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Mice with genetic and induced B-cell deficiency as a model for disseminated encephalitozoonosis

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ABSTRACT

Encephalitozoon cuniculi, an intracellular pathogen, lives in a balanced relationship with immunocompetent individuals based on the activity of T lymphocytes. We previously highlighted the greater susceptibility of B-1 cell-deficient mice (XID mice) to encephalitozoonosis. This study aimed to develop a model of disseminated and severe encephalitozoonosis in mice with combined immunodeficiency to elucidate the role of B cells. To address this objective, cyclophosphamide (Cy)-treated BALB/c and XID mice were inoculated with *E. cuniculi*, followed by the evaluation of the immune response and histopathological lesions. Immunosuppressed BALB/c mice manifested no clinical signs with an increase in the populations of T lymphocytes and macrophages in the spleen. Immunosuppressed and infected XID mice revealed elevated T cells, macrophages populations, and pro-inflammatory cytokines levels (IFN-γ, TNF-α, and IL-6) with the presence of abdominal effusion and lesions in multiple organs. These clinical characteristics are associated with extensive and severe encephalitozoonosis. The symptoms and lesion size were reduced, whereas B-2 and $CD4^+$ T cells populations were increased in the spleen by transferring B-2 cells adoptive to XID mice. Moreover, B-1 cells adoptive transfer upregulated the peritoneal populations of B-2 cells and macrophages but not T lymphocytes and decreased the symptoms. Herein, we speculated the consistency in the development of severe and disseminated encephalitozoonosis in mice with genetic deficiency of Bruton's tyrosine kinase (Btk) associated with Cy immunosuppression develop with that of the models with T cell deficiency. Taken together, these data emphasized the crucial role of B cells in the protective immune response against encephalitozoonosis.

1. Introduction

Eukaryotic microorganisms, Microsporidia, are obligate intracellular pathogens belonging to the phylum Microsporidia and kingdom Fungi, capable of infecting both vertebrates and invertebrates [14]. Out of more than 1200 species of Microsporidia described so far, the mammalian and human diseases are mostly known to be caused by the pathogens belonging to the genus *Encephalitozoon*, especially *E cuniculi*, *E. intestinalis*, and *E. hellem* [14]. Ultrastructural observations confirmed the capacity of the Microsporidia in infecting different types of cells. An injection structure called the polar tubule is responsible for injecting the sporoplasm, its infectious content, into the cytoplasm of infected cells [19,38].

Cellular immunity imparts a significant role in the survival of *E. cuniculi*-infected host, and $CD8^+$ T lymphocytes are involved in the targeted death of infected cells; thereby, preventing the spread of pathogens [23,3,4]. Depending on the route of infection, an efficient immune response against *E. cuniculi* is provided by $CD4^+$ T lymphocytes, as previously reported in the case of the oral route of infection [8]. Antibodies produced by B cells against *E. cuniculi* fail to elicit protection against the course of infection [13,21]. Although B lymphocytes are the central cells

Abbreviations: Cy, Cyclophosphamide; TEM, transmission electronic microscopy; Btk, Bruton's tyrosine kinase; RK, Rabbit Kidney 13; XID, X-linked immunodeficiency.

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of the immune response, primarily responsible for antibody production, they can also modulate the immune response by the production of pro-inflammatory as well as anti-inflammatory cytokines. Therefore, the role of B cells in microsporidiosis necessitates further investigation.

B cells play a crucial role in both innate and adaptive immune responses. Based on the pathogen and route of infection, the follicular or innate subtypes of B cells differentiate specifically in a manner that may be dependent or independent of T cells. *In vitro* studies of the immune response to microsporidia claim the function of antibodies in opsonizing and blocking the infection to non-phagocytic cells [34], while increased spores' phagocytosis and reduced *E. cuniculi* growth have been attributed to the presence of peritoneal macrophages [32,9]. The adoptive transfer of antibodies to nude or SCID mice was not enough to avert the death of *E. cuniculi*-infected animals [9], although the antibodies enabled the prolongation of the survival of previously $CD4^+$ reconstituted, perorally infected, and intraperitoneally monoclonal antibody-treated SCID mice [32].

Normal B cell responsiveness relies on Bruton's tyrosine kinase (Btk), the target of inactivating mutations in X-linked immunodeficiency diseases of mice (XID) and humans (XLA) [20]. XLA, the most common immunodeficiency in man, accounts for approximately 85% of the early B-cell defects [10]. In response to B cell receptor (BCR)-antigens' interactions, Btk activation induces a complex cell signaling resulting in B-cell survival, proliferation, differentiation, and the production of antibodies [20]. Disseminated encephalitozoonosis was described in a 4-year-old boy suffering from X-linked agammaglobulinemia. He had pneumonia and neurological symptoms, and the pathogen spores were obtained in the spinal fluid, feces, and aspirated paranasal and bronchial sinuses [40].

B lymphocytes are classified into B-1 cells, which are mostly localized in the peritoneal and pleural cavities, and B-2 lymphocytes, abundant in the lymph nodes, spleen, and bone marrow [2,36,37]. Furthermore, these two cell subtypes differed in cell surface markers, range of antibodies, distinct morphologies, and functions [15,16]. Our group reported the increased susceptibility of XID mice to encephalitozoonosis as compared to the WT BALB/c mice. The adoptive transfer of B-1 cells to XID mice facilitated enhanced numbers of immune system cells and more resistance to infection by the oral and peritoneal routes [7,8].

We also have demonstrated Cy-mediated immunosuppression, aggravating the susceptibility of BALB/c mice to widespread and lethal encephalitozoonosis [22]. Cyclophosphamide is an alkylating agent, impeding both the humoral and cellular immune responses. It has widespread application as a cytotoxic drug in anti-cancer therapy and as an immunosuppressive drug in autoimmune diseases and organ transplants, including prophylaxis of graft-versus-host disease after myeloablative allogeneic bone marrow transplantation [18]. Many models developed to study microsporidiosis involve animals without T cells, especially $CD8^+$ T cells. However, the immunodeficiencies, predisposing to these opportunistic infections, vary significantly and occur through HIV infections, anti-rejection treatment to transplants, chemotherapy, use of immunosuppressive drugs in autoimmune diseases, primary immunodeficiency disorder, among other situations [5]. To clarify the role of B cells, the present study aimed to develop a model of disseminated and severe encephalitozoonosis in mice with combined immunodeficiency, genetically (Btk deficiency) and pharmacologically induced by Cy.

