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Giardia Cathepsins and Their Role in Intestinal Disease

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Giardia Cathepsins and Their Role in Intestinal Disease

by

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A THESIS

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Abstract

Giardia duodenalis, a non-invasive protozoan parasite of the upper small intestine of mammals, including humans, closely associates with intestinal epithelial cells. The pathophysiology of giardiasis includes intestinal barrier dysfunction and cytoskeletal injury; however, the mechanisms or parasitic factors involved remain unclear. The *Giardia* genome contains genes for cathepsin-like cysteine proteases; however, their roles are unknown.

Using an *in vitro* model for studying host-parasite interactions, we illustrated that *G. duodenalis* trophozoites contain and release cathepsin B/L-like cysteine proteases. While cathepsin-like cysteine proteases are not involved in the tight junctional disruption caused by *G. duodenalis*, such proteases cleaved and disrupted cytoskeletal villin. This disruption of villin was sustained over time, at least in part, by host MLCK.

Overall, this study establishes a reliable model for studying roles of parasitic cysteine proteases during host-parasite interactions. Further understanding of these proteases may pave the way for therapeutic development.

Preface

Work from this thesis has contributed to the following publications:

Bhargava, A, Cotton JA, Dixon BR, Yates RM, Buret AG. *Giardia duodenalis* cathepsin-like cysteine proteases induce host pathophysiological responses in intestinal epithelial cells. *In preparation for International Journal for Parasitology*.

Cotton JA, **Bhargava A**, Ferraz JG, Yates RM, Beck PL, Buret AG. 2014 *Giardia duodenalis* cathepsin B proteases degrade intestinal epithelial interleukin-8 and attenuate interleukin-8-induced neutrophil chemotaxis. *Infect Immun*. (in press)

Beatty, JK, **Bhargava, A**, AG Buret. 2014. Post-infectious Irritable Bowel Syndrome: Mechanistic insights into chronic disturbances following enteric infection. *World Journal of Gastroenterology*. 20: 3976-3985.

Buret, AG, **Bhargava, A**. 2014. Modulatory mechanisms of enterocyte apoptosis by viral, bacterial and parasitic pathogens. *Critical Reviews in Microbiology*. 40(1):1-17.

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Dedication

To my parents, Sanjay and Leena.

You taught me that perseverance and determination are irreplaceable.

Thank you for your endless inspiration, love, and support.

&

To my grandparents, thank you for your kindness and constant encouragement!

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List of Abbreviations

AJC	Apical Junction Complex
CD	Crohn's Disease
EHEC	Enterohemorrhagic <i>Escherichia coli</i>
EPEC	Enteropathogenic <i>Escherichia coli</i>
GI	Gastrointestinal
HI-FBS	Heat Inactivated-Fetal Bovine Serum
HRP	Horseradish peroxidase
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
IBS-C	Irritable Bowel Syndrome-Constipation
IBS-D	Irritable Bowel Syndrome-Diarrhea
IEC	Intestinal Epithelial Cells
IFN- γ	Interferon gamma
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL	Interleukin
MDCK	Madin-Darby Canine Kidney cells
MEME	Minimal Essential Medium Eagle
MLC	Myosin Light Chain
MLCK	Myosin Light Chain Kinase
MMP	Matrix Metalloproteinase
PAR	Protease-activated Receptor
PARP	Poly(-ADP-ribose) Polymerase
PI-IBS	Post Infectious-Irritable Bowel Syndrome
SGLT	Sodium-Glucose Co-transporter
TNF- α	Tumor Necrosis Factor alpha
UC	Ulcerative Colitis
VSP	Variant-specific Surface Protein
ZFR-AMC	Benzyloxycarbonyl-L-Phenylalanyl-L-Arginine 4-Methyl-Coumaryl-7-Amide
ZO	Zonula Occludens
ZRR-AMC	Benzyloxycarbonyl-L-Arginine-L-Arginine 4-Methyl-Coumaryl-7-Amide

1. INTRODUCTION

1.1. The intestinal mucosal barrier

The intestinal mucosa serves as the first site of interaction between the host and its external environment. This physical barrier is vital in protecting against environmental antigens that may include food, and pathogenic or commensal microorganisms. This barrier is formed by a number of components, including the mucus layer and the intestinal epithelium. The extracellular layer of mucus contains mucin glycoproteins and trefoil factors, which are secreted by specialized goblet cells, along with defensins (released from Paneth cells). Beneath the mucus layer lays the intestinal epithelium, a heterogeneous layer of cells. In addition to critical absorptive functions, intestinal epithelial cells (IECs) also have secretory functions, and immune functions, which are important in protection against commensal and pathogenic microorganisms. One mechanism via which the epithelium fences out unwanted particles is through the membrane lipid composition of the apical side of these cells, which does not allow the movement of hydrophilic substances across this membrane in the absence of transporters [126]. Intestinal epithelial cells are connected via apical junctional complexes, including tight junctions and adherens junctions, which limit back-diffusion of absorbed materials from the lamina propria back into the lumen or vice versa. These junctions are closely associated with the cytoskeleton of IECs, and contribute to intestinal epithelial homeostasis. The intestinal epithelium must be continually renewed by pluripotent intestinal epithelial stem cells present in the crypts, as more senescent cells are extruded from the epithelium, a process known as epithelial turnover. Collectively, the physical barrier and cellular mechanisms employed at the intestinal mucosa serve to maintain a

homeostatic balance. Factors that contribute to the homeostasis of the intestinal epithelium and its barrier function will be discussed in following sections.

1.1.1. Homeostatic turnover of the intestinal epithelium

The intestinal epithelium is the largest mucosal surface of the human body, with nearly 400m² of surface area formed by a single heterogeneous layer of cells [56]. In the small intestine, this layer is organized into structures known as crypts and villi [101]. Crypts are tubular invaginations, while villi are finger-like protrusions; together they form the crypt-villus axis of the small intestinal epithelium [56]. On average, epithelial cells can live for 3-5 days and must be replaced by younger cells [18]. Undifferentiated pluripotent intestinal stem cells proliferate within the crypt region, followed by a number of rounds of cell division to form a pool of transit amplifying cells [35]. More senescent and terminally differentiated cells undergo a process of programmed cell death called apoptosis as they reach the tip of the villus. They are subsequently replaced by younger transit amplifying cells slowly differentiating from the stem cells and moving up the crypt-villus axis [56].

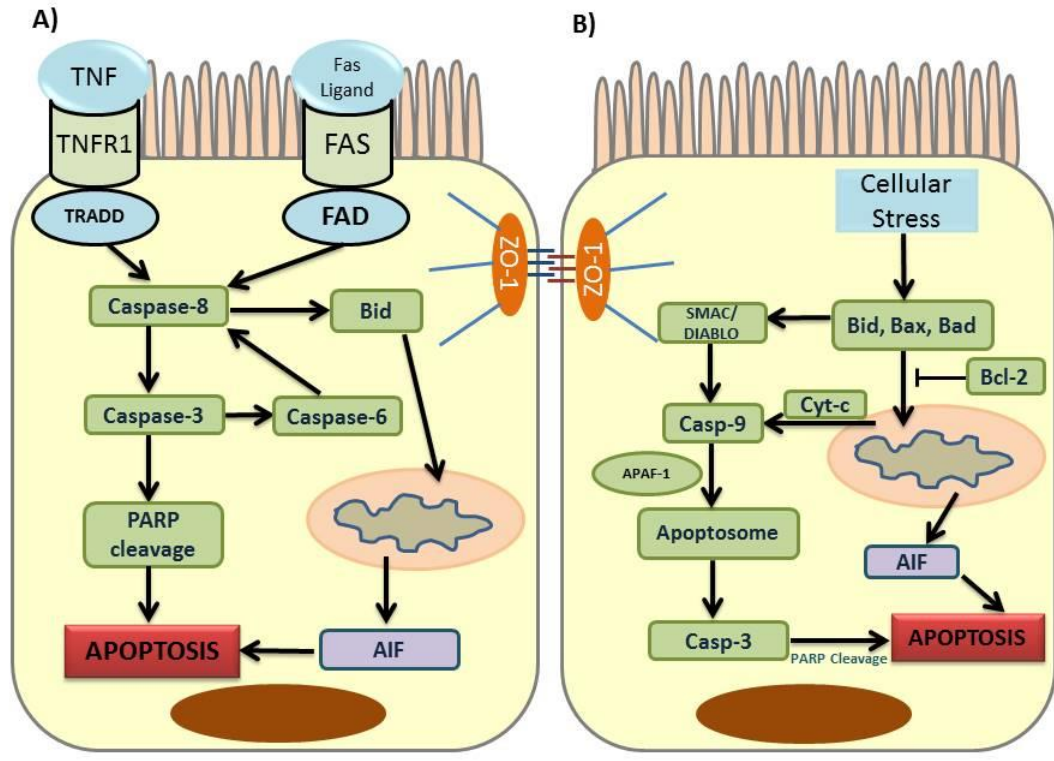
Homeostasis of the intestinal epithelium requires a regulated ratio of cell division to an appropriate rate of apoptosis. Apoptosis, or programmed cellular death, is a highly controlled process in which cellular membrane integrity is maintained, which prevents the spilling of cytotoxic cellular contents into the extracellular milieu [56]. Under healthy conditions, this delicate balance between cell proliferation and cell death does not result in a loss of barrier function [96, 151]. A study using Fas-induced apoptosis under normal conditions demonstrated that epithelial cells extend protrusions underneath shedding apoptotic cells to ensure the integrity of the epithelium [1]. This suggests that the

intestinal epithelium has well-developed mechanisms to counter the effects of cellular death in order to maintain barrier function, and while single cell death may not have negative effects on it, sustained enterocyte apoptosis may result in a loss of epithelial integrity through improper tight junction formation [41, 119]. The two hotspots for apoptotic cell death along the crypt-villus axis are at the villus tip and the crypt region [41]. Following apoptosis, these cells may be phagocytosed by resident macrophages, or may be expelled into the lumen through a process known as anoikis, which is induced by detachment from the matrix [56]. Too much cell death or too much proliferation can lead to pathophysiology of the intestinal mucosa, and there are number of microbial pathogens that may activate or enhance enterocyte apoptosis

Apoptosis is primarily carried out by caspases, although other factors such as apoptosis-inducing factor may also contribute to apoptotic cell death [18]. Key components of apoptosis are outlined below (Figure 1). There are two apoptotic pathways, both of which result in an activation of caspases. The “intrinsic” pathway, which may be activated by cellular stress, results in a translocation of Bid, Bad, and Bax from the cytosol to the mitochondria where they result in a release of cytochrome c into the cytosol. Liberated cytochrome c from mitochondria forms “apoptosomes” with apoptosis protease activating factor-1 and procaspase-9. Upon activation via auto-cleavage, caspase-9 can activate caspase-3, which results in the classical apoptotic death markers. The “extrinsic” pathway is activated by membrane-bound receptors for tumor necrosis factor – α (TNF- α) called TNFR-1, or Fas receptor that binds to the Fas ligand. This mechanism results in an activation of caspase-8, which activates caspase-3 directly

Figure 1. Schematic diagram of the mechanisms via which enterocytes undergo apoptosis.

Representation of extrinsic apoptosis, with external stimulation causing the activation of caspase-8 and eventually of caspase-3 (A). Representation of intrinsic apoptosis through the activation of Bid, Bad and Bax proteins, release of cytochrome c from the mitochondria and eventual activation of caspase-3 via caspase-9 (B). Prepared for and borrowed from Buret and Bhargava (2014) [18].



or feeds into the intrinsic pathway through activation of Bid that results in cytochrome c release from the mitochondria. In either case, caspase-3 is essential in cleaving substrates downstream, such as poly(-ADP-ribose) polymerase (PARP) leading to apoptotic cell death [18]. This process serves a key homeostatic purpose by facilitating a healthy turnover of the epithelium.

1.1.2 Intestinal epithelial cell apical junctions

Highly organized intercellular junctions known as apical junctional complexes (AJCs), desmosomes and gap junctions, seal the paracellular space between adjacent IECs [67]. This prevents the passage of unwanted pathogens and antigens into the underlying host tissues. AJCs are comprised of tight junctional proteins and adherens junction proteins, and contribute to the polarity of epithelial cells. These components also regulate the passive paracellular transport of ions, nutrients, and immune cells across the intestinal epithelium.

1.1.2.1 Physiology of intestinal tight junctions

Depending on their location in the human body, tight junctions are classified as “tight” or “leaky”. In the urinary bladder epithelium, tight junctions have to be “tight” and restrict the movement of ions across them; however, in the intestinal epithelium, these junctions are “leaky” to allow some paracellular flux for nutrient uptake and secretion [126]. Tight junctions (TJs) in the gastrointestinal (GI) tract form an important ion charge- and size-selective barrier between adjacent epithelial cells to separate the underlying host tissue from the external environment [124]. The size selectivity of the tight junction may be particular to the location and hypothesized to be under acute

regulation; for example, jejunal tight junctional pores in the crypt region are permeable to larger molecules than tight junctions in the jejunal villus [49, 86, 126].

