**UNIVERSIDADE PAULISTA – UNIP**

**PROGRAMA DE DOUTORADO EM PATOLOGIA AMBIENTAL E EXPERIMENTAL**

# B-1 CELL ROLE ON ACTIVITY OF PHAGOCYTIC CELLS IN *Encephalitozoon cuniculi* INFECTION

Tese apresentada ao Programa de Pós-Graduação em Patologia Ambiental e Experimental da Universidade Paulista – UNIP, para obtenção do título de Doutor em Patologia Ambiental e Experimental.

**ADRIANO PEREIRA**

**SÃO PAULO**

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**ADRIANO PEREIRA**

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**PAPER**

**CELL HOST & MICROBE**

# B-1 CELL ROLE ON ACTIVITY OF PHAGOCYTIC CELLS IN *Encephalitozoon cuniculi* INFECTION

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**Abstract**

Microsporidia are obligate intracellular fungal pathogens that infect a wide range of hosts in the animal kingdom. *Encephalitozoon cuniculi* was the first mammalian microsporidian successfully grown *in vitro* and infects macrophages, epithelial and endothelial cells and fibroblasts in a variety of mammals, including rodents, monkeys, carnivores, rabbits and humans. There are limited data on the host responses to these opportunistic pathogens and one study *in vivo* from our group describing B-1 cell involving in the decrease of susceptibility of mice to the *E. cuniculi* infection. To understand the role of B-1 cells in this issue we performed macrophages response to *E. cuniculi* in the presence or absence of B-1 cells. Adherent peritoneal cells from BALB/c (BALB/c APerC) and B-1 cell-deficient XID (XID APerC) mice and B-1 Cell-Derived Phagocyte (B-1 CDP) were simultaneously infected with *E. cuniculi*. The examination by light and transmission electronic microscopy demonstrated differences between BALB/c and XID APerC. Samples of BALB/C APerC after 1h and 48h revealed a large amount of lymphocytes in addition to macrophages and spores of *E. cuniculi* in great quantity outside the cells. In XID APerC samples the predominance were macrophages with a higher number of phagocytic cells with spores inside of cells. Also, an increased in IL-6, IL-10 and MCP-1 levels were observed in XID APerC. 96h and 144h in BALB/c APerC revealed a low cellularity with a great quantity of spores outside de cells indicating degeneration and death of phagocytes. Increased levels of MCP-1, TNF and IL-6 were detected in infected group in these cultures. Contrary, in XID APerC we observed a higher number of intact phagocytic cells with spores in degeneration and an increased TNF and NO production in infected group compared to control group. The findings suggest B-1 cells interfere with macrophage activity, increasing the microbicidal activity of macrophages and modulating cytokines secretion.

Key words: B-1 cell, *Encephalitozoon cuniculi*, innate immunity, macrophages

## INTRODUCTION

Microsporidia are an obligatory intracellular spore-forming microorganisms that infect a wide range of vertebrate and invertebrate species. These fungi have been recognized as human pathogen particularly in immunodeficient patients after the advent of HIV and AIDS interest in the *in vitro* culture researchers of them has been increased to study their biology and immune response against them (Ghosh and Weiss, 2012).

*Encephalitozoon cuniculi* is one of the most common microsporidian species, either human or animal, considered to be an emergent, zoonotic and opportunistic pathogen in immunocompromised and immunocompetent individuals (Al-Sadiand Al-Mahmood, 2014). Spores of *E. cuniculi* can survive in macrophages, spread throughout the host and cause various lesions in organs of urinary, digestive, respiratory and nervous systems (Abu-Akkada et al., 2015).

The adaptive immune response is essential for the elimination of *E. cuniculi*, but the innate immune response is responsible for initial defense mechanisms against these pathogens. *E. cuniculi* infection induces a $CD8^+$ cytotoxic T lymphocyte (CTL) response that lysing the infected cells by perforin-dependent mechanism (Ghosh and Weiss, 2012). There is an antibody response during *E. cuniculi* infection, but these humoral immunity is clearly not sufficient to prevent mortality or to cure infection and cell-mediated immunity is critical for the survival of *E. cuniculi* infection hosts (Khan et al., 2001). The survival and replication of some species of microsporidia inside macrophages may be associated with the absence of phagosome–lysosome fusion (Weidner and Sibley, 1985). Internalized microsporidian spores are normally destroyed within macrophages by the toxic activity of nitrogen species and reactive oxygen produced by the respiratory burst response, and cytokines released by macrophages may be an important role in protection against microsporidia (Franzen et al., 2005).

B-1 cells are a subtype of B cells that account for 35%–70% of B cells in the peritoneal cavity of mice and differ from B-2 cells in surface marker expression and function (Herzenberg and Kantor, 2013). B-1 cells act as antigen-presenting cells, phagocytes and express lymphoid markers (CD45/B220, CD19 and IgM) and CD11b, a myeloid marker, but not express CD23 like B-2 cells (Hardy, 2013).

The possible role of B-1 cells in the dynamics of the inflammatory process of various etiologies is unknownand researchers have demonstrated the influence of these cells for functional regulation of macrophages. B-1 cells secrete IL-10 and use it as an autocrine growth factor and Popi et al. (2004) demonstrated *in vitro* that this cytokine leads to a decrease in nitric

oxide and hydrogen peroxide production by macrophages, which lowers their phagocytic capacity. Additionally, Popi et al. (2008) demonstrated that BALB/c mice failed to control a *Paracoccidioides brasiliensis* infection when compared to XID (B-1 cells deficient) mice and attributed to IL-10 secreted by the B-1 cell as an impairment in macrophage function.

In a recent study from our group we demonstrated that XID mice (B-1 cell deficient) were more susceptible to experimental encephalitozoonosis than BALB/c mice as evidenced histologically with more prominent inflammatory lesions and parasite burden (Da Costa et al., 2017). These result suggested that B-1 cells may increase the resistance of BALB/c mice to encephalitozoonosis, evidencing for the first time the important role of B-1 lymphocytes in the control of microsporidia infection. For better understand the role of B-1 cells in the immune response against *E. cuniculi* infection we aimed to study *in vitro* the influence of B-1 cell on phagocyte activities of macrophages in an encephalitozoonosis model.

## METHODS

### Animals and Ethics Statements

Inbred BALB/c and BALB/c XID female mice with 6–8 weeks of age, specific pathogen free (SPF), were obtained from the animal facilities of Centro de Desenvolvimento de Modelos Experimentais (CEDEME), UNIFESP, Brazil. The animals were housed in polypropylene microisolator cages with a 12/12h light-dark cycle, maintained at 21 ± 2°C and >40% humidity and had standard chow and water *ad libitum*. All experimental procedures were performed in accordance with the animal care guidelines and were approved by the Ethics Committee in Animal Research at Paulista University under protocol number 385/15.

### *E. cuniculi* Spores

Spores of *E. cuniculi* (genotype I) (from Waterborne Inc., New Orleans, LA, USA) used in this experiment were previously cultivatedin a rabbit kidney cell lineage (RK-13, ATCC CCL-37) in Eagle supplemented with 10% of fetal calf serum (FCS) (Cultilab, Campinas, SP, Brazil), pyruvate, nonessential amino acids, and gentamicin at 37°C in 5% $CO_2$. The spores were purified by centrifugation and cellular debris were excluded by 50% Percoll (Pharmacia) as described previously (Didier et al., 1991)

**Adherent Peritoneal Cells (APerC) and B-1 Cell-Derived Phagocyte (B-1 CDP) Cultures**

Adherent Peritoneal Cells (APerC) cells were obtained from the peritoneal cavities (PerC) of BALB/c (BALB/c APerC) and B-1 cell-deficient XID (XID APerC) mice. PerC were washing with RPMI-1640 medium and cells ($0{,}5\times10^6$ cells/well) were dispensed onto 24-well plates and kept at 37°C in 5% $CO_2$ for 40 minutes. Non-adherent cells were discarded, and RPMI-1640 supplemented with 10% of FCS (R10) was added to the adherent fraction. To obtain B-1 CDP, APerC culture from BALB/c mice were performed and the enriched B-1 cells float portion were collected from third day (Alvares-Saraiva et al., 2015). $0{,}5 \times 10^6$ cells/well were re-suspended in R10 and re-cultured under the same conditions described above.

