Microbes and Infection 25 (2023) 105119

Contents lists available at ScienceDirect

Microbes and Infection

journal homepage: www.elsevier.com/locate/micinf

Original article

# *Candida albicans* causes brain regional invasion and necrosis, and activation of microglia during lethal neonatal neurocandidiasis

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ARTICLE INFO

*Article history:*
Received 2 December 2022
Accepted 31 January 2023
Available online 7 February 2023

*Keywords:*
*Candida albicans*
Neurocandidiasis
Candidemia
Newborn
Necrosis
Apoptosis
Microglia activation

ABSTRACT

Neurocandidiasis is a fungal infection that primarily affects neonates, which is associated with 70% case fatality rates, while pediatric patients who survive infection often have long-term neurological sequelae, making it a clinical requirement to understand the pathogenesis of neonatal neurocandidiasis. Currently, the brain regions to *Candida albicans* invasion during the neonatal period are not characterized. In this study, 0-day-old mice were infected with *C. albicans* intravenously to determine dissemination and invasion into the brain at different times post-infection by fungal burden assay and histopathological analysis, additionally cellular death and microglial activation were evaluated by flow cytometry. The results evidenced the dissemination of *C. albicans* within the first hour of infection in the brain. The meninges were the initial site of invasion during the first 6 hours post infection and then filamentous structures into the brain parenchyma increases during infection, the anatomic regions most susceptible to invasion being the cerebral cortex, thalamus, hypothalamus, midbrain, pons, and medulla oblongata. Furthermore, *C. albicans* invasion of brain tissue results in cell necrosis and activation of microglia as a consequence of fungal invasion.



*Candida albicans* is a commensal fungi of human mucous membranes and skin in humans, but it is also responsible for local and systemic infections under certain conditions: microbiota imbalance; long-term use of antibiotics; loss of epithelial barrier integrity; central venous catheter usage; gastrointestinal surgery; immunocompromised patients and premature newborns [1]. Systemic candidiasis refers to bloodstream infection and affects vital organs including the central nervous system (CNS), the spread of this pathogen to the CNS is more common in preterm newborns and full-term newborns than in children or adults [2,3]. Fifty percent of neonatal patients with systemic candidiasis have CNS fungal invasion, which is associated with a fatality rate of 70% [3–5]. In neonates, *C. albicans* is the species most frequently implicated in systemic candidiasis and neurocandidiasis. Neonatal neurocandidiasis usually results from systemic *Candida* infection mainly due to the use of intravascular catheters [6].

The clinical presentation of neurocandidiasis is meningitis, meningoencephalitis, brain abscesses and ventriculitis [3,7,8]. Brain abscesses are characterized by fungal invasion into the brain parenchyma surrounded by inflammatory cells; these brain abscesses have been found in cerebral cortex, basal ganglia, cerebellum and other brain regions at necropsy [9–11]. In premature infants, meningitis and brain abscesses are the most common clinical manifestation and are associated with a fatal outcome [7,12], while infants patients who survive mycosis often have long-term neurological sequelae [4,13]. Therefore, it is a clinical need to understand the pathogenesis of neonatal neurocandidiasis and the brain regions most susceptible to the invasion of *C. albicans* during the neonatal period.

Several *in vivo* studies of systemic candidiasis have evaluated the spread of *C. albicans* to CNS during the adult and neonatal period [14–16], as well as the immune response induced in the brain during mycosis [17,18], however these studies did not use 0-day-old newborn animals, neither did not evaluate the dissemination of *C. albicans* to the brain regions. Previously, we have developed a murine model of neonatal systemic candidiasis by infecting 0-day-old mice with *C. albicans* intravenously. The results

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https://doi.org/10.1016/j.micinf.2023.105119
1286-4579/

showed that regardless of the inoculum administered (lethal or non-lethal *C. albicans* doses), the brain and liver were the tissues most susceptible to invasion of fungal structures by histopathological analysis, and thus this experimental model emulates the dissemination of *C. albicans* to the CNS, a common clinical entity during systemic candidiasis in human neonates [19]. In this study, using our previously described neonatal murine model, we evaluated the course of *C. albicans* invasion into the brain parenchyma at different times of infection by fungal burden and histopathology assays, and also analyzed the type of cell death and microglial activation during neonatal neurocandidiasis.

## 2. Material and methods

### 2.1. Candida albicans

*C. albicans* ATCC 66027 was incubated 48 h at 37 °C in Sabouraud dextrose agar (Becton Dickinson, Heidelberg, Germany). After, the yeast cells were harvested by gently scraping the colonies with a microbiological loop, the cells were suspended in sterile 0.89% physiological saline solution (PSS) and then harvested by centrifugation at 2000 rpm for 10 min. The pellet was washed twice in 15 mL sterile PSS and suspended to a volume of 3 mL. Finally, the yeast concentration was obtained using a hemocytometer prior to infection procedures and confirmed by counting viable cells.

### 2.2. Mice

BALB/c adult mice were purchased from Envigo Company (Envigo, Mexico). Newborn mice were the result of planned crossed adult mice; the pregnant dams were monitored daily during the expected week of parturition to confirm age of pups. All experiments were performed with newborn mice <24 h old and were housed in a microisolator cage along with their mothers in 12 h light/dark cycles and fed with sterilized food and water *ad libitum*. Care, maintenance and handling of the animals were in accordance with the Mexican government license conditions for animal experimentation and the Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Research (Commission on Life Sciences, National Research Council). Experiments were authorized by the Ethics and Research Committee and the Institutional Committee for the Care and Use of Laboratory Animals, Facultad de Medicina, Universidad Autónoma de Nuevo León (registration number MB21-00001).

### 2.3. Intravenous infection

The neonatal intravenous infection was performed adapting the methodology described by Flores-Maldonado et al. [19] Briefly, groups of thirty newborn mice <24 h old were inoculated intravenously via the external jugular vein with 30 μL of $1 \times 10^5$ or $5 \times 10^5$ cfu of *C. albicans.* Additionally, another group of eighteen newborn mice were injected with 30 μL sterile PSS (uninfected mice). After injection, each experimental group of newborn mice were returned to a microisolator cage with their mother. In our previous study, it was found that injection with $1 \times 10^5$ cfu does not cause death of newborn animals during infection (non-lethal inoculum), whereas newborn mice infected with $5 \times 10^5$ cfu die starting 80 h after inoculation (lethal inoculum) [19].

