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Development of Anxiolytic and Depression-like Behavior in Mice Infected with *Mycobacterium lepraemurium*

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Abstract—Murine leprosy is a systemic infectious disease of mice caused by *Mycobacterium lepraemurium* (MLM) in which the central nervous system (CNS) is not infected; nevertheless, diseased animals show measurable cognitive alterations. For this reason, in this study, we explored the neurobehavioral changes in mice chronically infected with MLM. BALB/c mice were infected with MLM, and 120 days later, the alterations in mice were evaluated based on immunologic, histologic, endocrine, neurochemical, and behavioral traits. We found increases in the levels of IL-4 and IL-10 associated with high bacillary loads. We also found increase in the serum levels of corticosterone, epinephrine, and norepinephrine in the adrenal gland, suggesting neuroendocrine deregulation. Mice exhibited depression-like behavior in the tail suspension and forced swimming tests and anxiolytic behavior in the open field and elevated plus maze tests. The neurobehavioral alterations of mice were correlated with the histologic damage in the prefrontal cortex, ventral hippocampus, and amygdala, as well as with a blood–brain barrier disruption in the hippocampus. These results reveal an interrelated response of the neuroimmune–endocrino logical axis in unresolved chronic infections that result in neurocognitive deterioration.

Key words: anxiolytic behavior, depression-like behavior, chronic infection, central nervous system, murine leprosy.

INTRODUCTION

Neurobehavioral alterations associated with chronic infection processes are related to alterations in the nervous, endocrine, and immunological systems, where modifications in the levels of neurotransmitters, hormones, and cytokines can worsen disease symptoms and lead to behavioral disorders (Ashley and Demas, 2017; Bereshchenko et al., 2018; Wang et al.,

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2019). The role of the neuroimmune-endocrine response in chronic infections remains unclear, particularly in cases in which the central nervous system (CNS) is not infected. Therefore, using experimental models of chronic infections without involvement of the CNS will allow exploration of the intricate feedback-loop interactions connected with the disease (Demas and Carlton, 2015; Ashley and Demas, 2017; Del Rey and Besedovsky, 2017). These models have been used to investigate hypercortisolemia, neurodegeneration, neurocognitive impairment and anergy in both natural and experimental infections with prolonged periods of disease (O'Connor et al., 2009; Rodriguez-Zas et al., 2015; Hou et al., 2017; Mitchell et al., 2017; Becerril-Villanueva et al., 2018; D'Attilio et al., 2018; Lara-Espinosa et al., 2020). A fitting model of disease is murine leprosy, a chronic infectious disease caused by *Mycobacterium lepraemurium* (MLM) (Benjak et al., 2017), which in advanced stages, resembles lepromatous leprosy (Løvik, 1987; Rojas-Espinosa and Løvik, 2001; Rojas-Espinosa, 2009). MLM is an invasive microorganism that does not affect the CNS even in the latest stages of the disease. Murine leprosy, however, is a disease that affects working memory, suggesting that it has an effect on the CNS through soluble mediators, such as hormones, neurotransmitters and/or cytokines (Rojas-Espinosa et al., 2005; Becerril-Villanueva et al., 2018). Thus, murine leprosy is a model of chronic disease suitable to explore neurobehavioral disorders such as

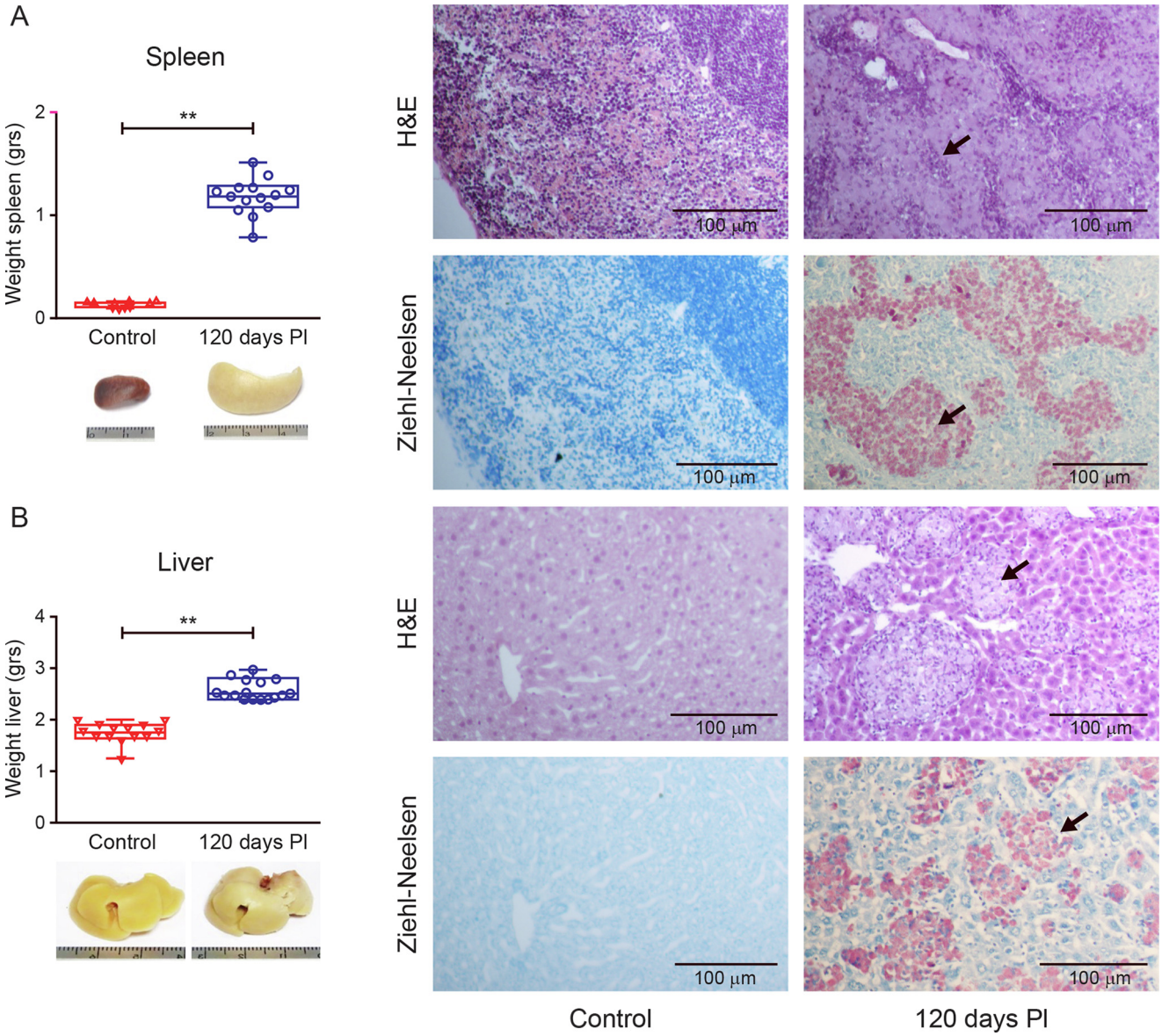


Fig. 1. Spleen and liver in mice infected with *Mycobacterium lepraemurium* (MLM) for 120 days. The figure illustrates the marked splenomegaly and hepatomegaly in the group of mice having a 120-day infection with MLM (120 days PI). **(A)** Histologically, mice in the 120-day PI group show atrophy of the periarteriolar lymphatic sheath and marginal zone, and extensive granuloma fraction in both the white and red pulp (arrows). **(B)** Hepatomegaly is also observed, with extensive granuloma fraction similar to the observed in the spleen representative images, (n = 14 per group) Hematoxylin-Eosin (HE) and Ziehl-Neelseen (ZN) stains, 40X. (**$p < 0.001$ where indicated). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

depression, anxiety, mental distress and fear, which have been reported in patients with lepromatous leprosy (Somar et al., 2020; van Dorst et al., 2020; Govindasamy et al., 2021). Therefore, the aim of this study was to identify the neuroimmuno-endocrine alterations in mice suffering from a long-lasting infection with MLM by evaluating the neurobehavioral changes associated with morphological damage in the prefrontal cortex-hippocampus-amygdala circuit, as well as peripheral hormonal and immunological alterations.

