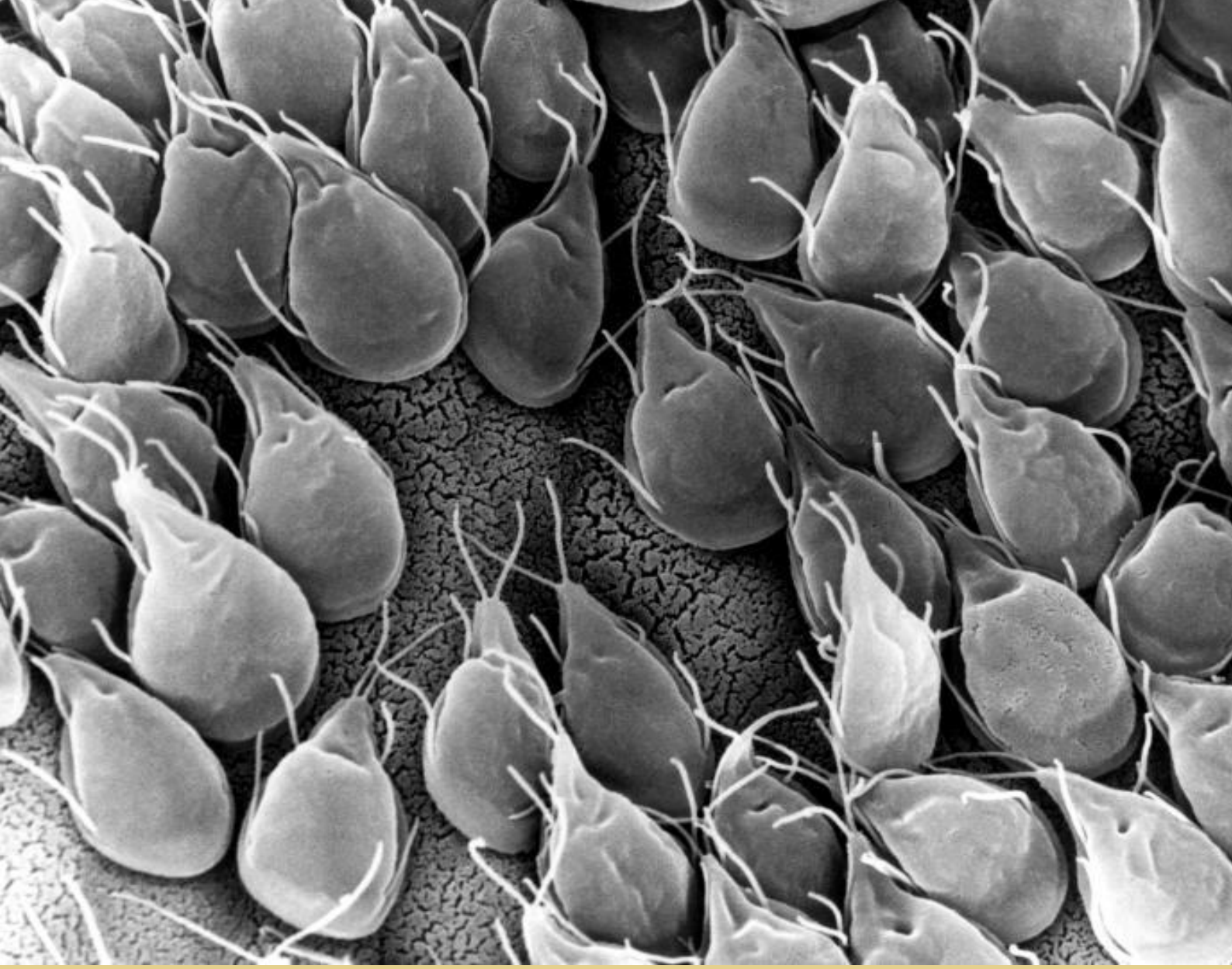

Ágata Filipa Bandeira Lourenço

The interaction of *Giardia lamblia* with macrophage cells: the action of parasite proteases on iNOS, COX-2 and $p65^{RelA}$ inflammatory proteins

Dissertação para a obtenção do grau de Mestre em Investigação Biomédica
sobe a orientação da Doutora Maria do Céu Sousa e co-orientação do Doutor
Henrique Girão apresentada à Faculdade de Medicina de Universidade de Coimbra

2016

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Universidade de Coimbra

Ágata Filipa Bandeira Lourenço

THE INTERACTION OF *Giardia lamblia* WITH MACROPHAGE CELLS: THE ACTION OF PARASITE PROTEASES ON INOS, COX-2 AND P65^{RELA} INFLAMMATORY PROTEINS

Dissertação de Mestrado em Investigação Biomédica, na especialidade de Infeção e Imunidade, apresentada à Faculdade de Medicina da Universidade de Coimbra para a obtenção do grau de Mestre

Orientadores: Doutora Maria do Céu Sousa e Doutor Henrique Girão

Coimbra, 2016

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UNIVERSIDADE DE COIMBRA

O trabalho apresentado nesta dissertação foi realizado no Laboratório de Microbiologia e Parasitologia da Faculdade de Farmácia da Universidade de Coimbra e no Instituto de Imagem Biomédica e Ciências da Vida (IBILI) da Faculdade de Medicina da Universidade de Coimbra, sob a orientação da Doutora Maria do Céu Sousa e do Doutor Henrique Girão, no âmbito do Mestrado em Investigação Biomédica (MIB) da Faculdade de Medicina da Universidade de Coimbra.

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Ágata

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Abbreviations

ADI	Arginine Deaminase
AEBSF	4-(2-aminoethyl)-benzenesulfonyl fluoride
AF	Anterior Flagella
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
BCA	Bicinchoninic Acid
BiP	Binding Immunoglobulin Protein
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium Ion
CD	Cluster of Differentiation
CF	Caudal Flagella
COX-2	Cyclooxygenase-2
CPAF	Chlamydial Protease-Like Activity Factor
CWPs	Cyst Wall Proteins
Cys	Cysteine
DCs	Dendritic Cells
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
DPP	Dipeptidyl Peptidase
DTT	Dithiothreitol
E-64	L-trans-epoxysuccinyl-Lleucylamido-(4-guanidino)-butane
EDTA	Ethylenediamine Tetraacetic Acid
eNOS	Endothelial NOS
ER	Endoplasmic Reticulum
ESVs	Encystation-Specific Vesicles
FAB	Fructose-1,6-biphosphate aldose
FBS	Fetal Bovine Serum
GHSP	*Giardia* Head Stalk Protein
GI	Gastrointestinal

gSPC	Subtilisin-Like Proprotein Convertase
GTA	*Giardia* Trophozoite Antigen
HCNCp	High-Cysteine Non-Variant Cyst Protein
HSPs	Heat-Shock Proteins
IECs	Intestinal Epithelial Cells
IFN-γ	Interferon Gamma
Ig	Immunoglobulin
IKK	Inhibitor of NF-κB Kinase
IL	Interleukin
iNOS	Inducible NOS
iRNA	Interfering RNA
IκB	Inhibitor of NF-κB
LEUP	Leupeptin
LPS	Lipopolysaccharide
MAPKs	Mitogen Activated Protein Kinases
MHC	Major Histocompatibility Complex
mRNA	Messenger RNA
MUC2	Mucin-2
NETs	Neutrophil Extracellular Traps
NF-kB	Nuclear Factor-kB
nNOS	Neuronal NOS
NO	Nitric Oxide
NOS	NO Synthase
OCT	Ornithine Carbamoyl Transferase
PEPA	Pepstatin A
PEPAP	PepA-Penetratin
PHEN	1,10-phenanthroline
PI3K	Phosphoinositide 3-Kinase
PLF	Posterior/Lateral Flagella
PMNs	Polymorphonuclear Leukocytes or Neutrophils
PMSF	Phenylmethylsulfonylfluoride

PPRs	Pattern Recognition Receptors
PVDF	Polyvinylidene Difluoride
SALP	Striated Fibre-Assembling-Like Protein
SDS	Sodium Dodecyl Sulfate
Ser	Serine
TBS	Tris-Buffered Saline
Th	T Helper cells
Thr	Threonine
TLR	Toll-Like Receptor
TNF-α	Tumor Necrosis Factor α
UPL	Uridine Phosphorylase
VF	Ventral Flagella
VSP	Variant Surface Proteins

Resumo

Giardia lamblia (*Giardia duodenalis* ou *Giardia intestinalis*) é um dos parasitas intestinais mais comuns do homem que causa doença gastrointestinal. Este parasita não é invasivo, mas consegue subverter/limitar a resposta inflamatória no intestino delgado permitindo a sua colonização. No entanto, o conhecimento dos mecanismos moleculares pelos quais *Giardia* modula as células imunitárias da mucosa intestinal, por exemplo os macrófagos, é limitado. Dado que as proteases de parasitas têm um papel importante nas interações parasita-hospedeiro, nomeadamente como efectores de mecanismos de evasão imunológica, investigamos o seu contributo nos efeitos imunomoduladores de *Giardia*. Para isso, estudaram-se os efeitos de extratos de *G. lamblia* na integridade de três proteínas chave da resposta inflamatória de macrófagos estimulados pelo LPS, iNOS, COX-2 e NF-kB $p65^{RelA}$. Os níveis de iNOS, COX-2 e NF-kB $p65^{RelA}$ foram avaliados por Western Blot e a atividade proteolítica em extractos de parasitas foi detetada por zimografia. Os inibidores selectivos das principais classes de proteases, incluindo a cisteína, serina, aspartato e metaloproteases foram utilizados para caracterizar a atividade proteolítica. Os resultados mostraram que os extratos de *G. lamblia* possuem elevada atividade proteolítica, em particular sobre a subunidade NF-kB $p65^{RelA}$. A zimografia detectou que os trofozoítos contêm, pelo menos, três proteases distintas: uma metaloprotease com 135 kDa, e duas proteases de cisteína com 75 e 63 kDa. Além disso, a clivagem de NF-kB $p65^{RelA}$ foi bloqueada principalmente por fenantrolina, E-64 e leupeptina, inibidores selectivos de metaloproteases e proteases de cisteína, respetivamente. No geral, os nossos resultados demonstram que as proteases presentes nos trofozoitos de *G. lamblia* estão envolvidas na inativação da NF-kB $p65^{RelA}$ dos macrófagos, sendo um possível mecanismo de subversão imunológica. Este conhecimento pode ser utilizado para o desenvolvimento de agentes terapêuticos que tenham como alvo proteases do parasita, permitindo, assim, o tratamento da giardíase. No entanto, são necessários mais estudos sobre o papel preciso das proteases de *Giardia* particularmente sobre os mecanismos pelos quais as células hospedeiras têm acesso a esses produtos de origem parasitária.

Abstract

Giardia lamblia (syn. *Giardia duodenalis* and *Giardia intestinalis*) is in humans one of the most common intestinal parasites responsible for gastrointestinal disease. Although *Giardia* is not an invasive parasite, it dampens the inflammatory response in small intestine by yet unidentified mechanisms, allowing its effective colonization. Given that parasite proteases have been suggested to play important roles in host-pathogen interactions, namely as effectors of immune escape mechanisms we sought to investigate in this work their contribution to *Giardia* immunomodulatory capacities. For this we addressed the direct effects of *G. lamblia* extracts on the integrity of macrophage iNOS, COX-2 and NF-κB $p65^{RelA}$, three key proteins of inflammatory response. The levels of iNOS, COX-2 and NF-κB $p65^{RelA}$ were evaluated by western blot analysis and the detection of proteolytic activities in parasite extracts was analysed by zymography. Selective inhibitors of the main classes of proteases including cysteine, serine, aspartic and metalloproteases were used in order to characterize the proteolytic activity. Our results showed that *G. lamblia* extracts possess high proteolytic activity, particularly over macrophages NF-κB $p65^{RelA}$ subunit. By zymography assays we found that *Giardia* trophozoites contain at least three distinct proteases: a 135 kDa metalloprotease, and two cysteine proteases with 75 and 63 kDa. Moreover, the cleavage of NF-κB $p65^{RelA}$ was mainly blocked by phenantroline, E-64 and leupeptin, selective inhibitors of metalloprotease and cysteine proteases, respectively. Overall our data demonstrate that the proteases present in the infective trophozoite form of *G. lamblia* are involved in host-cell NF-κB $p65^{RelA}$ inactivation, being a possible immune subversion mechanism. This knowledge may be used for the development of chemotherapeutic agents that specifically target parasite proteases allowing therefore the treatment of giardiasis. However, more studies are needed about the precise role of *Giardia* proteases particularly on the mechanisms by which the parasite delivers them to host cells.

Chapter I
Introduction

1. Introduction

Giardia lamblia (syn. *Giardia duodenalis* and *Giardia intestinalis*) is one of the most common intestinal parasites of humans that causes gastrointestinal disease; about 200 million people in Asia, Africa, and Latin America have symptomatic infections.[1] In developed countries, infection rates for giardiasis are about 1-7%, and in developing countries infection rates for *Giardia* fell into the range of 8% to 30%.[1]

Giardia parasites were described for the first time in 1681 by Antony van Leeuwenhoek when examining own diarrhoeal stools under the microscope. The next description only occurs in 1859 when parasites were observed by Vilem Dusan Lambl and described as *Cercomonas intestinalis*. The term *Lamblia intestinalis* was created in 1888 by Raphael Anatole Émile Blanchard. In 1915, Charles Wardell Stiles *et al.* introduced the name *Giardia lamblia* to honour the work done by Professor A. Giard in Paris and Dr. Lambl in Prague. Today, the organisms that infect humans are variously referred to as *G. lamblia*, *G. intestinalis* or *G. duodenalis*, indicating that intense debate continues to surround this intriguing protozoan parasite.[2]

1.1. *Giardia* Taxonomy

Giardia belongs to Phylum Sarcomastigophora, Subphylum Mastigophora (=Flagellata), Class Zoomastigophorea, Order Diplomonadida and Family Hexamitidae.[3]

Currently, six *Giardia* species are accepted by most researchers. Among them, *G. agilis*, *G. ardeae*, *G. muris*, *G. microti* and *G. psittaci* infect various animals, whereas *G. lamblia* infects humans and many mammals. Although *G. lamblia* is the only species found in humans and many other mammals, including pets and livestock, it is now considered a multispecies complex. Historically, allozyme analyses placed all isolates from humans into two genetic assemblages (assemblages A and B) encompassing at least four genetic clusters (groups I to IV). Additional lineages of *G. lamblia* from animals were identified: assemblages C and D from dogs, assemblage E from artiodactyls, assemblage F from cats, and assemblage G from rodents.[1]

1.2. Life Cycle of *Giardia*

Giardia has a relatively simple life cycle that consists of two different stages of structural and biochemical development: the trophozoite, vegetative form (Fig. 1a), which colonizes the host intestine, and the cyst, infective form (Fig. 1b), which is resistant to environmental conditions.[4]

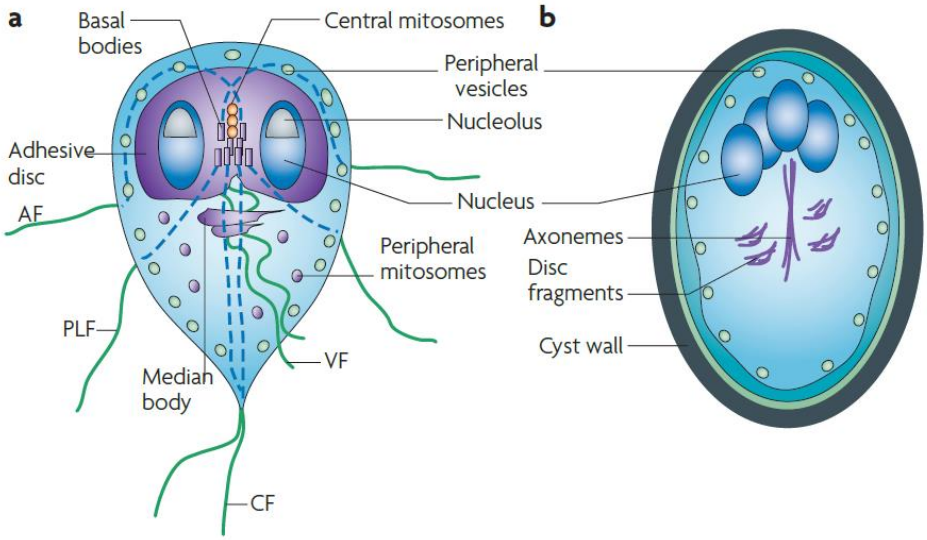


Figure 1 – Trophozoite and cyst of *Giardia*
a – The trophozoite is shown here viewed dorsally. AF: anterior flagella, VF: ventral flagella, PLF: posterior/lateral flagella, CF: caudal flagella.
b – *Giardia* cysts are non-motile and oval shaped, and they measure 8–12 µm long by 7–10 µm wide.
Figure from: Behind the smile: cell biology and disease mechanisms of Giardia species. Nat Rev Microbiol. 2010

1.2.1. Trophozoite

The trophozoites of *G. lamblia* are about 12–15 µm long and 5–9 µm wide. In comparison with other eukaryotes, *Giardia spp*. are unusual in that they have two nuclei and lack mitochondria, peroxisomes and a typical Golgi apparatus. Each trophozoite have eight flagella organized in four pairs: the anterior flagella (AF), ventral flagella (VF), posterior/lateral flagella (PLF) and caudal flagella (CF) (Fig. 1a). The basal bodies are the sites from which the flagella originate. The median body is a microtubular structure of unknown function. The adhesive disc is a large, rigid attachment structure composed of microtubules. There are several central and

peripheral mitosomes in the cell. Peripheral vesicles are lysosome-like vesicles that lie beneath the plasma membrane throughout the cell (Fig. 1a).[5]

The parasite is dependent on high mobility and a strong attachment to the enterocytes in the upper small intestine to avoid peristaltic elimination, and this is accomplished by the adhesive disc and the flagella.[5]

1.2.2. Cyst

Giardia cysts are non-motile and oval shaped, and they measure 8–12 µm long by 7–10 µm wide. Microscopic observations of *G. lamblia* cysts revealed a thick cyst wall and an enclosed excyzoite with four nuclei, ribbon-like microtubule structures extending from the disassembled adhesive disc, and flagella in the centre (Fig. 1b). The cyst wall is 0.3–0.5 µm thick, is lined by a double inner membrane and is mainly composed of N-acetylgalactosamine and three different cyst wall proteins (CWP1, CWP2 and CWP3).[5]