2. Material and methods

2.1. Animal protocol approval statement

This study was conducted in strict adherence with the recommendations in the Guide for the Care and Use of Laboratory Animals of Conselho Nacional de Controle de Experimentação Animal (CONCEA) and Comissão de Ética no Uso de Animais (CEUA) of Universidade Paulista (UNIP) approved the study protocol numbers 138/12 and 385/15.

2.2. Animals

Isogenic, female, 6–8 weeks old, *specific pathogen-free,* BALB/c and BALB/c XID mice were obtained from the "Centro de Desenvolvimento de Modelos Experimentais para Biologia e Medicina" (CEDEME) of the Universidade Federal of São Paulo (UNIFESP, in Portuguese). All the animals were segregated into groups and reared in sterilized isolators at the animal facility at Universidade Paulista-Unip, under controlled temperature and humidity with water and food ad libitum.

2.3. Adoptive transfer of B-2 cells

B-2 cells were adoptively transferred from the spleen of BALB/c mice to the XID mice. Tissue was dissociated, red blood cells lysed, and centrifuged. Thereafter, to block the Fc receptors, the resulting pellet was incubated with anti-CD16/32 for 15 min at 4 °C. The cells were subsequently washed with phosphate-buffered saline (PBS, Sigma, St Louis, MO, EUA) containing 1% Bovine Serum Albumin (BSA, Sigma, St Louis, MO, EUA) (PBS-BSA 1%). This was followed by labeling the B-2 cells, which was achieved by incubating the cells with Phycoerythrin (PE) Cyanin 7 (Cy7)-conjugated rat anti-mouse CD19 and PE-conjugated rat anti-mouse CD23 antibodies for 20 min at 4 °C. After labeling, the cells were sorted in a cytometer "*cell sorter*" FACSAria II at UNIFESP. The population of lymphocytes was selected based on *forward scatter* FSC versus *side scatter* SSC, and the B-2 cell populations were screened according to the $CD19^+CD23^+$phenotype. Finally, the centrifuged B-2 cells, resuspended in PBS (1×10^6 cells in 200 μL), were injected intraperitoneally in XID mice seven days prior to day zero of infection, constituting the XID+B-2 mice group.

2.4. Adoptive transfer of B-1 cells

To adoptively transfer B-1 cells to XID mice [1], peritoneal cells collected from BALB/c mice were sampled by successive washes with 2 mL of RPMI-1640 (Sigma, St. Louis, MO. USA) and cultured for 40 min at 37 °C with 5% CO_2. Discarding the supernatant, the adherent cells were washed and re-incubated with RPMI supplemented with 10% bovine fetal serum (BFS) (R10) under the same conditions for a further five days. Thereafter, the supernatant was collected, centrifuged, and resuspended in PBS. The centrifuged enriched population of B-1 cells was resuspended in PBS and finally injected intraperitoneally (1×10^6 cells in 200 μL) in XID mice seven days prior to day zero of infection, constituting the XID+B-1 mice group.

2.5. Pathogens and experimental infection

Rabbit Kidney (RK)−13 cells were employed to grow the spores of *Encephalitozoon cuniculi* of genotype I (bought from Waterborne® Inc. New Orleans, LA, USA) in the Cell Culture Laboratory at Universidade Paulista-Unip. The inoculated RK-13 cells were maintained in Eagle's medium supplemented with 10% BFS (Gibco, Grand Island, NY, EUA), 10% of non-essential amino acids, 10% pyruvate, and gentamicin (20 μg/mL), and the cultures were incubated in 5% CO_2 at 37 °C. Every seven days, the supernatant was sampled and centrifuged for 20 min at 500g to obtain spores. The spores of *E. cuniculi* were counted using a Neubauer chamber.

2.6. Cyclophosphamide (Cy) treatment

Cy (100 mg/kg, twice weekly) was administered to all the mice belonging to the infected and uninfected groups [22]. Treatment started on the day of infection until 14 or 21 days post-infection (dpi), respectively.

2.7. Experimental infection

On the day of the experimental infection, Cy-treated immunosuppressed animals were divided into 8 groups: infected BALB/c (n = 10), infected XID (n = 10), infected XID+B-1 (n = 10), infected XID+B-2 (n = 10), uninfected BALB/c (n = 6), uninfected XID (n = 6), uninfected XID+B-1 (n = 6), uninfected XID+B-2 (n = 6). Intraperitoneal injection of 1×10^7 *E. cuniculi* spores was administered to all the infected mice. Half of the animals were euthanized at 14 dpi, while the other half after 21 dpi.

2.8. Necropsy and tissue sampling

A mixture of ketamine (100 mg/mL), xylazine (20 mg/mL), and fentanyl (0.05 mg/mL) was used to euthanize the animals at 14 or 21 dpi. Firstly, a heart puncture enabled the collection of blood for serum cytokine dosage. Secondly, followed by a peritoneal wash, the spleen was processed for flow cytometry (see below). Finally, the tissue samples (liver, spleen, lungs, intestine) were processed for histopathology and TEM analyses.

2.9. Histological and morphometric analyses

After fixing the tissue samples of liver, lungs, small intestine, and spleen in 10% buffered formalin solution (pH 7.2–7.4), the samples were processed and embedded in paraffin to obtain sections of 4 μm thickness for hematoxylin and eosin staining. *MetaMorph Software* (Molecular Devices, California, EUA) was adopted to determine the inflammatory infiltrates in the liver. Taking ten random photomicrographs, the infiltrated area was manually delimited with the cursor and measured in pixels. The means of each group were considered for statistical analyses.

2.10. Fungal burden

Quantifying the number of free *E. cuniculi* spores in the peritoneal wash from each infected animal, the average number of spores from each animal was recorded as spores per milliliter of the sample and analyzed statistically.

2.11. Ultrastructural analysis by transmission electronic microscopy (TEM)

Glutaraldehyde (2%) in 0.2 M cacodylate buffer (pH 7.2) was used to fix the liver and spleen samples of 1 mm thickness from the infected XID mice in 2% at 4 °C for 10 h, followed by fixation in buffered 1% OsO_4 for 2 h. The samples were then routinely processed and embedded in Epon's resin. Semithin cuts were stained with Toluidine blue, and ultrathin cuts were double stained using aqueous uranyl and lead citrate. Finally, the samples were visualized under a TEM LEO EM 906 at 80 kV at the Butantan Institute.