EXPERIMENTAL PROCEDURES

This project was reviewed and approved by the Committee of Ethics in Research of the National School of Biological Sciences (CEI ZOO-010-2020). Mice were handled under the regulations of the Official Mexican Norm (NOM-062-ZOO-1999).

Mice

A total of 80 male eight-week-old BALB/c mice acquired from Harlan Mexico were used in this study. Mice were housed in 25 × 30 × 15 cm polystyrene cages (five mice per cage) maintained at a constant temperature (23–24 °C) under 12 × 12 h light–dark cycles and were fed purified water and food (Purina Chow, Mexico) *ad libitum.*

Mycobacterium collection and mouse infection

Mice were infected with *Mycobacterium lepraemurium* (MLM) isolated from the spleen and liver of a mouse with a 4-month infection. Isolation and purification of MLM were carried out as described by (Mendoza-Aguilar et al., 2012), and bacilli viability was assessed by the fluorescein diacetate-ethidium bromide method of (Jarnagin and Luchsinger, 1980).

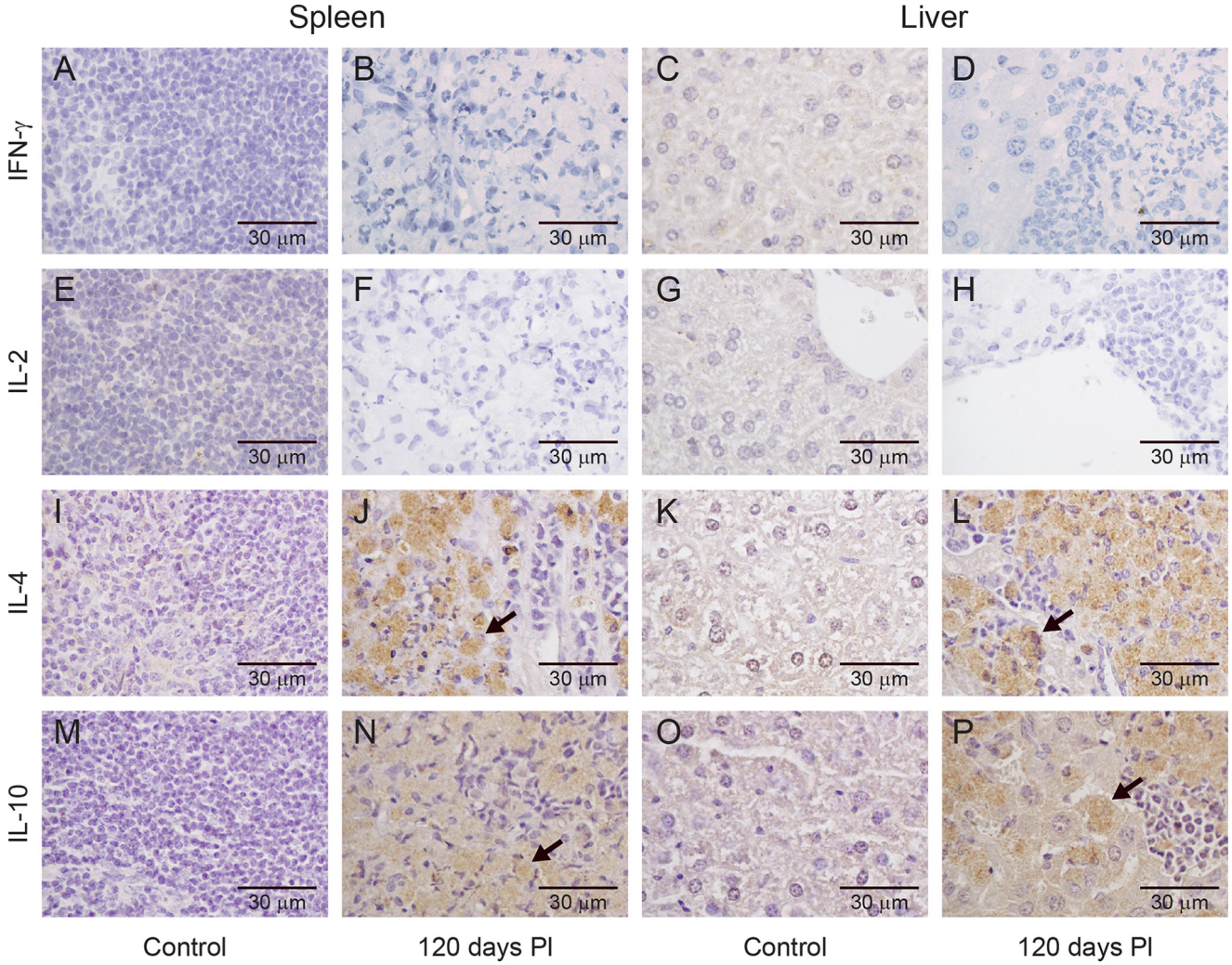


Fig. 2. Cytokines in the spleen and liver. The figure illustrates the presence of anti-inflammatory IL-4 and IL-10 cytokines (arrows), and the lack of proinflammatory IFN-γ and IL-2 cytokines in the spleen and liver of mice of the 120-day PI group (120 days PI). Most staining appears on the granuloma fraction in both organs. As expected, no proinflammatory or anti-inflammatory cytokines are expressed in the spleen and liver of mice in the control (non-infected) group representative images, (n = 5 per group). Immunoperoxidase and Hematoxylin stains 40×.

Forty mice were intraperitoneally inoculated with 20×10^6 MLM bacilli in 50 μl of physiological saline solution (PSS), and forty mice in the control group received plain PSS.

Histology and immunohistochemistry

To verify chronic infection, mice were sacrificed on day 120 postinfection (120-day PI) by intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). Then, they were intracardially infused with PSS, followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) (5 min each). The spleen, liver, brain, and adrenal glands were collected and kept in formalin for 5 days, and then, the organs were weighed and processed for inclusion in paraffin. Three-micron thick sections were produced and placed on poly-L-lysine-coated slides. Then, the sections were deparaffinized, rehydrated, and stained with Ziehl-Neelsen (ZN) and Hematoxylin–Eosin (H&E) stains. For the immunohistochemical analysis, the tissue sections were deparaffinized, the endogenous peroxidase was quenched (with 0.3% hydrogen peroxide in methanol/10 min), and the sites of unspecific binding were blocked with 3% skim milk for 2 h. Then, independent slides were incubated with rabbit anti-TNF-α (ab34674), anti-IFN-γ (ab216642), anti-IL-4 (PA5-25165) and anti-IL-10 (ab189392) antibodies and with anti-Iba-1 (ab153696) and anti-GFAP (ab7260) antibodies to detect neuroinflammation. All the primary antibodies were diluted 1:200 in PBS, while the secondary antibody (peroxidase-labeled goat anti-rabbit IgG) was diluted 1:1000. After immunostaining and extensive washing with PBS–Tween, the sections were stained with Hematoxylin, mounted in resin, and examined under a Nikon Eclipse E8000 microscope (Tokyo, Japan).