1.2.3. Differentiation

Infection begins with the ingestion of food or water that is contaminated with cysts. Following ingestion, the cyst becomes metabolically active and undergoes excystation. This rapid differentiation process takes place in only 15 minutes.[6]

Excystation is initially triggered by host stomach acids, and the cyst then passes into the small intestine before rupturing. Flagella first appear through an opening in one of the poles of the cyst, followed by the excyzoite body[7] (Fig.2). Cysteine proteases, released from the lysosome-like peripheral vesicles, are thought to have an important role in this process by degrading the cyst wall from the inside.[8]

The liberated excyzoite undergoes cytokinesis twice without intervening S phases, finally producing four trophozoites[9] (Fig. 2). During this division process, the excyzoite increases its metabolism and gene expression, segregates organelles, upregulates proteins associated with motility and assembles the adhesive disc, an attachment organelle specific to *Giardia spp*.(10, 11) The adhesive disc functions as a suction cup and binds surfaces nonspecifically.[11]

Trophozoites are resistant to removal via bulk flow in the intestinal lumen because they can attach to intestinal epithelium. When trophozoites migrate into the lower gastrointestinal tract, they encounter changes in the environment that can trigger encystation.[5]

Encystation is a long differentiation process that results in transformation of the motile trophozoite into the non-motile, infective cyst (Fig. 2). This process is induced in response to host-specific factors such as high levels of bile, low levels of cholesterol and a basic pH.[12] Early in encystation the trophozoite's flagella start to be internalized (Fig. 2). The parasite loses the ability to attach to the intestinal epithelium owing to fragmentation of the adhesive disc,[11] and the differentiating parasite gradually rounds up and enters hypometabolic dormancy.[10]

To date, three cyst wall proteins (CWPs) have been identified (CWP1, CWP2 and CWP3), all of which contain leucine-rich repeats and have positionally conserved cysteine residues.[13-15] A fourth protein, high-cysteine non-variant cyst protein (HCNCp), has been shown to be associated with the cyst wall.[16] Early in encystation, the synthesis of CWPs leads to the formation of new, large encystation-specific vesicles (ESVs).[17] It has been suggested that ESVs are unique, developmentally regulated Golgi-like organelles that are dedicated to the maturation and export of CWPs.[18]

Several enzymes are needed for post-translational modifications of the CWPs; these are protein disulphide isomerases,[19] cysteine proteinases[20, 21] and a Ca^{2+} -binding granule-specific protein.[22] Proteasomes localize close to the ESVs early during encystation and have been suggested to be involved in the maturation of ESV cargo.[23] Morphological studies suggest that ESVs may be a common mechanism for the maturation, transport and deposition of cyst wall components in protozoan parasites,[24] as similar structures have been seen in *Entamoeba invadens* and *Acanthamoeba castellani*.

The cysts are then released from the host through the faeces, and the transmission cycle is completed upon cyst infection of a new host.[5]

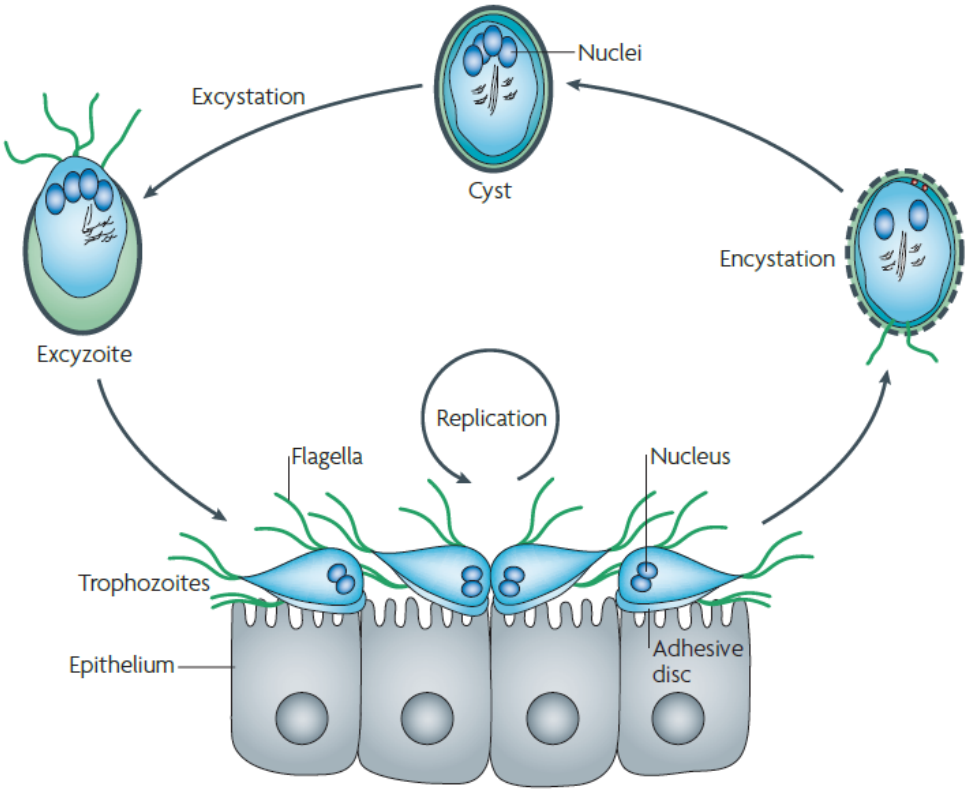


Figure 2 – Life cycle of *Giardia*

Giardia cysts are exposed to gastric acid during their passage through the host's stomach, triggering excystation. This entails a rapid differentiation of cysts into vegetative trophozoites via the short-lived excyzoite stage. Trophozoites attach to the intestinal epithelium with the adhesive disc and divide with a generation time of 6–12 hours *in vitro*. Trophozoites start to encyst *in vivo* when they migrate to the lower part of the small intestine. The cysts have a lower metabolic rate than the trophozoites and are highly resistant to environmental factors, being able to survive for several weeks in cold water outside the host.

Figure from: Behind the smile: cell biology and disease mechanisms of Giardia species. Nat Rev Microbiol. 2010

1.3. Disease Characteristics

G. lamblia is the aetiological agent of giardiasis, one of the most common gastrointestinal diseases worldwide. The clinical manifestations of giardiasis vary from no symptoms to acute or chronic diarrhoea with abdominal pain, flatulence, weight loss, intestinal lesions and malabsorption syndrome that can persist for several months.[(4)]

These symptoms appear 6–15 days after infection and the clinical impact is stronger in young children and in undernourished or immunodeficient individuals. Chronic infections are common but conversely, about half of the infections during epidemics are asymptomatic and the infection frequently resolves spontaneously.[(5)]

Giardia infections tend to be self-limiting in individuals with competent immune systems. A recent study in Brazilian children suggests that symptoms are less severe during re-infection, consistent with the hypothesis that if previous exposure does not always protect against future infections, it does at least reduce the severity of pathology.[25]

In addition to its acute symptoms, giardiasis may also cause anorexia and failure to thrive. Indeed, *G. lamblia* infections may have detrimental effects on nutritional status, growth status and cognitive function in humans.[26, 27]

Recent data indicate that functional gastrointestinal disorders such as irritable bowel syndrome can be associated with a previous *G. lamblia* infection. There is little insight into how *Giardia* spp. cause disease because they are not invasive and there are no information about secreted toxins.[5]

1.4. Pathophysiology of Giardiasis

The pathophysiological consequences of *Giardia* infection are clearly multifactorial, and involve both host and parasite factors, as well as immunological and non-immunological mucosal processes.[28]

After cyst ingestion in contaminated water or food, excystation occurs liberating four trophozoites, which adhere to the epithelial surface of the intestine via a ventral adhesive disk. This tight attachment between *Giardia* trophozoites and intestinal epithelial cells, as well as the production of yet incompletely characterized parasitic products, culminate in the production of diarrhea.[29]

As it is the case with other enteropathogens, induction of apoptosis in enterocytes by *Giardia* represents a key component in the pathogenesis of the infection.[30-32] Enterocytes apoptosis during giardiasis is caspase-3 and -9 dependent.[30, 31] While both host and parasite factors may modulate intestinal epithelial cell apoptosis, the products responsible for its activation during giardiasis have yet to be identified. In addition to promoting increased rates of enterocyte apoptosis, *Giardia* trophozoites may also block enterocyte cell-cycle progression *via* consumption of arginine, and up-regulation of cell-cycle inhibitory genes.[33]

Giardia-mediated increases in intestinal permeability result from alterations to the apical tight junctional complexes, including disruption of F-actin, zonula-occludens-1, claudin-1 and alpha-actinin, a component of the actomyosin ring that regulates paracellular flow.[31, 34-37] The role of *Giardia* proteinases in these effects is a topic of ongoing research.

Giardia-induced diffuse shortening of epithelial brush border microvilli represents a key factor in the production of diarrhoeal disease *via* malabsorption and maldigestion.[38, 39] Whether or not the diffuse loss of microvillous border surface area associated with giardiasis is related to the release of a "toxin" by the parasite, a phenomenon similar to the release of proteases in the bacterial overgrowth syndrome[40], remains poorly understood. Regardless, *G. lamblia* infection causes microvillous shortening in a lymphocyte-mediated manner which in turns impairs activities of disaccharidases.[38]

Bacterial components of the intestinal microbiota from *Giardia*-infected hosts may act as stimulatory factors for protozoan pathogenicity.[41] Indeed, micro-organisms isolated from the duodenal microbiota of patients with symptomatic giardiasis can stimulate the pathogenicity of *G. lamblia* in a gnotobiotic animal model.[41] The biological basis of this phenomenon remains unclear.

1.5. Host-Parasite Interaction

1.5.1. Immune Response to *Giardia*

Although current knowledge regarding the mechanisms of immunity to *Giardia* is limited, several studies have produced significant advances in the understanding of innate and adaptive host responses against the parasite (Fig. 3).[4]

The innate immune system is a rapid, nonspecific first line of defence against colonization by pathogens. *Giardia* primarily colonizes the duodenum and jejunum, both of which are hostile environments that limit the survival of microbes because of their high concentrations of digestive enzymes and bile. Therefore, *Giardia* must constantly adhere and detach to the epithelia to avoid being entrapped by mucus and eliminated by peristaltic movement. In chronic giardiasis, patients lose intestinal

barrier function, which results in reduced secretion of mucus and enzymes which can contribute to the clinical manifestation of the infection (Fig. 3, *1*).[4, 28]

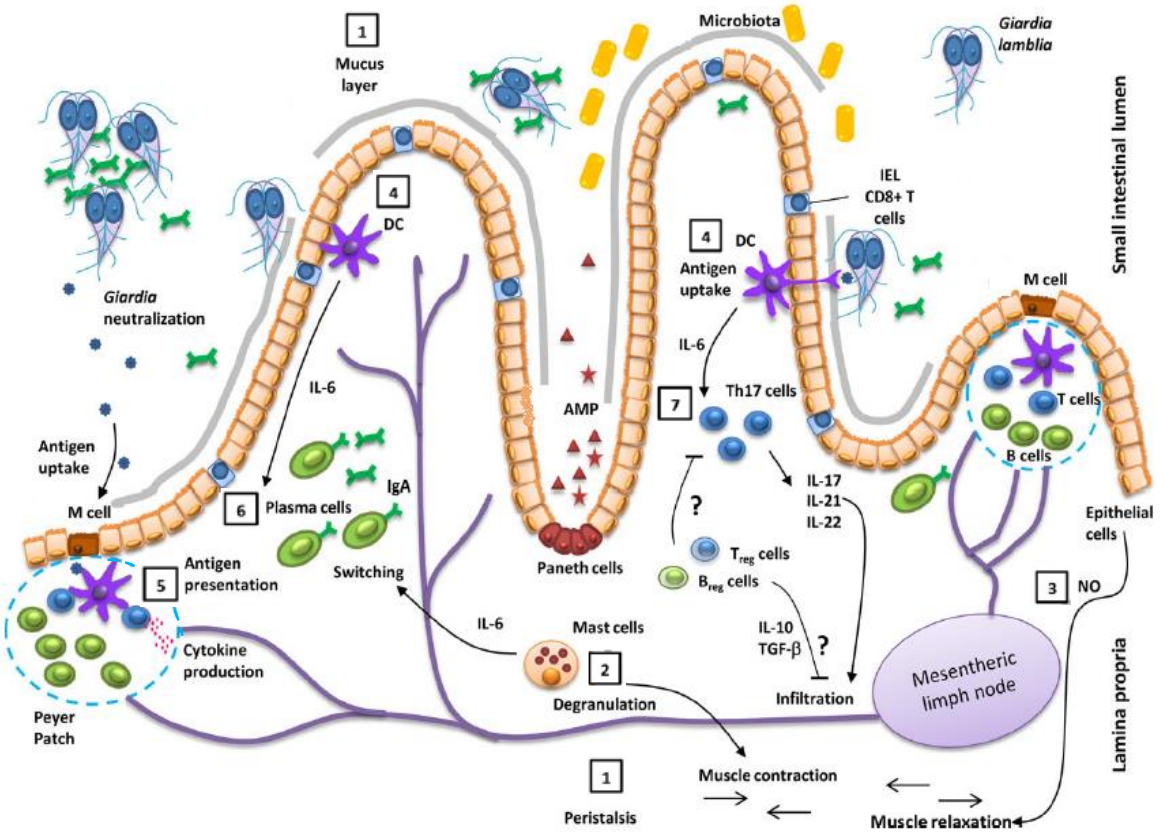


Figure 3 - Host defence mechanisms against *Giardia lamblia*. Innate and adaptive immune systems act in synchrony to control *Giardia* infection.

The mucus layer on the intestine surface and peristaltic movements constitute mechanical barriers to *Giardia* attachment *(1)*. Mast cells release pro-inflammatory cytokines such as IL-6; mast cell degranulation promotes peristalsis *(2)*. Gut epithelial and immune cells produce nitric oxide (NO), which has a cytostatic effect on *Giardia* trophozoites, inhibits excystation/encystation processes and contributes to peristalsis *(3)*. Dendritic cells (DC) play a role as a 'connector' between innate and adaptive immunity and are localized in lamina propria where can recognize antigens. Dendritic cells also can expand their dendrites into the intestinal lumen to take up antigens *(4)*. Dendritic cells ingest and process *Giardia* antigens for further presentation to naive T cells by MHC class II molecules (MHC-II). Activated T cells release a panel of cytokines, which modulate the anti-*Giardia* response *(5)*. IL-6 released by mast cell, DC or T cells is an important modulator of B-cell maturation, and it induces antibody class switching to produce IgA *(6)*. Th17 CD4+ T cells are activated during early adaptive immunity against *Giardia*, and release cytokines such as IL-17, IL-21, IL-22, which play a pro-inflammatory role *(7)*.

Adapted from: Host defences against Giardia lamblia. Parasite Immunol. 2015

Gut epithelial and immune cells can synthesize nitric oxide (NO), which has immunomodulatory and cytotoxic activities (Fig. 3, *3*). NO is produced enzymatically from L-arginine through the action of NO synthase (NOS), which can exist in three different isoforms: neuronal (nNOS), inducible (iNOS) and endothelial (eNOS). The major isoform that is expressed by intestinal epithelial cells, iNOS, is inducible by cytokines and microbial products.[42, 43] It has been observed that patients infected with *G. lamblia* exhibit increased NO levels.[44] Studies conducted *in vitro* have demonstrated that NO inhibits the excystation process and also induces a cytostatic effect on *G. lamblia* trophozoites.[45]

Mast cells are currently recognized as effector cells of the immune response against several parasites. In tissues from the small intestine that have been infected with *G. lamblia*, the most strongly induced transcripts are mast cell proteases.[46] *Mast cells* and NO may act together to induce peristalsis and can also contribute to B-cell survival, activation and differentiation into plasma cells, driving the development of IgA-oriented humoral immune responses (Fig. 3, *2*). The results suggested that mast cells play a significant role in the protective immune response against *Giardia*.[4, 47]

If innate immune mechanisms cannot eliminate the pathogen, it continues growing and expressing and releasing antigens, which triggers adaptive immune responses. Specific humoral and cellular immune responses play a role in *Giardia* clearance. Several reports have indicated that the clearance of *Giardia* in immunocompetent murine models occurs at approximately 3–5 weeks post-infection, which correlates with the establishment of humoral immunity.[42, 48] Host control of *Giardia* requires mucosal humoral and cellular immune responses, such as a balanced response of antigen-specific CD4+ T cells, the release of cytokines, including IL-6, TNF-α, IFN-γ and IL-4, and the production of specific IgA or IgG antibodies against parasite antigens.[4, 49-51]

Dendritic cells (DCs) also play a role in the clearance of giardiasis, although *G. lamblia* is a weak activator of murine and human DCs,[52] *Giardia* lysates, excretory-secretory products and other specific proteins can activate the maturation of DCs by causing increases in the expression of pro-inflammatory cytokines, such as IL-6, TNF-α and IL-12, and of surface molecules, such as CD80, CD86, MHC class II molecules (Fig. 3, *4*).[53] Accordingly, the presence of *Giardia* may activate the maturation and

migration of DC to the infection site and the subsequent release of immunomodulatory cytokines.[4]

Cytokines are small soluble proteins that modulate the differentiation, activation and proliferation of cells. They are produced by a variety of cell types, and several of them possess overlapping functions for regulating innate and adaptive immune responses. The cytokine that is mainly elevated during giardiasis is IL-6, a pro-inflammatory cytokine that induces innate cellular responses and also mediates B-cell switching to IgA and T-cell development into Th17 cells (Fig. 3, *5*).[54, 55] Although there are many sources of this cytokine, DC-derived IL-6 is fundamental to the clearance of *Giardia*. This is probably because the early production of IL-6 by DC is important for T-cell differentiation.[50, 56] The presence of IL-6 during initial contact between DC and T cells could be a key determinant of the type of response that ensues.

TNF-α is another pro-inflammatory cytokine that is released during *Giardia* infection.[57] TNF-α-deficient mice exhibit an obvious delay in the clearance of *Giardia*, suggesting that TNF-α plays an important role in giardiasis.[49] Nevertheless, the mechanisms by which TNF-α affects *Giardia* are still unknown.

