Can immunosuppressed mice control oral infection by the opportunistic pathogen *Encephalitozoon intestinalis*?

Encephalitozoon intestinalis infection in immunosuppressed mice

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24

25 **Abstract**

26 Intestinal mucosa (IM), or the outer surface of the intestine, serves at the primary site for the
 27 interaction of various pathogens that cause infection via the oral route. Thus, IM is crucial for
 28 developing an efficient adaptive immune response against pathogenic micro-organisms, thereby
 29 preventing their colonization and subsequent infection. In the present study, we investigated the
 30 immune response to *Encephalitozoon intestinalis*-caused infection in the IM and gut-associated
 31 lymphoid tissue (GALT) in C57BL/6 female mice. To mimic an immunosuppressive condition, the
 32 mice were treated with cyclophosphamide (Cy). Histopathology revealed lymphoplasmacytic
 33 enteritis at 7 and 14 days-post-infection (dpi) in all infected groups; however, inflammation
 34 diminished at 21 and 28 dpi. Cy treatment also led to a higher number of *E. intestinalis* spores and
 35 lesions, which reduced at 28 dpi. In addition, flow cytometry analysis demonstrated CD4⁺ and
 36 CD8⁺ T cells to be predominant immune cells, with a significant increase in both Th1 and Th2
 37 cytokines at 7 and 14 dpi, as demonstrated by histopathology. In conclusion, Cy treatment reduced
 38 GALT (Peyer's plaques and mesenteric lymph nodes) and peritoneum populations but increased the
 39 T-cell population in the intestinal mucosa and the production of pro-inflammatory cytokines, which
 40 were able to eliminate this opportunistic fungus and reduced the infection.

41

42 **Keywords:** *Encephalitozoon intestinalis*, microsporidia, mucosal immunity, intestinal inflammation

43

44 **Introduction**

45 Microsporidia are spore-forming intracellular pathogens, responsible for causing opportunistic
 46 infections in immunocompromised people, such as those with HIV infection and AIDS, cancer
 47 patients or individuals with autoimmune diseases taking immunosuppressive drugs, and in elderly
 48 and children (1-4). Two species are responsible for gastrointestinal infections: *Enterocytozoon*

49 *bieneusi* and *Encephalitozoon intestinalis* (5). Both microsporidia are transmitted by the oral route
50 and cause abdominal cramping, diarrhea, malabsorption, and weight loss in patients with AIDS (6).
51 *E. intestinalis* also infects and develops inside intestinal macrophages, allowing the infection to
52 spread from the intestine to other organs (7). A very limited number of drugs are available for
53 treating intestinal microsporidiosis; these include albendazole and fumagillin, which are at least
54 partially effective in reducing the parasite count (2,7). A strong immune response of an individual is
55 mainly responsible for controlling the pathogen. The compartmentalized response against *E.*
56 *intestinalis* infection is primarily mediated by CD8⁺ and CD4⁺ T cells together with interferon
57 (IFN) and interleukin (IL)-12 cytokines (8,9).
58 The intestinal mucosa (IM) acts as a host to a variable microflora, which plays an important role in
59 nutrition absorption and immune function, among others. The immune response of the IM plays a
60 critical role in maintaining the commensal homeostasis and protecting the host against pathogens
61 (10,11). The immune response of IM against *E. cuniculi* infection is majorly mediated by antigen-
62 specific intraepithelial lymphocytes (IELs) (12). Considering the entry point of microsporidia to be
63 intestinal mucosa, there is a lack of knowledge of local immunity, especially in individuals on
64 immunosuppressive medications.
65 The past decade has witnessed a surge in the use of immunosuppressive drugs for the treatment of
66 neoplastic and autoimmune disease patients, as well as in patients undergoing transplantation.
67 Cyclophosphamide (Cy) is one such immunosuppressive drug that has been recommended by the
68 World Health Organization (WHO) and is on the list of drugs most frequently used by the Public
69 and Private Health Systems owing to its efficacy, cost-effectiveness, and lesser side-effects than
70 other drugs with similar actions, such as dexamethasone (13). Cy is primarily used against
71 autoimmune and alloimmune diseases (14,15), in treating patients undergoing transplantation, such
72 as bone marrow recipients (16) and in cancer treatment (17,18).
73 Cyclophosphamide is a cytotoxic alkylating agent that binds to DNA; its major effects on the body
74 include cellular apoptosis and myelosuppression, and decreased lymphocyte, neutrophil, red blood

cell, and platelet count. However, it also possesses immunomodulatory effects, which have not yet been clarified, such as (i) expansion of antigen-specific T cells, (ii) expansion of T cell-specific cytokines (IFNs, IL-7, and IL-15), (iii) decrease in regulatory T cells (Treg), and (iv) increased mobilization of dendritic cells from bone marrow, with activation of intracellular machinery for antigen processing and presentation (19). Thus, this anticancer agent is known for inducing immunogenic cancer cell death, subverting immunosuppressive T cells, and promoting Th1 and Th17 cells that control cancer outgrowth (20, 21).

We have shown previously Cy to suppress the immune system of mice; intraperitoneal infection of mice with *E. cuniculi* resulted in a disseminated, acute, and fatal encephalitozoonosis. These results were associated with the immunosuppressive effects of Cy (22). This study was designed to describe how the immunosuppressive effects of cyclophosphamide compromise the intestinal immune response against *E. intestinalis*, one of the most prevalent microsporidia in opportunistic infections in humans. Herein, we show a higher number of CD8⁺ and CD4⁺ T lymphocytes in IM in association with pro-inflammatory cytokines to be responsible for resolution of *E. intestinalis* infection in both Cy immunosuppressed and immunocompetent mice, despite the immunosuppressive activity of Cy observed in cells populations of Peyer's plaques, mesenteric lymph nodes, peritoneum and even part of the immune population of the IM.