### 2.4. Fungal burden

Five newborn mice infected with $1 \times 10^5$ or $5 \times 10^5$ cfu of *C. albicans* or three mice inoculated with sterile PSS (uninfected mice) were euthanized at 1, 3, 6, 12, 24 and 72 hpi, then brain was aseptically removed, weighed and mechanically homogenized (Polytron-Aggregate, Kinematica) in 500 μL sterile PSS. Homogenates were used to prepare 1:10 serial dilutions, 50 μL of each dilution were plated by duplicate on mycosel agar plates (Becton Dickinson, Heidelberg, Germany). Colony-forming units were determined after 48 h of incubation at 37 °C, and results were expressed as log10 cfu/g tissue. No colonies were recovered from tissues of uninfected mice control groups. The experiment was performed independently at least twice.

### 2.5. Histopathological analysis

Infected or uninfected newborn mice were euthanized at 1, 3, 6, 12, 24 and 72 hpi (n = 5 mice/time point), the brain was aseptically removed and fixed in 4% formaldehyde for 24 h, then, a cut was made in the midline sagittal plane of brain to split into two hemispheres (right and left part). The hemispheres cerebral were dehydrated, embedded in paraffin, and sagittal 10 μm sections were cut in microtome Leica RM2125. Three sections for hemisphere were stained with Periodic Acid Schiff (PAS) (Merck Millipore, Darmstadt, Germany) following the manufacturer's specifications. A total of 30 sections per experimental group were observed. The experiment was performed independently at least twice.

### 2.6. Single-cell suspension from mouse brain

Infected and uninfected newborn mice were euthanized at 12, 24 and 72 hpi (n = 5 mice/time point). The brain was aseptically removed and finely minced and digested at 37 °C in digestion solution (RPMI 1640 without serum) containing 0.2 mg/mL of liberase TL (Roche, Chicago, USA) and 100 U/ml of DNAse I (Roche) for 30 min with intermittent shaking. Digested tissue was passed through a 70-μm filter (BD Biosciences), washed with RPMI 1640, and centrifuged at 1500 rpm for 5 min at 4 °C. The red blood cells were lysed with ACK lysing buffer (0.15 M $NH_4Cl$, 1.0 mM $KHCO_3$, 0.1 mM $Na_2$-EDTA) for 1 min, and the cells were passed through a 40-μm filter, washed with phosphate-buffered saline (PBS) and centrifuged, then, the cells were suspended in PBS solution and washed twice times in PBS solution.

### 2.7. Annexin V-FITC/7-AAD staining

Aliquots (50–70 μL) containing $1 \times 10^6$ cells of the brain single-cell suspensions were centrifuged and the cells were suspended in 1 mL of Annexin V-binding buffer. Then, cells were stained using the FITC Annexin V/7-AAD apoptosis detection kit (BD Pharmingen) according to the manufacturer's specifications. Stained cells were diluted in 400 μL of Annexin V-binding buffer. The acquisition of samples was performed on a BD Accuri C6 flow cytometer (BD Biosciences, California, USA), and data were analyzed using FlowJo software (Ashland, Oreg., USA). The experiment was performed independently at least twice.

### 2.8. Microglia activation

Aliquots (50–70 μL) containing $1 \times 10^6$ cells of the brain single-cell suspensions were centrifuged and the cells were suspended in 50 μL of FACS buffer, then, the cell suspension was incubated at 4 °C for 30 min with the following antibodies against specific microglia markers: FITC-anti-mouse CD45 (Clone 30-F11, BD Pharmingen), PE-anti-mouse CD11b (Clone M1/70, BD Pharmingen) PerCP-Cy5.5-anti-mouse MHC II (Clone M5/114.15.2, BD Pharmingen). After two washes with FACS buffer, cells were resuspended in 300 μL FACS buffer. The acquisition of samples was performed on a BD Accuri C6 flow cytometer (BD Biosciences, California, USA), and data were

analyzed using FlowJo software (Ashland, Oreg., USA). The experiment was performed independently at least twice.

### 2.9. Statistical analysis

All statistical analyses were performed in GraphPad Prism 7 (GraphPad Software Inc., La Jolla, California, USA). Differences in fungal burden were determined using the Mann–Whitney U-test. Analysis of cell death and microglia activation were analyzed using Kruskal-Wallis one way test. Significance was determined at $P \leq 0.05$.

## 3. Results

### 3.1. C. albicans *invades brain parenchyma during murine neonatal candidiasis*

In order to determine the dissemination and invasion of *C. albicans* to the neonatal mice brain, fungal burden assays were performed in the brain of newborn mice infected with $1 \times 10^5$ cfu (non-lethal inoculum) or $5 \times 10^5$ cfu (lethal inoculum) at different times of infection. The results evidenced the presence of *C. albicans* in the neonatal brain from hour 1 post-infection in newborn mice infected with both inocula, these levels of fungal burden in the brain remained constant during the first 12 hpi, whereas a marked increase in fungal burden was observed at 24 hpi, which continued to increase at 72 hpi in the brain of mice infected with both inocula, particularly, a higher fungal burden was observed in the brain of mice infected with the lethal inoculum at 72 hpi (Fig. 1).