There are two physical components of tight junctions, including cytosolic tight junctional plaque proteins and integral membrane proteins (Figure 2) [124]. Tight junctional plaque proteins are localized to the cytosol, interact and stabilize the transmembrane proteins at the junction, whereas the integral membrane proteins may have one, three or four transmembrane domains and define paracellular permeability [124].

The cytosolic plaque proteins consist of the zonula occludens (ZO) proteins. ZO-1 is a large ~220 kiloDalton (kDa) cytosolic, membrane-associated protein that was the first tight junction component to be discovered in 1986 [134]. ZO-1 has binding sites that provide for important interactions with transmembrane tight junctional components and regulatory actomyosin complexes [44, 66, 118, 156]. Taken together, the crucial role of ZO-1 in the maintenance of the function and organization of the tight junction is demonstrated through its interactions with other components of the tight junction. Indeed, a loss of ZO-1 expression leads to a loss of proper targeting of transmembrane proteins to the tight junction [90, 124]. McNeil *et al.* showed that gene silencing of ZO-1 delayed tight junction formation in Madin-Darby canine kidney (MDCK) cells [90].

Occludin and claudins are integral membrane proteins with transmembrane domains that interact with ZO-1. Occludin, a ~65kDa protein, was the first transmembrane protein to be identified [52]. Structurally, it is an intricate protein with four transmembrane domains, two extracellular loops, and N- and C-terminal cytoplasmic tails [124]. It has a vast number of interactions, including occludin itself, plaque proteins

ZO-1, -2, -3, and certain signalling molecules, including phosphatidylinositol-3-kinase [124]. A loss of occludin corresponds to a loss in barrier function *in vitro* [160]; however, mice lacking occludin are viable with normal TJ organization and epithelial barrier function, suggesting a compensatory role of other transmembrane proteins [113].

The compensation for the loss of occludin in TJ formation triggered significant research for the search into other tight junctional components, which resulted in the discovery of claudins [51]. Like occludin, proteins belonging to the claudin family can bind to ZO-1, -2 or -3, and are important for targeting to the TJ [66]. Functionally, claudins are important for charge selectivity in the pores they form, and this specificity can be attributed to the charge of amino acids that form the first extracellular loop [124]. Claudins are the major determinants of barrier properties of the TJ [85]. The mechanism by which claudins contribute to transport and barrier function both depend on the type of claudin that is in question. Claudin-2, for instance, forms pores that allows the passage cations, i.e. Ca^{2+} , and its expression is increased in Ulcerative Colitis (UC) patients [85]. Overall, TJs confer and contribute to the barrier function of the intestinal epithelium.

1.1.2.2 Regulation of intestinal tight junctions

Tight junctions in the small intestine are under a high degree of regulation that is required for the selective passage of molecules through the paracellular space. The cytoskeleton of enterocytes plays a critical role in the regulation of tight junctions. Contrary to static models, tight junctions must be dynamic in order to permit proper function and absorption of nutrients [127]. Sodium-glucose co-transporter (SGLT-1) activation results in myosin light chain (MLC) phosphorylation by myosin light chain kinase (MLCK), serving as an example of stimulation of the “leaky” phenotype of tight

junctions [144, 145]. The importance of MLCK in regulation of tight junctional permeability has been demonstrated by Shen and colleagues, as Caco-2 monolayers expressing constitutively active MLCK showed altered tight junction organization, including a marked redistribution of ZO-1 and occludin, and decreased barrier function [125]. Corroborating work in a murine model showed that mice expressing constitutively active MLCK had barrier dysfunctions, and had an increased permeability in the small intestine and the colon, strengthening the role of MLCK in regulation of tight junctions [135].

MLCK phosphorylates MLC in a Ca^{2+} -calmodulin-dependent fashion [71]. When the Ca^{2+} -calmodulin complex binds to MLCK, the C-terminal regulatory domain of the kinase is displaced from the catalytic region resulting in the activation of MLCK and phosphorylation of MLC [70]. MLC phosphorylation results in a contraction of actin filaments that are connected to plaque ZO-1 proteins at their actin-binding domains. This results in a distention of the transmembrane proteins resulting in an increase in epithelial permeability. Extrinsic regulators of tight junctional permeability will be discussed in section 1.1.4.2.

1.1.3 Intestinal epithelial cytoskeleton: role in homeostasis

The small intestinal epithelium serves a critical absorptive function, enhanced by its large surface area, one of the largest contributors to which are brush border microvilli. These are tiny protrusions that are found on the apical surface of the small intestinal epithelia. The epithelial cytoskeleton plays a major role in the proper maintenance of this structure, loss of which can result in malabsorption and reduction in important digestive enzymes [45]. The microvilli are extensions with uniform diameter with a core of cross-

linked actin filaments [72]. Undoubtedly, the organization and maintenance of actin confer structural integrity to enterocytes. The presence of only microfilaments of actin in the apical cytoskeleton of epithelial cells suggests that actin and actin-binding proteins have an important role in epithelial cell function and structure [72]. Furthermore, interactions of cytoskeletal proteins with membrane lipids point to a broader role for these proteins, particularly ezrin and villin – both of which have been shown to bind to phospholipids [62, 76]. Through their lipid bilayer-stabilizing interactions with the plasma membrane, actin-binding proteins have been suggested to be important for the maintenance of cell shape, and ion transport proteins [72]. Of particular importance to this study is the actin-binding protein, villin, which is an actin regulatory protein that is only expressed in epithelial cells.

1.1.3.1 Villin: A multi-functional cytoskeletal protein

Villin is a unique cytoskeletal protein that is expressed in GI, renal and urogenital epithelial cells, and plays a role in actin dynamics, signal transduction, cell morphology, cell migration, cell survival, cell polarity, and wound healing [8, 9, 72, 138, 149]. Villin is a 92kDa protein, found primarily in the microvilli in IECs, that has biochemical properties for both polymerizing and depolymerizing actin, with an ability to cap, sever, nucleate and bundle actin (Figure 2) [72]. Structurally, villin has a “core” that is conserved between the villin superfamily, which includes supervillin and gelsolin. The “core” has six repeated domains (S1-S6), along with a carboxylic acid-terminal headpiece [72].

Villin has specific sites in its core domains and headpiece that carry out its several molecular interactions. For example, the headpiece and regions on S1 and S2 domains

have actin-bundling sites, S1 is the capping site, S1 and S4 are nucleation sites, and finally S1 and S2 are severing sites [72]. This protein has at least two sites where it can bind with F-actin: one in the core and one in the headpiece. The regulation of villin's actin-modifying actions is controlled by calcium levels, phosphoinositides and by tyrosine phosphorylation of the protein [72]. Actin binding by villin is heavily dependent on Ca^{2+} , while villin's association with F-actin is inhibited by tyrosine phosphorylation [72].

The primary function of villin is its regulatory role in the organization of epithelial brush border microfilaments, and this role is exemplified by its leading apical localization during embryonic development [32]. Moreover, as enterocytes migrate and differentiate from the crypt towards the villus tip, the quantity of villin increases concurrently with the appearance of the brush border, supporting the crucial role of villin in microvilli formation [13]. While earlier *in vitro* studies demonstrated this, later *in vivo* work has determined that villin knockout mice still have microvilli, pointing to the role of other cytoskeletal proteins like fimbrin in the assembly of the brush border [8, 48]. The same study showed that Ca^{2+} -dependent villin activity is required for the reorganization of F-actin elicited by various signals, further pointing to villin's role in cell plasticity [48]. It is hypothesized that this property of villin may be involved in cell plasticity in response to cell injury [48, 146]. Indeed, a recent study showed that villin's F-actin severing function is important in gut wound healing and enterocyte polarity [146]. In mice that were missing the severing function of villin, wound healing in the colon was significantly reduced, and this was corroborated with cell culture data that further showed that the severing of microvillar actin filaments is required for proper cell migration,

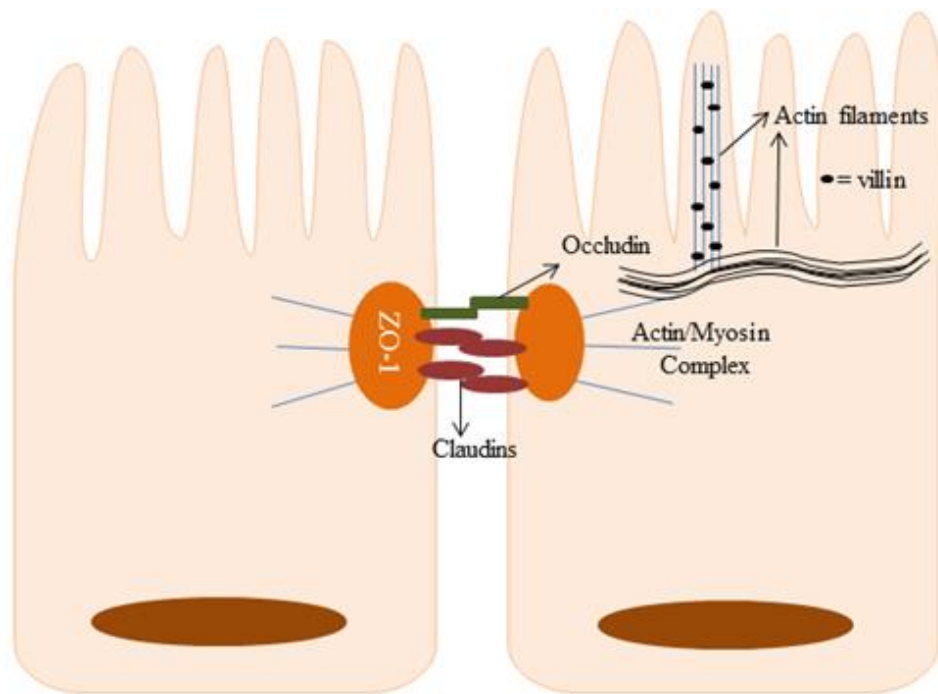
suggesting that microvillar disassembly is a crucial step in wound healing [146]. Villin's role in this is further supported by other cell culture data that showed its localization to the lamellipodium of migrating cells and enhancement of cell migration [139]. While villin-knockout mice have unaltered structural organization of microvilli as well as tight junctional proteins, villin may be required in mediating calcium ion-induced rearrangement of the actin microfilaments thereby regulating cell motility [48, 103].

Furthermore, previous evidence indicates that villin is a pro-survival protein, and its cleavage is suggested to generate pro-apoptotic fragments so as to assist with cellular extrusion at the tip of the villus [148]. In a study performed by Wang *et al.* in MDCK cells, an overexpression of villin resulted in delayed apoptosis in response to a camptothecin treatment [148]. Moreover, increased colonic epithelial apoptosis was observed in villin knockout mice when given a Dextran Sodium Sulfate treatment compared to their wild-type DSS-treated counterparts [148]. A collection of the amino-terminus cleaved fragment has been shown to be pro-apoptotic, while the collection of carboxylic acid-terminus fragments is pro-survival [148].

All of the above factors display that villin is a critical homeostatic protein with multiple functions that extend beyond the maintenance of the microvilli. The multifunctional purpose of this protein warrants more investigation in its role in pathophysiology.

Figure 2. Simplistic representation of enterocyte microvilli and tight junction composed of transmembrane claudins and occludins, and plaque ZO-1 protein.

The diagram represents enterocytes from the small intestinal epithelium. The blue lines in the microvilli represent actin, while the ● represents the actin-bundling cytoskeletal protein, villin. ZO-1 plaque protein is shown to be interacting with the actin/myosin complex, which is under the regulation of MLCK. Transmembrane proteins, occludin and claudins, interact with ZO-1 during and after the assembly of the tight junction.



1.1.4 Pathological disruption of epithelial homeostasis and barrier function

Several intestinal disorders are associated with a loss of barrier function resulting in a lack of homeostasis in the gut. Altered rates of cell death or cell proliferation, as well as tight junctional dysregulation can result in pathology. Both of these irregularities can allow the passage of commensal or pathogenic pathogens and antigens that could illicit an unwanted inflammatory response. Similarly, cytoskeletal disruption and breakdown can have implications for cell morphology and migration.

1.1.4.1 Modulation of enterocyte apoptosis and its pathological consequences

As previously mentioned, alterations to the rate of apoptosis in enterocytes or the rate of proliferation from the progenitor cells are part of pathophysiology of several diseases. Heightened rates of apoptosis, discussed herein, can result in the loss of the protective barrier function, and increased proliferation is associated with hyperplasia and tumorigenesis.

