The Brazilian Zika virus strain causes birth defects in experimental models

Fernanda R. Cugola1,*, Isabella R. Fernandes1,2,*, Fabiele B. Russo1,3,*, Beatriz C. Freitas2, João L. M. Dias1, Katia P. Guimarães1, Cecília Benazzato1, Nathalia Almeida1, Graciela C. Pignatari1,3, Sarah Romero2, Carolina M. Polonio1, Isabel Cunha4, Carla L. Freitas1, Wesley N. Brandão1, Cristiano Rossatto1, David G. Andrade4, Daniele de P. Faria5, Alexandre T. Garcez5, Carlos A. Buchpigel5, Carla T. Braconi6, Erica Mendes6, Amadou A. Sall7, Paolo M. de A. Zanotto6, Jean Pierre S. Peron4, Alysson R. Muotri2 & Patricia C. B. Beltrão-Braga1,8

Zika virus (ZIKV) is an arbovirus belonging to the genus Flavivirus (family Flaviviridae) and was first described in 1947 in Uganda following blood analyses of sentinel rhesus monkeys1. Until the twentieth century, the African and Asian lineages of the virus did not cause meaningful infections in humans. However, in 2007, vectored by Aedes aegypti mosquitoes, ZIKV caused the first noteworthy epidemic on Yap Island in Micronesia2. Patients experienced fever, skin rash, arthralgia and conjunctivitis7. From 2013 to 2015, the Asian lineage of the virus caused further massive outbreaks in New Caledonia and French Polynesia. In 2013, ZIKV reached Brazil, later spreading to other countries in South and Central America3. In Brazil, the virus has been linked to congenital malformations, including microcephaly and other severe neurological diseases, such as Guillain–Barré syndrome4,5. Despite clinical evidence, direct experimental evidence showing that the Brazilian ZIKV (ZIKVBR) strain causes birth defects remains absent8. Here we demonstrate that ZIKVBR infects fetuses, causing intrauterine growth restriction, including signs of microcephaly, in mice. Moreover, the virus infects human cortical progenitor cells in vitro, leading to an increase in cell death. We also report that the infection of human brain organoids results in a reduction of proliferative zones and disrupted cortical layers. These results indicate that ZIKVBR crosses the placenta and causes microcephaly by targeting cortical progenitor cells, inducing cell death by apoptosis and autophagy, and impairing neurodevelopment. Our data reinforce the growing body of evidence linking the ZIKVBR outbreak to the alarming number of cases of congenital brain malformations. Our model can be used to determine the efficacy of therapeutic approaches to counteract the harmful impact of ZIKVBR in human neurodevelopment.

The recent increase in microcephaly cases in Brazil has been associated with the outbreak of Zika virus (ZIKV)7, originating from an Asian-lineage strain that can be spread by Ae. aegypti mosquitoes8. The Brazilian ZIKV (ZIKVBR) has been detected in the placenta and amniotic fluid of two women with microcephalic fetuses9-11 and in the blood of microcephalic newborns10,12, suggesting that the virus can cross the placental membrane. The virus has also been identified in the brains and retinas of microcephalic newborns10,11,12. However, there is no direct evidence of the mechanism by which ZIKVBR might cause brain malformations. A previous study revealed that the African ZIKV (ZIKVAf, strain MR-766) has the ability to infect human skin cells14,15. More recently, ZIKVAf was also shown to infect human pluripotent stem cell (hPSC)-derived neural progenitor cells (NPCs) in vitro, which induced apoptotic cell death16. These studies were performed using the MR-766 ZIKVAf strain isolated in Uganda in 1947, which shares 87–90% sequence similarity with the Polynesian and Brazilian isolates3,17. Nevertheless, because severe congenital malformations were not reported for African isolates, there is a need to study the association of ZIKV with microcephaly and birth defects with isolates from affected localities, such as the ZIKVBR strain. Therefore, there is an urgent need to develop model systems to determine the relationship between infection with the ZIKVBR strain and birth defects.

We used ZIKVBR isolated from a febrile case in the state of Paraiba, in the northeast of Brazil in 2015 (see Methods). To evaluate the causal relationship between ZIKVBR and birth defects, including brain malformation during development, we first used a murine experimental model in which SJL and C57BL/6 pregnant mice were infected with ZIKVBR, evaluating newborns immediately after birth (Extended Data Fig. 1a). Notably, similar to ZIKVBR-infected human newborns18,19, pups born from the SJL ZIKVBR-infected pregnant females displayed clear evidence of whole-body growth delay or intrauterine growth restriction (IUGR)20 compared to pups born from the mock-infected controls (Fig. 1a, b). Using a qPCR assay, we confirmed the presence of ZIKVBR genomic RNA in several tissues of newborn animals, observing significantly more viral RNA in the brain, confirming the neurotropic nature of the virus (Fig. 1c).

Microcephaly is perhaps the most striking of the birth defects reported in ZIKVBR-infected newborns4,18,19. Mouse models often fail to reproduce the severely reduced brain size and pathological alterations found in human patients21,22, probably owing to differences in gestation time and brain development between the two species. Nevertheless, upon close inspection of the ZIKVBR-infected mouse brains, we noticed cortical malformations in the surviving animals, with reduced cell number and cortical layer thickness, signs associated with microcephaly in humans (Fig. 1d–f). At a cellular level, the neurons in the cortex, thalamus and hypothalamus displayed a ‘vacuolar nuclei’ appearance. This morphology was characterized by central emptiness and marginalized chromatin pattern with nuclear debris, suggesting ongoing cellular death (Fig. 1d and Extended Data Fig. 2). In addition, we also noticed apparent ocular abnormalities, reminiscent of that observed in human patients23 (Fig. 1g). Thus, SJL infected pups presented congenital malformations with similarities to those in ZIKVBR-infected human newborns. While the impact of ZIKVBR

