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Increased susceptibility to encephalitozoonosis associated with mixed Th1/Th2 profile and M1/M2 profile in mice immunosuppressed with cyclophosphamide

Amanda Miyuki Hidifira [a], Anuska Marcelino Alvares-Saraiva [a], Elizabeth Christina Perez [a], Diva Denelle Spadacci-Morena [b], Ronalda Silva de Araujo [c], Maria Anete Lallo [a,*]

[a] Programa de Patologia Ambiental e Experimental, Universidade Paulista (UNIP), São Paulo, Brazil
[b] Departmento de Fisiopatologia, Instituto Butantã, São Paulo, Brazil
[c] CETESB-Companhia Ambiental do Estado de São Paulo, Brazil

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ABSTRACT

Encephalitozoon cuniculi is a unicellular, spore-forming, obligate intracellular eukaryote belonging to the phylum Microsporidia. It is known to infect mainly immunocompromised and immunocompetent mammals, including humans. The parasite–host relationship has been evaluated using both *in vitro* cell culturing and animal models. For example, Balb/c and C57BL/6 mouse strains have been used interchangeably, although the latter has been considered more susceptible due to the higher fungal load observed after infection. In the present study, we identified the characteristics of the immune response of C57BL/6 mice treated or not with the immunosuppressant cyclophosphamide (Cy) and challenged with *E. cuniculi* by intraperitoneal route. After 14 days of infection, serum was collected to analyze Th1, Th2, and Th17 cytokine levels. In addition, peritoneal washes were performed, and the spleen sample was collected for immune cell phenotyping, whereas liver, spleen, kidney, lung, intestine, and central nervous system (CNS) samples were collected for histopathological analysis. Although infected mice displayed a reduced absolute number of macrophages, they showed an M1 profile, an elevated number of $CD4^+$T, $CD8^+$T, B-1, and B-2 lymphocytes, with a predominance of Th1 inflammatory cytokines (interferon [IFN]-γ, tumor necrosis factor [TNF]-α, and interleukin [IL]-2) and Th17. Furthermore, *Cy-Infected* mice showed a reduced absolute number of macrophages with an M1 profile but a reduced number of $CD4^+$T, $CD8^+$T, B-1, and B-2 lymphocytes, with a predominance of Th1 inflammatory cytokines (IFN-γ, TNF-α, and IL-2) and Th2 (IL-4). This group displayed a higher fungal burden as well and developed more severe encephalitozoonosis, which was associated with a reduced number of T and B lymphocytes and a mixed profile of Th1 and Th2 cytokines.

1. Introduction

Microsporidia are a group of obligate intracellular parasites comprising more than 1400 species. They are known to infect both vertebrates and invertebrates (Ruan et al., 2021). These fungi were described around 160 years ago with the identification of *Nosema bombycis,* which has been reported to cause pebrin in silkworms. More recently, these have been associated with localized or disseminated infections, sometimes fatal. These fungi are opportunistic pathogens, especially in immunosuppressed patients (Han et al., 2021; Seatamanoch et al., 2022). In immunocompromised individuals, infections can result in severe consequences, accompanied by persistent diarrhea, malabsorption, and weight loss. In addition, these pathogens can survive asymptomatically in immunocompetent individuals, with a spontaneously resolving course of infection (Pan et al., 2018).

These findings confirm that both innate and adaptative immunity are crucial to resist and protect against microsporidian infections. Several immune cells, including macrophages, dendritic cells, and $CD8^+$T cells, and cytokines such as interleukin (IL)-12 and interferon (IFN)-γ are activated and contribute to the immune defense (Han et al., 2020). These responses serve as the first line of defense and mostly involve inflammatory cells such as polymorphonuclear neutrophils (PMNs),

* Corresponding author.
E-mail addresses: anetelallo@hotmail.com, maria.lallo@docente.unip.br (M.A. Lallo).

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macrophages, and dendritic cells, and allow partial elimination of parasites (Dalboni et al., 2021). In addition, they condition the nature of the adaptive immune responses during host infection.

Mice infected with *Encephalitozoon cuniculi* have been used to study different aspects of the pathophysiology of microsporidiosis. As an opportunistic pathogen, *E. cuniculi* can cause disseminated and lethal infection in nude or severe combined immunodeficiency disease (SCID) mice; however, such conditions do not represent the most immunosuppressive states observed clinically (Han et al., 2019). Pharmacological immunosuppression animal models established using cyclophosphamide (Cy) (Lallo and Hirschfeld, 2012) or dexamethasone (An et al., 2020) have been used to determine more consistent changes and increase the susceptibility of animals to microsporidia infection. Although inbreeding is used to produce standardized model organisms, recent studies have demonstrated that factors such as ambient temperature (Kokolus et al., 2013), nutrition (Di Biase et al., 2016), and the microbiome (Garrett, 2015) can significantly impact scientific results; thus, thwarting all standardization efforts. Therefore, it is fundamental to identify the differences between animal models used in experimental studies.

A high spore load was demonstrated in the peritoneal washings of C57BL/6 mice infected intraperitoneally with *E. cuniculi*, thus, increasing the susceptibility of this strain to the pathogen compared to the Balb/c mice (Niederkorn et al., 1981). However, no additional experiments were performed to demonstrate or identify the causes of this susceptibility, and both strains (Balb/c and C57BL/6) have been used interchangeably for experimental studies. C57BL/6 mice are more resistant to infection by *Leishmania major*, whereas Balb/c mice died days after infection by the same pathogen (Scott and Farrell, 1982; Heinzel et al., 1988). In addition, heightened resistance was identified against *Mycobacterium tuberculosis, Pasteurella pneumotropica, Chlamydia*, and even melanoma metástases (Jiang et al., 2010; Bertolini et al., 2016; Foerster et al., 2017; Fornefett et al., 2018). Studies revealed that in contrast to C57BL/6 mice, which showed a Th1- and M1-dominant immune response, BALB/c mice displayed a more Th2- and M2-dominant immune response (Mills et al., 2000; Fornefett et al., 2018). Differences in susceptibility strongly indicated significant variations in the innate immune system function, which were eventually attributed to M1/M2 differences. Inbred mouse strains constitute the backbone of biomedical research. Thus, we identified the characteristics of the immune response of C57BL/6 mice treated or not with the immunosuppressant Cy and challenged with *E. cuniculi*.