Senescent enterocytes undergo a process of apoptosis and are eventually shed into the lumen. This process under homeostatic conditions is highly regulated so as not to allow for breaks in the barrier. There are reports (mentioned earlier) showing that single cell apoptosis does not cause breaks in the epithelium and when gaps do form *in vivo* they are remain impermeable [1, 151]. However, microbes including bacteria, viruses, and parasites have all been shown to increase rates of epithelial apoptosis [18]. For example, *Giardia duodenalis* (discussed extensively in section 1.2) causes increased rates of enterocyte apoptosis both *in vitro* and *in vivo* [27, 99]. Several other enteric pathogens, including Enterohemorrhagic *Escherichia coli* (EHEC), *Salmonella*, Norovirus, and *Clostridium difficile* induce apoptosis in enterocytes via a number of mechanisms

targeting the pro-apoptotic cellular machinery [18]. Enteropathogenic *E. coli* (EPEC), EHEC, *Salmonella*, *C. difficile* all use one of their virulence factors to induce apoptosis or other types of cell death [18]. This allows for bacterial invasion through the epithelium and can cause inflammation [106]. Indeed, *Salmonella* has been associated with post-infectious irritable bowel syndrome (PI-IBS), and its pro-apoptotic action may contribute to its pathophysiology.

In addition, inflammatory conditions also result in increased apoptotic rates in enterocytes as seen in the case of helminth-induced TNF- α and IFN- γ release leading to enterocyte apoptosis [29]. These two cytokines promote epithelial cell apoptosis and promote epithelial barrier dysfunction *in vitro* [14]. Similarly, in chronic diseases such as Ulcerative Colitis (UC) and Crohn's Disease (CD), increased rates of IEC death are also observed. Higher permeability in UC patients was found at sites of ulcers where increased rates of apoptosis were also observed [61, 115]. CD patients also display increased rates of epithelial apoptosis, and anti-TNF- α treatments are effective therapies against this disease by restoring the barrier function, including normalizing rates of apoptosis [162]. In both instances pro-inflammatory cytokine is associated with an increase in apoptosis; TNF- α in CD, and interleukin-13 (IL-13) in UC [61, 115].

Thus, sustained apoptosis of epithelial cells causes a loss of homeostasis and this can contribute to the loss of the critical architecture that is also important for absorption and secretion [41].

1.1.4.2 Pathology associated with tight junction and cytoskeletal dysfunction

Tight junctions are targets of a number of pathogens in the initiation of disease [96]. For example, EPEC dephosphorylates occludin to dissociate it from TJs [128].

Helicobacter pylori has also been shown to have similar effects, including occludin internalization through MLC phosphorylation, suggesting that microbial pathogens are able to hijack host machinery to disrupt tight junctions [47, 124]. Similarly, protozoan parasites such as *Giardia* spp. and *Entamoeba histolytica* disrupt tight junctions to cause an increase in permeability [19, 80].

There is a well-established relationship between a loss of barrier function and inflammation in chronic intestinal diseases [97]. Links between pro-inflammatory cytokines mediated increase in tight junctional permeability have been demonstrated; TNF- α induces the leak pathway by inducing a reorganization of the TJ and actomyosin ring through MLCK activation, while IL-13 induces the pore pathway by increasing claudin-2 expression [61, 152].

Alterations to the apical cytoskeletal structure can result in a loss or dysfunction of the brush border microvilli resulting in a loss of important digestive enzymes, including disaccharidases [45, 130]. The role of villin and its interactions with pathogens, particularly non-invasive ones, have yet to be fully elucidated. *Entamoeba histolytica*, a protozoan parasite, causes the proteolytic breakdown of villin resulting in a loss of the microvillar morphology, while *Shigella*, a bacterial pathogen, has been shown to require villin for cell-cell dissemination [7, 81]. Villin's role in protection and recovery during inflammatory conditions, such as IBD, remains to be further studied.

Overall, tight junctional dysregulation by pathogens or inflammation through MLCK-mediated mechanisms leads to increased permeability and can contribute to disease. In addition, understanding the role of cytoskeleton and the changes it undergoes

during pathology can help further our knowledge about the initiation of disease during which epithelial morphology is altered.

1.2 *Giardia duodenalis*

Giardia duodenalis (syn. *G. intestinalis*, *G. lamblia*) is non-invasive protozoan parasite of the upper small intestines of mammals, including humans. This parasite superficially interacts with the epithelium of the small gut where it causes disease. *G. duodenalis* is a common cause of parasitic diarrhoea, with a prevalence of 2-7% in developed countries and 20-30% in the majority of developing countries. In fact, there are an estimated 280 million cases of giardiasis annually worldwide and 20,000 reported cases per year in the US alone [3, 158]. Due to the high burden of *G. duodenalis*-related illnesses in the developing world, its impairment on development and socioeconomic improvements, and its close association with poverty, *Giardia* spp. was included in the World Health Organization (WHO) Neglected Diseases Initiative in 2006 [117]. In addition to the developing world, it was revealed in a study that addressed migration-associated illnesses in Europe that *G. duodenalis* was the second most common pathogen and the most common pathogen associated with GI illnesses [155]. This parasite also has a huge zoonotic potential, and in the developed world has been isolated from various animals, including livestock and nonhuman primates. Common household pets, like dogs and cats, may also contribute to zoonosis of *G. duodenalis* infections through interactions like pet-handling, sleeping together, licking and kissing [50]. The wide range of impact *G. duodenalis* has on society warrants a better understanding of the disease it causes.

In some cases, *G. duodenalis* infections are asymptomatic leading to carriage of the parasite, particularly in the developing world [69]. However, the symptoms of

giardiasis can be quite variable ranging from acute to chronic diarrheal disease. Other symptoms that may also present include abdominal pain, nausea, malabsorption, and subsequent weight loss [50]. In the case of healthy individuals with competent immune systems, *G. duodenalis* infections tend to be self-limiting and are cleared within two weeks. Metronidazole or tinidazole are prescribed for both immune-competent and immune-suppressed individuals to treat giardiasis; however, relapses and clinical failures have been known to occur, pointing to a possible emergence of resistant isolates of *G. duodenalis* [20]. Interestingly, a recent study on Brazilian children showed that while re-infection with *G. duodenalis* does occur, the second exposure had reduced pathology compared to the first infection [75]. Particularly in children, *Giardia* spp. causes iron-deficiency anemia, micronutrient deficiency and malabsorption that may contribute to adverse impact on child linear growth and psychomotor development [3].

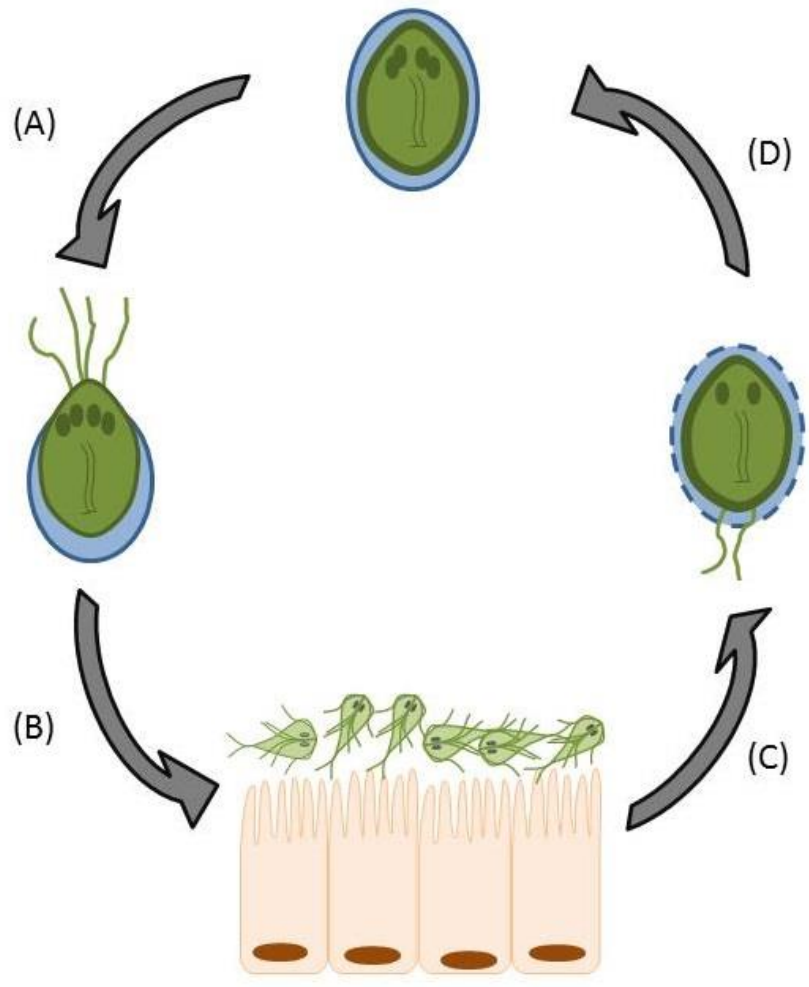
Extra-intestinal and long-term consequences of *G. duodenalis* infections have also been reported, including ocular pathologies, arthritis, stunting, impaired cognitive function, failure to thrive, chronic fatigue syndrome, allergies, PI-IBS [57]. These complications may present 2-3 years post-infection, and in some instances may only last a few weeks, but in other instances may have more chronic and long-term effects, particularly in the cases of chronic fatigue syndrome, failure to thrive, stunting and PI-IBS [57]. How giardiasis results in some of the more chronic long-term consequences remains to be explained.

1.2.1. Life Cycle

G. duodenalis has a simple life cycle, and only requires a definitive host with zoonotic potential (Figure 3). Its life cycle commences with the ingestion of highly

Figure 3. A Simplistic Illustration of the *Giardia duodenalis* Life Cycle.

The life cycle of *G. duodenalis* commences with the ingestion of infectious cysts in contaminated food or water, which undergo a process of excystation to release the disease causing trophozoites into the duodenal lumen (A). Trophozoites either adhere to IECs or swim around in the lumen (B). As trophozoites get to the distal small intestine or proximal colon, they begin to encyst (C). The cysts are subsequently released in the feces to further spread the parasite (D).



infectious cysts through the fecal-oral route, or through contaminated food or water. *G. duodenalis* is able to establish an infection with as few as 10 cysts [108]. These cysts, which are environmentally resistant, can persist in soil or water for months [3]. Upon reaching the small intestine, the cysts undergo excystation, a process triggered by host stomach acids, resulting in the release of two trophozoites per cyst [15]. The excysting cyst reaches the small intestine before rupturing to release the trophozoites [15].

The flagellated trophozoites then may colonize and attach themselves to the villous enterocytes in the small intestine or may remain free swimming. The trophozoite is the disease-causing stage of this parasite, and it has a structure called an adhesive disc, which acts as a suction cup that allows the trophozoite to attach to surfaces non-specifically [98]. Trophozoite motility via its four pairs of flagella, and host cell attachment contribute to its virulence, and prevent the parasite from being eliminated due to peristalsis [2, 43]. In addition to the classical cytoskeletal proteins, such as α - and β -tubulin, and the *Giardia* spp.-specific giardin family proteins, proper attachment of the trophozoites also requires parasitic lipid rafts [42, 64, 98, 153].

The next stage in the life cycle of *G. duodenalis* is called encystation, during which trophozoites undergo a transformation from the motile state to the non-motile and dormant state of the infectious cyst. Encystation of trophozoites is a process that is triggered by high levels of bile, low levels of cholesterol and a basic pH as the trophozoites move distally along the small intestine towards the colon [79]. This process begins with the trophozoites internalizing their flagella and the adhesive disc being fragmented [3, 98]. Strong interactions between carbohydrate chains as well as between cyst wall sugars and cyst wall proteins contribute to the cyst being very insoluble and

environmentally resistant [3]. The cyst is expelled into the external environment in the feces to further contaminate food or water.

1.2.2. Genetic Assemblages

To date, the *Giardia* spp. has been divided into eight total genotypes or assemblages, A through H. Out of the eight, only assemblages A and B are infective to humans. Assemblage C and D infect dogs, E infects hoofed animals, F infects cats, G infects rodents, and H infects seals [22, 77]. Genetic analysis has shown so far that there is a 78% amino acid identity between the two human assemblages, A and B [22]. For the two assemblages, most research has been done with the isolate WB from assemblage A, and GS/M from assemblage B. The isolate WB was obtained from human patients of giardiasis from Afghanistan, and GS/M was obtained from human patients from Alaska [3]. While a tremendous amount of work has been done in the laboratory with Assemblage A isolates, more recent epidemiological data suggests that assemblage B isolates may be more common in humans worldwide [22]. The idea that these two assemblages or even each genotype of *G. duodenalis* may be two different species is frequently debated; however, no consensus has been reached on that, or on whether pathogenicity is assemblage- or isolate-dependent. In addition to the phylogenetic analysis, further phenotypic studies looking at the pathogenesis of new isolates could provide further and better understanding about the assemblages. Heterogeneity among isolates and/or assemblages that are circulating in human populations may make certain isolates more prone to cause diarrheal disease over others [82]. This could also explain why asymptomatic *G. duodenalis* infections are observed.

1.2.3. Host-Parasite interactions and pathophysiology

The biology of *G. duodenalis* infections and interactions with the host remain incompletely understood. In this section, how the host mounts an immune reaction against this microscopic parasite will be discussed briefly; however, the focus will be on the intestinal pathophysiology induced by the parasite. To date, it has been established that *G. duodenalis* causes increased rates of enterocyte apoptosis, barrier function breakdown, shortening of brush border microvilli in a host lymphocyte dependent manner, anion hypersecretion, and increased rates of intestinal transit [33]. These will be discussed in following sub-sections.

