Immunobiology 227 (2022) 152194

Contents lists available at ScienceDirect

Immunobiology

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

Opportunistic pneumonia caused by *E. cuniculi* in mice immunosuppressed with cyclophosphamide

Iramirton Figuerêdo Moreira [a,b], Anuska Marcelino Alvares-Saraiva [a], Elizabeth Cristina Pérez [a], José Guilherme Xavier [a], Diva Denelle Spadacci-Morena [c], Ronalda Silva de Araújo [d], Paulo Ricardo Dell'Armelina Rocha [a], Maria Anete Lallo [a,*]

[a] Programa de Pós-Graduação em Patologia Ambiental e Experimental, Universidade Paulista (UNIP), Rua Dr. Bacelar 902, CEP 04057-000, São Paulo, SP, Brazil
[b] Faculdade de Medicina da Universidade Federal de Alagoas, Campus A. C. Simões Tabuleiro do Martins, CEP 57072-900, Maceió, AL, Brazil
[c] Departamento de Fisiopatologia, Instituto Butantan, Av. Vital Brasil 1500, CEP 05503-900, São Paulo, SP, Brazil
[d] Pesquisadora associada, Pós-Doutoranda da Companhia Ambiental do Estado de São Paulo - CETESB, Brazil

ARTICLE INFO

Keywords:
Cyclophosphamide
Encephalitozoon cuniculi
B lymphocytes
T lymphocytes
Alveolar macrophages
Pneumonia

ABSTRACT

Opportunistic fungal pneumonia is a cause of concern in immunocompromised patients due to its high morbidity and mortality rates. One such opportunistic agent affecting immunocompromised patients is the microsporidia called *Encephalitozoon cuniculi*. This study aimed to evaluate pneumonia caused by *E. cuniculi* in mice treated with the immunosuppressive agent cyclophosphamide (Cy). This study also aimed to describe the immune cells associated with the microsporidial pneumonia. C57BL/6 mice were infected intravenously with *E. cuniculi* spores and treated with Cy (75 mg/kg/week, intraperitoneally). Thirty days post-infection, the fungal burden (qPCR), histopathological lesions, cytokine production, and the phenotype of the immune cells in the lung parenchyma were evaluated. Histologically, interstitial pneumonia with lymphocytic infiltrate was observed in the infected animals. The infiltrate mainly consisted of $CD8^+$ and $CD4^+$ T lymphocytes, with reduced populations of B lymphocytes and macrophages. The production of tumor necrosis factor-alpha (TNF-α) was significant in the animals of the infected groups. Also, the fungal burden was higher in the Cy-treated animals, which was confirmed by the immunohistochemical observation of spores. These results demonstrated that *E. cuniculi* infection of C57BL/6 mice caused lymphocytic interstitial pneumonia (characterized by a predominant lymphocytic infiltrate), which was aggravated by Cy-induced immunosuppression. Thus, these results can be used to understand the different pathological, immunological, and therapeutic aspects of lymphocytic interstitial pneumonia.

1. Introduction

Pneumonia is considered the most aggressive pathological condition affecting the lower respiratory tract (Scott et al., 2012; Torres et al., 2019). Infectious agents causing pneumonia include bacteria, viruses, fungi, and protozoa; however, the vast majority of cases result from *Streptococcus pneumoniae* infections (Bradley et al., 2011; Gramegna et al., 2018). Interestingly, the etiology of pneumonia often remains undetected despite extensive diagnostic tests (Jain et al., 2015). The incidence, diagnosis, and clinical severity of pneumonia have increased in recent years, especially in immunocompromised patients, such as organ receptors, carriers of autoimmune diseases, patients infected with human immunodeficiency virus (HIV) and people on immunosuppressive drugs (Ghoyounchi et al., 2017). Among patients suffering from community-acquired pneumonia, 17.6% have some risk factor that compromises immunity, including chronic use of steroids, malignant hematological diseases, and chemotherapy (Di Pasquale et al., 2019). A significant rise in fungal infections was observed in the above-mentioned group of patients (Baker et al., 2020; Limper et al., 2011).

The phylum Microsporidia comprises more than 200 genera and 1400 species, with at least 16 species described in humans (Han et al., 2017). They are single-celled, obligatory intracellular fungi that are known to infect a range of vertebrate and invertebrate hosts (Sak et al., 2011; Kaczmarek and Boguś, 2021). Microsporidiosis is an opportunistic infection that occurs worldwide and affects immunocompromised

* Corresponding author at: Rua Dr. Bacelar 902, CEP 04057-000, São Paulo, SP, Brazil.
E-mail addresses: maria.lallo@docente.unip.br, anetelallo@hotmail.com (M. Anete Lallo).

https://doi.org/10.1016/j.imbio.2022.152194
Received 1 October 2021; Received in revised form 15 February 2022; Accepted 27 February 2022
Available online 6 March 2022

people, children, the elderly, travelers, etc. (Didier et al., 2009; Ghoyounchi et al., 2017).

Encephalitozoon cuniculi is a microsporidia causing severe and lethal systemic disease, without any effective treatment, in immunodeficient individuals. Encephalitozoonosis associated with pulmonary symptoms or pneumonia has been reported in HIV + patients (Weitzel et al., 2001), kidney transplant recipients (Kicia et al., 2019), and in a patient who received an allogeneic bone marrow transplant (Orenstein et al., 2005). Generally, such reports refer to several challenges in the diagnosis and treatment of patients, leading to lethal evolution and, therefore, poor prognosis. In a study based on BALB/c mice infected intraperitoneally with *E. cuniculi*, the involvement of the lower airways characterized by different degrees of degeneration, necrosis, detachment of the bronchiolar epithelium, presence of inflammatory infiltrates made up of mononuclear cells, and the existence of spores (both unassociated and associated with inflammatory cells) in the lung parenchyma was observed (Lallo et al., 2013). Other studies have demonstrated that *E. cuniculi* infection could determine granulomatous pneumonia and interstitial multifocal pneumonia, with the presence of peribronchiolar infiltrate and free spores on the walls of the alveoli (da Costa et al., 2017; Langanke Dos Santos et al., 2018).

Due to the importance of pneumonia in immunosuppressed patients, the development of animal models that assist in understanding and evaluating the role of immune cells against opportunistic agents is essential for making therapeutic decisions. Cyclophosphamide (Cy) is an immunosuppressive drug widely used in the treatment of autoimmune and alloimmune diseases (Brodsky, 2010, Dezern et al., 2013) and also in transplant procedures, such as bone marrow transplantation (Colvin, 1999). It is included in the list of the World Health Organization (WHO) among the essential medicines for health systems for its effectiveness, cost, and safety (WHO, 2013). Also, due to its therapeutic efficacy and the stimulation of the immune response, it is one of the most successful drugs in anticancer treatment (Emadi et al., 2009, Sistigu et al., 2011). This drug is used as an important tool in experimental studies as it is known to mimic clinical conditions (Sevko et al., 2013). In this context, the present study aimed to develop a model of pneumonia in mice immunosuppressed with Cy and infected with *E. cuniculi*. Additionally, the phenotypic immune components present in the lung parenchyma of these animals were also characterized.

2. Materials and methods

2.1. Animals and ethical statement

Pathogen-free six to eight-week-old C57BL/6 mice were purchased from the Federal University of Sao Paulo (CEDEME, UNIFESP), Brazil. Animals were housed in specific pathogen-free conditions, fed with food and water *ad libitum* at the Animal Facility of Paulista University, Sao Paulo, Brazil. All animal procedures were carried out in strict accordance with the Paulista University Ethics Committee (Project license 024/2018).

2.2. E. cuniculi cultivation

E. cuniculi spores were bought (Waterborne Inc., New Orleans, LA, USA) and propagated in rabbit kidney cells (RK-13) in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), pyruvate, non-essential amino acids, and gentamycin at 37 °C and 5% CO_2 (Visvesvara, 2002). Spores were collected from the supernatant, washed three times with phosphate-buffered saline (PBS), and counted in a Neubauer chamber.

2.3. Study design

Animals were divided into four experimental groups. 1. *Infected:* Mice infected with *E. cuniculi*; 2. *Uninfected:* Uninfected and non-treated (i.e., not treated with Cy) mice; 3. *Cy-Infected:* Mice treated with Cy and infected with *E. cuniculi*; and 4. *Cy-Uninfected:* Mice treated with Cy. A previously established protocol (Lallo and Hirschfeld, 2012) of Cy-treatment was followed, with intraperitoneal injections of 75 mg/kg twice a week (Genuxal®, Asta Medica Oncologia, São Paulo, Brazil) starting at the day of infection until 28 days post-infection (dpi). Mice were intravenously infected with 1×10^7 *E. cuniculi* spores, and the uninfected mice were included as controls.

2.4. Necropsy and tissue sampling

At 30 dpi, all animals were euthanized with a mixture of ketamine (100 mg/mL), xylazine (20 mg/mL), and fentanyl (0.05 mg/mL). The lung samples were collected and fixed in 10% buffered formalin for 72 h, and the microscopic analysis was performed for 4 μm hematoxylin and eosin (H&E)-stained tissue sections. For each animal, the morphometric analysis of the inflammatory infiltrate was performed for four selected "hot spots" in the histological field using an Opticam 0400S microscope fitted to a 10X eyepiece and a 40X objective. The area occupied by leukocytes was measured in pixels by the Image Analysis software (MetaMorph Software, Molecular Devices, California, USA), and the mean areas of each experimental group were used for statistical analysis.