2. Methods

2.1. Ethics statement

All experimental procedures were conducted in accordance with the Brazilian law on the use of experimental animals, safety, and use of pathogenic agents. The study was approved by the Universidade Paulista Ethics Committee for Animals (protocol number: 01016).

2.2. Animals

Specific-pathogen free (SPF), 8-week-old, C57BL/6 male mice were obtained from the "Centro de Desenvolvimento de Modelos Experimentais para Biologia e Medicina" from the Federal University of São Paulo, Brazil. During the experimental period of 35 days, animals were housed at the Paulista University Animal Facility and kept under SPF conditions with controlled temperature and humidity in microisolators.

2.3. Encephalitozoon cuniculi spores

E. cuniculi (genotype I) spores were obtained from Waterborne Inc. (New Orleans, LA, USA). The spores were grown in a rabbit kidney cell lineage (RK-13, ATCC CCL-37) in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal calf serum (FCS), pyruvate, non-essential amino acids, and gentamicin at 37 °C in 5% CO_2 and harvested from tissue culture supernatants. Spores were washed thrice in phosphate-buffered saline (PBS) and counted using a hemocytometer.

2.4. Treatment with cyclophosphamide

Mice in the immunosuppressed group were treated with 200 mg/kg Cy intraperitoneally once a week (Genuxal®, Asta Medica Oncologia, São Paulo, Brazil), starting on the day of infection (day 0) (Lallo and Hirschfeld, 2012).

2.5. Experimental design

C57BL/6 mice ($n = 5$ animals in each group, 20 animals in total) were infected or not with spores of *E. cuniculi* and treated or not with Cy throughout the experimental period (*Uninfected* = control; *Infected* = infected with *E. cuniculi*; *Cy-Unifected* = treated with Cy and uninfected; *Cy-Infected* = treated with Cy and infected with *E. cuniculi*) (Fig. 1). After 14 days of infection, serum was collected to analyze the levels of Th1, Th2, and Th17 cytokines. In addition, peritoneal lavage and spleen samples were collected for immune cell phenotyping, whereas liver, spleen, kidney, lung, intestine, and central nervous system (CNS) samples were collected for histopathological analysis.

2.6. Necropsy and sample collection

Mice were anesthetized by intraperitoneally injecting a solution of ketamine (50 mg/mL), xylazine (20 mg/mL), and fentanyl (0.05 mg/mL) to attain cardiorespiratory arrest. A cardiac puncture was performed to collect blood serum. Subsequently, peritoneal cells were obtained by successively washing the peritoneal cavity (PerC) with 10 mL of PBS (Sigma, St. Louis, MO, USA). Afterward, half of the spleen was processed, and red blood cells were removed using a lysis buffer. Cell suspension was centrifuged, and subsequently, cells were washed with PBS and re-suspended in 100 μL of PBS supplemented with 1% bovine serum albumin (BSA) (1% PBS-BSA) for phenotypic characterization. In addition, the liver, kidney, lung, brain, intestine, and half of the spleen were fixed in 10% buffered formalin for 72 h and routinely processed for histopathology.

2.7. Immunohistochemistry

Liver sections of approximately 3 μm in size were placed on silanized slides and deparaffinized. After incubation with trypsin (for antigen recovery) at 37 °C for 15 min, the sections were incubated in 3% hydrogen peroxide and methanol (50:50) twice for 10 min to block endogenous peroxidase. Subsequently, the slides were incubated in primary polyclonal antibody (Ab) against *E. cuniculi* (1:10,000 diluted rabbit IgG, Medicago, Uppsala, Sweden, catalog number 18–9001–1) for 18 h at 4 °C. Next, the slides were incubated with secondary anti-rabbit Ab for 30 min in an oven at 37 °C (ImmPRESS™ Universal Reagent, anti-mouse/rabbit IgG, Vector Laboratories, N. MP-7500). The slides were stained with the chromogen 3′,3′-diaminobenzidine (DAB) and counterstained with hematoxylin, and visualized and imaged under a light microscope (Leica DMLD, Greenville, SC, USA).

2.8. Light microscopy

Paraffin-embedded tissue samples were routinely prepared for histopathology. Tissue cuts were stained with hematoxylin–eosin (H&E), analyzed, and imaged by DM LS (Leica, German) light microscope, with DFC420 image pick-up (Leica) and Leica Application Suite version 3.1.0 image program. Histological sections of liver fragments obtained from each animal and stained with H&E in *infected* and *Cy-infected* groups were subjected to morphometric analysis. The MetaMorph Microscopy

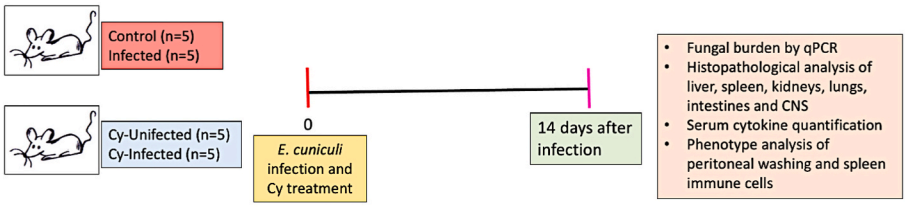

Fig. 1. Experimental design. C57BL/6 mice were infected or not infected with spores of *Encephalitozoon cuniculi* and treated or not with cyclophosphamide (Cy). After 14 days of infection, serum was collected to check the levels of Th1, Th2, and Th17. Peritoneal lavage and spleen samples were collected for immune cell phenotyping, whereas liver, spleen, kidney, lung, intestine, and central nervous system (CNS) samples were collected for histopathological analysis.