Blood–brain barrier permeability assay and confocal microscopy imaging

Damage to the blood–brain barrier (BBB) was evaluated in mice anesthetized with sodium pentobarbital (50 mg/kg body weight). Evans' Blue (EB) (2 mg/ml) was intracardially inoculated (0.2 ml/100 g of body weight) and allowed to circulate for 5 min. Then, mice were transcardially perfused with PSS, followed by 4% paraformaldehyde for 5 min. The brains were removed, postfixed for 24 h and washed with 10% sucrose for 24 h. Coronal 40-μm thick slices were subsequently prepared from the dorsal hippocampal region (Bregman −2.54 and −2.80), and the slices were washed with PBS and covered with VectaShield-DAPI mounting medium (Vector Laboratories, Inc., Burlingame CA, USA) for observation. Images were taken and analyzed under an Axioscop 2 mot plus confocal fluorescence microscope (Carl Zeiss, Mexico) at EC Plan-Neofluar 20X/0.5 ph2: LP650; BP 420–480; LP 505. Images were processed with Arviris Vision 4D software (Arviris Co., Germany, 2018).

Western blot

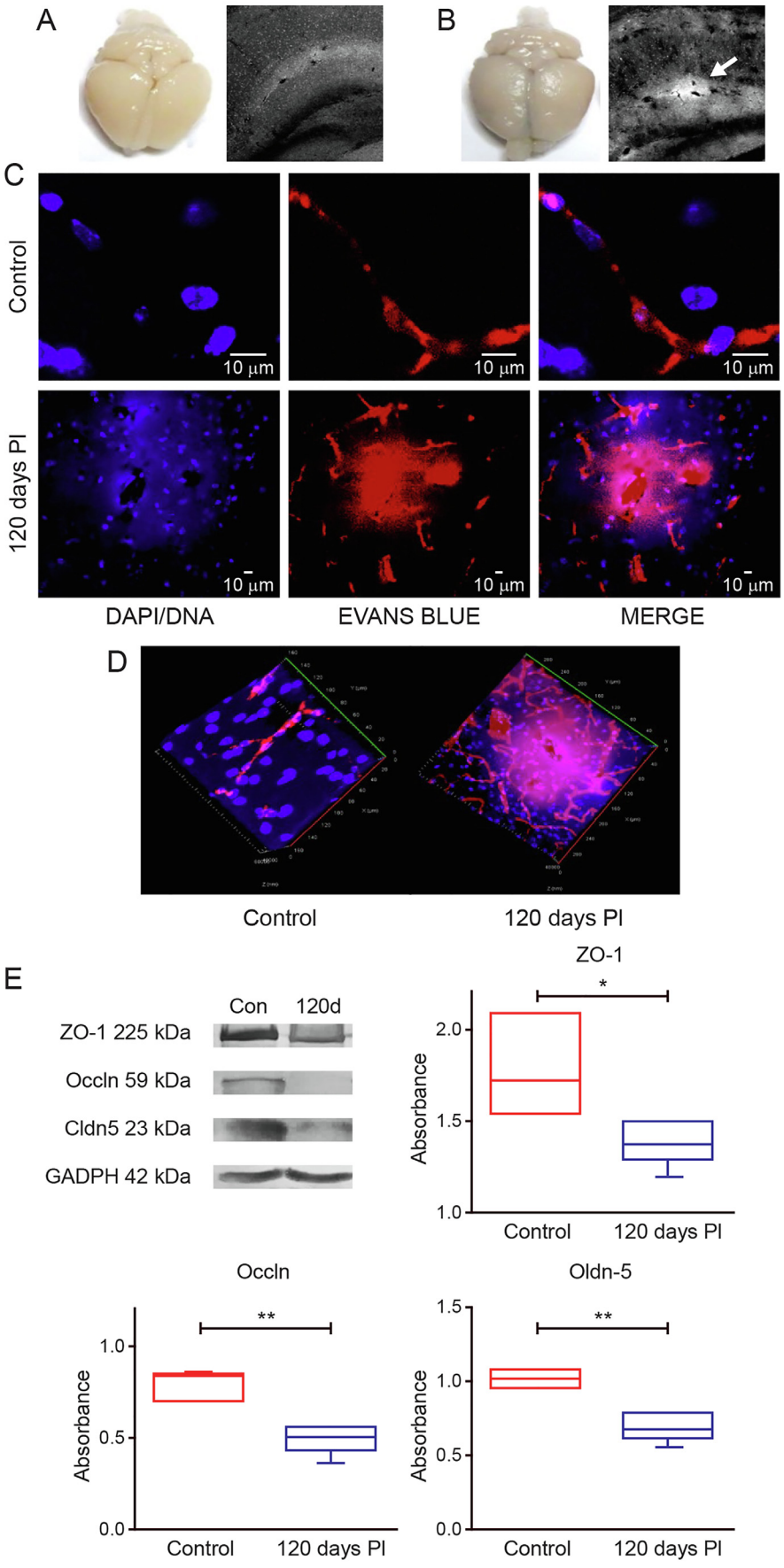
To assess the expression of proteins associated with the blood–brain barrier, five control mice and five 120-day PI mice time were killed by decapitation, and their brains were removed. The hippocampus was dissected and homogenized in RIPA buffer with protease inhibitors (S8820 Sigma) and centrifuged at 13,500 rpm for 10 min at 4 °C, and the supernatant was frozen at −80 °C until use. The protein concentration was determined by the Bradford method (BioRad), and 50-μg samples were resolved by denaturing polyacrylamide gel electrophoresis (10% SDS–PAGE). Then, the proteins were electrically transferred to nitrocellulose membranes for immunodetection. For immunodetection, the membranes were blocked for 2 h with 5% nonfat milk in Tris-buffer saline (150 mM NaCl, 50 mM Tris-HCl, pH 7.6.) and then incubated overnight at 4 °C with rabbit anti-claudin-5 (ab15106), occludin (ab167161), ZO-1 tight junction protein (ab96587) and GAPDH (ab8245) antibodies diluted 1:1000 in TBST (0.1% Tween-20 in TBS) with 5% BSA. The membranes were washed three times (10 min each) with TBST, incubated with a horseradish peroxidase-conjugated secondary polyclonal goat anti-rabbit IgG (ab205718 1:10,000) antibody, and revealed using a commercial chemiluminescence detection system (Amersham, RPN2232). Semiquantitative analysis was performed using the C-Digit program (LI-COR Image Studio, Version 3.1). GAPDH was used as a reference standard.

Morphology and neuroinflammation of brain regions

To estimate the damage associated with chronic MLM infection, the brains of 5 healthy mice and 5 120-day PI mice were processed as described above for histological and immunohistochemical analyses. The regions that were examined were the cortex, ventral hippocampus in (CA1, CA3, and Gyrus Dentatus), and amygdala; rabbit antibodies against Iba-1 (ab153696) and GFAP (ab7260) were used in the analysis, and five independent fields per region were evaluated under a Nikon Eclipse E8000 microscope (Tokyo, Japan).

Behavioral testing

Behavioral testing is a validated and widely used procedure to assess depression and anxiety-like behavior in rodents (Bailey, 2009; Castagné et al., 2011; Steimer, 2011). In this study, five tests were used to evaluate these parameters in six 120-day PI MLM mice and six control mice. These tests included the open field test (OFT), the elevated plus-maze (EPM) test (Bailey, 2009), the forced swimming test (FST), and the tail suspension (TS) test (Petit-Demouliere et al., 2004; Cryan et al., 2005; Kara et al., 2018). All tests were performed in individual, separate, mice to avoid cumulative stress due to manipulation and rotation of mice in multiple tests. The experimental groups were housed three days before testing in a sealed and acoustically isolated room under bright lighting (400 lux) for adaptation. All the behavioral trials were performed during the first 4 h of the dark phase



of the light cycle. Before each trial, the equipment was cleaned with 75% ethanol and allowed to dry to prevent olfactory cues from influencing mouse behavior. All of the behavioral tests were video recorded, and analysis of the videos was performed by two independent evaluators to eliminate subjective interpretations.