IFN-γ and IL-4 are the main cytokines that are produced during Th1 and Th2 responses, respectively, and they appear to play a role in giardiasis because they are elevated in infection models.[51, 57, 58] However, IFN-γ- and IL-4-deficient mice can eliminate the parasite suggesting that neither Th1 nor Th2 responses are absolutely necessary for the clearance of *Giardia*.[59]

It has been established that antibodies, particularly of the IgA isotype, contribute to the maintenance of protective immunity against giardiasis (Fig. 3, *6*). Mice deficient in antibody production, mainly of type IgA, fail to eliminate *G. lamblia*. Therefore, B cells and especially IgA antibodies are important for the control of *Giardia* infections.[4, 60]

T-cell-mediated immune responses play an essential role in the clearance of *Giardia* infections. In humans and animals, a decrease in $CD4^+$ T-cell populations contributes to the development of chronic giardiasis.[59] It is possible that $CD4^+$ T cells can cooperatively induce B-cell activation, maturation and differentiation into plasma cells to produce more specific and efficient immunoglobulins (Fig 3, *7*). B-cell-deficient mice can eliminate the vast majority of parasites when infected with *G. lamblia*,

whereas T-cell-deficient mice are not able to control these infections.[59] Although there is evidence of the important role that T cells play during the course of *Giardia* infection, the mechanisms of action are poorly understood.[4]

1.5.2. *Giardia* Immunogenic Proteins

We still have little knowledge regarding the immunogenic proteins of *Giardia* which can induce protective host humoral and cellular responses. The major proteins (Table 1) that have been identified as target antigens differ in their function and localization in the parasite and are variant surface proteins (VSP), giardins, tubulins (cytoskeletal proteins), heat-shock proteins (HSPs), cyst wall proteins (CWPs) and proteins related to the metabolism of the parasite, such as enolase-a, fructose-1,6-biphosphate aldose (FAB), arginine deaminase (ADI) and ornithine carbamoyl transferase (OCT), among others.[4]

Variant surface proteins are cysteine-rich proteins with molecular masses that range between 20 and 200 kDa. The most characterized VSP is VSPH7, a 56-kDa protein that is highly immunogenic.[61] Mechanisms of antigenic variation in *Giardia* are controlled by interfering RNA (iRNA) and alteration of this pathway generates trophozoites that simultaneously express numerous VSP. Furthermore, gerbils subjected to primary infection with *Giardia* expressing many VSP were protected from subsequent infections,[61] suggesting that antigenic variation is essential for immune evasion.

Giardins are small, structural, constitutive proteins (29–38 kDa) that can be classified into four groups: α-, β-, γ- and δ-giardins. They are associated with microtubules in the ventral disc and plasma membrane of trophozoites.[62] Further studies have demonstrated that α-1-giardin not only stimulates the production of anti-*Giardia* antibodies (IgA and IgG2a) but also establishes protection against posterior challenges.[63]Several metabolic proteins that have been identified in *Giardia* extracts act to activate humoral responses in infected humans and mice. The *Giardia* enzymes ADI and OCT use arginine to generate ATP and are considered to be *Giardia* virulence factors.[64] Arginine depletion modulates the immunophenotypes and cytokine secretions of DC during giardiasis.[65]

Table 1 – Main immunogenic proteins of *Giardia lamblia*

Name	Molecular weight (kDa)	Localization
Structural proteins		
α-1-Giardin	32	Ventral disc
α-2-Giardin	33	Ventral disc
α-7.3-Giardin	33	Ventral disc
α-7.1-Giardin	43*	Ventral disc
α-11-Giardin	35	Ventral disc
SALP-1	27	Ventral disc
β-Giardin	27	Cytoskeleton
α-2-Tubulin	50*	Cytoskeleton
β-Tubulin	55	Cytoskeleton
GHSP-115	115	Intracellular
Metabolism proteins		
ADI	64*	Intracellular
OCT	33-5	Intracellular
FBA	37	Intracellular
UPL-1	38	Intracellular
Enolase	50	Intracellular
Variant surface proteins		
VSPH7	57*	Membrane
VSP9B10, VSP1267, VSPA6, VSPS1, VSPS2, VSPS7, VSPS12 and VSPS6[a]	39–76	Membrane/Intracellular
TSA 417	25	Membrane
Heat-shock proteins		
BIP	71	ER/ESV
Cyst proteins		
CWP1	26	ESV
CWP2	39	Cyst wall
Others		
GTA-1	20	
GTA-2	27	Intracellular

[a] Simultaneous expression by antigenic variation disruption.
SALP, striated fibre-assembling-like protein; GHSP, *Giardia* head stalk protein 115; ADI, arginine deaminase; OCT, ornithine carbamoyl transferase; FBA, fructose-1,6-biphosphate aldose; UPL-1, uridine phosphorylase; VSP, variant surface protein; TSA, trophozoite surface antigen; BIP, binding immunoglobulin protein; CWP, cyst wall protein; GTA, *Giardia* trophozoite antigen; ESV, encystation-specific vesicles; ER, endoplasmic reticulum.
*Data obtained from *Giardia* database (www.giardiadb.org)
Adapted from: Host defences against Giardia lamblia. Parasite Immunol. 2015

Heat-shock proteins are chaperone proteins that are expressed in live cells. HSPs contribute to cell surveillance under conditions of intrinsic or extrinsic stress. The most well-studied HSP is HSP70 (70 kDa), which has been reported in other organisms as being capable of stimulating the innate immune system.[66, 67] Current knowledge regarding *Giardia* HSP70 is limited; they have been used as molecular markers of organelles (endoplasmic reticulum, ER)[68], encystation-specific vesicles (ESV)[23]), life cycle stages (encystation) and giardiasis diagnosis.[69-71]

Cyst wall proteins are expressed during the process of encystation and during the lifetime of a cyst, and they can be classified into two different groups.[4] Group I proteins are expressed during the early stages of encystation and are localized to ESV, whereas group II proteins are localized exclusively into cyst wall surface. The CWP2 protein is found in the cell wall of cysts and when mice were immunized with these, the production of anti-*Giardia* IgA and IgG2a was induced and the cyst release was inhibited.[72]

1.5.3. *Giardia* and Immunomodulation

Accumulating experimental evidence suggests that *Giardia* infections are also capable of modulating pro-inflammatory responses to stimuli via several mechanisms. Observations that *Giardia* infections can protect against the development of diarrheal disease are consistent with the immunomodulatory capabilities of the parasite. Indeed, acute gastrointestinal (GI) inflammatory responses represent a collection of cellular and humoral effector responses and involve a variety of different cell types and mediators; several of these have been shown to contribute to the development of diarrheal disease.[73]

The entire GI tract is lined with a layer of mucus of varying thickness with a structural backbone comprised of mucin glycoproteins dissolved in luminal water. In the intestinal tract, the primary mucus constituent is the mucin-2 (MUC2) protein.[74-76]

Cotton *et al.*[73] research has demonstrated *in vivo* that *Giardia* assemblage B infections in mice damages the small intestinal mucus layer by degrading the MUC2 protein and inducing the hypersecretion of mucus in the small intestine and colon. This culminated in a weakened mucus layer and facilitated disease (unpublished data).[73]

Recent studies have shown that *Giardia* infections may attenuate intestinal polymorphonuclear leukocytes or neutrophils (PMNs) recruitment. *Giardia* assemblage A decreased granulocyte infiltration and cytokines and chemokines involved in PMN recruitment after intra-rectal instillation of pro-inflammatory *Clostridium difficile* toxin A/B.[77] This study was also the first to demonstrate that co-incubation of *Giardia* trophozoites with inflamed colonic mucosal biopsy tissues from patients with active Crohn's disease decreased supernatant levels of numerous pro-inflammatory

mediators, including those involved in PMN recruitment.[77] Further studies went on to identify potential immunomodulatory molecules involved in this process.

The tissue accumulation of PMNs is a hallmark of numerous bacterial, viral, and parasitic infections. PMNs are myeloid-derived innate immune cells essential to host defence against a variety of bacterial and fungal pathogens, and they possess various anti-microbial mechanisms, including the ability to phagocytose infectious agents, secrete anti-microbial proteases, and release neutrophil extracellular traps (NETs).[78]

In the absence of pro-inflammatory stimuli, PMNs are kept in a non-activated state within the bone marrow and circulation. Importantly, PMN infiltration can induce pathophysiological responses that result in water and solute loss and, hence, diarrheal disease, and *in vivo* and *in vitro* experiments have suggested this may involve PMN-mediated intestinal barrier dysfunction and/or anion secretion.[79, 80] Collectively, these results demonstrate the importance PMNs have in contributing to diarrheal disease.

In intestinal epithelial cells (IECs), NO is largely produced by iNOS, and is upregulated following exposure to various host- or pathogen-derived pro-inflammatory stimulatory processes.[81, 82] NO has anti-microbial activity against numerous bacterial and parasitic pathogens,[83, 84] and in vitro experiments have demonstrated NO and its end-products are cytostatic to *Giardia* trophozoites and inhibit their encystation and excystation.[45, 85] Exposure to parasites resulted in the initial upregulation of iNOS mRNA in *in vitro* intestinal epithelial monolayers,[86] but human studies suggest that infection may also result in the downregulation of iNOS expression.[87]

Despite the potential effects of NO on *Giardia* trophozoites, the parasite has developed strategies to evade this host defence mechanism. Trophozoites downregulate the expression of iNOS in intestinal epithelial cells.[86] *Giardia* can inhibit the production of epithelial NO by taking up and consuming arginine as a source of energy.[45, 85] Arginine depletion is known to induce apoptosis in human cell lines.(33, 85) Interestingly, human giardiasis exhibits an increased rate of apoptosis of intestinal epithelial cells, which is one of the pathogenic mechanisms of the disease.[31, 88]

It remains to be determined how the consumption of L-arginine and the concomitant loss of NO during *Giardia* infections may contribute to the modulation of host immune responses and/or susceptibility to co-infecting GI pathogens. Additional

experiments are required to determine how the *Giardia*-mediated inhibition of NO production may, potentially, modulate host inflammatory responses and/or susceptibility to GI infection.[73]

Changes in intestinal epithelial proliferation are essential responses to GI infection and facilitate the removal of damaged cells and/or pathogen-infected cells; however, GI pathogens can alter the kinetics of epithelial cell death and turnover to facilitate their colonization and subsequent invasion.[73]

Giardia infections can inhibit intestinal epithelial proliferation and, subsequently, induce epithelial apoptosis; however, the pathophysiological processes may differ from those of other GI pathogens. L-arginine is involved in cellular proliferation via its conversion into polyamines,[89] and *Giardia* arginine deiminase-mediated consumption of arginine has been associated with the inhibition of in vitro IEC proliferation;[33] this consumption was proposed to reduce intestinal epithelial cell turnover and create a more stable environment for the parasite.[33] Other reports have demonstrated that *Giardia* trophozoites induce IEC apoptosis via the activation of cysteinyl aspartate proteases (caspases) through mechanisms that remain incompletely understood.[30-32] However, it remains to be determined how these pathophysiologic processes induced by *Giardia* potentially modulate host immune responses and their interaction during GI co-infections.

DCs are essential to the induction of adaptive immune responses and/or tolerance. Research to date has produced conflicting results on how *Giardia* trophozoites affect DC activation and their ability to induce and/or modulate effector immune responses. The co-incubation of *Giardia* assemblage B GS/M trophozoite extracts and murine bone marrow-derived DCs *in vitro* resulted in the upregulation of co-stimulatory CD40, and to a lesser extent, CD80 and CD86. Moreover, these extracts altered DC responses to toll-like receptor (TLR) ligands, whereby parasites reduced the expression of MHC Class II, CD80, and C86, decreased the secretion of IL-12, and enhanced IL-10 production via activation of the phosphoinositide 3-kinase (PI3K) pathway.[90] In contrast, separate experiments found that the *Giardia* homolog of immunoglobulin protein (BIP) triggered the expression of MHC Class II molecules and concomitantly resulted in the secretion of TNFα, IL-12, and IL-6 via several pro-

inflammatory signalling cascades in in vitro murine dendritic cells.[53] These results demonstrate that *Giardia* trophozoites are capable of modulating DC cell function.

Very little research has examined how *Giardia* infections modulate macrophage phenotypes during infection, and only one study has shown that *in vivo Giardia* assemblage B infections result in the accumulation of macrophages positive for both iNOS and Arginase-1.[91] Future studies are also required to assess whether a *Giardia*-induced switch to macrophage phenotype, if present, may alter susceptibility to GI co-infection. *Giardia*-induced changes to macrophage phenotypes may significantly affect susceptibility to a variety of infections. For example, the intracellular replication of *Salmonella typhimurium* is greatly impaired in monocyte-derived macrophages with an M1 phenotype.[92] Moreover, macrophage Arginase-1 expression has been found to limit helper Th2-mediated immune responses and fibrosis during *in vivo Schistosoma mansonii* infection.[93] These studies highlight the need for additional research examining the interaction between *Giardia* and host macrophages.

Therefore, future studies need to assess the immunomodulatory mechanisms of *Giardia* infections in the context of the attenuation of diarrheal disease.[73]

1.6. Nuclear Factor-kB (NF-kB)

The transcription factor nuclear factor-kB (NF-kB) is a central element to the innate and acquired immune responses to pathogens, controlling the expression of pro-inflammatory effector molecules and coordinating cellular responses.[94]

The NF-kB pathway is triggered by bacterial and viral infections, as well as pro-inflammatory cytokines, all of which activate the IKK complex (Fig. 4). This complex is composed of two catalytic subunits, IKK-α (also known as IKK1) and IKK-β (also known as IKK2), and a regulatory subunit, IKK-γ (also known as NEMO).[95]

The IKK complex phosphorylates NF-κB-bound IκBs, thereby targeting them for proteasomal degradation and liberating NF-κB dimers that are composed of REL-A (also known as p65), REL (also known as cREL) and p50 subunits to enter the nucleus and mediate transcription of target genes (Fig. 4). This event mostly depends on the catalytic subunit IKK-β, which carries out IκB phosphorylation.[95]

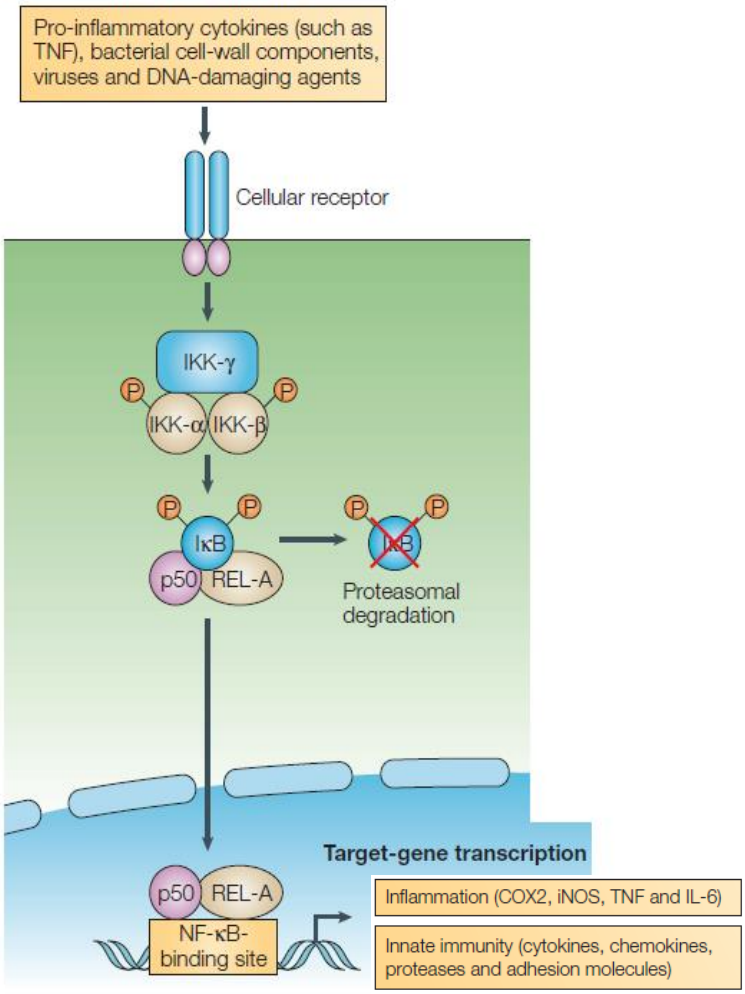


Figure 4 – Signalling pathway that lead to the activation of nuclear factor-κB transcription factor

The pathway is triggered by pro-inflammatory stimuli and genotoxic stress, including the following: cytokines, such as tumour-necrosis factor (TNF) and interleukin-1 (IL-1); bacterial cell-wall components, such as lipopolysaccharide; viruses; and DNA-damaging agents. The stimuli lead to the IKK-β-dependent and IKK-γ-dependent phosphorylation of IκBs, which results in their proteasomal degradation, and to the subsequent liberation of NF-κB dimers (which are mostly p50–REL-A dimers).