Results

Immunosuppressed mice showed transitory enteritis with fast resolution. All infected mice survived the oral infection by *E. intestinalis*, with no evidence of symptoms and macroscopic lesions in the gut. Microscopically, lymphoplasmacytic enteritis was observed in infected animals (*infected* and *Cy-infected*), mostly affecting the duodenum and ileum, especially at 7 and 14 dpi (Fig. 1a, b). In addition to the inflammatory infiltrate, mucosal ulceration caused exposure of the underlying lamina propria (Fig. 1b), apical and mural multifocal necrosis, and villus-based epithelial proliferation. At 21 and 28 dpi, young cells and an increased number of mitoses were

observed in the small intestine, suggesting a restoration of tissue integrity, via resolution of the inflammatory process and tissue remodeling. *E. intestinalis* spore clusters were observed on the glandular base (Fig. 1c), with a higher fungal burden observed in the *Cy-infected* than the *infected* (Fig. 1d) group. However, in both groups, the fungal burden and the lesions progressively decreased from 7 to 28 dpi (Fig. 1d). No *E. intestinalis* spores were observed in uninfected mice. Thus, infection by *E. intestinalis* via oral route caused lymphoplasmacytic enteritis, accompanied by favorable proliferation in both immunosuppressed and non-immunosuppressed groups; however, the fungal burden was higher in the Cy-treated mice. Macroscopically, the infection led to the enlargement of the spleen and intestinal lymph nodes in the infected animals.

CD8⁺ T lymphocytes increased in intestinal mucosa in infected mice. The *infected* and *Cy-infected* groups showed a significant increase in CD8⁺ T lymphocyte population as compared to controls at 7, 14, and 28 dpi (Fig. 2). This is in line with a previous study that demonstrated CD4⁺ T and CD8⁺ T lymphocyte subpopulations to play a substantive role in protecting against peroral infection of *E. intestinalis* (8). Another study reported a significant increase in the CD8αα subset of IELs in response to oral *E. cuniculi* infection (12).

Immunosuppressive therapy with Cy could not cause a significant difference in CD8⁺ T lymphocyte population among the infected mice. Moreover, CD8⁺ T lymphocyte population witnessed a 10-time decrease, when compared 7 with 28 dpi (Supplementary Fig. 1). Together, the results showed that CD8⁺ T lymphocyte peak was observed at 7 dpi and was associated with a high number of *E. intestinalis* spores and lymphoplasmacytic enteritis. At the same time, there was a gradual reduction in the CD8⁺ T cell population, fungal burden, and histological lesions in infected (*infected* and *Cy-infected*) animals at 14, 21, and 28 dpi, indicating the resolution of infection in both immunosuppressed and non-immunosuppressed groups. The treatment with Cy resulted in a significant increase in the CD4⁺ T lymphocyte population in the uninfected group. In addition, *infected* and *Cy-infected* groups also showed an increase in this population at 14 and 28 dpi as

compared to the untreated control (Fig. 2), suggesting that *E. intestinalis* stimulated CD4⁺ T expansion.

Further, the NKT cells decreased significantly in the *infected*, *Cy-infected*, and *Cy-uninfected* groups as compared to the *uninfected* control at 7 and 14 dpi (Fig. 2). However, these cells increased in the *infected* and *Cy-infected* groups in the later stages of infection (Supplementary 1).

A significant reduction in B-1 cells was reported in the *infected*, *Cy-infected*, and *Cy-uninfected* groups as compared to the *uninfected* control at 14, 21, and 28 dpi (Fig. 2). Moreover, B-2 cells decreased in the *infected* and *Cy-infected* groups as compared to the *uninfected* control at 14 dpi but increased significantly in the *infected* group at 28 dpi (Fig. 2). Another notable observation was a decrease in the dendritic cells in PP in the *infected* and *Cy-infected* groups (Fig. 3a). PP in the *infected* group was more evident, with a marked increase in the germinal center activity, thereby expanding the lymphoid center (Fig. 3b). Moreover, PP from *Cy-infected* mice showed a rarified lymphoid tissue (Fig. 3b), enlarged lymphatics, and clusters of *E. intestinalis* spores (data not shown).

Interestingly, Cy treatment (*Cy-uninfected*) showed an immunomodulatory effect on lymphocyte populations in the intestinal mucosa. Overall, elevated levels of CD8⁺ T, CD4⁺ T, and B-2 cell populations were observed as compared to other groups (Fig. 2).

T and B lymphocytes decreased in mesenteric lymph nodes of *Cy-infected* mice. The populations of B-2, CD4⁺ T, CD8⁺ T, and NKT cells decreased at 21 and 28 dpi in the *Cy-infected* group as compared to the *infected* group, suggesting the immunosuppressive effect of Cy. At 14 dpi, a higher percentage of macrophages was present in the *Cy-infected* group as compared to the *infected* group; however, this witnessed a decrease at 21 and 28 dpi (Fig. 4).

T and B lymphocytes decreased in the peritoneum of *E. intestinalis*-infected mice. The CD4⁺ T, CD8⁺ T, and NKT cells decreased significantly in all *infected* groups as compared to the *uninfected*

control (Fig. 5). Moreover, CD8⁺ T, B-1, and B-2 lymphocytes decreased significantly in all Cy-treated groups at 7 and 28 dpi (Fig. 5). At 14 dpi, both B-1 and B-2 cell populations and macrophages increased in the *infected* group as compared to the *Cy-infected* group (Fig. 5). However, the macrophage number decreased in the *infected*, *Cy-infected*, and *Cy-uninfected* groups at 14 and 28 dpi (Fig. 5). Overall, the infected mice showed a decreased number of B-1, B-2, CD4⁺ T, and CD8⁺ T cells, with a statistical difference at 14 dpi (Supplementary Fig. 2). This difference was maintained at 21 and 28 dpi for B cells, but not for T cells.