***E. cuniculi* Infection**

The culture of BALB/c APerC, XID APerC and B-1 CDP were infected simultaneously with *E. cuniculi* ($1\times10^6$ spores/ml) in the proportion of two spores per cell (2:1). The cultures were incubated at 37°C in 5% $CO_2$ and after 30 min, 1h, 48h, 96h and 144h of infection the supernatants were collected and stored at -80°C to measured nitric Oxide (NO) and cytokines. Cultures uninfected with *E. cuniculi* were incubated in the same times and used as a group control.

**Nitric Oxide Production Measure**

NO production was measured by a colorimetric detection of nitrite (nitrate was prior converted to nitrite by nitrate reductase) as product of the Griess reaction (R&D Systems) in the supernatants of cell cultures. Briefly, supernatants were mixed with Griess reagent [equal volumes of 0.2 % (w/v) naphthylethylenediamine in 60% of acetic acid and 2 % (w/v) sulfanilamide in 30 % (v/v) acetic acid] and incubated at room temperature for 10 min. A microplate reader was used to measure the absorbance at 540 nm and fresh culture medium was used as a blank in all experiments. The amount of nitrite in the test samples was calculated from a sodium nitrite standard curve (0,78 – 100 µM).

**Cytokines Quantification**

Cytokines were measured in culture supernatants with BD Cytometric Bead Array (CBA) Mouse inflammation Kit (BD Bioscience, San Jose, CA, USA). The kit was used for the simultaneous detection of mouse monocyte chemoattractant protein-1 (MCP-1), interleukin-4 (IL-4), IL-6, IFN-γ, tumor necrosis factor (TNF-α), IL-10 and IL-12p70 according to the manufacturer's instruction. Briefly, supernatants samples were added to capture allophycocyanin

(APC)-conjugated beads specific for the cytokines listed above and phycoerythrin (PE)-conjugated antibodies. Samples were incubated for 2 hours at room temperature in the dark and then they were measured using BD FACS Canto II Flow Cytometer and analyzed by FCAP Array™ Software (BD Bioscience), version 3.0. Individual cytokine concentrations (pg/Ml) were indicated by PE fluorescence intensity and cytokine standard curves.

**Light and Transmission Electron Microscopy Analyses**

Cell volume of BALB/c APerC, XID APerC and B-1 CDP cells as described above was adjusted to $1x10^7$ cells which were transferred to 25 $cm^2$ bottles each one and incubated in the same medium plus 10% FCS (R 10) at 37°C with 5% $CO_2$ for 40 min. After this time, the culture medium was removed and new R10 medium containing *E. cuniculi* spores (2:1) was introduced into the bottles. After 1h, 48h and 144h, the cultures were collected and fixed in 2% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.2) at 4°C for 10 h, and post-fixed in 1% $OsO_4$ buffered for 2 h. Semi-thin sections stained with toluidine blue to be viewed by light microscope and ultrathin to TEM were made.

**Statistical Analysis**

Differences between groups were determined using the two-way analysis of variance and the significance of mean difference within and between the groups was evaluated with multiple comparisons Bonferroni or Tukey posttests. All experimental data are expressed as mean ± standard error mean, indicated by bars in the figures and $p$ value of $<0.05$ was considered significant. All the graph and statistical analyses were performed using GraphPad Prism software version 6.0 for Windows (GraphPad Software, San Diego, California, USA).

**RESULTS**

**Phagocytic and Microbicidal Activity of BALB/c and XID APerC**

To study B-1 cells influence to macrophage activity we obtained APerC cultures from PerC washes of BALB/c and XID mice. These cultures were infected with *E. cuniculi* spores and evaluated under light and electron microscopy. After 1 hour, in the culture of BALB/c APerC we observed a high number of spores of *E. cuniculi* outside the cells and in XID APerC there were few spores observed outside the cells and a great quantity of them inside of macrophages (Figure 1).

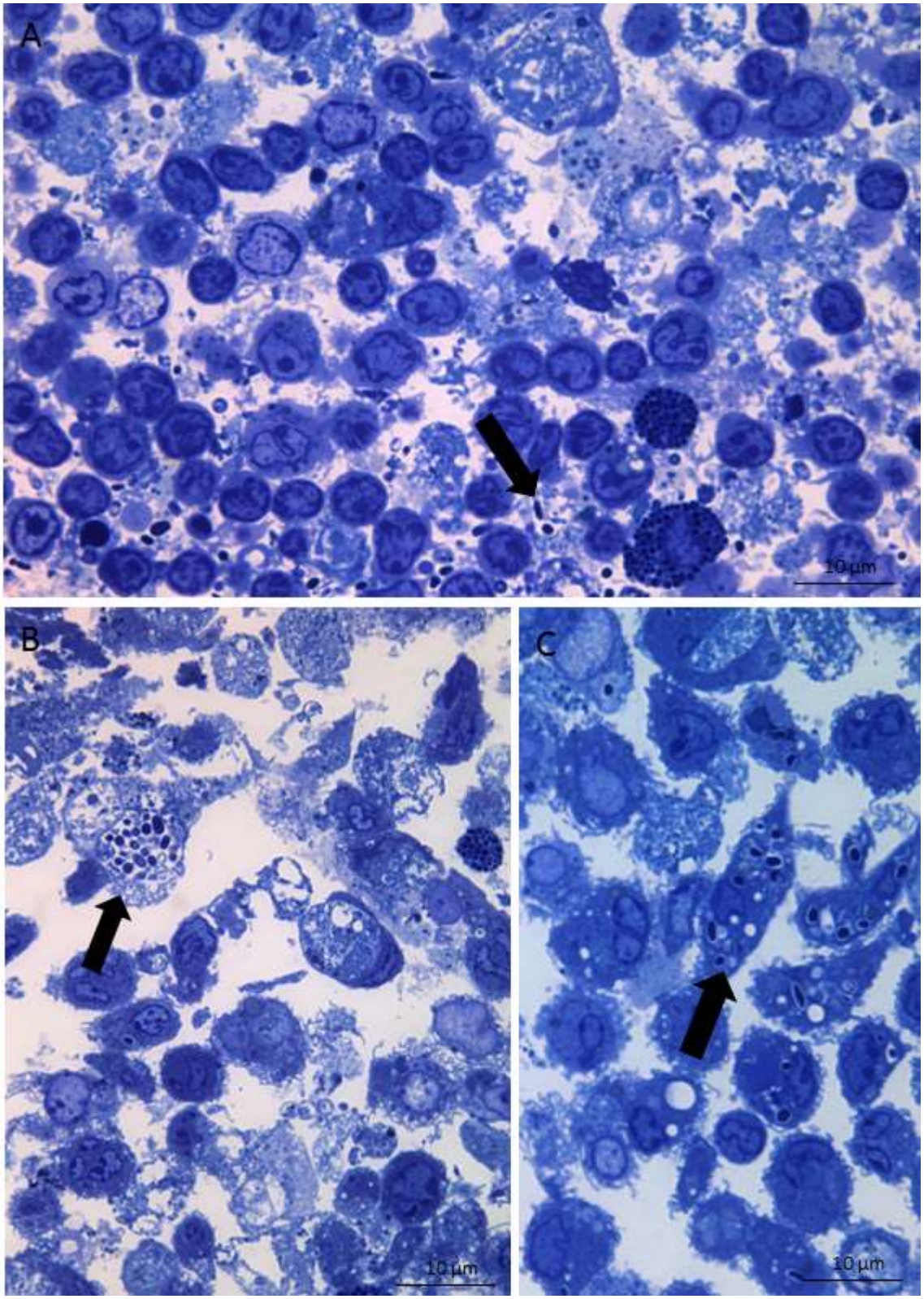


Figure 1. Photomicrography of semi-thin sections stained with toluidine blue of BALB/c and XID APerC after 1 hour of infection with *E. cuniculi* spores. (A) Spores of *E. cuniculi* (arrow) outside the cells in BALB/c APerC (B, C) Phagocytic cells with spores inside them (arrow) in XID APerC.

The phagocytosis of the spores occurred in both groups, being evidenced projections of the cellular membrane of macrophages near or involving the spores (Figures 2A and B).

The microbicidal activity was identified by the presence of a large amount of amorphous and electrodensing material inside phagocytic vacuoles in the macrophages and also being exocytosed (Figures 2C and D). Another important finding was the presence of degenerating macrophages (Figure 2D). Internalized spores surrounded by a vacuolar membrane forming a typical phagosome were observed (Figures 2 E and F).

