Original Article

Effects of Cytokines IFN-γ and TGF-β on the Functional Activity of Blood Mononuclear Cells against *Giardia lamblia*

Maximilian Wilhelm Brune [1,2], Eduardo Luzía França [1], Lucélia Campelo Albuquerque Moraes [1], Victor Pena Ribeiro [1], Maria Aparecida Gomes [2], *Adenilda Cristina Honorio-França [1]

1. *Institute of Biological and Health Science, Federal University of Mato Grosso, Barra do Garças, MT, Brazil*
2. *Department of Parasitology, Institute of Biological Science, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil*

Abstract
Background: This study aimed to analyze cultures of mononuclear (MN) cells with *Giardia lamblia* to determine the levels of the cytokines IFN-γ and TGF-β and the functional activity of MN cells after incubation with cytokines.
Methods: This study was conducted in 2018 in Barra do Garças, Mato Grosso State, Brazil. Blood samples were collected from 60 healthy volunteer donors to obtain leukocytes. The levels of IFN-γ and TGF-β were quantified in trophozoite cell culture supernatants. Superoxide release, phagocytosis, microbicidal activity, apoptosis and intracellular calcium release were analyzed.
Results: The cytokines evaluated were detected in the culture supernatant of MN cells and *G. lamblia*. Regardless of the type of cytokine, MN cells increased superoxide release in the presence of *G. lamblia*. Phagocytosis, microbicidal activity and apoptosis were higher when MN phagocytes were treated with cytokines. The highest microbicidal activity and apoptosis rates were observed in MN cells cultured with TGF-β. IFN-γ increased the release of intracellular calcium by MN phagocytes.
Conclusion: Cytokines play a beneficial role in the host by activating MN cells against G. lamblia. In addition, phagocytosis causes *G. lamblia* death and that the modulation of the functional activity of blood MN phagocytes by cytokines is an alternative mechanism for eliminating *G. lamblia*.

Available at: http://ijpa.tums.ac.ir

Introduction

The high prevalence of protozoan infections remains a public health problem given the adverse effects on human health (1). *G. lamblia*, a flagellate protozoan that inhabits the upper small intestine of the host, is one of the most common intestinal parasites in humans (2,3).

Giardiasis affects thousands of people worldwide, and poor sanitation contributes to the high infection rate (4). Although asymptomatic infections appear to be very common, giardiasis may also persist for several months as a chronic infection. Symptoms such as acute or chronic diarrhea, abdominal cramps, nausea, distension, weight loss, intestinal malabsorption, and reduction in brush-border disaccharidases (3,5,6) have been documented in *G. lamblia* infections, but the underlying cellular and molecular mechanisms that lead to disease are poorly understood.

The immunological response to *G. lamblia* involves a number of processes, including non-specific mechanisms such as phagocytic cell activity. Monocytes and macrophages, immune cells involved in phagocytosis, are primary agents of microorganism destruction (7-10). Macrophages are recruited in *G. lamblia* infections, suggesting that these cells play an important role in the control of the parasite. These cells also produce free radicals and are known to be the first line of host defense against various infections, including protozoa (7,8,10). However, how phagocytic cells respond to cytokine modulation during infection has yet to be elucidated.

Activation of the adaptive immune response involves the participation of T lymphocytes and B lymphocytes (7). In giardiasis, CD4$^+$ T lymphocytes participate in the control of the infection, likely through increased cytokine secretion (12,13). The presence of cytokines with inflammatory characteristics can cause damage to the parasite, accelerate intestinal peristalsis and facilitate eradication of the parasite by the production of mucus in the intestine, which is indispensable for parasite eradication (14).

Thus, the role of cytokines is directly related to the immunity profile, and depending on the stimulus, cytokines can determine differentiated immune responses against the parasite. TGF-β acts in the production of regulatory cells (15) and plays a beneficial role in the host by activating mononuclear cells against protozoa (16), whereas IFN-γ has been linked to an increase in the production of active metabolites of oxygen by macrophages in *Giardia* spp. Studies suggest that high IFN-γ production by phagocytes may contribute to the control and elimination of *G. lamblia* infection (17).