Automation & Image Analysis software (Molecular Devices, California, USA) was used to randomly measure the area of 10 lesions of each animal to obtain the mean. Next, the mean of each experimental group was calculated. We evaluated the mean area of lesions to compare the microsporidian infection in animals between the two groups.

2.9. Phenotypic characterization of immune cells

To block the Fc receptors, cells were incubated with anti-CD16/CD32 antibody (diluted in PBS supplemented with 1% albumin-BSA) for 20 min. Afterward, the cells were washed and further incubated with the following monoclonal antibodies*:* peridinin chlorophyll (PerCP)- or allophycocyanin (APC)-conjugated rat anti-mouse CD19, fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD23, PerCP-conjugated rat anti-mouse CD4, FITC-conjugated rat anti-mouse CD8, APC-conjugated rat anti-mouse F4/80, and pacific blue-conjugated rat anti-mouse CD11b (BD-Pharmingen, San Diego, CA). Cell phenotypes were determined as follows: $CD4^+$T cells ($CD19^-/CD4^+$), $CD8^+$T cells ($CD19^-/CD8^+$), macrophages ($CD19^-CD11b^+F4/80^+$), B-1 cells ($CD23^-/CD19^+$), and B-2 cells ($CD23^+/CD19^+$). After 30 min at 4 °C, the cells were washed and re-suspended in 200 μL of PBS for flow cytometry. Data were acquired with BD Accuri™ C6 (BD Biosciences, Mountain View, CA).

2.10. Cytokine quantification

The serum was obtained from the total blood from each mouse and stored at −80 °C. Next, the blood samples were thawed and the levels of IL-2, IL-4, IL-6, IFN-γ, TNF-α, IL-17, and IL-10 were measured using the BD CBA Mouse Th1/Th2/Th17 Cytokine Kit (BD Biosciences, CA, USA) according to the manufactures' instructions. Briefly, 25 μL of each sample was added to capture the beads specific to the cytokines and PE (phycoerythrin)-labeled secondary antibodies. The samples were incubated for 2 h at room temperature in the dark. Two-color flow cytometric analysis was performed using BD Accuri™ C6 (BD Biosciences).

2.11. Fungal burden

The fungal burden was determined by real-time polymerase chain reaction (qPCR)-based quantification of the fungal genomic DNA in the liver tissue. After euthanasia, the liver was weighed, and a constant amount of the liver lobe was frozen in RNAlater. The DNeasy Blood & Tissue extraction kit (Qiagen®) was used to extract the DNA as per the manufacturer's instructions and the protocol described by Moretto et al. (2015). The qPCRs were performed using the QuantStudio™ 5 System Real-Time PCR (Applied Biosystems, Foster City, CA, USA) and the Syber Green systems (Promega Corporation©, USA). Amplification reaction included a total volume of 25 μL/reaction containing 12.5 μL of the GoTaq® qPCR Master MixT® kit (Promega Corporation©, USA) along with 5 μL of the template DNA and respective primers (20 pmol). Quality controls included positive DNA samples obtained from purified suspensions and non-template controls (NTC).

Synthetic DNA fragments (gBlock® Gene Fragment, IDT, Iowa, USA) were used to establish standard curves. The 326-bp target region was extracted from the *18S rRNA* gene of the reference strain, *Donovan E. cuniculi* (NCBI: txid X98470.1). Ten-fold serial dilutions of gBlock® were used to construct a standard curve, reaching from 2.8×10^8 to $2{,}8 \times 10^2$ gene copies per μL (GC/μL) in each amplification cycle (Cq) for the *18S rRNA* gene of *E. cuniculi*. The standard curve was generated using linear regression of the relationship between the quantitation cycle value and copy number. After amplification, the number of copies (GC/μL) was converted to the number of spores/mg, considering the number of genomic copies already determined of 22 copies for the *18S* rRNA (Katinka et al., 2001).

2.12. Statistical analysis

The normality was calculated using the Shapiro–Wilk test. The homogeneity of the variance between the groups was verified using the Levene test. One-way analysis of the variance (ANOVA) was performed with Tukey's post-test. Confidence intervals were 95% of the media, and analysis was performed using the *bootstrap* tool. In all cases, the level of significance was $\alpha < 0.05$. All analyses were performed using the GraphPad Prism software (GraphPad Software Inc, La Jolla, CA, USA), version 7.0 for Windows ®.

3. Results

3.1. E. cuniculi infection determined clinical signs and inflammatory infiltrate in the liver

The infected animals (*Infected* and *Cy-Infected*) developed clinical signs, namely, wasting and lethargy. No deaths were recorded during this period. Necropsy revealed discrete ascites, hepatomegaly, and splenomegaly in the *Infected* group; however, clinical findings were more severe in the *Cy-Infected* group. Granulomatous multifocal hepatitis was observed randomly in all *E. cuniculi*-infected groups. Furthermore, spore clusters were associated or not with inflammatory infiltrates (Fig. 2A, B, C, D). Despite the immunosuppressive effect of Cy, morphometric analysis of the areas of hepatic inflammatory infiltrate measured and compared between infected animals revealed no differences (Fig. 2E). In addition, qPCR indicated a higher fungal burden in the liver of animals treated with Cy (*Cy-Infected*) than in the infected animals (Fig. 2F).

Interstitial multifocal pneumonia was observed in all infected groups. These lesions were more severe in *Cy-Infected* animals (Supplementary Fig. 1). Renal lesions included pyelonephritis, multifocal interstitial nephritis, and inflammatory infiltrate in the perirenal fat (Supplementary Fig. 1).