COX2, cyclooxygenase-2; iNOS, inducible nitric-oxide synthase

Adapted from: NF-kappaB: linking inflammation and immunity to cancer development and progression. Nat Rev Immunol. 2005

The NF-kB activation induces an expression of proinflammatory cytokines such as TNF-α, IL-6 and cyclooxygenase-2 (COX-2). NF-kB also regulates iNOS expression, which produces the antimicrobial radical NO.[94]

1.6.1. Impairment of NF-KB Pathway as an immune evading mechanism

Cascade signals downstream pattern recognition receptors (PPRs) activation mainly converge to two key signalling pathways: the transcription factor NF-κB and the mitogen activated protein kinases (MAPKs). NF-κB is a cornerstone of innate immunity and inflammatory responses, controlling the expression of effector molecules, such as pro-inflammatory cytokines/chemokines, anti-apoptotic factors and defensins; MAPKs are also signalling cascades intimately connected to the regulation of in numerous aspects of immunity.[96] However, as part of their pathogenic strategies, several microorganisms evade immune system by circumventing, or distorting, these signalling pathways and creating, therefore, conditions that facilitate their replication and spreading in the host. In the last few years great efforts have been made to understand the molecular mechanisms behind this subversion, and various signalling cascades were identified as main targets of pathogens and virulence factors.[96]

Yersinia species use a type III secretion system that allows inject into the cytosol of the host cell six different bacteria outer proteins. These effector proteins interfere with signalling pathways involved in the regulation of the actin cytoskeleton, phagocytosis, apoptosis and the inflammatory response, favouring survival of the bacteria.[97] *Salmonella* delivers effector proteins into host cell, suppressing cellular immune response through blockade of NF-κB and MAPKs cascades.[98] *Shigella flexneri* has evolved the capacity to precisely modulate host cell epigenetic "information", interfering with MAPKs and NF-κB pathways at several points.[99, 100]

The obligate intracellular bacterial parasite *Chlamydia* avoids host inflammatory response, partially by disrupting the NF-κB signal resultant from the PPR recognition of bacterial component such lipopolysaccharide (LPS).[101] This blockage was shown to result from the selective cleavage of the $p65^{RelA}$ subunit of NF-κB by the chlamydial protease-like activity factor (CPAF).[101] Also *E.coli* decreases production of pro-inflammatory cytokines and reduces macrophage bactericidal activity, by targeting NF-κB signal transduction at multiple points.[102] Infection by *E coli* induces a host caspase 3-mediated cleavage of $p65^{RelA}$, by a mechanism not completely defined, but thought to be mediated through the mitochondrial pathway of apoptosis.[102]

However, some pathogens, such *Toxoplasma gondii* and *Leishmania spp* have evolved distinct processes to block this central signalling pathway. *Toxoplasma gondii*

impairs in macrophages the NF-κB signalling, limiting the production of IL-12, TNF-α and NO.[103] This blockage was shown to occur independently of infection-induced IKK-dependent degradation of IκB-α, resulting in specific impairment of NF-κB nuclear translocation. The termination of NF-κB signalling was therefore associated with reduced phosphorylation of p65/RelA subunit, an event involved in the ability of NF-κB to translocate to the nucleus and to bind DNA.[103]

One of the most important virulence factors of *Leishmania* is the surface metalloprotease GP63 that was shown to cleave host protein tyrosine phosphatases.[104] GP63 is also responsible for the observed cleavage of NF-κB $p65^{RelA}$ subunit in *L. mexicana* and *L.infantum* -infected macrophages and dendritic cells. From this cleavage results a fragment of approximately 35 kDa that is rapidly translocated into the nucleus where it has some transcriptional activity. It was postulated that the resulting $p35^{RelA}$ fragment may represent an important mediator by which *Leishmania* induce several chemokines without inducing other NF-κB-regulated genes, such as iNOS and IL-12 that are detrimental for parasite survival.[105, 106]

1.7. Proteases

Proteases, also called peptidases or proteolytic enzymes, constitute a large group of enzymes that catalyse the hydrolysis of peptide bonds. The occurrence of proteases in all living organisms indicates their critical role in essential metabolic and regulatory functions in many biological processes.[107]

Proteases are important in the production of nutrients for growth and proliferation and perform critical regulatory functions in numerous physiological processes since they regulate the fate, localization and activity of many proteins, modulate protein–protein interactions and contribute to the generation, transduction and amplification of molecular signals.[107] A wide span of cellular and metabolic processes, including regulation of gene expression, DNA replication, transport of proteins, cell growth and differentiation, cell cycle, heat shock response, SOS response to DNA damage, misfolded protein response, oxidative stress response and programmed cell death are closely regulated by proteases. Moreover, in animals, proteases are involved in tissue morphogenesis and remodelling, angiogenesis,

neurogenesis, ovulation, fertilization, wound repair, stem cell mobilization, haemostasis, blood coagulation, inflammation, immunity, autophagy and senescence.[107]

Cleavage of peptide bonds can be general, leading to complete degradation of protein substrates into their constituent amino acids, or it can be specific, leading to selective protein cleavage for post-translational modification and processing.[107]

Regardless of their specific roles, this class of enzymes catalyses the cleavage of peptide bonds through a common mechanistic pathway. The aspartic, glutamic, and metalloproteases activate a water molecule that nucleophilically attacks the peptide bond of the protein substrate.[108] The serine, cysteine, and threonine proteases utilize their respective amino acids (Ser, Cys, and Thr) as the nucleophile to attack the peptide bond, and since the nucleophiles come from the protease active site, these proteases can be covalently modified (either reversibly or irreversibly).[108]

Cysteine proteases produced by microorganisms do not only contribute to general protein turnover and nutrient processing, but often constitute important virulence factors during host invasion.[109] Parasitic protists accumulate cysteine proteases in various cellular compartments, like vacuoles, endosomes, lysosomes, cytoplasmic vesicles and granules, in cell membrane and internal membranes, and in Golgi apparatus.[110-113] The vast majority of these proteases exhibit a cathepsin-like structure and exert numerous pathological effects on the host, including hydrolysis of the extracellular matrix, haemoglobin, transferrin and immunoglobulins, release of pro-inflammatory cytokins and kinins, activation or inactivation of the complement system, degradation of the mucus layer, destruction of the colonic epithelium with its accompanying tight junctions and macrophage infection.[110-113]

There is also the downside of proteases as some are important virulence factors of many pathogenic bacteria, parasites and virus. These proteases are involved in acquiring nutrients for growth and proliferation through host tissue degradation. In addition to colonizing and facilitating dissemination functions, they are also involved in evading the host immune system by interrupting the cascade pathways, disrupting the cytokine network, excising cell surface receptors and inactivating host protease inhibitors.[107]

When a given protease has been shown to play a key role in a given infection and/or pathology, one potential therapeutic approach is to target the protease with a small molecule protease-specific inhibitor. Protease inhibitors constitute a very important mechanism for regulating proteolytic activity. They can be classified approximately according to the class of proteases they inhibit (for example, serine or cysteine protease inhibitors).[107, 108]

There are several protease inhibitors that show broad inhibitory specificity and inhibit proteases of different classes.[114, 115] Several inhibit both serine and cysteine proteases (antipain, chymostatin, leupeptin), serine and metalloproteases (bestatin, puromycin), metallo- and cysteine proteases (amastatin) or metallo-, cysteine and serine proteases (bacitracin A). The best known cysteine protease inhibitor is E-64 (1-[*L*-*N*-(*trans*-epoxysuccinyl) leucyl] amino-4-guanidinobutane), an irreversible inhibitor originally isolated from *Aspergillus japonicus* and routinely used as a class-specific cysteine protease inhibitor.[116] The only natural inhibitor of aspartic proteases is pepstatin, originally isolated from various species of actinomycetes,[117] which inhibits several families of aspartic proteases (an hexa-peptide containing the unusual amino acid statine).

1.7.1. *Giardia* Proteases

Protease activity from cultured *Giardia* trophozoites includes cysteine proteases, serine proteases, aspartic proteases, metalloproteases, and aminopeptidases.[118-121]

Cysteine proteases are the main proteases in trophozoites and are localized in endosome-lysosome vacuoles where they function is to degrade endocytosed proteins. The best-characterized roles of *Giardia* cysteine proteases are in the encystation and excystation processes.[108, 122] Ward *et al.*[8] inhibited the excystation process in cultured *Giardia muris* trophozoites with E-64 and three different fluoromethyl ketone-derivatized dipeptides. They demonstrated that cysteine protease activity was localized in cytoplasmic vacuoles, which upon excystation, release their contents into the space between the trophozoite and cyst wall for cyst wall protein degradation. The cysteine protease inhibitors did not affected trophozoite

growth, motility, or cyst viability. A specific cysteine protease, GlCP2, was implicated in the degradation of cyst wall proteins.[8, 108] Dubois and collaborators [20] showed that the GlCP2 gene transcript was also highly expressed in the vegetative and encysting stages of *G. lamblia* trophozoites, and co-localized to encystation-specific vesicles. These vesicles contain the precursor materials needed for cyst wall formation, including cyst wall proteins that need to be proteolytically processed before being incorporated into the cyst wall.[20, 21, 108]

Two serine proteases have also been partially characterized in *Giardia*. One is the membrane associated dipeptidyl peptidase (DPP) IV[123] and the other is the subtilisin-like proprotein convertase (gSPC)[124]. Each may play a role in encystation (DPP IV and gSPC) and excystation (gSPC). *Giardia* DPP IV was identified in cultured trophozoites in the presence of bestatin (aminopeptidases inhibitor). The inhibitor blocked cyst formation and abolished the expression of cyst wall proteins without having an effect on trophozoite growth.[123] The exact function of this protease in trophozoite differentiation into cysts is still unknown. Regarding gSPC its endogenous activity was inhibited using AEBSF (a specific serine protease inhibitor) resulting in arrest of encystation and excystation.[124]

Giardia parasites may actively subvert/limit the inflammatory response in small intestine allowing its effective colonization. However, molecular mechanisms by which *Giardia* parasites modulate innate immune cells of intestinal mucosa such as macrophages remain scarce.

Preliminary results suggest that the interaction of *Giardia* with macrophage leads to impairment of NF-κB $p65^{RelA}$ subunit in LPS-activated macrophages. The mechanisms of this interaction are however not completely understood.[125]

In this context, we propose to study the effects of *Giardia* on the modulation of inflammatory response in LPS-stimulated macrophages having a special focus on iNOS, COX-2 and p65 proteins.

Chapter II
Objectives

2. Objectives

The present work aimed to study the direct effects of *Giardia* protein extracts on the levels of LPS-evoked inflammatory proteins in macrophages.

In view of the main purpose, a work plan was designed with the following objectives:

- Evaluation of the effects of *G. lamblia* extracts on the cleavage of LPS-activated macrophages proteins, in particular of iNOS, COX-2 and NF-κB $p65^{RelA}$, by western blot analysis;
- Identification of proteinase activities in *G. lamblia* by zymographic assays;
- Characterization, with specific inhibitors, of the main classes of proteases present in *Giardia* trophozoites*;*
- Identification of the proteases responsible for the cleavage of LPS-activated macrophages proteins, in particular of iNOS, COX-2 and $p65^{RelA}$

Chapter III
Materials and Methods

3. Materials and Methods

3.1. Chemicals

L-trans-epoxysuccinyl-Lleucylamido-(4-guanidino)-butane (E-64), Leupeptin (LEUP), Pepstatin A (PEPA) and PepA-penetratin (PEPAP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Phenylmethylsulfonylfluoride (PMSF), ethylenediamine tetraacetic acid (EDTA), 1,10-phenanthroline (PHEN) and 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) were purchased from Calbiochem (Darmstadt, Germany).

3.2. *Giardia lamblia* trophozoites culture

Giardia lamblia (strain WB, clone 6 [ATCC 30957]) was obtained from the American Type Culture Collection (Manssasas, VA, USA). Trophozoite forms were maintained in axenic culture at 37°C in 10ml of Keister's modified TYI-S-33 medium[126] (Support Information, Table S.1). Penicillin (100U/ml) and streptomycin (100µg/ml) were added during routine culture.

3.3. Macrophage cell culture

Raw 264.7 is a mouse leukemic monocyte macrophage cell line from American Type Culture Collection (ATCC TIB-71). The cells were growth in Dulbecco's modified Eagle's medium (DMEM) (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% non-inactivated fetal bovine serum (FBS) (Invitrogen, Paisley, UK), 100U/ml penicillin, and 100µg/ml streptomycin (Sigma Chemical Co., St. Louis, MO, USA) at 37°C in a humidified atmosphere containing 95% air and 5% CO_2.

3.4. *Giardia lamblia* trophozoite extracts

Trophozoites were harvested in log-phase growth within 72-96 h postinoculation after chilling in bath ice for 15 min and collect by centrifugation at 500 x g for 5 min at 4°C. Pooled trophozoites were resuspended in 0.25M sucrose and

washed thrice by centrifugation.[120] Suspensions were frozen at −80°C during 1-2 days, thawed at room temperature and disrupted at 4°C by ten 10-s periods of ultrasonic treatment in Vibra Cell sonicator (Sonics & Material INC.). The lysates were centrifuged at 5000 x g, 20 min at 4°C and recovered supernatants were stored at −80°C.[120]

3.5. Macrophage LPS-activation and whole cell protein extraction

Raw 264.7 cells (1 x 10^6) were cultured in 12-well microplates in DMEM growth medium at 37°C for 14h. Following this period, medium was replaced and LPS (1 μg/ml) was used to activate the macrophages (30 min for NF-κB $p65^{RelA}$ and 24h for iNOS and COX2). To obtain the lysates, cells were washed in cold PBS and harvested in RIPA buffer (50 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS and 2 mM EDTA) freshly supplemented with 1 mM DTT, protease and phosphatase inhibitor cocktails (Roche, Mannheim, Germany) and sonicated (three times for 4s at 40 μm peak to peak) in Vibra Cell sonicator (Sonics & Material INC.) to decrease viscosity. The nuclei and the insoluble cells debris were removed by centrifugation at 12000 x g for 10min at 4°C. The postnuclear extracts were collected and used as total cell lysates.

3.6. Protein quantification

Protein concentration of macrophages lysates and *Giardia* extracts was estimated by the bicinchoninic acid protein assay (BCA Protein Assay Reagent Kit, Pierce), according to manufacturer's instructions, employing bovine serum albumin (BSA) as standard (Support Information, Figure S.1). Each sample was assayed in duplicate and blanks were included in all assays.

3.7. Effect of *Giardia* extracts on macrophage inflammatory proteins

Macrophage lysates (40 μg) and *Giardia* extracts (30 μg, 20 μg, 10 μg, 5 μg, 2.5 μg and 1 μg) were thawed and incubated together 1 hour at room temperature. Following this period cells lysates were denatured at 95°C, for 10 min, in sample buffer

(0.125mM Tris, pH 6.8, 2% (w/v) SDS, 100mM DTT, 10% glycerol and bromophenol blue). Western blot analysis for quantification of COX-2, iNOS and NF-κB $p65^{RelA}$ was performed.

3.8. Effects of protease inhibitors on *Giardia* extracts proteolytic activity

In order to determine if *Giardia* proteases are involved on proteolytic cleavage of studied proteins, protease inhibitors were tested in *Giardia* and macrophage interaction assays. EDTA (5 mM), PHEN (10 mM), E-64 (10 μM), LEUP (50 μM), PEPA (1 μM), PEPAP (1 μM), PMSF (1 mM), and AEBSF (1 mM) were incubated with *Giardia* extracts (10 μg) at room temperature during 30 min. Then 30 μg of macrophage lysate were added and incubated for 1 hour at room temperature. Following this period, denaturing buffer was added and samples denatured 6 min at 95°C. Western blot analysis was performed as follow.

3.9. Western blot analysis

Western blot analysis was performed to evaluate the effects of *G. lamblia* extracts on NF-κB signalling pathways and to evaluate the levels of iNOS and COX-2. Proteins were electrophoretically separated on a 10% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, Bedford, MA). The membranes were blocked with 5% (w/v) fat-free dry milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T), for 1 h, at room temperature. Blots were incubated overnight at 4ºC with the primary antibodies against the different proteins to be studied as follow: COX-2 (1:10.000) (Abcam, Cambridge, UK), iNOS (1:1000) (R&D System, Mineapolis, MN, USA) and NF-κB $p65^{RelA}$ (1:1000) (Cell Signaling Technologies, Danvers, MA, USA). After washing three times with TBS-T, membranes were incubated for 2 h at room temperature with alkaline phosphatase-conjugated anti-rabbit or anti-mouse antibodies (1:20.000) GE Healthcare (Chalfont St. Giles, UK). Protein detection was performed using enhanced chemifluorescence system and the membranes were scanned for blue excited fluorescence on the Typhoon imager (GE Healthcare). The generated signals were analysed using software TotalLab TL120, Nonlinear Dynamics.

To demonstrate equivalent protein loading, membranes were stripped and reprobed with anti-tubulin antibody (Sigma Chemical Co., St. Louis, MO, USA).

3.10. Substrate-gel electrophoresis

Proteinase activities in *Giardia* extracts were examined by zymographic assays. The *Giardia* extracts were analysed in 10% SDS-PAGE gels, containing protein substrates as gelatin (0.1%) and BSA (0.1%). *Giardia* extracts (50 µg, 20 µg and 10 µg) were solubilized in zymography sample buffer and loaded on gelatin or BSA copolymerized gels, respectively. Following electrophoresis, gels were renatured by washing twice for 30 min with 2.5% Triton X-100 to remove SDS, washed with distilled water before incubating for 16 h at 37°C in digestion buffer (0.1 M phosphate buffer pH 7.4, 5 mM Ca^{2+}). Gels were stained with Coomassie Blue R250 0.25% in 50% methanol and 10% acetic acid and destaining with 25% methanol and 5% acetic acid solution. Proteolysis was visualized as clear bands against a blue background. Molecular weight marker (NZYTech) was used.

In order to characterize the proteolytic gelatinase activity, *Giardia* extracts (10 µg) were pre-incubated with each one of the protease inhibitors EDTA (5 mM), PHEN (10 mM), E-64 (10 µM), LEUP (50 µM), PEPA (1 µM), PEPAP (1 µM), PMSF (1 mM), and AEBSF (1 mM) and loaded on gels. After electrophoresis and Triton X-100 treatment, the gels were incubated in the digestion buffer supplemented with each inhibitor. The assay was conducted as described above.

3.11. Statistical analysis

The results of Western Blot are expressed as mean ± SEM from at least three independent experiments. The results were analysed by one-way analysis of variance (ANOVA), followed by Dunnett´s test and t test, using GraphPad Prism, version 6.0d (GraphPad Software, San Diego, CA, USA). The significance level was ***p<0.001 and ****p<0.0001, when compared to M (LPS-activate macrophages) and ###P<0.0002 and ####P<0.0001 when compared to G (LPS-activate macrophages with *Giardia* extract).

Chapter IV
Results

4. Results

4.1. *Giardia lamblia* extracts significantly decreases the total levels of NF-κB p65^{RelA} subunit

The effect of *Giardia* extracts on macrophage NF-κB p65^{RelA} was analysed by Western blot using a specific anti-p65^{RelA} antibody. For this, lysates of LPS-activated macrophages (40 µg protein) were treated for 1 hour with different amounts of parasite extracts (30µg, 20µg, 10µg, 5µg, 2.5µg and 1µg) (Fig. 5).

As expected, LPS stimulation of Raw 264.7 cells results in a strong NF-κB p65^{RelA} subunit protein expression (*lane 1*, M). Strikingly, levels of NF-κB p65^{RelA} were strongly decrease in the presence of *Giardia* extracts being the effects significant for 10 µg ($p<0.001$; *lane 4*), 20 µg ($p<0.0001$; *lane 3*) and 30 µg ($p<0.0001$; *lane 2*).