Pro- and anti-inflammatory cytokines detected in the ileum of infected mice. *E. intestinalis* infection was associated with increased levels of various pro- and inflammatory cytokines, including IL-10, IL-17a, TNF- α , IFN- γ , IL-6, IL-4, and IL-2 at 14 dpi (Fig. 6). High levels of TNF- α persisted at 21 and 28 dpi, whereas other cytokines were not detected at these dpi (Fig. 6). These results implied that Cy treatment increased the production of IL-10 and IFN- γ at 7 dpi in the *Cy-uninfected* group as compared to the *uninfected* group. The highest level of cytokines was observed in the *Cy-infected* mice and included mostly IL-2, IL-6 and IL-4. The cytokine IL-17a was detected in all groups in all experimental animals although no statistical difference was observed between them. Moreover, the *uninfected* group showed enhanced levels of TNF- α (Fig. 6). Overall, the cytokines levels in the present study after *E. intestinalis* infection corroborate with the histopathological results obtained in all *infected* groups at 7 and 14 dpi, reflecting the immune system-mediated attempt to counter the intestinal infection.

Transmission electron microscopy. Transmission electron microscopy (TEM) results showed *E. intestinalis* spores of varying shapes, from oval to piriform, to be present close to the microvillousities and in the cytoplasm of enterocytes (Fig. 7) of the *Cy-infected* group. Additionally, two patterns of the morphological invasion were observed: endocytosis (Fig. 7 a,b) and injection of sporoplasm by polar tubule into the host cells (Fig. 7 c,d). The discontinuous microvillousities,

present next to spores, showed invaginations, manifesting their attempt to engulf spores, a mechanism similar to phagocytosis. However, no phagocytic vacuoles were observed (Fig. 7 a,b,c). The cytoplasm surrounding the spores was found to be electrodense with a high number of mitochondria (Fig. 7 a,b,c). Moreover, surrounding the *E. intestinalis* sporoplasm injection demonstrated a loss of microvillousities and cytoplasmic membrane projections involving the extruded polar tubule that appeared to form a channel for its entry into the electrodense cytoplasm (Fig. 7 d).

Discussion

E. intestinalis invades and develops in the cytoplasm of enterocytes, causing persistent diarrhea in humans with AIDS (7) or in individuals immunocompromised by cancer or chemotherapy (23,24). Microsporidia also infect the macrophages of the lamina propria that help in its dissemination to kidneys and the hepatobiliary tract (25). In the present study, we observed oral infection with *E. intestinalis* not to be associated with clinical signs, such as diarrhea, even in the Cy-immunosuppressed mice. However, microscopically, lymphoplasmacytic enteritis was observed in association with *E. intestinalis* spores.

The adaptive immune response is very crucial for containing microsporidiosis. For example, T cells-deficient mice (athymic/nude or SCID) are incapable of successfully killing the pathogen and die due to encephalitozoonosis as opposed to immunocompetent mice (1,26). Similarly, IFN- γ ^{-/-} mice lacking major proinflammatory cytokines showed hepatomegaly, colicystitis, splenomegaly, intestinal enlargement, and ascites three to four weeks post infection with *E. intestinalis*. Moreover, these mice could live only up to six weeks post infection (25). Therefore, physical or genetic deletion of immune cells or cytokines represents a situation that does not correspond to clinical observations observed in individuals immunosuppressed by drugs. Since immunosuppressive drugs act on different cells of the immune system, leading to a diversity of immunocompromised states, we used Cy to mimic the physiological immunosuppression situations.

205 We observed that *Cy-infected* mice showed a higher number of spores, reflecting the increased
 206 susceptibility to pathogen upon treatment with Cy, as previously reported by our group in
 207 experimental *E. cuniculi* infection in mice (22). Unexpectedly, the present results showed a
 208 decrease in fungal burden and histopathological lesions in immunosuppressed mice at 21 and 28
 209 dpi, indicating successful elimination of pathogen. Cy treatment is lymphotoxic and rapidly
 210 decreases the T and B cell populations (14). However, a study reported it to cause an extensive
 211 mobilization of immune cells from the bone marrow and other lymphoid organs (18). In the present
 212 study, we observed an increase in CD4⁺ T and CD8⁺ T cells population in the IM of Cy-treated
 213 mice. These results may be associated to two possible explanations: i) the displacement of immune
 214 cells from nearby immune sites such as Peyer's patches, lymph nodes and peritoneum favored the
 215 assembly of an immune response in the gut capable of controlling infection, however, in this case, it
 216 should be considered that such reduction could be linked to the suppressive effect of Cy on these
 217 immune sites, or ii) low action on the immune population of the intestinal mucosa, especially T
 218 cells, data that may be linked to a possible immunomodulatory activity of Cy, which should be
 219 further explored

220 Another observation that point toward resolution of infection is the increased presence of mitosis in
 221 the intestinal crypts of the small intestine associated with enteritis in all infected animals, indicating
 222 tissue remodeling in the intestine under both pathological and physiological conditions (27). Since
 223 enterocytes serve as the first physical barrier against pathogens in intestine, maintenance of a strong
 224 epithelial barrier is crucial for normal physiology and evasion of infections. Moreover, epithelial
 225 cells secrete important chemoattractants that are involved in cell chemotaxis (28). The IELs
 226 constitute a sub-population of T lymphocytes in the IM that has been implicated in both intestinal
 227 homeostasis and inflammation, as observed in intestinal toxoplasmosis. Another important category
 228 of immune cells is dendritic cells that reside in the lamina propria beneath the IM and act as antigen
 229 presenting cells. They play a crucial role in containing toxoplasmosis. Lamina propria also

comprises CD4⁺ T, CD8⁺ T, and B lymphocytes; B cells are known for producing secretory IgA that is responsible for the exclusion of environmental antigens (29).

There are reports stating an effective immune response against microsporidia to involve majorly CD8⁺ T cells (30,31). Interestingly, other studies report CD8⁺ T cells and not CD4⁺ T cells to be indispensable in successfully eliminating pathogens in intraperitoneal infection by *E. cuniculi* (30,32). In fact, the dichotomic role of CD4⁺ T cells has been shown to be associated with the infection route. It has been shown that the oral infection with *E. cuniculi* stimulates a synergistic effect of CD8⁺ and CD4⁺ T cells (12). The present study also reported an increased number of CD4⁺ T cells in the intestines of infected mice, contributing to successful *E. intestinalis* elimination. We believe this increase in CD4⁺ T cells to be attributed to the immunomodulatory effect of Cy in the intestinal mucosa.

