Patologia: Doenças Virais

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APRESENTAÇÃO

No volume I da coleção Patologia intitulado: Doenças Virais, apresentamos em capítulos, diversos artigos de pesquisas realizadas em diferentes regiões. A temática inclui estudos sobre infecções virais por adenovírus, retrovírus e arbovírus; dados epidemiológicos, diagnósticos e tratamentos, bem como temáticas correlacionadas.

Os vírus são microscópicos agentes infecciosos acelulares, formados em sua maioria por uma cápsula proteica envolvendo o material genético, que necessitam do metabolismo de células hospedeiras para realizarem atividades como: nutrição, reprodução e propagação. Em muitos casos os vírus modificam o metabolismo da célula que parasitam, podendo provocar a sua degeneração; o que pode acarretar riscos potenciais à saúde do organismo como um todo.

As infecções podem acometer desde seres unicelulares até pluricelulares, como os humanos. Em humanos, é responsável por várias doenças em que a transmissão, sintomas e tratamentos são peculiares ao respectivo agente patogênico. Além disso, existe uma complexa interação entre o hospedeiro, reservatórios e vetores a ser explorada para que novas abordagens sejam colocadas em prática.

O estudo dos aspectos relacionados às infecções virais, bem como de suas incidências regionais, constitui-se uma importante ferramenta para ações de prevenção, diagnóstico e tratamento. Neste volume I, buscamos ampliar o conhecimento destas patologias e seus dados epidemiológicos, contribuindo assim para a formulação de políticas públicas de apoio dirigidas às macro e micro regiões.

A obra é fruto do esforço e dedicação das pesquisas dos autores e colaboradores de cada capítulo e da Atena Editora em elaborar este projeto de disseminação de conhecimento e da pesquisa brasileira. Espero que este livro possa somar conhecimentos e permitir uma visão critica e contextualizada; além de inspirar os leitores a contribuírem com pesquisas para a promoção de saúde e bem estar social.

Yvanna Carla de Souza Salgado

SUMÁRIO

CAPÍTULO 18

PLACENTAL INFLAMMATION AND FETAL INJURY IN A RARE ZIKA CASE ASSOCIATED WITH GUILLAIN-BARRÉ SYNDROME AND ABORTION

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ABSTRACT: Zika virus (ZIKV) is an emerging virus involved in recent outbreaks in Brazil. The association between the virus and Guillain-Barré syndrome (GBS) or congenital disorders has raised a worldwide concern. In this work, we investigated a rare Zika case, which was associated with GBS and spontaneous retained abortion. Using specific anti-ZIKV staining, the virus was identified in placenta (mainly in Hofbauer cells) and in several fetal tissues, such as brain, lungs, kidneys, skin and liver. Histological analyses of the placenta and fetal organs revealed different types of tissue abnormalities, which included inflammation, hemorrhage, edema and necrosis in placenta, as well as tissue disorganization in the fetus. Increased cellularity (Hofbauer cells and TCD8^{+} lymphocytes), expression of local pro-inflammatory cytokines such as IFN-γ and TNF-α, and other markers, such as RANTES/CCL5 and

VEGFR2, supported placental inflammation and dysfunction. The commitment of the maternal-fetal link in association with fetal damage gave rise to a discussion regarding the influence of the maternal immunity towards the fetal development. Findings presented in this work may help understanding the ZIKV immunopathogenesis under the rare contexts of spontaneous abortions in association with Guillain-Barré syndrome.
KEYWORDS: Zika virus, immune response, Guillain-Barré syndrome, fetal infection, histopathology

INTRODUCTION

Zika virus (ZIKV) is an emerging mosquito-borne pathogen that belongs to the Spondweni serocomplex of the Flavivirus genus, Flaviviridae family (Didier Musso and Gublerb, 2016). Zika fever emerged in Latin America in 2015–2016 and rapidly became a worldwide public health concern (Singh et al., 2016; Slavov et al., 2016). This massive outbreak highlighted possible correlations between the infection and dangerous complications such as Guillain-Barré syndrome (GBS) (Araujo et al., 2016; Malkki, 2016) and congenital microcephaly (Araujo et al., 2016; Garcez et al., 2016). In the absence of specific treatment or vaccine, only in Brazil the number of accumulated infections was estimated between 440,000 and 1,300,000 (Bogoch et al., 2016), with a prevalence of microcephaly of nearly 100 cases per 100,000 live births (Ventura et al., 2016). As an outcome, several unanswered questions, especially regarding the circumstances that may explain a possible connection between the infection and these complications, became a relevant matter of debate.

Initial attempts to model the vertical ZIKV transmission included investigations using immunocompetent mice (Cugola et al., 2016; Vermillion et al., 2017). As these animals are in general resistant to the infection due to virus inability to circumvent the interferon-α/β response (Grant et al., 2016), these models are limited in providing mechanistic explanations to describe pathogenesis. In alternative approaches, genetically modified animals, which are knockout for interferon receptors (IFNARs) or downstream signaling targets, such as IRF3 and IRF7, were employed for investigation (Miner et al., 2016; Yockey et al., 2016). In these reports, despite the characterization of injuries in the fetal brain and the viral escape through the trans-placental route, the animal immunological restriction limited the comprehension of the host response upon infection. In a recent report, an animal model of ZIKV infection involving pregnant non-human primates revealed that, upon prolonged viremia, several fetal tissues, as well as the maternal-fetal interface, were affected (Nguyen et al., 2017). Subjects were found to respond to ZIKV with proliferation of CD16+ NK cells and CD8+ effector T cells. In addition, the maternal-fetal interface was marked by suppurative placentitis and deciduitis with variable mineralization and necrosis. While reports based on ZIKV-infected non-human primates are valuable due to deep similarities between macaques

and human pregnancies, for a better description of the immunopathological events and their impact towards the maternal-fetal link it would be ideal to explore human case samples.

BACKGROUND

Clinical presentation

All procedures in this work were approved by the FIOCRUZ Ethics Committee for studies with Zika case and control (CAEE: 65924217.4.0000.5248). The legal representative (mother) of the involved patient provided written consent for the publication of data.