The sections with 48 hours reveled the same findings of 1 hour in BALB/c and XID APerC, but we observed a higher number of spores in degeneration surrounded by a vacuolar membrane, forming a typical phagosome (Figure 3A) and macrophage degeneration was visualized. Myelin figures were noted indicating spore degeneration (Figure 3B). The examination not revealed intracellular proliferative stages of *E. cuniculi* (meronts, sporonts or sporoblast).

Macrophages of XID APerC showed large numbers of vacuoles in the cytoplasm and many membrane projections (pseudopodies) indicating spreading and intense phagocytic activity (Figure 2 A, B). BALB/c APerC showed a delay in the elimination of phagocytosed spores, although phagocytosis was also intense in these cultures. The exocytosis of the amorphous material was observed (Figure 2C). These same findings were seen at 48 hours (Figure 3A) with enhancement of spore in degeneration and with 144 hours few intact spores were observed inside the phagocytic cells (Figure 4A) and in lyses (Figure 4B). The examination with 144h of BALB/c APerC revealed the absence of macrophages and mature spores of *E. cuniculi* outside the cells (Figures 4C and D) and degenerated lymphocytes. These findings suggest intense microbicidal activity in both cultures at 144 hours.

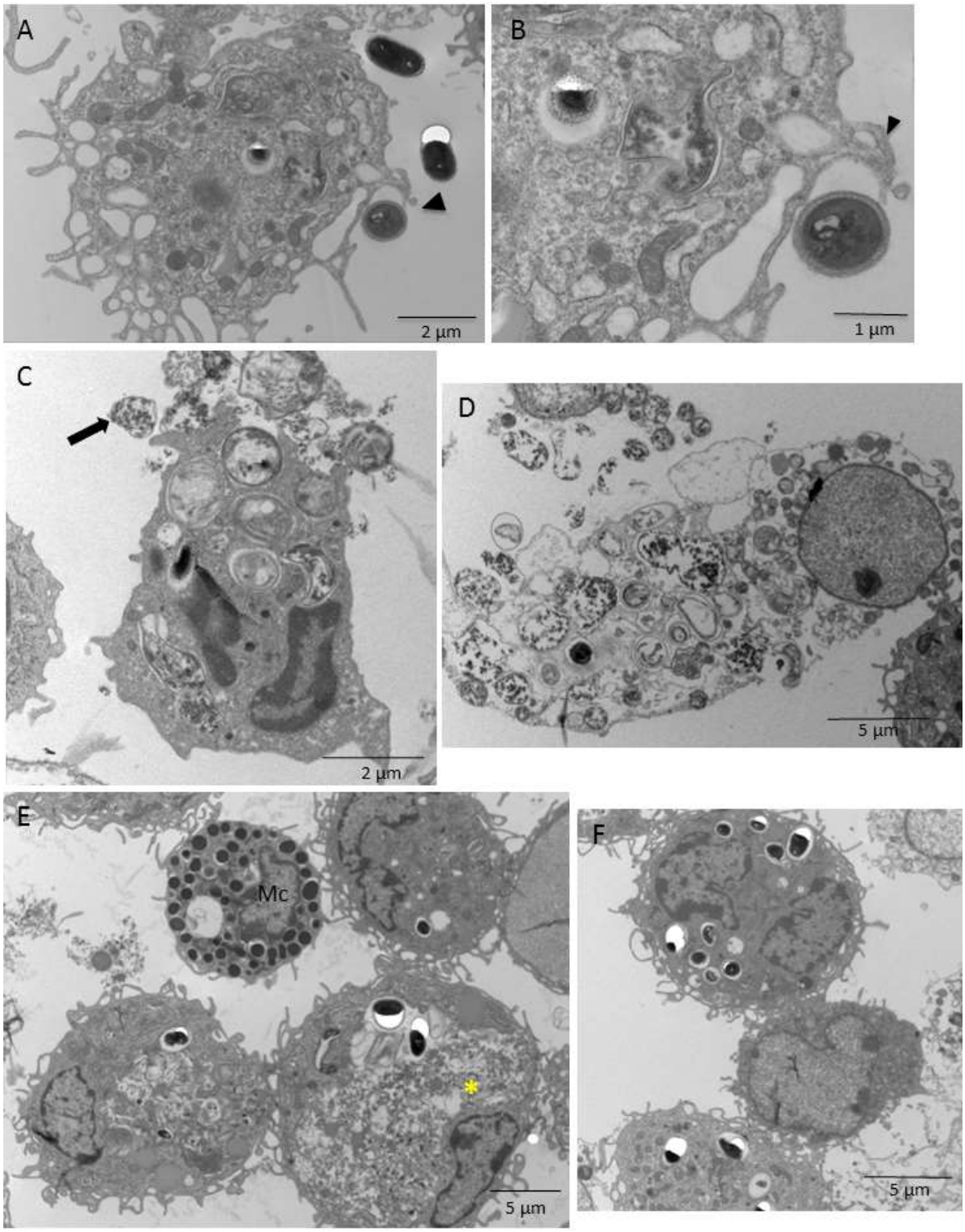


Figure 2. Ultramicrography of BALB/c and XID APerC after 1 hour of infection with *E. cuniculi* spores. (A, B) Projections of the cellular membrane of macrophages near or involving the spores (head arrows) of *E. cuniculi* in XID APerC. (C) Amorphous and electrodensing material inside phagocytic being exocytosed (arrow) in BALB/c APerC. (D) in XID APerC. (E, F) Intact mature spores and amorphous material (*) inside que cells of XID APerC. Note mast cell (Mc) in contact with macrophages.

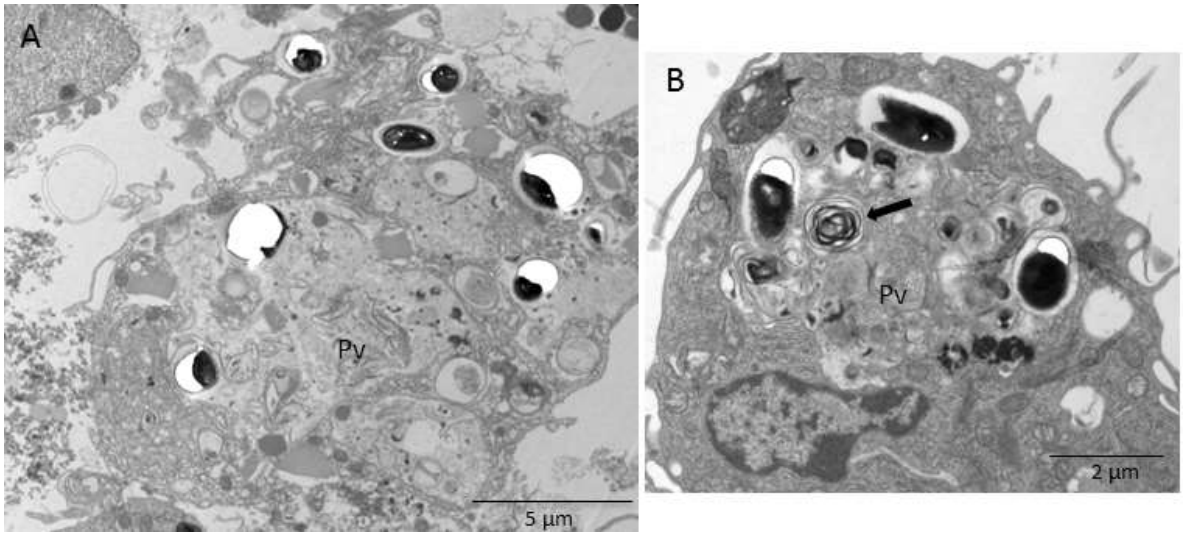


Figure 3. Ultramicrography of BALB/c and XID APerC after 48 hour of infection with *E. cuniculi* spores. (A) Amorphous and electrodensing material inside phagocytic vacuoles (Pv) in the macrophages *E. cuniculi* in APerC BALB/c. (B) Myelin figure (arrow) in phagocytic cell in APerC XID.