2.5. Immunohistochemistry

Approximately 3 μm histological sections of the lungs were placed on salinized slides and deparaffinized. For antigen recovery, the sections were incubated with trypsin (proteolytic enzyme) at 37 °C for 15 min, with subsequent blocking of endogenous peroxidase by incubating the slides for 10 min in 3% hydrogen peroxide and methanol (50:50) twice. The slides were then incubated in primary polyclonal antibody (Ab), which was 1:10,000 diluted rabbit IgG (against *E. cuniculi*) (MEDICAGO, UPPSALA, SWEDEN, Catalogue number 18–9001–1), for 18 h at 4 °C. Secondary anti-rabbit Ab was applied for 30 min in an oven at 37 °C (ImmPRESS ™ Universal Reagent Anti-Mouse/Rabbit IgG, Vector Laboratories, N. MP-7500). For visualization, the slides were stained with the chromogen 3′, 3′-diaminobenzidine (DAB) and counterstained with hematoxylin; the slides were observed and photographed under a light microscope (Leica DMLD, Greenville, SC, USA).

2.6. Fungal burden

The fungal burden was determined by real-time PCR (qPCR)-based quantification of the fungal genomic DNA present in the lung tissue. After euthanasia, the lung samples were weighed, and a constant amount of pulmonary lobe was frozen in RNAlater for a maximum of 30 days. The DNeasy Blood & Tissue extraction kit (Qiagen®) was used as per the manufacturer's instructions and the protocol described by Moretto et al. (2015). The lung fragments were thawed and cut into smaller pieces, with the subsequent addition of the buffer and incubation at 56 °C in a water bath. Every 15 min, homogenization was performed until the tissue was completely lysed. Subsequently, 1 mL of the lysed material was transferred to a new tube; 200 μL of the ATL buffer was added, mixed in a vortex for 30 s, and incubated in a water bath at 70 °C for 10 min. Afterward, 200 μL of 100% ethanol was added, and the tissue was homogenized again in the vortex. The extracted DNA was transferred to another tube with the addition of 500 μL of buffer AW1, centrifuged for 1 min at 6000 g, and stored at –20 °C for qPCR analysis.

2.7. Phenotypic characterization of the immunological components of the lung parenchyma

The lungs were removed and incubated at 37 °C in a water bath for 30 min. Afterward, the samples were mechanically dissociated (BD Medimachine, TO, ITALY), and the resulting cell suspensions were filtered through a 70 μm cell strainer and treated with hemolytic buffer.

The lung samples were transferred to a 15 mL falcon tube, and the supernatant was removed after centrifuging at 500*g*, 4 °C for 7 min. To the remaining pellet, 1 mL of hemolytic buffer was added and incubated for 90 s at 4 °C to eliminate red blood cells. After centrifuging at 421 g, 4 °C for 7 min, 10 mL of PBS solution was added and again centrifuged at 500*g*, 4 °C for 7 min. To block the Fc receptors, the samples were incubated for 20 min in an ice bath with anti-CD16/CD32 antibodies in a PBS solution plus 1% bovine serum albumin (PBS-BSA) (20 μL). Subsequently, the cells were washed and incubated for 30 min at 4 °C with the following monoclonal antibodies: anti-mouse CD19 conjugated to allophycocyanin (APC) (eBioscience), anti-mouse CD45 conjugated to phycoerythrin (PE) (Bio Albra), mouse anti-CD4 conjugated to peridinin-chlorophyll-protein complex (PerCP), mouse anti-CD8 conjugated to fluorescein isothiocyanate (FITC), anti-mouse F4/80 conjugated to PE Cy7a (eBioscience), and anti-mouse CD11b conjugated to APC Cy7 (BD Biosciences). Then, the cells were washed and resuspended in 100 μL of PBS, centrifuged at 4 °C for 5 min at 500 g; the supernatant was discarded, and the cells were fixed in a 1% paraformaldehyde-buffered solution (PFA). To remove the PFA, the samples were centrifuged at 4 °C for 5 min at 2000 rpm and resuspended in PBS (200 μL) for reading on a BD Accuri™ C6 Flow cytometer (BD Biosciences).

Phenotypically, macrophages were characterized as follows: total ($F4/80^{+}CD11b^{+}$), interstitial ($F4/80^{+}CD11b^{+}SIGLEC\text{-}F^{-}$), and alveolar macrophages ($F4/80^{+}CD11b^{-}SIGLEC\text{-}F^{+}$) (Hussell and Bell, 2014). In addition, the remaining cells were identified according to the following phenotypes: $CD4^{+}$ T lymphocytes ($CD45^{+}CD4^{+}$), $CD8^{+}$ T lymphocytes ($CD45^{+}CD8^{+}$), and B lymphocytes ($CD45^{+}CD19^{+}$).

2.8. Cytokines quantification

Half of the lungs obtained after the necropsy was refrigerated with a protease inhibitor (Protease Inhibitor Cocktail, Sigma-Aldrich, Saint Louis, USA). After grinding, the material was centrifuged, and the supernatant was stored at − 80 °C. Subsequently, after thawing, the lung was processed in PreCellys for three cycles for 20 s and passed through a cell strainer for removal of debris. Cytokines were measured by flow cytometry using the CBA Mouse Th1/Th2/Th17 Cytokine Kit (BD Biosciences, CA, EUA) for detection of IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ, and TNF-α, according to the manufacturer's instructions. In brief, 25 μL of each sample was added to capture beads specific for the cytokines and PE-labeled secondary antibodies and incubated for 2 h at room temperature in the dark. Two-color flow cytometric analysis was performed using FACSCanto II (BD Biosciences, Mountain View, CA) and analyzed using FCAP Array 1.0 software.

2.9. Statistical analysis

Variance analysis (ANOVA) and Tukey and Bonferroni multiple comparison post-tests were performed. All values were represented as mean ± standard error mean (SEM), with the significance of $\alpha = 0.05$ ($p < 0.05$). Graphs were plotted using "GraphPad Prism" version 5.0 for Windows ® (GraphPad Software Inc, La Jolla, CA, EUA).

3. Results

3.1. E. cuniculi induces interstitial pneumonia in C57BL/6 mice

Results indicated zero deaths up to 30dpi of C57BL/6 mice infected with *E. cuniculi* in both the Cy-treated and untreated groups. Interstitial pneumonia was observed in the lungs of the animals belonging to the *Infected* and *Cy-Infected* groups, with thickening of the alveolar walls associated with lymphoplasmacytic infiltration. Based on the morphometric analysis, it was concluded that statistically significant differences do not exist between the groups regarding the area occupied by the alveolar infiltrate. The histological sections of the lungs of the infected mice demonstrated preservation of pulmonary compartmentalization, with reduction of the exchange areas associated with thickening of the alveolar walls. The morphometric analysis of infiltrative lesions in the lungs did not indicate any significant difference between the infected groups. The presence of multiple leukocyte infiltration sites in the alveolar wall, with a lymphoplasmacytic predominance, is noteworthy. The presence of vascular congestion, eventual hemorrhage, and histologically preserved pneumocytes completed the picture observed through experimental infection (Fig. 1).

3.2. Treatment with Cy resulted in a higher pulmonary fungal burden

The results of qPCR indicated a higher fungal burden in the lungs of animals treated with Cy (*Cy-Infected*) compared to that of the infected animals (Fig. 2). The immunohistochemical technique (IHC) allowed the observation of *E. cuniculi* spores in the pulmonary interstitium (free spores) and, possibly, within the macrophages and epithelial cells, of the animals of the *Cy-Infected* and *Infected* groups (Fig. 1).

3.3. Interstitial inflammatory infiltrate was characterized by the predominance of T lymphocytes

An upward trend was identified, although with no statistical difference, in the $CD8^{+}$ T lymphocyte population in the lungs of animals belonging to the *Infected* and *Cy-Uninfected* groups, compared to the *Uninfected* and *Cy-Infected* groups (Fig. 3A). As for the number of $CD4^{+}$ T lymphocytes, it was observed that the reduction in this population was predominantly linked with the application of Cy, since the *Cy-Uninfected* group had a smaller population than the *Infected* and *Uninfected* control groups (Fig. 3B). The ratio of $CD4^{+}$ and $CD8^{+}$ T lymphocytes was higher in the *Infected* group (Fig. 3C), suggesting that the increase in the $CD4^{+}$ T population is directly related to the presence of *E. cuniculi*, as demonstrated in a previous study (da Costa et al., 2017).

Regarding the population of B lymphocytes ($CD45^{+}$and $CD19^{+}$) present in the lungs, a decrease was observed in all infected animals (*Infected* and *Cy-Infected*) and in the *Cy-Uninfected* group compared to the *Uninfected* control. Also, in the *Cy-Infected* group, the number of B lymphocytes was lower than in the *Cy-Uninfected* and *Infected* groups (Fig. 3D).