Depression-like behavior

Forced swim test. This test was performed in transparent Plexiglas cylinders (height, 21 cm; diameter, 14.5 cm) filled with water (22–24 ± 0.5 °C, 15 cm depth) in 5-min sessions. Immobility behavior was scored when mice remained floating and treading just enough to keep their nose above the water. After the swimming sessions, the mice were removed from the cylinder, gently dried, placed in warm cages for 20 min, and returned to their housing cages.

Tail suspension. For this test, mice were individually placed in an acrylic box (20 cm wide × 20 cm deep × 30 cm high) and fastened by their tail with adhesive tape placed 1 cm from the tip of the tail. Each mouse was suspended for 5 min, and the immobility behavior was scored when the mouse remained passively hung and completely motionless.

Anxiolytic behavior

Open field test. This test was performed during 5 min per animal in a cubic device (50 × 50 × 50 cm) with transparent acrylic walls and a black floor. White lines drawn on the black floor divided the area into sixteen, 12.5 × 12.5 cm squares. Four squares in the central area, surrounded by 12 peripheral squares, were selected as the probe area. The number of entries into the central area and the time spent there (4 paws inside), were registered with a digital video camera to assess the anxiolytic-like behavior.

Elevated plus maze. This test consisted of four wooden arms of equal size (30 cm long, 5 cm wide) in which two closed arms were perpendicular to two open arms. The intersecting open and closed arms formed a central 5 × 5 cm square platform. The two closed arms had 40-cm high dark walls, and the two open arms had 0.5-cm high protection ledges. The arms were arranged to form a "plus" (+) sign. The maze was elevated 50 cm above the floor. This test was performed for 5 min per animal and the time spent and the number of entries into an open arm were registered with a digital video camera. An entry was scored when all four paws were inside of either an open or a closed arm.

Corticosterone levels

Corticosterone levels in serum were determined by using a commercial ELISA kit (ADI-900-097 Enzo Life Science) following the manufacturer's instructions. Absorbance was measured at 450 nm in a Sunrise TECAN microplate reader, and the corticosterone concentration was calculated from a standard curve fit to a semilog plot in Excel (Microsoft, Redmond, WA, USA).

Neurotransmitters quantification

To quantify norepinephrine (NE) and epinephrine (EP), the adrenal gland was homogenized in 400 μl of a solution containing 5% ascorbic acid, 200 mM sodium phosphate, 2.5 mM L-cysteine, and 2.5 mM EDTA. Then, protein was precipitated by adding 100 μl of 0.4 M perchloric acid, followed by incubation at 20 °C for 20 min. After centrifugation at 12,000 rpm for 10 min at 4 °C, the supernatants were collected and used to quantify the levels of NE and EP by reversed-phase high-pressure liquid chromatography (RP-HPLC) following the standard procedure (Becerril-Villanueva et al., 2018; Barbosa Méndez and Salazar-Juárez, 2019; Lara-Espinosa et al., 2020; Maldonado-García et al., 2021).

Statistical analysis

In all cases, a Shapiro–Wilk normality analysis was performed. The comparison between groups was carried out by a one-way analysis of variance (ANOVA) test, followed by a post-doc test (Bonferroni), using the SPSS v21 and SigmaStat v11 programs. The value of n represents the number of individuals used in each test. The correlation analysis between neuronal damage and behavioral disturbances was performed using a Spearman correlation analysis. A value of $p < 0.05$ was considered statistically significant in all cases. Graphs were made using Prism 9 software. Data are shown in box plots and whiskers plots. In the box plots, a black line within the box marks the median. The boundary of the box indicates the 25th and 75th percentiles. Whiskers above and below the box indicate the 5th and 95th percentiles.

Fig. 3. Disruption of the blood–brain barrier in the hippocampus of mice at 120 post infection days with *MLM.* Image from the control group shows a histologically preserved hippocampal structure without parenchymal infiltrate **(A)**. Atrophy in the molecular layer of hippocampus is observed in the 120-day PI group **(B)**. Confocal images illustrate Evans' Blue distribution in the hippocampal regions. Evans' blue is observed within the vascular compartment in the control group, while it is observed within the hippocampus parenchyma in the 120-day PI group owing to vessels disruption **(C)**. The 3D reconstructions illustrate Evans' Blue flow through the vessels' lumen **(D)**. Diffusion of the dye to the tissue parenchyma is observed in the 120-day PI group. Representative Western blot result of hippocampal extracts from control and 120-day PI groups showing a reduction in the expression of the tight junction proteins zonula occludens (ZO-1), occludin (Ocldn) and claudin-5 (Cldn-5) in the 120-day PI group **(E)**. Representative results, ($n = 5$ per group). (*$p < 0.05$, **$p < 0.001$, where indicated).

RESULTS

Histopathological changes in the liver and spleen

Compared to normal mice, chronically infected mice showed splenomegaly (8.5-fold increase, $F = (1,26)$ 487.15 $p < 0.001$) and hepatomegaly (0.6-fold increase, $F = (1,26)$ 127.19 $p < 0.001$). Atrophy was observed in several organs, including bone marrow, the liver, and the spleen. In these organs, extensive coalescing granulomas composed of AFB-laden macrophages and epithelioid and multinucleated cells were observed (arrows) (Fig. 1A, B).

Cytokine expression in the liver and spleen

No expression of the type-1 cytokines IFN-γ and IL-2 was observed in the control group or the 120-day PI group in either the spleen or liver (Fig. 2A–H). However, the type-2 cytokines IL-4 and IL-10 were present in the 120-day PI group, mainly in macrophages and epithelioid cells spread throughout the tissue parenchyma (Fig. 2 I-P).

Blood–brain-barrier permeability

Chronic infection selectively increased the permeability of the BBB in the 120-day PI group (Fig. 3B). Confocal microscopy allowed to see the tracing dye distribution in blood vessels; in the micrographs, a conserved morphology is observed in the control group which does not allow the entrance of Evans blue (EB) into the tissue parenchyma (Fig. 3C). The 120-day PI group shows ruptured blood vessels and the entrance of the dye into the parenchyma (red) (Fig. 3C). The 3D reconstruction image shows the diffusion of EB through the lacunosum-molecular layer, molecular layer, and the hippocampus hilus can be observed (Fig. 3D). The expression of tight junction proteins associated with BBB permeability was notably reduced in the 120-day PI group claudin-5$F = (1,8)$ 37.29 $p < 0.001$, occludin $F = (1,8)$ 32.64 $p < 0.001$, ZO-1$F = (1,8)$ 8.51 $p < 0.05$ (Fig. 3E).