Evidence from literature reports CD4⁺ and CD8⁺ T cells, associated with IFN- γ and IL-12 cytokines, to be important players in eliminating *E. intestinalis* infection (8,9). The present results reinforce this hypothesis; as evident by a significant increase in CD8⁺ T cells in all infected groups. A subsequent decrease in CD8⁺ T cell population was associated with a decrease in spore numbers and histopathological lesions. At 7 and 14 dpi, the increased production of TNF- α , IFN- γ , and IL-10 cytokines was noticed in the ileum of infected mice, together with an increase in IL-2 and IL-4 in *Cy-infected* mice. The results suggest that besides IFN- γ (8,9), other cytokines, including anti-inflammatory cytokines, play a crucial role in mediating an intestinal immune response against *E. intestinalis*, for the resolution of enteritis and pathogen killing.

The IELs comprise a heterogeneous population, predominantly composed of CD8⁺ T (CD8 $\alpha\alpha$ and CD8 $\alpha\beta$) cells and a few CD4⁺ T lymphocytes and known to play an important role in oral infections. Upon infection, an expansion in the number of these cells causes increased production of IFN- γ , cytolytic properties of which inhibit intestinal *E. cuniculi* proliferation and dissemination (12). In fact, our results showed an increased CD8⁺ T IELs in intestinal *E. intestinalis* infection. However, CD8⁺ T cells decreased in the peritoneal cavity, suggesting their migration to the site of

256 pathogen proliferation. This finding is in corroboration to previous studies that have already shown
 257 cells in the peritoneal cavity to migrate to other sites (33,34).

258 Our group has previously shown B-1 cells to be an important player in the generation of the
 259 immune response against *E. cuniculi* infection (35,36). B-1 cells are important in generating
 260 adaptive immune response and antibody production, which is independent of T cells (37). However,
 261 B-1 cells are also dependent on T cells for generating an effective immune response, such as cell-
 262 mediated hypersensitivity (38,39) and rejection of allografts (40). Moreover, adoptive transfer of
 263 B-1 cells activates T cells that produce IFN- γ (41). Here, we showed a decrease in B-1 cell
 264 frequency in *infected* animals as compared to controls of both intestine and peritoneal cavity. We
 265 hypothesized that B-1 cells from the peritoneal cavity differentiated into B-1 cell-derived phagocyte
 266 (B-1 CDP) in infected mice, which, in turn, promoted phagocytosis of *E. intestinalis* spores. *In vitro*
 267 studies showed that B-1 cells may also differentiate into mononuclear phagocytes, which upon
 268 attachment to a substrate, acquire a myeloid phenotype (33). Moreover, *Propionibacterium acnes*
 269 infection of B-1 cells of myeloid lineage induced only differentiation into phagocytes (42);
 270 however, the role of B-1 cells in the intestines warrants further understanding of microsporidia
 271 infection.

272 We also found a decrease in the NKT cells in the intestine and mesenteric lymph nodes in all
 273 infected mice. NKT cells are known to play a protective role in *Toxoplasma gondii* oral infection
 274 although these cells are susceptible to direct invasion by the parasite (43). In fact, NKT cells
 275 develop a hypermotility phenotype *in vivo* during *T. gondii* oral infection, suggesting manipulation
 276 of motility of immune cells by *T. gondii*, which assist in the spread of the causative organism
 277 (44,45), as already shown in macrophages (45). *E. cuniculi* uses macrophages as a Trojan horse
 278 (46); however, whether this phenomenon occurs with NKT cells in microsporidiosis remains largely
 279 unknown.

280 While an effective immune response against microsporidia is predominantly driven by T cells,
 281 dendritic cells have also been shown to play an important and critical role in stimulating these cells.

282 Dendritic cells of the IM act as antigen-presenting cells and are responsible for priming T naive
 283 cells in effectors and memory cells upon infection (47). The secretion of cytokines and chemokines
 284 from infected enterocytes results in migration of these cells from PP to the mucosa (28). On the
 285 other hand, a study reported *in vitro* inhibition of dendritic cell differentiation by *E. intestinalis*
 286 (48). On the same lines, we, in the present study, demonstrate a reduction in dendritic cells in PP of
 287 infected animals at 7 and 14 dpi, which was associated with a higher number of fungal spores and
 288 histopathological lesions, suggesting the migration of these cells to the site of infection.

289 In the gastrointestinal tract, dendritic cells play an important role in suppression of colitis
 290 development, inducing the traffic of T regulatory cells in the intestine, and inducing IgA secretion
 291 from B cells in the intestine (28). These are also involved in inducing oral tolerance and preventing
 292 inflammatory response mediated by gut microbiota and ingested antigens (28). The breach in this
 293 function may decrease the number of T regulatory cells and increase the number of cells producing
 294 *Th1* and *Th17* cytokines (28). A lower frequency of dendritic cells observed in the present study is
 295 related to a higher parasite burden; however, this is yet to be clarified.

296 It has been previously shown that microsporidia spores infect new cells mostly by injection of
 297 sporoplasm into the host cells via the polar tubule (49). However, different cell lineages are capable
 298 of phagocytosing microsporidia spores (50,51), suggesting endocytosis to be an important
 299 mechanism in intestinal microsporidiosis. In order to evade this protective mechanism,
 300 microorganisms have developed complex systems to subvert endocytosis by host cells, allowing
 301 invasion of even those cells that generally do not phagocytose (52). Previous studies have suggested
 302 microsporidia to induce invaginations of the cell membrane of host cells close to the polar tubule in
 303 a process similar to endocytosis, causing injection of sporoplasm into the host cell. The results of
 304 TEM showed that a phenomenon similar to phagocytosis occur more frequently in *E. intestinalis*
 305 infection.

306 The results of this study showed that the increase in the CD8⁺ and CD4⁺ T cell population in the
 307 intestinal mucosa of Cy immunosuppressed mice and orally infected with *E. intestinalis*, in

association with the presence of pro-inflammatory cytokines, controlled the infection by the opportunistic fungus, although reduction of cellular populations at immune sites has been observed, confirming a state of immunosuppression, reinforcing that the selective effect of Cy should be better understood.