Then, we performed histological analysis to demonstrate the course of *C. albicans* invasion into the brain of neonatal mice. No fungal structures were observed in the neonatal brain at 1 and 3 hpi in mice infected with both inocula, but yeast cells were evident in the meninges of infected mice at 6 hpi, interestingly, some yeasts in the meninges initiated germination of fungal cells (germ tube) at 6 hpi, possibly initiating invasion into the brain parenchyma (Fig. 2). Following the course of infection, scarce yeast and hyphae of *C. albicans* were found invading brain tissue at 12 hpi in neonatal mice infected with both inocula. A moderate invasion of fungal filaments in different anatomical regions of the brain of neonatal mice infected with $1 \times 10^5$ (non-lethal inoculum) was evidenced at 72 hpi. In contrast, massive invasion of fungal filaments in different anatomical regions of the brain of neonatal mice infected with $5 \times 10^5$ (lethal inoculum) was observed at 72 hpi (Fig. 2). These results show early detection of *C. albicans* in the neonatal brain (1 h post-infection) regardless of the inoculum by tissue colony count. Invasion of *C. albicans* into the neonatal brain occurs up to 6 hpi, being the meninges the first site of invasion, then subsequent invasion of fungal filaments to other brain regions occurs. The course of *C. albicans* presence and invasion into the brain of neonatal mice after 24 hpi is inoculum-dependent.

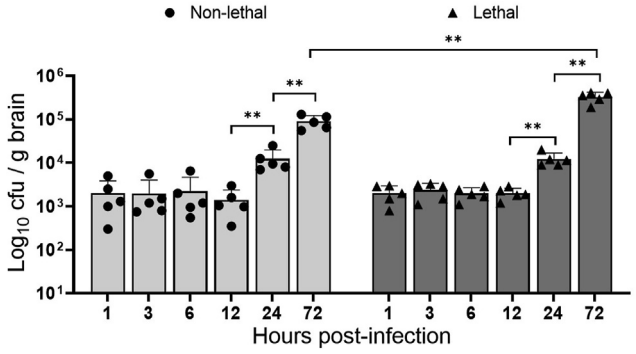


**Fig. 1.** Fungal burden in brain of newborn mice during systemic candidiasis. Fungal burden in brain of neonatal mice infected with $1 \times 10^5$ cfu (non-lethal inoculum) or $5 \times 10^5$ cfu (lethal inoculum) of *C. albicans*. Fungal count was determined at 1, 3, 6, 12, 24 and 72 h post-infection (hpi). Each symbol indicates single mouse data per time point. Mann–Whitney U-test (*$P < 0.05$, **$P < 0.01$).

Since histological analysis showed that *C. albicans* invades different anatomical regions of the brain during neurocandidiasis, we performed a quantitative analysis of the degree of fungal invasion in neonatal brain regions which were identified as shown in Supplementary Figure 1. Fungal invasion was categorized as follow, mild invasion (+), when fungal structures were observed in 1–10 histologic sections per anatomic region; moderate invasion (++), with prevalence of fungal structures in 11–20 histologic sections per anatomic region; and severe invasion (+++), with fungal structures in >20 histologic sections per anatomic region. Table 1 summarizes the results of the degree of invasion of *C. albicans* into the brain. Briefly, moderate invasion of *C. albicans* was found in the meninges of neonatal mice infected with both inocula at 6 hpi. Then, fungal mild invasion was determined at 12 hpi in prosencephalon (cortex, thalamus and hypothalamus), midbrain and rhombencephalon of neonatal mice infected with both inocula. The same pattern was observed at 24 hpi in the brain regions of neonatal mice infected with the non-lethal inoculum. However, in this group fungal invasion increased to moderate in thalamus, hypothalamus, mesencephalon, pons and medulla oblongata at 72 hpi. In contrast, at 24 hpi moderate fungal invasion was observed in thalamus, hypothalamus, midbrain and medulla oblongata in mice infected with the lethal inoculum. Moreover, at 72 hpi severe fungal invasion was determined in most brain regions; cortex, thalamus, hypothalamus, midbrain, pons and medulla oblongata of mice infected with the lethal inoculum (Table 1). These results show differences in the degree of invasion of *C. albicans* in the neonatal brain regions depending on the inoculum administered, furthermore the cerebral cortex, thalamus, hypothalamus, midbrain, pons and medulla oblongata were shown to be the anatomical regions most susceptible to invasion by *C. albicans* during fungemia.

### 3.2. Cell death in the brain of neonatal mice with neurocandidosis

Previously we have demonstrated that *C. albicans* invasion of the brain causes inflammatory tissue damage [19]. Therefore, the type of cell death that occurs in the brain of neonatal mice during mycosis was analyzed. Necrosis (7-AAD$^+$ cells), and apoptosis (Annexin-V$^+$ cells) was assessed through flow cytometry. Fig. 3A shows the flow cytometry gating strategy used to determine the type of cell death occurring in the brain of neonatal mice with neurocandidiasis. Necrosis was the predominant type of cell death in the brain of neonatal mice during *C. albicans* invasion indistinctly of the inoculum (Fig. 3B). However, the brain of newborn mice infected with the lethal inoculum had ~25% 7-AAD$^+$ cells at 72 hpi, opposite to only ~9% 7-AAD$^+$ cells in the brain of newborn mice infected with the non-lethal inoculum (Fig. 3B and C). Whereas apoptosis was the cell death type that occurred in the lowest percentage (~8 of Annexin-V$^+$ cells) in the brain of mice infected with both inocula at 72 hpi (Fig. 3B and D). Since the levels of necrosis in the brain were highest at 72 hpi, we analyzed the type of cell death in different brain regions at 72 hpi. The prosencephalon and mesencephalon were the regions that showed the highest levels of necrosis, particularly, the prosencephalon and mesencephalon of newborn mice injected with the lethal inoculum had ~28% and ~18% necrotic cells, respectively, and less than 10% apoptotic cells in contrast to the rhombencephalon which was the brain region that showed the lowest levels of necrosis and apoptosis with both inoculums (Supplementary figure 2). These results demonstrate that