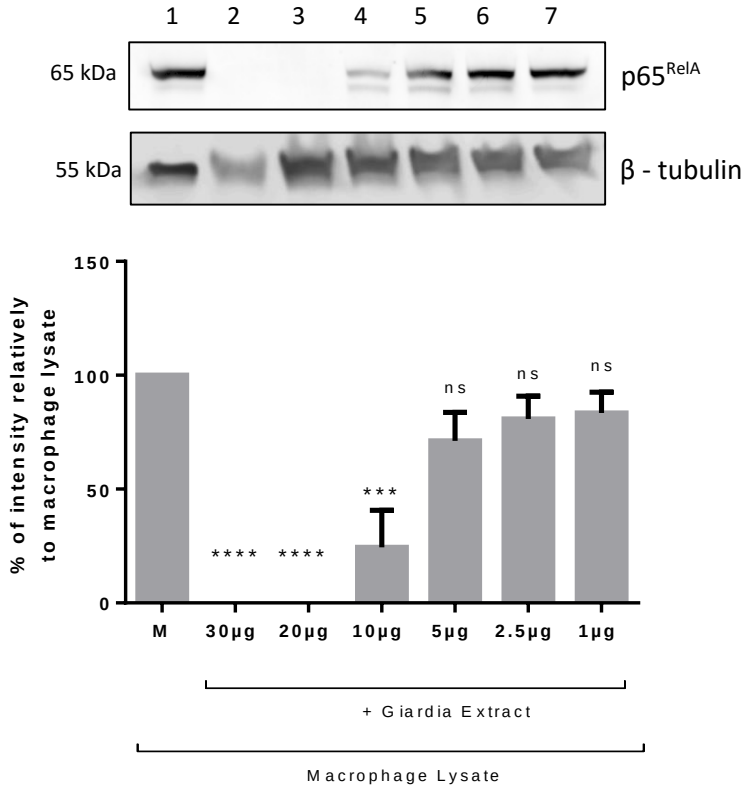


Figure 5 – Effect of *G. lamblia* on the total levels of NF-κB p65^{RelA} in LPS-activate macrophages

LPS-activated macrophages lysates (*lane 1*) were incubated with *G. lamblia* extracts in concentrations of 30 µg (*lane 2*), 20 µg (lane 3), 10 µg (*lane 4*), 5 µg (*lane 5*), 2.5 µg (*lane 6*) and 1.0 µg (*lane 7*). NF-κB p65^{RelA} subunit levels was analysed by Western blot using a specific anti-p65 antibody and anti-tubulin antibody. The blot shown is representative of three blots yielding similar results. Each value represents the mean ± SEM from at least 3 experiments (ns, not significant; ****p <0.0001, ***P <0.001 compared to LPS-activate macrophages, M).

4.2. *Giardia lamblia* extracts does not modify the total levels of iNOS and COX-2

The effect of parasite extracts on macrophage iNOS and COX-2 proteins was also investigated. Similarly to the observed for NF-κB $p65^{RelA}$ expression, LPS treatment caused a strong expression of iNOS (Fig. 6, *lane 1*, M) and COX-2 (Fig. 7, *lane 1*, M) in Raw 264.7 cells. However, contrarily to the observed for NF-κB $p65^{RelA}$ treatment with f *Giardia* extracts (20 µg, 10 µg and 5 µg), does not have significant effects on the total levels of iNOS (Fig. 6, *lanes 2-4*) and COX-2 (Fig. 7, *lanes 2-4*).

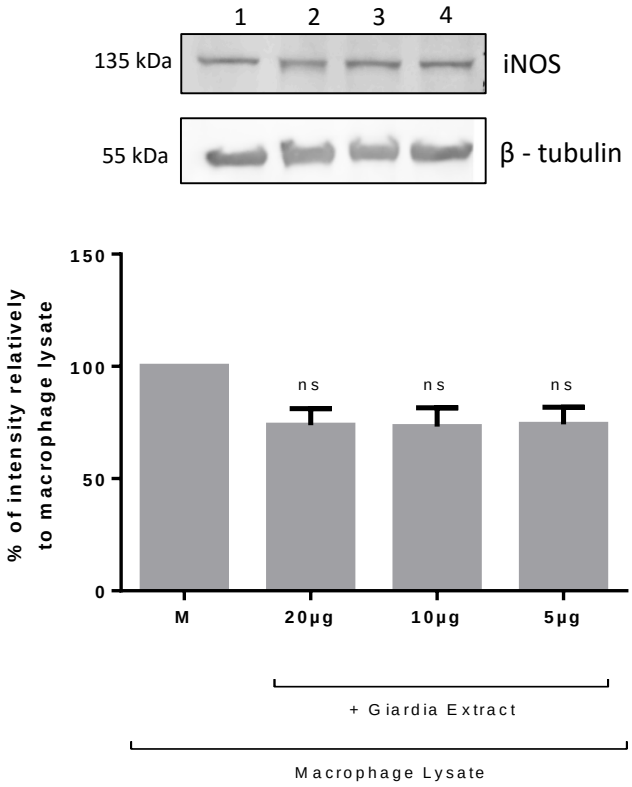


Figure 6 – Effect of *G. lamblia* on the total levels of iNOS in LPS-activated macrophages.
LPS-activated macrophages lysates (*lane 1*) were incubated with *G. lamblia* extracts in concentrations of 20 µg (*lane 2*), 10 µg (*lane 3*) and 5 µg (*lane 4*). iNOS levels was analysed by Western blot using a specific anti-iNOS antibody and anti-tubulin antibody. The blot shown is representative of three blots yielding similar results. Each value represents the mean ± SEM from at least 3 experiments (ns, not significant; M, LPS-activated macrophages).

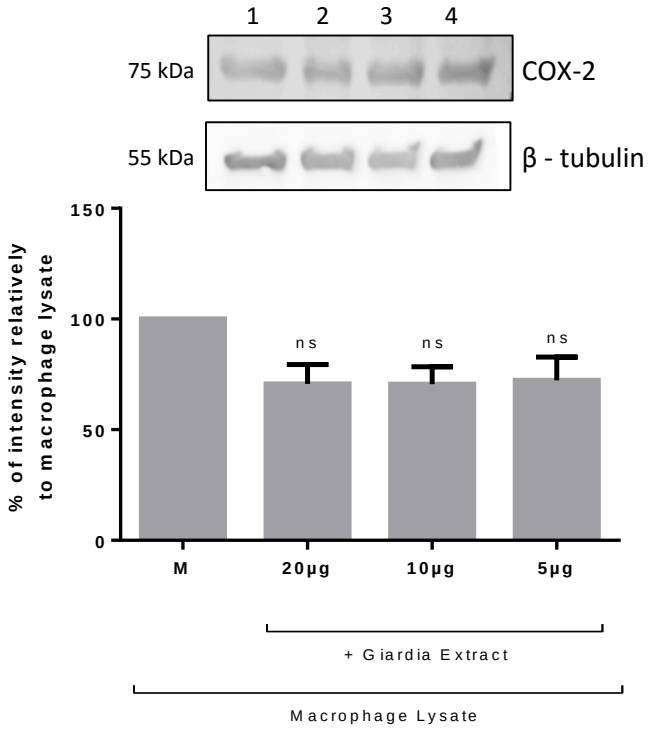


Figure 7 – Effect of *G. lamblia* on the total levels of COX-2 in LPS-activated macrophages.

LPS-activated macrophages lysates (lane 1) were incubated with *G. lamblia* extracts in concentrations of 20 µg (lane 2), 10 µg (lane 3) and 5 µg (lane 4). COX-2 levels w analysed by Western blot using a specific anti-COX-2 antibody and anti-tubulin antibody. The blot shown is representative of three blots yielding similar results. Each value represents the mean ± SEM from at least 3 experiments (ns, not significant; M, LPS-activated macrophages).

4.3. Investigation of proteolytic activity of *Giardia lamblia* extracts

In order to determine whether the effects of *Giardia* on NF-κB p65^{RelA} subunit occurs via degradation by parasite proteases, a serial of experiments were conducted. *Giardia* proteases activity was analysed using proteins impregnated SDS-PAGE. These experiments allowed the identification of subtract and establish the *Giardia* protein concentration to be applied in subsequent work for the protease activity characterization.

The proteolytic activity of *G. lamblia* was identified by SDS-PAGE gels containing gelatin (0.1%) or BSA (0.1%) (data not shown) as substrates and using several protein concentration of parasite extracts (20 µg, 10 µg, 5 µg and 1 µg). Proteinase activity was mainly observed on gelatin gels in concentrations > 10µg (*lanes 1 and 2*) (Fig. 8). The zymogram revealed proteolytic activity, distributed in the migration region of 135 to 63 kDa. Among the profiles, the main pronounced zones of proteolysis were distinguished at 100, 75 and 63 kDa.

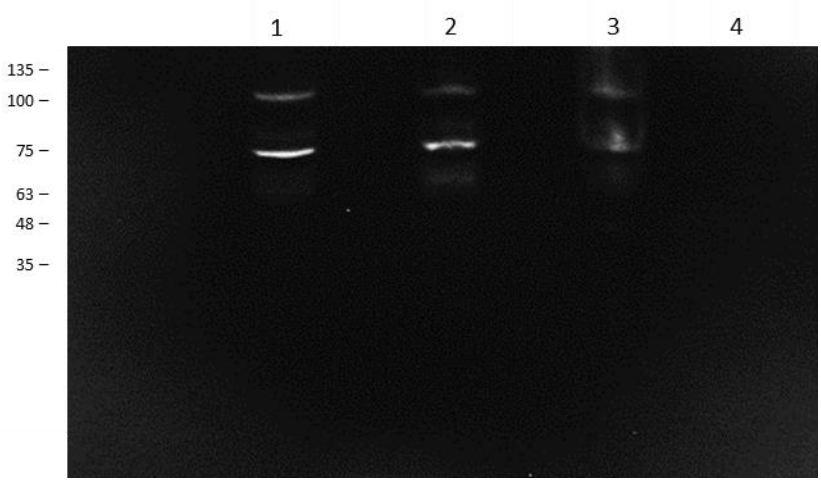


Figure 8 – Gelatin-SDS-PAGE analysis of *G. lamblia* proteases activity. *Giardia* extracts were loaded in the gel at different protein concentrations: 20 µg (*lane 1*), 10 µg (*lane 2*), 5 µg (*lane 3*) and 1 µg (*lane 4*). Molecular mass markers are shown on the left in kiloddaltons (kDa).

4.4. Investigation of the protease classes responsible for *Giardia lamblia* gelatinolityc activity

The characterization of the *Giardia* proteases evolved on gelatinolytic activity, was based on inhibition assays employing inhibitors for the main classes of proteases (cysteine, serine, metallo and aspartic proteases). The effect of inhibitors was evaluated by gelatin zymography using 10 µg of *Giardia* extracts. Samples were incubated with protease inhibitors: EDTA (5 mM), PHEN (10 mM), E-64 (10 µM), LEUP (50 µM), PEPA (1 µM), PEPAP (1 µM), PMSF (1 mM), and AEBSF (1 mM).

As observed in previous experiments, the proteolytic activity was visible at a range from 135 to 63 kDa (Fig. 9 - C, control of proteolytic activity) with prominent bands observed at 100, 75 and 63 kDa. As can be seen in Figure 9, the proteolytic activities were mainly blocked by E-64 (cysteine protease inhibitor) at 100, 75 and 63 kDa, LEUP (cysteine-serine protease inhibitor) at 100 and 75 kDa and PHEN (metalloprotease inhibitor) at 100 kDa.

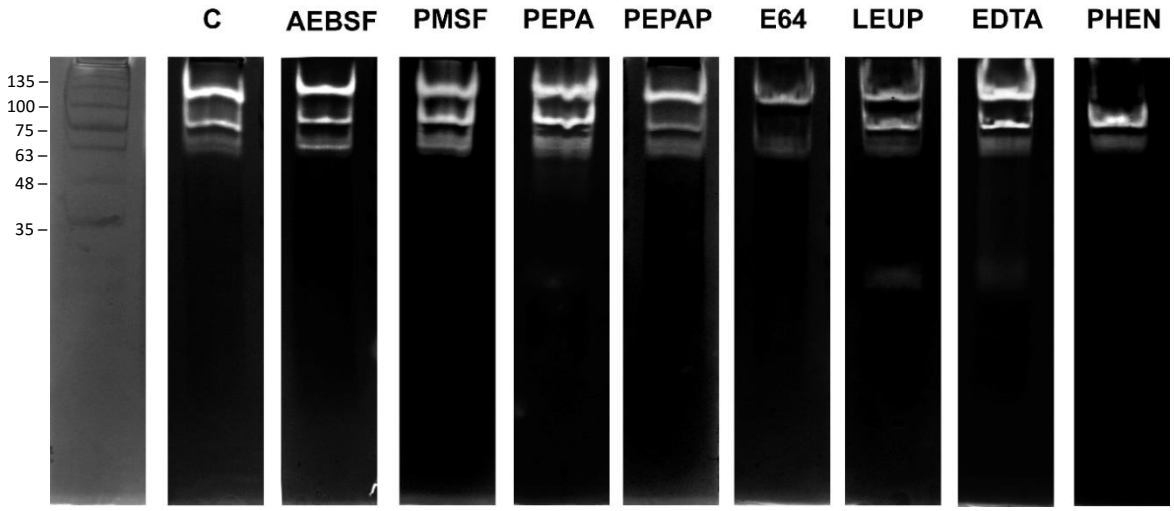


Figure 9 – Gelatin-SDS-PAGE analysis of *G. lamblia* protease activities in presence of protease inhibitors.
Zymograms of *Giardia* lysate 10µg (C; control of proteolytic activity) with protease inhibitors EDTA (5 mM), PHEN (10 mM), E-64 (10 µM), LEUP (50 µM), PEPA (1 µM), PEPAP (1 µM), PMSF (1 mM), and AEBSF (1 mM). Molecular mass markers are shown on the left in kilodaltons (kDa)

4.5. *Giardia lamblia* cysteine, serine and metalloproteases are responsible for NF-κB p65RelA proteolysis

In order to determine whether the effects of *Giardia* on NF-κB $p65^{RelA}$ subunit occurs via degradation by parasite proteases, we addressed the interaction of *Giardia*-macrophage in the presence of protease inhibitors.

The effect of protease inhibitors in NF-κB $p65^{RelA}$ proteolysis was studied using *Giardia* extracts (10 µg of protein) and LPS-activated macrophages (30 µg of protein), pre incubated with protease inhibitors: EDTA (5 mM), PHEN (10 mM), E-64 (10µM), LEUP (50µM) (Fig. 9), PEPA (1µM), PEPAP (1µM), PMSF (1mM), and AEBSF (1mM) (Fig. 10).

As expected, interaction of LPS-activated macrophages with *Giardia* extract results on a significant decrease of NF-κB $p65^{RelA}$ ($p<0.0001$ (Fig. 10 C-, G). When protease inhibitors were used, the NF-κB $p65^{RelA}$ proteolysis was significantly reversed by the metalloprotease inhibitor PHEN ($p<0.001$) (Fig. 10, *lane 2*), the cysteine inhibitor E-64 ($p<0.0001$) (Fig. 10, *lane 3*) and the cysteine-serine inhibitor LEUP ($p<0.0001$) (Fig. 10, *lane 4*).

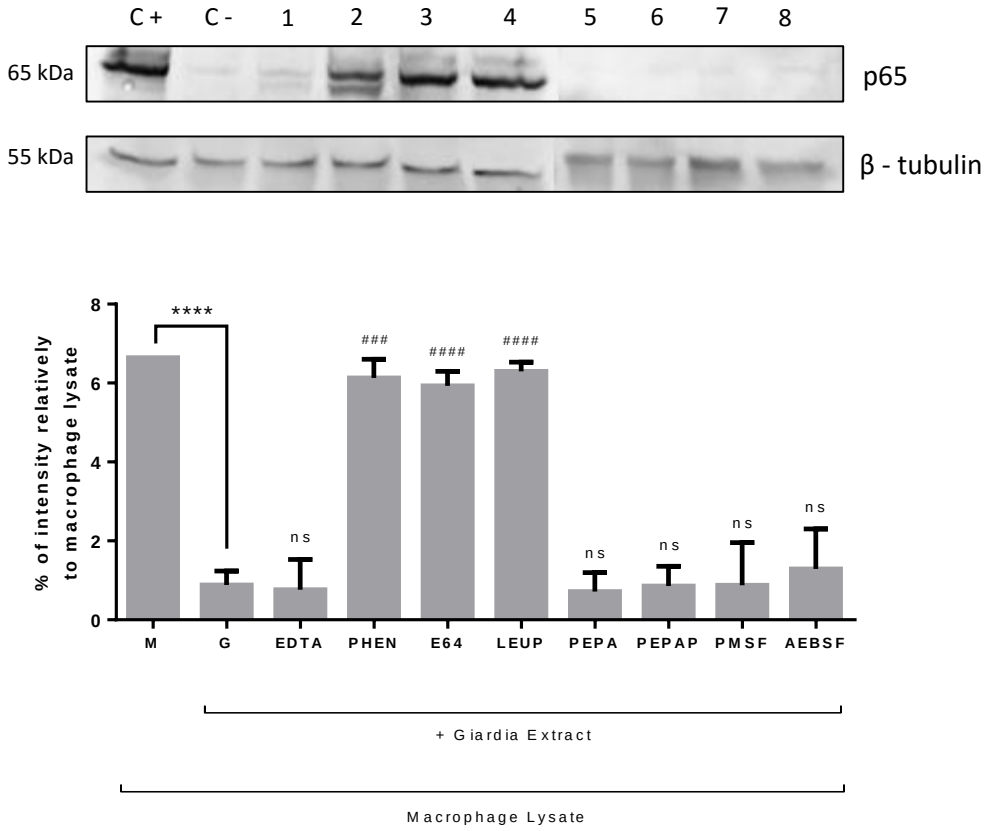


Figure 10 – Western blot analysis of total levels of NF-κB p65RelA in LPS-activate macrophages treated with *G. lamblia* extracts and protease inhibitors.
LPS-activated macrophages lysates (M, C+) were incubated with *G. lamblia* extracts in concentrations of 10 µg (G, *lane C*-) and with protease inhibitors EDTA (*lane 1*), PHEN (*lane 2*), E-64 (*lane 3*) and LEUP (*lane 4*), PEPA (*lane 5*), PEPAP *(lane 6*), PMSF (*lane 7*) and AEBSF (*lane 8*). NF-κB p65RelA subunit levels was analysed by Western blot using a specific anti-p65 antibody and anti-tubulin antibody. The blot shown is representative of three blots yielding similar results. Each value represents the mean ± SEM from at least 3 experiments (ns, not significant; ****p <0.0001, LPS-activated macrophages (M) vs *Giardia* treated extracts (G), M; ###p <0.001, ####p <0.0001, inhibitors vs G).