3.2. E. cuniculi infection reduced the macrophage population and cy modified its profile

Studies have demonstrated that the C57BL/6 mouse strain is more susceptible to encephalitozoonosis than Balb/c mice. However, there is a lack of information to explain the reason for this. In addition, little is

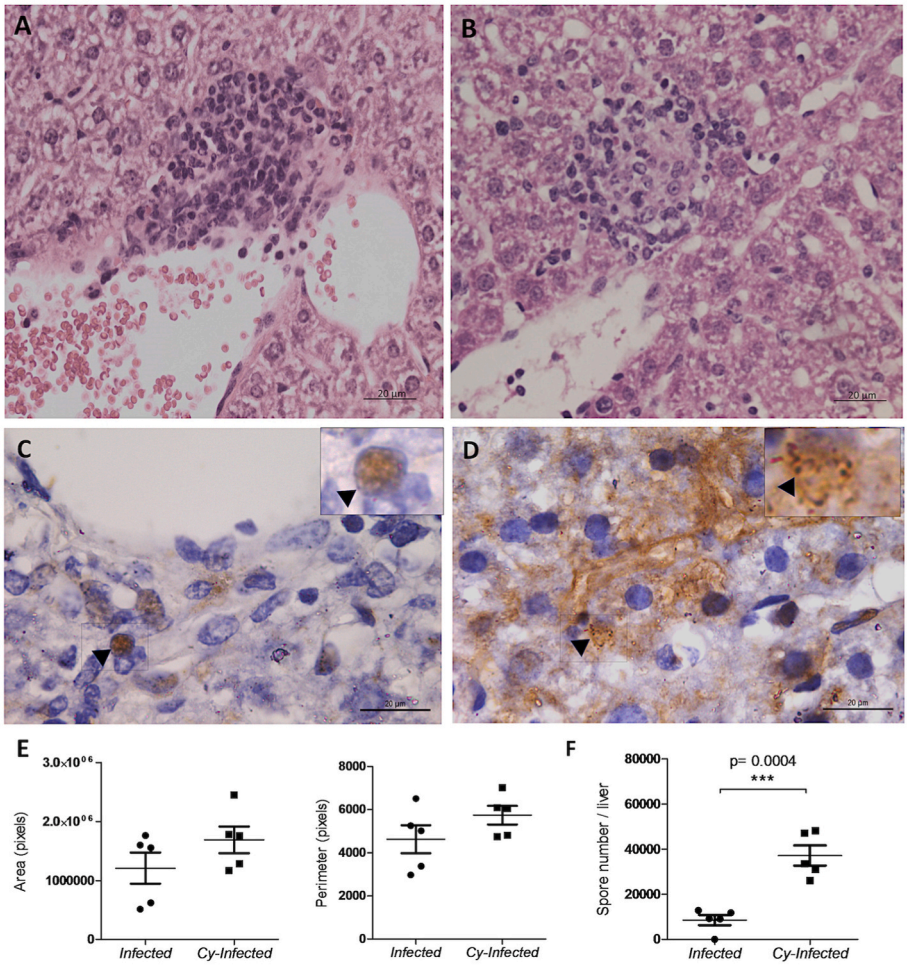

Fig. 2. Photomicrographs and lesion area of the hepatic parenchyma of mice infected with *E. cuniculi* spores and treated with cyclophosphamide (Cy) or not. (A) Hepatic granuloma in mice infected with *E. cuniculi* spores (hematoxylin and eosin [H&E] staining). (B) Hepatic granuloma in mice infected with *E. cuniculi* spores and treated with cyclophosphamide (H&E staining). (C) Collection of spores of *E. cuniculi* in the liver of infected mice (3,3′-diaminobenzidine [DAB] chromogen staining; hematoxylin counterstained). (D) Large collection of spores of *E. cuniculi* in Cy-infected mice (DAB chromogen staining; hematoxylin counterstained). (E) Morphometric analyses (area and perimeter) of liver lesions in infected (mice infected with *E. cuniculi* spores) or Cy-infected (mice infected with *E. cuniculi* spores and treated with Cy) (unpaired *t*-test). (F) Quantification of fungal burden in the liver parenchyma of mice belonging to the *Infected* and *Cy-Infected* groups using real-time PCR (qPCR) technique (Student's *t*-test, $p < 0.05$*).

known about the characteristics of the immune response associated with immunosuppression caused by Cy. Here, we demonstrated a significant reduction in the number of peritoneal macrophages at the site of *E. cuniculi* inoculation in infected mice (*Infected and Cy-Infected* groups) compared to their uninfected controls (Fig. 3A and B). The expression of costimulatory molecules such as CD80 and CD86 was increased in the macrophages of the *Infected* group compared to the other groups, that is, uninfected, *Cy-Uninfected*, and *Cy-Infected*. Moreover, our results displayed an increased expression of CD40 and CD206 in the peritoneum and spleen macrophages of groups treated with Cy (Fig. 3A, C, D). Increased M1 (CD40 ^{high})/M2 (CD206 ^{high}) ratio was noted in *Cy-Infected* versus *Cy-Uninfected* in the peritoneum (Fig. 3A) and in the *Infected* versus other groups (Fig. 3D). In summary, although *E. cuniculi* infection reduced the population of peritoneal and splenic macrophages, a higher expression of CD80 and CD86 (*Infected* group) was associated with the predominance of M1 macrophage profile following Cy treatment.

3.3. Cy-Infected mice had reduced T and B lymphocytes

$CD8^+$T lymphocytes are the main effector cells responsible for the elimination of *E. cuniculi*. We found an increased population of $CD8^+$T lymphocytes in the peritoneum and spleen of mice in the *Infected* group. However, a comparative analysis showed that Cy treatment reduced the number of $CD8^+$ T lymphocytes in *Cy-Infected* mice than in *Infected* mice (Fig. 4). The number of $CD4^+$T cells increased in the spleen and peritoneum of *Infected* mice, but decreased in *Cy-Infected* animals; thus, showing a similar behavior to $CD8^+$T lymphocytes. A decrease in the percentage of B-1 cells was noted in mice following pathogen inoculation and Cy treatment. The controls had 40% of the relative population of B-1 cells, whereas groups treated with Cy had B-1 cell indices below 10% in the peritoneum and spleen (Fig. 5). However, infection with *E. cuniculi* did not modify the number of B-1 cells in the peritoneum compared to the *Uninfected* group, with increased number of these cells reported in the spleen (Fig. 5). The absolute number of B-2 lymphocytes increased in the peritoneum and spleen following infection with *E. cuniculi* as compared to the other groups, which was confirmed by the higher percentage of B-2 cells in the spleen of the *Infected* group than in the *Cy-Infected* group (Fig. 5). In summary, lymphocyte populations of $CD8^+$, $CD4^+$, and B-2 and B-1 lymphocytes increased in mice following infection and these same populations decreased in *Cy-Infected* animals.