In a previous study, IFN-γ and TGF-β play beneficial roles in the host by activating MN cells against *E. histolytica* (16). However, the effector mechanisms of these cytokines in cellular activation as well as the functions of these signaling pathways in these cells during interactions with *G. lamblia* remain unclear. Although phagocytic cells play an important role in giardiasis, the immunomodulatory effects of cytokines on phagocytes in protozoan infections are only partially understood. The present study analyzed the supernatants of cultures of MN cells with *G. lamblia* to determine the levels of the cytokines IFN-γ and TGF-β and the functional activity of MN cells after incubation with cytokines.

Materials and Methods

Ethics statement

This study was conducted in 2018 in Barra do Garças, Mato Grosso State, Brazil and was approved by the Institutional Research Ethics Committee of Araguaia University Center under protocol nº684.338, and all subjects gave written informed consent before participation.

Blood sampling and MN cell separation

Blood samples (8 mL) were collected from 60 volunteer donors in tubes with the anticoagulant EDTA. The samples were centrifuged at 160×g for 15 min to separate the plasma from the cells. The cells were separated over a Ficoll-Paque gradient (Pharmacia, Uppsala, Sweden) and analyzed by light microscopy. MN cells were resuspended independently in serum-free medium 199 at a final concentration of 2×10^6 cells/mL (18). MN cells were used immediately for superoxide release, phagocytosis, microbicidal activity, intracellular calcium release and apoptosis assays.

Parasites

G. lamblia trophozoites, Portland 1 [P1, ATCC 30.888], were used in all experiments. The parasites were cultivated axenically in TYI-S-33 modified medium (19). The parasites were cultivated for 2 to 5 days. Before the experiments, the culture tubes were centrifuged at 250×g for 5 min at 4 °C. The trophozoites were washed out and resuspended in serum-free medium 199.

Viability of the parasites in serum-free medium 199

As described by Hill and Pearson (20), the capacity of the parasites for survival in serum-free medium 199 was determined by incubating $1/10^6$ trophozoites/ml with 10 ml of serum-free medium 199 or TYI-S-33 medium at 37 °C. After 2 h of incubation, trophozoite movement and flagellum mobility were assessed to determine the viability of the parasite. These experiments were performed in triplicate.

Incubation of MN cells with cytokines

To assess the functional activity of mononuclear phagocytes, MN cells (2×10^6 cells/mL) were incubated with IFN-γ or TGF-β at a concentration of 100 ng/mL (Sigma ST Louis, USA) (21) for 1 h at 37 °C. The MN cells were then washed once with serum-free medium 199 at 4 °C and immediately used in the assays. A control was performed with only serum-free medium 199.

Cultures of MN cells and G. lamblia

MN cells were centrifuged and resuspended in RPMI culture medium supplemented with 10% fetal bovine serum. The cells (2×10^6 cells/mL) treated or not treated with cytokines were incubated with *G. lamblia* (1×10^6 parasites/mL) for 2 h at 37 °C with 5% CO_2. After this period, the cultures were centrifuged for 10 min at 160×g. The cells were used for superoxide release, phagocytosis and microbicidal assays, apoptosis and intracellular calcium release assays, and the supernatant was reserved for cytokine quantification.

Cytokine detection by ELISA (enzyme-linked immunosorbent assay) and flow cytometry

The IFN-γ concentrations in the supernatant of cultures of MN cells with *G. lamblia* were determined by flow cytometry using a cytometric bead array kit (CBA, BD Biosciences, USA) according to the manufacturer's procedures. A flow cytometer was used for these analyses [FACSCalibur, BD Biosciences, USA]. The data were analyzed using FCAP Array 1.0 [CBA, BD Biosciences, USA]. TGF-β concentrations were analyzed using an ELISA kit from Enzo® Life Sciences (United Kingdom) according to the manufacturer's procedures. The reaction rates were measured by absorbance in a spectrophotometer with a 450 nm filter. The results were calculated by reference to a standard curve and presented as pg/mL.