Methods

Animals

Specific pathogen-free, 6–8-week-old C57BL/6 mice were purchased from the Federal University of São Paulo (CEDEME, UNIFESP), Brazil. Animals were housed under sterile conditions at the Animal Facility of Paulista University, São Paulo, Brazil, and given food and water *ad libitum*. All animal procedures were performed in strict accordance with the Paulista University Ethics Committee (project license no. 313/15).

Encephalitozoon intestinalis cultivation and experimental infection

Spores of *E. intestinalis* were purchased from Waterborne Inc., New Orleans, LA, United States. These were cultivated in rabbit kidney cells (RK–13) in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), pyruvate, non-essential amino acids, and gentamicin followed by incubation at 37 °C and 5% CO₂. Spores were collected from the supernatant, washed thrice in phosphate-buffered saline (PBS), and counted using a Neubauer chamber.

Study design

Mice were divided into four experimental groups: *infected*, mice infected with *E. intestinalis*; *uninfected*, non-infected and non-treated mice; *Cy-infected*, mice treated with cyclophosphamide and infected with *E. intestinalis*; and *Cy-uninfected*, mice treated with cyclophosphamide. The Cy-treatment protocol was previously established (22) and consisted of intraperitoneal injection of 100

mg/kg twice a week (Genuxal; Asta Medica Oncologia, São Paulo, Brazil). The treatment started at the day of infection until 28 days post infection (dpi). Mice were orally infected by gavage with 5×10^7 *E. intestinalis* spores. Non-infected mice served as control.

Necropsy and tissue sampling

At 7, 14, 21, and 28 dpi, five animals from each group were euthanized with a mixture of ketamine (100 mg/mL), xylazine (20 mg/mL), and fentanyl (0.05 mg/mL). The samples of the intestine (duodenum, ileum), liver, kidneys, and lungs were collected and fixed in 10% buffered formalin for 72 h, routinely processed for histopathology, and stained with hematoxylin-eosin (HE) and Giemsa.

Ultrastructural analysis by transmission electronic microscopy

Ileum samples of 1 mm thickness from Cy-infected mice were fixed in 2% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.2) at 4 °C for 10 h. These were then fixed in buffered 1% OsO₄ for 3 h. Subsequently, the samples were embedded in EPON resin, sliced into semi-thin cuts, and stained with toluidine blue. Then, ultrathin sections were double stained using uranyl and lead citrate and observed under the TEM LEO EM 906 at 80 kV at the Butantan Institute.

Fungal burden

The paraffinized ileum was cut into 5-µm thick sections and evaluated histopathologically using HE staining to determine the fungal number. Fungal spores were counted randomly in at least 10 fields under the light microscope (40× objective magnification). The average number of spores from each mice was recorded and statistically analyzed.

Phenotypic analysis of immune intestinal mucosal cells

Cells of the IM were obtained as previously described,⁵³ with minor modifications. The small intestine was washed with 50 mL of Hanks' balanced salt solution (HBSS)–2% FBS solution,

longitudinally cut and separated into 2-cm segments. The mucosa was grated and submerged in HBSS–2% FBS solution supplemented with 0.1 M ethylenediaminetetraacetic acid (EDTA) at 37°C for 20 min. Then, samples were vortexed for 15 s and filtered using a cell strainer to remove the cell debris. Percoll gradient (70% and 40%) centrifugation was used to isolate the IM cells. After centrifugation, cells were washed with HBSS–2% FBS and resuspended in 100 μ L of PBS–1% bovine serum albumin (BSA). Mesenteric lymph nodes (MLN) and Peyer’s patches (PP) were isolated from the intestines using a scalped blade and washed in a cell strainer with HBSS–2% FBS. In addition, cells from the peritoneal cavity (PerC) were obtained by successive washes with at least 10 mL of HBSS–2% FBS. Finally, cell suspensions from PerC, MLN, and IM were washed with HBSS–2% FBS and resuspended in 100 μ L of PBS–1% BSA. After centrifugation at 500 $\times$ g for 5 min, each sample was incubated at 4 °C for 20 min with the anti-CD16/CD32 antibody. After incubation, cells were divided into two aliquots and resuspended in PBS–1% BSA, followed by incubation with monoclonal antibodies: APC-conjugated anti-mouse CD19, FITC, or PE-conjugated anti-mouse CD23, PerCP-conjugated anti-mouse CD4, FITC-conjugated anti-mouse CD8, APC-Cy7-conjugated anti-mouse CD11b, and PE-conjugated anti-mice CD11c (BD Pharmingen; San Diego, CA, United States). Finally, cell suspensions were run on the flow cytometer FACS Canto II (BD Biosciences; Mountain View, CA, United States). The cells were characterized according to their phenotypes in CD8 T (CD19[–] CD4[–] CD8⁺), CD4 T (CD19[–] CD8[–] CD4⁺), NKT cells (CD19[–] CD4⁺ NK1.1⁺), B-1 (CD23[–] CD19⁺), B-2 cells (CD23⁺ CD19⁺) and dendritic cells (CD11c⁺); and analyzed with the software FlowJo (FlowJo LLC; data analysis software, Ashland, OR, United States).

Cytokine quantification

For quantification of intestinal cytokines, 100 mg of ileum was sampled and treated with 1 mL of protease inhibitor (Sigma-Aldrich; St. Louis, MO, United States) at –80 °C. The sample was thawed and processed using a Precellys homogenizer for three cycles, 20 s each, filtering the

homogenate using a cell strainer to remove the debris. Cytokines (IL-2, IL-4, IL-6, IL-10, IL-17, IFN- γ , and TNF- α) in the homogenate were measured using the CBA Mouse Th1/Th2/Th17 cytokine kit (BD Biosciences; CA, United States) according to manufacturer's instructions. The kit consists of fluorescent beads coated with antibodies specific to cytokines. Briefly, 25 μ L of each sample was added to capture beads and PE-labeled secondary antibodies. The samples were incubated for 2 h at room temperature in the dark, following which two-color flow cytometry analysis was performed on the FACS Canto II flow cytometer (BD Biosciences; Mountain View, CA, United States) and analyzed using the FCAP Array 1.0 software (BD Biosciences; CA, United States).