Neuroinflammation

Regarding the neuroinflammatory status, an increase in immunostaining for GFAP was observed in the 120-day PI group compared with the control group: cortex $F = (1,38)$ 8.00 $p < 0.05$, hippocampus $F = (1,38)$

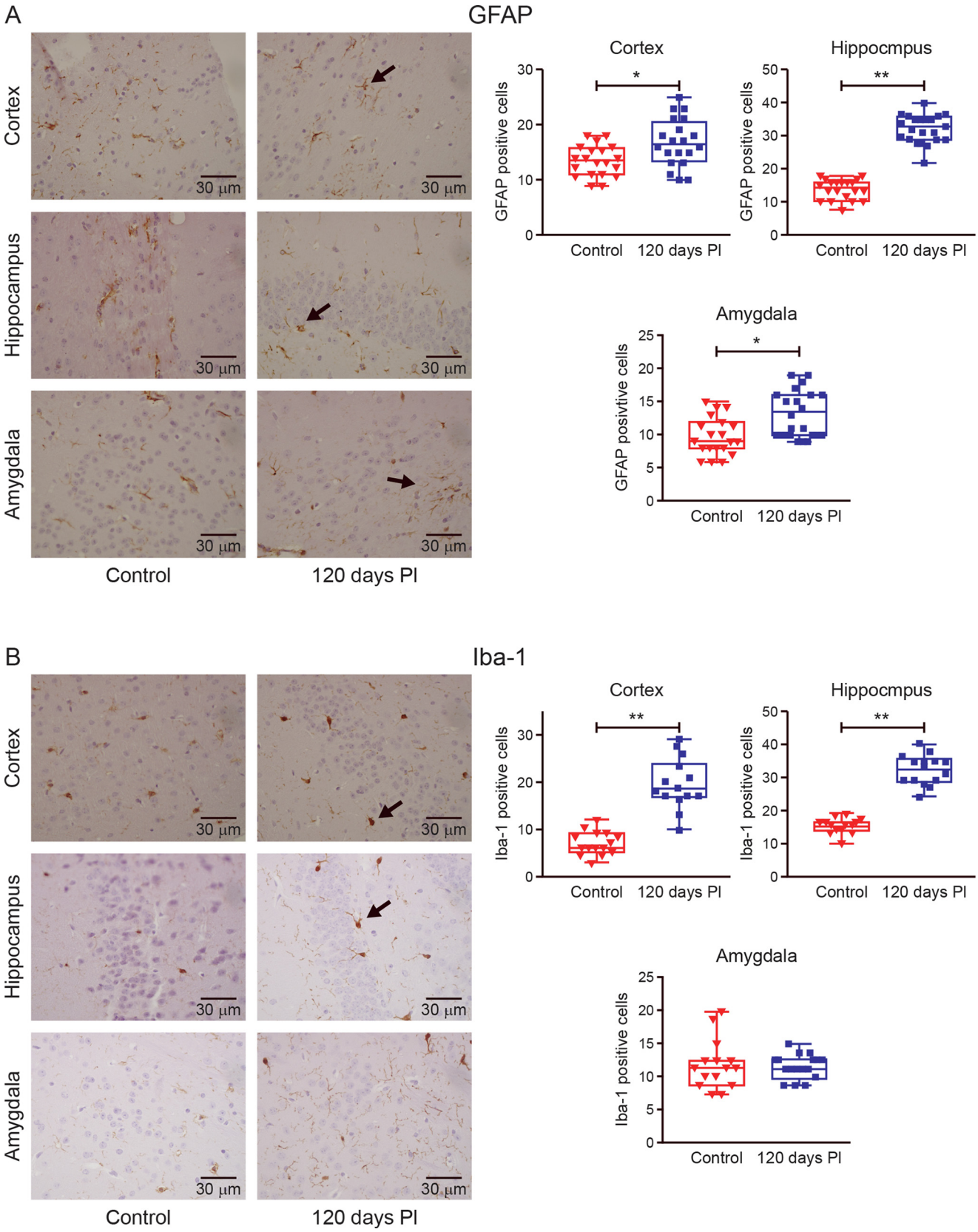


Fig. 4. Analysis of glia in brain regions during chronic infection with MLM. Representative immunostaining images for GFAP and Iba1 in the cortex, hippocampus, and amygdala. GFAP-positive glial cells (arrows) are present in the three regions in the 120-day PI group **(A)**. Immunostaining for Iba-1 is stronger in the cortex and hippocampus region (arrows) in the 120-day PI group **(B)**. Percent damage was calculated by evaluating 25 independent 400× fields ($n = 5$ per group) (*$p < 0.05$, **$p < 0.001$, where indicated).

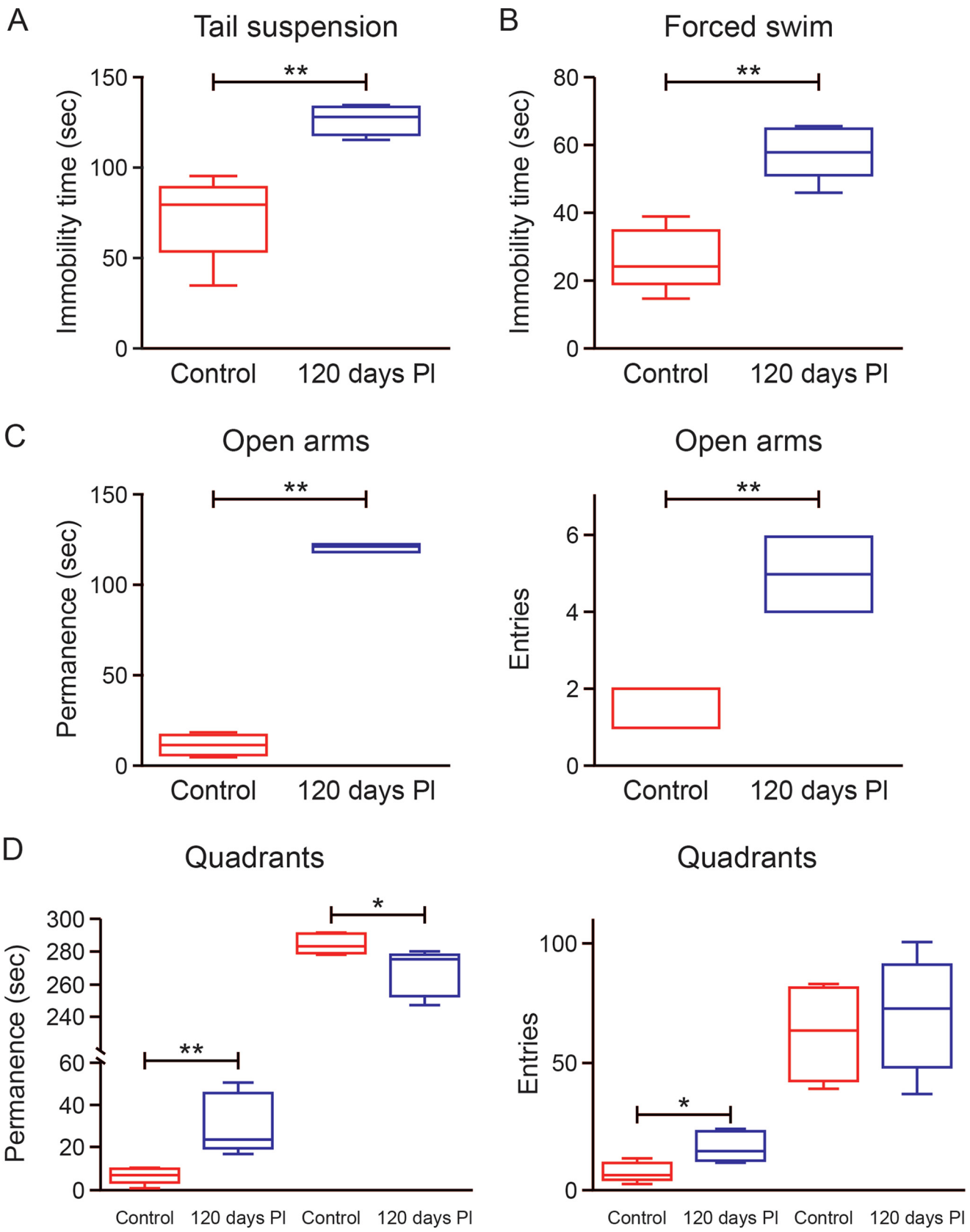


Fig. 5. Neurobehavioral disorders associated to chronic infection with MLM. Depression-like behavior in the 120-day PI group increases the time of immobility in the tail suspension **(A)** ($n = 5$ per group) and the forced swimming **(B)** tests ($n = 6$ per group). In the elevated plus maze test, mice in the 120-day PI group showed increased entrance and permanence ratios into the open arms **(C)** ($n = 6$ per group). Mice in the 120-day PI group showed an increased entrance ratio to the central quadrant as well as an increased residence time in the open field test ($n = 5$ per group) **(D)**. All tests were performed on individual mice to avoid cumulative stress ($^*p < 0.05$, $^{**}p < 0.001$, where indicated).