Release of superoxide anion

Superoxide was determined by cytochrome C reduction (19,22). Suspensions of MN cells (2×10^6 cells/mL) were mixed with *G. lamblia* trophozoites (1×10^6 parasites/mL), followed by incubation with shaking for 2 h at 37 °C. The suspension was resuspended in 0.5 mL of

ferricytochrome C (Sigma, St. Louis USA) at a concentration of 2 mg/mL. A control containing only cells was performed to verify the spontaneous release of superoxide anion by MN cells. MN cells were also incubated with cytokines in the absence of *G. lamblia*. The suspensions (100 µL) were incubated for 60 min at 37 °C on culture plates. The absorbance of the reaction was measured in a spectrophotometer at 550 nm. The concentration of the superoxide anion was calculated from the following relation: concentration O_2^- (nmol)=OD/6.3×100. All experiments were performed in duplicate.

Phagocytosis and microbicidal activity

Phagocytosis and microbicidal activity were evaluated by the acridine orange (Acros Organics, New Jersey, USA) method (23). Equal volumes of parasite ($1×10^6$ parasites/mL) and cytokine-treated or untreated MN cells ($2×10^6$ cells/mL) were incubated at 37 °C for 2 h under continuous shaking. Phagocytosis was stopped by incubation on ice. The suspensions were washed twice (160×g, 10 min, 4 °C). The suspension was resuspended in serum-free medium 199 and centrifuged. The supernatant was discarded, and the sediment was stained with 200 µL of acridine orange (Sigma, St. Louis, USA; 14.4 g/L) for 1 min. The sediment was resuspended in cold serum-free medium 199 and washed twice. Phagocytosis and the death of parasite and MN cells were determined by fluorescence microscopy at 400× and 1000× magnification. One hundred parasites were counted per slide.

The phagocytosis index was calculated by counting the number of cells that phagocytosed *G. lamblia* in a total of 100 cells. The microbicide index was obtained from the cell count containing parasites. The phagocytosed parasites with orange staining were counted as dead, and the parasites phagocytosed by MN cells but with green coloration were considered alive (24). All experiments were performed in duplicate.

Apoptosis assay

Annexin V staining was performed to assess apoptosis (25). Untreated cells were used as a negative control, and cells treated with staurosporine [Sigma, St. Louis, USA] to induce apoptosis were used as a positive control. The controls and MN cells treated with cytokines and incubated with *G. lamblia* were resuspended in 500 µL of binding buffer containing 5 µL of annexin V-FITC (Annexin V-FITC Apoptosis Detection Kit, Alexis™, San Diego, USA) and then incubated for 10 min at room temperature. The fluorescence of the cells was analyzed by flow cytometry (FACS Calibur system, BD, San Jose, USA). The resultant data were analyzed using CellQuest software.

Intracellular Ca2+ release determination

To assess intracellular Ca_2^+ release in MN cells, fluorescence staining was performed on a FACS Calibur system (BD, San Jose, USA; 16). Cells were loaded with the fluorescent radiometric calcium indicator Fluo3-Acetoxymethyl (Fluo3-AM, Sigma, St. Louis, USA). Cell suspensions were pretreated with or without 5 µL of cytokines (Sigma, final concentration of 100 ng/mL), mixed and incubated at 37 °C for 30 min under continuous shaking. The suspensions were centrifuged twice (160×g, 10 min, 4 °C) and resuspended in PBS containing BSA (5 mg/mL). This suspension was incubated with 5 µL of Fluo-3 (1 µg/mL) for 30 min at 37 °C. After incubation, the MN cells were washed twice in PBS containing BSA (5 mg/mL; 160×g, 10 min, 4 °C) and then analyzed by flow cytometry (FACS Calibur system, BD, San Jose, USA). The rate of intracellular Ca_2^+ release was expressed as the geometric mean fluorescence intensity of Fluo-3.

Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Analysis of variance (ANOVA) was used to evaluate statistically significant differences in superoxide anion release, phagocytosis, microbicidal index, apoptosis

and intracellular Ca_2^+ release in the presence or absence of cytokines. Differences were considered statistically significant at *P*-values less than 0.05.

Results

Cytokine release was observed during the interaction between MN phagocytes and *G. lamblia*. Of the cytokines studied, IFN-γ exhibited the highest concentration (Table 1).