1University of São Paulo, Department of Surgery, Stem Cell Laboratory, São Paulo, São Paulo 05508-270, Brazil. 2University of California San Diego, School of Medicine, Department of Pediatrics/ Rady Children’s Hospital San Diego, Department of Cellular & Molecular Medicine, Stem Cell Program, La Jolla, California 92037-0695, USA. 3Tissuemo, The Biotech Company, São Paulo, São Paulo 01401-000, Brazil. 4University of São Paulo, Department of Immunology, Neuroimmune Interactions Laboratory, São Paulo, São Paulo 05508-000, Brazil. 5University of São Paulo, Department of Radiology and Oncology, USP School of Medicine, São Paulo, São Paulo 05403-010, Brazil. 6University of São Paulo, Department of Microbiology, Institute of Microbiology Sciences, Laboratory of Molecular Evolution and Bioinformatics, São Paulo, São Paulo 05508-000, Brazil. 7Institute Pasteur in Dakar, Dakar 220, Sénégal. 8University of São Paulo, School of Arts, Sciences and Humanities, Department of Obstetrics, São Paulo, São Paulo 03828-000, Brazil.

*These authors contributed equally to this work.
Figure 1 | ZIKVBR infection in SJL mice. a, SJL pups born with IUGR. Scale bar, 1 cm. b, Total body weight, crown–rump and skull measurements in pups born from infected animals (n = 6 pups, comprising 3 mice from 2 separate litters; error bars, s.e.m.; t-test, ***P < 0.001). c, ZIKVBR RNA detected in SJL pup tissues (n = 6 pups, comprising 3 mice from 2 separate litters; error bars, s.e.m.; t-test). d, Histopathological aspect of the cortical organization (brackets) in infected brains, including intranuclear vacuoles, and 'empty' nuclei aspect with chromatin margination in neurons (arrowheads). Scale bar, 100 μm (left panels), 50 μm (middle panels) and 10 μm (right panels). e, ZIKVBR-infected brains displayed a reduced cortical layer thickness (n = 6 pups, comprising 3 mice from 2 separate litters; error bars, s.e.m.; t-test, ***P < 0.001). Infected brains have fewer cells per layer (n = 6 pups, comprising 3 mice from 2 separate litters; error bars, s.e.m.; t-test, **P < 0.1). f, ZIKVBR-infected cortical neurons have pronounced nuclei (diameter) (cortical, n = 31; deep cortical, n = 21 and medulla, n = 41 nuclei; error bars, s.e.m.; two-way ANOVA, ****P < 0.001). g, Ocular malformations (arrow) in the ZIKVBR-infected pups. h, Cell death gene expression signature in the brains of ZIKVBR-infected pups (n = 2 mice per group; threshold = twofold).
Figure 2 | ZIKV infection in vitro. a, Relative expression of TAM receptors (n = 2 technical replicates from two pooled different donors; error bars, s.e.m.; t-test; ***P < 0.001). b, Expression of TAM receptors in NPCs after ZIKVBR infection (MOI = 10) at 48 h post-infection (n = 2 technical replicates from two pooled different donors; error bars, s.e.m.; one-way ANOVA, P > 0.05). c, TEM detection of ZIKVBR viral particles 24 h post-infection at MOI = 10 (red arrowheads) inside NPCs (top panels) and neurons (bottom panels). Yellow arrowheads, viral factories; white arrowheads, immature viral particles. Scale bars, 0.5 μm (top left); 200 nm (top right); 0.2 μm (bottom left); 50 nm (bottom right). d, Immunofluorescence revealed susceptibility to infection in NPC and neurons with the ZIKVBR (MOI = 10) at 24 h post-infection. Scale bar, 25 μm. e, ZIKVBR replication dynamics in NPCs (MOI = 10; n = 2 technical replicates from RNA of two different donors). f, ZIKVBR replication dynamics in neurons (MOI = 10; n = 2 technical replicates from RNA of two different donors). g, NPC death measured by FACS with two different cell gating sizes (P1 and P2). Apoptosis (left panel), necrosis (middle panel) and late apoptosis (right panel) (MOI = 10; 48 h post-infection; n = 2 technical replicates from two different donors; error bars, s.e.m.; two-way ANOVA, *P < 0.05). PI, propidium iodide. h, Representative images of human neurospheres infected with ZIKVBR (MOI = 10; 96 h post-infection). Scale bar, 200 μm. i, Alterations in neurosphere diameter over time (MOI = 10; n = 25 neurospheres from two different donors for each time point; error bars, s.e.m.; ANOVA, +++P < 0.0001). j, ZIKV replication dynamics in neurospheres (MOI = 10; n = 2 technical replicates from two different donors).

Next, we challenged two three-dimensional neuronal cell culture systems, neurospheres and cerebral organoids, with ZIKVBR and ZIKVAF. We generated neurospheres by growing human NPCs in suspension. While the mock-infected control neurospheres continued to grow over time, the ZIKVBR-infected neurospheres (MOI = 10) displayed evident morphological abnormalities with signs of cell death (Fig. 2h). The sizes of the neurospheres infected with ZIKVBR were significantly smaller than the mock-control and ZIKVAF-infected at 96 h post-infection (Fig. 2i). A less dramatic effect is observed at an MOI of 1, where both ZIKVBR and ZIKVAF infection reduced the size of the neurospheres compared to mock-infected controls (Extended Data Fig. 4a, b). These observations were paired with increased ZIKVBR replication in these cultures at both MOIs of 10 and 1 (Fig. 2j) and Extended Data Fig. 4c). These results suggest that ZIKVBR induces cell death in human NPCs, impairing the growth and morphogenesis of healthy neurospheres (Extended Data Fig. 4d–f).