As for the macrophage population, results indicated that the total pulmonary macrophages ($F4/80^{+}CD11b^{+}$) decreased in all infected animals (*Infected* and *Cy-Infected*) and in the *Cy-Uninfected* group compared to the *Uninfected* control (Fig. 4A). By phenotypically differentiating the populations of macrophages present in the lungs into interstitial ($F4/80^{+}CD11b^{+}SIGLEC\text{-}F^{-}$) and alveolar ($F4/80^{+}CD11b^{+}SIGLEC\text{-}F^{+}$), it was observed that the two subpopulations followed the same pattern of behavior, that is, there was a reduction of this population in infected animals (*Infected* and *Cy-Infected*) and in the *Cy-Uninfected* group compared to the *Uninfected* control (Fig. 4B and 4C).

3.4. Increase of TNF-α in the lungs and serum of mice with E. cuniculi-induced pneumonia

Results indicated a significant increase of TNF-α in the lung tissue of *E. cuniculi*-infected animals treated with Cy compared to the other groups (Fig. 5A). In serum, an increase in TNF-α levels was detected in infected animals; however, a statistically significant difference was observed only in the *Cy-Infected* group compared to the *Cy-Uninfected* and *Uninfected* groups (Fig. 5B). The cytokines IL-2, IL-4, IL-6, IL-10, IL-17A, and IFN-γ were not detected in the serum and lung tissues of these mice (data not shown).

4. Discussion

Pneumonia caused by opportunistic fungi, such as microsporidia, is currently an important public health concern as it affects individuals with chronic health problems or weakened immune systems (Limper

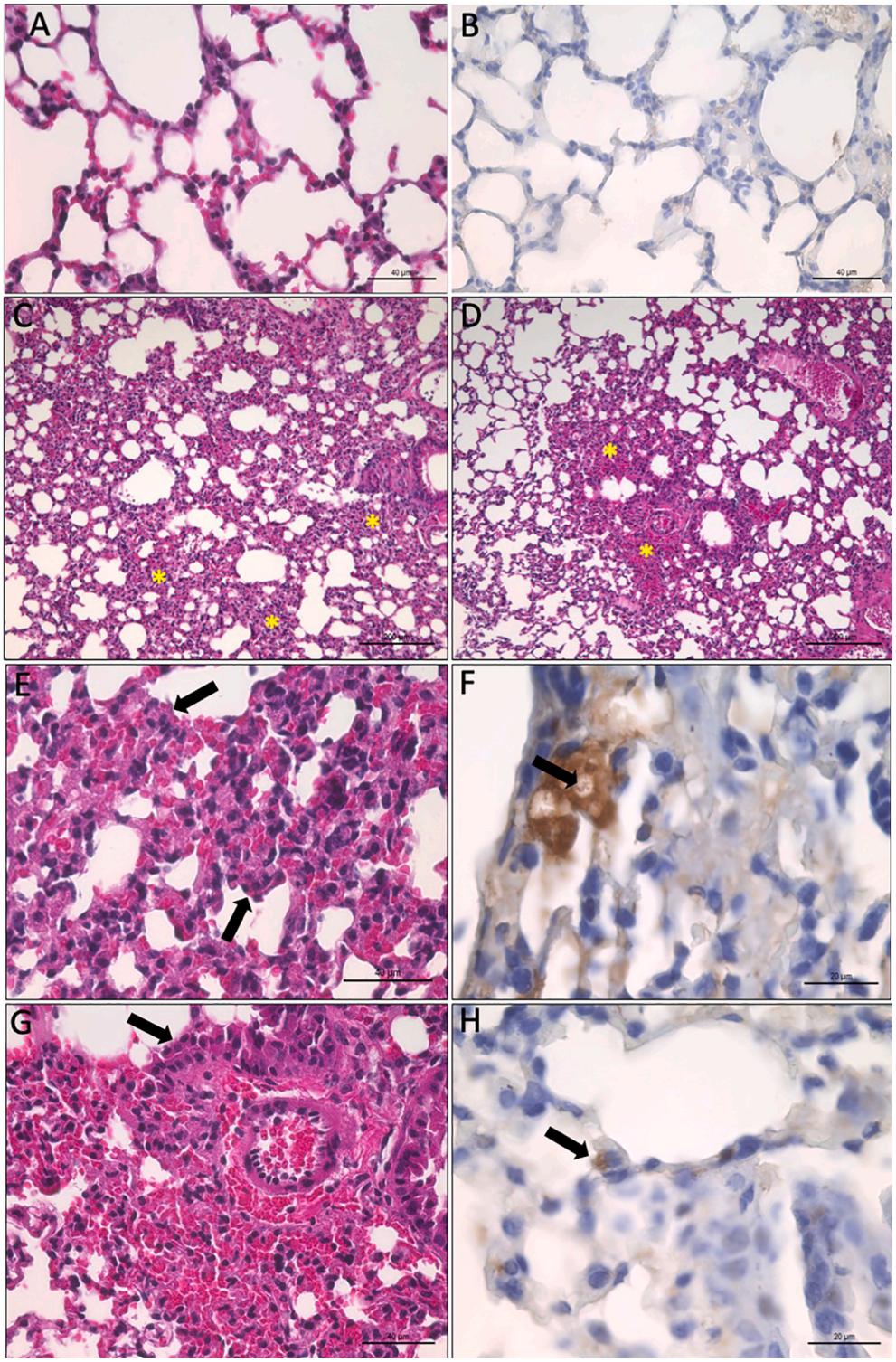

Fig. 1. Photomicrographs of the lung parenchyma of mice belonging to the *Uninfected, Infected, Cy-Infected and Cy-Uninfected* groups. Note the presence of interstitial pneumonia in animals of the *Infected* and *Cy-Infected* groups, evidenced by leukocyte infiltrate and alveolar wall thickening (arrow). (A) *Uninfected* without pneumonia and spores (H&E staining); (B) *Uninfected* (DAB chromogen staining; hematoxylin counterstained); (C) *Cy-Infected* with a severe interstitial pneumonia, evidenced by leukocyte infiltrate and alveolar wall thickening (*) (H&E staining); (D) *Infected* with more localized interstitial pneumonia (*) (H&E staining); (E) *Cy-Infected* evidenced by leukocyte infiltrate and alveolar wall thickening (arrow) (H&E staining); (F) Note the higher number of *E. cuniculi* spores in the *Cy-Infected* animals (DAB chromogen staining; hematoxylin counterstained); (G) *Infected* evidenced by leukocyte infiltrate and alveolar wall thickening (arrow); (H) Note the rare presence of *E. cuniculi* spores in the *Infected* animals (DAB chromogen staining; hematoxylin counterstained).

et al., 2011; Girmenia and Iori, 2012). This is supported by the fact that the number of microsporidiosis cases in immunosuppressed and immunocompetent patients has increased in recent years (Kotkova et al., 2013; Kicia et al., 2016; Kicia et al., 2018). Since the available therapies are long, toxic, and ineffective, the infection greatly worsens the condition of the affected host (Kotkova et al., 2013; Özkoç et al., 2016; Kicia et al., 2019). Recently, an 8% prevalence of microsporidia was identified in kidney transplant patients treated with immunosuppressants, and a positive association was identified between pneumonia and *E. cuniculi* infection (Kicia et al., 2019). In the present study, C57BL/6 mice were infected with spores of the opportunistic pathogen *E. cuniculi* and treated with Cy to mimic the conditions of immunosuppression commonly observed in transplant recipients, carriers of autoimmune diseases, or patients undergoing anticancer therapy. The infected animals, with or without Cy treatment, presented interstitial pneumonia with infiltrate, characterized by the increased presence of T lymphocytes.

As microsporidia are intracellular pathogens, studies have identified that the protective immune response against encephalitozoonosis is

A

Sample	Spores number in 200 µL	Sample weight/lung total weight (mg)	Total spores in lung
Infected 1	2730	30/154	14014
Infected 2	4500	43/145	15174
Infected 3	3100	30/178	18393
Infected 4	4030	30/181	24314
Cy-Infected 5	5310	30/156	27612
Cy-Infected 6	18000	45/144	57600
Cy-Infected 7	5610	30/162	30294
Cy-Infected 8	7780	30/163	42271
control	0,00	0	0

B

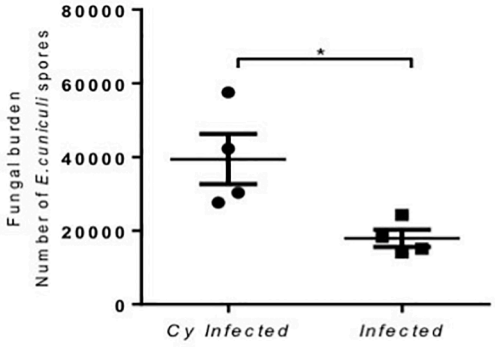

Fig. 2. Quantification of the fungal burden by real-time PCR (qPCR) technique in the lung parenchyma of mice belonging to the *Infected*, *Cy-Infected, Uninfected, and Cy-Uninfected* groups. A) Results observed per sample per animal; 1– 4: *Infected* animals; 5–8: *Cy-infected* animals and uninfected control; B) The higher fungal burden of animals treated with cyclophosphamide. Student's *t*-test, $p < 0.05$*.