Table 1: Cytokine (IFN-γ, TGF-β) concentrations (pg/mL) in cultures of mononuclear cells and *G. lamblia*

Cytokines	MN	MN and G. lamblia
IFN-γ	4.4± 1.0	14.5±3.1*
TGF-β	5.1±1.7	9.44±0.82*

Notes: The results are presented as the mean and standard deviation *P*<005*Indicates differences between MN (mononuclear) cells [control] and MN cells incubated with *G. lamblia*

The release of superoxide anion by the cytokine-stimulated MN phagocytes in the presence of *G. lamblia* is shown in Table 2. An increase in the release of superoxide was observed when MN phagocytes were stimulated by both cytokines. The highest concentrations of superoxide were observed when MN phagocytes were pretreated with TGF-β.

Table 2: Superoxide release in the interaction between MN phagocytes and *G lamblia* in the absence and presence of cytokines (IFN-γ, TGF-β) as determined by cytochrome C reduction

MN phagocytes incubated with	Superoxide (nmol)
PBS	1.8±0.8
G lamblia	2.1±0.7
IFN-γ	4.3±0.9*
TGF-β	6.2±0.6*
G lamblia + IFN-γ	7.1±1.2*#
G lamblia + TGF-β	12.6±2.6*#

Notes: The results are presented as the mean and standard deviation *P*<005 *Indicates differences between untreated phagocytes (control) and those treated with cytokines. #Indicates differences between MN phagocytes incubated with untreated *G lamblia* (control) and those incubated with *G lamblia* treated with cytokines

The phagocytic (Fig. 1) and microbicidal activity (Fig. 2) of MN cells against *G. lamblia* were higher when these cells were treated with cytokines. The highest phagocytosis index of microbicidal activity against the parasite was observed when MN cells were treated with TGF-β [Figs. 1 and 2].

The apoptosis rates of MN phagocytes in the presence of *G. lamblia* are shown in Fig. 3. MN phagocytes had low apoptotic indices in the absence of *G. lamblia*. When these cells were incubated with *G. lamblia*, death by apoptosis increased. Apoptosis rates rose when MN phagocytes were treated with cytokines and incubated with *G. lamblia*. These apoptosis rates were higher when MN phagocytes were treated with TGF-β. MN phagocytes treated with IFN-γ showed apoptosis rates similar to those observed when MN phagocytes were not treated with cytokines but incubated with the parasite and higher than those observed for MN phagocytes not treated with cytokines and not incubated with the parasite.
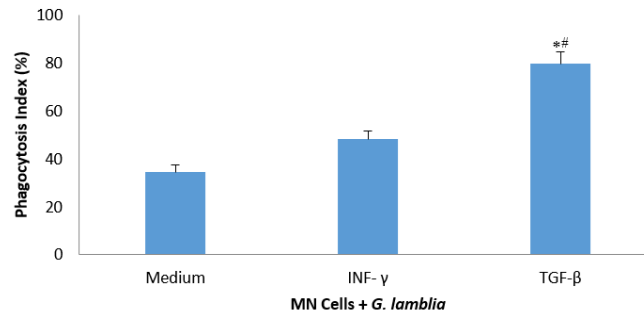
Fig. 1: Phagocytosis index for the interaction of MN + *G lamblia* in the presence of cytokinesas determined by the acridine orange method. Phagocytosis and MN cells were determined by fluorescence microscopy. *P*<005 *Indicates differences in relation to the untreated (control) MN phagocytes and those treated with cytokines; #Differences between the MN phagocytes of the cytokine-treated groups
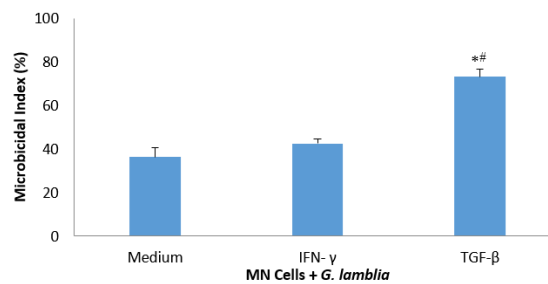