A 28-year-old woman, pregnant, black, housekeeper, from São Francisco do Itabapoana, RJ, was admitted to the hospital on June 29th 2016, presenting weakness in the lower limbs and a consequent inability to ambulate for the previous week. The patient affirmed not having experienced similar episodes previously and claimed to be free of comorbidities. The patient reported rash on her limbs, pruritus and vomiting approximately one month before admission to the hospital. Initial obstetric examinations showed globular abdomen, unlimited uterus and absence of vaginal bleeding. The fetal heartbeat was not detected in the Sonar Doppler. Transvaginal ultrasonography evidenced a single fetus with longitudinal status and cephalic circumference suggesting 15 weeks of gestational age, normohydramnios, lack of heartbeat, and non-apparent active movements, which lead to the diagnosis of death and retained fetus (stillbirth). The patient was admitted to curettage and remained in hospital for 30 days. Analysis of the cerebrospinal fluid obtained by lumbar puncture and exams of the patient's peripheral blood revealed normal parameters (Table S1). The IgM serology for Dengue, Chikungunya, Zika, Epstein Barr and Cytomegalovirus were non-reactive (in further sections of this paper, diagnosis of ZIKV infection was confirmed by specific staining in placental and fetal tissues). The patient evolved to ascending and symmetrical flaccid tetraparesis, paresthesia, areflexia, presenting hands with pendular movement, dysautonomia (resting tachycardia and hypertension) and signs of respiratory insufficiency (mild dyspnea at rest which worsens upon effort), characterizing the Guillain-Barré syndrome. The patient was further treated with intravenous immunoglobulin (30g/day) for five days, atenolol 50mg 12/12h, amlodipine 5mg 24h, motor rehabilitation, respiratory physiotherapy and psychological intervention. In the second week of disease evolution, the neurological examination showed symmetric flaccid tetraparesis, motor incoordination, global areflexia and sensitive disorders (tactile and thermal distal hypoesthesia). Neurological follow up during and after the hospitalization period are described in Table S2. During the fourth week of disease progress, the patient was admitted to electroneuromyography, which revealed

peripheral, acquired, chronic, symmetrical, diffuse, myelin and axonal (predominantly myelin), sensory and motor polyneuropathy: a condition that affected the peripheral nerves of the upper and lower limbs. In addition to that, the patient showed signs of neurogenic myopathy and muscular denervation. Together, these findings were compatible with polyradiculoneuropathy. By the end of the fourth week, the patient was discharged from the hospital under prescription of antihypertensive drugs and still being followed up by motor physical therapy. After 40 days of hospital discharge, the patient was orthostatic, walking-dependent for small distances and with pain sequels in the lower limbs. Five months later, residual sequels were still present with autonomous difficult scarvant gait. After twelve months, the gait patterns were regular; however, the speed and execution of upward and downward movements remained affected. Finally, the patient returned to her daily activities. Methods performed in this work using the placenta and fetal organs are described in Supplementary Material.

RESULTS

Investigation of the placental tissue

ZIKV infection leads to histopathological damage in placenta

The histopathological analysis considering the patient's placenta showed relevant damage in the membrane, maternal decidua and chorionic villi. We can highlight large areas of hemorrhage, diffuse fibrinoid necrosis and inflammatory infiltrates formed by mononuclear cells. In addition, regions with cell degeneration and macrophages with clear cytoplasm in membrane and chorionic villi, decidual edema and macrophages were also found. We also noted a decrease in blood vessels of chorionic villi. The decidual region showed dense calcification, which is commonly observed only during the third trimester of gestation (Fig. 1D-K). As expected, control samples showed regular arrangement of decidual parenchyma. Controls also exhibited normal chorionic villi, syncytotrophoblasts, cytotrophoblasts and endothelial cells (Fig. 1A-C).

The patient's placental tissue was screened for the detection of ZIKV NS1 protein and E protein using immunohistochemistry. Of note, the anti-NS1 antibody used in these assays is ZIKV specific, thus, is able to differentiate ZIKV from other flaviviruses. While the viral antigens were detected in samples from the affected patient, no immunostaining was observed in samples considering the control placenta (Fig. 1L, M, P and Q). E structural viral proteins were detected in decidual cells of the maternal portion, cytotrophoblasts and mesenchymal cells of chorionic villi (Fig. 1N and O). In the placental portion towards the fetal side, the NS1 protein was detected in cytotrophoblasts, Hofbauer cells of chorionic villi and also in decidual cells (Fig. 1R, S and T). Viral detection occurred mainly within the cytoplasmic region of cells with minor to indistinguishable staining in the nuclear area. This staining pattern strongly suggests

that viral replication occurred in these target cells.

Characterization of cell subpopulations, colocalization with virus and quantification of cytokine-producing cells

Since the histopathological analysis showed inflammatory infiltrates in both maternal and fetal areas, we proceeded with immunohistochemical characterization of the cell types present in this tissue. For this, we used anti-CD68 antibodies to stain the Hofbauer cells and anti-CD8/ anti-CD4 antibodies for phenotype arriving lymphocytes. Staining with anti-CD68 revealed an increase in hyperplasic Hofbauer cells spread in chorionic villi and decidua basalis (Fig. 2 C-D). While $CD8^+$ cells were found in the same areas (Fig. 2 H-I), $CD4^+$ cells were not detected within the tissue. The control tissue showed low density of positive cells (Fig. 2 A-B, F-G). After quantification considering 50 distinct fields, the numbers of both $CD68^+$ and $CD8^+$ cells were significantly increased (6 and 4 fold, respectively) in the placenta of the Zika patient, when compared to the control (Fig. 2E and J).

Further evidence for ZIKV infection in specific cell subpopulations was provided by immunofluorescence assay. In this case, $CD11b^+$ cells (red fluorescence, which we considered as the mononuclear cells) costained with anti-ZIKV NS1 signals (green fluorescence) within several areas of the patient's placenta (Fig. 2L). Under this analysis, ZIKV NS1 was also detected in placental cells that were negative for anti-CD11b. As expected, no positive reactions against NS1 were observed in the control tissue (Fig. 2K).