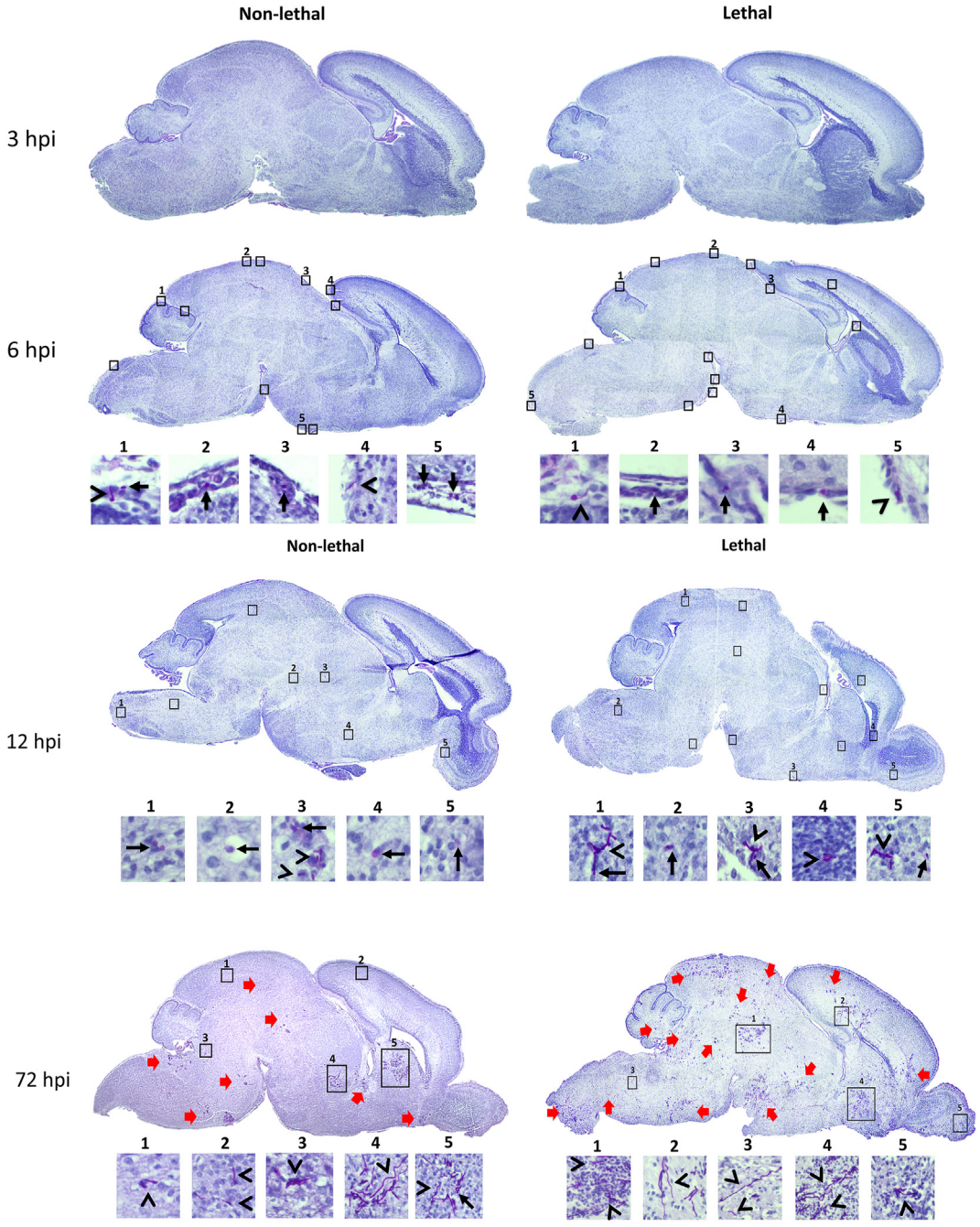


**Fig. 2.** Invasion of *C. albicans* into the brain of newborn mice with systemic candidiasis. Periodic acid-Schiff (PAS)-stained sagittal brain sections of newborn mice infected with $1 \times 10^5$ cfu (non-lethal inoculum) or $5 \times 10^5$ cfu (lethal inoculum) of *C. albicans* at 3, 6, 12 and 72 hpi. Black boxes evidence the presence of fungal structures, arrow with continuous line shows yeasts, arrowhead shows fungal filaments hyphae and pseudohyphae, red arrows show clusters of fungal structures.

invasion of *C. albicans* into the neonatal brain induces cell death, such as necrosis and apoptosis. Necrosis is associated with increased invasion of *C. albicans* into the tissue and possibly contributes to brain damage and death of neonatal animals, as observed in the lethal inoculum, whereas low levels of necrosis and apoptosis are associated with low-to-moderate *C. albicans* invasion into the brain and survival of neonatal animals, as observed in the non-lethal inoculum. Furthermore, mesencephalon and prosencephalon were the regions with the highest cell death rates during mycosis.

### *3.3. Activation of microglia in the brains of neonatal mice with neurocandidiasis*

The activation of microglia in the brain of neonatal mice during mycosis was assessed by flow cytometry. Fig. 4A shows the flow cytometry gatting strategy used to determine the microglia activation ($CD11b^{+}CD45^{low}MHCII^{+}$). Low microglia activation (11% $CD11b^{+}CD45^{low}MHCII^{+}$ cells) was found in the brain of neonatal mice infected with the lethal inoculum at 24 hpi compared to the vehicle group and animals infected with the non-lethal inoculum

**Table 1**
Fungal invasion of *C. albicans* in the brain of newborn mice during neurocandidiasis.

| Anatomical areas | 3 hpi | | 6 hpi | | 12 hpi | | 24 hpi | | 72 hpi | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $1 \times 10^5$ | $5 \times 10^5$ | $1 \times 10^5$ | $5 \times 10^5$ | $1 \times 10^5$ | $5 \times 10^5$ | $1 \times 10^5$ | $5 \times 10^5$ | $1 \times 10^5$ | $5 \times 10^5$ |
| **Meninges** | ND | ND | ++ | ++ | ND | ND | ND | ND | ND | ND |
| **Prosencephalon** | | | | | | | | | | |
| Olfactory bulb | ND | ND | ND | ND | ND | + | + | + | + | ++ |
| Cortex | ND | ND | ND | ND | + | + | + | + | + | +++ |
| Hippocampus | ND | ND | ND | ND | ND | ND | ND | ND | + | + |
| Septum | ND | ND | ND | ND | ND | ND | + | + | + | ++ |
| Thalamus | ND | ND | ND | ND | + | + | + | ++ | ++ | +++ |
| Hypothalamus | ND | ND | ND | ND | + | + | + | ++ | ++ | +++ |
| **Mesencephalon (midbrain)** | ND | ND | ND | ND | + | + | + | ++ | ++ | +++ |
| **Rhombencephalon** | | | | | | | | | | |
| Cerebellum | ND | ND | ND | ND | + | + | + | + | + | ++ |
| Pons | ND | ND | ND | ND | + | + | + | + | ++ | +++ |
| Medulla oblongata | ND | ND | ND | ND | + | + | + | ++ | ++ | +++ |