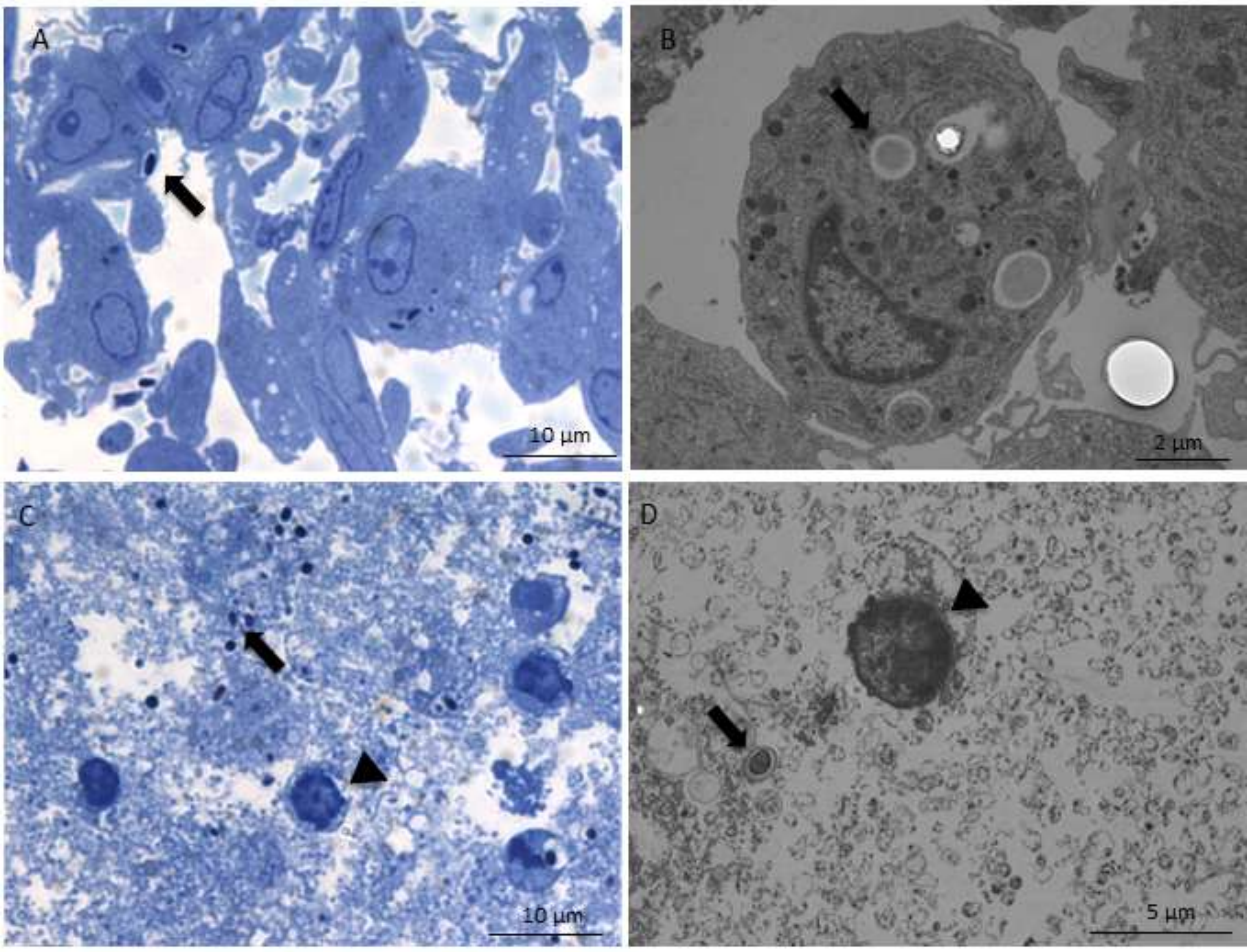


Figure 4. Photomicrography of semi-thin sections stained with toluidine blue and ultramicrography of BALB/c APerC and XID APerC after 144 hour of infection with *E. cuniculi* spores.

(A) Photomicrography of phagocytic cells with *E. cuniculi* spores inside them (arrow) in APerC XID. (B) Ultramicrography of a phagocytic cell with *E. cuniculi* spores in lysis (arrow) in APerC XID. (C) Photomicrography of APerC BALB/c with absence of macrophages and mature spores of *E. cuniculi* outside the cells (arrow) and degenerated lymphocytes (head arrow). (D) Ultramicrography of APerC BALB/c with absence of macrophages and mature spores of *E. cuniculi* outside the cells (arrow) and degenerated lymphocytes (head arrow) with pyknotic nucleous. 144 hours.

The internalized spores by macrophages in APerC XID cultures had a contact area between the phagocytic vacuole membrane of the cell and the spore wall (Figures 5A and B), suggesting communication between them. We observed the extrusion of the filament polar of intracellular mature spores with 1h (Figures 5C and D).

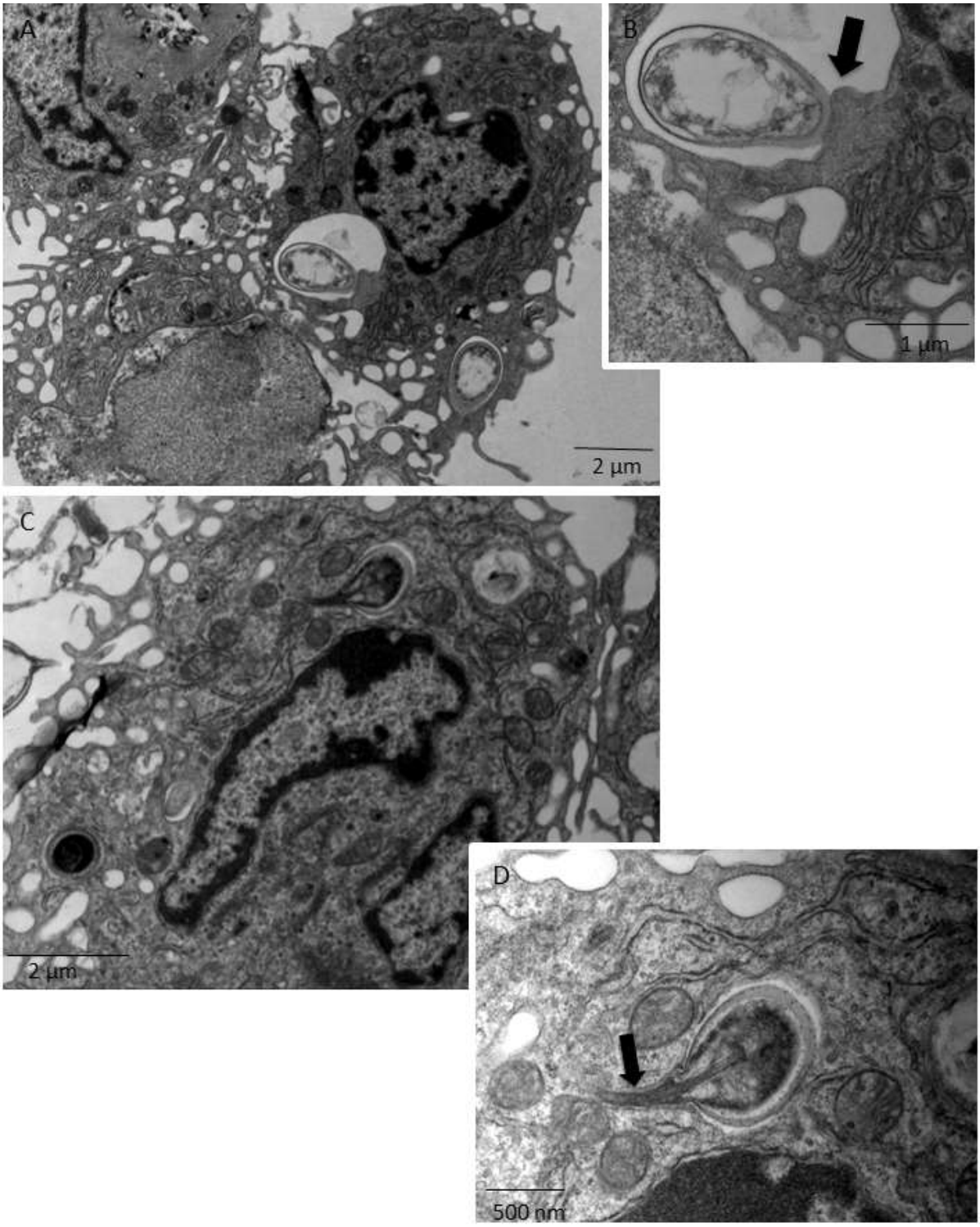


Figure 5. Ultramicrography of XID APerC after 1 hour of infection with *E. cuniculi* spores with observation of contact area between the phagocytic vacuole membrane and spore wall and the extrusion of the filament polar of intracellular mature spores.