The majority of the described cases of ZIKVBR-infected newborns (95%) had malformations of cortical development4. Thus, we also used brain organoids generated from hPSCs and human embryonic stem cells, to evaluate the impact of ZIKVBR on human cortical development. In the following experiments, alongside the ZIKVAF and mock infection, we added the Yellow Fever virus (YFV), a slow replicating attenuated live-vaccine flavivirus that has a low risk of causing neuropathy. Cerebral organoids are three-dimensional, self-organized, stem-cell-derived models that recapitulate the first trimester of human neurodevelopment, including the molecular and cellular architecture reminiscent of the fetal cortex15. Organoids show some degree of lamination and resembled the human neocortex in terms of the spatial relationships of the progenitor populations, defined here as a proliferative ventricular zone, post-migratory neurons in cortical plate and a marginal zone (Fig. 3a–c). We infected organoids with ZIKVBR, ZIKVAF and YFV.
using an MOI of 0.1 and compared to mock-infected organoids at 24 and 96 h post-infection. We quantified the percentage of different subtypes of cortical neurons, TBR1-positive or CTIP2-positive cells (deep-layer V/VI) in the cortical plate, finding a significant reduction in their number and respective cortical plate thickness in the ZIKV<sup>BR</sup>-infected organoids compared to the others. A significant reduction in TBR1-positive cells was observed in the ZIKV<sup>BR</sup>-infected organoids at 96 h post-infection, while CTIP2-positive cells were significantly reduced in both ZIKV<sup>BR</sup>- and ZIKV<sup.AF</sup>-infected organoids at the same time point (Fig. 3d, e and Extended Data Fig. 5a–f). Consistent with the reduced population of cortical cells, we observed a significant decrease in PAX6-positive cells (dorsal forebrain progenitor cells) following ZIKV infection (Fig. 3f and Extended Data Fig. 5d). Dividing cells in the ventricular zone, detected by the population of Ki67- and SOX2-positive cells, were only significantly reduced in the ZIKV<sup>BR</sup>-infected organoids (Fig. 3g, h and Extended Data Fig. 5d). As observed in our other <i>in vitro</i> models, the number of apoptotic cells (cleaved caspase 3- and TUNEL-positive cells) was increased in organoids infected with ZIKV<sup>BR</sup>, possibly explaining the decrease in the NPC population (Fig. 3i and Extended Data Fig. 5g, h).

ZIKV<sup.AF</sup> was derived from a zoonotic agent associated with primates in Africa, whereas ZIKV<sup>BR</sup> is an isolate from a lineage adapted to human-to-human transmission for the past 70 years. As an entry point to establishing the potential mechanistic adaptive differences between ZIKV<sup>BR</sup> and ZIKV<sup.AF</sup> towards human cells, we also generated brain organoids from non-human primate (chimpanzee) pluripotent stem cells. We infected these chimpanzee's cerebral organoids (MOI of 0.1) and measured the impact on cortical neurons at 24 and 96 h post-infection. ZIKV<sup>BR</sup> failed to reduce the percentage of either TBR1- or CTIP2-positive cells in non-human primates (Extended Data Fig. 5i, j). Consistently, the kinetics of infection were different between the two ZIKV isolates. While ZIKV<sup>BR</sup> did not replicate in the chimpanzee organoids, ZIKV<sup.AF</sup> seemed well adapted to these cells (Extended Data Fig. 5k).

To evaluate the causal relationship between ZIKV congenital infection and birth defects, we used a murine experimental model, in which pregnant SJL and C57BL/6 mice were infected with ZIKV<sup>BR</sup>. Notably, the SJL strain was susceptible to viral infection of fetal tissues, causing severe IUGR that resembled the affected Brazilian newborns, including signs of microcephaly, such as cortical malformations. We also showed that ZIKV<sup>BR</sup> induced apoptosis and autophagy in the mouse neural tissue. This is in accordance with the literature, as it has been previously demonstrated that ZIKV induces and localizes in autophagic phagosomes. To our knowledge, this is the first report showing a gene expression profile that correlates to cell death in the brains of microcephalic newborn ZIKV<sup>BR</sup>-infected mice, corroborating a causal relationship. It is unclear why the virus could not cross the placenta of C57BL/6 mice, but this result may be due to the robust anti-viral
immune response of this mouse strain, which secretes significant levels of type I/II interferon, known to confer resistance to ZIKV14,26. These data suggest that genetic differences could explain in part why some ZIKV-infected pregnant women give birth to newborns with detectable congenital brain malformations37. Nonetheless, our murine model is a valuable tool for future pre-clinical studies, such as vaccine development. The presence of major cortical histological abnormalities in the pups with UPRG prompted us to use an hPSC model to study the impact of ZIKV on neurodevelopment. ZIKV infects cells at different stages of brain maturation leading to alterations in the cortical layer organization. While this manuscript was under review, two other papers revealed the impact of previously established ZIKV strains on human organoids, confirming our observations with ZIKV158 (refs 28, 29). Finally, our data using a non-human primate organoids suggested that the ZIKV158 might have experienced adaptive changes in human cells. It has been demonstrated that the Asian lineage of ZIKV is undergoing codon usage adaptation towards biases observed in highly expressed human genes30. Our findings support the hypothesis that microcephaly is a distinctive feature of recent ZIKV Asian-lineage virus, which originated in the Pacific and is now spreading in South and Central America.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

Received 19 March; accepted 4 May 2016.

Published online 11 May 2016.