Fungal invasion was categorized as mild invasion (+), when fungal structures were observed in 1–10 histologic sections per anatomic region; moderate invasion (++), when we observed fungal structures in 11–20 histologic sections per anatomic region; and severe invasion (+++), when we observed fungal structures in >20 histologic sections per anatomic region. 30 sections per experimental group were observed. ND No detected.

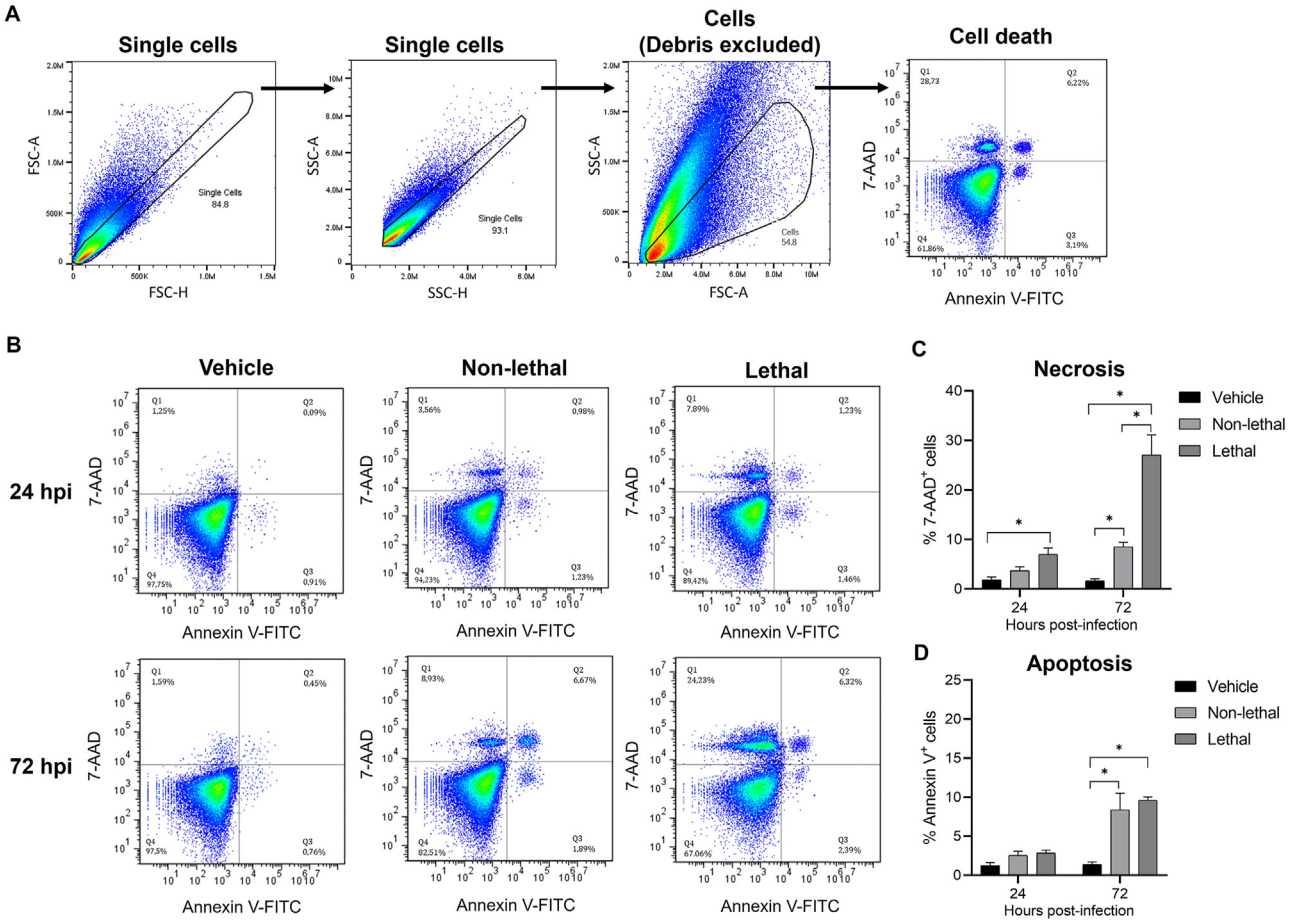


**Fig. 3.** Cell death in the brain of newborn mice during neurocandidiasis. (A) Flow cytometry gating strategy used to determine cell death, (B) flow cytometry plots of necrosis and apotosis detection by annexin V-FITC/7-AAD staining, (C) percentage of necrotic cells (7-AAD$^+$) and (D) percentage of apoptotic cells (Annexin V$^+$) in the brain of newborn mice infected with $1 \times 10^5$ cfu (non-lethal inoculum) or $5 \times 10^5$ cfu (lethal inoculum) of *C. albicans* at 24 and 72 hpi. Kruskal–Wallis one way test (*$P < 0.05$).

(Fig. 4B and C). Whereas analysis at 72 hpi evidenced a marked increase in microglia activation (35% CD11b$^+$CD45$^{low}$MHCII$^+$ cells) in the brain of mice infected with the lethal inoculum in contrast to low microglia activation in the brain of mice infected with the non-lethal inoculum (Fig. 4B and C). These results show that the invasion of *C. albicans* into the neonatal brain allows microglia activation, and that microglia activation is dependent on the inoculum administered.