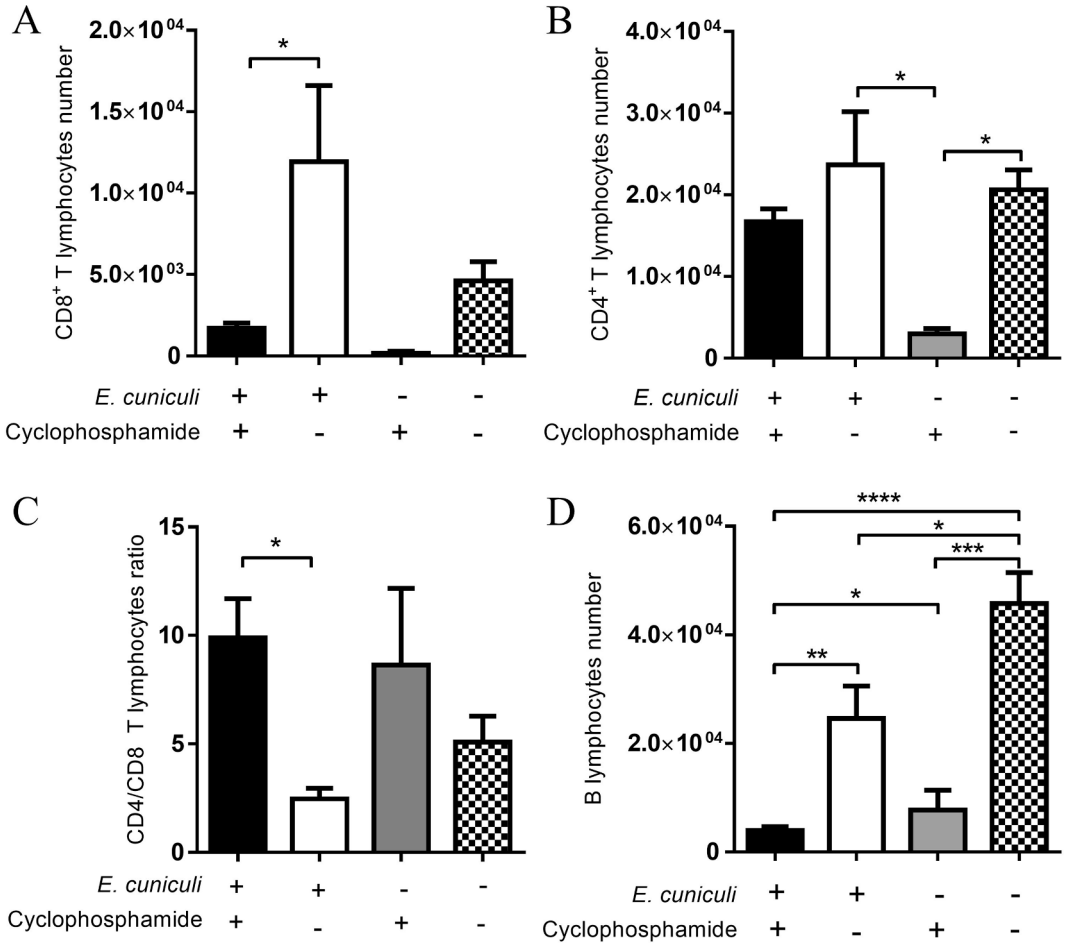

Fig. 3. Evaluation of lymphocytes present in the lung parenchyma of C57BL/6 mice, inoculated (+) or not (−) with *E. cuniculi* and treated (+) or not (−) with cyclophosphamide. A) $CD45^+CD8^+$ T lymphocyte. B) $CD45^+CD4^+$ T lymphocyte. C) $CD45^+CD4^+/CD45^+CD8^+$ ratio. D) $CD45^+CD19^+$ B lymphocytes. One-way analysis of variance (ANOVA) with Tukey post-test revealed $p < 0.05$*, $p < 0.01$**, $p < 0.001$***, and $p < 0.0001$****.

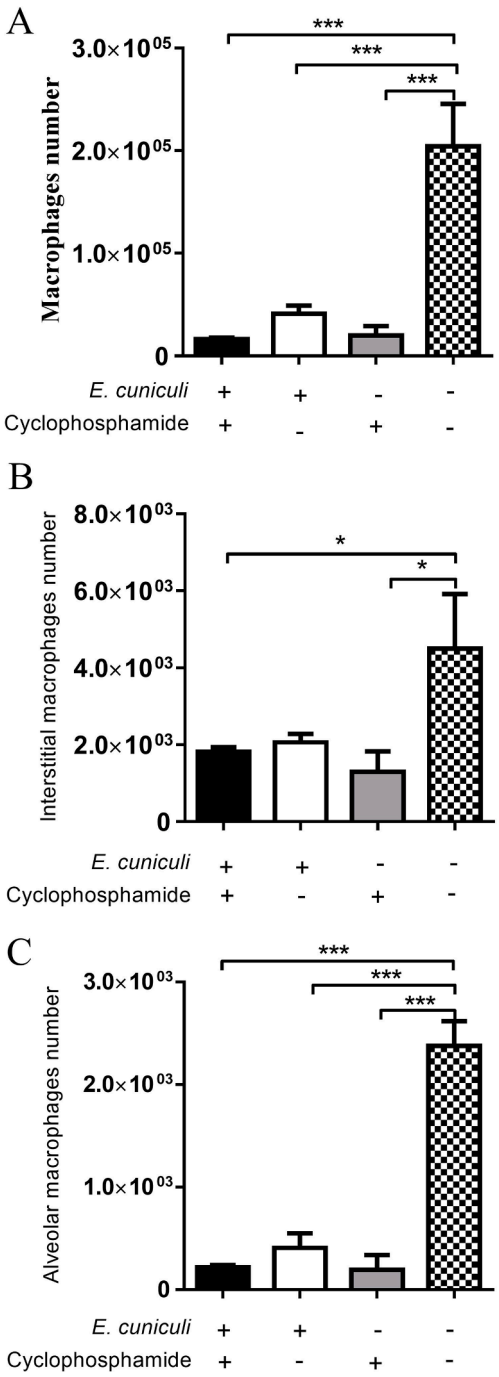

Fig. 4. Evaluation of macrophages present in the lung parenchyma of C57BL/6 mice, inoculated (+) or not (−) with *E. cuniculi* and treated (+) or not (−) with cyclophosphamide. A) The total lung macrophage population (F4/80$^+$). B) The population of interstitial macrophages expressing (F4/80$^+$CD11b$^+$SIGLEC-F$^-$). C) Alveolar macrophage population (F4/80$^+$CD11b$^-$SIGLEC-F$^+$). One-way analysis of variance (ANOVA) with Tukey post-test revealed $p < 0.05$* and $p < 0.001$***.

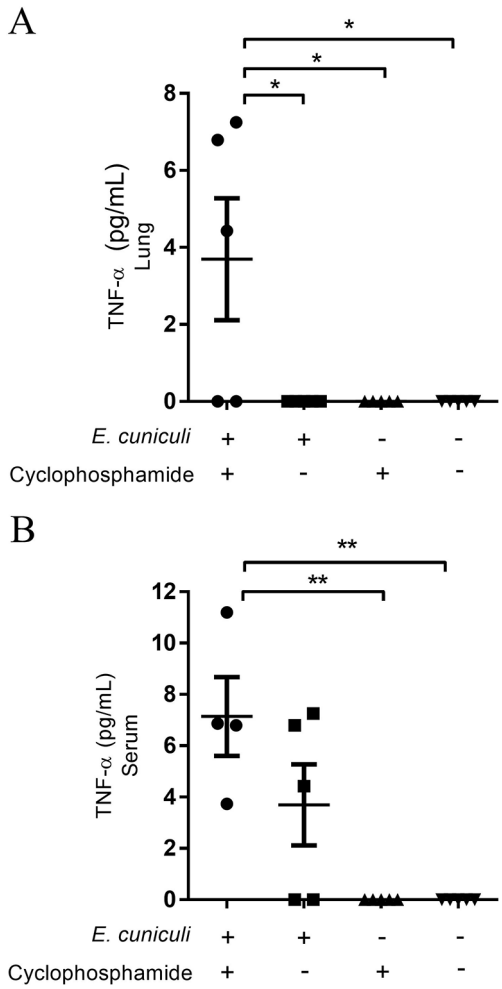

Fig. 5. TNF-α levels detected in the lung parenchyma (A) and serum (B) of C57BL/6 mice inoculated (+) or not (−) with *E. cuniculi* and treated (+) or not (−) with cyclophosphamide. One-way ANOVA analysis of variance with Bonferroni post-test revealed statistically significant differences; $p < 0.05$* and $p < 0.01$**.

mediated mainly by CD8$^+$ T lymphocytes; they act directly by secreting perforins and granzymes, inducing the apoptosis of infected cells (Braunfuchsová et al., 1999; Moretto et al., 2001). Our findings indicated an increase in the population of CD8$^+$ T lymphocytes in the lungs of animals from the *Infected* and *Cy-Uninfected* groups, compared to the *Uninfected* and *Cy-Infected* groups, corroborating the data in the literature. The increase in the number of CD8$^+$ T cells in the lungs and the expression of perforins by these cells were evidenced in a murine model of pulmonary infection with *M. tuberculosis* by inhalation and intravenous methods; the increase after four weeks of infection suggested that the release of cytotoxic granules by CD8$^+$ T cells during infection explains the mechanism of death of *M. tuberculosis* (Serbina et al., 2000). In this sense, the increase in the population of CD8$^+$ T cells in the lungs of mice not treated with Cy corroborates the effector, and cytotoxic function previously described; however, this population was characterized in the lungs in an unprecedented way.