Statistical analysis

Analysis of variance (ANOVA) tests and Tukey's or Bonferroni's multiple comparison post-tests were used for the statistical analysis of data. All values are expressed as mean $\pm$ standard error mean (SEM) with significance of $\alpha = 0.05$ ($p < 0.05$). All graphs were generated using the "GraphPad Prism" version 5.0 for Windows (GraphPad Software Inc; La Jolla, CA, United States).

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Competing interests

The authors declare no competing interests

References

1. Moretto M, Khan IA, Weiss LM. 2012. Gastrointestinal cell mediated immunity and the microsporidia. Plos Pathogen 8: e.100 2765.

- 412 2. Didier ES, Weiss LM. 2011. Microsporidiosis: not just in AIDS patients. *Curr Opin Infect Dis*
- 413 24:490–495.
- 414 3. Kicia M, Wesolowska M, Jakuszko K, Kopacz Z, Sak B, Květonova D. Krajewska M, Kváč
- 415 M. 2014. Concurrent infection of the urinary tract with *Encephalitozoon cuniculi* and
- 416 *Enterocytozoon bieneusi* in a renal transplant recipient. *J Clin Microbiol* 52:1780–1782.
- 417 4. Hinney B, Sak B, Joachim A, Kváč M. 2016. More than a rabbit's tale e *Encephalitozoon* spp. in
- 418 wild mammals and birds. *Int J Parasitol* 5:76–87.
- 419 5. Field AS, Milner DA. 2015. Intestinal microsporidiosis. *Clin Lab Med* 35:445–459.
- 420 6. Han B, Weiss LM. 2017. Microsporidia: Obligate Intracellular Pathogens within the Fungal
- 421 Kingdom. *Microbiol Spectr* 5. doi:10.1128/microbiolspec. FUNK-0018-2016.
- 422 7. Didier ES. 2005. Microsporidiosis: an emerging and opportunistic infection in human and
- 423 animals. *Acta Trop.* 94:61–76.
- 424 8. Salát J, Sak B, Le T, Kopecký J. 2004. Susceptibility of IFN- gamma or IL-12 knock-out and
- 425 SCID mice to infection with two microsporidian species, *Encephalitozoon cuniculi* and *E.*
- 426 *intestinalis*. *Folia Parasitol* 51:275–282.
- 427 9. Salát J, Horká H, Sak B, Kopecký J. 2006. Pure CD4⁺ T-lymphocytes fail to protect perorally
- 428 infected SCID mice from lethal microsporidiosis caused by *Encephalitozoon cuniculi*. *Parasitol Res*
- 429 99:682–686.
- 430 10. Allaire JM, Crowley SM, Law HT, Chang S, Ko H, Vallance BA. 2018. The intestinal
- 431 epithelium: central coordinator of mucosal immunity. *Trends Immunol.* 39:677-696.
- 432 <https://doi.org/10.1016/j.it.2018.04.002>.
- 433 11. Mangan NE, Fung, KY. 2012. Type I interferons in regulation of mucosal immunity. *Immunol*
- 434 *Cell Biol* 90:510–519.
- 435 12. Moretto M, Weiss LM, Khan IA. 2004. Induction of a rapid and strong antigen-specific
- 436 intraepithelial lymphocyte response during oral *Encephalitozoon cuniculi* infection. *J Immunol*
- 437 172:4402–4409.

- 438 13. World Health Organization. 2013. WHO Model list of Essential Medicine.
439 http://www.who.int/medicines/publications/essentialmedicines/18th_EML.pdf.
- 440 14. Brodsky RA. 2010. High-dose cyclophosphamide for autoimmunity and alloimmunity.
441 *Immunol Res* 47:79–84.
- 442 15. Dezern AE, Styler MJ, Drachman DB, Hummers LK, Jones RJ, Brodsky RA. 2013. Repeated
443 treatment with high dose cyclophosphamide for severe autoimmune diseases. *Am J Blood Res*
444 3:84–90.
- 445 16. Colvin OM. 1999. An overview of cyclophosphamide development and clinical applications.
446 *Curr Pharm Des* 5:555–560.
- 447 17. Emadi AL, Jones RJ, Brodsky RA. 2009. Cyclophosphamide and cancer: golden anniversary.
448 *Nat Rev Clin Oncol* 6:638–647.
- 449 18. Sistigu A, Viaud S, Chaput N, Bracci L, Proietti E, Zitvogel L. 2011. Immunomodulatory
450 effects of cyclophosphamide and implementations for vaccine design. *Semin Immunopathol*
451 33:369–383.
- 452 19. Sevko A, Sade-Feldman M, Kanterman J, Michels T, Falk CS, Umnsky L, Ramacher M, Kato
453 M, Schadendorf D, Baniyash M, Umansky V. 2013. Cyclophosphamide promotes chronic
454 inflammation-dependent immunosuppression and prevents antitumor response in melanoma. *J*
455 *Invest Dermatol* 133:1610–1619.
- 456 20. Schiavoni G, Sistigu A, Valentini M, Mattei F, XSestili P, Spadaro F, Sanchez M, Lorenzi S,
457 D’Urso MT, Belardelli F, Gabriele L, Proietti E, Braccio L. 2011. Cyclophosphamide synergizes
458 with type I interferons through systemic dendritic cell reactivation and induction of immunogenic
459 tumor apoptosis. *Cancer Res* 71:768–778.
- 460 21. Viaud S, Flament C, Zoubir M, Pautier P, LeCesne A, Ribrag V, Soria JC, Marty V, Vielh P,
461 Robert C, Chaput N, Zitvogel L. 2011. Cyclophosphamide induces differentiation of Th17 cells in
462 cancer patients. *Cancer Res.* 71:661–665.