Fig. 2: Microbicidal activity of MN phagocytes treated with cytokines (IFN-γ and TGF-β) in the presence of *G lamblia* as determined by the acridine orange method. Death of the parasite and MN cells was determined by fluorescence microscopy *P*<005. *Indicates a difference between the control group (without cytokines) and the cytokine-treated groups; #Indicates differences between the cytokine-treated groups
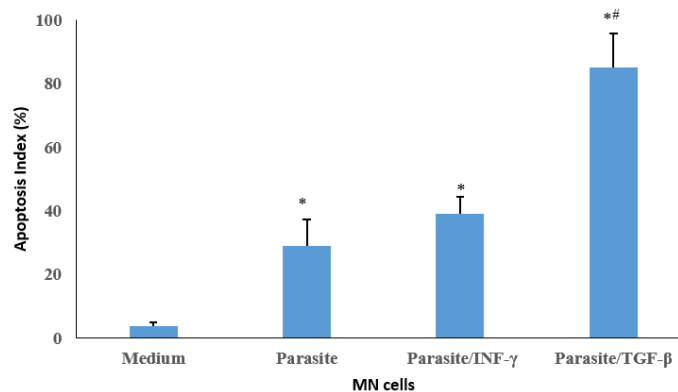

Fig. 3: Apoptosis index of MN phagocytes treated with cytokines (IFN-γ and TGF-β) during interactions with *G lamblia.* Annexin V staining was used to assess apoptosis, and the data were analyzed using CellQuest software. *P*<005. *Indicates differences between the control group (without cytokines) and the cytokine-treated groups; #Indicates differences between the cytokine-treated groups

There was increased release of intracellular calcium from IFN-γ-treated MN phagocytes.

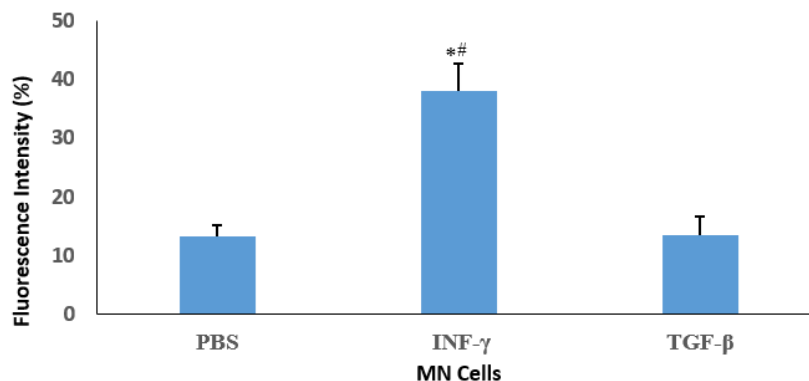TGF-β did not alter the release of intracellular calcium by MN phagocytes (Fig. 4).

Fig. 4: Intracellular Ca_2^+ release by mononuclear (MN) cells as indicated by the geometric mean fluorescence intensity. Cells were stained with Fluo-3, and immunofluorescence analyses were performed by flow cytometry (FACS Calibur, Becton Dickinson, USA). MN cells were preincubated with or without cytokines. *Indicates statistically significant differences between MN cells incubated with cytokines and the control [PBS]; #Indicates differences between the cytokine-treated groups

Discussion

Changes in the host immune response are fundamental in determining the course and symptomatology of *G. lamblia* infection. The clinical course of infection is detected by a combination of factors related to parasite virulence, pathogenicity and number of cysts ingested in the presence of concomitant infections, nutritional status, age and immunological condition of the host (26). This work describes the concentrations of the cytokines IFN-γ and TGF-β in the supernatant of cultures of human blood MN phagocytes and *G. lamblia* and how these cytokines modulate the functional activity of MN phagocytes during interactions with the parasite.

The role of MN cells and effector mechanisms in the response to *G. lamblia* has been reported in several studies (19,23). A correlation of cytokines with the cytotoxic activity of MN cells against *G. lamblia* has been suggested since cells stimulated by these molecules exhibit increased microbicidal activity. In the present study, the cytokines IFN-γ and TGF-β

were detected in supernatant analysis, and IFN-γ, which showed the highest levels, was mostly released in cultures of MN phagocytes with trophozoites.