Table 1. Behavior and neuronal damage correlation in cortex, ventral hippocampus (CA1, CA3 Dentate gyrus) and amygdala

	Tail Suspension Immobility (s)	Forced Swim Immobility (s)	Elevated Plus Open Arms Permanence (s)	Open Fiel Quadrants Central Permanence (s)
	r_s	r_s	r_s	r_s
Cortex	0.8333*	0.7142	0.7784*	0.8333*
CA1	0.9285**	0.5714	0.9221**	0.7142
CA3	0.7619*	0.7857*	0.6467	0.6666
Dentate gyrus	0.8095*	0.8571*	0.7425*	0.8333*
Amygdala	0.6904*	0.8333*	0.6347	0.7142

Spearman correlation test of Shapiro-Wilk test Normalized-data. Statistical significance is represented as *$p < 0.05$, ** $p < 0.005$ (two tailed).

243.66 $p < 0.001$ and amygdala $F = (1,38)$ 13.79 $p < 0.001$ (Fig. 4A). The expression of the marker Iba-1 was found increased in two of the analyzed regions: cortex $F = (1,27)_{68.54}$ $p < 0.001$ and hippocampus $F = (1,26)_{150.54}$ $p < 0.001$ but no differences were observed in the case of amygdala (Fig. 4).

Alterations in depression and anxiety behavior

Tests for depression and anxiety behavior were performed in separate groups of animals to avoid cumulative stress between mice. A significant difference in immobility between the control and 120-day PI groups was observed in both tests forced swimming, $F = (1,10)_{42.78}$ $p < 0.001$ (Fig. 5A) and tail suspension, $F = (1,8)$ 23.53 $p < 0.001$ (Fig. 5B). Immobility in both tests is correlated with depression-like behavior.

Anxiolytic behavior was evaluated with the elevated plus maze (EPM) test and open field test (OFT). In the EMP test, compared with those in the control group, the 120-day PI group showed an increase in the time permanence in the open arms (sec) $F = (1,10)$ 28.62 $p < 0.001$ and in the number of entries to the maze's open arms $F = (1,10)$ 66.81 $p < 0.001$ (Fig. 5C), while in the OFT, the 120-day PI group demonstrated an increased permanence time (sec) in the central quadrants $F = (1,8)$ 12.91 $p < 0.01$, which was correlated with the increase in the number of entrances to the central zone $F = (1,8)$ 10.24 $p < 0.05$ (Fig. 5D).

Neural atrophy parameters

To assess CNS damage, the cortex, amygdala, and hippocampal ventral regions (CA1, CA3 and gyrus dentatus, GD) were analyzed. The changes indicative of neuronal damage included basophilic pyknotic nuclei, acidophilic cytoplasm, and fragmented nuclei. Neurons with normal characteristics were observed in the three regions analyzed in the control group. In the 120-day PI group, the cortex showed an increased number of cells with pyknotic nuclei and cytoplasmic contraction $F = (1,42)$ 1154.72 $p < 0.001$. In amygdala, an increased percentage of morphologically damaged cells was observed $F = (1,38)$ 116.84 $p < 0.001$. In the hippocampus, the CA1 region showed the greatest number of cells with nuclear and cytoplasmic atrophy $F = (1,34)$ 394.94 $p < 0.001$, followed by the CA3 $F = (1,34)$ 345.5 $p < 0.001$ and GD regions $F = (1,38)$ 308.78 $p < 0.001$. Despite these alterations, no acid-fast bacilli or inflammatory infiltration was observed in these regions (Fig. 6).

Correlations between behavioral changes and morphological alterations were evaluated with the Spearman–Shapiro–Wilk correlation test. It was found that the depression-like behavior tests, TST and FS, positively correlated with morphological alterations in all evaluated regions: Cortex, CA1, CA3, dentate gyrus and amygdala ($p < 0.05$ to < 0.005), and this was also so in the tests for anxiolytic behavior EPM and OP ($p < 0.05$ to < 0.005) (Table 1).

Alterations in adrenal gland and serum corticosterone

General atrophy was observed in the 120-day PI group, including thickening of and fibrosis in the adrenal capsule, reduction of the thickness of the cortex layers, cells with a vacuolated cytoplasm, and loss of cells. Macrophages harboring mycobacteria but not granulomas were present in the parenchyma gland (Fig. 7A). The levels of epinephrine $F = (1,6)$ 114.36 $p < 0.001$ and norepinephrine $F = (1,6)$ 162.75 $p < 0.001$ (Fig. 7B), as was the level of corticosterone in serum $F = (1.8)$ 37.76 $p < 0.001$ (Fig. 7C).

DISCUSSION

Murine leprosy is a chronic granulomatous disease characterized by loss of the cellular immune response, which leads to anergy. This immunological alteration allows sustained growth of the bacillus (*M. lepraemurium*) and the development of a systemic infection that affects the viscera and skin. The lesions in chronic murine leprosy are similar to those observed in patients with untreated lepromatous leprosy (LL) (Rojas-Espinosa and Løvik, 2001; Rojas-Espinosa, 2009; Juarez-Ortega et al., 2015; Rojas-Espinosa et al., 2020). Despite being a systemic infection, the disease does not affect peripheral nerves or the central nervous system (CNS) (Rojas-Espinosa et al., 2005; Becerril-Villanueva et al., 2018), which makes murine leprosy an adequate model to explore the morphologic and behavioral changes involved in the anxiolytic and depression-like behaviors observed in diseased animals.