2.12. Phenotyping of immune cells from the peritoneal cavity and spleen

Peritoneal cells were obtained as described. After mechanical dissociation of the spleen, it was filtered using a 70 μm cell strainer, and a hemolytic buffer was used to remove the red blood cells. Following centrifugation and washing with PBS, the cells were resuspended in 100 μL of PBS–1% BSA. Incubation of each sample with anti-CD16/CD32 antibody was conducted at 4 °C for 20 min. Thereafter, dividing the cells into two aliquots, they were resuspended in PBS-1% BSA and subsequently incubated with the monoclonal antibodies Allophycocyanin (APC)-conjugated rat anti-mouse CD19, Fluorescein Isothiocyanate (FITC) or PE-conjugated rat anti-mouse CD23, Peridinin Chlorophyll Protein Complex (PerCP)-conjugated rat anti-mouse CD4, FITC-conjugated rat anti-mouse CD8, PE-conjugated rat anti-mouse F4/80 and Pacific Blue or APC-Cy 7-conjugated rat anti-mouse CD11b (BD-Pharmingen, San Diego, CA). Finally, the cell suspensions were evaluated in the flow cytometer FACS Canto II (BD Biosciences, Mountain View, CA, USA). The cells were characterized according to their phenotypes: B-1 cells ($CD23^-$ $CD19^+$), B-2 cells ($CD23^+$ $CD19^+$), $CD4^+$ T cells ($CD19^-$ $CD4^+$), $CD8^+$ T cells ($CD19^-$ $CD8^+$), macrophages ($CD19^-$ $CD11b^+$ $F4/80^+$). The FlowJo software (FlowJo LLC, Data Analysis Software, Ashland, OR) was exploited for analysis.

2.13. Quantification of cytokines

Serum was extracted from the blood and stored at −80 °C until use.

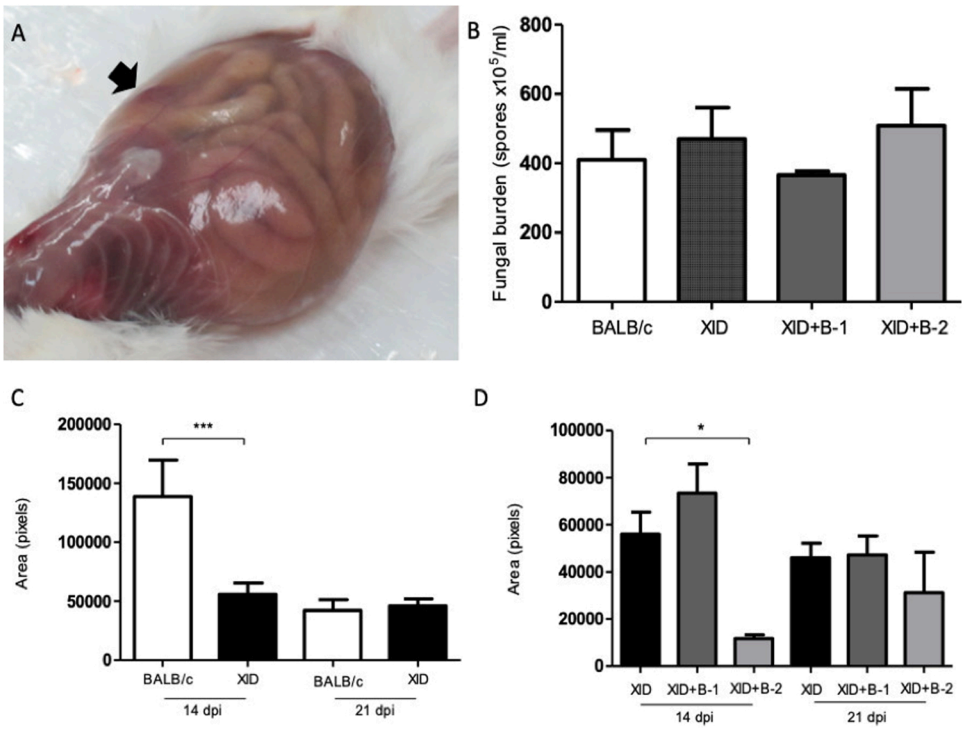

Fig. 1. Fungal burden. A) The serous-bloody effusion in the abdominal cavity of the XID mice infected with *E. cuniculi*. B) Evaluation of the spores from peritoneal washes in BALB/c, XID, XID+B-1 (adoptively transferred with B-1 cells), and XID+B-2 (adoptively transferred with B-2 cells) mice infected with *E. cuniculi*. One-way ANOVA followed by Bonferroni multiple comparison post-test failed to elucidate any significance. C) Comparison of the means of the lesion areas in the liver of BALB/c, XID, XID+B-1, and XID+B-2 mice, in pixels, estimated by morphometric analysis. One-way ANOVA with the Bonferroni multiple comparison post-test revealed *$p < 0.5$; * ** $p < 0.001$.