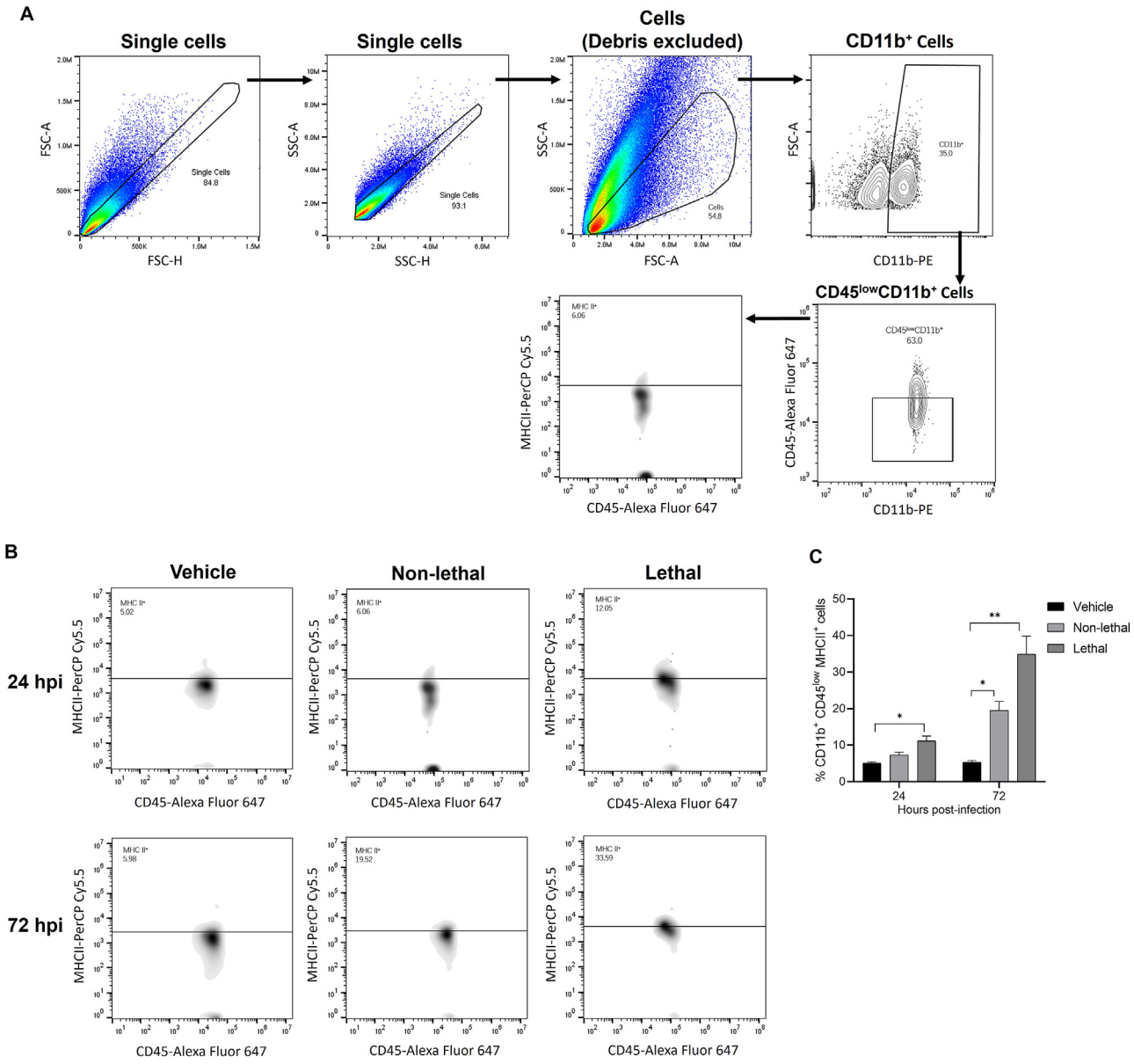


**Fig. 4.** Microglia activation in the brain of newborn mice during neurocandidiasis. (A) Flow cytometry gating strategy used to determine microglia activation, (B) flow cytometry plots to detect microglia activation ($CD11b^+$, $CD45^{low}$, $MHCII^+$) and (C) percentage of microglia activation ($CD11b^+$, $CD45^{low}$, $MHCII^+$) in the brain of newborn mice infected with $1 \times 10^5$ cfu (non-lethal inoculum) or $5 \times 10^5$ cfu (lethal inoculum) of *C. albicans* at 24 and 72 h. One-way Kruskal–Wallis test ($*P < 0.05$).

## 4. Discussion

Although experimental models of neonatal systemic candidiasis have been described, some studies did not evaluate the presence of *C. albicans* into the brain of neonatal animals [20], while another study did not detected dissemination of the fungus in the brain parenchyma [21]. In this work, *C. albicans* was found in the brain of neonatal mice from 1 h of infection by colony count. Studies of systemic candidiasis in adult mice have shown the presence of *C. albicans* fungal burden in the brain at 1 hpi, which is maintained at similar levels during the first 24 h of infection [18], as in this study. On the other hand, adult animals infected intravenously with *Cryptococcus neoformans*, the fungus was found at 1 hpi by a fungal burden assay [22]. Therefore, the detection of pathogenic yeasts such as *C. albicans* to the brain occurs early times after infection, probably due to the transport of blood flow to the brain and are likely limited to the vascular compartment. To invade the brain parenchyma, *C. albicans* must adhere to and invade the endothelial cells lining the cerebral blood vessels [11,14]. Navarathna et al. in their model of disseminated candidiasis using adult mice showed that *C. albicans* invades the cerebral vasculature at 30 min after infection, and also reported presence of yeast in the meninges at 2 hpi [15]. In this study, the first anatomical site of the brain that invades the fungus are the meninges at 6 hpi. Meningitis caused by *C. albicans* is common in preterm newborns [3,4,13]; however, the immunopathogenesis of meningitis during the neonatal period is poorly studied.