To better characterize the inflammatory process in the patient's placenta, we also investigated the local expression of cytokines related to inflammation. Under this approach, we verified the expression of: TNF-α and IFN-γ, due to their well-known participation in a pro-inflammatory context; and VEGFR2 and RANTES/CCL5, since these markers are implicated with altered vascular permeability (Chen et al., 2008; Dalrymple and Mackow, 2012). TNF-α was detected in Hofbauer cells of decidua and chorionic villi (Fig. 2N), while expression of IFN-γ was found mostly in macrophages of membrane and decidua (Fig.2Q). The expression of VEGFR2 was found also in macrophages throughout the placental membrane (Fig. 2T). The chemokine RANTES/CCL5 was detected mainly in the endothelium and in Hofbauer cells located within the chorionic villi and decidua (Fig. 2X). Cells expressing all these cytokines were found in the control tissue, but in smaller amounts (Fig. 2M, P, S and V). The numbers of cells expressing all these considered markers were significantly increased in the Zika patient's placenta, when contrasted with the non-Zika control tissues (Fig. 2O, R, U and Z).

INVESTIGATION OF THE FETAL TISSUES

Histopathological alterations in fetal organs caused by ZIKV

The histopathological analysis of the brain tissues collected from the Zika patient's fetus revealed diffuse areas of edema, disorganization of the cerebral cortex layers, mainly in the layer of polymorphic cells and degenerate nerve fibers (Fig. 3B). The analysis of the lung tissues revealed several damaged areas with disorganization of the bronchioles architecture associated with focal areas of hyaline membrane. Other alterations in the lungs included regions of septal thickening, increased cellularity, necrosis in the respiratory epithelium accompanied by cell detachments and the presence of mononuclear cell inflammatory infiltrates (Fig. 3D). The skin from the affected fetus presented diffuse areas of edema associated with perivascular lymphocytic infiltrate in the dermis region (Fig. 3F). In the kidneys, some areas of glomeruli ischemia were observed causing loss of the tubule architecture and its degeneration. This observation was associated with focal mononuclear cell infiltrates (Fig. 3H). Liver fetal samples from the Zika patient exhibited severe parenchyma and circulatory disorganization. In this organ, the most prominent lesions were the cellular and matrix degenerations that were associated with increased numbers of Kupffer cells (Fig. 3J). As expected, when considering the samples extracted from the control fetus, all the analyzed sites (brain, lungs, skin, kidneys and liver) presented regular structures (Fig. 3A, C, E, G and I).

Detection of ZIKV antigens in fetal organs

Using IHC technique to investigate the Zika patient's fetus we detected the flaviviral-E and ZIKV-NS1 proteins in microglial cells and neurons within the cerebral cortex of the brain tissue (Fig. 4B, L). In the lung, these proteins were detected in alveolar macrophages (Fig. 4D, N), in mononuclear cells of skin (Fig. 4F, P), in macrophages of the kidneys (Fig. 6H, R), and, finally, in hepatocytes and Kupffer cells of the liver (Fig. 4J, T). No E or NS1 immunostaining was observed in samples from the control organs (Fig. 4A, C, E, G, I, K, M, O, Q, S).

DISCUSSION

The incidence of GBS in Brazil has been evidently increasing after the ZIKV outbreak (Oehler et al., 2014). A recent study showed ZIKV cases with neurological alterations similar to those found in our study, defined as GBS. In these cases, the viremia appeared to persist for longer than normal (Ferreira et al., 2017). While the relationship between Zika fever and GBS still relies solely on epidemiological data, the description of the viral influence towards congenital malformations has become less enigmatic. In this work, we investigated a rare Zika case, which was associated with GBS and spontaneous retained abortion during the 15th week of fetal development. Viral infection was characterized in placental and in several fetal tissues. As found previously (Schaub et al., 2017), this scenario suggested that the case was involved with a high or persistent viremia. A limitation in our study was the unavailability of

cerebrospinal (CSF) and amniotic fluid to analize the presence of viral RNA to confirm the high viremia. However, the histological analysis of the patient's placenta and fetal organs revealed different types of tissue abnormalities, which included inflammation, hemorrhage, edema and necrosis in placenta and tissue disorganization in the fetus. The patient presented negative IgM serology for several viruses, including ZIKV; however, ZIKV could be detected directly in tissue samples. Based on this, any of the tested viruses (dengue, chikungunya, Epstein-Barr and Human Citomegalovirus) could also potentially be contributing to the outcome. Nonetheless, given the peculiarity of the clinical presentation, the epidemiological aspect involved, and obviously, the characterization of ZIKV in several areas, we believe that ZIKV may have contributed as the major component for the observed alterations. Of note, the present case happened in an area that had no incidence of yellow fever, which also reduces a probable influence of this disease in the observed results.

In the particular case of the observed placental alterations, one fact that drew our attention was the local increase of numbers of Hofbauer cells in association with the viral infection and pro-inflammatory cytokine production. Infection of Hofbauer cells during the antenatal period not only reflects the critical failure of the protective arrangement, but also highlights a potential pathway for ZIKV vertical transmission. In fact, several research groups have demonstrated either histologically (Noronha et al., 2016; Rabelo et al., 2017; Rosenberg et al., 2016)the intrauterine fetal exposure to ZIKV was associated with a significant risk of developing microcephaly and neurological disorders in the infected infants. ZIKV-associated disease has since been reported in 24 countries in the Americas. At present, definitive evidence is lacking regarding the intrauterine co-exposure to ZIKV and other viral infections and whether the coinfection impacts the risk of acquiring either infection or disease severity. Here, we provide evidence of intrauterine exposure to both ZIKV and human immunodeficiency virus (HIV or by isolated cultures/explants (Jurado et al., 2016; Quicke et al., 2016; Tabata et al., 2016) that these placental macrophages are highly permissive to ZIKV replication. This observation matches the findings from Rosenberg and colleagues, that in a histological study of a Zika case also detected proliferation and hyperplasia of such resident placental cells (Rosenberg et al., 2016). Another indication that ZIKV-infected placental cells were targeted by immune activation was the detection of $TCD8^{+}$ cell infiltrates within the tissue. As broadly investigated previously, TCD8-mediated cellular immunity is apparently critical for host's defense against ZIKV infection (Huang et al., 2017; Ngono et al., 2017; Pardy et al., 2017; Wen et al., 2017). Since we found elevated expression of local pro-inflammatory cytokines (IFN-γ and TNF-α), one hypothesis to explain this scenario is that ZIKV-infected Hofbauer cells may have contributed to the establishment of a chemotactic environment for the arrival of specific lymphocytes.