(A, B) Contact area between the phagocytic vacuole membrane of the macrophages and the internalized spore wall (arrow). (C and D) Extrusion of the filament polar of intracellular mature spores (arrow).

The levels of NO in BALB/c APerC infected with *E. cuniculi* were not different of uninfected group at all times. There was a higher NO production with 144 h, regardless of infection, therefore, this production is probably related to the death of macrophages and NO release. In supernatants of XID APerC with 96h and 144h infected with *E. cuniculi* were significantly higher than in uninfected group (Figure 6). These data suggest that the presence of B-1 cells has a downregulation in NO production by macrophages.

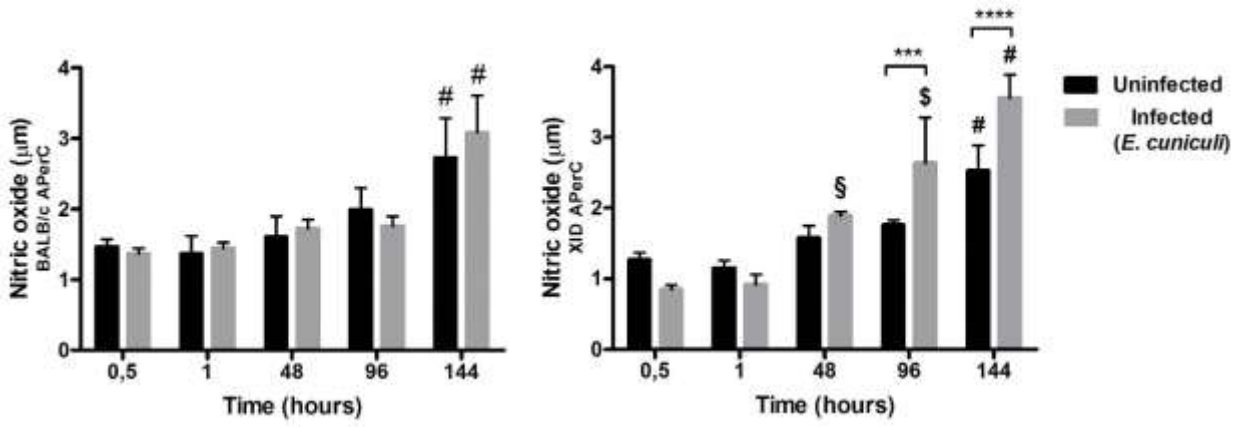


Figure 6. Levels of NO in supernatants of BALB/c and XID APerC. Two-way ANOVA test with Tukey posttests shows ***p<0,001, ****p<0,0001, # p<0,01 compared to other times, $ - p<0,01 compared to 48h and 1h, § - p<0,001 compared to 1h and 30 min.

Levels of IL-6 in BALB/c AperC in infected group were low in 30 min. and 48h in relation to the controls, however, this cytokine increased with 96h and 144h (Figure 7). MCP-1 decreased after 48 hours in both groups (uninfected and infected with *E. cuniculi*). After 96h in XID APerC the levels of IL-6 decreased in both groups, the infection promoted a decrease of TNF and IL-10 and MPC-1 were not detected.

The cytokine IL-12 was tested in this study but detectable levels of them were not found. IFN-γ levels were not statistically (data not shown).

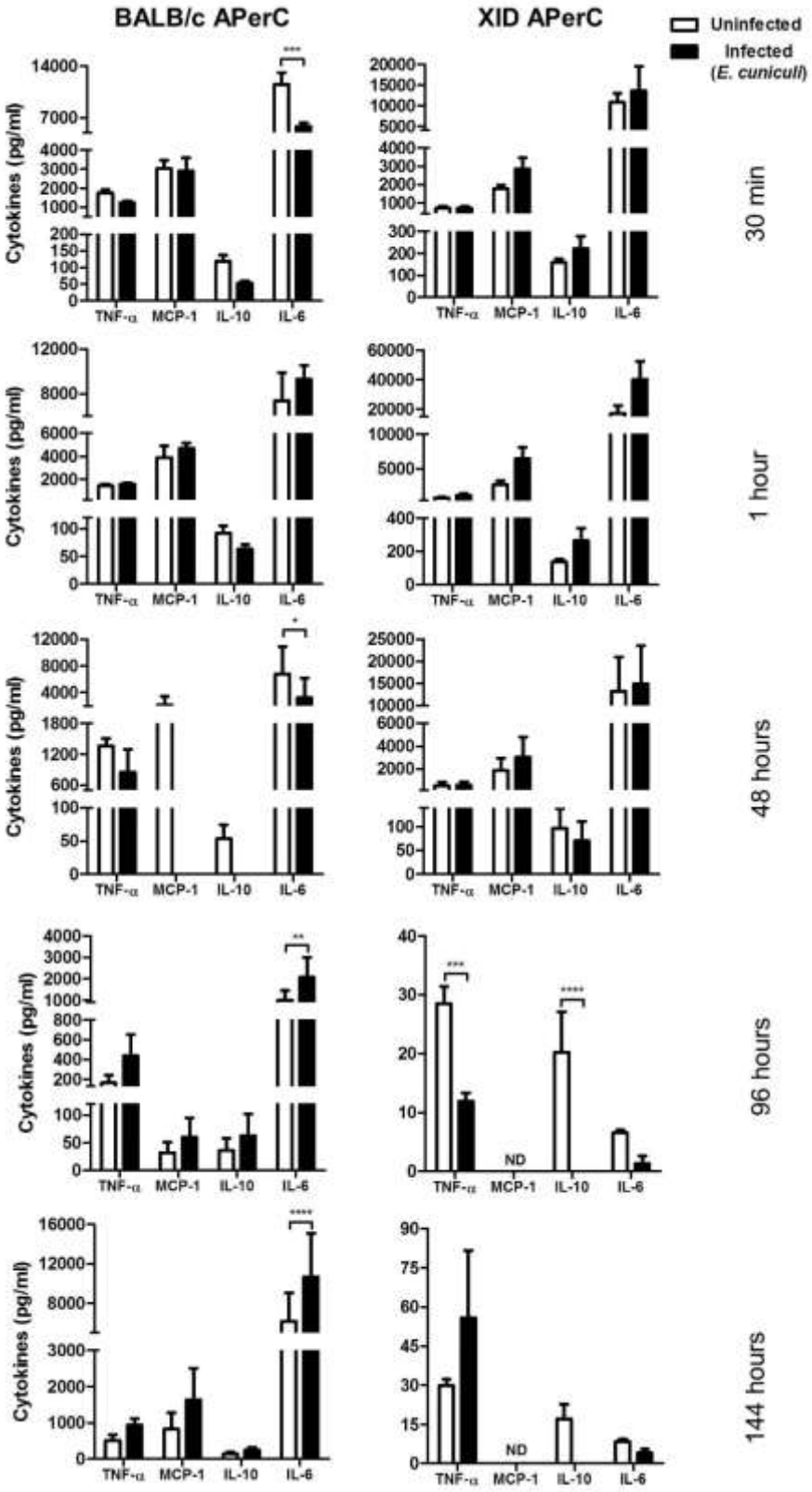


Figure 7. Cytokines levels in the supernatants of BALB/c and XID APerC. Two-way ANOVA test with multiple comparisons Bonferroni posttests shows *p<0,05, **p<0,01, ***p<0,001, ****p<0,0001 and ND = not detected.

**Contact and Interaction Between Peritoneal Cells**

The B-1 cells were identified in BALB/c AperC by the ultrastructural morphological characteristic of these cells as described by Abrahão et al. (2003) such as lobular organization of the nucleus with bridges of the nuclear membrane joining the lobules, a well-developed endoplasmic reticulum with few mitochondria (Figure 8A). Often intercellular communication involved other types of lymphocytes (Figure 8 A), mast cells in BALB/c (Figure 8B) and XID AperC (Figure 2E), and plasma cells (Figure 8C) in BALB/c AperC.

These cells were frequently observed in the process of communicating with macrophages by cell membrane projections (pseudopodia) or adhered to them with evidence of intima relationship (Figure 9A and B). We also evidenced the presence of cytoplasmic projections of macrophages and B-1 lymphocytes adhered or near extracellular *E. cuniculi* spores in BALB/c AperC (Figure 9C).

