RVHI), or RV- infected conditions (RVinf), including cells with weak positive-strand RV RNA (RVinf, (+)<8) or cells with stronger positive-strand RV RNA ((RVinf, (+)>=8). Two biological replicates (bHR60 and bHR61), n indicates number of cells counted.



While we could not detect an increase in the viral particles from microglia mixed cultures, we confirmed the presence of GFP from the RV-GFP reporter construct, and we believe it serves as a proof that the virus can infect microglia cells and lead to production of functional viral protein (see Author response image 20, Figure 1E-F of the current manuscript)

Author response image 20.



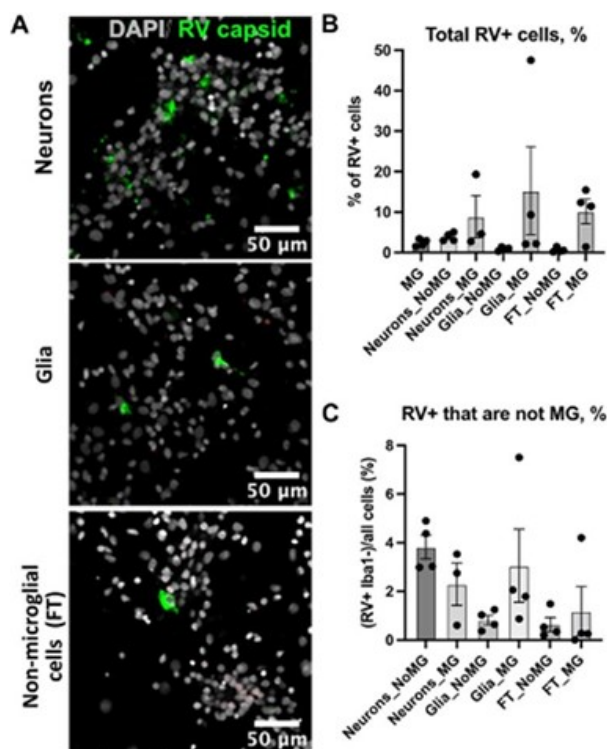
Thus, overall we detect replication of viral RNA and protein (qPCR, RV-GFP), but not an appreciable increase in released newly-made virions. The discussion now reflects this more clearly in the current manuscript.

1. The claim of requiring a diffusible factor to enable RV infection requires additional data. A suggestion would be to include further characterization of affinity-purified cells to define the levels of cell enrichment and to determine which other cell types are present, It is also important to test the RV infection of the fractionated cell types alone before adding to the microglia, in order to demonstrate whether RV is replicating in cell types other than microglia.

We performed quantifications of RV capsid-positive cells in each of the affinity-purified cell populations: neuron-enriched (purified with PSA-NCAM beads), glia-enriched (PSA-NCAM depleted cell fraction), or non-microglia fraction ("Flow through", depleted of CD11b+ cells). We show that across each condition, we have low infectivity (ranging from ~1 to 4% of total cell population) after 72 hours post-infection. We include these data in Supplementary Figure 3.

Author response image 21.

Rubella infection in non-microglia cells. A. Representative images of different cell types depleted of microglia. Cell cultures were stained RV capsid (green) and DAPI. B. Quantification of total cells that are positive for RV capsid across conditions. C. Quantification of RV+ cells that are not microglia across different cell populations. No statistically significant difference was detected in RV infectivity in cells c-cultured with or without microglia.



Another approach to limit cell heterogeneity would be to use iPSC-derived cells, which are highly enriched as a single cell type as a specific cell type, to test the requirement for additional cell types to achieve RV infection of microglia.

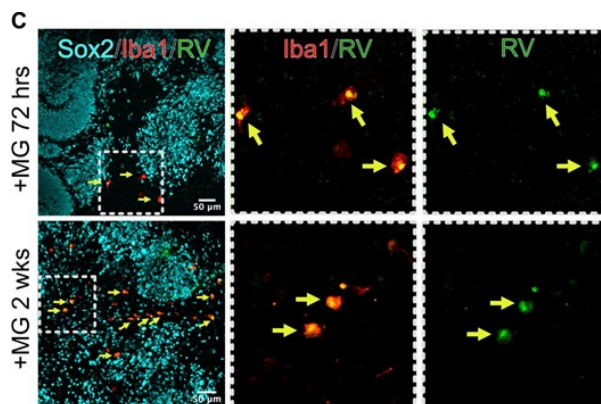
In our prior publication (Popova et al. 2021) we have identified a number of molecular differences between primary and iPSC derived microglia. iPSC derived microglia like cells could show differences in infection tropism from primary microglia, and those results may be difficult to interpret biologically. We agree with the reviewer that iPSC derived cells would be an interesting model, there are now several distinct protocols for deriving microglia like cells from pluripotent stem cells and we feel that embarking on a protocol comparison project would fall outside the scope of the current manuscript.

1. Consider a longer organoid infection. The authors did not identify viral RNA transcripts in their organoid scRNAseq data after a 72-hour infection. Although the 72-hour time point seems right for cells in 2D culture, it's possible that the infection in the organoids is slower because the virus has to spread inwardly. It would be worth trying a time course out to 2 weeks, collecting organoids every few days and then imaging and doing pcr or plaque assays. Zoomed-out views that show immunofluorescence of the entire organoid would also be beneficial in assessing organoid quality and immunofluorescent staining to identify cell types,

We performed longer RV infection for two weeks and now present data on RV capsid in microglia in 72 hrs and 2 weeks post-infection (Author response image 22, Figure 4C of the current manuscript). We have also validated one of the scRNAseq-generated gene candidates in combination with different cell type markers and present data on whole organoids immunostained with NeuN for neurons and EOMES for intermediate progenitor cells that demonstrate the overall structure of the organoids (Author response image 23; Figure 6 of the current manuscript).

Author response image 22.

Microglia in organoids co-localize with RV capsid staining. Organoid with microglia were exposed to RV for 72 hrs or two weeks.



Author response image 23.

Organoids labeled with splice regulator NOVA1 (magenta), neuronal marker NeuN (green) and intermediate progenitor cell marker EOMES (cyan).