The considerations exposed above gave rise to a little explored discussion when considering the maternal-fetal link under a viral influence: the maternal immune activation (MIA). Initial thoughts considered pregnancy as a temporary immunosuppressed

condition that would be necessary to allow a successful fetal development (Medawar, 1948; Silasi et al., 2015). Nowadays, MIA is thought to be a complex process that changes in a dynamical fashion as the pregnancy evolves (Mor and Cardenas, 2010; Racicot et al., 2014; Silasi et al., 2015). Hypothetically, this entire process culminates in a maternal environment designed to sustain and to protect the pregnancy. In general, when the placenta is targeted by viruses, this organ presents an outstanding capacity to hold back infection, and consequently, to prevent the virus from spreading towards the developing fetus (Bayer et al., 2015; Cardenas et al., 2010; Ouyang et al., 2014; Romero et al., 2007). Conversely, what we saw in the Zika case exposed in this work is much closer to an exception of the above outlook. Due to a yet unknown mechanism, ZIKV seems to hold a unique capacity to circumvent MIA and therefore promote relevant infection and inflammation throughout the placental tissue. Considering the patient's placental conditions, we hypothesize that this fact probably created a bridge between the maternal infection and the effects observed in the developing fetus.

The patient's placental dysfunction caused by ZIKV infection, given the local inflammation and possible altered vascular permeability (as evidenced by the over-expression of RANTES/CCL5 and VEGFR2), may have impaired the normal balance of this hormonal distribution and consequently negatively contributed to fetal development. Other authors have also proposed that placental and decidual inflammation by ZIKV, or other viruses, would critically impact in the normal development of the fetus (Mor, 2016; Silasi et al., 2015). The overexpression of VEGFRs has previously been associated with pathophysiological damage in placentae, while RANTES expression has been found in large quantities in the acute phase of ZIKV infection (Tappe et al., 2016; Tsatsaris et al., 2003)placentas, and placental bed biopsies were collected. The mRNA levels of VEGF-A, PlGF, and their receptors were quantified in placentas and placental beds. Levels of VEGF-A, PlGF, and soluble VEGF receptor (VEGFR. In this sense, a new assumption is proposed for the circumstances by which ZIKV is able to break through the biological placental barrier and to debilitate the pregnancy as a whole.

Although the fetal control tissue samples were at 15 weeks of embryological development and the organs were not yet mature, we observed large histopathological differences between these and tissue samples infected by ZIKV. These injuries are probably due to prolonged viremia in the mother, leading to GBS, fetal involvement and consequently retained abortion. IHC analysis of the patient's fetal brain revealed that this organ was targeted by ZIKV infection, in special the microglial cell types. Several other reports showed that the developing fetal central nervous system (CNS) is highly permissive to ZIKV replication (Kuivanen et al., 2017; Lin et al., 2017; Qian et al., 2017; Rosenfeld et al., 2017). However, despite the well-known tropism of ZIKV to the developing CNS, our findings showed that in the peculiar case of the studied fetus, the infection went beyond the cerebral structure and was found in several peripheral tissues. The commitment of other fetal sites such as the lungs, skin, kidneys and liver supported an idea that the patient was under high or persistent viremia. At the same

time, this observation highlights the possibility of novel target tissues when considering an extreme situation, as noted by the clinical features of the studied patient.

CONCLUDING REMARKS

This work describes placental and fetal abnormalities found in a rare Zika case involved with GBS and spontaneous abortion. The clinical scenario gave rise to a novel discussion regarding the influence of maternal immunity towards fetal development. Given the unrecognized prevalence of such an uncommon clinical presentation, samples used in this work are valuable for studying the parallel between the infection and the occurrence of GBS and abortion. Findings from this work may add to the current description of ZIKV congenital pathogenesis.

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AUTHOR CONTRIBUTIONS

KR, MP, and JC designed the study. LJS, ML, PS, FR and LPS, collected samples and performed clinical exams. KR, NGS, and RO performed all research experiments for placental and fetal evaluation. KR and EO wrote the manuscript. KR, MVP and JC analyzed the experimental results. All authors gave final approval in the manuscript.