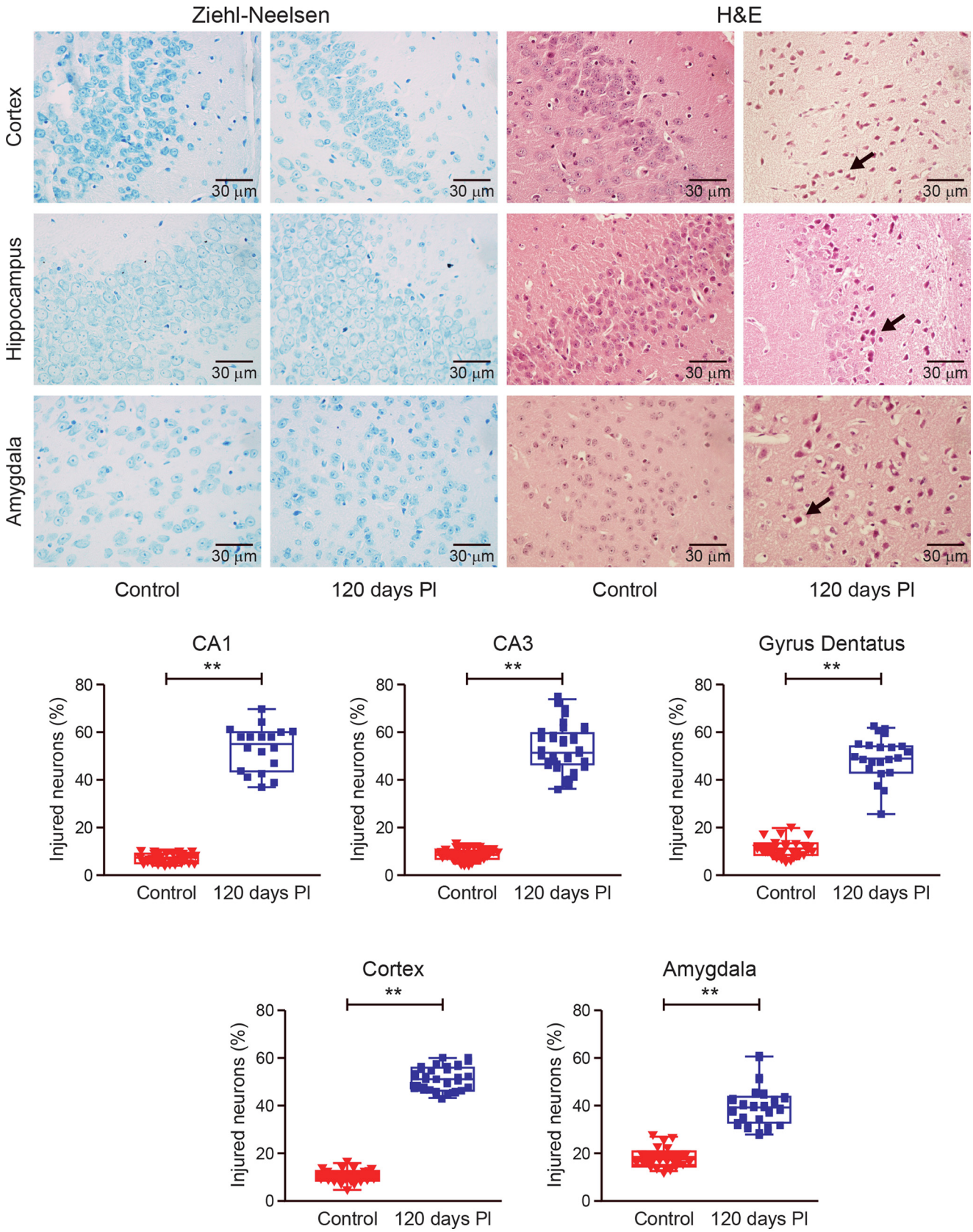
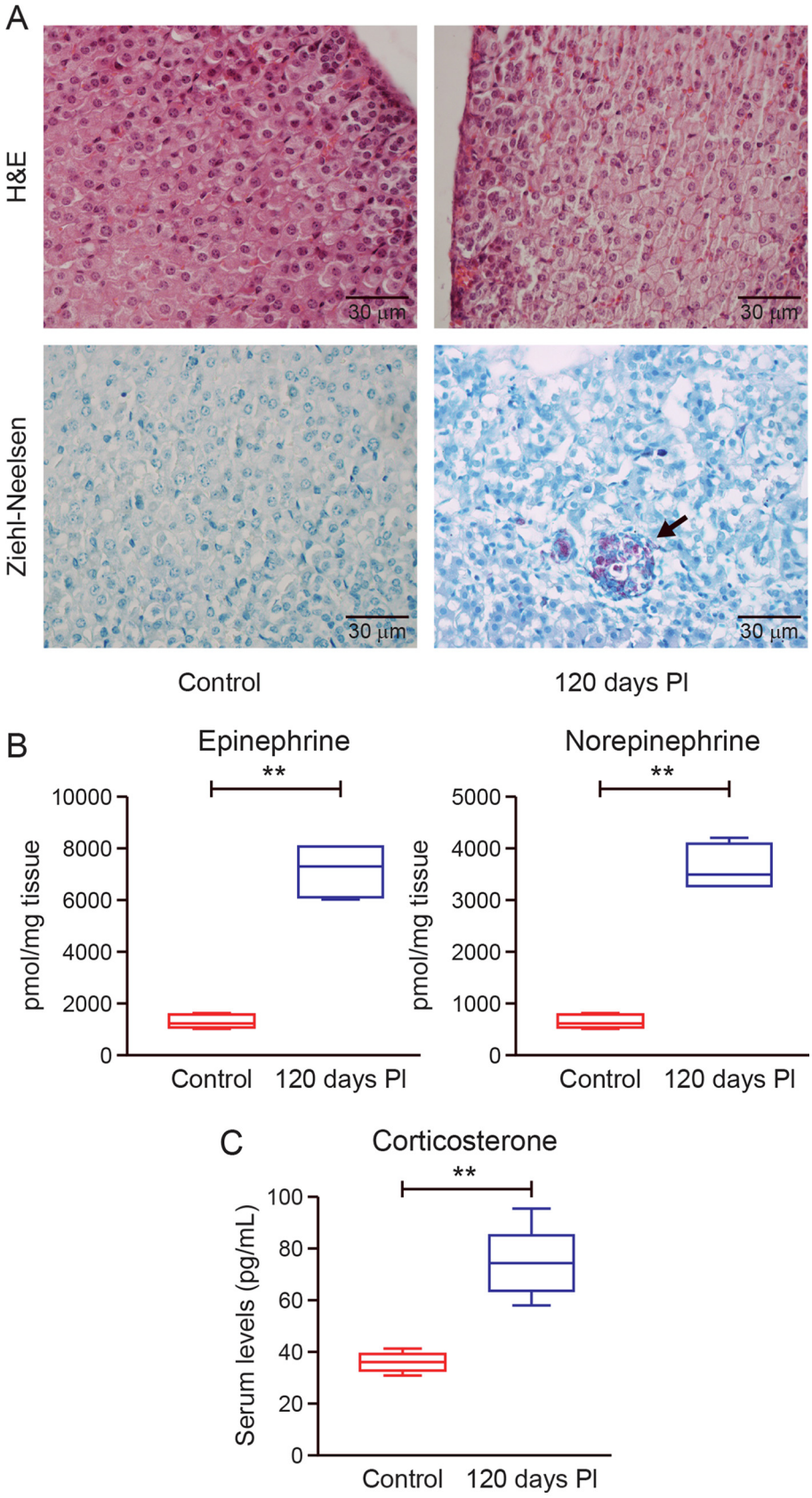


Fig. 6. Cellular atrophy in nervous tissue in the absence of MLM. Representative images of the cortex, hippocampus, and amygdala regions in the 120-day PI group showing neurons with contracted cytoplasm, pyknosis, and fragmented and acidophilic nuclei (arrows). No inflammatory lesions or bacilli were found in the analyzed regions (n = 5 per group). The percent damage was calculated by evaluating 25 independent 40× fields. Significant differences from the control group were observed in the hippocampal ventral CA1, CA3, GD, cortex and amygdala regions (**p < 0.001). HE and ZN stains.