In adult mice with candidiasis, the invasion of *C. albicans* into the cerebral parenchyma has been shown to occur between day 1–3 of infection [15,18]. Here, we reported invasion of fungal structures in the neonatal brain parenchyma within 24 h of infection. Necropsy studies of patients diagnosed with disseminated candidiasis

showed the formation of brain abscesses, these fungal abscesses were frequently found in the cerebral cortex, basal ganglia, supratentorial region, cerebellum and corticomedullary junction [23,24]. Our histological analysis showed that most susceptible regions of neonatal brain were cerebral cortex, thalamus, hypothalamus, midbrain, pons and medulla oblongata. Similarly, cases report of preterm infants with neurocandidiasis showed the presence of fungal abscesses in the prosencephalon, especially in the thalamus and hypothalamus [7,8,10]. Kondo et al. evaluated in their rodent model the effect of transient ischemia in the prosencephalon, they demonstrated that neuronal cell death caused cognitive alterations [25]. On the other hand, Bellot et al. investigated the functions of the midbrain, pons, medulla oblongata and cerebellum, using a model of hypoxia-ischemia-induced encephalopathy in 7-day-old mice, they observed alterations such as decreased body weight, as well as defects in the motor control of feeding, sucking, swallowing and breathing [26]. Possibly the spread of *C. albicans* to these anatomical regions of the brain contributes to the neurological sequelae in neonatal patients who survive neurocandidiasis. However, studies are needed to understand the neurological sequelae during candidiasis.

Previously, we demonstrated that the spread of *C. albicans* to the brain induce an inflammatory immune response, which was associated with tissue damage [19]. Previous studies have shown that invasion of *C. albicans* in the brain causes lesions with foci of necrosis [6]. In this study, we observed that the invasion of *C. albicans* in the brain induces necrosis and apoptosis, interestingly, necrosis was the main type of cell death during mycosis and tissue necrosis levels is inoculum-dependent. Thus, necrosis is associated with increased invasion of *C. albicans* into the tissue and possibly contributes to brain damage and death of neonatal animals. Several studies have shown that penetration of *C. albicans* into endothelial cells of cerebral blood vessels causes endothelial cell lysis accompanied by local necrotic lesions [15,27]. Also, Pais et al. they observed that necrotic neurons induced activation of microglia characterized by overexpression of MHCII, CD11b, proinflammatory cytokines and nitric oxide synthase [28]. Here, we observed that *C. albicans* invasion in the neonatal brain induce microglia activation. Lionakis et al. in their model of invasive candidiasis using adult animals, they showed activation of microglia by expression of markers such as Iba1 and MHCII after fungal invasion of the brain [18]. Also, Gandhi et al. observed cell activation and increased production of proinflammatory cytokine in the microglia cell line CHME-3 stimulated with *C. albicans* [29]. The possible role of microglia activation in neurotoxicity and neuroinflammation has been proposed [30]. On other hand, microglial activation has been associated with anti-*Candida* responses [18,31], since microglia phagocytose and eliminate *C. albicans*.

In conclusion, this is the first study to demonstrate the kinetics of dissemination of *C. albicans* into the neonatal brain during candidiasis. We showed that after *C. albicans* entry into the bloodstream, it spreads during the first hour of infection to the brain. Then, invasion occurs initially in the meninges during the first 6 h of infection and then the presence of filamentous structures to the brain increases during infection, the most susceptible regions to invasion were cerebral cortex, thalamus, hypothalamus, midbrain, pons and medulla oblongata. In addition, *C. albicans* invasion of brain tissue causes cell necrosis and activation of microglia as a consequence of fungal invasion.

## Author contributions

Flores-Maldonado: Conceptualization, Methodology, Formal analysis, Investigation, Writing-original draft, Visualization. González G.: Methodology, Validation, Formal analysis, Resources, Writing-review & editing. Enríquez-Bañuelos: Methodology, Formal analysis, Visualization, Andrade Angel.: Methodology, Investigation, Writing-original draft. Treviño-Rangel: Methodology, Investigation. Becerril-García: Conceptualization, Methodology, Validation, Resources, Writing-review & editing, Supervision, Project administration, Funding acquisition.

## Funding

This work was supported by Consejo Nacional de Ciencia y Tecnología [CONACYT-INFRA-2015-01-251142] and Programa de Apoyo a la Investigación Científica y Tecnológica [PAICYT SA1907-21].

## Declaration of competing interest

The authors declare no financial or commercial conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

## Acknowledgments

We thank for the valuable help of Luis A. Rodríguez-Gámez and Liliana Guadalupe Olvera Quijano for animal housing. Enríquez-Bañuelos was fellow holder from CONACYT. Flores-Maldonado, Gonzalez G., Andrade A., Treviño R. and Becerril García are members of National System of Researchers, SNI-CONACYT.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micinf.2023.105119.