Previous research indicates that the increase in CD8$^+$ T cell population in the intestinal mucosa was related to the elimination of the pathogen and greater resistance to infection (Moretto et al., 2004, Langanke dos Santos et al., 2018). Thus, the reduction in the CD8$^+$ T lymphocyte population in mice of the *Cy-Infected* group is associated with a greater fungal load, which reinforces the role of cytotoxic lymphocytes in controlling microsporidiosis. Since the reduction of CD8$^+$ T cells was observed only in the *Infected* and *Cy-Infected* groups and not in the *Cy-Uninfected* group, it is evident that the decreased numbers are not an effect of the drug.

Among the various roles of CD8$^+$ T cells in the immune response, an interesting phenomenon was evidenced in a study with *M. tuberculosis*. In this study, simultaneous production of IFN-γ and TNF-α, similar to CD4$^+$ T cells, was observed, along with a cytotoxic action leading to the death of infected macrophages (Serbina et al., 2000). In infection with other pathogens, such as *Chlamydia trachomatis* and *Listeria*

monocytogenes, $CD8^+$ T cells induce protection by producing IFN-γ (Lampe et al., 1998; Harty et al., 1992). In our study, among the evaluated cytokines, a significant increase was observed only for TNF-α in the lungs of the *Cy-Infected* animals and in the serum of the *Cy-Infected* and *Infected* groups. Studies have indicated that in the aerial infection of *C. neoformans*, the alveolar macrophages produce TNF-α (Huffnagle et al., 1985; Levitz et al., 1994; Herring et al., 2002). Other cells of the innate immune response, such as neutrophils and natural killer cells, can also produce TNF-α (Uezu et al., 2004). According to Herring et al. (2002), TNF-α is expressed during the first week of infection by alveolar macrophages via a T cell-independent mechanism, without increasing the antifungal activity of pulmonary phagocytes.

A study with *C. neoformans* in CBA mice described an increase in the number of $CD4^+$ T lymphocytes after the second week of infection, with an increase in IFN-γ production in the lungs and without any change in the number of $CD8^+$ T lymphocytes (Lindell et al., 2006). Previous studies have demonstrated the role of $CD4^+$ T lymphocytes in cryptococcal infection, in which, Th1 cytokines, such as IFN-γ, IL-12, IL-18, and TNF-α, were shown to be essential for host protection (Kawakami et al., 2000; Gordon, 2002). In the present study, a reduction in the $CD4^+$ T lymphocyte population was observed only in the *Cy-Uninfected* group as a direct result of the drug's action. In the *Cy-Infected* and *Infected* groups, the populations were similar to that of the *Uninfected* group. Previous studies have shown that protective immunity against *E. cuniculi* infection via the intraperitoneal route is exclusively dependent on $CD8^+$ T cells; however, when inoculated orally, the participation of $CD8^+$ and $CD4^+$ T lymphocytes is equally important to confer resistance to the infection. (Ghosh and Weiss, 2012; Moretto et al., 2004). The results of this study demonstrated that $CD8^+$ T lymphocytes play a predominant role in the immune response in lung tissue.

Cy is a cytotoxic alkylant, biotransformed by cytochrome P450, and prevents cell division, facilitates apoptosis, delays and suppresses the response of T, B cells, and pulmonary macrophages, favors myelosuppression, and affects the function and number of neutrophils, lymphocytes, red blood cells, and platelets (LeVine et al., 1999; Brodsky, 2010; Emadi et al., 2009). In the present study, compared to the *Uninfected* group, a decrease in B lymphocytes was observed in all the groups, a finding that can be attributed to the effects of Cy. However, the *Infected* group exhibited more B lymphocytes than the *Cy-Infected* and *Cy-Uninfected* groups, suggesting that the *E. cuniculi* infection mobilized the B lymphocyte response. In studies based on B-2 mice infected intraperitoneally (da Costa et al., 2017) or orally (Langanke dos Santos et al., 2018) with *E. cuniculi*, lower resistance to infection was observed; a fact that reinforces our findings that *Cy-Infected* mice had a higher fungal load. Other studies have demonstrated that the infection of rabbits and foxes with *E. cuniculi* stimulated an inflammatory reaction and the production of higher levels of antibodies, a fact that highlights the importance of these lymphocytes in microsporidiosis (Leipig et al., 2013; Akerstedt et al., 2002). In the pulmonary infection of mice with *M. pulmonis*, greater infiltration of B lymphocytes and a large number of specific immunoglobulins against mycoplasma were observed (Xu et al., 2006). Therefore, the function of B cells, which allows the differentiation of their subtypes and measurement of immunoglobulins, should be further explored in future studies.

Macrophages are essential for linking the innate and acquired immune response; hence, their presence is important at the body's entry points (resident macrophages), such as in the pulmonary alveoli. After antigen recognition, these cells release chemokines, cytokines, nitric oxide, and free radicals. During the innate and adaptive immune responses that occur against pneumonia-causing pathogens, alveolar and interstitial macrophages play a fundamental role by modulating the expression of $CD4^+$ T cells; when activated, these cells control the infection and participate in the formation of granulomas (Voelz et al., 2009). They also play an important role in maintaining lung homeostasis and removing pathogens and harmful particles without inducing inflammation or recruiting monocytes and neutrophils (Lee, 2012). In bacterial infections, macrophages are an important indicator of the inflammatory response as they are the first line of defense against infection (Weiss and Schaible, 2015; Storek and Monack, 2015).

In experimental models of respiratory infection with *Mycoplasma pneumoniae*, an increase in the number of dendritic cells and lung macrophages was observed after 14 dpi, i.e., predominantly in the inflammatory process (Sun et al., 2013). However, in this study, the pulmonary populations of the total, interstitial, and alveolar macrophages were reduced after *E. cuniculi* infection and Cy treatment. In experimental models of viral pneumonia induced by the influenza virus, alveolar macrophage deficiency favored the occurrence of diffuse and severe alveolar damage, with lethal respiratory compromise, indicating the protective role of alveolar macrophages (Cardani et al., 2017). These results corroborate the findings of this study, according to which a reduction in macrophages associated with the presence of histological lesions was observed. These lesions were caused by the presence of multiple sites of leukocyte infiltration in the alveolar wall, with a predominance of lymphoplasmacytic infiltration.

A study performed on mice infected intratracheally with *C. albicans* on the 5th day and treated with Cy (150 mg/kg/day, 4 days, and intraperitoneally) demonstrated increased susceptibility to pulmonary infection, with a decrease in the number of macrophages in the bronchoalveolar lavage and dissemination of the infection with consequent death of the animals. In vitro experiments conducted in the same study indicated that the alveolar macrophages from mice belonging to the control group were able to eliminate approximately 70% of *C. albicans* in 3 h. However, in the group of animals treated with Cy, only 20% of the pathogens were eliminated, indicating that an increased number of macrophages is necessary for the complete elimination of *C. albicans* from the lung tissue (Sawyer and Harmse, 1989).

A study carried out with C57BL/6 mice treated subcutaneously with Cy for 5 days, at a dose of 150 mg/kg on the first day and 100 mg/kg on the other days, demonstrated an association between peripheral blood leukopenia and decreased the number of alveolar macrophages in the lung. However, no association was observed between the increased number of leukocytes in the peripheral blood and alveolar macrophages after treatment with granulocyte–macrophage colony-stimulating factor (GM-CSF). Also, Cy administration did not affect the production of TNF-α and NO, along with no effect on bacterial phagocytosis, suggesting that Cy has a direct and persistent action on the number of macrophages, but not on their function (Santosuosso et al., 2002). Since macrophages perform intense spore phagocytosis and constitute important antigen-presenting cells in the lungs that are the targets of this activity, we speculate that the decrease in macrophage populations can be attributed to the cytotoxic activity of $CD8^+$ T lymphocytes.

The results obtained here allowed us to conclude that the infection of C57BL/6 mice with *E. cuniculi* allowed the development of interstitial pneumonia characterized by a predominant lymphocytic infiltrate in both Cy-treated and untreated mice. The inflammatory infiltrate was predominantly made up of $CD8^+$ and $CD4^+$ T lymphocytes. Also, immunosuppression aggravated pneumonia and increased the fungal burden. Therefore, this model can be utilized to understand the different pathological, immunological, and therapeutic aspects of lymphocytic interstitial pneumonia.