Declaration of interest

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Figure legends

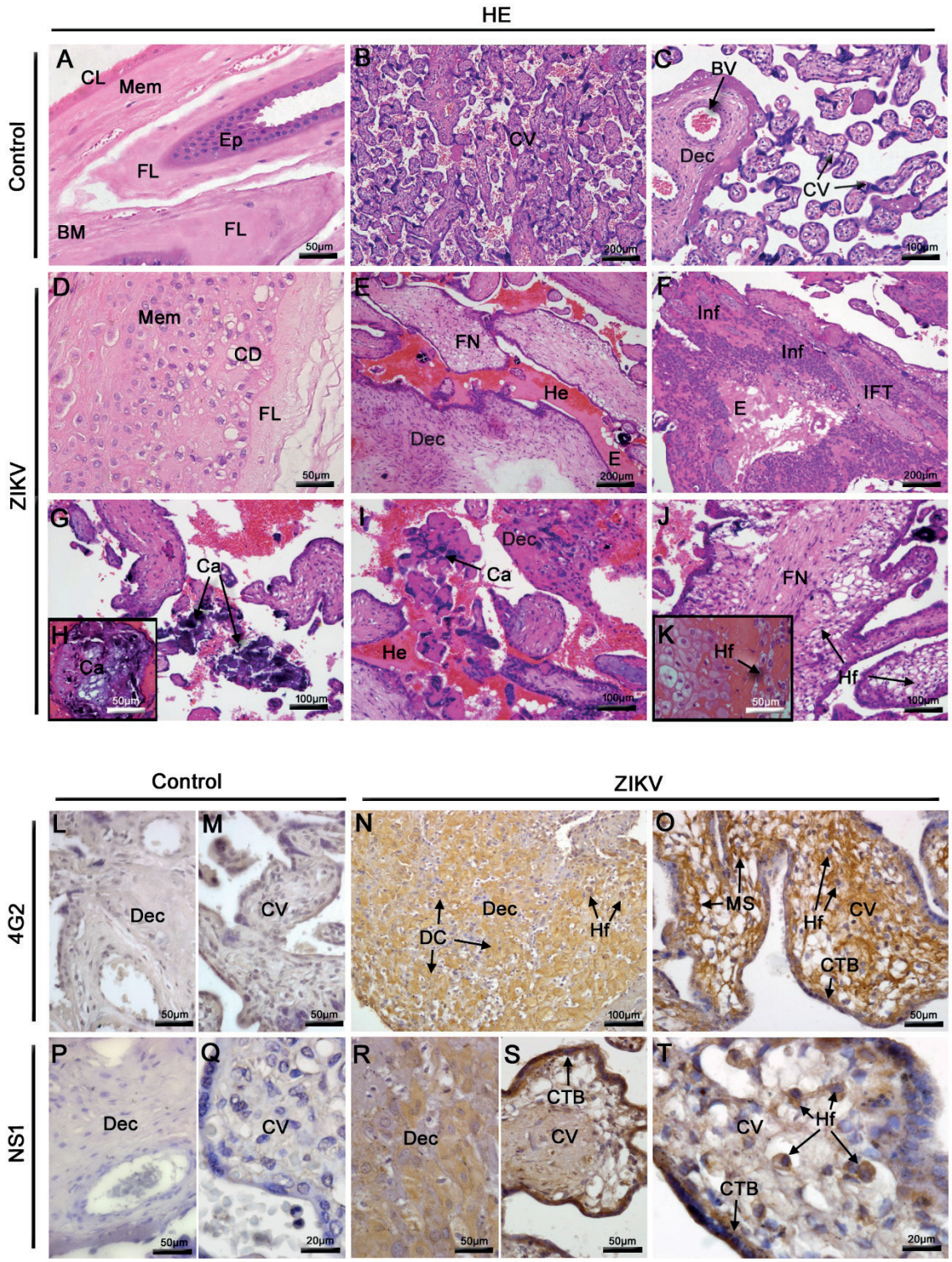

Figure 1: Histopathological analysis of the placenta and detection of ZIKV. (A- C) Placenta of a non-ZIKV patient stained with H.E. and presenting normal features: membrane (Mem), (MB) basal membrane, (FL) fibroblastic layer, (CL) compact layer, (Ep) epithelium, chorionic villi (CV), maternal decidua (Dec), and blood vessels (BV). (**D- K**) Sections of ZIKV-infected placental tissue stained with H.E., showing abnormalities in membrane, with cellular degeneration (CD), in the decidua and chorionic villi, including fibrinoid necrosis (FN), hemorrhage (He), mononuclear inflammatory infiltrate (Inf), infarct (IFT), calcification (Ca) and Hofbauer cells with clear cytoplasm (Hf). (**L-M, P-Q**) The flavivirus E protein and NS1 antigens of ZIKV were not detected by immunohistochemistry in the control placenta. (**N-O**) Detection of ZIKV E protein in decidual cells (DC), cytotrophoblasts (CTB), mesenchymal cells (MS) and Hofbauer cells (Hf) of the infected placenta. (**R-T**) The NS1 protein of ZIKV was also detected by immunohistochemistry in decidual cells (DC), cytotrophoblasts (CTB) and Hofbauer cells (Hf).