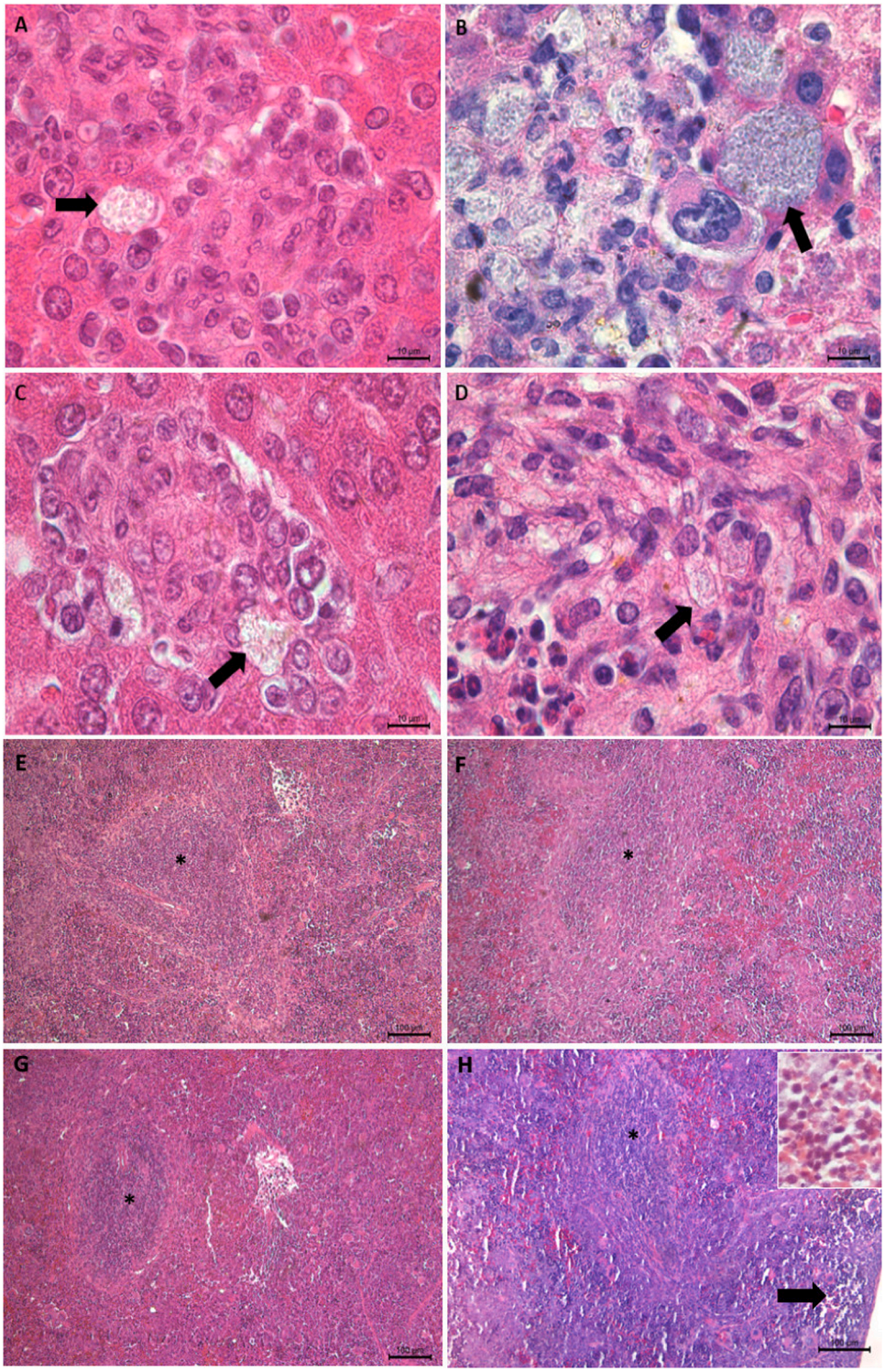

Fig. 2. Photomicrography of hepatic and spleen lesions of *E. cuniculi*-infected mice. Spores (arrow) associated with inflammatory infiltrates in liver tissue from BALB/c (A), XID (B), XID+B-1 (C), and XID+B-2 (D) mice. Rarefaction in the lymphoid white pulp of spleen (*) in BALB/c (E), XID (F), and XID + B-1 (G) mice, and lymphocyte infiltration (arrow) in red pulp region in XID+B-2 mice (see detail in insert) (H). HE stains.

The cytokine dosage was determined with thawed serum samples by flow cytometry using CBA Mouse Th1/Th2/Th17 Cytokine Kit (BD Biosciences, CA, EUA), according to the manufacturer's instructions. A mixture of 25 μL of each sample, 25 μL of mixed beads containing specific capture antibodies, and 25 μL of PE-conjugated detection antibody was incubated for 2 h at room temperature in the dark. Subsequently, the samples were centrifuged, washed, and resuspended for analysis by two-color flow cytometry using FACS Canto II (BD Biosciences, Mountain View, CA, USA). The software FCAP Array 3.0 was adopted for analysis.

2.14. Statistical analysis

One-way or two-way analysis of variance (ANOVA) followed by Tukey's or Bonferroni multiple comparison post-test was employed for comparisons. All the values were expressed as mean ± standard error mean (SEM) with the significance of $\alpha = 0.05$ ($p < 0.05$). All the

statistical figures were constructed using "GraphPad Prism" version 5.0 for Windows® (GraphPad Software Inc, La Jolla, CA, EUA).

3. Results

3.1. Immunosuppressed XID mice develop severe disseminated E. cuniculi infection

The immunosuppressed XID mice infected with *E. cuniculi* at 21 dpi expressed clinical-pathological symptoms including difficulty in locomotion, ruffled coat, a large volume of abdominal serous-bloody effusion, hepatomegaly, splenomegaly, and extended intestinal segments; however, none of the mice died (Fig. 1A). The immunosuppressed BALB/c mice failed to portray any symptoms. Consistency was recorded in the spore burden using peritoneal washes among all the mice groups (Fig. 1B). Microscopically, granulomatous lesions with multifocal mononuclear inflammatory infiltrate were observed in the liver, lungs, spleen, and small intestine. Granulocytes were infrequent. At 14 dpi, the BALB/c mice exhibited a greater area of lesions relative to the XID mice (Fig. 1C). Although the lesion area was smaller in XID mice, degenerative lesions in the liver substantiated the presence of vacuoles, pyknotic nuclei, and spores. Inflammatory infiltrates were most abundant at the periportal space as well as in the liver tissue adjacent to the subcapsular space associated with spores. *E. cuniculi* spores were noted in the liver associated with the cellular infiltrate (Fig. 2A–D). The spleen of infected BALB/c and XID demonstrated the rarefaction of the white pulp (Fig. 2E–F) mice. Multifocal interstitial pneumonia, associated with mononuclear, predominantly lymphoplasmacytic infiltrate and alveolar collapse, was documented in the infected mice (Supplementary Fig. 1A–D). Furthermore, all the infected groups revealed lymphoplasmacytic mild enteritis, and spores were rare (Supplementary Fig. 1E–H).

Adoptive transfer of B-1 or B-2 cells to the XID mice manifested fewer clinical signs with discrete peritoneal effusion. A significant decrease and increase in the lesion area were observed following the adoptive transfer of B-2 cells (XID+B-2) and B-1 cells (XID+B-1), respectively (Fig. 1C). While XID+B-1 mice exhibited rarefaction in the white pulp