1.2.3.1 Immunological host responses against *Giardia duodenalis* infections.

The host immune system involved in the clearance of *Giardia*, like other pathogens, relies on innate and adaptive components. IgA antibodies contribute to the clearance of *G. duodenalis* infections [129]. In addition, B cells and IgG antibodies are also implicated in helping with the clearance of infection. No overt inflammatory cell infiltrate is found during giardiasis with the exception of a mild increase in intra-epithelial lymphocyte numbers [121, 142]. Recent work in our laboratory has shown that *G. duodenalis* cathepsin B proteases are able to cleave interleukin-8 (CXCL-8), a potent chemoattractant for neutrophils, to cause reduced neutrophil chemotaxis to the site of injury [34]. In addition to this immuno-modulatory effect, *G. duodenalis* trophozoites are able to evade antibodies through their ability to change their surface proteins called variant-specific surface proteins (VSPs) [94]. In addition, mast cells may play a role by helping drive B cells to switch to a production of IgA, which may contribute to the clearance of the parasite [91, 129]. Similarly, *in vitro* work has also shown that mast cells

can be activated and stimulated by *G. duodenalis* antigens to produce histamine, TNF- α and IL-6 [93]. A murine model has also demonstrated the importance of mast cells in the clearance of *G. duodenalis* through the release of IL-6 [84]. Some of this immune activation may contribute to acute, and some long term effects of giardiasis, including PI-IBS [57].

1.2.3.2 *Giardia duodenalis* causes increased rates of enterocyte apoptosis.

As discussed in section 1.1.1, a homeostatic turnover of the small intestinal epithelium requires a fine balance between proliferation and cell death. The rates of enterocyte apoptosis are significantly increased shortly after exposure to *G. duodenalis* both *in vitro* and in patients [27, 99, 142]. Chin *et al.* showed that *G. duodenalis* induces caspase-3 dependent apoptosis in non-transformed canine duodenal epithelial cell line, SCBN, after a co-incubation for 24 hours [27]. These changes are isolate-dependent, which is rather interesting as the isolates all belong to assemblage A [27]. Yet another study assessing the apoptotic potential of *G. duodenalis* on an intestinal epithelial cell line, HCT-8, showed that the WB isolate (Assemblage A) of *G. duodenalis* is able to induce apoptosis on this cell line [99]. Interestingly, sonicates of the WB isolate failed to induce apoptosis in SCBN cells when looked at by Chin *et al.* (2002) [27]. This may suggest that the induction of apoptosis may be occurring via different mechanisms by one isolate versus another, and the WB isolate may require live and direct interactions with host enterocytes to induce apoptosis. Troeger *et al.* (2007) also observed increased apoptosis in patients of chronic giardiasis; however, the authors suggests that due to the chronic colonization of *Giardia* in these patients, it may also be the low-level release of pro-inflammatory cytokines such as TNF- α or IFN- γ that may contribute to increased

apoptosis [142]. Indeed, activation of pro-caspase-3 and -9, increased expression of pro-apoptotic Bax, decreased expression of anti-apoptotic Bcl-2 and an increased cleavage of PARP have all been shown to be involved in *G. duodenalis*-induced increased rate of apoptosis [27, 99]. In addition to intrinsic apoptosis via caspase-9 activation, *G. duodenalis* trophozoites also induce apoptosis in an extrinsic fashion, as seen by caspase-8 activation [99]. However, more work is needed to explain whether the activation of caspase-8 is a direct effect of giardiasis or if it is activated as a result of caspase-3 and -6 activation [33]. Whether any parasitic factors may be involved in the induction of apoptosis by *G. duodenalis* remains unclear. There are also protective mechanisms that act against such pro-apoptotic stimuli; indeed, Yu *et al.* showed that increased glucose uptake via the sodium-glucose co-transporter-1 (SGLT-1) may have cytoprotective roles against apoptosis induced by *G. duodenalis* [161]. In addition to causing apoptotic cell death, *G. duodenalis* may also halt enterocyte cell-cycle progression via its consumption of arginine and an up-regulation of cell-cycle inhibitory genes [131]. The induction of increased rates of apoptosis may contribute to a loss of barrier function, particularly in conjunction with *G. duodenalis*' effects on tight junctions.

1.2.3.3 Disrupted barrier function

A breakdown or disruption of tight junctional proteins can contribute to increased permeability across the epithelium; indeed, this has been shown to occur during giardiasis. *In vivo* studies have demonstrated that *G. duodenalis* infections result in elevated intestinal permeability during the peak of colonization, which is followed by a return to baseline upon clearance of infection [59, 120]. Chronic giardiasis patients also have increased intestinal permeability, in part due to increased apoptosis but also due to

disruptions of a key tight junctional protein, claudin-1 [142]. *G. duodenalis* induces increased intestinal permeability via direct alterations to the apical junctional complex, including disruptions to F-actin, ZO-1, and α -actinin, and downregulation of claudin-1, all of which are key components in sealing the paracellular space between enterocytes [19, 120, 137, 142]. Interestingly, relocalization of ZO-1 and F-actin seem to be mediated at least in part to the activation of caspase-3, suggesting a direct link between tight junctional disruptions and induction of apoptosis [27]. The disruption of tight junctional ZO-1 and F-actin is also MLCK-dependent [120]; however, whether this MLCK activation is caspase-3-dependent or –independent is not known. Recent findings also suggest that occludin, another transmembrane protein is cleaved in mice during giardiasis with the GS/M isolate from assemblage B, and the levels of cleaved occludin still persist even after the infection has been cleared [26]. This finding was contradictory to what was seen in the chronic giardiasis study that showed no occludin changes in biopsies from giardiasis patients compared to the control group [142]. This may point to assemblage-, isolate-, or host-specific pathophysiology during giardiasis. A downregulation of claudin-1 during chronic giardiasis and the presence of cleaved occludin even after the clearance of *G. duodenalis* suggests the involvement of host immunity and/or the gut microbiota in the breakdown of the epithelial barrier function, which may also contribute to one or more of the long-term consequences of giardiasis [26, 57, 142].

1.2.3.4 Diffuse shortening of brush border microvilli and other pathology

G. duodenalis induces diffuse shortening of epithelial brush border microvilli, and this represents a key mechanism for *G. duodenalis*-induced malabsorption and maldigestion [16, 121]. The total duodenal villous surface area is also decreased due to

the loss of microvilli in patients with chronic giardiasis, as assessed in human biopsies [142]. The processes of microvillar disaccharidase deficiency and brush border microvilli shortening are both mediated by activated CD8⁺ lymphocytes [121]. This, in part, supports the idea that immunopathophysiology in response to *G. duodenalis* takes place once the intestinal barrier defects have taken place [33]. Another factor that may contribute to malabsorption during giardiasis is villous atrophy in the small intestine; however, this may be an isolate-dependent phenomenon and may be contributing to the loss of absorptive surface area in the duodenum independently of the microvilli brush border shortening [33, 142]. Recent studies have implicated lymphocyte-dependent changes to cytoskeletal proteins, villin and ezrin, that are important for proper morphology of the intestinal epithelium in disaccharidase deficiency following giardiasis in mice [130]. This is proposed to happen via these cytoskeletal proteins contributing to proliferation of immature enterocytes increasing the ratio of immature to mature enterocytes resulting in lower disaccharidase levels [130], an idea supported by previous findings that demonstrate the presence of crypt hyperplasia leading to an increase in crypt/villus ratio during giardiasis [3, 6].

This loss of the absorptive surface area not only results in malabsorption, but also contributes to the osmotic gradient formed by the increased concentration of nutrients and electrolytes, such as glucose and sodium, resulting in water being drawn out into the lumen resulting in intestinal distention and increased peristalsis [33, 55]. Other factors that contribute to the increased transit and diarrhea during giardiasis include increased mast cell degranulation and adaptive immune responses, and increased chloride secretion by enterocytes [25, 55, 83].

1.2.4. Parasitic Products/Factors

One of the biggest gaps in our knowledge about the pathophysiology of *G. duodenalis* is the involvement of any parasitic products that may initiate disease. For example, we understand that caspase-3 is activated and this causes apoptosis in enterocytes, but we still do not understand how caspase-3 is activated. It is incompletely understood whether it is parasitic factors or host factors alone that contribute to enterocyte apoptosis. Similarly, MLCK-dependent tight junctional disruption is incompletely understood with the main question being what it is that activates MLCK: caspase-3 or another factor. A very small number of parasitic virulence factors have been identified thus far, and of these the major ones are the ventral adhesive disc and surface lectins that ensure attachment, the four pairs of flagella that confer re-colonization and movement, and VSPs to evade host IgA-directed clearance [2, 3, 98]. Due to the number of mechanisms that have been suggested to be important for the induction of symptoms, it has been suggested that the cause of giardiasis is likely multifactorial [3].

There are also parasitic products that are released by the trophozoites. *G. duodenalis* has been shown to release arginine deiminase (ADI) and ornithine carbamoyl transferase upon interaction with host cells [110]. These products allow *G. duodenalis* to evade host immune responses as both of these arginine-metabolizing enzymes are able to consume endogenous arginine, which is a critical metabolite for the production of epithelial nitric oxide (NO) [40]. NO has been shown to be cytostatic to *G. duodenalis*, suggesting that arginine deiminase may play a key role in the establishment of an infection [33]. Indeed, a recent study showed that *G. duodenalis* ADI causes arginine depletion, which in turn modulates host dendritic cell cytokine secretion [10]. TNF- α , a

pro-inflammatory and pro-apoptotic cytokine, release was increased from these dendritic cells, while anti-inflammatory IL-10 release was decreased as a result of ADI-mediated arginine depletion [10]. ADI is also able to contribute to antigenic variability, as its role in VSP-modification has been described [140]. In addition, ADI from mycoplasmas and streptococci has been shown to be an apoptosis-inducing virulence factor [17, 110]. Another parasitic product, deemed an unknown 58kDa “enterotoxin” produced by *G. duodenalis* has a role in excessive ion secretion and intestinal fluid accumulation through the induction of several signal transduction pathways [123]. Cysteine proteases are vital in the pathogenesis and pathophysiology of several protozoan parasites, and *G. duodenalis* also has genes for such proteases. Very little is known about these proteases in terms of their characterization and role in disease. These potentially crucial parasitic products will be discussed in detail in section 1.3.4.

1.3 Cysteine Proteases: their role in physiology and pathophysiology

Proteases are a class of enzymes that catalyze the cleavage of peptide bonds in the presence of water through a process called hydrolysis [23]. The process of hydrolysis requires that the target substrate peptide interact with the active site of the enzyme, and specificity of an enzyme is conferred not only by the amino acid residues in the active site but also through the residues neighbouring the active site [114]. Parasitic cysteine proteases are of importance in this study due to their relevance in the initiation of disease by protozoan parasites, including *Entamoeba*, *Leishmania*, and *Plasmodium*.

Cysteine proteases require a cysteine residue in the active site for hydrolysis. Cysteine proteases have a thiol group that has an enhanced ability to act as a nucleophile through the presence of a histidine residue that acts as a proton donor [114]. The

interaction between the sulfhydryl group of the cysteine residue and the imidazole group of the histidine residue create a nucleophilic thiolate-imidazolium charge relay diad [114]. This creates a complex that has two ionisable groups, which is what gives this type of protease its broad pH range for enzymatic activity (4.0 – 8.5) [114]. Cysteine proteases are divided into two main groups called clans CA and CD. Clan CA comprises of proteases that are papain-like, whereas clan CD has calpain-like proteases. Of particular interest to this study are cathepsins B and L-like cysteine proteases, both of which belong to Clan CA. One of the features of Clan CA peptides is their sensitivity to E64 (L-*trans*-epoxysuccinyl-leucyl-amido (4-guanidino) butane) and its derivatives, including E64d [104]. In the mammalian system, substrate specificity is also defined by the S₂ pocket of the enzyme, which is the region two amino acid residues closer to the N-terminus than the active site. In cathepsin B, an ‘occluding loop’ is present in the catalytic domain that confers specificity [114]. Cathepsin L in the same site, has an ERFNIN motif but lacks the peptide loop seen in cathepsin B [114]. Ca-074Me is a cathepsin B-selective irreversible inhibitor that targets the occluding loop of cathepsin B [141].

1.3.1 Proteases in intestinal homeostasis

Under normal homeostatic conditions, the small intestinal epithelium is in contact with proteases from a number of sources at low concentrations, including bacterial, pancreatic proteases, and brush border digestive enzymes produced by epithelial cells on the luminal side, and immune and mesenchymal proteases in the lamina propria [11]. Proteases, under normal conditions, play a big role in the turnover of gut barrier components as well as physiological migration of immune and non-immune cells, and also in the absorptive function of the small intestinal epithelium. Under inflammatory and

pathological conditions, a variety of these proteases are dysregulated leading to structural and functional changes of the epithelium that may further facilitate the inflammatory process [100, 122]. Proteases from pathogens have been shown to induce key physiological changes for the pathogenesis of acute infection, and studies assessing the long term consequences of such proteolytic activity are warranted. There are several types of proteases involved in homeostasis, including matrix metalloproteinases, aspartic proteases, serine proteases, and cysteine proteases.