Chapter V
Discussion

5. Discussion

Recent evidences suggested that *Giardia* parasites may actively subvert/limit the inflammatory response in small intestine allowing its effective colonization.[4, 73] One of the hypothesis proposed suggests that *Giardia* may compromise NF-κB signalling pathway in macrophages resulting in attenuation of COX-2 and iNOS expression. The inhibition of LPS-induced iNOS expressed by Giardia impairs NO production, *in vitro*.[125]

However, the molecular mechanisms by which *Giardia* parasites modulate innate immune cells of intestinal mucosa such as macrophages remain scarce. Therefore, we sought to investigate the effects of *Giardia* lysates on inflammatory response of LPS-stimulated macrophages having a special focus on iNOS, COX-2 and NF-κB p65 inflammatory proteins.

We first addressed the direct effects of *Giardia* on the cleavage of macrophage NF-κB $p65^{RelA}$ subunit. For this, lysates from LPS-activated macrophages were exposed to *Giardia* extracts and analysed by western blot for NF-κB $p65^{RelA}$ quantification. We demonstrated that 10 µg of *Giardia* protein extract were sufficient to cause a decrease of about 80% on the levels of NF-κB $p65^{RelA}$ subunit and higher concentrations lead to total disappearance of $p65^{RelA}$. This result led us hypothesize that *Giardia* may contain proteases that directly cleave NF-κB $p65^{RelA}$ causing its inactivation. This is consistent with a preliminary report on *Giardia* capacity to cleave NF-κB $p65^{RelA}$ [125]. Accordingly, numerous microorganisms as *Toxoplasma gondii,*[103] *Chlamydia*,[101] *Samonella*,[98] *Shigella flexneri*[99, 100] *E. coli*[102] and *Leishmania*[105, 106] have as immune evading mechanism the impairment of NF-κB signalling pathway. The NF-kB pathway is a central element in immune responses to infection given that its activation induces the expression of pro-inflammatory cytokines and coordinates cellular responses in the presence pathogens. Therefore the blockage or inhibition of this pathway facilitates parasite replication and spreading in the host.

As we observed a cleavage of NF-κB $p65^{RelA}$ subunit by *Giardia* extracts, we addressed if the same occurs with pro inflammatory proteins such as iNOS and COX-2. In literature there are evidences that several pathogens such as *Toxoplasma gondii*[103] and *Leishmania spp*.,[105, 106] have the ability to downregulate pro inflammatory

proteins, creating conditions that facilitate their permanence in the host. Furthermore, preliminary results from our lab showed that, *in vitro*, infection of LPS-stimulated macrophages with *Giardia* strongly impairs NO production, suggesting a possible interference with iNOS.[125] However, contrary to the observed for $p65^{RelA}$ subunit, we found no degradation of iNOS or COX-2 when macrophage extracts were exposed to the different concentrations of *Giardia* proteins.

We then focus our attention on the cleavage of $p65^{RelA}$ subunit and on the possible molecules present in *Giardia* extract that can be responsible for this activity. The production of proteolytic enzymes by parasites have attracted considerable interest of researchers given that they can mediate crucial events of host-pathogen interactions. With respect to *Giardia*, non-invasive protozoa, the fact that trophozoites contain and/or release proteases appears to be of special importance in giardiasis pathogenesis. There are evidences that these enzymes may be implicated in epithelial injury during the infection[121].

In order to study the putative proteinase activities in *Giardia* we performed zymographic assays with *Giardia* extracts at different concentrations, using BSA and gelatin as substrates. We only detected proteolytic activity over gelatin subtract, being this result consistent with previous findings reported by Coradi *et al.* and another authors[120, 121, 127]. Our results revealed proteolytic activity at a range from 135 to 63 kDa, with at least three prominent bands observed at 100, 75 and 63 kDa.

With the purpose to define the class of proteases responsible for this gelatinolytic activity, *Giardia* extracts were incubated with inhibitors of the main classes (cysteine, serine, aspartic and metalloproteases) and zymographic analysis was performed. We observed that the bands of proteolytic activity were differently affected by the inhibitors used. The cysteine-serine protease inhibitor LEUP slightly reduced the activity of the 3 observed bands similarly to the specific cysteine protease inhibitor E64. However, E64 showed a prominent effect over the 75 kDa band causing its complete inhibition. This can indicate that the protease present here may be of the cysteine class. Accordingly, several studies reported the blockade of *Giardia* gelatin hydrolysis by cysteine and serine proteases inhibitors as E-64 and LEUP.[120, 121, 127] Finally, phenantroline (PHEN), a ZN^{2+} chelating agent, specifically blocked the activity of the band at 135 kDa. All the other inhibitors tested do not caused substantial

effects. These results indicate that the main proteases present in *Giardia* extract belong to cysteine and metalloprotease classes.

Protease activity in cultured trophozoites is dominated by cysteine proteases (localized in endosome-lysosome vacuoles) and these cysteine proteases are well characterized in the encystation and excystation processes.[108, 122] Therefore it is reasonable that the main proteolytic activity observed in our experiments came from cysteine and serine protease, given that these proteases are essential for the parasite life cycle and differentiation and maybe are in larger amount. Ward and collaborators[8] showed that cysteine protease inhibitors inhibited the excystation process in cultured *Giardia muris* trophozoites , while trophozoite growth, motility or cyst viability were not affected. Despite the cysteine and serine proteases are important for encystation and excystation, this fact shows that maybe there are other proteases that which may be involved in *Giardia* intestinal colonization and proliferation.

We next addressed how the different protease inhibitors affect the degradation of macrophage NF-κB $p65^{RelA}$ subunit. For that, *Giardia* lysates were incubated with each one of protease inhibitors and then macrophage extract was added, being the effects analysed by western blot. We demonstrated that the cleavage of NF-κB $p65^{RelA}$ subunit was completely reverted by protease inhibitors E-64, LEUP and PHEN. This is consistent with the results obtained in zymography assays. We can then conclude that *Giardia* cysteine, serine and metalloproteases are involved on NF-κB $p65^{RelA}$ proteolysis.

Proteases are important virulence factors of many pathogenic bacteria, parasites and virus. These proteases are involved in acquiring nutrients for growth and proliferation through host tissue degradation. In addition to colonizing and facilitating dissemination functions, they are also involved in evading the host immune system by interrupting the cascade pathways, disrupting the cytokine network and excising cell surface.[108] Cysteine proteases have been associated with the pathogenesis of trypanosomiasis; cruzipain of *Trypanosoma cruzi* and brucipain or rhodesain of *Trypanosoma brucei*. Cruzipain (or cruzain) has been extensively studied since it is critical for parasite viability in all stages of infection, especially for nutrition acquisition, tissue invasion and host immune response evasion.[128] Other cysteine proteases have

been implicated in the pathogenesis of *Leishmania* species and are major virulence factors as they substantially modify the immune response.[129] Surface metalloprotease GP63 has been shown to be essential for establishing and maintaining the infection and responsible for the observed cleavage of NF-κB $p65^{RelA}$ subunit in *L. mexicana* and *L. infantum* -infected macrophages and dendritic cells.[130] The transcriptional activation is impaired and consequently the ability of the macrophage to mount a pro-inflammatory IL-12-driven response is also impaired.[131]

Another studies showed that *L. infantum* promastigotes was unable to activate the NF-κB transcription factor, as shown by the inability of parasites to promote phosphorylation and posterior degradation of the NF-κB inhibitory protein, IκB-α.[106] The parasites actively counteracted the LPS-triggered activation of NF-κB cascade by reducing IκB-α phosphorylation and extensively cleaving the NF-κB $p65^{RelA}$ subunit, resulting in a major new fragment of approximately 35 kDa.[106]

Attending to our results, we think that potentially *Giardia* can promote proteolytically cleave of NF-Kb $p65^{RelA}$ subunit resulting in non-transcription of target genes that induces an expression of pro-inflammatory cytokines, impairing recruitment of several chemokines such TNF-α, IL-6, COX-2 and iNOS.

Blocking parasite proteases may therefore be an attractive pharmacological approach given that this will impair parasite evasion of immune system and lead to a most efficient elimination.

As *Giardia* are extracellular parasites the effects of their proteases over cytosolic proteins such as NF-κB $p65^{RelA}$ may be the consequence of their release as soluble forms or included in secreted/excreted vesicles. Through reported studies we know that parasites as *Leishmania* and *Trypanosoma cruzi* produce extracellular vesicles in response to external factors as host inflammatory immune response. Silverman and co-workers[132] described the release of exosome-like vesicles by promastigotes of *Leishmania donovani*. Exosomes are delivered to the cytoplasm of infected macrophages, as a response to the environmental changes between the insect midgut and the acidic vacuole in vertebrate macrophages. Moreover, the authors demonstrated that these *Leishmania* exosomes have an immunosuppressive effect on host cells.[133] Microvesicles release by *T. cruzi* were first described in tissue-culture-derived trypomastigotes, as a mechanism of spontaneously shedding of

surface antigens into the culture medium.[134] These vesicles released spontaneously are adsorbed to non-infected mammalian cell membranes, increasing the expression of extracellular matrix components that will facilitate infection.[135] Moreover pre-immunization of mice using trypomastigote microvesicles induce severe heart pathology with intense inflammatory reaction and higher number of amastigote nests in cardiac tissue.[136]

The role of extracellular vesicles in *Giardia* infectivity and capacity to modulate host immune system is still poorly understood. We believe that *Giardia* may release vesicles/exosomes containing pathogen effectors such as the proteases identified in present work. The endocytosis of these vesicles by epithelial and phagocytic cells may then promote important events such as the impairment of intracellular signalling pathways. We are currently exploring this hypothesis at our lab.

Overall, our results demonstrate that *Giardia* extracts significantly decrease the total levels of NF-κB p65RelA subunit in macrophages, a mechanism dependent on parasite cysteine, serine and metalloproteases. More experiments are needed to clarify the role of these proteases in *Giardia* infections and their exploitation as potential chemotherapeutic targets for the treatment of giardiasis. On the other hand, these proteases, due their anti-inflammatory activity, could have potential to be used for the attenuation of diarrheal and of intestinal inflammatory diseases.

References

References

1. Feng Y, Xiao L. Zoonotic Potential and Molecular Epidemiology of *Giardia* Species and Giardiasis. Clinical Microbiology Reviews. 2011;24(1):110-40.

2. Ford BJ. The Discovery of *Giardia*. The Microscope. 2005;53(4):147-53.

3. Plutzer J, Ongerth J, Karanis P. *Giardia* taxonomy, phylogeny and epidemiology: Facts and open questions. Int J Hyg Environ Health. 2010 Sep;213(5):321-33. PubMed PMID: 20619729. Epub 2010/07/14. eng.

4. Lopez-Romero G, Quintero J, Astiazaran-Garcia H, Velazquez C. Host defences against *Giardia lamblia*. Parasite Immunol. 2015 Aug;37(8):394-406. PubMed PMID: 26072999. Epub 2015/06/16. eng.

5. Ankarklev J, Jerlstrom-Hultqvist J, Ringqvist E, Troell K, Svard SG. Behind the smile: cell biology and disease mechanisms of *Giardia* species. Nat Rev Microbiol. 2010 Jun;8(6):413-22. PubMed PMID: 20400969. Epub 2010/04/20. eng.

6. Hetsko ML, McCaffery JM, Svard SG, Meng TC, Que X, Gillin FD. Cellular and transcriptional changes during excystation of *Giardia lamblia* in vitro. Exp Parasitol. 1998 Mar;88(3):172-83. PubMed PMID: 9562420. Epub 1998/04/30. eng.

7. Buchel LA, Gorenflot A, Chochillon C, Savel J, Gobert JG. In vitro excystation of *Giardia* from humans: a scanning electron microscopy study. J Parasitol. 1987 Jun;73(3):487-93. PubMed PMID: 3598798. Epub 1987/06/01. eng.

8. Ward W, Alvarado L, Rawlings ND, Engel JC, Franklin C, McKerrow JH. A primitive enzyme for a primitive cell: the protease required for excystation of *Giardia*. Cell. 1997 May 2;89(3):437-44. PubMed PMID: 9150143. Epub 1997/05/02. eng.

9. Bernander R, Palm JE, Svard SG. Genome ploidy in different stages of the *Giardia lamblia* life cycle. Cell Microbiol. 2001 Jan;3(1):55-62. PubMed PMID: 11207620. Epub 2001/02/24. eng.

10. Paget TA, Macechko PT, Jarroll EL. Metabolic changes in *Giardia intestinalis* during differentiation. J Parasitol. 1998 Apr;84(2):222-6. PubMed PMID: 9576491. Epub 1998/05/12. eng.

11. Palm D, Weiland M, McArthur AG, Winiecka-Krusnell J, Cipriano MJ, Birkeland SR, et al. Developmental changes in the adhesive disk during *Giardia* differentiation. Mol Biochem Parasitol. 2005 Jun;141(2):199-207. PubMed PMID: 15850703. Epub 2005/04/27. eng.

12. Lauwaet T, Davids BJ, Reiner DS, Gillin FD. Encystation of *Giardia lamblia*: a model for other parasites. Curr Opin Microbiol. 2007 Dec;10(6):554-9. PubMed PMID: 17981075. PMCID: PMC2709507. Epub 2007/11/06. eng.

13. Mowatt MR, Lujan HD, Cotten DB, Bowers B, Yee J, Nash TE, et al. Developmentally regulated expression of a *Giardia lamblia* cyst wall protein gene. Mol Microbiol. 1995 Mar;15(5):955-63. PubMed PMID: 7596296. Epub 1995/03/01. eng.

14. Lujan HD, Mowatt MR, Conrad JT, Bowers B, Nash TE. Identification of a novel *Giardia lamblia* cyst wall protein with leucine-rich repeats. Implications for secretory granule formation and protein assembly into the cyst wall. J Biol Chem. 1995 Dec 8;270(49):29307-13. PubMed PMID: 7493963. Epub 1995/12/08. eng.

15. Sun CH, McCaffery JM, Reiner DS, Gillin FD. Mining the *Giardia lamblia* genome for new cyst wall proteins. J Biol Chem. 2003 Jun 13;278(24):21701-8. PubMed PMID: 12686559. Epub 2003/04/11. eng.

16. Davids BJ, Reiner DS, Birkeland SR, Preheim SP, Cipriano MJ, McArthur AG, et al. A new family of giardial cysteine-rich non-VSP protein genes and a novel cyst protein. PLoS One. 2006;1:e44. PubMed PMID: 17183673. PMCID: PMC1762436. Epub 2006/12/22. eng.

17. Reiner DS, Douglas H, Gillin FD. Identification and localization of cyst-specific antigens of *Giardia lamblia*. Infect Immun. 1989 Mar;57(3):963-8. PubMed PMID: 2917795. PMCID: PMC313206. eng.

18. Marti M, Hehl AB. Encystation-specific vesicles in *Giardia*: a primordial Golgi or just another secretory compartment? Trends Parasitol. 2003 Oct;19(10):440-6. PubMed PMID: 14519581. Epub 2003/10/02. eng.

19. Knodler LA, Noiva R, Mehta K, McCaffery JM, Aley SB, Svard SG, et al. Novel protein-disulfide isomerases from the early-diverging protist *Giardia lamblia*. J Biol Chem. 1999 Oct 15;274(42):29805-11. PubMed PMID: 10514458. Epub 1999/10/09. eng.

20. DuBois KN, Abodeely M, Sakanari J, Craik CS, Lee M, McKerrow JH, et al. Identification of the major cysteine protease of *Giardia* and its role in encystation. J Biol Chem. 2008 Jun 27;283(26):18024-31. PubMed PMID: 18445589. PMCID: PMC2440617. Epub 2008/05/01. eng.

21. Touz MC, Nores MJ, Slavin I, Carmona C, Conrad JT, Mowatt MR, et al. The activity of a developmentally regulated cysteine proteinase is required for cyst wall formation in the primitive eukaryote *Giardia lamblia*. J Biol Chem. 2002 Mar 8;277(10):8474-81. PubMed PMID: 11773053. Epub 2002/01/05. eng.

22. Touz MC, Gottig N, Nash TE, Lujan HD. Identification and characterization of a novel secretory granule calcium-binding protein from the early branching eukaryote *Giardia lamblia*. J Biol Chem. 2002 Dec 27;277(52):50557-63. PubMed PMID: 12354777. Epub 2002/10/02. eng.

23. Stefanic S, Palm D, Svard SG, Hehl AB. Organelle proteomics reveals cargo maturation mechanisms associated with Golgi-like encystation vesicles in the early-diverged protozoan *Giardia lamblia*. J Biol Chem. 2006 Mar 17;281(11):7595-604. PubMed PMID: 16407213. Epub 2006/01/13. eng.

24. Chavez-Munguia B, Omana-Molina M, Gonzalez-Lazaro M, Gonzalez-Robles A, Cedillo-Rivera R, Bonilla P, et al. Ultrastructure of cyst differentiation in parasitic protozoa. Parasitol Res. 2007 May;100(6):1169-75. PubMed PMID: 17252271. Epub 2007/01/26. eng.

25. Kohli A, Bushen OY, Pinkerton RC, Houpt E, Newman RD, Sears CL, et al. *Giardia duodenalis* assemblage, clinical presentation and markers of intestinal inflammation in Brazilian children. Trans R Soc Trop Med Hyg. 2008 Jul;102(7):718-25. PubMed PMID: 18485429. PMCID: PMC2963065. eng.

26. Berkman DS, Lescano AG, Gilman RH, Lopez SL, Black MM. Effects of stunting, diarrhoeal disease, and parasitic infection during infancy on cognition in late childhood: a follow-up study. Lancet. 2002 Feb 16;359(9306):564-71. PubMed PMID: 11867110. Epub 2002/02/28. eng.