3.4. Increased inflammatory cytokines in infected mice

Mice infected with *E. cuniculi* displayed increased levels of serum inflammatory cytokines, that is, IFN-γ, TNF-α, and IL-2 compared to the respective non-infected controls (Fig. 6). Only the *Infected* group reported an increase in the serum levels of IL-17, a cytokine closely related to the immune response against fungi. In addition, the production of IFN-γ and TNF-α was higher in the *Cy-Infected* group than in the *Infected* group. The *Infected* group showed elevated levels of anti-inflammatory cytokine IL-4 (Fig. 6). We could not detect IL-10 in any group.

In summary, although mice in the *Infected* group showed a reduced absolute number of macrophages, they displayed an M1 profile, elevated numbers of $CD4^+$T, $CD8^+$T, B-1, and B-2 lymphocytes in association with a predominance of Th1 inflammatory cytokines (IFN-γ, TNF-α, and

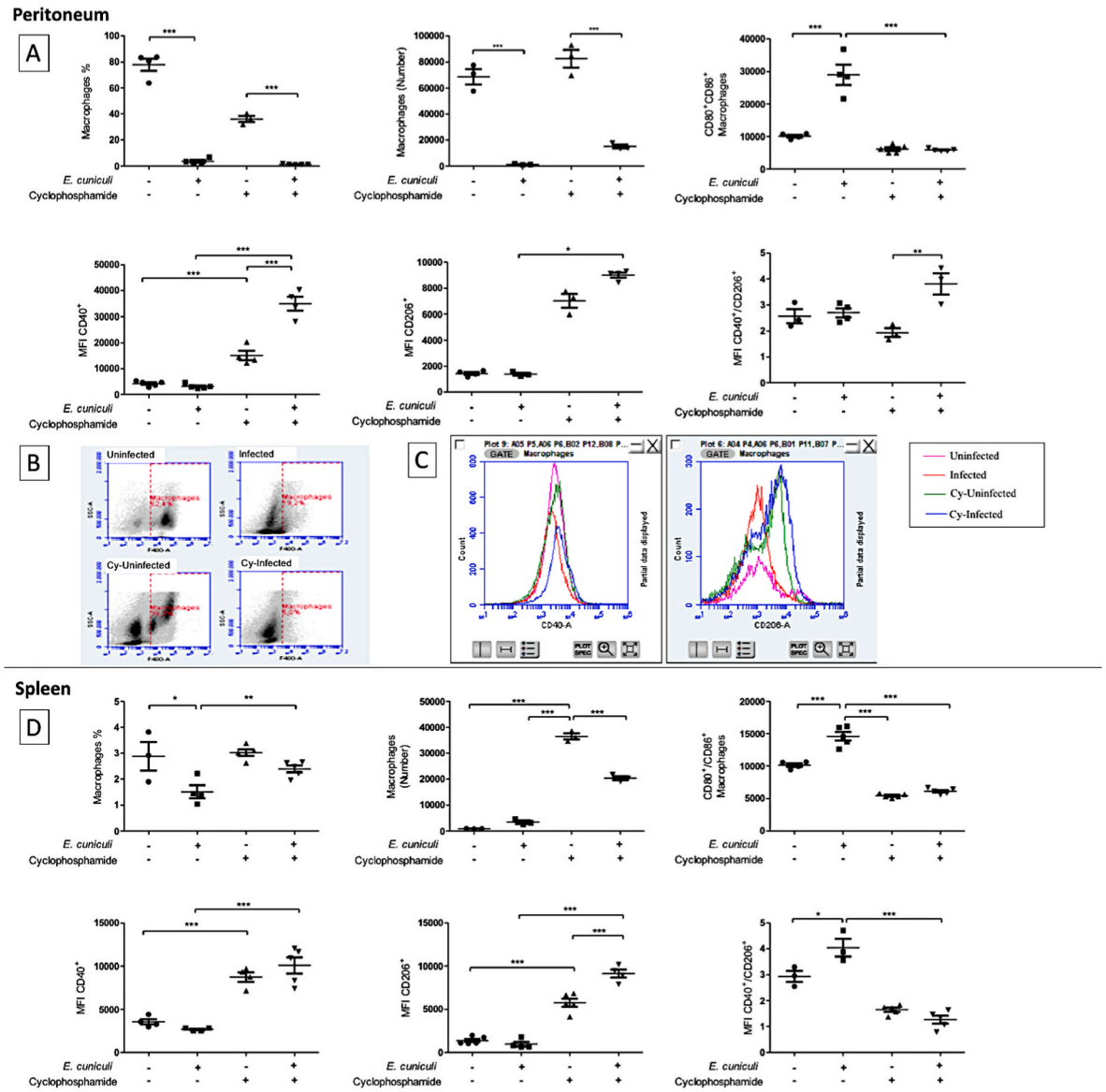

Fig. 3. Activation phenotype of macrophages obtained from the peritoneal cavity (peritoneum) and spleen of C57BL/6 mice infected with *E. cuniculi* spores (+) or not (−) and treated with cyclophosphamide (Cy) (+) or not (−). (A) **Peritoneum:** Total percentage (%) and number of F4/80$^+$CD11b$^+$ macrophages, mean fluorescence intensity (MFI) of CD80$^+$ and CD86$^+$ costimulatory molecules on macrophages, proportion of F4/80$^+$CD11b$^+$ macrophages expressing CD40$^+$, proportion of F4/80$^+$CD11b$^+$ macrophages expressing CD206, and MFI ratio of CD40/CD206 molecules expression on F4/80$^+$CD11b$^+$ macrophages. (B) Dot plots showing the percentage of F4/80$^+$ macrophages. (C) Histogram demonstrating the mean surface fluorescence intensity of peritoneal macrophages for CD40 and CD206. (D) **Spleen:** Total percentage (%) and number of F4/80$^+$CD11b$^+$ macrophages, mean fluorescence intensity (MFI) of CD80$^+$ and CD86$^+$ costimulatory molecules on macrophages, proportion of F4/80$^+$CD11b$^+$ macrophages expressing CD40$^+$, proportion of F4/80$^+$CD11b$^+$ macrophages expressing CD206, and MFI ratio of CD40/CD206 molecules expression on F4/80$^+$CD11b$^+$ macrophages. The data are represented as mean ± standard error of the mean (SEM) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, one-way analysis of variance (ANOVA) with multiple comparisons and Tukey's post-test).