1. Dick, G. W., Kitchen, S. F. & Haddow, A. J. Zika virus. I. Isolations and serological specificity. Trans. R. Soc. Trop. Med. Hyg. 46, 509–520 (1952).
2. Lanciotti, R. S. et al. Genomic and serologic properties of Zika virus associated with an epidermal, Yap State, Micronesia, 2007. Emerg. Infect. Dis. 14, 1232–1239 (2008).
3. Faria, N. R. et al. Zika virus in the Americas: early epidemiological and genetic findings. Science 352, 345–349 (2016).
4. de Fatima Vasco Aragao, M. et al. Clinical features and neuroimaging (CT and MRI) findings in presumed Zika virus related congenital infection and microcephaly: retrospective case series study. Br. Med. J. 353, 1901 (2016).
5. Cao-Lormeau, V. M. et al. Guillain-Barré Syndrome outbreak associated with Zika virus infection in French Polynesia: a case-control study. Lancet 387, 1531–1539 (2016).
6. Rasmussen, S. A., Jamieson, D. J., Honein, M. A. & Petersen, L. R. Zika virus and birth defects - reviewing the evidence for causality. N. Engl. J. Med. 374, 1981–1987 (2016).
7. Campos, G. S., Bandeira, A. C. & Sardi, S. I. Zika virus outbreak, Bahia, Brazil. Emerg. Infect. Dis. 21, 1885–1886 (2015).
8. Hayes, E. B. Zika virus outside Africa. Emerg. Infect. Dis. 15, 1347–1350 (2009).
9. Sarno, M. et al. Zika Virus infection and stillbirths: a case of hydrops fetalis, hydrencephaly and fetal demise. PLoS Negl. Trop. Dis. 10, e0004517 (2016).
10. Caixas, G. et al. Detection and sequencing of Zika virus from amniotic fluid of fetuses with microcephaly in Brazil: a case study. Lancet Infect. Dis. http://dx.doi.org/10.1016/S1473-3099(16)00095-5 (2016).
11. Martines, R. B. et al. Notes from the field: evidence of Zika virus infection in brain and placental tissues from two congenitally infected newborns and two fetal losses - Brazil, 2015. MMWR Mortal. Wkly. Rep. 65, 159–160 (2016).
12. Miklar, J. et al. Zika virus associated with microcephaly. N. Engl. J. Med. 374, 951–958 (2016).
13. Ventura, C. V., Maia, M., Bravo-Filho, V., Góis, A. L. & Belfort, R., Jr. Zika virus in Brazil and macular atrophy in a child with microcephaly. Lancet 387, 226 (2016).
14. Hamel, R. et al. Biology of Zika virus infection in human skin cells. J. Virol. 89, 8880–8896 (2015).
15. Bell, T. M., Field, E. J. & Narang, H. K. Zika virus infection of the central nervous system of mice. Arch. Gesamte Virusforsch. 35, 183–193 (1971).
16. Tang, H. et al. Zika virus infects human cortical neural progenitors and attenuates their growth. Cell Stem Cell 18, 578–590 (2016).
17. Faye, O. et al. Molecular evolution of Zika virus during its emergence in the 20(th) century. PLoS Negl. Trop. Dis. 8, e2636 (2014).
18. Brasil, P. et al. Zika virus outbreak in Rio de Janeiro, Brazil: clinical characterization, epidemiological and virological aspects. PLoS Negl. Trop. Dis. 10, e0004636 (2016).
19. Brasil, P. et al. Zika virus infection in pregnant women in Rio de Janeiro - preliminary report. N. Engl. J. Med. http://dx.doi.org/10.1056/NEJMoA1602412 (2016).
20. Jang, E. A., Longo, L. D. & Goyal, R. Antenatal maternal hypoxia: criterion for fetal growth restriction in infants. Front. Physiol. 6, 1 (2015).
21. Lazzarino, S. B. et al. Cdk5rap2 regulates centrosome function and chromosome segregation in neuronal progenitors. Development 137, 1907–1917 (2010).
22. Pulvers, J. N. et al. Mutations in mouse Aspm (abnormal spindelike microcephaly associated) cause not only microcephaly but also major defects in the germline. Proc. Natl Acad. Sci. USA 107, 16595–16600 (2010).
23. de Paula Freitas, B. et al. Ocular findings in infants with microcephaly associated with presumed Zika virus congenital infection in Salvador, Brazil. JAMA Ophthalmol. 134, 529–535 (2016).
24. Nowakowski, T. J. et al. Expression analysis highlights AXL as a candidate Zika virus entry receptor in neural stem cells. Cell Stem Cell 18, 591–596 (2016).
25. Lancaster, M. A. et al. Cerebral organoids model human brain development and microcephaly. Nature 501, 373–379 (2013).
26. Rossi, S. L. et al. Characterization of a novel murine model to study Zika virus. Am. J. Trop. Med. Hyg. http://dx.doi.org/10.4269/ajtmh.16-0111 (2016).
27. Cauchemez, S. et al. Association between Zika virus and microcephaly in French Polynesia, 2013–15: a retrospective study. Lancet 387, 2125–2132 (2016).
28. Garcez, P. P. et al. Zika virus impairs growth in human neurospheres and brain organoids. Science 352, 816–818 (2016).
29. Qian, X. et al. Brain-region-specific organoids using mini-bioreactors for modeling ZIKV exposure. Cell 165, 1–17 (2016).
30. Freire, C. C. et al. Spread of the pandemic Zika virus lineage is associated with NS1 codon usage adaptation in humans. Preprint at bioRxiv.org/content/early/2015/11/25/032839 (2015).
METHODS

No statistical methods were used to predetermine sample size. The experiments were not randomized and the investigators were not blinded to allocation during experiments and outcome assessment.

Viral culture and amplification. A lyophilized ZIKV isolate from a clinical case in Brazil (ZIKVBR), provided by the Evandro Chagas Institute in Belém, Pará, was reconstituted in 0.5 ml of sterile DEPC water. The African-lineage MR-766 (ZIKVAE), a reference strain isolated in Uganda in 1947 and the Yellow Fever vaccine strain (YFV-17D)19, both used here for controls, were provided by the Institute Pasteur in Dakar, Senegal. Aedes albopictus mosquito cells (C6/36 cells) were previously prepared to culture the three viruses. C6/36 cell culture was maintained by Leibovitz’s L-15 medium supplemented with 10% fetal bovine serum (FBS) (Gibco), 1% non-essential amino acids (Gibco), 1% sodium pyruvate (Gibco), 1% penicillin/streptomycin (Gibco), 0.05% of amphotericin B (Gibco) and kept at 27 °C in the absence of CO2. After reaching an approximately 70% confluent monolayer, 50 μl of each viral sample was inoculated into C6/36 with an hour of adsorption, with gentle shaking every 10 min to allow the homogeneous adsorption of the viruses. At the end of the adsorption period, 5 ml of the culture media was added, plus 2% FBS, 1% non-essential amino acids and 1% sodium pyruvate. The cultures were then incubated under the same adsorption conditions.