An important source of proteases to consider in the gut is the microbiota or commensal microbes. Commensals can produce a variety of proteases including all of the ones discussed above. The role of microbial proteases in disease is reviewed extensively in [23]. One particular study that is of key interest is one where *Enterococcus faecalis* (*E. faecalis*), a gelatinase producing commensal bacterial species, was shown to induce inflammation in IL-10^{-/-} mice [73]. This protease from *E. faecalis* was shown to have a critical role in the initiation of inflammation as mutants lacking this gelatinase showed significantly less colonic inflammation [132]. It was also shown that this enzyme results in a reduction of E-cadherin expression, pointing to poor adhesion between epithelial cells, and further suggesting that a role for commensal bacterial proteases in the initiation of disease by targeting the barrier function of the epithelium layer [132]. Another study confirms such findings where serine proteases in fecal supernatants from diarrhea-predominant IBS (IBS-D) patients increased paracellular permeability in mice, and this was shown to be through an increase in phosphorylation of MLCK resulting in delayed ZO-1 distribution to the membrane [53]. A recent study established a link between increased fecal cysteine protease activity in patients with constipation-predominant IBS

(IBS-C) and occludin breakdown, and one of the suggested sources of such proteases was the microbiota [4]. This suggests that altered proteolytic profile can induce different mechanisms leading to disease. Overall, under homeostatic conditions, bacterial proteases are present but kept under control; however, during chronic diseases or in immunosuppressed hosts these bacterial proteases may contribute to further disease pathophysiology.

1.3.2 Parasitic proteases and their role in pathophysiology

Parasitic cysteine proteases have gained a lot of attention over the last decade. Cysteine proteases are the most common type of protease in protozoan and primitive metazoan parasites [88]. Indeed, it has been suggested that proteases serve as promising chemotherapeutic or vaccine targets against certain parasites [89, 114]. There are a number of factors that make them an attractive area of research, and this includes their adaptability to different substrates and their stability in different biological environments [114]. In certain cases, parasitic proteases are critical to the host-parasite interaction and the establishment of an infection due to their roles in immune evasion, virulence, and tissue and cellular invasion [114]. For instance, another protozoan parasite, *Entamoeba histolytica*, has several cysteine proteases that are involved in the degradation of extracellular matrix components of the colon, including fibronectin, laminin, and collagen [88, 105, 114]. In addition, their roles in immune evasion through a degradation of complement system, C3a and C5a, and IgA and IgG have been described, pointing further to the importance of these proteases in the establishment of an infection during amebiasis [74]. Clan CA proteases, particularly belonging to a subdivision named family C1, are targets for drug development of such proteases in *E. histolytica*, and

hemoglobinolytic falcipains from *Plasmodium* and cruzain from *Trypanosoma cruzi* [54, 60, 88, 112]. Within Clan CA, and particularly within the papain-family (C1), parasitic proteases such as cathepsin B and L have a high degree of homology in the active site with the mammalian proteases [114]. As such, some of the techniques for studying human cathepsins can be employed as a beginning tool towards assessment of the role of proteases in parasitic pathogenesis. Understanding the role of proteases in parasitic diseases as well as differences between host and parasitic proteases can help pave the way for drug therapy development.

1.3.3. *Giardia duodenalis* cathepsin-like cysteine proteases

The parasitic products that are involved in the pathogenesis of this parasite are still unknown. Indeed, *G. duodenalis* trophozoites produce a number of products as discussed in section 1.2.4, and one set of these factors, cysteine proteases, and their role in pathogenesis still remains understudied. *G. duodenalis* cultures have been shown to have proteolytic activity originating from cysteine, serine, aspartic proteases, metalloproteases, and aminopeptidases [31, 37, 111, 154]. Furthermore, *G. duodenalis* has been shown to express 23 genes for cathepsin-like cysteine proteases, including genes for cathepsin B-like, cathepsin C-like, and cathepsin K/L-like cysteine proteases [38]. There are structural differences between these proteases and the mammalian proteases, however. The aforementioned occluding loop in the mammalian cathepsin B provides proteases with peptidyl dipeptidase activity; however, the *Giardia* cathepsin B-like proteases lack this occluding loop, and therefore expected to only have endopeptidase activity [65].

Thus far, one of the roles for these proteases that has been established is in encystation and excystation, where a cysteine protease called GICP2 was shown to degrade cell wall proteins [150]. Later, it was shown that this protease is highly expressed during the vegetative and encysting stages of the parasite's life cycle [39]. *G. duodenalis* excretory/secretory products have been shown to degrade gelatin and collagen in a cysteine protease dependent manner, which was inhibited by E64 [37]. A recent study from our lab has demonstrated that a *G. duodenalis* cathepsin B-like cysteine protease cleaves interleukin-8, leading to reduced neutrophil chemotaxis, as measured by myeloperoxidase activity [34]. However, the effect of these cathepsin-like cysteine proteases on live enterocytes has yet to be evaluated. A better understanding of such proteases in the initiation of disease, particularly in enterocyte homeostasis, can further our knowledge about *G. duodenalis* pathogenesis, and might also open doors for drug development through the targeting of specific proteases instead of the traditional metronidazole treatment, which has proven issues with resistance [20].

1.4 Summary

G. duodenalis trophozoites induce a wide variety of pathophysiological responses including increased rates of apoptosis, tight junctional disruption and barrier dysfunction, and a loss of the microvilli brush border leading to malabsorption. *G. duodenalis* has genes for 23 cathepsin-like cysteine proteases with largely unknown roles. Elucidating roles for these proteases could fill one of the biggest gaps in the study of giardiasis and pave the way for drug development. This study aims at uncovering the role for *G. duodenalis* proteases during host IEC-parasite interactions to assess their effects on important homeostatic proteins, including tight junctional and cytoskeletal proteins.

1.5 Hypothesis and Objectives

1.5.1 Hypothesis

G. duodenalis trophozoites contain and release cathepsin-like cysteine proteases. These proteases contribute to the disruption of tight junctional and cytoskeletal proteins.

1.5.2. Objectives and aims

The objective of this study is to characterize cysteine cathepsin-like activity during *G. duodenalis*-host interactions, as well as to assess the role of these cathepsin-like cysteine proteases in *G. duodenalis* pathophysiology.

Specific aims are as follows:

1. To characterize cysteine cathepsin-like protease activity during parasite-host co-cultures with IECs.
2. To determine if *G. duodenalis* cathepsin-like cysteine proteases are involved in the breakdown of barrier function through the alteration of tight junctional proteins, particularly in the disruption of occludin, claudin-1, and ZO-1. If so, then to determine the mechanisms via which this disruption occurs.
3. To determine if *G. duodenalis* cathepsin-like cysteine proteases are involved in cytoskeletal disruption and degradation, particularly of actin and villin. If so, then to determine the mechanisms via which this disruption occurs.

2. METHODS AND MATERIALS

2.1 Reagents, Inhibitors and Antibodies

In some experiments, Formononetin (10 μ M) purchased from Sigma-Aldrich (Oakville, ON, Canada) was used to detach trophozoites from enterocytes following co-incubations.

For the detection of cathepsin B/L activity, a fluorogenic substrate, Benzyloxycarbonyl-L-Phenylalanyl-L-Arginine 4-Methyl-Coumaryl-7-Amide (Z-Phe-Arg-AMC) (200 μ M), from Peptides International (Louisville, KY, USA) was used. Cathepsin B/L activity was measured based on the liberation of the fluorescent 7-amino-4-methylcoumarin (AMC) group over time. A cathepsin B-selective substrate, Benzyloxycarbonyl-L-Arginine-L-Arginine 4-Methyl-Coumaryl-7-Amide (Z-Arg-Arg-AMC) (Peptides International), was also used at the same concentration of 200 μ M. All cathepsin assays were kinetic assays and were run in the presence of a sodium acetate buffer (100mM sodium acetate, 10mM DTT, 0.1% Triton X-100, 1mM EDTA, 0.5% DMSO).

The inhibitors used in this study included a cathepsin B-selective inhibitor, L-3-trans-(Propylcarbamoyl)Oxirane-2-Carbonyl)-L-Isoleucyl-L-Proline Methyl Ester (Ca-074Me) (10 μ M or 200 μ M; Peptides International) or a broad spectrum Clan CA cysteine protease inhibitor, (2S,3S)-*trans*-Epoxy succinyl-L-leucylamido-3-methylbutane ethyl ester (E64d) (10 μ M or 200 μ M; Sigma-Aldrich). *G. duodenalis* trophozoites were either pre-incubated with these inhibitors prior to exposure to Caco-2 monolayers or each of the inhibitors was added in a co-incubation setting. Also, ML-9 (40 μ M; Sigma-Aldrich), an

inhibitor of MLCK was used. A caspase-3 inhibitor, Z-DEVD-FMK (caspase-3 inhibitor II) (50 μ M), from EMD Millipore (Billerica, MA) was used as well.

The following antibodies were used for immunoblotting and immunofluorescence: ZO-1 anti-mouse monoclonal antibody (1:500) from Invitrogen Life Technologies (Burlington, ON), Occludin anti-mouse monoclonal antibody (1:500; Invitrogen Life Technologies), Claudin-1 anti-mouse monoclonal antibody (1:500; Invitrogen Life Technologies), Villin anti-mouse monoclonal antibody (1:500) from Santa Cruz Biotechnologies (Dallas, TX), and Actin anti-mouse monoclonal antibody (1:500; Santa Cruz). Mouse and rabbit antibodies (1:1000 for both) from Cell Signaling Technologies (Beverly, MA) conjugated with horseradish peroxidase (HRP) were used for Western blotting.

2.2 Cell Culture

Previous work in our lab and elsewhere has shown that the Caco-2 cell line serves as a reliable model for studying giardiasis [34, 46, 92, 137]. Caco-2 cells (obtained from American Type Culture Collection, Manassas, VA) were grown into monolayers in 25cm² culture flasks between passages 22 and 34, and were supplemented with Minimum Essential Media Eagle (MEME), 100 μ g/ml streptomycin, 100 Units/ml penicillin, 200mM L-glutamine, 5mM sodium pyruvate (all from Sigma-Aldrich), and 20% heat-inactivated fetal bovine serum (FBS) (VWR, Radnor, PA). The cells were subcultured using 2x Trypsin-EDTA into 6-well plates (Becton Dickinson, Sparks, MD) or Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL) when the flasks were at approximately 80% confluence. The media in the subcultures and flasks was replaced every 2-3 days. The cells were incubated at 37°C, 5% CO₂, and 96% humidity.

2.3 Propagation of *Giardia duodenalis* trophozoites in axenic culture

The NF *G. duodenalis* isolate, belonging to assemblage A (unpublished data), was obtained following an epidemic of human giardiasis in Newfoundland, Canada [27]. The S2 isolate of *G. duodenalis* also belonging to assemblage A (unpublished data) was obtained from a sheep [27]. The Assemblage B isolate, GS/M, of *G. duodenalis* was obtained from the American Type Culture Collection (ATCC).

Trophozoites of *G. duodenalis* from Assemblage A isolates (NF and S2), and Assemblage B isolate (GS/M) were cultured axenically in Diamond's TY1-S-33 medium supplemented with piperacillin (Sigma-Aldrich). Trophozoites were grown and passaged in 15mL polystyrene conical tubes (Benton Dickinson Falcon) at 37°C under anaerobic conditions.

2.4 *Giardia duodenalis* trophozoite co-incubation

When confluent, the trophozoite tubes were placed on ice for 30 minutes to cold shock the trophozoites. Following this, the 15mL tubes were pooled into 50mL polypropylene tubes and centrifuged at 500xg for 10 minutes at 4°C and the supernatant was aspirated. The pellets were resuspended and collected in 10mL of chilled sterile 1x Phosphate Buffered Saline (PBS). This solution was centrifuged with the same settings as above, and the resulting pellet was resuspended in infection medium (same as the Caco-2 culture media). The trophozoites were enumerated using a hemocytometer, and their concentration was adjusted to fit experimental conditions. Three different types of infection models were used.

1) Co-incubation Model: Multiplicity of infection for *in vitro* experiments with Caco-2 monolayers was maintained at 10 trophozoites : 1 host cell. Old Caco-2 medium

was replaced with fresh medium. Trophozoites that had been pre-treated with either E64d or Ca-074Me or DMSO alone (section 2.8) were co-incubated with Caco-2 monolayers. Alternatively, trophozoites were co-incubated with Caco-2 cells in the presence or absence of ML-9 (40 μ M). Similarly, trophozoites were also co-incubated with Caco-2 cells in the presence or absence of Z-DEVD-FMK (50 μ M). The co-incubations were limited to 2 or 24 hours. The incubation conditions were maintained at 37°C, 5% CO₂, and 96% humidity for the entirety of the co-incubations. Caco-2 monolayers were collected and processed for Western blotting (section 2.5).