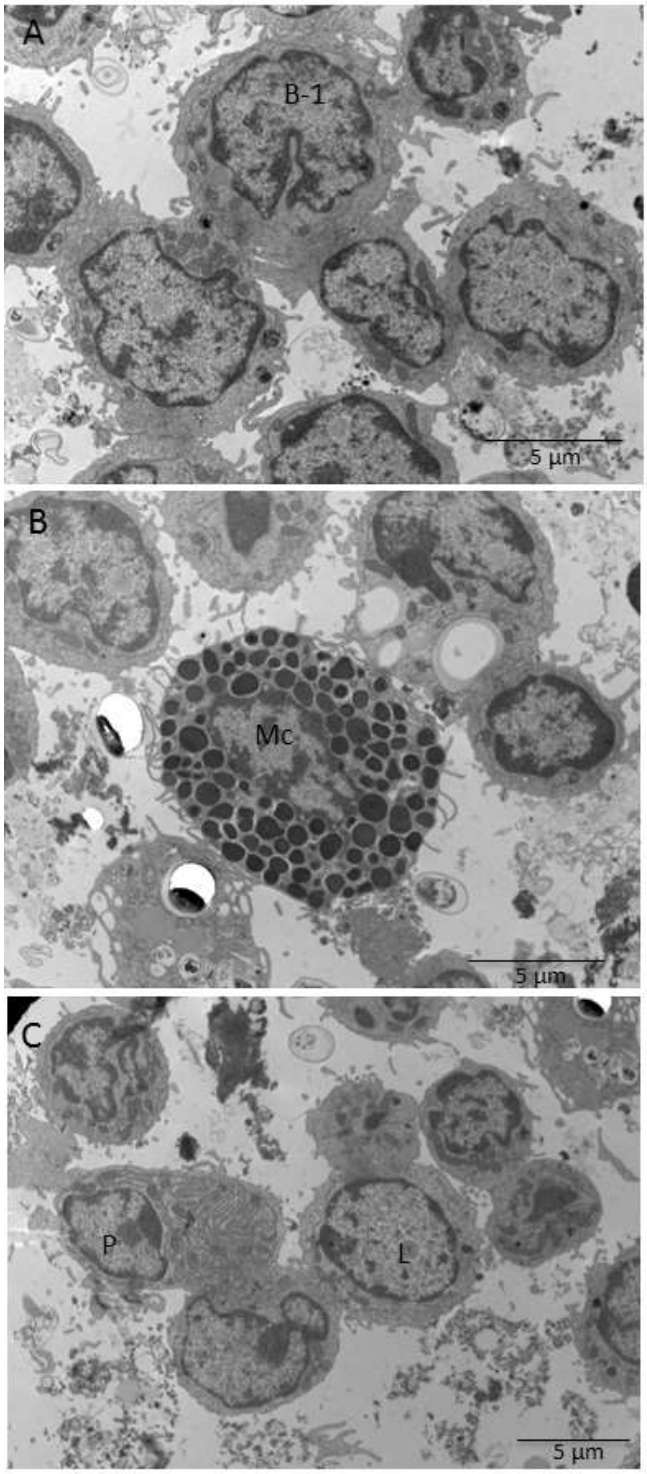


Figure 8. Ultramicrography of BALB/c AperC after 48 hour of infection with *E. cuniculi* spores showing contact and interaction between peritoneal cells.

(A) Typical B-1 cell (B-1) with a lobular organization of the nucleus in communication with others lymphocytes. (B) Mast cell (Mc)in communication with macrophages. (C) Plasma cell (P) in communication with lymphocytes (L).

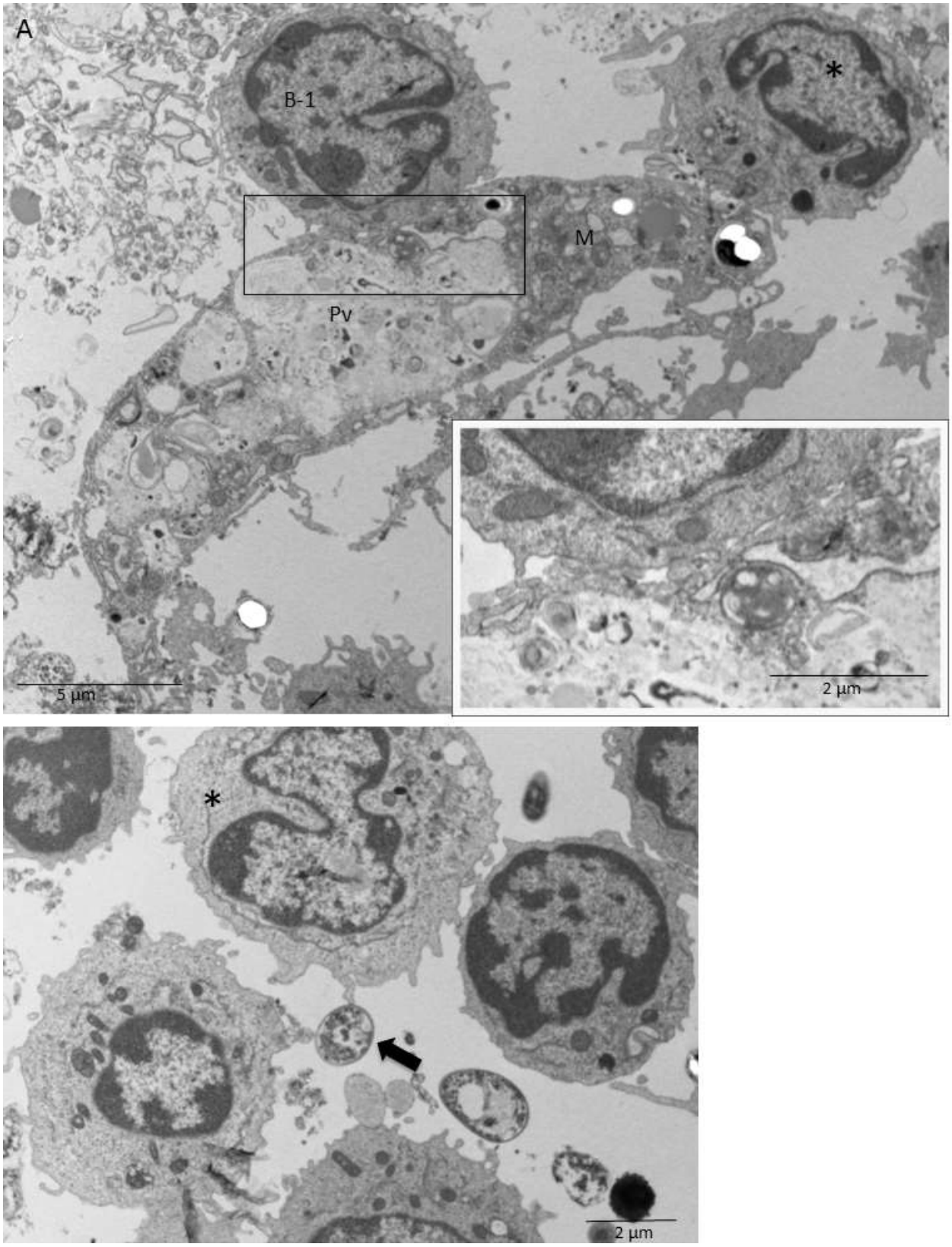


Figure 9. Ultramicrography of BALB/c AperC after 1 hour of infection with *E. cuniculi* spores showing contact and interaction between peritoneal cells.

(A) B-1 cells (B-1) and pre-B-1 CDP (*) in communication with macrophage with amorphous material inside phagocytic vacuoles (Pv). (B) Pre-B-1 CDP (*) in contact with *E. cuniculi* spores (arrow).

**Activity of B-1 Cell-Derived Phagocyte**

In cultures of B-1 CDP after 1h and 48h we found phagocytes (B-1CDP) and cells with preserved characteristics of B-1 lymphocytes. Another interesting finding was the contact of spores with lymphocytes (Figure 10A). There was B-1 CDP with spores of *E. cuniculi* in the process of lysis inside phagocytic vacuoles, presence of amorphous material (Figure10B) and intact spores were also visualized. Spores outside the cells were identified showing low index of phagocytosis after one hour (Figure 10C). The ultrastructure of *E. cuniculi* spores was typical of non-germinated mature spores and consisted of a thick wall composed of an electron-dense outer layer (exospore), an electron-lucent inner layer (endospore) and a plasma membrane enclosing the cytoplasm (Figure 10C). B-1 cells and B-1 CDP had abundant microvesicles in their membranes (Figures 10D and E) indicating cell communication.