## References

[1] Pappas PG, Lionakis MS, Arendrup MC, Ostrosky-Zeichner L, Kullberg BJ. Invasive candidiasis. Nat Rev Dis Prim 2018;4(1):18026.
[2] Chakrabarti A. Epidemiology of central nervous system mycoses. Neurol India 2007;55(3):191–7.
[3] Faix RG, Chapman RL. Central nervous system candidiasis in the high-risk neonate. Semin Perinatol 2003;27(5):384–92.
[4] Benjamin Jr DK, Stoll BJ, Fanaroff AA, McDonald SA, William O, Higgins RD, et al. Neonatal candidiasis among extremely low birth weight infants: risk factors, mortality rates, and neurodevelopmental outcomes at 18 to 22 months. Pediatrics 2006;117(1):84–92.
[5] Murthy JM, Sundaram C. Fungal infections of the central nervous system. Handb Clin Neurol 2014;121:1383–401.
[6] Sánchez–Portocarrero J, Pérez-Cecilia E, Corral O, Romero-Vivas J, Picazo JJ. The central nervous system and infection by *Candida* species. Diagn Microbiol Infect Dis 2000;37(3):169–79.
[7] Ancalle IM, Rivera JA, García I, García L, Valcárcel M. *Candida albicans* meningitis and brain abscesses in a neonate: a case report. Bol Asoc Med P R 2010;102(1):45–8.
[8] Baradkar VP, Mathur M, Kumar S. Cerebral *Candida* abscess in an infant. Indian J Med Microbiol 2009;27(1):70–2.
[9] Yoshida K, Matsuda N, Sato T, Watanabe T, Nakamura K, Saito K, et al. *Candida* brain abscesses in a patient with anorexia nervosa receiving total parenteral nutrition. Clin Neurol Neurosurg 2022;212:107058.
[10] Pahud BA, Greenhow TL, Piecuch B, Weintrub PS. Preterm neonates with candidal brain microabscesses: a case series. J Perinatol 2009;29(4):323–6.
[11] Vigezzi C, Riera FO, Rodriguez E, Icelya PA, Miró MS, Figueredo CM, et al. Candidiasis invasora: un enfoque a la infección en el sistema nervioso central. Rev Argent Microbiol 2021;53(2):171–8.
[12] Zhu Y, Gong X, Li Z, Wang D, Yan C. Clinical analysis of intravenous and oral sequential treatment with voriconazole for *Candida* central nervous system infection in six premature infants. Front Pharmacol 2021;12:631293.
[13] Friedman S, Richardson SE, Jacobs SE, O'Brien K. Systemic *Candida* infection in extremely low birth weight infants: short term morbidity and long term neurodevelopmental outcome. Pediatr Infect Dis J 2000;19(6):499–504.
[14] Lossinsky AS, Jong A, Fiala M, Mukhtar M, Buttle KF, Ingram M. The histopathology of *Candida albicans* invasion in neonatal rat tissues and in the human

blood-brain barrier in culture revealed by light, scanning, transmission and immunoelectron microscopy. Histol Histopathol 2006;21(10):1029–41.
[15] Navarathna DH, Munasinghe J, Lizak MJ, Nayak D, McGavern DB, Roberts. D.D. MRI confirms loss of blood-brain barrier integrity in a mouse model of disseminated candidiasis. NMR Biomed 2013;26(9):1125–34.
[16] Sanches MD, Mimura AN, Oliveira RC, Ishikawa LW, Garces HG, Bagagli E, et al. Differential behavior of non-*albicans Candida* species in the central nervous system of immunocompetent and immunosuppressed mice. Front Microbiol 2018;9:2968.
[17] Ophelders DR, Gussenhoven R, Lammens M, Küsters B, Kemp MW, Newnham JP, et al. Neuroinflammation and structural injury of the fetal ovine brain following intra-amniotic *Candida albicans* exposure. J Neuroinflammation 2016;13:29.
[18] Lionakis MS, Lim JK, Lee CC, Murphy PM. Organ-specific innate immune responses in a mouse model of invasive candidiasis. J Innate Immun 2011;3(2):180–99.
[19] Flores-Maldonado OE, González GM, Andrade-Torres Á, Treviño-Rangel R, Donis-Maturano L, Silva-Sánchez A, et al. Distinct innate immune responses between sublethal and lethal models of disseminated candidiasis in newborn BALB/c mice. Microb Pathog 2021;158:105061.
[20] Trofa D, Soghier L, Long C, Nosanchuk JD, Gacser A, Goldman DL. A rat model of neonatal candidiasis demonstrates the importance of lipases as virulence factors for *Candida albicans* and *Candida parapsilosis*. Mycopathologia 2011;172(3):169–78.
[21] Tsai NY, Laforce-Nesbitt SS, Tucker R, Bliss JM. A murine model for disseminated candidiasis in neonates. Pediatr Res 2011;69(3):189–93.
[22] Charlier C, Nielsen K, Daou S, Brigitte M, Chretien F, Dromer F. Evidence of a role for monocytes in dissemination and brain invasion by *Cryptococcus neoformans*. Infect Immun 2009;77(1):120–7.
[23] Nakayama H, Shibuya K, Kimura M, Ueda M, Iwabuchi S. Histopathological study of candidal infection in the central nervous system. Nippon Ishinkin Gakkai Zasshi 2010;51(1):31–45.
[24] Pendlebury WW, Perl DP, Munoz DG. Multiple microabscesses in the central nervous system: a clinicopathologic study. J Neuropathol Exp Neurol 1989;48(3):290–300.
[25] Kondo T, Yoshida S, Nagai H, Takeshita A, Mino M, Morioka H, et al. Transient forebrain ischemia induces impairment in cognitive performance prior to extensive neuronal cell death in Mongolian gerbil (Meriones unguiculatus). J Vet Sci 2018;19(4):505–11.
[26] Bellot B, Peyronnet-Roux J, Gire C, Simeoni U, Vinay L, Viemari JC. Deficits of brainstem and spinal cord functions after neonatal hypoxia-ischemia in mice. Pediatr Res 2014;75(6):723–30.
[27] Góralska K, Blaszkowska J, Dzikowiec M. Neuroinfections caused by fungi. Infection 2018;46(4):443–59.
[28] Pais TF, Figueiredo C, Peixoto R, Braz MH, Chatterjee S. Necrotic neurons enhance microglial neurotoxicity through induction of glutaminase by a MyD88-dependent pathway. J Neuroinflammation 2008;5:43.
[29] Gandhi J, Naik P, Kaur I, Kumar A, Joseph J. Microglial response to *Aspergillus flavus* and *Candida albicans*: implications in endophthalmitis. J Fungi 2020;6(3).
[30] Serdar M, Kempe K, Rizazad M, Herz J, Bendix I, Felderhoff-Müser U, et al. Early pro-inflammatory microglia activation after inflammation-sensitized hypoxic-ischemic brain injury in neonatal rats. Front Cell Neurosci 2019;13:237.
[31] Blasi E, Mazzolla R, Barluzzi R, Mosci P, Bartoli A, Bistoni F. Intracerebral transfer of an in vitro established microglial cell line: local induction of a protective state against lethal challenge with *Candida albicans*. J Neuroimmunol 1991;32(3):249–57.