- 463 22. Lallo MA, Hirschfeld MMP. 2012. Encephalitozoonosis in pharmacologically
464 immunosuppressed mice. *Exp Parasitol* 131:339–343.
- 465 23. Chabchoub N, Abdelmalek R, Mellouli F, Kanoun F, Thellier M, Bouratbine A, Aoun K. 2009.
466 Genetic identification of intestinal microsporidia species in immunocompromised patients in
467 Tunisia. *Am J Trop Med Hyg* 80:24–27.
- 468 24. Hamamci B, Çetinkaya Ü, Berk V, Kaynar L. 2015. Prevalence of *Encephalitozoon intestinalis*
469 and *Enterocytozoon bieneusi* in cancer patients under chemotherapy.
470 *Mikrobiyol Bul* 49:105–113.
- 471 25. El Fakhry Y, Achbarou A, Franetich JF, Desportes-Livage I, Mazier D. 2001. Dissemination of
472 *Encephalitozoon intestinalis*, a causative agent of human microsporidiosis, in IFN-gamma receptor
473 knockout mice. *Parasite Immunol* 23:19–25.
- 474 26. Gannon J. 1980. The course of infection of *Encephalitozoon cuniculi* in immunodeficient and
475 immunocompetent mice. *Lab Anim* 14:189–192.
- 476 27. Fuss IJ, Heller F, Boirivant M, Leon F, Yoshida M, Fichtner-Feigl S, Yang Z, Exley M, Kitani
477 A, Blumberg RS, Mannon P, Stöber W. 2004. Nonclassical CD1d-restricted NK T cells that
478 produce IL-13 characterize an atypical Th2 response in ulcerative colitis. *J Clin Invest* 114:1490–
479 1497.
- 480 28. Goodman WA, Pizarro TT. 2013. Regulatory cell populations in the intestinal mucosa. *Curr*
481 *Opin Gastroenterol* 29:614–620.
- 482 29. Cohen SB, Denkers EY. 2015. The gut mucosal immune response to *Toxoplasma gondii*.
483 *Parasite Immunol* 37:108–117.
- 484 30. Braunfuchsová P, Salát J, Kopecký J. 2001. CD8⁺ T-lymphocytes protect SCID mice against
485 *Encephalitozoon cuniculi* infection. *Int J Parasitol* 31:681–686.
- 486 31. Khan IA, Schwartzman JD, Kasper LH, Moretto M. 1999. CD8⁺CTLs are essential for
487 protective against *Encephalitozoon cuniculi* infection. *J Immunol* 162:6086–6091.

488 32. Moretto M, Casciotti L, Durell B, Khan IA. 2000. Lack of CD4⁺ T cells does not affect
489 induction of CD8⁺ T-cell immunity against *Encephalitozoon cuniculi* infection. Infect Immun
490 68:6223–6232.

491 33. Almeida SR, Aroeira LS, Frymuller E, Dias MA, Bogsan CS, Lopes JD, Mariano M. 2001.
492 Mouse B-1 cell-derived mononuclear phagocyte, a novel cellular component of acute non-specific
493 inflammatory exudate. Int Immunol 13:1193–201.

494 34. Palos MC, Azevedo MC, Thies FG, Osugui L, Alveres AM, Laurindo MF, Russo RT, Popi AF,
495 Mariano M. 2012. Different inflammatory stimuli in the footpad of mice influence the kinetics of
496 resident peritoneal cells. Inflamm Res 61:1187–1194.

497 35. Da Costa LF, Alvares-Saraiva AM, Dell'Armeline Rocha PR, Spadacci-Morena DD, Perez EC,
498 Mariano M, Lallo MA. 2017. B-1 cell decreases susceptibility to encephalitozoonosis in mice.
499 Immunobiol 222:218–227.

500 36. Langanke dos Santos D, Alvares-Saraiva AM, Xavier, JG, Spadacci-Morena DD, Peres GB,
501 Dell'Armeline Rocha PR, Perez EC, Lallo MA. 2018. B-1 cells upregulate CD8 T lymphocytes and
502 increase proinflammatory cytokines serum levels in oral encephalitozoonosis. Microbes Infect
503 20:196–204.

504 37. Choi YS., Baumgarth N. 2008. Dual role for B-1a cells in immunity to influenza virus infection.
505 J Exp Med 205:3053–3064.

506 38. De Lorenzo B, Brito RR, Godoy LC, Lopes JD, Mariano M. 2007. Tolerogenic property of B-
507 1b cells in a model of allergic reaction. Immunol Lett 114:110–118.

508 39. Szczepanik M, Akahira-Azuma M, Bryniarski K, Tsuji RF, Kawikova I, Ptak W, Kiener C,
509 Campos RA, Askenase PW. 2003. B-1 B cells mediate required early T cell recruitment to elicit
510 protein-induced delayed-type hypersensitivity. J Immunol 171:6625–6635.

511 40. Nogueira-Martins MF, Mariano M. 2010. B-1 cell participation in T-cell-mediated alloimmune
512 response. Immunobiol 215,:264–274.

513 41. Margry B, Wieland WH, van Kooten PJ, van Eden W, Broere F. 2013. Peritoneal cavity B-1a
514 cells promote peripheral CD4⁺ T-cell activation. *Eur J Immunol* 43:2317–2326.

515 42. Mussalem JS, Squaiella-Baptistão CC, Teixeira D, Yendo TM, Thies FG, Popi AF, Mariano M,
516 Longo-Mauregi I. 2012. Adjuvant effect of killed *Propionibacterium acnes* on mouse peritoneal B-
517 1 lymphocytes and their early phagocyte differentiation. *Plos One* 7: e33955.
518 doi:10.1371/journal.pone.0033955.

519 43. Persson CM, Lambert H, Vutova PP, Dellacasa-Lindberg I, Nederby J, Yagita H, Ljunggren
520 HG, Gradien A, Barragan A, Chambers BJ. 2009. Transmission of *Toxoplasma gondii* from
521 infected dendritic cells to natural killer cells. *Infect Immun* 77:970–976.

522 44. Harker KS, Ueno N, Wang T, Bonhomme C, Liu W, Lodoen MB. 2013. *Toxoplasma gondii*
523 modulates the dynamics of human monocyte adhesion to vascular endothelium under fluidic shear
524 stress. *J Leukoc Biol* 93:789–800.