2) Co-Lysates Model: Culture media was removed from confluent Caco-2 monolayers, followed by a PBS wash. All of the solution was aspirated off, and the Caco-2 monolayers were collected in 200 μ L protease inhibitor-free RIPA in microcentrifuge tubes and sonicated for 5 seconds at level 3. Caco-2 lysates protein concentrations were adjusted to 3mg/mL using the Bradford assay method (BioRad Laboratories, Hercules, CA). 1x10⁷ trophozoites/mL (sonicated at level 4 for 30 seconds) were then added to the normalized Caco-2 lysates in the presence or absence of cysteine protease inhibitors (E64d or Ca-074Me) as discussed in the reagents section at 200 μ M for 2 hours under the same conditions as in method 1 in the presence of 10mM DTT. Samples were processed for Western blotting.

3) Transwell Model: Caco-2 monolayers were grown in 12 well plates until confluence, and old medium was replaced prior to incubation with trophozoites. Transwell filter units (0.4 μ m pore size) were installed and used to separate *G. duodenalis* NF trophozoites from the underlying Caco-2 monolayers. *G. duodenalis* NF

trophozoites were co-incubated in the presence or absence of Transwells for 24 hours. Subsequently, Caco-2 cells were lysed for Western blotting.

2.5 Whole cell protein extraction for electrophoresis

At the end of the co-incubation for 2 or 24 hours, trophozoites were removed from the co-culture via three ice-cold PBS washes. The Caco-2 monolayers were collected in RIPA buffer supplemented with a protease inhibitor cocktail tablet (Complete-Mini, Roche Diagnostics, Laval, QC). After a 30 minute incubation at 4°C, lysates were sonicated (level 3 for 5 seconds) and centrifuged at 10,000xg for 10 minutes at 4°C. Cell lysate protein concentrations were normalized to 1mg/mL using the Bradford assay method. Following normalization, 2x electrophoresis buffer was added to the samples to further dilute the samples to 0.5mg/mL. The samples were then heated at 95°C for 3 minutes. The samples were stored at -20°C until further analysis.

2.6 Gel electrophoresis and transfer

Once thawed, protein samples were separated via SDS-PAGE (7-12%) and transferred to nitrocellulose membranes (Whatman, Buckinghamshire, England) over 1h and at 100V. The membranes were blocked using 5% fat-free milk solution in 1x Tris-buffered saline + 0.1% Tween (TBS-T) for 1 hour. Primary antibodies were diluted in the same 5% milk solution and incubated with the membranes overnight at 4°C. After three 15-minute washes with TBS-T, HRP-conjugated secondary antibodies, also diluted in 5% milk solution, were added to the membranes for 1 hour at room temperature. The membranes were washed three times with TBS-T for 15 minutes each. The membranes were visualized using ECL-plus chemifluorescence detection system (GE Healthcare, Pittsburgh, PA). ECL-plus was added the membranes for 5 minutes. The membranes

were visualized on ECL film (GE Healthcare). The films were scanned for densitometric analysis using the software ImageJ (<http://rsbweb.nih.gov/ij/>). The membranes were stripped using 0.5M acetic acid (45 minute incubation) and 0.2M NaOH (5 minute incubation), and re-probed with a GAPDH antibody to ensure equal loading of the gels. In all instances, GAPDH was used as the loading control.

2.7 Immunofluorescence

Caco-2 monolayers were grown in Lab-Tek chamber slides to confluence (8-10 days), at which point, they were co-incubated with *G. duodenalis* NF trophozoites (1×10^7 trophozoites) that had been pretreated for 3 hours with E64d (10 μ M) for 2 or 24 hours at 37°C and under 5% CO₂ conditions. In another set of experiments, Caco-2 monolayers were treated with ML-9 (40 μ M) for 30 minutes prior to co-incubation with *G. duodenalis* NF trophozoites for 2 or 24 hours. At the end of the co-incubation, the infection medium was aspirated off out of each chamber, followed by two ice-cold PBS washes. To fix/permeabilize the cells, ice-cold methanol was then added to each well and the chamber slides were subsequently incubated at 4°C for 30 minutes. After two more PBS washes, the cells were blocked with heat inactivated-fetal bovine serum (HI-FBS; VWR) for 15 minutes at room temperature. Primary antibodies were prepared in a solution of 2% FBS-PBS at appropriate concentrations, and the cells were incubated with these for 1 hour at 37°C. Following two PBS washes, the cells were incubated with fluorescent secondary antibodies (also prepared in 2% FBS-PBS) for 1 hour at 37°C. The cells were subsequently washed twice with PBS. Nuclei were counterstained with 1 μ M Hoechst fluorescent staining 33258 (Invitrogen Life Technologies) for 30 minutes at 37°C. The cells were washed with PBS once and the slides were mounted with Aqua Poly/Mount

(Polyscience Inc.; Warrington, PA). Micrographs were obtained using a Leica DMR Microscope, with a Retiga 2000R (Q Imaging, BC) at 400x. All images were collected with the same gain and exposure time lengths. Micrographs presented in the Results section are representative images of 2 replicate monolayers from three separate experiments.

2.8 Cathepsin activity assays

After co-incubating *G. duodenalis* trophozoites with Caco-2 monolayers for 24 hours according to model 1 outlined in section 2.3, the infection media, *G. duodenalis* trophozoites, and Caco-2 monolayers were isolated and collected. The supernatant from the co-culture was collected and centrifuged at 500xg at 4°C for 10 minutes to separate the trophozoites from the supernatant. The supernatant from the centrifuged solution was carefully collected without disturbing the pellet. The pellet consisting of *G. duodenalis* trophozoites was resuspended in ice-cold PBS and centrifuged again at 500xg for 10 minutes at 4°C. The PBS was aspirated off and the pellet was resuspended in RIPA buffer.

In order to completely separate *G. duodenalis* trophozoites from Caco-2 monolayers, a sterile 10µM formononetin (reconstituted in sterile DMSO) solution was made in the MEME Caco-2 media, and this solution was added to the monolayers previously incubated with *G. duodenalis* trophozoites. Formononetin has been shown previously to cause a rapid detachment of *Giardia* trophozoites off of glass, epithelial cells and in mice [78]. The monolayers were then placed in infection conditions for 60 minutes. The formononetin solution was aspirated off, and monolayers were washed with ice-cold PBS three times. The monolayers were then scraped and collected in the RIPA

lysis buffer. RIPA buffer was not supplemented with a protease inhibitor cocktail tablet during this collection. All samples were snap frozen in liquid nitrogen and stored at -70°C.

2.8.1 Supernatant cathepsin activity

To assess the cathepsin activity in the supernatant, the samples were thawed and incubated with a cathepsin assay sodium acetate buffer and a cathepsin B/L substrate, Z-Phe-Arg-AMC (ZFR-AMC) (200µM) or cathepsin B-selective substrate, Z-Arg-Arg-AMC (ZRR-AMC) (200µM) in 96 well, clear and flat bottom plates [136]. The ratio of sample to buffer and substrate was 1:2. Microplates were incubated at 37°C for 10 minutes during a kinetic read using a plate reader (SpectraMax M2^e, Molecular Devices, Sunnyvale, CA) at an excitation and emission at 354nm and 445nm respectively. The rate at which the fluorescence changed as a result of the AMC group being cleaved and liberated, and read by the plate reader determined the amount of cathepsin activity [136]. In other words, the slope of fluorescence change over time was the cathepsin B/L activity in the sample.

2.8.2 Intra-trophozoite cathepsin activity

The trophozoite lysate samples were thawed and sonicated at level 3 for 5 seconds. They were then centrifuged for 10 minutes at 10,000xg at 4°C. Following this, a Bradford assay was performed and all samples were normalized to 1 mg/mL. Once normalized, the samples were plated in a 96 well clear and flat bottom plate in a 1:3 ratio of sample to NaAcetate buffer at a pH of 7.2 in the presence ZFR-AMC or ZRR-AMC (both at 200µM). The plate was read over 5 minutes at an excitation and emission at

354nm and 445nm respectively. The rate at which fluorescence was given out determined the amount of cathepsin activity in each sample.

2.8.3 *Intra-enterocyte cathepsin activity*

The Caco-2 lysate samples were thawed and sonicated at level 3 for 5 seconds. They were then centrifuged for 10 minutes at 10,000xg at 4°C. Following this, a Bradford assay was performed and all samples were normalized to 3-4 mg/mL. Once normalized, the samples were plated in a 96 well clear and flat bottom plate in a 1:3 ratio of sample to NaAcetate buffer at a pH of 7.2 in the presence ZFR-AMC or ZRR-AMC (both at 200µM). The plate was read over 5 minutes at an excitation and emission at 354nm and 445nm respectively using a plate reader. The rate at which fluorescence was given out determined the amount of cathepsin activity in each sample.

2.9 Inhibition of *Giardia duodenalis* cysteine protease activity

The cysteine protease inhibitors used in this study listed in *Reagents and Antibodies* (2.1) were either co-incubated in the co-cultures of Caco-2 cells and *G. duodenalis*; or *G. duodenalis* trophozoites were pre-treated with one of them prior to co-incubation with Caco-2 cells. The concentrations of both inhibitors were maintained at 10µM. At this concentration, neither E64d nor Ca-074Me has toxic effects on *G. duodenalis* trophozoites, while also showing inhibition of cathepsin-like cysteine protease activity [24, 34].

2.9.1 *Co-treatment with cysteine protease inhibitors*

Ca-074Me (10µM) or E64d (10µM) was added to the Caco-2 monolayers briefly before the addition of trophozoites. *G. duodenalis* trophozoites were enumerated, and subsequently added to the monolayers at a ratio of 10 trophozoites : 1 enterocyte. After a

24 hour co-incubation of Caco-2 monolayers with *G. duodenalis* trophozoites, Caco-2 cells were isolated and processed for Western blotting after 24 hours using the protocol outlined in section 2.5.

2.9.2 Pre-treatment with cysteine protease inhibitors

E64d (10 μ M) or Ca-074Me (10 μ M) or vehicle control (DMSO) was added to the *G. duodenalis* trophozoite culture tubes for 3 hours. At higher concentrations (50 μ M and 100 μ M) of E64d, *G. duodenalis* trophozoites lose their ability to adhere to culture tubes [24]. The tubes were subsequently placed on ice, appropriate groups pooled, and centrifuged at 500xg at 4°C for 10 minutes. The supernatant was aspirated and the pellet was re-suspended in ice-cold PBS. Following being centrifuged at 500xg at 4°C for 10 minutes, the pellet was resuspended in infection medium. Trophozoites were enumerated, and then co-incubated with Caco-2 monolayers for 2 or 24 hours at a ratio of 10 trophozoites : 1 enterocyte. Caco-2 lysates were isolated and collected for Western blotting.

2.10 Statistical analysis

All data were from three separate experiments, and were expressed as means \pm SEM, where applicable. All statistical analysis was performed using the software, Graphpad Prism 6, which ensures normality of data prior to analysis. Comparisons between groups were made using Student T-Test or One-way ANOVA, followed by Tukey's test for multiple comparison analysis. Statistical significance was established at $p < 0.05$.

3. RESULTS

3.1 Characterization of *Giardia duodenalis* cathepsin-like cysteine protease activity during co-incubation with Caco-2 monolayers

It has previously been established that *Giardia* spp. contain 23 genes for cathepsin-like cysteine proteases, including cathepsin B-like, cathepsin K/L-like, and cathepsin C-like cysteine proteases [38]. Based on these observations made previously, we investigated whether *G. duodenalis* trophozoites have active proteases and whether the amount of proteolytic activity is isolate-dependent or dependent on the presence of intestinal epithelial cells. All of the characterization experiments were conducted in the presence or absence of Caco-2 monolayers.

3.1.1 *G. duodenalis* trophozoites contain cathepsin B/L activity

G. duodenalis trophozoites contain cathepsin-like cysteine proteases, and their activity can be quantified using fluorogenic substrates (ZFR-AMC or ZRR-AMC). Lysates of *G. duodenalis* NF trophozoite isolated from co-cultures with Caco-2 monolayers (24 hours) were co-incubated with ZFR-AMC at 37°C resulted in the liberation of AMC, which was detected over time as seen as reflective fluorescent units (RFUs) (Figure 4A). The slope of this graph, calculated by SoftMax Pro, is indicative of the amount of cathepsin activity, and is expressed as cathepsin activity based on ZFR-AMC (Figure 4B) or ZRR-AMC (Figure 4C) processing. All three isolates (NF, S2 or GS/M) displayed cathepsin activity at the end of a 24 hour co-incubation with Caco-2 monolayers (Figure 4B, 4C). No differences in intra-trophozoite cathepsin B/L activity were observed in the presence or absence of Caco-2 monolayers (Figure 4B). No differences were observed in the cathepsin B/L activity between lysates from the three

isolates or the two assemblages (Figure 4B). Interestingly, the total amount of cathepsin B-like activity was lower as assessed by ZRR-AMC cleavage than the corresponding group processing ZFR-AMC (Figure 4C). Overall, *G. duodenalis* trophozoites from all of the isolates that were tested contained cathepsin B/L-like cysteine proteases.