Funding

This work was supported by Fundação de Amparo à Ciência do Estado de São Paulo – Fapesp (Process number 2015/25948–2) and Coordenação de Aperfeiçoamento Pessoal de Nível Superior-Capes.

CRediT authorship contribution statement

Iramirton Figuerêdo Moreira: Methodology, Data curation, Investigation, Supervision. **Anuska Marcelino Alvares-Saraiva:** Methodology, Data curation, Investigation, Supervision. **Elizabeth Cristin Pérez:**

Methodology, Data curation, Investigation, Supervision. **José Guilherme Xavier:** Methodology, Data curation, Investigation. **Diva Denelle Spadacci-Morena:** Methodology, Data curation, Investigation. **Ronalda Silva de Araújo:** Methodology, Data curation, Investigation. **Paulo Ricardo Dell'Armelina Rocha:** Methodology, Data curation, Investigation. **Maria Anete Lallo:** Methodology, Data curation, Investigation, Supervision, Funding acquisition, Project administration, Resources.

Declaration of Competing Interest

The authors Iramirton Figuerêdo Moreira, Anuska Marcelino Alvares-Saraiva, Elizabeth Cristina Pérez, José Guilherme Xavier, Diva Denelle Spadacci-Morena, Ronalda Silva de Araújo, Paulo Ricardo Dell'Armelina Rocha, Maria Anete Lallo declare no Competing interests in papers publication "Opportunistic pneumonia caused by E. cuniculi in mice immunosuppressed with cyclophosphamide".

References

Akerstedt, J., Nordstoga, K., Mathis, A., Smeds, E., Deplazes, P., 2002. Fox encephalitozoonosis: isolation of the agent from an outbreak in farmed blue foxes (*Alopex lagopus*) in Finland and some hitherto unreported pathologic lesions. J. Vet. Med. 49, 400–405. https://doi.org/10.1046/j.1439-0450.2002.00588.

Baker, A.W., Maziarz, E.K., Arnold, C.J., Johnson, M.D., Workman, A.D., Reynolds, J.M., Perfect, J.R., Alexander, B.D., 2020. Invasive fungal infection after lung transplantation: epidemiology in the setting of antifungal prophylaxis. Clin. Infect. Dis. 70 (1), 30–39.

Bradley, J.S., Byington, C.L., Shah, S.S., Alverson, B., Carter, E.R., Harrison, C., Kaplan, S. L., Mace, S.E., McCracken, G.H., Moore, M.R., St Peter, S.D., Stockwell, J.A., Swanson, J.T., 2011. The management of community-acquired pneumonia in infants and children older than 3 months of age: clinical practice guidelines by the Pediatric Infectious Diseases Society and the Infectious Diseases Society of America. Clin. Infect. Dis. 53 (7), e25–e76.

Braunfuchsová, P., Kopecký, J., Ditrich, O., Koudela, B., 1999. Cytokine response to infection with the microsporidian, *Encephalitozoon cuniculi*. Folia Parasitol. 46, 91–95. PMID: 10425742.

Brodsky, R.A., 2010. High-dose cyclophosphamide for autoimmunity and alloimmunity. Immunol. Res. 47, 179–184. https://doi.org/10.1007/s12026-009-8149-y.

Cardani, A., Boulton, A., Kim, T.S., Braciale, T.J., Thomas, P.G., 2017. Alveolar macrophages prevent lethal influenza pneumonia by inhibiting infection of type-1 alveolar epithelial cells. PlosPathog. 13 (1), 1–25. https://doi.org/10.1371/journal.ppat.1006140.

Colvin, O.M., 1999. An overview of cyclophosphamide development and clinical applications. Curr. Pharm. Des. 5, 555–560. PMID: 10469891.

da Costa, L.F.V., Alvares-Saraiva, A.M., Dell'Armelina Rocha, P.R., Spadacci-Morena, D. D., Perez, E.C., Mariano, M., Lallo, M.A., 2017. B-1 cell decreases susceptibility to encephalitozoonosis in mice. Immunobiol. 222 (2), 218–227.

Dezern, A.E., Styler, M.J., Drachman, D.B., Hummers, L.K., Jones, R.J., Brodsky, R.A., 2013. Repeated treatment with high dose cyclophosphamide for severe autoimmune diseases. Am J Blood Res. 3, 84–90. PMID: 23358715; PMCID: PMC3555191.

Di Pasquale, M.F., Sotgiu, G., Gramegna, A., Radovanovic, D., Terraneo, S., Reyes, L.F., Rupp, J., González del Castillo, J., Blasi, F., Aliberti, S., Restrepo, M.I., Aruj, P.K., Attorri, S., Barimboim, E., Caeiro, J.P., Garzón, M.I., Cambursano, V.H., Cazaux, A., Ceccato, A., Chertcoff, J., Lascar, F., Tulio, F.D., Díaz, A.C., de Vedia, L., Ganaha, M. C., Lambert, S., Lopardo, G., Luna, C.M., Malberti, A.G., Morcillo, N., Tartara, S., Cetrangolo, A.A., Pensotti, C., Pereyra, B., Scapellato, P.G., Stagnaro, J.P., Shah, S., Lötsch, F., Thalhammer, F., Anseeuw, K., Francois, C.A., Van Braeckel, E., Vincent, J. L., Djimon, M.Z., Bashi, J., Dodo, R., Nouér, S.A., Chipev, P., Encheva, M., Miteva, D., Petkova, D., Balkissou, A.D., Yone, E.W.P., Ngahane, B.H.M., Shen, N., Xu, J.-f., Rico, C.A.B., Buitrago, R., Paternina, F.J.P., Ntumba, J.-M., Carevic, V.V., Jakopovic, M., Jankovic, M., Matkovic, Z., Mitrecic, I., Jacobsson, M.-L., Christensen, A.B., Bødtger, U.C.H., Meyer, C.N., Jensen, A.V., Baunbæk-Knudsen, G., Petersen, P.T., Andersen, S., El-Wahhab, I.-S., Morsy, N.E., Shafiek, H., Sobh, E., Abdulsemed, K.A., Bertrand, F., Brun-Buisson, C., de Montmollin, E., Fartoukh, M., Messika, J., Tattevin, P., Khoury, A., Ebruke, B., Dreher, M., Kolditz, M., Meisinger, M., Niederlausitz, K., Pletz, M.W., Hagel, S., Rupp, J., Schaberg, T., Spielmanns, M., Creutz, P., Suttorp, N., Siaw-Lartey, B., Dimakou, K., Papapetrou, D., Tsigou, E., Ampazis, D., Kaimakamis, E., Bhatia, M., Dhar, R., D'Souza, G., Garg, R., Koul, P.A., Mahesh, P.A., Jayaraj, B.S., Narayan, K.V., Udnur, H.B., Krishnamurthy, S.B., Kant, S., Swarnakar, R., Limaye, S., Salvi, S., Golshani, K., Keatings, V.M., Martin-Loeches, I., Maor, Y., Strahilevitz, J., Battaglia, S., Carrabba, M., Ceriana, P., Confalonieri, M., Monforte, A.D., Prato, B.D., Rosa, M.D., Fantini, R., Fiorentino, G., Gammino, M.A., Menzella, F., Milani, G., Nava, S., Palmiero, G., Petrino, R., Gabrielli, B., Rossi, P., Sorino, C., Steinhilber, G., Zanforlin, A., Franzetti, F., Carugati, M., Morosi, M., Monge, E., Carone, M., Patella, V., Scarlata, S., Comel, A., Kurahashi, K., Bacha, Z.A., Ugalde, D.B., Zuñiga, O.C., Villegas, J.F., Medenica, M., van de Garde, E.M.W., Mihsra, D.R., Shrestha, P., Ridgeon, E., Awokola, B.I., Nwankwo, O.N.O., Olufunlola, A.B., Olumide, S., Ukwaja, K.N., Irfan, M., Minarowski, L., Szymon, S., Froes, F., Leuschner, P., Meireles, M., Ferrão, C., Leuschner, P., Neves, J., Ravara, S.B., Brocovschii, V., Ion, C., Rusu, D., Toma, C., Chirita, D., Dorobat, C.M., Birkun, A., Kaluzhenina, A., Almotairi, A., Bukhary, Z.A.A., Edathodu, J., Fathy, A., Enani, A.M. A., Mohamed, N.E., Memon, J.U., Bella, A., Bogdanović, N., Milenkovic, B., Pesut, D., Borderìas, L., Garcia, N.M.B., Alarcón, H.C., Cilloniz, C., Torres, A., Diaz-Brito, V., Casas, X., González, A.E., Fernández-Almira, M.L., Gallego, M., Gaspar-GarcÍa, I., del Castillo, J.G., Victoria, P.J., Martínez, E.L., de Molina, R.M., Marcos, P. J., Menéndez, R., Pando-Sandoval, A., Aymerich, C.P., de la Torre, A.L., García-Olivé, I., Rello, J., Moyano, S., Sanz, F., Sibila, O., Rodrigo-Troyano, A., Solé-Violán, J., Uranga, A., van Boven, J.F.M., Torra, E.V., Pujol, J.A., Feldman, C., Yum, H.K., Fiogbe, A.A., Yangui, F., Bilaceroglu, S., Dalar, L., Yilmaz, U., Bogomolov, A., Elahi, N., Dhasmana, D.J., Feneley, A., Ions, R., Skeemer, J., Woltmann, G., Hancock, C., Hill, A.T., Rudran, B., Ruiz-Buitrago, S., Campbell, M., Whitaker, P., Youzguin, A., Singanayagam, A., Allen, K.S., Brito, V., Dietz, J., Dysart, C.E., Kellie, S.M., Franco-Sadud, R.A., Meier, G., Gaga, M., Holland, T.L., Bergin, S.P., Kheir, F., Landmeier, M., Lois, M., Nair, G.B., Patel, H., Reyes, K., Rodriguez-Cintron, W., Saito, S., Soni, N.J., Noda, J., Hinojosa, C.I., Levine, S.M., Angel, L.F., Anzueto, A., Whitlow, K.S., Hipskind, J., Sukhija, K., Totten, V., Wunderink, R.G., Shah, R.D., Mateyo, K.J., Noriega, L., Alvarado, E., Aman, M., Labra, L., 2019. Prevalence and etiology of community-acquired pneumonia in immunocompromised patients. Clin. Infect. Dis. 68 (9), 1482–1493.