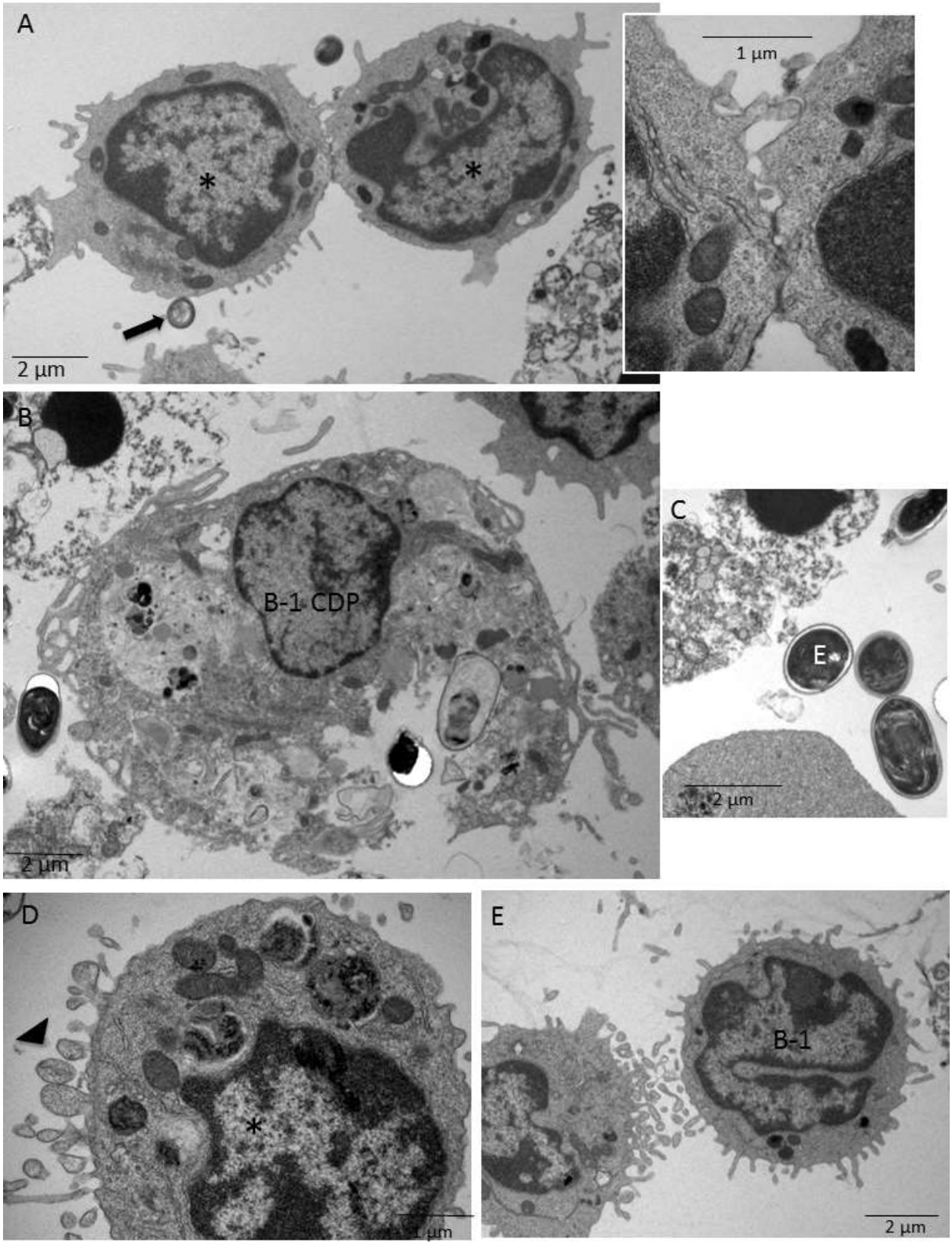


Figure 10. Ultramicrography of B-1 CDP culture after 1 hour and 48 hour of infection with *E. cuniculi* spores showing spores inside and outside the cells and abundant microvesicles in the membranes of B-1 cell and B-1 CDP indicating cell communication.

(A) Pre-B-1 CDP (*) in communication between them and with *E. cuniculi* spores (arrow). Amplified view of interaction between B-1 cells. (B) B-1 CDP with presence of amorphous material inside phagocytic vacuoles (Pv). (C) Non-germinated spores outside the cells with a thick wall composed of two layers and a plasma membrane. (D) Microvesicles in membrane of pre-B-1 CDP (*) with presence of amorphous material inside. (E) Microvesicles in membranes of pre-B-1 CDP.

The levels of NO in supernatants of B-1 CDP cultures infected with *E. cuniculi* were not different of uninfected group at all times (Figure 11A). TNF-α levels were increased in infected group after 30 min. and 1h and decrease after 96h and 144h. In both groups (infected and uninfected) MCP-1 was not detected after 30 min. and 1h and the levels of this cytokines increased after 48h, 96 and 144h (Figure 11B). The cytokine IL-12 was tested in this study but detectable levels of them were not found. IFN-γ levels were not statistically (data not shown).

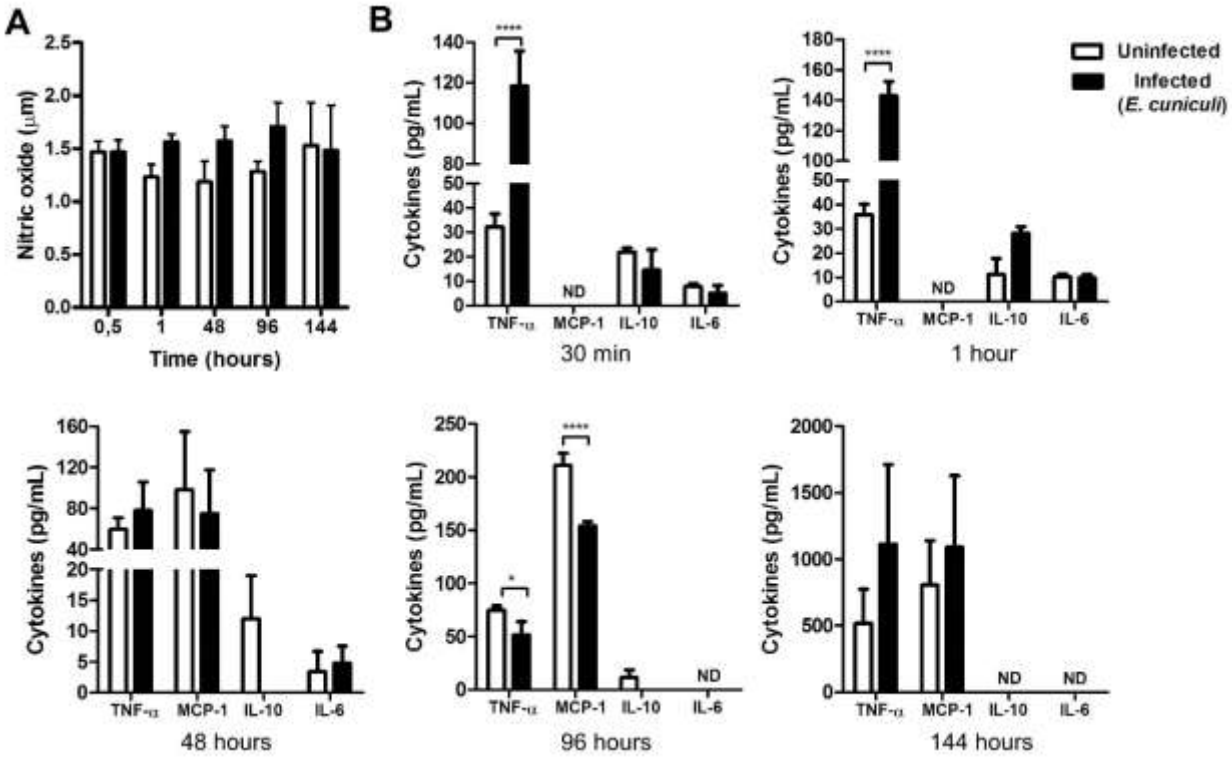


Figure 11. NO and cytokines levels in the supernatants from B-1 CDP cultures.
(A) NO levels. Two-way ANOVA test with Tukey posttests. (B) Cytokines levels. Two-way ANOVA test with multiple comparisons Bonferroni posttests shows *p<0,05, ****p<0,0001 and ND = not detected.