In the first subculture (T1), the infected cells were less confluent compared to the control cells but had few noticeable morphological changes. On the fourth day after infection, the second subculture (T2) was made blindly by transferring 500 μl of the T1 supernatant, followed by the third subculture, which was collected on the eighth day after infection (T3). Pronounced cytopathic effects were perceived beginning at T2. The supernatants were harvested, titrated and T3 was used for the experiment inoculation.

Virus titration. Titration (in PFU ml−1) of each C6/36 subculture was obtained by plaque assay, as described. The amount of infectious viral particles (PFU). The virus titration was performed in porcine kidney epithelial (PS) cells and in L15 medium with 5% FBS. Briefly, the virus titration was done using 200 μl of L15 medium (5% FBS, 1% penicillin/streptomycin, and 1% glucose) in a 24-well plate. Then, a serial dilution of each virus stock from ZIKVBR, ZIKVAE and YFV-17D in L15 medium was performed, from 10−4 to 10−11. Then, 200 μl of each dilution was added in each 24-well plate. After this, 1×105 PS cells were seeded in each well of a 24-well plate for at least 3 h at 37 °C to allow virus adsorption and PS cell adherence. Later, each well was overlaid with complete carboxymethyl cellulose (CMC) medium (0.6% in L15 supplemented with 3% FBS). After 5 days of incubation at 37 °C, the plaque visualization was made using blue-black staining solution. The most appropriate viral dilution was estimated to determine the amount of infected cells visible (PFU ml−1). For ZIKVBR, the first C6/36 subculture had a titre of 6×109. The following subcultures had titres of 7.5×109 (T2) and 4×1012 (T3). All the subculture aliquots were stored in cryovials and maintained in liquid nitrogen or were distributed to the ZIKV São Paulo task force.

In vivo infection. Pregnant mice, 6–8 weeks of age, C57BL/6 or SJL (JAX), were infected intravenously with 200 μl of ZIKVBR−infected C6/36 cell supernatant containing 1×104, 4×1010 or 1×1012 PFU ml−1 of virus on day 10–13 of gestation. The animals were observed daily. All the experiments were performed with the approval of the Institute of Biomedical Sciences Ethics Committee protocol number 05/2016.

Real-time PCR. RNA was extracted from each sample (cells, supernatant of cell culture or mouse tissue) using the QIAamp UltraSens Virus Kit (Qiagen) or TRIzol reagent (Invitrogen). All RNA pellets were resuspended in 30 μl of RNase-free distilled water, quantified using a NanoDrop spectrophotometer (NanoDrop Technologies) and stored at −80 °C. The set of primers/probes specific for ZIKV were synthesized by Sigma Life Science, with 5-FAM as the reporter dye for the detection and quantification of viral RNA, the real-time PCR of each sample was performed by the QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems). For normalization ACTB was used as a housekeeping gene.

For the real-time analysis, the microgram of total RNA from brains of 4 pups, 2 pooled mock and 2 pooled ZIKVBR−infected from SJL mothers were submitted to gene expression analysis for cell death target genes using the RT2 Profiler PCR Array Mouse Cell Death PathwayFinder (cat. no. PAMM-2122ZA- Qiagen) according to the manufacturer’s protocols. qPCR was performed in the QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems). To evaluate gene expression, we established a fold change threshold of at least twofold up- or downregulation compared to mock infected samples. Statistical analysis was performed using the RT2 profiler RT–PCR array data analysis software v3.5.

NPCs, neurons, neurospheres and organoids. We used three human and two chimpanzee iPSC lines that were previously characterized in the Beltrão-Braga and Mooturi laboratories42–45 plus H9 human embryonic stem cells (hESCs) for all the experiments using pluripotent stem cells. All the cell lines tested negative for mycoplasma contamination. Briefly, high passages of iPSC/hESC colonies on feeder-free plates were maintained for 5 days with mTeSR media (Stem Cell Technologies). On the fifth day, the medium was changed to N2 media (DMEM/ F12 medium supplemented with 1X N2 supplement (Invitrogen) and the dual SMAD inhibitors, 1μM dorsomorphin (Tocris) and 1μM SB431542 (Stemgent), for 48 h. Further, the colonies were detached from the plate and cultured in suspension as embryoid bodies (EBs) for 5 days at 90 r.p.m. in N2 media with the above SMAD inhibitors. The EBs were plated on matrigel-coated plates with N2 media composed of the following: DMEM/F12 media supplemented with 0.5X N2, 0.5X B27 supplement (Gibco), 20 ng ml−1 of FGF2 and 1% penicillin/streptomycin. The emergent rosettes containing the NPCs were manually picked, dissociated and plated in a double-coated plate with pol-ornithine (10 μg ml−1, Sigma–Aldrich) and laminin (2.5 μg ml−1, Gibco). The NPC population was expanded using N2B2 medium. The neuronal differentiation induction protocol consisted of treating the confluent NPC plate with 10μM ROCK inhibitor for 48 h (Y-27632, Calbiochem) in the absence of FGF in the media, with regular media changes every 3 or 4 days. Neurons were considered completely differentiated and ready to experiments after 28 days. To produce neurospheres, NPC were scrapped from the plates and submitted to continuous shaking for 5–7 days at 90 r.p.m. in NFB media. Cerebral organoids were generated as previously described42,43. All experiments were performed with the approval of the Institute of Biomedical Sciences Ethics Committee protocol number 1001.