Didier, E.S., Weiss, L.M., Cali, A., Marciano-Cabral, F., 2009. Overview of the presentations on microsporidia and free-living amebae at the 10th International Workshops on Opportunistic Protists. Eukaryot. Cell 6, 441–445. https://doi.org/10.1128/EC.00302-08.

Emadi, A., Jones, R.J., Robert, A., Brodsky, R.A., 2009. Cyclophosphamide and cancer: golden anniversary. Nat. Rev. Clin. Oncol. 6, 638–647. https://doi.org/10.1038/nrclinonc.2009.146.

Ghosh, K., Weiss, L.M., 2012. T cell response and persistence of the microsporidia. FEMS Microbiol. Rev. 36, 748–760. https://doi.org/10.1111/j.1574-6976.2011.00318.x.

Ghoyounchi, R., Ahmadpour, E., Spotin, A., Mahami-Oskouei, M., Rezamand, A., Aminisani, N., Ghojazadeh, M., Berahmat, R., Mikaeili-Galeh, T., 2017. Microsporidiosis in Iran: a systematic review and meta-analysis. Asian Pac. J. Trop. Med. 10 (4), 341–350.

Girmenia, C., Iori, A.P., 2012. Safety and interactions of new antifungals in stem cell transplant recipients. Expert Opin. Drug Saf. 11, 803–818. https://doi.org/10.1517/14740338.2012.712111.

Gordon, S., 2002. Pattern recognition receptors: doubling up for the innate immune response. Cell 111, 927–930. https://doi.org/10.1016/S0092-8674(02)01201-1.

Gramegna, A., Sotgiu, G., Di Pasquale, M., Radovanovic, D., Terraneo, S., Reyes, L.F., Vendrell, E., Neves, J., Menzella, F., Blasi, F., Aliberti, S., Restrepo, M.I., 2018. Atypical pathogens in hospitalized patients with community-acquired pneumonia: a worldwide perspective. BMC Infect. Dis. 18 (1) https://doi.org/10.1186/s12879-018-3565-z. Erratum. In: BMC Infect Dis. 2020 Oct 13;20(1):748. PMID: 30563504; PMCID: PMC6299604.

Han, B., Weiss, L.M., Heitman, J., Stukenbrock, E.H., 2017. Microsporidia: obligate intracellular pathogens within the fungal kingdom. Microbiol. Spectr. 5 (2) https://doi.org/10.1128/microbiolspec.FUNK-0018-2016. PMID: 28944750; PMCID: PMC5613672.

Harty, J.T., Schreiber, R.D., Bevan, M.J., 1992. CD8 T cell can protect against an intracellular bacterium in an interferon g-independent fashion. Proc. Natl. Acad. Sci. U. S. A. 89, 11612–11616. https://doi.org/10.1073/pnas.89.23.11612.

Herring, A.C., Lee, J., Mcdonald, R.A., Toews, G.B., Huffnagle, G.B., 2002. Induction of Interleukin-12 and Gamma Interferon equires Tumor Necrosis Factor Alpha for Protective T1-Cell-Mediated Immunity to Pulmonary *Cryptococcus neoformans* Infection. Infect. Immun. 70, 2959–2964. https://doi.org/10.1128/IAI.70.6.2959-2964.2002.

Huffnagle, G.B., Chen, G.H., Curtis, J.L., McDonald, R.L., Strieter, R.M., Toews, G.B., 1985. Downregulation of the afferent phase of T cell-mediated pulmonary inflammation and immunity by a high melanin-producing strain of *Cryptococcus neoformans*. J. Immunol. 155, 3507–3516. PMID: 7561046.

Hussell, T., Bell, T.J., 2014. Alveolar macrophages: plasticity in a tissue-specific context. Nat. Rev. Immunol. 14, 81–93. https://doi.org/10.1038/nri3600.

Jain, S., Self, W.H., Wunderink, R.G., Fakhran, S., Balk, R., Bramley, A.M., Reed, C., Grijalva, C.G., Anderson, E.J., Courtney, D.M., Chappell, J.D., Qi, C., Hart, E.M., Carroll, F., Trabue, C., Donnelly, H.K., Williams, D.J., Zhu, Y., Arnold, S.R., Ampofo, K., Waterer, G.W., Levine, M., Lindstrom, S., Winchell, J.M., Katz, J.M., Erdman, D., Schneider, E., Hicks, L.A., McCullers, J.A., Pavia, A.T., Edwards, K.M., Finelli, L., 2015. Community-acquired pneumonia requiring hospitalization among U.S. adults. N. Engl. J. Med. 373 (5), 415–427.

Kaczmarek, A., Boguś, M.I., 2021. Fungi of entomopathogenic potential in Chytridiomycota and Blastocladiomycota, and in fungal allies of the Oomycota and Microsporidia. IMA Fungus 12 (1). https://doi.org/10.1186/s43008–021–00074-y. PMC8504053.

Kawakami, K., Koguchi, Y., Qureshi, M.H., Miyazato, A., Yara, S., Kinjo, Y., Iwakura, Y., Takeda, K., Akira, S., Kurimoto, M., Saito, A., 2000. IL-18 contributes to host resistance against infection with *Cryptococcus neoformans* in mice with defective IL-12 synthesis through induction of IFN γ production by NK cells. J. Immunol. 165 (2), 941–947.

Kicia, M., Wesolowska, M., Kopacz, Z., Jakuszko, K., Sak, B., Květonová, D., Krajewska, M., Kváč, M., 2016. Prevalence and molecular characteristics of urinary and intestinal microsporidia infections in renal transplant recipients. Clin. Microbiol. Infect. 22 (5), 462.e5–462.e9. https://doi.org/10.1016/j.cmi.2016.01.014.

Kicia, M., Wesolowska, M., Kopacz, Z., Kváč, M., Sak, B., Sokulska, M., Cebulski, K., Hendrich, A.B., Pozowski, A., 2018. Disseminated Infection of *Encephalitozoon*