27. Niehaus MD, Moore SR, Patrick PD, Derr LL, Lorntz B, Lima AA, et al. Early childhood diarrhea is associated with diminished cognitive function 4 to 7 years later in children in a northeast Brazilian shantytown. Am J Trop Med Hyg. 2002 May;66(5):590-3. PubMed PMID: 12201596. Epub 2002/08/31. eng.

28. Halliez MC, Buret AG. Extra-intestinal and long term consequences of *Giardia duodenalis* infections. World J Gastroenterol. 2013 Dec 21;19(47):8974-85. PubMed PMID: 24379622. PMCID: PMC3870550. Epub 2014/01/01. eng.

29. Cotton JA, Beatty JK, Buret AG. Host parasite interactions and pathophysiology in *Giardia* infections. Int J Parasitol. 2011 Aug 1;41(9):925-33. PubMed PMID: 21683702. Epub 2011/06/21. eng.

30. Chin AC, Teoh DA, Scott KG, Meddings JB, Macnaughton WK, Buret AG. Strain-dependent induction of enterocyte apoptosis by *Giardia lamblia* disrupts epithelial barrier function in a caspase-3-dependent manner. Infect Immun. 2002 Jul;70(7):3673-80. PubMed PMID: 12065509. PMCID: PMC128105. Epub 2002/06/18. eng.

31. Troeger H, Epple HJ, Schneider T, Wahnschaffe U, Ullrich R, Burchard GD, et al. Effect of chronic *Giardia lamblia* infection on epithelial transport and barrier function in human duodenum. Gut. 2007 Mar;56(3):328-35. PubMed PMID: 16935925. PMCID: PMC1856804. Epub 2006/08/29. eng.

32. Panaro MA, Cianciulli A, Mitolo V, Mitolo CI, Acquafredda A, Brandonisio O, et al. Caspase-dependent apoptosis of the HCT-8 epithelial cell line induced by the parasite *Giardia intestinalis.* FEMS Immunol Med Microbiol. 2007 Nov;51(2):302-9. PubMed PMID: 17714487. Epub 2007/08/24. eng.

33. Stadelmann B, Merino MC, Persson L, Svard SG. Arginine consumption by the intestinal parasite *Giardia intestinalis* reduces proliferation of intestinal epithelial cells. PLoS One. 2012;7(9):e45325. PubMed PMID: 23028934. PMCID: PMC3446895. Epub 2012/10/03. eng.

34. Scott KG, Meddings JB, Kirk DR, Lees-Miller SP, Buret AG. Intestinal infection with *Giardia spp.* reduces epithelial barrier function in a myosin light chain kinase-dependent fashion. Gastroenterology. 2002 Oct;123(4):1179-90. PubMed PMID: 12360480. Epub 2002/10/03. eng.

35. Maia-Brigagao C, Morgado-Diaz JA, De Souza W. *Giardia* disrupts the arrangement of tight, adherens and desmosomal junction proteins of intestinal cells. Parasitol Int. 2012 Jun;61(2):280-7. PubMed PMID: 22146155. Epub 2011/12/08. eng.

36. Buret AG, Mitchell K, Muench DG, Scott KG. *Giardia lamblia* disrupts tight junctional ZO-1 and increases permeability in non-transformed human small intestinal epithelial monolayers: effects of epidermal growth factor. Parasitology. 2002 Jul;125(Pt 1):11-9. PubMed PMID: 12166516. Epub 2002/08/09. eng.

37. Bhargava A, Cotton JA, Dixon BR, Gedamu L, Yates RM, Buret AG. *Giardia duodenalis* Surface Cysteine Proteases Induce Cleavage of the Intestinal Epithelial Cytoskeletal Protein Villin via Myosin Light Chain Kinase. PLoS One. 2015;10(9):e0136102. PubMed PMID: 26334299. PMCID: PMC4559405. Epub 2015/09/04. eng.

38. Scott KG, Yu LC, Buret AG. Role of CD8+ and CD4+ T lymphocytes in jejunal mucosal injury during murine *giardia*sis. Infect Immun. 2004 Jun;72(6):3536-42. PubMed PMID: 15155662. PMCID: PMC415705. Epub 2004/05/25. eng.

39. Buret A, Gall DG, Olson ME. Growth, activities of enzymes in the small intestine, and ultrastructure of microvillous border in gerbils infected with *Giardia duodenalis*. Parasitol Res. 1991;77(2):109-14. PubMed PMID: 2027878. Epub 1991/01/01. eng.

40. Jonas A, Flanagan PR, Forstner GG. Pathogenesis of mucosal injury in the blind loop syndrome. Brush border enzyme activity and glycoprotein degradation. J Clin Invest. 1977 Dec;60(6):1321-30. PubMed PMID: 410830. PMCID: PMC372488. Epub 1977/12/01. eng.

41. Torres MF, Uetanabaro AP, Costa AF, Alves CA, Farias LM, Bambirra EA, et al. Influence of bacteria from the duodenal microbiota of patients with symptomatic giardiasis on the pathogenicity of *Giardia duodenalis* in gnotoxenic mice. J Med Microbiol. 2000 Mar;49(3):209-15. PubMed PMID: 10707940. Epub 2000/03/09. eng.

42. Eckmann L. *Mucosal defences against Giardia.* Parasite Immunol. 2003 May;25(5):259-70. PubMed PMID: 12969444. Epub 2003/09/13. eng.

43. Muller N, von Allmen N. Recent insights into the mucosal reactions associated with *Giardia lamblia* infections. Int J Parasitol. 2005 Nov;35(13):1339-47. PubMed PMID: 16182298. Epub 2005/09/27. eng.

44. Matowicka-Karna J, Kralisz M, Kemona H. Assessment of the levels of nitric oxide (NO) and cytokines (IL-5, IL-6, IL-13, TNF, IFN-gamma) in giardiosis. Folia Histochem Cytobiol. 2011;49(2):280-4. PubMed PMID: 21744329. Epub 2011/07/12. eng.

45. Eckmann L, Laurent F, Langford TD, Hetsko ML, Smith JR, Kagnoff MF, et al. Nitric oxide production by human intestinal epithelial cells and competition for arginine as potential determinants of host defense against the lumen-dwelling pathogen *Giardia lamblia*. J Immunol. 2000 Feb 1;164(3):1478-87. PubMed PMID: 10640765. Epub 2000/01/21. eng.

46. Tako EA, Hassimi MF, Li E, Singer SM. Transcriptomic analysis of the host response to *Giardia duodenalis* infection reveals redundant mechanisms for parasite control. MBio. 2013;4(6):e00660-13. PubMed PMID: 24194537. PMCID: PMC3892777. Epub 2013/11/07. eng.

47. Merluzzi S, Frossi B, Gri G, Parusso S, Tripodo C, Pucillo C. Mast cells enhance proliferation of B lymphocytes and drive their differentiation toward IgA-secreting plasma cells. Blood. 2010 Apr 8;115(14):2810-7. PubMed PMID: 20101023. Epub 2010/01/27. eng.

48. Velazquez C, Beltran M, Ontiveros N, Rascon L, Figueroa DC, Granados AJ, et al. *Giardia lamblia* infection induces different secretory and systemic antibody responses in mice. Parasite Immunol. 2005 Sep;27(9):351-6. PubMed PMID: 16149993. Epub 2005/09/10. eng.

49. Zhou P, Li E, Shea-Donohue T, Singer SM. Tumour necrosis factor alpha contributes to protection against *Giardia lamblia* infection in mice. Parasite Immunol. 2007 Jul;29(7):367-74. PubMed PMID: 17576366. PMCID: PMC2443547. Epub 2007/06/20. eng.

50. Kamda JD, Nash TE, Singer SM. *Giardia duodenalis*: dendritic cell defects in IL-6 deficient mice contribute to susceptibility to intestinal infection. Exp Parasitol. 2012 Mar;130(3):288-91. PubMed PMID: 22248985. PMCID: Pmc3289762. Epub 2012/01/18. eng.

51. Jimenez JC, Fontaine J, Creusy C, Fleurisse L, Grzych JM, Capron M, et al. Antibody and cytokine responses to *Giardia* excretory/secretory proteins in *Giardia intestinalis*-infected BALB/c mice. Parasitol Res. 2014 Jul;113(7):2709-18. PubMed PMID: 24867815. Epub 2014/05/29. eng.

52. Obendorf J, Renner Viveros P, Fehlings M, Klotz C, Aebischer T, Ignatius R. Increased expression of CD25, CD83, and CD86, and secretion of IL-12, IL-23, and IL-10 by human dendritic cells incubated in the presence of Toll-like receptor 2 ligands and *Giardia duodenalis*. Parasites & Vectors. 2013;6(1):317. PubMed PMID: doi:10.1186/1756-3305-6-317.

53. Lee HY, Kim J, Noh HJ, Kim HP, Park SJ. *Giardia lamblia* binding immunoglobulin protein triggers maturation of dendritic cells via activation of TLR4-MyD88-p38 and ERK1/2 MAPKs.

54. Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. Biochim Biophys Acta. 2011 May;1813(5):878-88. PubMed PMID: 21296109. Epub 2011/02/08. eng.

55. Hou W, Jin YH, Kang HS, Kim BS. Interleukin-6 (IL-6) and IL-17 synergistically promote viral persistence by inhibiting cellular apoptosis and cytotoxic T cell function. J Virol. 2014 Aug;88(15):8479-89. PubMed PMID: 24829345. PMCID: PMC4135960. Epub 2014/05/16. eng.

56. Jones SA. Directing transition from innate to acquired immunity: defining a role for IL-6. J Immunol. 2005 Sep 15;175(6):3463-8. PubMed PMID: 16148087. Epub 2005/09/09. eng.

57. Solaymani-Mohammadi S, Singer SM. Host immunity and pathogen strain contribute to intestinal disaccharidase impairment following gut infection. J Immunol. 2011 Oct 1;187(7):3769-75. PubMed PMID: 21873528. PMCID: PMC3178714. Epub 2011/08/30. eng.

58. Bienz M, Dai WJ, Welle M, Gottstein B, Muller N. Interleukin-6-deficient mice are highly susceptible to *Giardia lamblia* infection but exhibit normal intestinal immunoglobulin A responses against the parasite. Infect Immun. 2003 Mar;71(3):1569-73. PubMed PMID: 12595479. PMCID: PMC148820. Epub 2003/02/22. eng.

59. Singer SM, Nash TE. T-cell-dependent control of acute *Giardia lamblia* infections in mice. Infect Immun. 2000 Jan;68(1):170-5. PubMed PMID: 10603384. PMCID: PMC97117. Epub 1999/12/22. eng.

60. Langford TD, Housley MP, Boes M, Chen J, Kagnoff MF, Gillin FD, et al. Central importance of immunoglobulin A in host defense against *Giardia spp*. Infect Immun. 2002 Jan;70(1):11-8. PubMed PMID: 11748158. PMCID: PMC127595. Epub 2001/12/19. eng.

61. Rivero FD, Saura A, Prucca CG, Carranza PG, Torri A, Lujan HD. Disruption of antigenic variation is crucial for effective parasite vaccine. Nat Med. 2010 May;16(5):551-7, 1p following 7. PubMed PMID: 20418884. Epub 2010/04/27. eng.

62. Faubert G. Immune Response to *Giardia duodenalis*. Clin Microbiol Rev. 132000. p. 35-54.

63. Jenikova G, Hruz P, Andersson KM, Tejman-Yarden N, Ferreira PCD, Andersen YS, et al. α1-giardin based live heterologous vaccine protects against *Giardia lamblia* infection in a murine model. Vaccine. 2011 Nov 28;29(51):9529-37. PubMed PMID: 22001876. PMCID: PMC4045459. eng.

64. Palm JE, Weiland ME, Griffiths WJ, Ljungstrom I, Svard SG. Identification of immunoreactive proteins during acute human giardiasis. J Infect Dis. 2003 Jun 15;187(12):1849-59. PubMed PMID: 12792861. Epub 2003/06/07. eng.

65. Banik S, Renner Viveros P, Seeber F, Klotz C, Ignatius R, Aebischer T. *Giardia duodenalis* arginine deiminase modulates the phenotype and cytokine secretion of human dendritic cells by depletion of arginine and formation of ammonia. Infect Immun. 2013 Jul;81(7):2309-17. PubMed PMID: 23589577. PMCID: PMC3697621. Epub 2013/04/17. eng.

66. Dhakal J, Brah GS, Agrawal RK, Pawar HN, Kaur D, Verma R. Over-expression of gene encoding heat shock protein 70 from Mycobacterium tuberculosis and its evaluation as vaccine adjuvant. Indian J Med Microbiol. 2013 Apr-Jun;31(2):123-9. PubMed PMID: 23867667. Epub 2013/07/23. eng.

67. Segal BH, Wang XY, Dennis CG, Youn R, Repasky EA, Manjili MH, et al. Heat shock proteins as vaccine adjuvants in infections and cancer. Drug Discov Today. 2006 Jun;11(11-12):534-40. PubMed PMID: 16713905. Epub 2006/05/23. eng.

68. Soltys BJ, Falah M, Gupta RS. Identification of endoplasmic reticulum in the primitive eukaryote *Giardia lamblia* using cryoelectron microscopy and antibody to Bip. J Cell Sci. 1996 Jul;109 (Pt 7):1909-17. PubMed PMID: 8832413. Epub 1996/07/01. eng.

69. Lee GC, Nam SH, Chae JC, Lee CH. *Giardia duodenalis*: improved detection of viable cysts by reverse transcription-PCR of heat shock-inducible hsp70 gene. Exp Parasitol. 2009 Dec;123(4):377-80. PubMed PMID: 19703445. Epub 2009/08/26. eng.

70. Kim J, Bae SS, Sung MH, Lee KH, Park SJ. Comparative proteomic analysis of trophozoites versus cysts of *Giardia lamblia*. Parasitol Res. 2009 Jan;104(2):475-9. PubMed PMID: 18853189. Epub 2008/10/15. eng.

71. Zhang Y, Liu R, Ni M, Gill P, Lee AS. Cell surface relocalization of the endoplasmic reticulum chaperone and unfolded protein response regulator GRP78/BiP. J Biol Chem. 2010 May 14;285(20):15065-75. PubMed PMID: 20208072. PMCID: PMC2865300. Epub 2010/03/09. eng.

72. Larocque R, Nakagaki K, Lee P, Abdul-Wahid A, Faubert GM. Oral immunization of BALB/c mice with *Giardia duodenalis* recombinant cyst wall protein inhibits shedding of cysts. Infect Immun. 2003 Oct;71(10):5662-9. PubMed PMID: 14500486. PMCID: PMC201086. Epub 2003/09/23. eng.

73. Cotton JA, Amat CB, Buret AG. Disruptions of Host Immunity and Inflammation by *Giardia Duodenalis*: Potential Consequences for Co-Infections in the Gastro-Intestinal Tract. Pathogens. 2015;4(4):764-92. PubMed PMID: 26569316. PMCID: PMC4693164. Epub 2015/11/17. eng.

74. Johansson ME, Phillipson M, Petersson J, Velcich A, Holm L, Hansson GC. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. Proc Natl Acad Sci U S A. 2008 Sep 30;105(39):15064-9. PubMed PMID: 18806221. PMCID: PMC2567493. Epub 2008/09/23. eng.

75. Johansson ME, Larsson JM, Hansson GC. The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host-microbial interactions. Proc Natl Acad Sci U S A. 2011 Mar 15;108 Suppl 1:4659-65. PubMed PMID: 20615996. PMCID: PMC3063600. Epub 2010/07/10. eng.

76. Gum JR, Jr., Hicks JW, Toribara NW, Siddiki B, Kim YS. Molecular cloning of human intestinal mucin (MUC2) cDNA. Identification of the amino terminus and overall sequence similarity to prepro-von Willebrand factor. J Biol Chem. 1994 Jan 28;269(4):2440-6. PubMed PMID: 8300571. Epub 1994/01/28. eng.

77. Cotton JA, Motta JP, Schenck LP, Hirota SA, Beck PL, Buret AG. *Giardia duodenalis* infection reduces granulocyte infiltration in an in vivo model of bacterial toxin-induced colitis and attenuates inflammation in human intestinal tissue. PLoS One. 2014;9(10):e109087. PubMed PMID: 25289678. PMCID: PMC4188619. Epub 2014/10/08. eng.

78. Amulic B, Cazalet C, Hayes GL, Metzler KD, Zychlinsky A. Neutrophil function: from mechanisms to disease. Annu Rev Immunol. 2012;30:459-89. PubMed PMID: 22224774. Epub 2012/01/10. eng.

79. Madara JL, Patapoff TW, Gillece-Castro B, Colgan SP, Parkos CA, Delp C, et al. 5'-adenosine monophosphate is the neutrophil-derived paracrine factor that elicits chloride secretion from T84 intestinal epithelial cell monolayers. J Clin Invest. 1993 May;91(5):2320-5. PubMed PMID: 8486793. PMCID: PMC288238. Epub 1993/05/01. eng.

80. Madara JL, Parkos C, Colgan S, MacLeod RJ, Nash S, Matthews J, et al. Cl- secretion in a model intestinal epithelium induced by a neutrophil-derived secretagogue. J Clin Invest. 1992 Jun;89(6):1938-44. PubMed PMID: 1602001. PMCID: PMC295894. eng.

81. Salzman A, Denenberg AG, Ueta I, O'Connor M, Linn SC, Szabo C. Induction and activity of nitric oxide synthase in cultured human intestinal epithelial monolayers. Am J Physiol. 1996 Apr;270(4 Pt 1):G565-73. PubMed PMID: 8928785. Epub 1996/04/01. eng.

82. Witthoft T, Eckmann L, Kim JM, Kagnoff MF. Enteroinvasive bacteria directly activate expression of iNOS and NO production in human colon epithelial cells. Am J Physiol. 1998 Sep;275(3 Pt 1):G564-71. PubMed PMID: 9724270. Epub 1998/09/02. eng.

83. Fang FC. Perspectives series: host/pathogen interactions. Mechanisms of nitric oxide-related antimicrobial activity. J Clin Invest. 1997 Jun 15;99(12):2818-25. PubMed PMID: 9185502. PMCID: PMC508130. eng.

84. James SL. Role of nitric oxide in parasitic infections. Microbiol Rev. 1995 Dec;59(4):533-47. PubMed PMID: 8531884. PMCID: PMC239385. Epub 1995/12/01. eng.