525 45. Seipel D, Oliveira BC, Resende TL, Schuindt SH, Pimentel PM, Kanashiro MM, Amholdt AC.
526 2010. *Toxoplasma gondii* infection positively modulates the macrophages migratory molecular
527 complex by increasing matrix metalloproteinases, CD44 and alpha v beta 3 integrin. *Vet Parasitol*
528 169:312–319.

529 46. Mathews A, Hotard A, Hale-Donze H. 2009. Innate immune responses to *Encephalitozoon*
530 species infections. *Microbes Infect.* 11:905–911.

531 47. Steimnman RM, Hemmi H. 2006. Dendritic cells: translating innate to adaptive immunity. *Curr*
532 *Top Microbiol Immunol* 311:17–58.

533 48. Bernal JA, Andrés M, Palmero MF. 2016. Bruton’s tyrosine kinase inhibitors could induce
534 rheumatoid arthritis-like manifestations: A comment on the article by Nyhoff et al. *Arthritis*
535 *Rheumatol* 16:475.

536 49. Leitch GJ, Ward TL, Shaw AP, Newman G. 2005. Apical spore phagocytosis is not a significant
537 route of infection of differentiated enterocytes by *Encephalitozoon intestinalis*. *Infect Immun*
538 73:7697–7704.

50. Couzinet S, Cejas E, Schittny J, Deplazes P, Weber R, Zimmerli S. 2000. Phagocytic up take of *Encephalitozoon cuniculi* by non professional phagocytes. Infect Immun 68:6939–6945.
51. Franzen C, Muller A, Hartmann P, Salzberger B. 2005. Cell invasion and intracellular fate of *Encephalitozoon cuniculi* (Microsporidia). Parasitol 130:285–292.
52. Cossart P, Sansonetti PJ. 2004. Bacterial invasion: the paradigms of entero invasive pathogens. Science 304:242–248.
53. Guy-Grand D, Griscelli C, Vassali P. 1978. The mouse gut T lymphocyte, a novel type of T cell: nature, origin, and traffic in mice in normal and graft-versus-host conditions. J Exp Med 148:1661–1677.

Figure legends

- Figure 1. a) Photomicrograph of lymphoplasmacytic enteritis in the small intestine (arrowhead) in *Infected* mice. b) Ulceration (arrow) of intestinal mucosa of *Cy-Infected* mice. c) Clusters of *E. intestinalis* (arrow) at the glandular region in the mucosa of the ileum of *Cy-Infected* mice. d) Spores counting in the *Infected* and *Cy-Infected* mice. HE staining. ANOVA test with Tukey's posttest showed $p < 0.01^{**}$ and $p < 0.001^{***}$.
- Figure 2. Evaluation of T and B cell population in the intestinal mucosa of the mice infected with *E. intestinalis* and treated or not with Cy at 7, 14, 21 and 28 dpi. Percentage of lymphocytes CD8 T ($CD19^{-} CD4^{-} CD8^{+}$), CD4 T ($CD19^{-} CD8^{-} CD4^{+}$), NKT cells ($CD19^{-} CD4^{+} NK1.1^{+}$), B-1 ($CD23^{-} CD19^{+}$) and B-2 cells ($CD23^{+} CD19^{+}$). ANOVA test with Tukey's post-test showed $p < 0.05^{*}$, $p < 0.01^{**}$ and $p < 0.001^{***}$ compared to non-infected controls.
- Figure 3. Peyer's patch analyzed of the mice infected with *E. intestinalis* and treated or not with Cy for 7, 14, 21 and 28 dpi. a) Percentage of dendritic cells ($CD11c^{+}$) in Peyer's Patch. b) Photomicrograph of Peyer's Patch with lymphoid expansion in *Infected* group or rarified lymphoid

tissue in *Cy-Infected* group. HE staining. ANOVA test with Tukey's post-test showed $p < 0.05^*$, $< 0.01^{**}$ and $< 0.001^{***}$ compared to non-infected controls.

Figure 4. Cell population in the mesenteric lymph nodes of the mice infected with *E. intestinalis* and treated or not with Cy for 7, 14, 21 and 28 dpi. Percentage of B-2 cells ($CD23^+ CD19^+$), macrophages ($CD19^- CD11b^+$), CD4 T cells ($CD19^- CD8^- CD4^+$), CD8 T cells ($CD19^- CD4^- CD8^+$) and NKT cells ($CD19^- CD4^+ NK1.1^+$). ANOVA test with Tukey's post-test showed $p < 0.05^*$ and $p < 0.01^{**}$.

Figure 5. T and B population in the peritoneum of the mice infected with *E. intestinalis* and treated or not with Cy for 7, 14, 21 and 28 dpi. The number of CD8 T cells ($CD19^- CD4^- CD8^+$), CD4 T cells ($CD19^- CD8^- CD4^+$), NKT cells ($CD19^- CD4^+ NK1.1^+$), B-1 cells ($CD23^- CD19^+$), B-2 ($CD23^+ CD19^+$) cells, and macrophages ($CD19^- CD11b^+$). ANOVA test with Tukey's post-test showed $p < 0.05^*$, $p < 0.01^{**}$ and $p < 0.001^{***}$ compared to non-infected controls.

Figure 6. Proinflammatory cytokines (IL-2, IL-6, IFN- γ , and TNF- α) and anti-inflammatory cytokines (IL-4, IL-10, and IL-17A) levels in the intestine of mice infected with *E. intestinalis* and treated or not with Cy for 7, 14, 21, and 28 dpi. ANOVA test with Tukey's posttest.

Figure 7. TEM from the intestine of *Cy-Infected* mice. a, b, and c) *E. intestinalis* spores adhered to and enterocyte (*). Note that microvillousities disappeared, the membrane shows invaginations associated with an electron dense area in the cytoplasm close to the spore (*). d) Injection of the sporoplasm with loss of microvillousities (arrow). Projections of cytoplasmic membrane involving the extruded polar tubule (arrow).

d

Intestine

Peyer's patch

a

b

Infected

Cy-Infected

Mesenteric lymph nodes

Peritoneum