In vitro infection. NPCs, neurons, neurospheres and organoids were infected with ZIKVBR, ZIKVAE, YFV and mock (culture supernatant from uninfected C6/36 cells). NPCs were seeded in plates in 24-well plates and after 24 h viral samples were diluted to the desired MOI (0.1, 1 or 10) and added to the cells. For viral adsorption, cells were incubated at 37 °C with gentle agitation every 10 min. After the desired 0.1 MOI. For mock controls, the same volume of supernatant was added. Cells were incubated in the absence of FGF in the media, with regular media changes every 3 or 4 days. Neurons were considered completely differentiated and ready to experiments after 28 days. To produce neurospheres, NPC were scrapped from the plates and submitted to continuous shaking for 5–7 days at 90 r.p.m. in NFB media. Cerebral organoids were generated as previously described42,43. All experiments were performed with the approval of the Institute of Biomedical Sciences Ethics Committee protocol number 1001.

In vitro infection. NPCs, neurons, neurospheres and organoids were infected with ZIKVBR, ZIKVAE, YFV and mock (culture supernatant from uninfected C6/36 cells). NPCs were seeded in plates in 24-well plates and after 24 h viral samples were diluted to the desired MOI (0.1, 1 or 10) and added to the cells. For viral adsorption, cells were incubated at 37 °C with gentle agitation every 10 min. After the desired 0.1 MOI. For mock controls, the same volume of supernatant was added. Cells were incubated in the absence of FGF in the media, with regular media changes every 3 or 4 days. Neurons were considered completely differentiated and ready to experiments after 28 days. To produce neurospheres, NPC were scrapped from the plates and submitted to continuous shaking for 5–7 days at 90 r.p.m. in NFB media. Cerebral organoids were generated as previously described42,43. All experiments were performed with the approval of the Institute of Biomedical Sciences Ethics Committee protocol number 1001.

Immunofluorescence. Cells were fixed using paraformaldehyde, 4% in PBS, for 15 min at room temperature. After washing, the cells were permeabilized with 0.1% Triton X-100 (Promega) diluted in PBS for 15 min. After blocking with 2% of BSA (Sigma–Aldrich) for 4 h, primary antibodies directed against the following were added: anti-ZIKV (polyclonal mouse, Institute Pasteur in Dakar, 1:80), anti-Flavivirus D1–4G2–4–15 (polyclonal mouse, Millipore, 1:100), 1:50, anti-MAP2 (chicken, Abcam ab5392, 1:200), anti-cleaved-caspase-3 (rabbit, Cell Signaling #9661, 1:400), anti-Sox2 (mouse, Abcam ab97959), anti-GFAP (rabbit, Abcam, 1:500) and anti-Mushashi1 (rabbit, Abcam, 1:1000) (Extended Data Table 2). The cells were incubated in the absence of FGF in the media, with regular media changes every 3 or 4 days. Neurons were considered completely differentiated and ready to experiments after 28 days. To produce neurospheres, NPC were scrapped from the plates and submitted to continuous shaking for 5–7 days at 90 r.p.m. in NFB media. Cerebral organoids were generated as previously described42,43. All experiments were performed with the approval of the Institute of Biomedical Sciences Ethics Committee protocol number 1001.
Cerebral organoids analyses. Human and chimp organoids were infected with an MOI of 0.1 and analysed after 24 and 96 h post-infection. Organoids were cryosectioned at 20 μm. Immunofluorescence was performed after blocking sections in a solution with 0.1% Triton and 3% BSA (Gemini) for 1 h at room temperature. The primary antibodies were diluted in a solution with 0.1% Triton and 3% BSA, and the sections were incubated with following antibodies: anti-ZIKV, anti-Flavivirus, anti-MAP2, anti-cleaved-caspase-3 and anti-SOX2, all mentioned above, and anti-PAX6 (rabbit, Covance PRB-278P; 1:100), anti-TBR1 (rabbit, Abcam ab31940, 1:300), CTIP2 (rat, Abcam ab18465, 1:100) and KI67 (rabbit, Abcam ab15580, 1:100). The sections were blocked with 0.1% Triton (Sigma-Aldrich) and 3% BSA for 30 min at room temperature and the secondary antibodies previously diluted, the same mentioned above, were added. The nuclei were stained with DAPI, as mentioned above and slides were mounted with DPX (Sigma-Aldrich).

Transmission electron microscopy. Cell pellet was fixed using a 3% glutaraldehyde solution (Merck) at 4 °C for 2 h, rinsed in three changes of PBS for 1 h, and incubated for 16 h at 4 °C. The next day, post-fixation was performed with 1% of osmium tetroxide for 30 min at room temperature. Dehydration was carried out gradually with a series of ethanol concentrations: 70%, 95% and 100%. Sample was taken through two changes of propylene oxide and placed at a 1:1 ratio with embedding medium for 1 h in a rotary mixer followed by 100% embedding medium at room temperature for 24 h. Fresh embedding medium was placed overnight at 37 °C and polymerized in oven to 24 h. Ultrathin sections were cut and stained with uranyl acetate and lead citrate. The cells were visualized with a transmission electron microscope (JEOL, JEM 1011, Peabody, Massachusetts, USA). All experimental analyses were performed blinded to the treatment.

Flow cytometry. Cells were infected with an MOI of 10 or 1, prepared using supernatants from infected C6/36, and equal volume of mock. Cellular infection occurred at 4 °C for 1 h with cell homogenization every 10 min. After that, the cells were washed once and then maintained at 37 °C in CO2 incubators with medium, as described before. After 24, 48, 72 and 96 h post-infection the cells were harvested and then submitted to a staining protocol for annexin V and propidium iodide (PI) (BD Biosciences). The cells were washed twice with PBS and were harvested with 200 μl of trypsin 0.25% (LG) for 10 min at 37 °C. Next, the cell suspensions were washed in DMEM with 10% of FBS and centrifuged for 5 min at 450 and 4 °C. The cells were then resuspended in 20 μl of annexin V binding buffer in 96-well round-bottom plates and with 1 μl of FITC–annexin V + 1 μl of PI and then incubated at room temperature for 15 min protected from light. After incubation period, the samples were added to 80 μl of binding buffer and acquired in the BD FACSC Accuri C6 (BD Biosciences) flow cytometer.