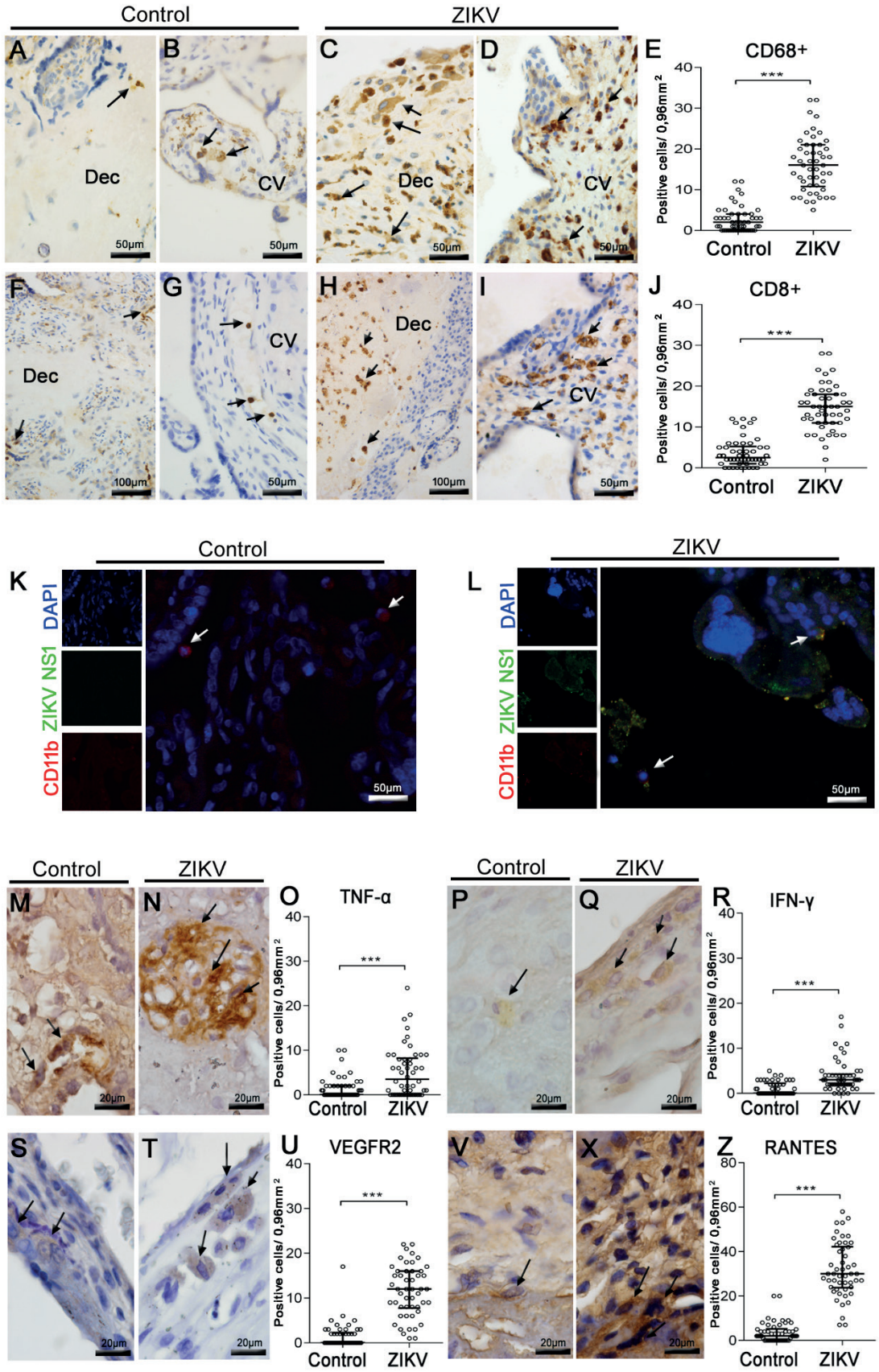

Figure 2: Characterization of mononuclear cell subpopulations in ZIKV-infected placental tissue, colocalization with virus and cytokine-producing cell profile. (A, B) Detection of CD68⁺ cells (Hofbauer cells) by immunohistochemistry in decidua and chorionic villi of control placenta, respectively. (**C, D**) Hofbauer cells in decidua and chorionic villi of ZIKV infected placental tissue, respectively. (**F, G**) Detection of CD8⁺ cells by immunohistochemistry in decidua and chorionic villi of control placenta, respectively. (**H, I**) CD8⁺ cells immunostained in decidua and chorionic villi of ZIKV-infected placental tissue, respectively. (**E, J**) Quantification of CD68⁺ and CD8⁺ cells in ZIKV case and control, respectively. (**K- L**) Colocalization by immunofluorescence of the NS1 protein (fluorescent green) and CD11b for identification of

leukocytes (fluorescent red). Nuclei were stained using DAPI (fluorescent blue). (**K**) ZIKV NS1 antigen was not detected in the control placenta. (**L**) Cells presenting dual staining (green and red) were observed in the ZIKV-infected placenta. (**M, N**). Detection of TNF-α in cells of chorionic villi of control and ZIKV infected placenta by immunohistochemistry, respectively. (**P, Q**) Production of IFN-γ in cells of membrane of control and ZIKV infected placenta, respectively. (**S, T**) VEGFR2-expressing cells of decidua in control and ZIKV infected placenta, respectively. (**V, X**) Detection of CCL5/RANTES in cells of chorionic villi of control and ZIKV infected placenta, respectively. (**O, R, U and Z**) Quantification of the number of cells expressing TNF-α, IFN-γ, VEGFR2 and CCL5/RANTES, in ZIKV case and control, respectively. Asterisks indicate differences that are statistically significant between groups (***$p < 0.001$).