IFN-γ is particularly relevant to phagocytic activity because it promotes their microbicidal potential, increasing the expression of surface receptors and phagocytosis rates (18). The high production of IFN-γ may contribute to the early control of *G. lamblia* infection by eliminating parasites (27), given that the cytokine induces the production of mediators capable of activating phagocytic cells.

In the present study, the presence of TGF-β in cultures of MN cells and *G. lamblia* suggests that the cytokine is an important mediator of immunity that regulates macrophage proliferation (6) and plays a beneficial role in the host by activating MN cells (16,20). However, the importance of this cytokine during MN phagocyte-parasite interactions may be related to its activity in mucosal immunity and direct effects on phagocytes (20) and IgA production (28). The production of TGF-β during MN cell-parasite interactions suggests that this cy-

tokine, in addition to determining cell recruitment to the infection site (29), can stimulate these cells, which is consistent with earlier findings (16,20). Once activated, these MN cells may increase secretory IgA production in the intestinal mucosa and act directly on parasite elimination.

Microbicidal activity efficiency is associated with oxidative stress and the formation of reactive oxygen species (ROS). The destruction of microorganisms after phagocytosis can be mediated by oxidative metabolism with the production of reactive oxygen metabolites such as the superoxide anion (16,30,31). In the present study, cytokines modulated the release of superoxide anion. There was an increase in the release of superoxide anion by MN phagocytes in the presence of *G. lamblia*. This increase was more significant when the phagocytes were treated with cytokines.

The release of the superoxide anion positively influenced the microbicidal capacity of the cells. Cytokines were able to modulate the phagocytosis of *G. lamblia* trophozoites. Phagocytosis and cellular microbicidal activity, with the production of active oxygen metabolites, are important defense mechanisms against several bacterial infections (32-34) and protozoa (16,33,35). Cytokines such as IFN-γ first act on monocytes/macrophages by activating their phagocytosis and microbicidal mechanisms (36). In vitro studies have shown that MN cells have greater microbicidal activity upon activation by cytokines such as IFN-γ (37).

The microbicidal activity of phagocytes was influenced by cytokines. TGF-β was more effective in this process since it potentiated cellular oxidative stress, resulting in the release of high concentrations of superoxide anion. The increase in superoxide may be related to the increase in apoptosis (38,39).

There was an increase in microbicidal activity and the apoptosis index during MN phagocyte-parasite interactions. These indices were higher in the presence of TGF-β. *G. lamblia*-induced apoptosis is related to a loss of epi-

thelial cells in small intestinal monolayers, and increased permeability is a caspase-3-dependent process (11). Trophozoites are capable of inducing apoptosis, suggesting a possible role of molecules released by the parasite, particularly antigens with proteolytic activity (40).

The induction of apoptosis may contribute to the pathogenesis of giardiasis due to a loss of intestinal epithelial barrier function and increased permeability via caspase-dependent mechanisms (11), and the high rates of apoptosis are likely associated with effective phagocytosis mechanisms that culminate in parasite death (24). The action of cytokines is also associated with a number of processes, such as changes in intracellular Ca_2^+ by phagocytes (25). Despite increasing superoxide release and apoptosis rates, TGF-β and IL-17 did not alter intracellular Ca_2^+ release in MN phagocytes. In contrast, IFN-γ induced increased superoxide release, phagocytosis, apoptosis and intracellular calcium, suggesting that these cytokines specify different microbicidal mechanisms in MN phagocytes in the presence of the parasite.

In light of the parasite's habitat, the results of the present study reinforce the essential role of the host's immunity integrity, both systemic and mucosal, during *G. lamblia* infections. Additional studies using experimental models or specific cytokine inhibitors are needed to clarify the mechanisms involved in host interactions with *G. lamblia*.

Conclusion

The present evidence of a modulating role of cytokines in phagocyte functional activity suggests the importance of interactions with blood cells and soluble components, particularly cytokines. The cytokines IFN-γ and TGF-β act on the functional activity of MN blood phagocytes, and this activity appears to be essential in the elimination of *G. lamblia*.

Acknowledgments

Conflict of interest

The authors declare no conflict of interest and non-financial competing interests regarding the publication of this article.