3.1.2 *G. duodenalis* trophozoites release cathepsin B/L activity

Supernatants from cultures of *G. duodenalis* trophozoites in the presence or absence of Caco-2 monolayers were assessed for cathepsin activity using ZFR-AMC, or ZRR-AMC substrates. Control supernatants did not have any cathepsin activity present as no change in RFUs was observed over time; however, in the presence of *G. duodenalis* NF trophozoites we observed liberation of the AMC group from the substrate increased over time and was detected by the plate reader indicative of cathepsin activity both in the presence or absence of Caco-2 monolayers (Figure 5A). The slope or change in RFUs over time (RFU/s) was calculated by the software SoftMax Pro similar to section 3.1.1. All isolates displayed similar amounts of cathepsin activity in the presence of Caco-2 cells for the processing of ZFR-AMC or ZRR-AMC (Figure 5B, 5C). However, cathepsin activity in the presence of Caco-2 cells was observed to be higher than in supernatants from trophozoites alone for the assemblage A isolates (NF and S2). This was not the case for the assemblage B isolate, GS/M (Figure 5B). This effect was not present when supernatant cathepsin B-like cysteine protease activity was assessed using the selective substrate, ZRR-AMC (Figure 5C). Overall, *G. duodenalis* trophozoites release cathepsin B/L-like cysteine proteases, which may be stimulated by the presence of Caco-2 cells in Assemblage A, but not Assemblage B, isolates.

Figure 4. *G. duodenalis* trophozoites constitutively contain intra-trophozoite cathepsin B/L activity in an isolate-independent manner.

G. duodenalis trophozoites from Assemblage A (isolates NF, S2) or Assemblage B (isolate GS/M) were incubated in the presence or absence of Caco-2 monolayers for 24 hours. Isolate NF trophozoite lysates were assessed for cathepsin activity using Z-Phe-Arg-AMC (cathepsin B/L substrate) and the release of AMC was recorded as RFUs over 5 minutes as a representative line graph for the rest of the isolates (A). The slopes or cathepsin B/L activity were calculated and represented as histograms for the different trophozoite lysates tested for Z-Phe-Arg-AMC processing. There was no significant difference between the isolates in cathepsin B/L activity (B) or for Z-Arg-Arg-AMC (cathepsin B-selective substrate) processing, and no differences between isolates were observed (C). In both instances, the presence of Caco-2 monolayers did not affect the total amount of cathepsin B/L or cathepsin B activity within the trophozoites from any isolate. Data are mean \pm SEM, n = 3.

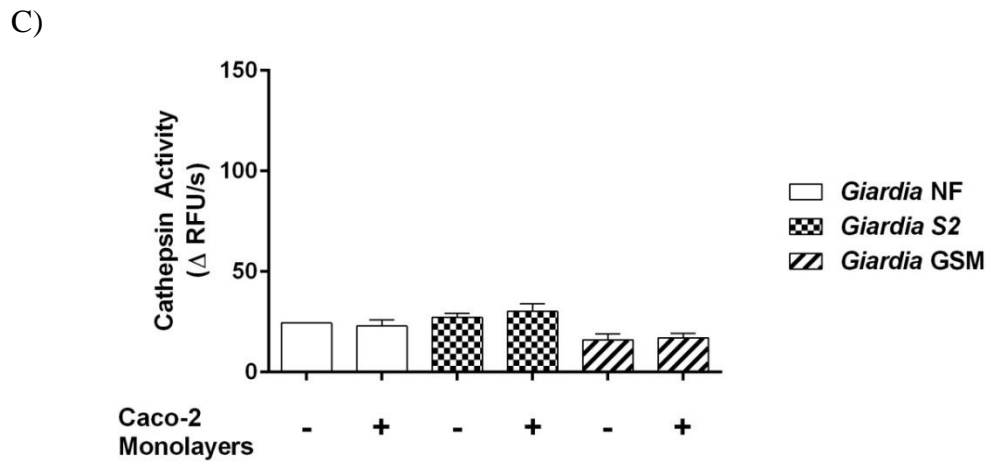
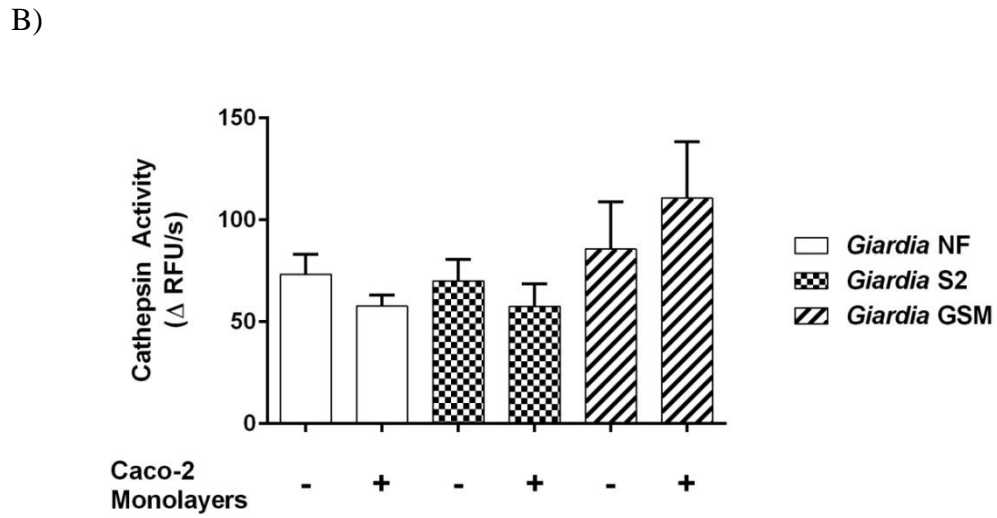
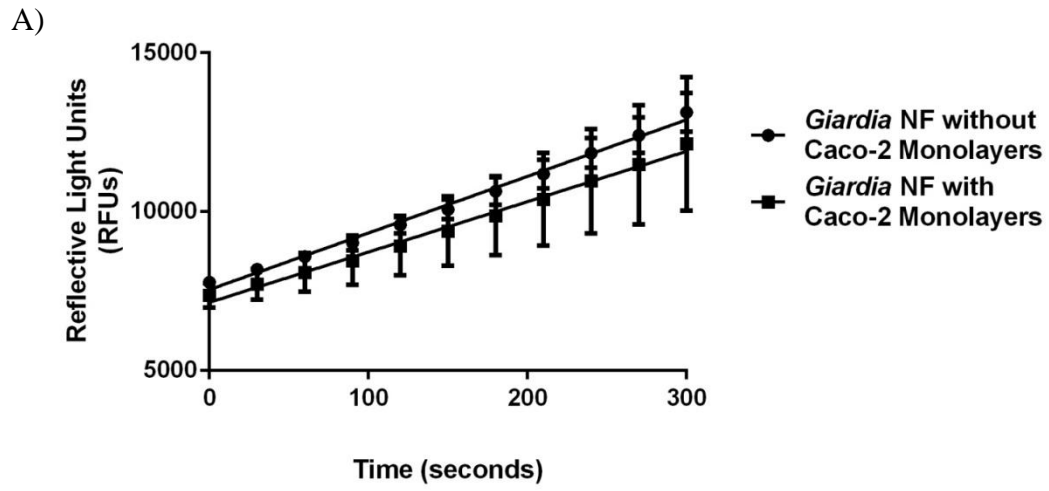
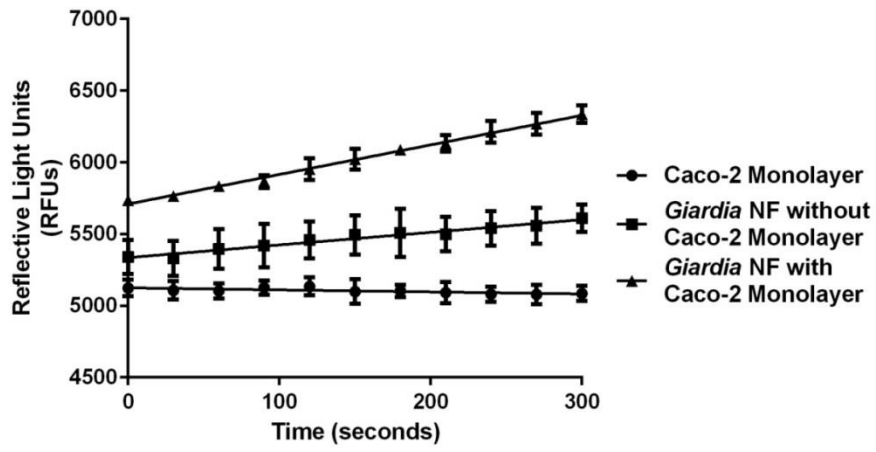


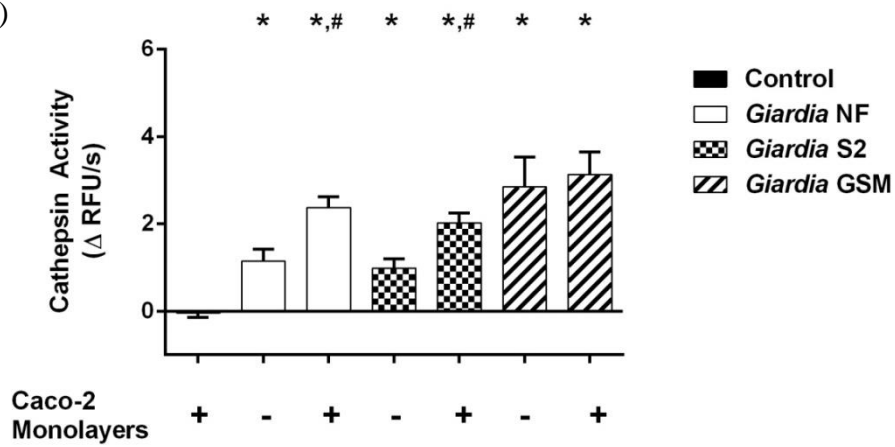
Figure 5. *G. duodenalis* trophozoites release cathepsin B/L activity.

G. duodenalis trophozoites from Assemblage A (isolates NF, S2) or Assemblage B (isolate GS/M) were incubated in the presence or absence of Caco-2 monolayers for 24 hours. Supernatants from isolate NF incubations were assessed for cathepsin activity using Z-Phe-Arg-AMC and the release of AMC was recorded as RFUs over 5 minutes as a representative line graph for the rest of the isolates (A). The slopes or cathepsin B/L activity were calculated and represented as histograms for the different trophozoite lysates tested. ZFR-AMC cathepsin B/L activities were not significantly different between isolates, but there was an increase in released cathepsin B/L activity in the presence of Caco-2 monolayers for *G. duodenalis* isolates NF and S2 (B). ZRR-AMC processing was not significantly different between isolates, and was not affected by the presence of Caco-2 monolayers (C). * $p < 0.05$ vs Control monolayers; # $p < 0.05$ vs corresponding isolate incubated without Caco-2 monolayers. Data are mean \pm -SEM, n = 3.

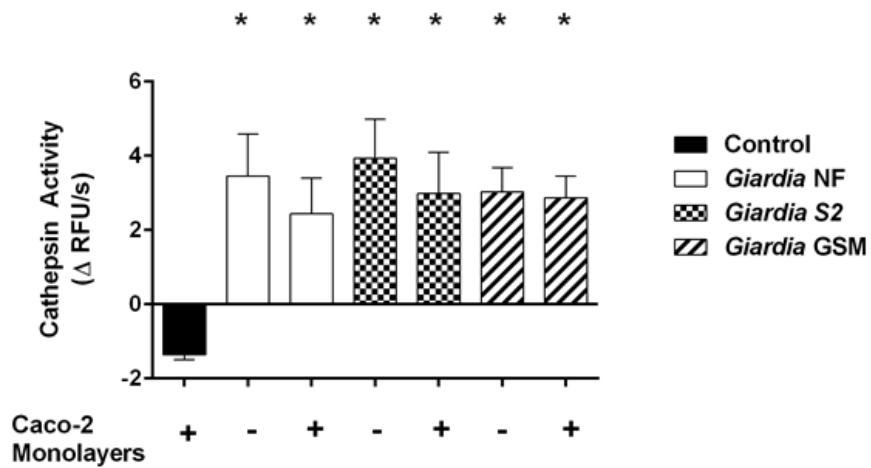
A)



B)



C)



3.1.3 Intra-enterocyte cathepsin B/L activity is not altered in the presence of G. duodenalis trophozoites

Having shown that *G. duodenalis* trophozoites contain cathepsins and release them into the supernatant, we wanted to assess whether host Caco-2 monolayers displayed altered cathepsin activity in the presence of *G. duodenalis*. Caco-2 monolayers exposed to *G. duodenalis* were assessed for cathepsin activity using ZFR-AMC or ZRR-AMC to determine their ability to process these substrates at the luminal pH of 7.2. In the presence of any of the isolates regardless of the assemblage, little to no cathepsin activity was observed in Caco-2 lysates of monolayers previously exposed to *G. duodenalis* trophozoites for 24 hours (Figure 6). A line graph showing change in RFUs over time in Caco-2 cells that were exposed to *G. duodenalis* isolate NF demonstrated a flat or downward slope (Figure 6A). Processing of ZFR-AMC or ZRR-AMC by Caco-2 lysates was assessed and no cathepsin B/L activity was noticed upon exposure to any of the isolates tested (Figure 6B, 6C). The presence of *G. duodenalis* trophozoites does not induce cathepsin B/L-like cysteine protease activity within Caco-2 monolayers.