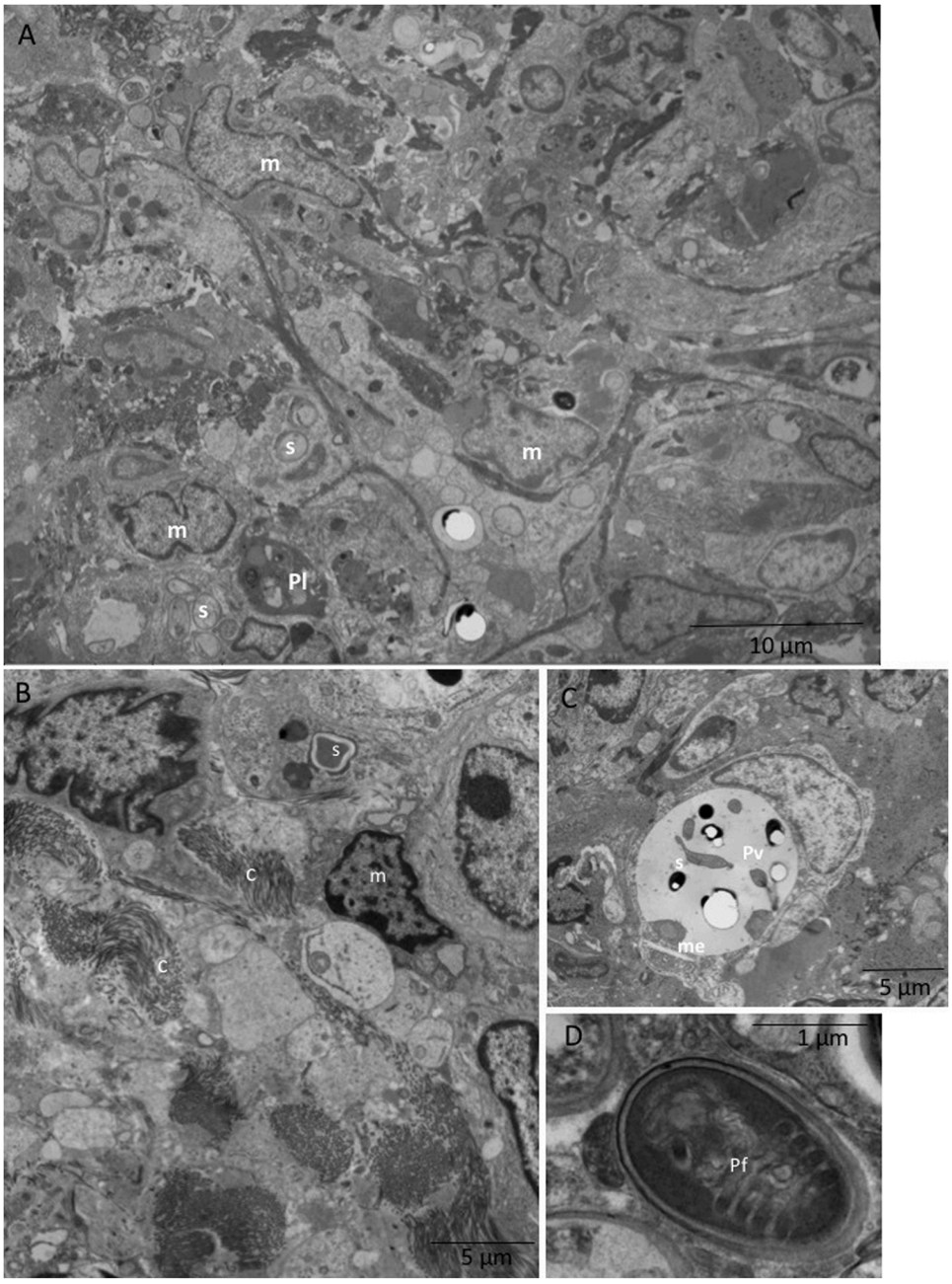

Fig. 3. Ultramicrography of the liver from XID mice treated with cyclophosphamide and infected with *E. cuniculi*. (A) The inflammatory infiltrates with predominant macrophages (m) with phagolysosome vacuoles (Pl) containing the remains of spores or intact spores (e). (B) The inflammatory infiltrates deposited with collagen (c), spores (e), and macrophages (m). (C) Parasitophorous vacuoles (Pv) with proliferative forms such as meronts and (me) e spore (s). (D) detail of a free spore in the tissue parenchyma, note the polar filament (Pf).

(Fig. 2F–G), considerable lymphocytic infiltrate was evident in the spleen of XID+B-2 mice (Fig. 2H). These indicated less susceptibility compared to the immunosuppressed XID mice.

3.2. TEM revealed parasitophorous vacuoles in the liver and spleen from XID mice

TEM imaging of the liver of XID mice emphasized the predominance of macrophages in the mononuclear infiltrates with fungal spores in the lysis and parasitophorous vacuoles (Fig. 3A–B), wherein different stages of parasitic development, such as meronts, sporoblasts, and mature spores, were spotted (Fig. 3C). Results also highlighted intact spores in the cytoplasm of hepatocytes and intercellular spaces, with evidence of the polar tubule (Fig. 3D). These findings substantiated the development of the pathogen in the tissue regardless of the presence of immune cells. Collagen was also detected in the vicinity of the lesion areas (Fig. 3B). The splenic cells from XID mice confirmed the existence of the different developmental stages of *E. cuniculi* spores, such as meronts in the binary division, sporoblasts, and sporonts (Supplementary Fig. 2), reinforcing the growth of the pathogen, even in lymphoid tissue.

3.3. Increase of immune cells in the peritoneal cavity and spleen of infected XID mice

Phenotypic analysis of immune cells from the peritoneal cavity reflected escalation in the numbers of $CD8^+$ and $CD4^+$ T lymphocytes and macrophages in infected XID mice compared to the uninfected control (Fig. 4A). Increased macrophage population in the peritoneum at 14 dpi was evident in the infected BALB/c mice relative to the uninfected control. However, this population decreased at 21 dpi (Fig. 4A). Infected BALB/c mice also manifested decreased population of B-2 cells. Overall, compared to the uninfected control, the infected XID mice confirmed increased populations of the peritoneal $CD8^+$ and $CD4^+$ T lymphocytes, B-2 lymphocytes, and macrophages, at both 14 and 21 dpi (Fig. 4A).

An increase in the $CD8^+$ and $CD4^+$ T lymphocytes and macrophages in the spleen of infected BALB/c mice was recorded in comparison to the uninfected control (Fig. 4B). Nonetheless, the XID mice revealed no difference in the populations of immune cells compared to the uninfected control, except B-2 cells, which were found to be decreased in XID mice at 14 dpi (Fig. 4B). These results indicated important differences in the assembly of the immune response in the spleen of XID and BALB/c mice, which could contribute to the growth and spread of *E. cuniculi* in immunosuppressed XID mice.

In animals transferred with B cells, reduced $CD8^+$ and $CD4^+$ T lymphocytes were noted in the peritoneal cavity from the XID+B-2 mice group compared to other XID groups (XID and XID+B-1) (Fig. 5A). Contradictorily, the adoptive transfer of B-1 cells to the XID mice (XID+B-1 group) resulted in a significant increase in B-2 cells and macrophages than the other XID mice (Fig. 5A). Meanwhile, findings of this study also confirmed a decline in the numbers of macrophages and an increase in those of $CD4^+$ T and B-2 lymphocytes in the spleen of XID+B-2 mice, compared to the XID and XID+B-1 groups (Fig. 5B).