## DISCUSSION

The role of B-1 cell immunity against fungi, protozoa, bacteria and helminth infections has been described by our laboratory and other laboratories. Popi et al. (2008) demonstrated that BALB/c mice are more susceptible to experimental infection with *Paracoccidioides brasiliensis* than XID mice, suggesting that B-1 cells may favor infection. This is consistent with other experimental infections, such as *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) (Russo and Mariano, 2010) and the *Trypanosoma cruzi* (Minoprio et al., 1983) However, XID mice were more susceptible to *Cryptococcus neoformans* (Szymczak et al., 2013) and *Shistosoma mansoni* infection (Gaubert et al., 1999). Also currently available literature published by our group demonstrated that XID mice were more susceptible to encephalitozoonosis than BALB/c mice, suggesting that the pathogenecity caused by *E. cuniculi* depends upon the relationship between the parasite multiplication and the host's immune response(Da Costa et al., 2017).Our results showed the B-1 cell may initially compromise the phagocytosis process by macrophages with delay, but not compromise the ability of macrophages to eliminate spores meaning microbicidal capacity.

B-1 cells from mouse peritoneal cavity or from cultures of adherent peritoneal cells can be clearly identified on the basis of their distinct morphology and cell surface phenotype. The main morphological characteristic of these cells resides in bridges of the nuclear membrane, suggesting a lobular organization of the nucleus. In addition, B-1 cells are characterized by small membrane projections and a large number of ribosomes, a predominance of euchromatin in B-1 cell nuclei and a more condensed chromatin in the nuclear periphery (Abrahão et al., 2003). In this study we identified B-1 cells by TEM in BALB/c APerC and B-1 CDP cultures.

Cell-to-cell communication is required to guarantee proper coordination among different cell types within tissues. Studies have suggested that cells may also communicate by circular membrane fragments named microvesicles that released from the endosomal compartment as exosomes or shed from the surface membranes of most cell types (Ratajczak et al., 2006). In our study we identified phagocytic cells of B-1 CDP culture with abundant microvesicles in their membranes indicating cell communication. In addition, we observed cells in the process of communicating by cell membrane projections (pseudopodia) or adhered cell-to-cell membrane between different types of cells in BALB/c APerC.

The infection process of *E. cuniculi* involves the forcible eversion of a coiled hollow polar filament that pierces the host cell membrane, allowing the passage of infectious sporoplasm into the host cell cytoplasm. If a spore is phagocytosed by a host cell, germination

will occur and the polar tube can pierce the phagocytic vacuole, delivering the sporoplasm into the host cell cytoplasm (Ghosh and Weiss, 2012). In APerC XID with 1h we observed the extrusion of the filament polar of intracellular mature spores, suggesting that spores may germinate after phagocytosis and thus escape from the phagolysosome. This extrusion of the polar tubule is described in the literature, but there was no ultramicrography showing this process, therefore, this image is unpublished by TEM and unprecedented in the literature. These findings indicate an intimate relationship between spores and the phagocytic vacuole, suggesting that some APerC XID macrophages have less microbicidal activity. We speculate that these macrophages can be polarized to M-2 in the absence of B-1 cells and promote the maintenance of *E. cuniculi*.

After infecting the host cell, *E. cuniculi* undergoes further development within a parasitophorous vacuole. The developmental stages consist of meronts, sporonts, sporoblasts, and spores (Ghosh and Weiss, 2012). The examination of our cultures not revealed intracellular proliferative stages of *E. cuniculi* but only intact mature intracellular spores and spores in a lytic process of degeneration, suggesting intense phagocytic and microbicidal activity.

NO production by macrophages has an important contribution to kill intracellular parasites (Murrayand Nathan 1999) and the literature has shown that reactive nitrogen intermediates contribute to kill *E. cuniculi* of peritoneal macrophages of mice by IFN-γ-activated and LPS (Didier and Shadduck, 1994; Didier, 1995). On the other hand, Frazenand Salzberger (2005) demonstrated that viable microsporidian spores did not induce a NO response in monocyte-derived human macrophages and there was a negative correlation between the number of intracellular spores and the amount of NO. The authors suggest that a modulation of NO response by intracellular microsporidia may contribute to the survival of microsporidia within the macrophage by a mechanism yet unknown. The role of NO in *E. cuniculi* immunity was excluded by Khan and Moretto (1999) considering that they evaluated the importance of NO in protection against *E. cuniculi* infection using $iNOS^{-/-}$ mice. None of these animals died or exhibited any signs or symptoms of disease throughout the course of experiment and appeared clinically indistinct from the wild-type controls. Induction of NO in macrophages requires TNF that is an important cytokine to the defense against intracellular pathogens, favoring phagocytosis and intracellular killing (Frazen and Salzberger, 2005). In this study there were no significance changes in TNF and no difference in NO production between infected and non-infected cultures after 30 minutes, 1 hour and 48 hours in all groups.

NO is probably not the major mechanism for controlling the *E. cuniculi* infection. NO in supernatants of XID APerC (96h and 144h) infected with *E. cuniculi* were significantly higher

than in uninfected culture. In contrast, the levels of NO in BALB/c APerC inoculated with *E. cuniculi* were not different from uninfected control. These data suggest that B-1 cells can induce down-regulation of macrophage NO production *in vitro*.

Th2 cytokines have been demonstrated in *E. cuniculi* infection (Khan and Moretto, 1999; Da Costa et al., 2017).and increase in the mRNA for IL-10 was observed in the splenocytes of the infected animals (Khan and Moretto,1999). This cytokine has been reported to be involved in regulation of Th1 immune response against *Toxoplasma gondii* (Gazzinelli et al., 1994) and it is possible to have the similar role in *E. cuniculi* infection. In an *in vitro* study with cell culture of human macrophages incubated with *E. cuniculi* spores was seen an increase in IL-10 in supernatants of cultures and this cytokine was below the limit of detection in the group control (non-infected cultures). Our findings demonstrated an increased in IL-10 in the infected group of XID mice after 30 min. and 1h of cultures infection, however this correlation was statistically not significant.

Chemokines are a group of small molecules that regulate cell trafficking of leukocytes. They mainly act on monocytes, lymphocytes, neutrophils and eosinophils and play an important role in host defense mechanisms (Zlotnikand Yoshie, 2000). MCP-1, also known as CCL2, was the first human chemokine to be characterized (Zlotnikand Yoshie, 2000)and this molecule attracts cells of the monocyte lineage including macrophages, monocytes, and microglia(Yoshimura and Leonard, 1992). Mice deficient in MCP-1 are reportedly unable to effectively recruit monocytes in response to an inflammatory stimulus, despite the presence of normal circulating leukocyte numbers (Lu et al., 1998). There is a documentation that chemokine production is induced by microsporidian infections in human macrophages. Primary human macrophages culture of peripheral blood mononuclear cells was infected with *E. cuniculi* and revealed several chemokines involved in the inflammatory responses, including MCP-1(Fischer et al., 2007). Our findings demonstrated an increased in MCP-1 in the supernatants of infected group of XID APerC after 30 min, 1h and 48h of infection, however this correlation was statistically not significant. After 96h this cytokine was not detected.

IL-6 is a proinflammatory cytokine. In a research in our laboratory we demonstrated that Diabetes mellitus (DM) increased the susceptibility to encephalitozoonosis in C57BL/6 mice and DM mice infected with *E. cuniculi* showed a high level in IL-6 than DM-*uninfected* mice, suggesting that DM may also modulate a pro-inflammatory state of the organism (Francisco Neto et al., 2017). In our results, that was an increased in IL-6 in XID APerC after 30 min., 1h

and 48h of cultures infection. This cytokine was increased in BALB/c APerC after 96h and 144h of infection.

## CONCLUSION

The findings study conclude that B-1 cells interfere with macrophage activity. As we hypothesized in the *in vivo* study of encephalitozoonosis, the presence of B-1 cells increases the microbicidal activity of macrophages.

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