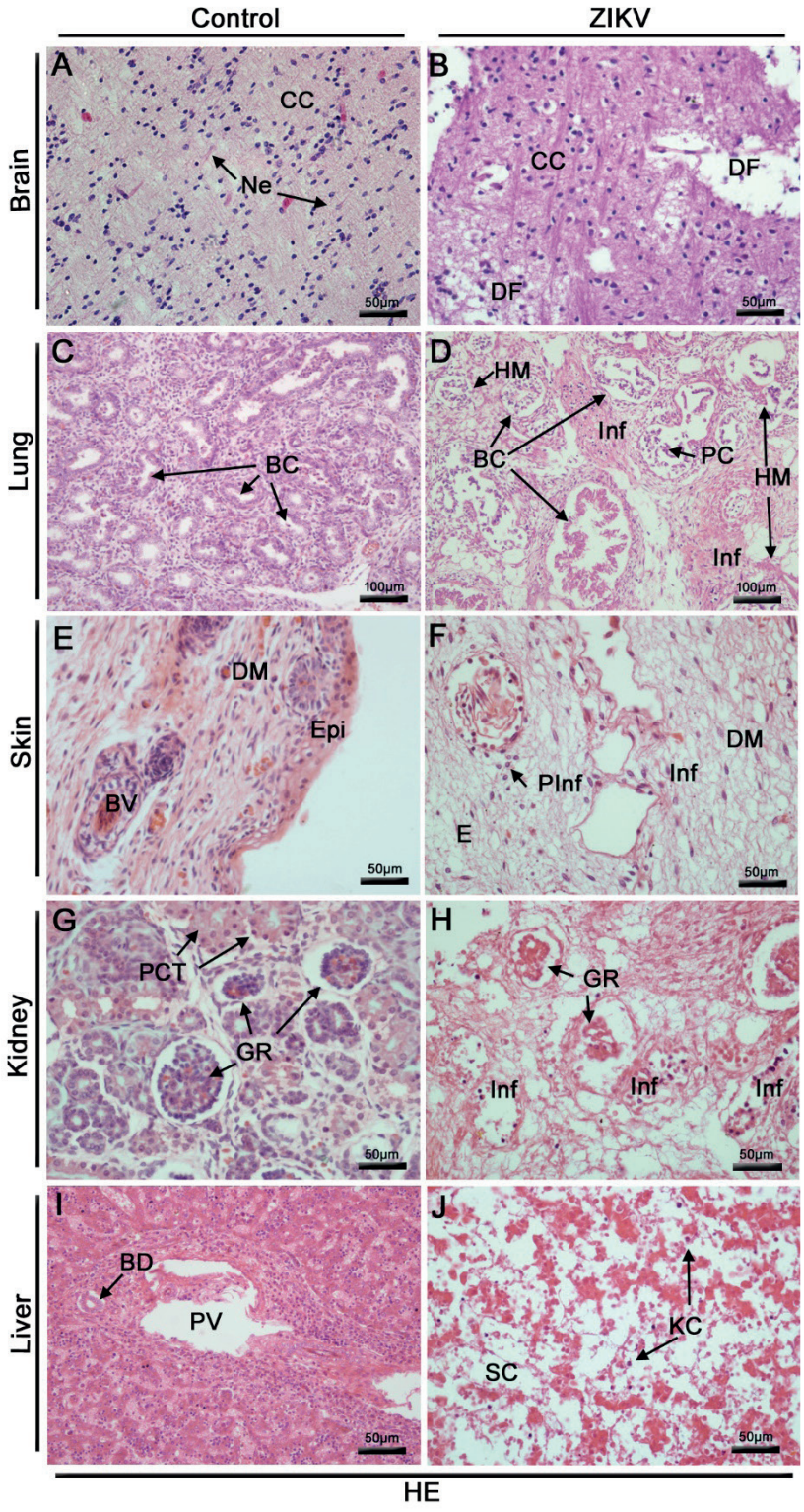

Figure 3: **Histopathological analysis of the fetal organs.** (**A-J**) All tissues were stained with H. E. (**A**) Brain of a non-ZIKV case presenting normal aspect: cerebral cortex (CC) and neurons (Ne). (**B**) Brain of a ZIKV infected fetus, presenting areas of degenerated nerve fibers (DF). (**C**) Lung section of a control fetus showing normal bronchioles (BC). (**D**) Injuries in fetal lung infected by ZIKV: disorganized bronchioles (DBC) with loss of cylindrical appearance, focal areas of hyaline membrane (HM), diffuse mononuclear infiltrates (Inf) and peeled cells of respiratory epithelium (PC). (**E**) Skin dermis (DM) of a non-ZIKV case presenting normal aspect, epidermis (Epi), blood vessel (BV). (**F**) Skin dermis of a ZIKV infected fetus, with perivascular and mononuclear infiltrate (PInf and Inf) and areas of edema (E). (**G**) Kidney of a non-ZIKV case presenting normal aspect, with normal glomerulus (GR) and proximal contorted tubules (PCT). (**H**) Kidney sections showing injuries, including: disorganized renal glomerulus (GR), tubular disarrangement and inflammatory infiltrate (Inf). (**I**) Section of a control liver, with normal bile duct (BD) and portal vein (PV). (**J**) Liver of a ZIKV infected fetus, presenting dilatation of sinusoidal capillaries (SC), hepatic parenchymal disorganization and Kupffer cells (KC).

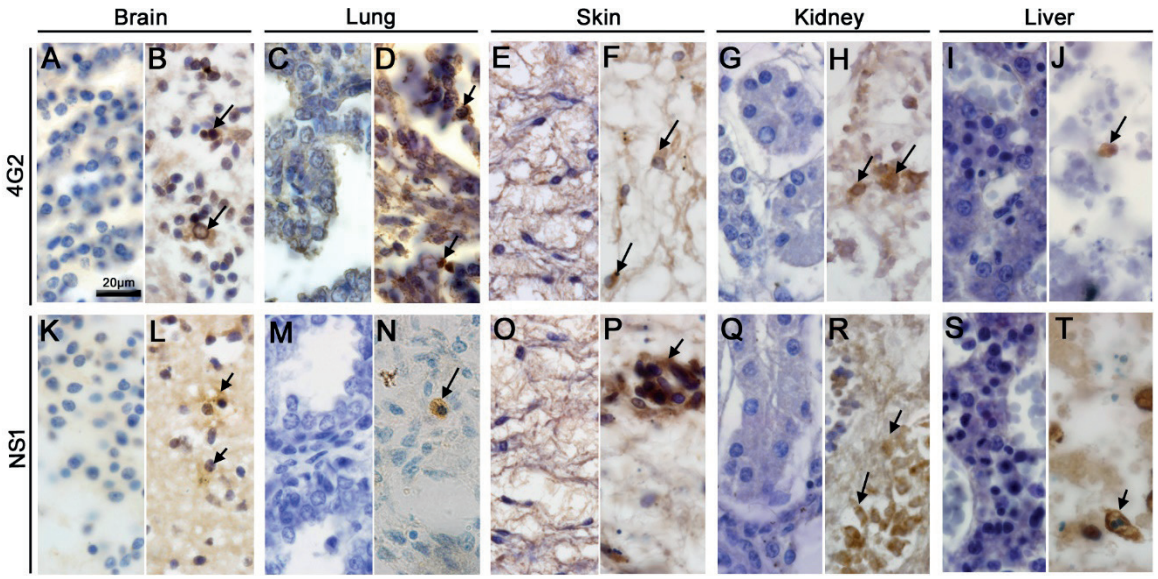

Figure 4: **Detection of ZIKV antigens in fetal organs.** (**B, D, F, H, J, L, N, P, R, T**) The flaviviral E and ZIKV-NS1 protein were detected in all tissues studied. (**B, L**) Microglial cells and neurons of the brain tissue were positive for ZIKV E protein and NS1, respectively. (**D, N**) ZIKV E and NS1 proteins were detected in alveolar macrophages. (**F, P**) Section of a skin dermis showed mononuclear cells of an inflammatory infiltrate and endothelial cells stained respectively to ZIKV E and NS1 proteins. (**H, R**) ZIKV E protein and NS1 detection in macrophages of the kidney. (**J, T**) Detection of ZIKV E protein and NS1 in hepatocytes, respectively. (**A, C, E, G, I, K, M, O, Q, S**) The E and NS1 antigens of ZIKV were not detected by immunohistochemistry in the control fetus.

Supplementary material

Material and Methods

Ethics and sample collection

All procedures performed during this work were approved by the Ethics Committee of the Oswaldo Cruz Foundation/FIOCRUZ for studies with Zika case and control (CAEE: 65924217.4.0000.5248). The legal representative (mother) of the involved patient provided written consent and permission for the publication of data and images.

The delivery and specialized care of the patient were performed in Plantadores de Cana Hospital, in Campos dos Goytacazes, Brazil. The placenta and fetus were immediately collected and fixed accordingly to the techniques described below. Consent and permission were obtained from the patient and the institution for comparison purposes (controls) the following samples were considered: placental tissue obtained from a non-Zika case; and a fetus originated from spontaneous abortion at the same stage of development found in the Zika case. The fetus did not present any other infectious disease.