3.4. E. cuniculi infection-mediated increase in the pro-inflammatory cytokine levels in XID mice

The quantification of cytokines from the serum indicated that, compared to the control and BALB/c groups, the infection with *E. cuniculi* elevated the levels of interferon-gamma (IFN-γ), tumor necrosis factor (TNF-α), and interleukin–6 (IL-6) in the XID mice (Fig. 6). Moreover, these cytokine levels were higher in XID mice than in those receiving the adoptive transfer of B-1 and B-2 cells (Fig. 6).No statistically significant difference was identified in the IL-4 cytokine dosage among all groups (data not shown). The cytokines IL-2, IL-10, and IL-17 were not detected.

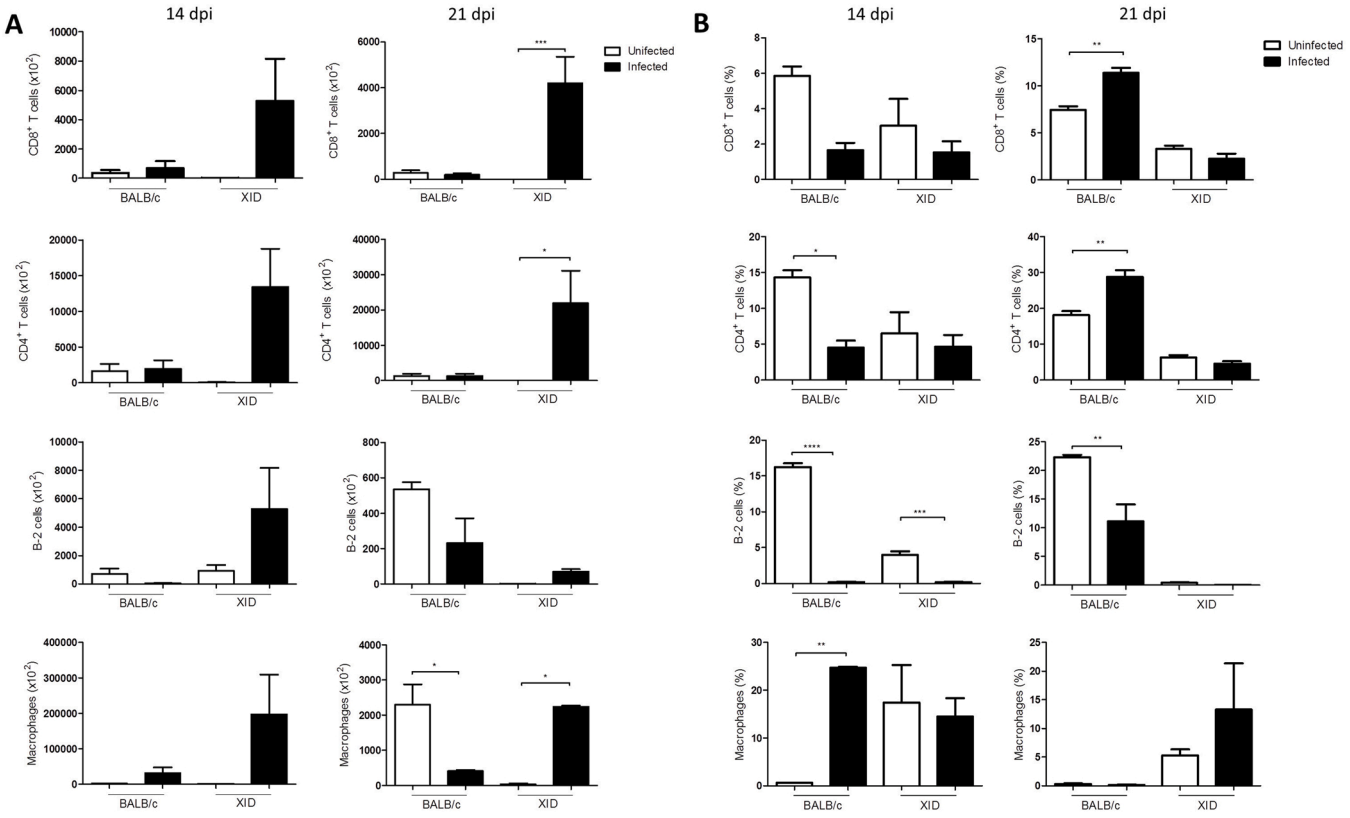

Fig. 4. Evaluation of the peritoneal and splenic populations of $CD8^+$ and $CD4^+$ T lymphocytes, B-2 cells, and macrophages derived from cyclophosphamide-induced immunosuppressed mice infected or not with *E. cuniculi* at 14-or 21-days post-infection (dpi). (A) Peritoneal populations, (B) Splenic populations from BALB/c and XID mice. One-way ANOVA with the Bonferroni multiple comparison post-test highlighted *p < 0.5, * *p < 0.01, * **p < 0.001.