cuniculi associated with osteolysis of hip periprosthetic tissue. Clin. Infect. Dis. 67 (8), 1228–1234. https://doi.org/10.1093/cid/ciy256.
Kicia, M., Szydłowicz, M., Cebulski, K., Jakuszko, K., Piesiak, P., Kowal, A., Sak, B., Krajewska, M., Hendrich, A.B., Kváč, M., Kopacz, Ż., 2019. Symptomatic respiratory *Encephalitozoon cuniculi* infection in renal transplant recipients. Int. J. Infect. Dis. 79, 21–25. https://doi.org/10.1016/j.ijid.2018.10.016.
Kotkova, M., Sak, B., Kvetonova, D., Kvac, M., Didier, E., 2013. Latent microsporidiosis caused by *Encephalitozoon cuniculi* in immunocompetent hosts: a murine model demonstrating the ineffectiveness of the immune system and treatment with albendazole. PLoS ONE 8 (4), e60941.
Lallo, M.A., Hirschfeld, M.M.P., 2012. Encephalitozoonosis in pharmacologically immunosuppressed mice. Exp. Parasitol. 131 (3), 339–343.
Lallo, M.A., da Costa, L.F.V., de Castro, J.M., 2013. Effect of three drugs against *Encephalitozoon cuniculi* infection in immunosuppressed mice. Antimicrobial Agents Chemother. 57, 3067–3071. https://doi.org/10.1128/AAC.00157-13.
Lampe, M.F., Wilson, C.B., Bevan, M.J., Starnbach, M.N., 1998. Gamma Interferon production by cytotoxic T lymphocytes is required for resolution of *Chlamydia trachomatis* infection. Infect. Immun. 66 (11), 5457–5461.
Langanke Dos Santos, D., Alvares-Saraiva, A.M., Xavier, J.G., Spadacci-Morena, D.D., Peres, G.B., Rocha, P.R.D.A., et al., 2018. B-1 cells upregulate CD8 T lymphocytes and increase proinflammatory cytokines serum levels in oral encephalitozoonosis. Microbes Infect. 20 (3), 196–204. https://doi.org/10.1016/j.micinf.2017.11.004.
Lee, J.S., 2012. Heterogeneity of lung mononuclear phagocytes in chronic obstructive pulmonar disease. J. Innate Immun. 4, 489–497. https://doi.org/10.1159/000337434.
Leipig, M., Matiasek, K., Rinder, H., Janik, D., Emrich, D., Baiker, K., Hermanns, W., 2013. Value of histopathology, immunohistochemistry, and real-time polymerase chainreaction in the confirmatory diagnosis of *Encephalitozoon cuniculi* infection in rabbits. J. Vet. Diagn. Invest. 25 (1), 16–26.
LeVine, A.M., Reed, J.A., Kurak, K.E., Cianciolo, E., Whitsett, J.A., 1999. GM-CSF–deficient mice are susceptible to pulmonary group B streptococcal infection. J. Clin. Invest. 103, 563–569. https://doi.org/10.1172/JCI5212.
Levitz, S.M., Tabuni, A., Kornfeld, H., Reardon, C.C., Golenbock, D.T., 1994. Production of tumor necrosis factor alpha in human leukocytes stimulated by *Cryptococcus neoformans*. Infect. Immun. 62, 1975–1981. https://doi.org/10.1128/iai.62.5.1975-1981.1994.
Limper, A.H., Knox, K.S., Sarosi, G.A., Ampel, N.M., Bennett, J.E., Catanzaro, A., Davies, S.F., Dismukes, W.E., Hage, C.A., Marr, K.A., Mody, C.H., Perfect, J.R., Stevens, D.A., 2011. An official American Thoracic Society statement: treatment of fungal infections in adult pulmonary and critical care patients. Am. J. Respir. Crit. Care Med. 183 (1), 96–128.
Lindell, D.M., Moore, T.A., McDonald, R.A., Toews, G.B., Huffnagle, G.B., 2006. Compartmentalization of $CD4^+$ T-Cell effector function versus proliferative capacity during pulmonary cryptococcosis. Am. J. Pathol. 168, 847–855. https://doi.org/10.2353/ajpath.2006.050522.
Moretto, M.M., Durell, B., Schwartzman, J.D., Khan, I.A., 2001. γδ T cell-deficient mice have a down-regulated CD8+ T cell immune response against *Encephalitozoon cuniculi* infection. J. Immunol. 166, 7389–7397. https://doi.org/10.4049/jimmunol.166.12.7389.
Moretto, M.M., Weiss, L.M., Khan, I.A., 2004. Induction of a rapid and strong antigen-specific intraepithelial lymphocyte response during oral *Encephalitozoon cuniculi* infection. J. Immunol. 172, 4402–4409. https://doi.org/10.4049/jimmunol.172.7.4402.
Moretto, M.M., Harrow, D.I., Hawley, T.S., Khan, I.A., Deepe, G.S., 2015. Interleukin-12-producing CD103+ CD11b− CD8+ dendritic cells are responsible for eliciting gut intraepithelial lymphocyte response against *Encephalitozoon cuniculi*. Infect. Immun. 83 (12), 4719–4730. https://doi.org/10.1128/IAI.00820–15.
Orenstein, J.M., Russo, P., Didier, E.D., Bowers, C., Bunin, N., Teachey, D.T., 2005. Fatal pulmonary microsporidiosis due to *Encephalitozoon cuniculi* following allogeneic bone marrow transplantation for acute myelogenous leukemia. Ultrastruct. Pathol. 29, 269–276. https://doi.org/10.1080/01913120590951257.
Özkoç, S., Bayram delibaş, S., Akisü, Ç., 2016. Evaluation of pulmonary microsporidiosis in iatrogenically immunosuppressed patients. Tuberk Toraks. 64 (1), 9–16. https://doi.org/10.5578/tt.10207.
Sak, B., Brady, D., Pelikanova, M., Kvetonova, D., Rost, M., Kostka, M., Tolarova, V., Huzova, Z., Kvac, M., 2011. Unapparent microsporidial infection among immunocompetent humans in the Czech Republic. J. Clin. Microbiol. 49 (3), 1064–1070. https://doi.org/10.1128/JCM.01147–10.
Santosuosso, M., Divangahi, M., Zganiacz, A., Xing, Z., 2002. Reduced tissue macrophage population in the lung by anticancer agent cyclophosphamide: restoration by local granulocyte macrophage–colony-stimulating factor gene transfer. Blood 99, 1246–1252. https://doi.org/10.1182/blood.V99.4.1246.
Sawyer, R.T., Harmse, A.G., 1989. The relative contribution of resident pulmonary alveolar macrophage and inflammatory polymorphonuclear neutrophils in host resistance to pulmonary infection by *Candida albicans*. Mycopathol. 108, 95–105. https://doi.org/10.1007/BF00436059.
Scott, J.A., Wonodi, C., Moïsi, J.C., Deloria-Knoll, M., DeLuca, A.N., Karron, R.A., et al., 2012. The definition of pneumonia, the assessment of severity, and clinical standardization in the Pneumonia Etiology Research for Child Health study. Clin Infect Dis. 54 (Suppl 2), S109–116. doi: 10.1093/cid/cir1065. PMID: 22403224; PMCID: PMC3297550.
Serbina, N.V., Liu, C.C., Scanga, C.A., Flynn, J.L., 2000. $CD8^+$ CTL from Lungs of *Mycobacterium tuberculosis*-infected mice express perforin in vivo and lyse infected macrophages. J. Immunol. 165, 353–363. https://doi.org/10.4049/jimmunol.165.1.353.
Sevko, A., Sade-Feldman, M., Kanterman, J., Michels, T., Falk, C.S., Umansky, L., Ramacher, M., Kato, M., Schadendorf, D., Baniyash, M., Umansky, V., 2013. Cyclophosphamide promotes chronic inflammation-dependent immunosuppression and prevents antitumor response in melanoma. J, Invest. Dermatol. 133 (6), 1610–1619. https://doi.org/10.1038/jid.2012.444.
Sistigu, A., Viaud, S., Chaput, N., Bracci, L., Proietti, E., Zitvogel, L., et al., 2011. Immunomodulatory effects of cyclophosphamide and implementations for vaccine design. Semin. Immunopathol. 33, 369–383. https://doi.org/10.1007/s00281-011-0245-0.
Storek, K.M., Monack, D.M., 2015. Bacterial recognition pathways that lead to inflammasome activation. Immunol. Rev. 265, 112–129. https://doi.org/10.1111/imr.12289.
Sun, X., Jones, H.P., Dobbs, N., Bodhankar, S., Simecka, J.W., Shams, H., 2013. Dendritic cells are the major antigen presenting cells in inflammatory lesions of murine mycoplasma respiratory disease. PLoSOne. 8 (2), e55984.
Torres, A., Chalmers, J.D., Dela Cruz, C.S., Dominedò, C., Kollef, M., Martin-Loeches, I., Niederman, M., Wunderink, R.G., 2019. Challenges in severe community-acquired pneumonia: a point-of-view review. Intensive Care Med. 45 (2), 159–171. https://doi.org/10.1007/s00134–019–05519-y.
Uezu, K., Kawakami, K., Miyagi, K., Kinjo, Y., Kinjo, T., Ishikawa, H., et al., 2004. Accumulation of T Cells in the Lungs and Their Regulatory Roles in Th1 Response and Host Defense against Pulmonary Infection with *Cryptococcus neoformans*. J. Immunol. 172, 7629–7634. https://doi.org/10.4049/jimmunol.172.12.7629.
Visvesvara, G.S., 2002. In vitro cultivation of microsporidia of clinical importance. Clin. Microbiol. Rev. 15, 401–413. https://doi.org/10.1128/CMR.15.3.401-413.2002.
Voelz, K., Lammas, D.A., May, R.C., 2009. Cytokine signaling regulates the outcome of intracellular macrophage parasitism by *Cryptococcus neoformans*. Infect. Immun. 77, 3450–3457. https://doi.org/10.1128/IAI.00297-09.
Weiss, G., Schaible, U.E., 2015. Macrophage defense mechanisms against intracellular bacteria. Immunol. Rev. 264 (1), 182–203. https://doi.org/10.1111/imr.12266.
Weitzel, T., Wolff, M., Dabanch, J., Levy, I., Schmetz, C., Visvesvara, G.S., et al., 2001. Dual Microsporidial Infection with *Encephalitozoon cuniculi* and *Enterocytozoon bieneusi* in an HIV-Positive Patient. Infect. 29, 237–239. https://doi.org/10.1007/s15010-001-1164-0.
World Health Organization WHO Model list of Essential Medicine 2013 http://www.who.int/medicines/publications/essentialmedicines/18th_EML.pdf.
Xu, X., Zhang, D., Lyubynska, N., Wolters, P.J., Killeen, N.P., Baluk, P., McDonald, D.M., Hawgood, S., Caughey, G.H., 2006. Mast cells protect mice from *Mycoplasma* pneumonia. Am. J. Respir. Crit. Care Med. 173 (2), 219–225. https://doi.org/10.1164/rccm.200507–1034OC.