IL-2) and Th17 (Table 1). Similarly, although *Cy-Infected* mice displayed a reduced absolute number of macrophages, they had an M1 profile and reduced populations of CD4$^+$T, CD8$^+$T, B-1, and B-2 lymphocytes, in association with a predominance of Th1 inflammatory cytokines (IFN- γ, TNF-α, and IL-2) and Th2 (IL-4) (Table 1), and this group displayed a higher fungal burden.

4. Discussion

Encephalitozoon cuniculi is an opportunistic pathogen that is known to cause disseminated infection in immunosuppressed patients. In certain mammals, such as rabbits, the infection is evidenced by high levels of antibodies and the presence of an inflammatory process involving the CNS and kidneys. It has been reported that *E. cuniculi* infection causes ascites and death after dissemination of the pathogen, with large amounts of microsporidian spores identified in the peritoneum, liver, and spleen of SCID mice (Salát et al., 2002, 2004, 2006; Koudela et al., 2004), nude mice (Gannon, 1980; Niederkorn et al., 1981; Liu et al., 1989), and CD8$^+$T-lymphocyte knockout mice (Khan et al., 1999; Moretto et al., 2001). In addition, Niederkorn et al. (1981) demonstrated higher recovery of *E. cuniculi* spores in peritoneal washings following intraperitoneal pathogen infection of inbred mice of the C57BL/6 strain, indicating the increased susceptibility of this strain to the pathogen. Herein, we demonstrated that C57BL/6 mice infected intraperitoneally with spores of *E. cuniculi* displayed symptoms of systemic inflammation (lethargy and wasting) associated with the presence of granulomatous inflammatory infiltrate distributed in several organs, especially in the liver. These findings are corroborated by Lallo and Hirschfeld (2012)

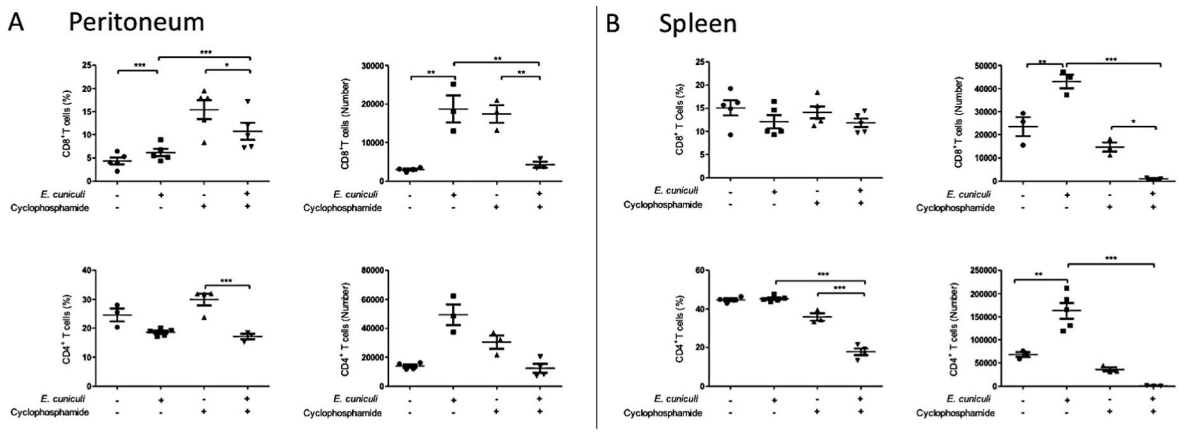

Fig. 4. Evaluation of total percentage (%) and number of $CD8^+$ and $CD4^+$ T cell populations in the peritoneal cavity and spleen of C57 BL/6 mice infected with *E. cuniculi* spores (+) or not (−) and treated with cyclophosphamide (Cy) (+) or not (−). (A) Peritoneum. (B) Spleen. The data are represented as mean ± standard error of the mean (SEM) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, one-way analysis of variance (ANOVA) with multiple comparisons and Tukey's post-test).

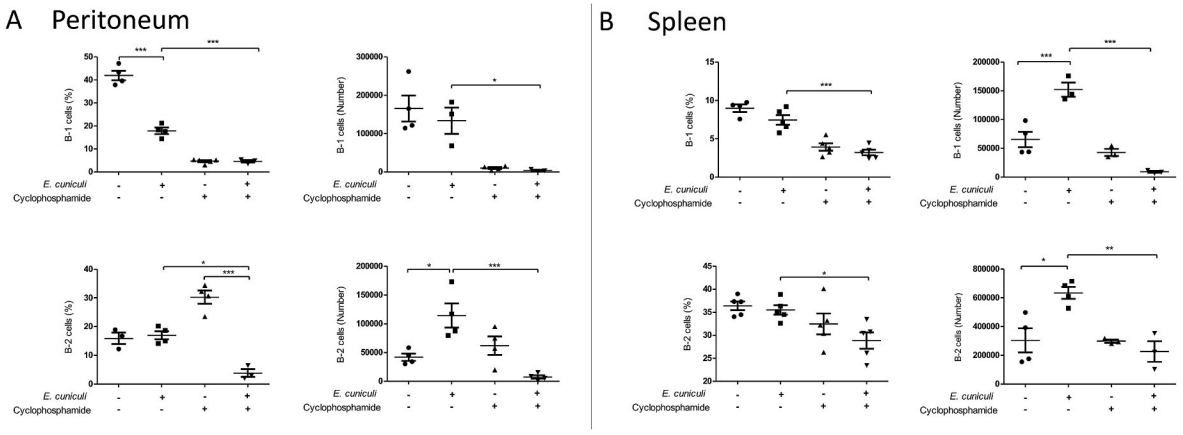

Fig. 5. Evaluation of total percentage (%) and number of B-1 and B-2 cell populations in the peritoneal cavity and spleen of C57 BL/6 mice infected with *E. cuniculi* spores (+) or not (−) and treated with cyclophosphamide (Cy) (+) or not (−). (A) Peritoneum. (B) Spleen. The data are represented as mean ± standard error of the mean (SEM) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, one-way analysis of variance (ANOVA) with multiple comparisons and Tukey's post-test).