85. Ringqvist E, Palm JE, Skarin H, Hehl AB, Weiland M, Davids BJ, et al. Release of metabolic enzymes by *Giardia* in response to interaction with intestinal epithelial cells. Mol Biochem Parasitol. 2008 Jun;159(2):85-91. PubMed PMID: 18359106. PMCID: PMC3658456. Epub 2008/03/25. eng.

86. Stadelmann B, Hanevik K, Andersson MK, Bruserud O, Svard SG. The role of arginine and arginine-metabolizing enzymes during *Giardia* - host cell interactions in vitro. BMC Microbiol. 2013;13:256. PubMed PMID: 24228819. PMCID: Pmc4225669. Epub 2013/11/16. eng.

87. Mokrzycka M, Kolasa A, Kosierkiewicz A, Wiszniewska B. Inducible nitric oxide synthase in duodenum of children with *Giardia lamblia* infection. Folia Histochem Cytobiol. 2010 Jan;48(2):191-6. PubMed PMID: 20675273. Epub 2010/08/03. eng.

88. Fisher BS, Estraño CE, Cole JA. Modeling Long-Term Host Cell-*Giardia lamblia* Interactions in an In Vitro Co-Culture System. In: Talamas-Rohana P, editor. PLoS One. 8. San Francisco, USA2013.

89. Alm K, Oredsson S. Cells and polyamines do it cyclically. Essays Biochem. 2009;46:63-76. PubMed PMID: 20095970. Epub 2010/01/26. eng.

90. Kamda J, Singer SM. Phosphoinositide 3-Kinase-Dependent Inhibition of Dendritic Cell Interleukin-12 Production by *Giardia lamblia*. Infect Immun. 772009. p. 685-93.

91. Maloney J, Keselman A, Li E, Singer SM. Macrophages expressing arginase 1 and nitric oxide synthase 2 accumulate in the small intestine during *Giardia lamblia* infection. Microbes Infect. 2015 Jun;17(6):462-7. PubMed PMID: 25797399. PMCID: PMC4461514. Epub 2015/03/24. eng.

92. Lathrop SK, Binder KA, Starr T, Cooper KG, Chong A, Carmody AB, et al. Replication of Salmonella enterica Serovar Typhimurium in Human Monocyte-Derived Macrophages. Infect Immun. 2015 Jul;83(7):2661-71. PubMed PMID: 25895967. PMCID: PMC4468555. Epub 2015/04/22. eng.

93. Pesce JT, Ramalingam TR, Mentink-Kane MM, Wilson MS, El Kasmi KC, Smith AM, et al. Arginase-1-expressing macrophages suppress Th2 cytokine-driven inflammation and fibrosis. PLoS Pathog. 2009 Apr;5(4):e1000371. PubMed PMID: 19360123. PMCID: PMC2660425. Epub 2009/04/11. eng.

94. Hall BS, Tam W, Sen R, Pereira MEA. Cell-specific Activation of Nuclear Factor-κB by the Parasite Trypanosoma cruzi Promotes Resistance to Intracellular Infection. In: Silver P, editor. Mol Biol Cell. 112000. p. 153-60.

95. Karin M, Greten FR. NF-kappaB: linking inflammation and immunity to cancer development and progression. Nat Rev Immunol. 2005 Oct;5(10):749-59. PubMed PMID: 16175180. Epub 2005/09/22. eng.

96. Neves BM, Lopes MC, Cruz MT. Pathogen Strategies to Evade Innate Immune Response: A Signaling Point of View. Dr. Gabriela Da Silva Xavier (Ed.), InTech; 2012

97. Viboud GI, Bliska JB. Yersinia outer proteins: role in modulation of host cell signaling responses and pathogenesis. Annu Rev Microbiol. 2005;59:69-89. PubMed PMID: 15847602. Epub 2005/04/26. eng.

98. McGhie EJ, Brawn LC, Hume PJ, Humphreys D, Koronakis V. Salmonella takes control: effector-driven manipulation of the host. Curr Opin Microbiol. 2009 Feb;12(1):117-24. PubMed PMID: 19157959. PMCID: PMC2647982. Epub 2009/01/23. eng.

99. Kim DW, Lenzen G, Page AL, Legrain P, Sansonetti PJ, Parsot C. The Shigella flexneri effector OspG interferes with innate immune responses by targeting ubiquitin-conjugating enzymes. Proc Natl Acad Sci U S A. 1022005. p. 14046-51.

100. Reiterer V, Grossniklaus L, Tschon T, Kasper CA, Sorg I, Arrieumerlou C. Shigella flexneri type III secreted effector OspF reveals new crosstalks of proinflammatory signaling pathways during bacterial infection. Cell Signal. 2011 Jul;23(7):1188-96. PubMed PMID: 21402152. Epub 2011/03/16. eng.

101. Christian J, Vier J, Paschen SA, Hacker G. Cleavage of the NF-kappaB family protein p65/RelA by the chlamydial protease-like activity factor (CPAF) impairs proinflammatory signaling in cells infected with Chlamydiae. J Biol Chem. 2010 Dec 31;285(53):41320-7. PubMed PMID: 21041296. PMCID: PMC3009857. Epub 2010/11/03. eng.

102. Albee L, Perlman H. E. coli infection induces caspase dependent degradation of NF-kappaB and reduces the inflammatory response in macrophages. Inflamm Res. 2006 Jan;55(1):2-9. PubMed PMID: 16328105. Epub 2005/12/06. eng.

103. Shapira S, Harb OS, Margarit J, Matrajt M, Han J, Hoffmann A, et al. Initiation and termination of NF-kappaB signaling by the intracellular protozoan parasite Toxoplasma gondii. J Cell Sci. 2005 Aug 1;118(Pt 15):3501-8. PubMed PMID: 16079291. Epub 2005/08/05. eng.

104. Gomez MA, Contreras I, Halle M, Tremblay ML, McMaster RW, Olivier M. Leishmania GP63 alters host signaling through cleavage-activated protein tyrosine phosphatases. Sci Signal. 2009;2(90):ra58. PubMed PMID: 19797268. Epub 2009/10/03. eng.

105. Gregory DJ, Godbout M, Contreras I, Forget G, Olivier M. A novel form of NF-kappaB is induced by Leishmania infection: involvement in macrophage gene expression. Eur J Immunol. 2008 Apr;38(4):1071-81. PubMed PMID: 18383035. Epub 2008/04/03. eng.

106. Neves BM, Silvestre R, Resende M, Ouaissi A, Cunha J, Tavares J, et al. Activation of Phosphatidylinositol 3-Kinase/Akt and Impairment of Nuclear Factor-κB : Molecular Mechanisms Behind the Arrested Maturation/Activation State of Leishmania infantum-Infected Dendritic Cells. Am J Pathol. 2010 Dec;177(6):2898-911. PubMed PMID: 21037075. PMCID: Pmc2993270. eng.

107. Sabotic J, Kos J. Microbial and fungal protease inhibitors--current and potential applications. Appl Microbiol Biotechnol. 2012 Feb;93(4):1351-75. PubMed PMID: 22218770. Epub 2012/01/06. eng.

108. Rascon AA, Jr., McKerrow JH. Synthetic and natural protease inhibitors provide insights into parasite development, virulence and pathogenesis. Curr Med Chem. 2013;20(25):3078-102. PubMed PMID: 23514418. Epub 2013/03/22. eng.

109. Sajid M, McKerrow JH. Cysteine proteases of parasitic organisms. Mol Biochem Parasitol. 2002 Mar;120(1):1-21. PubMed PMID: 11849701. Epub 2002/02/19. eng.

110. Caffrey CR, Lima AP, Steverding D. Cysteine peptidases of kinetoplastid parasites. Adv Exp Med Biol. 2011;712:84-99. PubMed PMID: 21660660. Epub 2011/06/11. eng.

111. Kissoon-Singh V, Mortimer L, Chadee K. Entamoeba histolytica cathepsin-like enzymes : interactions with the host gut. Adv Exp Med Biol. 2011;712:62-83. PubMed PMID: 21660659. Epub 2011/06/11. eng.

112. Rosenthal PJ. Falcipains and other cysteine proteases of malaria parasites. Adv Exp Med Biol. 2011;712:30-48. PubMed PMID: 21660657. Epub 2011/06/11. eng.

113. Sajid M, Robertson SA, Brinen LS, McKerrow JH. Cruzain : the path from target validation to the clinic. Adv Exp Med Biol. 2011;712:100-15. PubMed PMID: 21660661. Epub 2011/06/11. eng.

114. Rawlings ND. Peptidase inhibitors in the MEROPS database. Biochimie. 2010 Nov;92(11):1463-83. PubMed PMID: 20430064. Epub 2010/05/01. eng.

115. Rawlings ND, Barrett AJ, Bateman A. MEROPS: the peptidase database. Nucleic Acids Res. 2010 Jan;38(Database issue):D227-33. PubMed PMID: 19892822. PMCID: PMC2808883. Epub 2009/11/07. eng.

116. Hanada K, Tamai M, Yamagishi M, Ohmura S, Sawada J, Tanaka I. Isolation and Characterization of E–64, a New Thiol Protease Inhibitor. Agricultural and Biological Chemistry. 1978;42(3).

117. Umezawa H, Aoyagi T, Morishima H, Matsuzaki M, Hamada M. Pepstatin, a new pepsin inhibitor produced by Actinomycetes. J Antibiot (Tokyo). 1970 May;23(5):259-62. PubMed PMID: 4912600. Epub 1970/05/01. eng.

118. Williams AG, Coombs GH. Multiple protease activities in *Giardia intestinalis* trophozoites. Int J Parasitol. 1995 Jul;25(7):771-8. PubMed PMID: 7558562. Epub 1995/07/01. eng.

119. Rodriguez-Fuentes GB, Cedillo-Rivera R, Fonseca-Linan R, Arguello-Garcia R, Munoz O, Ortega-Pierres G, et al. *Giardia duodenalis*: analysis of secreted proteases upon trophozoite-epithelial cell interaction in vitro. Mem Inst Oswaldo Cruz. 2006 Sep;101(6):693-6. PubMed PMID: 17072486. Epub 2006/10/31. eng.

120. Coradi ST, Guimaraes S. *Giardia duodenalis*: protein substrates degradation by trophozoite proteases. Parasitol Res. 2006 Jul;99(2):131-6. PubMed PMID: 16521040. Epub 2006/03/08. eng.

121. Carvalho TB, David EB, Coradi ST, Guimaraes S. Protease activity in extracellular products secreted in vitro by trophozoites of *Giardia duodenalis*. Parasitol Res. 2008 Dec;104(1):185-90. PubMed PMID: 18797927. Epub 2008/09/18. eng.

122. Adam RD. Biology of *Giardia lamblia*. Clin Microbiol Rev. 2001 Jul;14(3):447-75. PubMed PMID: 11432808. PMCID: PMC88984. Epub 2001/07/04. eng.

123. Touz MC, Nores MJ, Slavin I, Piacenza L, Acosta D, Carmona C, et al. Membrane-associated dipeptidyl peptidase IV is involved in encystation-specific gene expression during *Giardia* differentiation. Biochem J. 2002 Jun 15;364(Pt 3):703-10. PubMed PMID: 12049634. PMCID: PMC1222619. Epub 2002/06/07. eng.

124. Davids BJ, Gilbert MA, Liu Q, Reiner DS, Smith AJ, Lauwaet T, et al. An atypical proprotein convertase in *Giardia lamblia* differentiation. Mol Biochem Parasitol. 2011 Feb;175(2):169-80. PubMed PMID: 21075147. PMCID: PMC3018286. Epub 2010/11/16. eng.

125. Faria CP, Silva A, Martins JD, Cruz MT, Pereira S, Zanini GM, et al. *Giardia lamblia* triggers NF-kappaB cleavage and suppresses LPS-induced pro-inflammatory response in macrophages. ECCMID (Poster Presentation); Barcelona, Spain. 2014.

126. 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. PubMed PMID: 6636276. Epub 1983/01/01. eng.

127. David EB, Guimaraes S, Ribolla PE, Coradi ST, Alonso DP. Partial characterization of proteolytic activity in *Giardia duodenalis* purified proteins. Rev Inst Med Trop Sao Paulo. 2007 Nov-Dec;49(6):385-8. PubMed PMID: 18157406. Epub 2007/12/25. eng.

128. Duschak VG, Couto AS. Cruzipain, the major cysteine protease of Trypanosoma cruzi: a sulfated glycoprotein antigen as relevant candidate for vaccine development and drug target. A review. Curr Med Chem. 2009;16(24):3174-202. PubMed PMID: 19689291. Epub 2009/08/20. eng.

129. Olivier M, Hassani K. Protease inhibitors as prophylaxis against leishmaniasis: new hope from the major surface protease gp63. Future Med Chem. 2010 Apr;2(4):539-42. PubMed PMID: 21426002. Epub 2011/03/24. eng.

130. Yao C. Major Surface Protease of Trypanosomatids: One Size Fits All? Infect Immun. 782010. p. 22-31.

131. Cameron P, McGachy A, Anderson M, Paul A, Coombs GH, Mottram JC, et al. Inhibition of lipopolysaccharide-induced macrophage IL-12 production by Leishmania mexicana amastigotes: the role of cysteine peptidases and the NF-kappaB signaling pathway. J Immunol. 2004 Sep 1;173(5):3297-304. PubMed PMID: 15322192. Epub 2004/08/24. eng.

132. Silverman JM, Chan SK, Robinson DP, Dwyer DM, Nandan D, Foster LJ, et al. Proteomic analysis of the secretome of Leishmania donovani. Genome Biol. 2008;9(2):R35. PubMed PMID: 18282296. PMCID: PMC2374696. Epub 2008/02/20. eng.

133. Silverman JM, Clos J, de'Oliveira CC, Shirvani O, Fang Y, Wang C, et al. An exosome-based secretion pathway is responsible for protein export from Leishmania and communication with macrophages. J Cell Sci. 2010 Mar 15;123(Pt 6):842-52. PubMed PMID: 20159964. Epub 2010/02/18. eng.

134. Goncalves MF, Umezawa ES, Katzin AM, de Souza W, Alves MJ, Zingales B, et al. Trypanosoma cruzi: shedding of surface antigens as membrane vesicles. Exp Parasitol. 1991 Jan;72(1):43-53. PubMed PMID: 1993464. Epub 1991/01/01. eng.

135. Pinho RT, Vannier-Santos MA, Alves CR, Marino AP, Castello Branco LR, Lannes-Vieira J. Effect of Trypanosoma cruzi released antigens binding to non-infected cells on anti-parasite antibody recognition and expression of extracellular matrix components. Acta Trop. 2002 Aug;83(2):103-15. PubMed PMID: 12088851. Epub 2002/06/29. eng.

136. Trocoli Torrecilhas AC, Tonelli RR, Pavanelli WR, da Silva JS, Schumacher RI, de Souza W, et al. Trypanosoma cruzi: parasite shed vesicles increase heart parasitism and generate an intense inflammatory response. Microbes Infect. 2009 Jan;11(1):29-39. PubMed PMID: 19028594. Epub 2008/11/26. eng.

Support Information

Table S.1

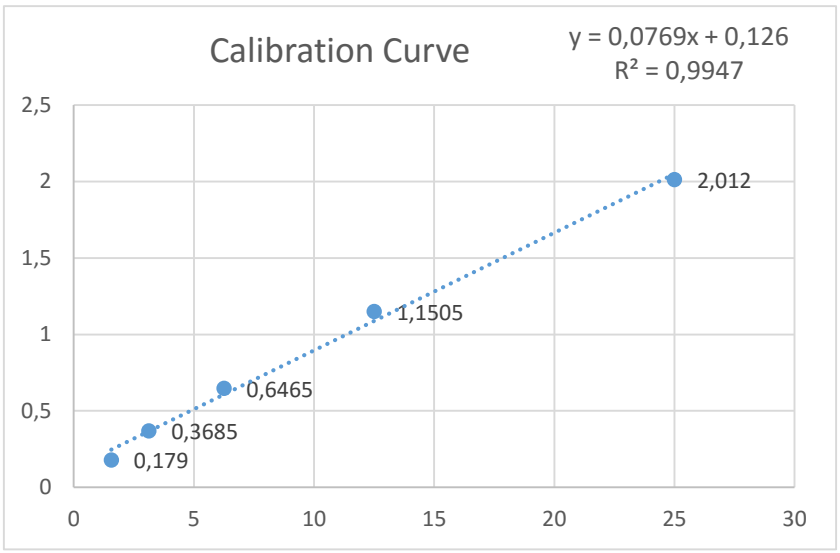
Culture medium for *Giardia*

Keister's modified TYI-S-33 medium	
A. Nutrient broth	
K_2HPO_4	1 g
KH_2PO_4	0.6 g
NaCl	2 g
Casitone	20 g
Yeast Extract	10 g
Glucose	10 g
Ascorbic Acid	0.2 g
Ferric Ammonium Citrate	0.023 g
Distilled H_2O	800 ml
Dissolve ingredients, adjust pH to 6.8 with NaOH 1*N* sterilize by filtration. Broth can be stored at -20°C up to 6 months.	
B. Cysteine-bile solution	
L-Cysteine HCl	2 g
Distilled H_2O	100 ml
When dissolved add:	
Bile bovine	0.6 g
NaOH 1*N*	1 ml
Mix to clear the solution, sterilize by filtration and immediately add to the broth	
C. Complete Medium	
Mix aseptically:	
Broth (A)	800 ml
Heat-inactivated Bovine Serum	100 ml
Cysteine-bile solution (B)	100 ml
Add Antibiotic solution (penicillin 100 U/ml and streptomycin 100 µg/ml)	

Adapted from: Jaroslav Kulda and Eva Nohýnková. *Giardia in Humans and Animals*. *In* Parasitic Protozoa. Vol. 10. 1995. Ed. Julius P. Kreier.

Figure S.2

Calibration curve for protein quantification



Standards	Protein	Absorbance		Mean	Mean - Blank
Blank	0	0,098	0,098	0,098	0
P1	**25**	2,084	2,136	2,11	**2,012**
P2	**12,5**	1,212	1,285	1,2485	**1,1505**
P3	**6,25**	0,742	0,747	0,7445	**0,6465**
P4	**3,125**	0,478	0,455	0,4665	**0,3685**
P5	**1,5625**	0,28	0,274	0,277	**0,179**