References

1. Geurden T, Vercruysse J, Claerebout E. Is Giardia a significant pathogen in production animals? Exp Parasitol. 2010; 124(1):98-106.
2. Astiazaran GH, Quintero J, Veja R, et al. Identification of T-cell stimulating antigens from *Giardia lamblia* by using *Giardia*-specific T-cell hybridomas. Parasite Immunol. 2009; 31(3):132-9.
3. Castillo-Villanueva A, Rufino-Gonzálezb Y, Méndeza ST, et al. Disulfiram as a novel inactivator of *Giardia lamblia* triosephosphate isomerase with antigiardial potential. Int J Parasitol Drugs Drug Resist. 2017: 7(3): 425–432.
4. Bartelt LA, Platts-Mills JA. *Giardia*: a pathogen or commensal for children in high prevalence settings? Curr Opin Infect Dis. 2016; 29(5): 502–507.
5. Cotton JA, Beatty JK, Buret AG. Host parasite interactions and pathophysiology in *Giardia* infections. Int J Parasitol. 2011; 41(9):925-33.
6. Fan K, Ruan Q, Sensenbrenner L, et al. Transforming growth factor-b bifunctionally regulates murine macrophage proliferation. Blood. 1992; 79(7):1679-85.
7. Roxstrom-Lindquist K, Palm D, et al. *Giardia* immunity--an update. Trends Parasitol. 2006; 22(1):26-31.
8. Teoh DA, Kamieniecki D, Pang G, et al. *Giardia lamblia* rearranjes F-actin and a-actinin in human colonic and duodenal monolayers and reduces transepithelial electrical resistanc. J Parasitol. 2000; 86(4):800-6.
9. Panaro MA, Cianciulli A, Mitolo V, et al. Caspase-dependent apoptosis of the HCT-8 epithelial cell line induced by the parasite *Giardia intestinalis*. FEMS Immunol Med Microbiol. 2007; 51(2):302-9.
10. Troeger H, Epple HJ, Schneider T, et al. Effect of chronic *Giardia lamblia* infection on epithelial transport and barrier function in human duodenum. Gut. 2007; 56(3): 328–335.
11. Chin AC, Teoh DA, Scott KGE, et al. Strain-dependent induction of enterocyte apoptosis by *Giardia lamblia* disrupts epithelial barrier function in a caspase-3-dependent manner. Infect Immun. 2002; 70(7):3673-80.
12. Singer SM, Nash TE. T-Cell-dependent control of acute *Giardia lamblia* infections in mice. Infect Immun. 2000; 68(1):170-5.
13. Fink MY, Singer SM. The Intersection of Immune Responses, Microbiota, and Pathogenesis in Giardiasis. Trends Parasitol. 2017; 33(11):901-913.
14. Hawrelak J. Giardiasis: pathophysiology and management. Altern Med Rev. 2003; 8(2):129-42.
15. Aluvihare VR, Kallikourdis M, Betz AG. Regulatory T cells mediate maternal tolerance to the fetus. Nature Immunol. 2004; 5(3):266-71.
16. Moraes LCA, França EL, Pessoa RS, et al. The effect of IFN-γ and TGF-β in the functional activity of mononuclear cells in the presence of *Entamoeba histolytica*. Parasit Vectors. 2015; 8: 413.
17. Jimenez JC, Morelle W, Michalsky JC, et al. Excreted/secreted glycoproteins of G intestinalis play an essential role in the antibody response. Parasitol Res. 2007; 100(4):715-20.
18. Honorio-França AC, Carvalho MP, Isaac L, et al. Colostral mononuclear phagocytes are able to kill Enteropathogenic *Escherichia coli* opsonized with colostral IgA. Scand J Immunol. 1997; 46(1):59-66.
19. Keister DB. Axenic culture of *Giardia lamblia* in TYI-S-33 medium supplemented with bile. Trans R Soc Trop Med Hyg. 1983; 77(4):487-8.
20. Hill DR, Pearson RD. Ingestion of *Giardia lamblia* trophozoites by human mononuclear

phagocytes. Infect Immun. 1987; 55(12):3155-61.

21. Fagundes DLG, França EL, Morceli G, et al. The Role of Cytokines in the Functional Activity of Phagocytes in Blood and Colostrum of Diabetic Mothers. Clin Develop Immunol. 2013; 2013:590190.