who reported similar findings in Balb/c mice treated with Cy at the same dose and time (Lallo and Hirschfeld, 2012). However, in both mice strains, immunosuppressive treatment with Cy is insufficient to determine a similar course of infection to that observed in nude mice (Niederkorn et al., 1981), SCID mice (Salát et al., 2002), and $CD8^{-/-}$ T cell mice (Khan et al., 1999a,b; Moretto et al., 2001a,b) or even Xid mice treated with Cy (Moysés et al., 2022). The reduced resistance of these animals to infection by *E. cuniculi* due to the immunosuppressive effects of Cy was identified, as evidenced by a higher fungal burden in *Cy-Infected* animals than in the *Infected* group animals.

We reported a reduced number of peritoneal and splenic macrophages following infection as compared to Balb/c mice that demonstrated an elevated number of peritoneal macrophages after infection (da Costa et al., 2017; Moysés et al., 2022), even with treatment with Cy. However, these macrophages were predominantly M1. A few studies have demonstrated the functional profile of macrophages or other innate immune cells in microsporidial infections (Carriere et al., 2023). *In vitro* studies have demonstrated a predominance of adherent peritoneal macrophages with an M1 profile and release of proinflammatory cytokines in the presence of B-1 cells, whereas peritoneal macrophages showed an M2 profile, with a delay in microbicidal activity and fewer cell death in the absence of B-1 cells (Pereira et al., 2019). In addition, we reported a positive connection between inflammation induction and a significant increase in *E. cuniculi* genotypes I and III in inflammatory foci in both immunocompetent BALB/c and immunodeficient SCID mice in the acute phase of infection (Sak et al., 2022).

The C57BL/6 strain has a genetic alteration that compromises the function of B lymphocytes, resulting in the production of less efficient antibodies in these animals (Watkiss et al., 2013; Mahajan et al., 2016). Here, we demonstrated a reduced population of B-1 cells in *Cy-Infected* mice. Based on the findings of Pereira et al. (2019), who identified the M1 pattern associated with the presence of B-1 cells, we hypothesized that a reduction in the macrophage population could be linked to a decrease in the number of B-1 cells.

$CD8^+$T lymphocytes are the primary cells involved in immunity against *E. cuniculi* (Khan et al., 1999a,b; Moretto et al., 2000, 2001). We observed an increase in the population of $CD8^+$T lymphocytes in mice infected with the pathogen; however, an opposite trend was noted in the *Cy-Infected* group due to the severity of the infection. $CD8^+$T-cell-deficient animals are highly susceptible to this pathogen, and infected mice exhibit severe morbidity before death. An inability to clear *E. Cuniculi* infection has been demonstrated for perforin knockout mice, suggesting the significant contribution of the cytotoxic function of $CD8^+$T cells against the pathogen (Khan et al., 1999a,b).

Increased serum levels of IFN-γ, TNF-β, and IL-2 were noted in infected animals, a finding that corroborates the idea that the C57BL/6 strain has a Th1 profile. In general, Balb/c mice display classic Th2-biased responses (e.g., IL-4, IL-6, IL-10, and IL-13) to several intracellular pathogens, increasing their susceptibility to severe infections compared to C57BL/6 mice, thereby demonstrating protective Th1-