Computed tomography. Mice were properly anaesthetized with isoﬂurane and immobilized on their right side on the bed with a piece of gauze and positioned with the whole body in the field of view (FOV). CT images were acquired using small animal imaging equipment (Triumph Tri-modality Gamma Medica Ideas) with 45 kVp, 0.4 mA and 2.13 min of X-ray exposition (512 projections over 360°).

The images were reconstructed using the FBP (filtered back projection) algorithm, a matrix of 512 × 512, smoothing filter and a pixel size of 92–117 μm (according to the animal size). Experienced evaluators, who were blinded to the animal group assignments, performed images analyses using the AMIDE 1.0.4, General Public License software. Fiducial markers were added to measure the distance between points considering, visually, the larger axis of the brain in the sagittal plane for posterior—anterior and superior—inferior distances and the coronal plane for the lateral right–left. For measuring whole–body length, the distance between the superior point of the brain to the first vertebra of the tail was used. The thorax measure was made from the third rib (right–left) in the coronal plane to the spinal cord level. The results are presented in mm.

Histologic processing. Tissue histology was performed using a dehydrating protocol with two alcohol baths of 95% (the first for 1 h and 15 min and the second for 30 min), three absolute alcohol baths (1 h for the first followed by 3 h for the second and 2 h for the third), followed by clarification with three baths of xylene (the first for 30 min and the next two for 1 h each). Finally, the material was added to three paraffin baths (the first for 30 min and the second two for 1 h each). Then the material was immersed in paraffin and cut with a microtome to a thickness of 5 μm. The deparaffinization protocol consisted of three xylene baths heated in an oven for 30 min each, two baths of absolute alcohol for 2 min each, two baths of 95% alcohol lasting 2 min each, an alcohol in water bath (85%) for 2 min and the last bath in 70% alcohol for 2 min. The haematoxylin and eosin staining protocol began with two quick baths in running water, followed by a 2-min bath in distilled water, a 2-min bath in haematoxylin, a 5-min bath in running water, a 1-min bath in eosin, followed by 1 min in a fast-flowing water bath, two baths of 95% ethanol for 2 min each, two baths of absolute ethanol for 2 min each, ending with three baths in xylene for 2 min each. Slides were mounted using Permound (Sigma–Aldrich) and analysed on multiple coronal slices in glass slides using light microscopy (Olympus BX40, ZEISS KS400) on a genotype–blinded fashion.