22. Pick E, Mizel D. Rapid microassays for the measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automatic enzyme immunoassay reader. J Immunol Methods. 1981; 46(2):211-26.

23. França-Botelho AC, Honorio-França AC, França EL, et al. Phagocytosis of *Giardia lamblia* trophozoites by human colostral leukocytes. Acta Paediatr. 2006; 95(4):438-43.

24. França EL, Bittencourt RV, Fujimori M, et al . Human colostral phagocytes eliminate enterotoxigenic *Escherichia coli* opsonized by colostrum supernatant. J Microbiol Immunol Infect. 2011; 44(1):1-7.

25. Hernandes MRG, Albuquerque LC, Ribeiro EB, et al . In vitro immunomodulatory effects of microemulsions with levamisole delivery systems on blood phagocytes interacting with *Giardia lamblia*. Parasitol Int. 2017; 66(3):299-304.

26. Boreham PFL. Giardiasis and its control. Pharm J. 1991; 234: 271–274.

27. Baeza IW, Alcantara-Hernandez M, Mancilla-Herrera, et al. The Role of Lipopeptidophosphoglycan in the Immune Response to *Entamoeba histolytica*. J Biomed Biotechnol. 2010; 2010:254521.

28. Corthésy B. Roundtrip ticket for secretory IgA: role in mucosal homeostasis? J Immunol. 2007; 178(1):27-32.

29. Celada A, Maki RA. Transforming growth factor-βl enhances the M-CSF and GM-CSF-stimulated proliferation of macrophages. J Immunol. 1992: 148(4):1102-5.

30. França-Botelho AC, França JL, Oliveira FMS, et al. Melatonin reduces the severity of experimental amoebiasis. Parasit Vectors. 2011; 4:62.

31. Pereira QLC, Hara CCP, Silva RT, et al . Human colostrum action against *Giardia lamblia*

infection influenced by hormones and advanced maternal age. Parasitol Res. 2018; 117(6):1783-1791.

32. Casares S, Richie TL. Immune evasion by Malaria parasites: a challenge for vaccine development. Currt Opin Immunol. 2009; 21(3):321-30.

33. Pacheco-Yepez J, Jarillo-Luna RA, Gutierrez-Meza M, et al. Peroxynitrite and peroxiredoxin in the pathogenesis of experimental amebic liver abscess. Biomed Res Int. 2014; 2014:324230.

34. Fagundes DLG, França EL,Gonzatti MB, et al . The modulatory role of cytokines IL-4 and IL-17 in the functional activity of phagocytes in diabetic pregnant women. APMIS. 2018; 126(1):56-64.

35. Ribeiro E B, Kelly P, Lanes D, et al. Microemulsions with Levamisole Delivery Systems as Novel Immunomodulating Agents with Potential for Amebiasis Therapies. Science of Advanced Materials. 2015; 7(1): 15–27.

36. Dickson-Gonzalez SM, De-Uribe ML, Rodriguez-Morales AJ. Polymorphonuclear neutrophil infiltration intensity as consequence of *Entamoeba histolytica* density in amebic colitis. Surg Infect (Larchmt). 2009; 10(2):91-7.

37. Chadee K, Heerovitch E. *Entamoeba histolytica*: diffuse liver inflammation in gerbils (*Meriones unguiculatus*) with experimentally induced amebic liver abscesso. J Protozool. 1989; 36(2):154-8.

38. Novelli EL, Rodríguez NL, França EL, et al . High Dietary Carbohydrate and Panceatic lesion. Braz J Med Biol Res. 1993; 26(1):31-6.

39. Espino J, Bejarano I, Redondo PC, et al. Melatonin Reduces Apoptosis Induced by Calcium Signaling in Human Leukocytes: Evidence for the Involvement of Mitochondria and Bax Activation. J Memb Biol. 2010; 233(1-3):105-18.

40. Jiménez JC, Fontaine J, Grzych JM, et al . Systemic and mucosal responses to oral administration of excretory and secretory antigens from *Giardia intestinalis*. Clin Diag Lab Immunol. 2004: 11(1): 152–160.