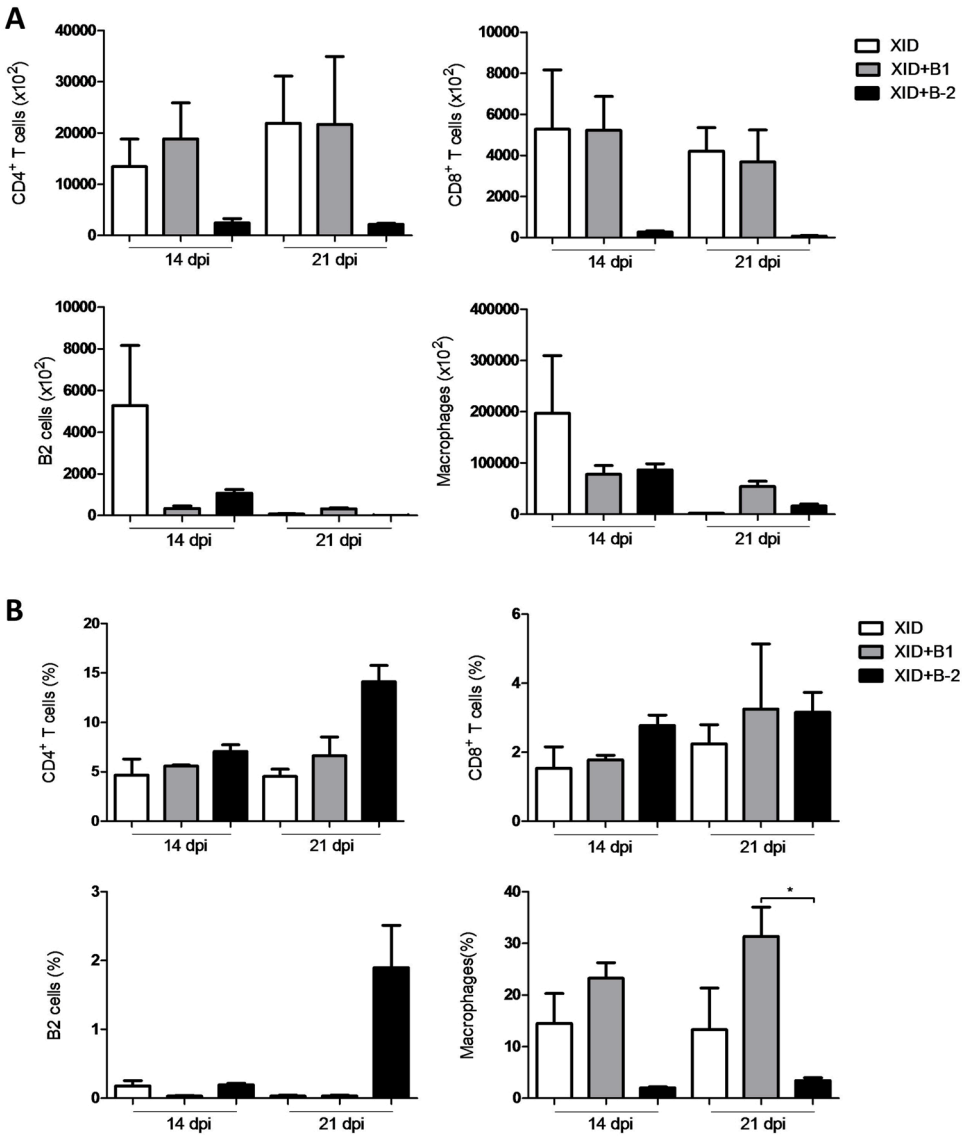

Fig. 5. Comparison of the peritoneal and splenic populations of $CD8^+$ and $CD4^+$ T lymphocytes, B-2 cells, and macrophages obtained from XID, XID+B-1, and XID+B-2 mice immunosuppressed with cyclophosphamide and infected *E. cuniculi* at 14-or 21-days post-infection (dpi). (A) Peritoneal populations (B) Splenic populations from XID, XID+B-1, and XID+B-2 mice. One-way ANOVA followed by the Bonferroni multiple comparison post-test revealed *$p < 0.5$.

4. Discussion

An adequate function of the innate and adaptive immune response dictates the efficiency of the defense system of the host against pathogenic fungi. $CD8^{-/-}$ mice inoculated with the pathogen manifested severe and widespread disease, characterized by lethargy, ascites, hepatitis, pneumonia, and spleen lesions, which lead to the death of the animals. This confirmed the relevance of the $CD8^+$ T cells for the survival of *E. cuniculi*-infected host [21,39].

Our previous results highlighted the less resistance of immunosuppressed XID mice to experimental infection by *E. cuniculi* [7,8], demonstrating more tissue damage and less immune response than BALB/c mice. Herein, we highlighted severe and widespread encephalitozoonosis in X-linked immunodeficiency (XID) mice treated with cyclophosphamide, which was consistent with the models with T cell deficiency, emphasizing the involvement of B cells in the immune response against *E. cuniculi*.

Although XID mice are frequently employed to ascertain the role of B-1 cells, the mutation in Bruton's tyrosine kinase also affects the development of the B-2 cell population; therefore, such mice also have B-2 cell deficiency. Reduced immune populations in the peritoneal cavity and downregulated serum Th1, Th2, and Th17-related cytokines were found to be attributed to the transfer of B-2 cells. On the contrary, the XID+B-1 mice revealed upregulated peritoneal cells and serum cytokines. In the spleen, B-2 populations enhanced in the XID+B-2 and reduced in the XID+B-1, while the macrophage populations decreased in the XID+B-2 and increased in the XID+B-1. Though antibody production is the major function of B-2 lymphocytes in the immune response, they are also engaged in modulating the immune response by the secretion of pro-inflammatory as well as anti-inflammatory cytokines. As reported by other studies [32,34,9], the massive production of antibodies is not sufficient to combat the infection, but its participation as an active element of immunity against encephalitozoonosis was reinforced in this article using XID mice model.

Cy-immunosuppressed XID mice exhibited symptoms as severe as $CD8^{-/-}$ mice; however, increased peritoneal $CD8^+$ and $CD4^+$ T cells witnessed in these animals, was not evident in the case of BALB/c mice. The results claimed a deficient response of T cells in the XID mice. Previous studies established the ability of B-1 cells to regulate other cell functions. B-1 cells may alter the composition of the granuloma in the lungs caused by BCG in mice [31]. B-1 cells also escalate the migration of $CD8^+$ T cells in grafts [28]. Moreover, by activating T cells, the