In line with previous reports on murine and lepromatous leprosy, we observed marked splenohepatomegaly in mice bearing a 120-day infection with MLM that was caused by the accumulation of macrophages with a large number of bacilli. Extensive granuloma fractions composed of macrophages full of bacilli were observed in the residual parenchyma of both organs (Herrerias et al., 1980; Hickey et al., 2015; Becerril-Villanueva et al., 2018; Massoth and Abner Louissaint, 2020; Rojas-Espinosa et al., 2020). The absence of IL-2 and IFN-γ expression was observed in both organs, but IL-4 and IL-10 were highly expressed. These results are consistent with the findings in other models of chronic infection with mycobacteria in which cellular anergy is also elicited (Hernandez-Pando et al., 1996; Rojas-Espinosa and Løvik, 2001; Hurtado Ortiz et al., 2009; Rojas-Espinosa, 2009; Juarez-Ortega et al., 2015; Mitchell et al., 2017; Becerril-Villanueva et al., 2018; Rojas-Espinosa et al., 2020; Röltgen et al., 2020). IL-4 and IL-10 are cytokines that are involved in phagolysosomal maturation processes and the microbicidal activity of macrophages and polarize the response towards a Th2 profile through activation of the Janus kinase 1 (JAK1)/Tyk2/STAT3/JAK2/STAT6 pathway; their anti-inflammatory activity is, however, a homeostatic host response that restrains the severe effects of chronic inflammation. Our finding of a lack of fibrotic lesions in the spleen and liver in the experimental group was consistent with those of previous studies (Van Meegeren et al., 2012; Wynn and Vannella, 2016; Steen et al., 2020). The integrity of the blood–brain barrier (BBB) plays a key role in preventing potentially neurotoxic molecules from accessing the CNS (Zhao et al., 2015; Besedovsky, 2019). We found an increase in BBB permeability in the molecular layer of the hippocampus of mice in the 120-day PI group, together with a decrease in the expression of the tight junction proteins ZO1, Cldn5 and Ocldn. These data suggest that specific BBB disruptions are associated with chronic inflammatory processes, as has been



reported in infection with *M. tuberculosis* (Hurtado-Alvarado et al., 2017, 2018; Nwafor et al., 2019; Xu et al., 2019; Lara-Espinosa et al., 2020). The role of IL-4/IL-10 in the opening of the BBB is not entirely clear. An increase in gliosis markers is commonly observed in BBB disruptions, although it can occur without BBB alterations; accordingly, we found increases in GFAP and Iba-1 expression in the cortex, ventral hippocampus (vHPC), and amygdala in the experimental group. IL-4 may increase BBB permeability in the hippocampus as it does in neonatal mice (Wang et al., 2018). IL-10, on the other hand, is an anti-inflammatory cytokine; its marked expression in the CNS of mice in the 120-day PI group suggests that it plays a role in controlling the damage caused by neuroinflammation (Lin et al., 2018; Porro et al., 2020).

Inflammatory events in the CNS can be associated with neurobehavioral impairment. In our study, mice in the 120-day PI group showed significant alterations indicative of depression and anxiolytic-like behaviors, similar to those described in other models of chronic stress and infections with other mycobacteria (Rodriguez-Zas et al., 2015; Becerril-Villanueva et al., 2018; Cui et al., 2020; Lara-Espinosa et al., 2020).

In the tail suspension and forced swim tests, mice in the 120-day PI group showed a significant increase in immobility time, indicative of depression-like behavior; this neurobehavioral alteration was correlated with the histologic damage observed in the prefrontal cortex, including thinning due to neuronal atrophy and alterations of emotional balance (Drevets, 2007; Christoffel et al., 2011; Späti et al., 2015). Lesions in the CA1, CA3, and GD regions of the vHPC were also associated with behavioral disturbances. Similar studies in humans and other animal models have also studied the relationships of the decrease in size of the hippocampus, neuronal atrophy and decreased neurogenesis with disturbances related to depression-like behavior (Videbech and Ravnkilde, 2004; Fanselow and Dong, 2010; Christoffel et al., 2011; Schoenfeld et al., 2017; Fang et al., 2018; Gulyaeva, 2018).

Mice from the 120-day PI group exhibited increases in the time of permanence and in the number of entries to the open arms zone and central quadrants in the elevated plus maze (EPM) test. The open field test (OFT), which reflects a decrease in fear and protection against a threat, revealed a hyperanxiolytic state in the 120-day PI group that has been related to dysregulation of the basolateral amygdala circuit (BLA), vHPC and prefrontal cortex (PFC) (LeDoux, 2000a; Adhikari, 2014; Tovote et al., 2015). The atrophy in the PFC observed in the 120-day PI group was consistent with cytotoxic lesions and selective inhibition of the PFC-vHPC and PFC-BLA circuits. The weakening of the functional connectivity between these areas explains the breakdown of this interconnection, which results in biochemical, molecular, and electrophysiological abnormalities, and in the breakdown of the excitation/inhibition (E/I) relationship of some neuronal groups (Deacon et al., 2003; Franklin et al., 2017; Kim and Cho, 2017; Spalding, 2018; Liu et al., 2020). In addition, there is evidence that experimental lesions in the vHPC favor an increase of time spent in the anxiolytic zone in the EPM and OFT (Kjelstrup et al., 2002; Felix-Ortiz et al., 2013; Felix-Ortiz and Tye, 2014; Pi et al., 2020). On the hand, the amygdala maintains a dynamic interconnection with the fear circuit and is a safeguard against threats (LeDoux, 2000b; Felix-Ortiz et al., 2013; Adhikari, 2014; Felix-Ortiz and Tye, 2014; Tovote et al., 2015; Korn et al., 2017; McDonald and Mott, 2017; Liu et al., 2020). Thus, the cellular atrophy in the PFC-vHPC-amygdala circuit could explain the hyperanxiolytic state of the 120-day PI group versus that of the control group.

Our results indicate dysregulations in the endocrine system (ES) and sympathetic nervous system (SNS). In addition to the morphological lesions in the cortical zone, the increase in the serum levels of corticosterone (CORT) in the 120-day PI group is consistent with reports on increased CORT and IL-4/10 levels associated to increased Th2 responses in other mycobacterial infections (Auphan et al., 1995; Hernandez-pando et al., 1998; Hernandez et al., 2013; D'Attilio et al., 2018). In the CNS, depression-like behavior has been associated with an increase in CORT, which induces atrophy of the PFC, HPC and BLA (Videbech and Ravnkilde, 2004; Baptista and Andrade, 2018; Fang et al., 2018; Gulyaeva, 2018). In addition, there is evidence of macrophages with the M2 b/c phenotype that secrete CORT (data to be published). The increase in NE and EPI in the 120-day PI group could play important roles in increasing the bacillary load through inhibition of nitric oxide (NO) production and differentiation of M2 macrophages (Sigola and Zinyama, 2000; Grailer et al., 2013, 2014; Lamkin et al., 2019; Gotovina et al., 2020).

In summary, the tridirectionally communication among the CNS, endocrine, and immunologic systems is a complex biological multisystem responsible for the maintenance of harmony and homeostasis (Ponce-Regalado et al., 2022). Infections are usually resolved because of an efficient host response; however, infections that reach a chronic stage are excellent experimental models to study the key steps of the regulation/deregulation of this multisystem and to discover critical steps that can be modified to interfere with the progression of chronic diseases.

Fig. 7. Atrophy in adrenal glands of mice chronically infected with MLM. Representative images of adrenal glands of mice in the 120-day PI, group showing fibrotic lesions in the capsule and atrophy in the cortical area together with cytoplasmic vacuolization and thinning of the cortical layer (arrows) (n = 5 per group). Few AFB-containing macrophages were observed without granulomatous lesions (arrows) **(A)**. Epinephrine and norepinephrine levels in tissue (n = 4 per group) **(B)** and corticosterone in serum (n = 5 per group) were increased compared to levels in the control group **(C)**. HE and ZN stains, 40× (**$p < 0.001$, where indicated).

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AUTHOR CONTRIBUTIONS

E B-V and O. R-E designed the study and wrote the protocol and manuscript. MD P-R, A C-O, G H-A and G P-S performed the experimental work. MD P-R, A S-J, P A-P, G-P MI, P-R L, ME A-S and H-P R supervised the project and discussed the results. All the authors approved the final manuscript and its submission for publication.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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APPENDIX A. SUPPLEMENTARY DATA

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