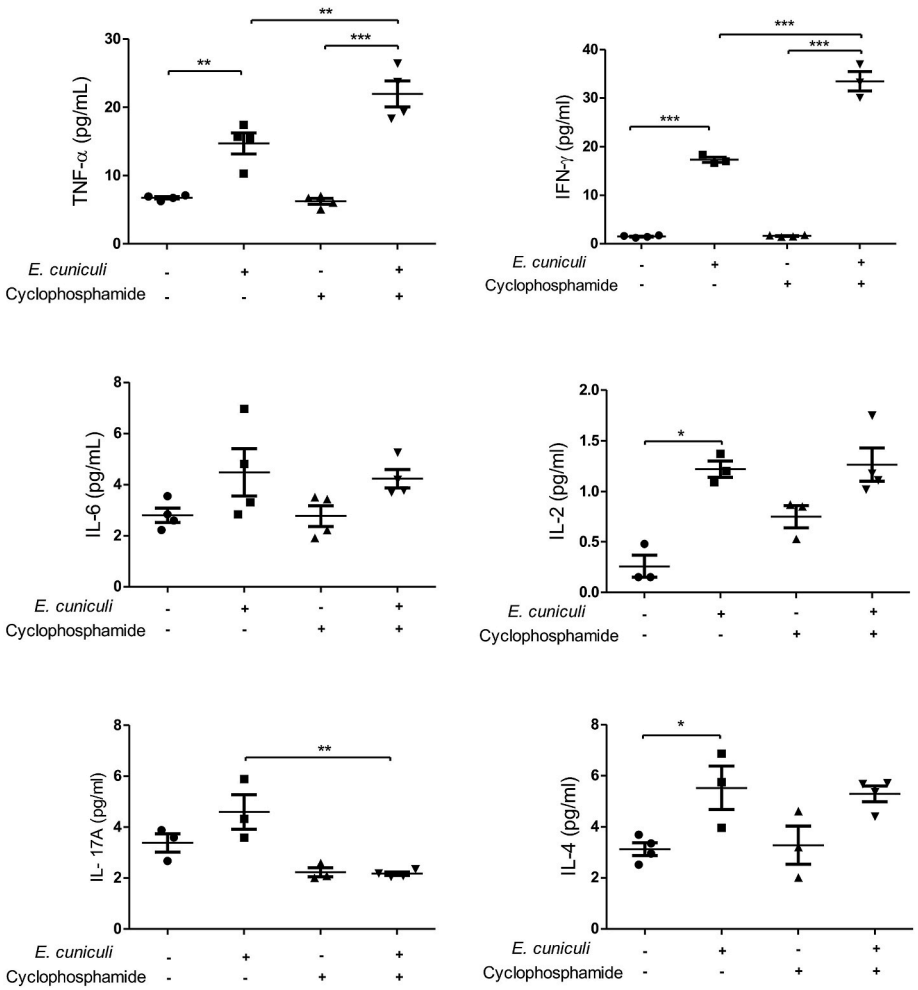

Fig. 6. Serum levels of TNF-α, IFN-γ, IL-6, IL-2, IL-17A, and IL-4 cytokines in C57BL/6 mice infected with *E. cuniculi* spores (+) or not (−) and treated with cyclophosphamide (Cy) (+) or not (−). Data are representative as mean ± standard error of the mean (SEM) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, one-way analysis of variance (ANOVA) with multiple comparisons and Tukey's post-test).

Table 1
Differences in immune response measured by macrophages, T and B lymphocytes, and cytokines between the *Infected* (infected with *E. cuniculi*) and *Cy-Infected* (treated with cyclophosphamide and infected with *E. cuniculi*) groups.

	Macrophages	Lymphocytes	Cytokines
Infected	↓ in spleen and peritoneum ↑ CD 80/86 expression ↑ CD40 (M1)	↑ CD4 T, CD8 T and B-2 in spleen and peritoneum ↑B-1 cell spleen ↓ B-1 cell peritoneum	↑ IFN-γ, TNF-α, IL-2 and IL-17
Cy-Infected	↓ in spleen and peritoneum ↑ CD40 (M1)	↓ CD4 T, CD8 T, B-2 and B-1 cells in spleen and peritoneum	↑ IFN-γ, TNF-α, IL-2 and IL-4.

biased responses (TNF-β and IFN-γ). Higher levels of pro-inflammatory cytokines with Th1 profile were observed in the serum of C57BL/6 mice, which is in line with the clinical signs of more severe disease and corroborates the hypothesis that C57BL/6 mice have a profile similar to the XID mice, as previously proposed (Moysés et al., 2022). In contrast, elevated levels of IL-2 were observed in the infected groups; IL-2 is a cytokine not shown to be fundamental in Balb/c and Xid mice immunosuppressed with Cy and infected with *E. cuniculi*. Th17 cytokines are divided into six IL-17 subtypes, A through F, which can be produced by a broad spectrum of cell populations, including γδT, natural killer T (NKT), $CD8^+$T cells, neutrophils, microglia, and mast cells (Khan et al., 1999; Chiricozzi et al., 2011; Moretto & Khan, 2022). The production and release of IL-17A are associated with several fungal infections, for example, infections caused by *Aspergillus fumigatus, Pneumocystis carinii, Cryptococcus neoformans*, and *Candida albicans* (Rudner et al., 2007; Murdock et al., 2014; Sparber et al. al., 2015; Huang et al., 2016; Levy et al., 2016; Guerra et al., 2017). However, IL-17C can exacerbate the secretion of proinflammatory cytokines, contributing to the worsening of inflammation in systemic fungal infections (Huang et al., 2016). Under the conditions described in our study, elevated serum levels of IL-17 observed in the *Infected* group were associated with a lower fungal burden, implicating this cytokine as reported for other fungal infections (Rudner et al., 2007; Murdock et al., 2014; Sparber et al., 2015; Huang et al., 2016; Levy et al., 2016; Guerra et al., 2017). Here, we hypothesize that $CD4^+$T lymphocytes, although in reduced numbers, could be responsible for the production and release of this inflammatory cytokine. In addition, we highlighted the increased production of IL-4 in the *Cy-Infected* animals as an anti-inflammatory cytokine. Its presence could explain the inflammatory infiltration in the liver in similar proportions to those observed in the *Infected* group.

5. Conclusion

In conclusion, mice in the *Cy-Infected* group developed more severe encephalitozoonosis associated with reduced populations of T and B lymphocytes, a mixed profile of Th1 and Th2 cytokines as compared with the mice in the *Infected* group. Despite a reduction in the number of macrophages, an elevated M1 population was observed. We can attribute the higher resistance of non-immunosuppressed C57BL/6 mice (*Infected*) to the increased number of $CD8^{+}$T and $CD4^{+}$T lymphocytes. Despite a reduction in the population of macrophages, the M-1 profile was predominant, with higher expression of CD80/CD86 and a mixed profile of proinflammatory cytokines, with a maintained B-1 cell population. Compared to Balb/c mice, variations in the population of peritoneal macrophages are the most striking difference observed in the immune response of C57BL/6 mice against encephalitozoonosis.

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Author statement

Maria Anete Lallo: conceived and designed the study, methodology, data curation, investigation, supervision, writing paper, funding acquisition, project administration and resources. **Amanda Miyuki Hidifira, Anuska Marcelino Alvares-Saraiva, Elizabeth Christina Perez, Diva Denelle Spadacci-Morena, Ronalda Silva de Araujo:** methodology, data curation, investigation and writing paper.

Declaration of competing interest

The authors Amanda Miyuki Hidifira; Anuska Marcelino Alvares-Saraiva; Elizabeth Christina Perez; Diva Denelle Spadacci-Morena; Ronalda Silva de Araujo; Maria Anete declare no Competing interests in papers publication “Increased susceptibility to encephalitozoonosis associated with mixed Th1/Th2 profile and M1/M2 profile in mice immunosuppressed with cyclophosphamide”.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exppara.2023.108606.

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