31. Stock, N. K. et al. Biological and phylogenetic characteristics of yellow fever virus lineages from West Africa. J. Virol. 87, 2895–2907 (2013).
32. de Jonge, H. J. et al. Evidence based selection of housekeeping genes. PLoS One 2, e898 (2007).
33. Marchetto, M. C. et al. A model for neural development and treatment of Rett syndrome using human induced pluripotent stem cells. Cell 143, 527–539 (2010).
34. Marchetto, M. C. et al. Differential L1 regulation in pluripotent stem cells of humans and apes. Nature 503, 525–529 (2013).
35. Beltrao-Braga, P. L. et al. Feeder-free derivation of induced pluripotent stem cells from human immature dental pulp stem cells. Cell Transplant. http://dx.doi.org/10.3727/096368911X566235 (2011).
36. Lancaster, M. A. & Knoblich, J. A. Organogenesis in a dish: modeling development and disease using organoid technologies. Science 345, 1247125 (2014).
Extended Data Figure 1 | Impact of ZIKV<sup>BR</sup> infection in the C57BL/6 and SJL mice. a, Scheme for infecting mice and the follow-up analyses. Pregnant females at approximately day 10–13 of gestation were challenged with $4 \times 10^{10}$ PFU of ZIKV<sup>BR</sup> via an intravenous route. Their pups were analysed immediately after birth for signs of malformation.
b, A representative pup from mock-infected and the ZIKV<sup>BR</sup>-infected C57BL/6 mice. Scale bar, 1 cm.
c, C57BL/6 pups born with no gross morphological changes or size differences compared to mock controls ($n = 21$ pups from three separate litters, error bars, s.e.m, $t$-test).
d, e, CT analysis confirmed lack of anatomical alterations ($n = 21$ pups from three separate litters, error bars, s.e.m, $t$-test). For scale, the crosses indicate 1.2 mm (top left; top right; bottom left) and 0.6 mm (bottom right).
f, ZIKV<sup>BR</sup> RNA was not detected in the brains of six C57BL/6 pups.
g, Cell death pathway signature revealed by qPCR gene expression in the brains of the ZIKV<sup>BR</sup>-infected SJL pups ($n = 2$ technical replicates of pooled RNA from two pups each group; threshold = twofold).
h, Heat map representation of misregulated genes in g.
Extended Data Figure 2 | Histopathological analysis of brains from ZIKV<sup>BR</sup>-infected SJL pups. Morphological aspect of hippocampus, thalamus, hypothalamus and cerebellum from brains of pups born from mothers infected with ZIKV<sup>BR</sup>. Arrowheads indicate intranuclear vacuoles and ‘empty’ nuclei aspect with chromatin margination observed in thalamus and hypothalamus. Scale bar from left to right: 100 μm, 100 μm, 50 μm and 10 μm.
Extended Data Figure 3 | Impact of ZIKV infection in human NPCs and neurons. a, Scheme of the *in vitro* experiments using hPSCs. The cells were differentiated into NPCs, neurons, neurospheres and cerebral organoids to test the impact of ZIKV BR over time. b, Infection of NPCs with the ZIKV BR and ZIKV AF (MOI = 1) at 24 and 96 h post-infection. Scale bar, 25 μm. c, Aspects of iPSC-derived human neurons after ZIKV infection (MOI = 1) at 24 and 96 h post-infection. Scale bars, 200 μm (bright field); 25 μm (immunofluorescence). d, Viral replication dynamics in human NPCs over time (MOI = 1) (n = 2 technical replicates from two different donors; error bars, s.e.m.). e, Viral replication dynamics in human neurons over time (MOI = 1) (n = 2 technical replicates from two different donors; error bars, s.e.m.). f, Dynamics of NPCs toxicity over time after ZIKV infection (MOI = 1), indicating no significant differences among the different viruses (n = 2 technical replicates from two different donors; error bars, s.e.m.). g, h, Viral replication dynamics of ZIKV in human neurons over time at MOI = 10 and MOI = 1, respectively (n = 2 technical replicates from two different donors; error bars, s.e.m.; one-way ANOVA).
Extended Data Figure 4 | Impact of ZIKV infection in human neurospheres. a, Representative bright-field images of ZIKV infection (MOI = 1) at 24 and 96 h post-infection. Scale bar, 400 μm. b, Alterations in neurospheres diameter over time (MOI = 1; n = 22 neurospheres from two different donors for each time point in each condition; error bars, s.e.m.; ANOVA, ****P < 0.0001). c, ZIKV replication dynamics in neurospheres (MOI = 1; n = 3 technical replicates from two different donors). d, Representative bright-field images of ZIKV infection (MOI = 1) at 96 h post-infection. Scale bars, 400 μm and 1,000 μm (mock). The dotted circle describes the neurospheres borders indicating the immunostained regions in e and f. e, f, Immunostaining of neurospheres infected with ZIKV at 96 h post-infection at MOI = 1 (e) or MOI = 10 (f), revealing a qualitative reduction of proliferative cell migration from Mushashi (Mus)-positive cells. Scale bars, 50 μm.
Extended Data Figure 5  |  See next page for caption.
Extended Data Figure 5 | Human and chimp cerebral organoids infected with ZIKV. a, Representative image of an entire cross-section of a cerebral human organoid infected with the ZIKV\textsuperscript{BR} (MOI = 0.1, 24 h post-infection). Scale bar, 200 µm. b, Detail of the surface of a human organoid infected with the ZIKV\textsuperscript{BR} at 24 h post-infection (MOI = 0.1). Marginal zone (MZ) and cortical plate (CP) delineated by dotted white lines. Scale bar, 200 µm. c, Detail of the surface of a human organoid infected with the ZIKV\textsuperscript{BR} at 96 h post-infection (MOI = 0.1). Notice the significant tissue damage and reduction in the CP at 24 h post-infection. Scale bar, 200 µm. d, A representative characterization of CP and ventricular zone (VZ) in human organoid infected with the ZIKV\textsuperscript{BR} at 24 and 96 h post-infection (MOI = 0.1). Scale bar, 50 µm. e, Reduction in the cortical thickness measured by the extension of TBR1-positive layer of cells in human organoids at 96 h post-infection (MOI = 0.1; n = 3 replicates from three human cell lines; error bars, s.e.m.; unpaired t-test, **P = 0.0005). g, Nuclear size (diameter) of cleaved-caspase-3-positive apoptotic cells in human organoids at 96 h post-infection (MOI = 0.1; n = 10 organoids/slides from three human cell lines; error bars, s.e.m.; unpaired t-test, ***P = 0.0004). h, Percentage of TUNEL-positive cells in relation to controls (dotted line) at 24 and 96 h post-infection (MOI = 0.1; n = 10 organoids/slides from three human cell lines; error bars, s.e.m.; ANOVA, ***P = 0.0042). i, Percentage of TBR1-positive cells in non-primate organoids (chimp) in relation to controls (dotted line) at 24 and 96 h post-infection (MOI = 0.1; n = 3 organoids from two donors, error bars, s.e.m.; ANOVA). j, Percentage of CTIP2-positive cells in non-primate organoids (chimp) in relation to controls (dotted line) at 24 and 96 h post-infection (MOI = 0.1; n = 3 organoids from two donors; error bars, s.e.m.; ANOVA). k, Viral replication dynamics in chimpanzee organoids over time (MOI = 0.1; n = 3 replicates from two donors; error bars, s.e.m.; ANOVA).
**Extended Data Table 1 | Probes used for TAM receptors detection**

| Target  | Species | Code            |
|---------|---------|-----------------|
| Tyro-3  | Human   | Hs_00170723_m1  |
| Axl     | Human   | Hs_01064444_m1  |
| MertK   | Human   | Hs_01031973_m1  |
| DC-Sign | Human   | Hs_01588349_m1  |
| β-actin | Human   | Hs_01060665-m1  |
### Antibodies used in this study, related to experimental procedures

| Cell type/tissue                  | Antigen          | Host            | Supplier                  | Cat number   | Dilution |
|----------------------------------|------------------|-----------------|---------------------------|--------------|----------|
| Zika virus                       | ZIKV             | Mouse polyclonal| Institute Pasteur in Dakar| MAB10216     | 1:50     |
| Flavivirus                       | Flavivirus       | Mouse polyclonal| Millipore                 | ab5392       | 1:200    |
| Neurons                          | MAP2             | Chicken polyclonal| Abcam                  | ab5392       | 1:200    |
| Apoptosis                        | Cleaved caspase-3| Rabbit polyclonal| Cell Signaling           | 9661         | 1:400    |
| Progenitor cell                  | Sox2             | Mouse monoclonal| Abcam                    | ab79351      | 1:200    |
| Astrocytes                       | GFAP             | Rabbit polyclonal| Abcam                    | ab7260       | 1:500    |
| Neuronal progenitor cells        | Mushashi1        | Rabbit polyclonal| Abcam                    | ab21618      | 1:200    |
| Neuronal progenitor cells        | PAX6             | Rabbit polyclonal| Covance                  | PRB-278P     | 1:100    |
| Pre-plate/deep-layer cortical neurons | TBR1            | rabbit polyclonal| Abcam                    | ab31940      | 1:400    |
| Deep-layer cortical neurons      | CTIP2            | rat monoclonal  | Abcam                    | ab18465      | 1:100    |
| Cell proliferation               | Ki67             | rabbit polyclonal| Abcam                    | ab15580      | 1:100    |
| Neurons                          | TUJ1             | mouse polyclonal| Covance                  | MMS-435P     | 1:500    |