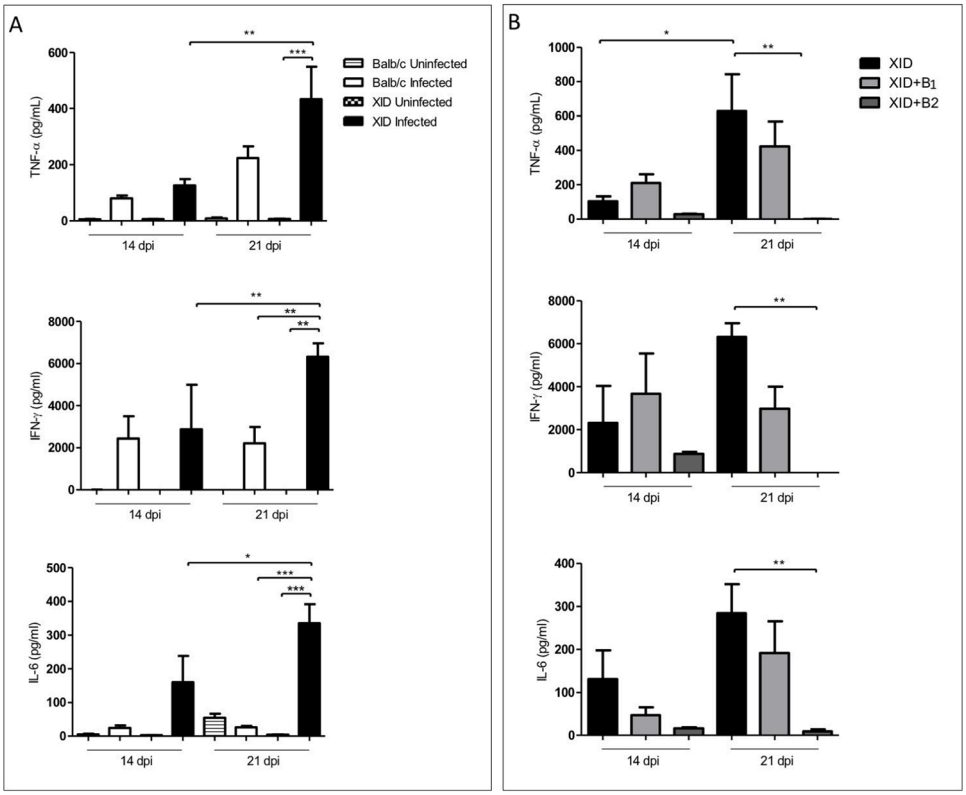

Fig. 6. Exploration of TNF-α, IFN-γ, and IL-6 cytokines in the serum samples of BALB/c and XID mice (A) and of XID, XID+B-1, and XID+B-2 (B) immunosuppressed with cyclophosphamide and infected or not with *E. cuniculi* at 14-and 21-days post-infection (dpi). One-way ANOVA with the Bonferroni multiple comparison post-test, *p<0.5; **p<0.5; ***p<0.001.

peritoneal B-1 cells may also influence the immune response without needing to migrate to the targeted lymphoid organ [26]. Thus, we suggest that B-1 cells deficiency could affect the cytotoxic capacity of these lymphocytes in XID mice. Further experimentation is being conducted in our lab to better explain this hypothesis.

The activity of peritoneal lymphocytes remains to be fully elucidated. Despite having a BALB/c background, the XID mice may present a Th1 response like C57BL/6. In these mice, IFN-γ-induced activated peritoneal macrophages may promote the suppression of peritoneal T cells by arginine utilization [6]. In contrast, activation of the peritoneal T cells in co-cultures with macrophages was observed in BALB/c mice, which present a Th2 profile and low production of IFN-γ [6]. This phenomenon could justify the response noted in XID mice in the present experiment. Nonetheless, the presence of the pathogen may also stimulate the proliferation of $CD8^+$ T lymphocytes, rationalizing their increase in number. The presence of macrophages and high levels of IFN-γ may also impede the function of these cells, whereas in BALB/c mice, the lower peritoneal population of $CD8^+$ T cells associated with milder infection could suggest effective activation of these cells. Furthermore, the increase in splenic $CD4^+$ and $CD8^+$ T cells of BALB/c mice presumes the origin and activation of these cells in the spleen. A similar profile identified in XID+B-1 mice further emphasized the role of B-1 cells in the mounting of an immune response.

An increased population of macrophages was reported in the peritoneum of infected XID mice. Our in vitro findings confirmed the predominance of the M2 profile, with high phagocytic and low microbicidal activities, in the peritoneal macrophages from XID mice [29]. Acting as a Trojan horse, macrophages with an M2 profile may favor the dissemination of the pathogen, which might explain the severity of the disease in XID mice. Post-infection, the areas of mononuclear inflammatory infiltrate into the liver were significantly larger in BALB/c mice than those in XID, proposing partial restriction of the spread of the pathogen mediated by this inflammatory response.

Microsporidia are among the most pathogenic agents adapted for intracellular parasitism. Following *E. cuniculi* infection, usually by the oral route, many mammals suffer from progressive disease after weeks or months. This infection is characterized by the prevalence of high levels of antibodies coupled with a continuous inflammatory infiltration [35]. The susceptibility to *E. cuniculi* infection is more prominent among certain mice strains, such as C57BL/6 [27] and as observed in this and other studies [7,8], in XID mice, differently of wild-type BALB/c mice. It was demonstrated that a genetic alteration in the C57BL/6 strain compromises the function of B lymphocytes, thereby justifying the low efficiency of the antibodies produced by these animals [25]. Despite having high levels of antibodies detected after 14 DPI with *E. cuniculi*, the C57BL/6 mice exhibited significant lesions in the liver and brain that were associated with the presence of spores of the pathogen [12]. It is also evident that cytokines production was deferred in this mouse strain as compared to that in BALB/c mice, with a distinct delay in the mobilization of the immune response [24].

In line with the clinical signs of more severe disease, higher levels of pro-inflammatory cytokines or Th1 profile were observed in the serum of immunosuppressed XID when compared to that of the immunosuppressed BALB/c mice. This finding corroborates the hypothesis that the XID mice have a profile like the C57BL/6 mice. Similar to the observations previously obtained from *E. cuniculi*-infected but not Cy-immunosuppressed mice [24,33], IFN-γ, TNF-α, and IL-6 cytokines were identified. *In vivo* and in vitro models substantiated the significance of Th1 cytokines against microsporidian *E. cuniculi*-infection but not in the Cy-immunosuppressed mice model. The level of IFN-γ may also increase and play a crucial role in immunosuppressed mice [17,24]. Enhanced IFN-γ in diabetic mice immunosuppressed with Cy, but not in diabetic non-immunosuppressed mice, implied the immunomodulatory effect of Cy in the production of this cytokine during encephalitozoonosis [11]. The results presented herein reveal that *E. cuniculi* infection, together with Cy-immunosuppression, contributed to the increased

IFN-γ levels, both in XID and BALB/c mice. Cy is implicated with increased serum IFN-γ and other pro-inflammatory cytokines [30].

5. Conclusions

Thus to summarize, the described murine model demonstrates an increasing susceptibility to *E. cuniculi* infection and severity of the fungal infection associated with immunosuppression resulting from the genetic deficiency in Btk and Cy, particularly with B cell involvement. Meanwhile, the transfer of B-1 and B-2 cells confirmed their relevance in developing immunity against encephalitozoonosis. B-1 cells are involved in modulating the immune system, although the role of B-2 cells remains ambiguous. We hypothesize the vital role of the antibodies produced by these cells in the resolution of the infection, which needs to be explored in further detail.

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Declaration of Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The authors alone are responsible for the content and the writing of the paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.cimid.2021.101742.

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