Histopathological analysis

Samples from the placentae and fetal organs were fixed in formalin (10%),

dehydrated in ethanol, clarified in xylene and blocked in paraffin resin. Tissue sections were cut (4 μm thick), deparaffinized in three baths of xylene and rehydrated with decreasing concentrations of ethanol (100, 90, 80 and 70 %). Sections were stained with hematoxylin and eosin for 2 min, for histological examination. Stained specimens were visualized by light microscopy (Olympus BX 53F, Japan) and digital images obtained by Image Pro Plus software (Version 4.5). All analyses were performed without prior knowledge of the nature of the samples (blind test).

Immunohistochemical procedure

For immunohistochemical studies, the paraffin-embedded tissues were cut (4 μm thick), deparaffinized in xylene and rehydrated with alcohol. Antigen retrieval was performed by heating the tissue in the presence of citrate buffer. Next, tissues were blocked for endogenous peroxidase with 3% hydrogen peroxidase in methanol and rinsed in Tris-HCl (pH 7.4). To reduce non-specific binding, sections were incubated in Protein Blocker solution (Spring Bioscience, USA) for 5 min at room temperature. Placental and fetal samples were then incubated overnight at 4 ºC with anti-human monoclonal antibodies that recognize flavivirus E protein (4G2 - produced in house as described in (Henchal et al., 1982)), Zika NS1 (Arigo, USA), diluted 1:200. This step was also performed to placental tissue with CD8 (DAKOCytomation, USA), CD68 (Biocare Medical, USA), RANTES/CCL5 (Santa Cruz Biotechnology, USA), TNF-α (Abbiotec, USA), IFNγ (Abbiotec), VEGFR2 (Spring Bioscience, USA), all diluted 1:200. In the next day, sections were incubated with a rabbit anti-mouse IgG-HRP conjugate (Spring Bioscience) for 40 min at room temperature. For negative controls, samples were incubated with both antibodies or only with the secondary HRP conjugated antibody. Reactions were revealed with diaminobenzidine (Dako, USA) as chromogen and the sections were counterstained in Meyer's hematoxylin (Dako).

Quantification of positive cells by immunohistochemistry

Slides were evaluated using an Olympus BX 53F microscope. For each specific antibody, 50 images (fields) were randomly acquired at 1000x magnification using the software Image Pro version 4.5 from placentae (zika infected and control). After collecting the frames, positive cells were quantified in each of the 50 fields in every organ and the median of positive cell number was determined. All analyzes were accomplished in a blind test without prior knowledge of the studied groups. After quantification, frames exhibited in figures were selected as to be more informative.

Immunofluorescence assay and co-staining of NS1 protein/ phenotypic cell markers:

The paraffin-embedded tissues were cut (4 μm thick), deparaffinized in xylene

and rehydrated with decreasing alcohol series. Antigen retrieval was performed by heating the tissue in presence of citrate buffer. In sequence, tissues were blocked with 1% bovine serum albumin (BSA) for 30 minutes and permeabilized with 0.5% Triton X-100 at room temperature. Samples were incubated overnight at 4 ºC with anti-human monoclonal antibodies that recognize Zika NS1 protein (Arigo, USA) and anti-CD11b (Abcam, UK) diluted 1:200 for co-localization. In the next day, sections were incubated with Alexa 488 rabbit anti-mouse IgG (Thermo Scientific) to bind anti-NS1 antibodies and Alexa 555 mouse anti-rabbit IgG (Thermo Scientific, USA) to bind anti-CD11b antibodies. Slides were analyzed under a confocal microscope (Zeiss LSM 510 Meta, Germany).

Statistical analyses

Data were analyzed with GraphPad prism software v 6.0 (La Jolla, USA) using Mann-Whitney non-parametric statistical tests. Significant differences between groups were determined considering $***p < 0.001$.

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Table S1**.** Analysis of the cerebrospinal fluid obtained by lumbar puncture and exams of the patient's peripheral blood.

Liquor		
Test	**Results**	**Reference value**
Cytology	Global: 10 cel/mm^3	-
	Specific: 10% PMN/ 90% MNC/ 0% EOS	-
Total proteins	39 mg/dL	10 - 45 mg/dL
LDH	148 U/L	71- 207 U/L
Glucose	50 mg/dL	50 - 80 mg/dL
Peripheral Blood		
	29/06/2016	**08/07/2016**
Erythrocytes	2.52	3.51	4.0 – 5.2 millions/ mm^3
Hemoglobin	8.3	11.2	12 - 16 g/dL
Hematocrit	24,1	32,4	35 - 47%
Leucocites	11,000	5,500	4,000 – 11,000/mm^3

Platelets	365,000	336,000	150.000 – 400,000/ mm^3
Erythrocyte sedimentation rate	60	37	0 - 20 mm/h
C-reactive protein	-	2.05	< 0.80 mg/dL
Potassium	3.6	4.2	3.5 - 5.0 mEql/L
Sodium	142	141	135 - 150 mEq/L
Urea	23	14	15 - 45 mg/dL
Creatinin	0.50	0.67	0.4 - 1.4 mg/dL
Aspartate aminotransferase	-	32	10 - 37 U/L
Alanine aminotransferase	-	13	10 - 37 U/L
Total proteins	6.9	9.50	6.0 – 8.0 g/dL
Albumin	4.0	3.31	3.5 – 5.5 g/dL

Table S2. Evaluation of the degree of upper and lower limbs muscle strength (upper and lower limbs, respectively) during period of hospitalization and follow up after hospitalization.

Time of disease evolution (progression)	**Force quantification**				**Functional Severity Scale**
	Upper limbs muscle		Lower limbs muscle		**Hughes Clinic**
	Proximal	Distal	Proximal	Distal	
14 days	III	II	II	0	04
21 days	IV	II	III	0	05
35 days	IV	III	III	0	04
46 days	IV	III	III	0	04
75 days	V	III	IV	II	03
123 days	V	IV	V	II	03
188 days	V	V	V	IV	02
417 days	V	V	V	IV	02

SOBRE A ORGANIZADORA

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