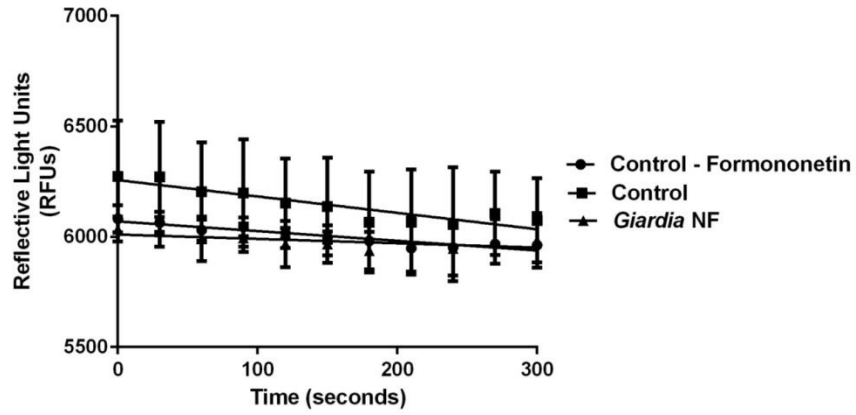
3.1.4 E64d and Ca-074Me inhibit intra-trophozoite and secretory-excretory cathepsin B/L-like cysteine proteases without cytotoxic effects.

G. duodenalis NF trophozoites were used for the rest of the study to assess the role of the cathepsin B/L proteases whose activity was assessed previously. NF trophozoites were pretreated with E64d or Ca-074Me or DMSO (vehicle) for 3 hours prior to co-incubation with Caco-2 monolayers. After the 3 hour incubation, the proportion of trophozoites that were swimming or twitching was assessed as a measure of

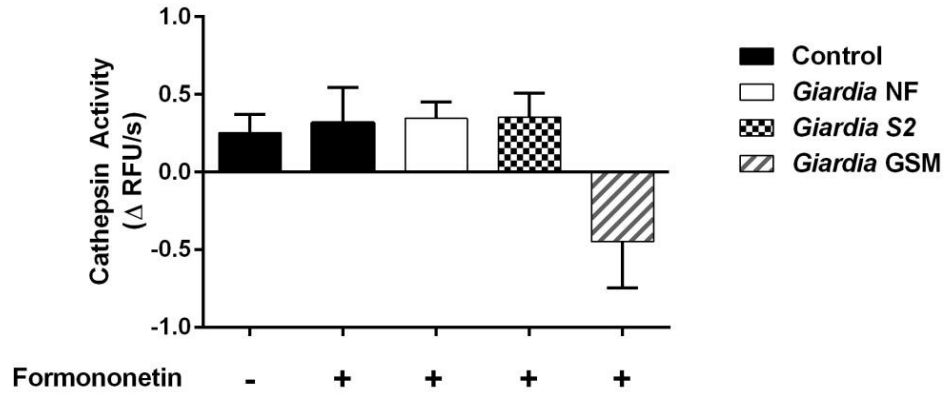
Figure 6. *G. duodenalis* do not induce cathepsin B/L activity inside of Caco-2 cells.

G. duodenalis trophozoites from Assemblage A (isolates NF, S2) or Assemblage B (isolate GS/M) were incubated with Caco-2 monolayers for 24 hours. Prior to collection, Caco-2 monolayers were treated with 10 μ M Formononetin for 1 hour. Caco-2 lysates incubated with isolate NF were assessed for cathepsin activity using ZFR-AMC and the release of AMC was recorded as RFUs over 5 minutes as a representative line graph for the rest of the isolates (A). The slopes or cathepsin B/L activity were calculated and represented as histograms for Caco-2 lysates incubated with trophozoites tested. ZFR-AMC cathepsin B/L activities were not different in the presence of any of the *G. duodenalis* isolates compared to controls (B). ZRR-AMC cathepsin B activities were also not significantly different from control monolayers (C). Data are mean \pm SEM, n = 3.

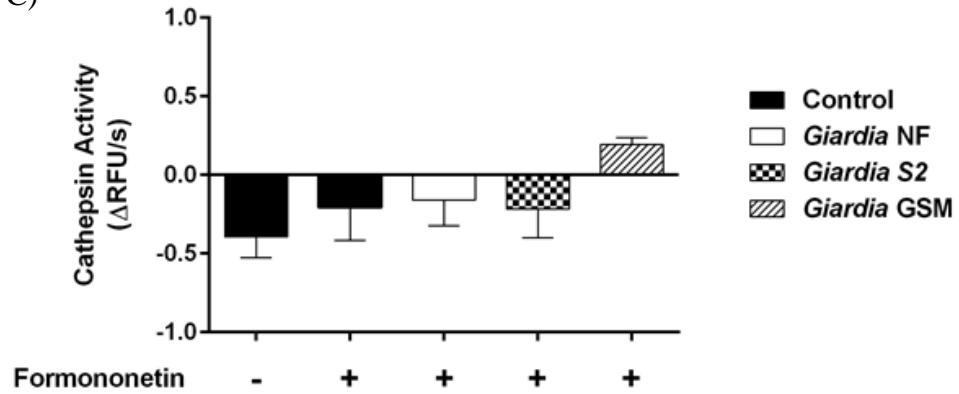
A)



B)



C)



cytotoxic effects of these inhibitors on trophozoites. No differences were found in the proportion of trophozoites that were swimming or twitching in the trophozoites that were treated with E64d or Ca-074Me or DMSO compared to control *G. duodenalis* trophozoites that received no treatment (Figure 7). Caco-2 monolayers were co-incubated with *G. duodenalis* NF trophozoites that were pretreated with E64d or Ca-074Me for 2 or 24 hours. Cathepsin B/L activity, assessed by ZFR-AMC processing, was significantly reduced after a 2 hour incubation with Caco-2 monolayers in lysates of NF trophozoites pretreated with E64d (Figure 8A). The supernatant cathepsin B/L activity after a 2 hour incubation was also significantly reduced in E64d pretreated NF trophozoites compared to untreated trophozoites or vehicle controlled trophozoites (Figure 8B). Cathepsin B/L activity, as assessed by ZFR-AMC processing, was significantly reduced in lysates of trophozoites pretreated with E64d or Ca-074Me isolated from a 24 hour co-incubation with Caco-2 cells (Figure 9A). Similarly, supernatant cathepsin B/L activity (using ZFR-AMC) was assessed, and this showed significant cathepsin B/L activity reduction in supernatants of co-cultures that were incubated with NF trophozoites pretreated with E64d or Ca-074Me (Figure 9B). Overall, E64d and Ca074Me are able to inhibit cathepsin B/L-like cysteine protease activity against ZFR-AMC without having a cytotoxic effect on trophozoites.

Figure 7. E64d and Ca074Me do not alter *G. duodenalis* NF trophozoite viability (as assessed via motility) after a 3 hour incubation.

G. duodenalis isolate NF trophozoites were treated with E64d (10 μ M) or Ca074Me (10 μ M) vehicle control, DMSO for 3 hours prior to being incubated with Caco-2 monolayers. The proportion of swimming or twitching trophozoites did not change in the presence of either inhibitor compared to control trophozoites. Data are mean +/- SEM, n=3.

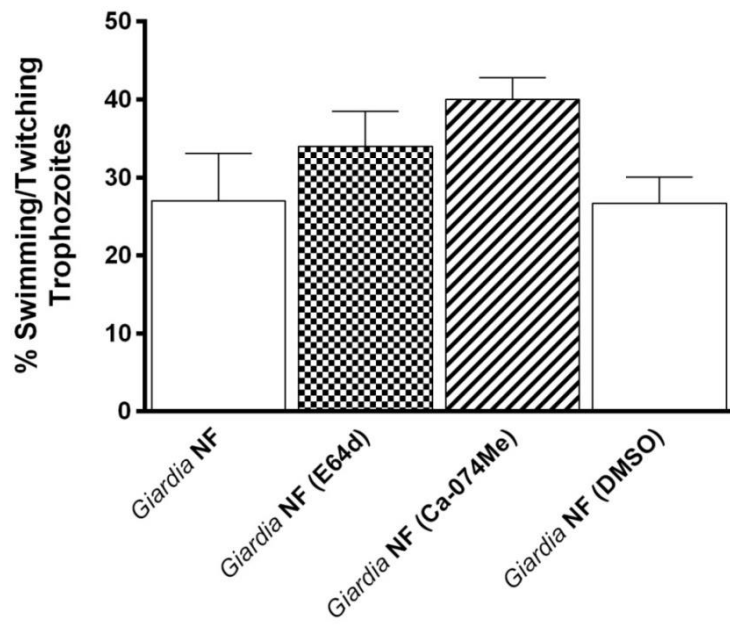


Figure 8. Pre-treatment with E64d inhibits cathepsin B/L activity both within *G. duodenalis* NF trophozoites and the released activity after a 2 hour incubation with Caco-2 monolayers.

G. duodenalis isolate NF trophozoites were pre-treated with E64d (10 μ M) or vehicle control, DMSO, prior to co-incubation with Caco-2 monolayers for 2 hours. Trophozoites were isolated from the co-culture and their lysates were assessed for cathepsin B/L activity using ZFR-AMC as the substrate (A). Supernatants from the co-culture were also collected and assessed for cathepsin B/L activity using ZFR-AMC as the substrate (B).

*p<0.05 vs Control cells; #p<0.05 vs *Giardia* NF. Data are mean +/- SEM, n=3.

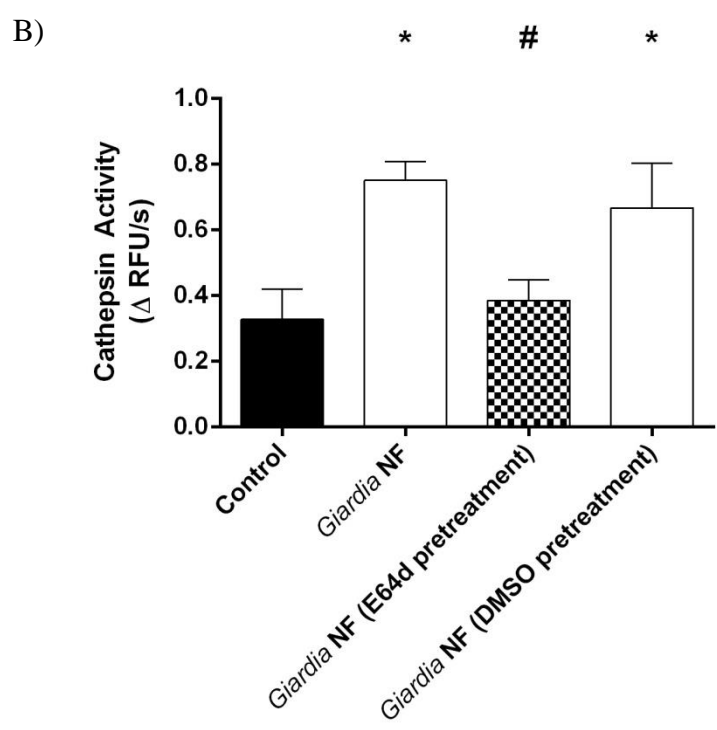
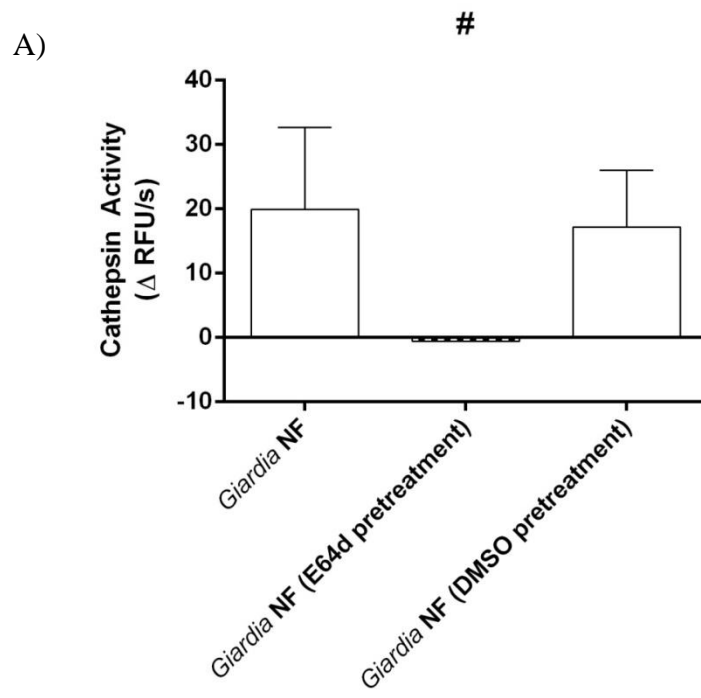
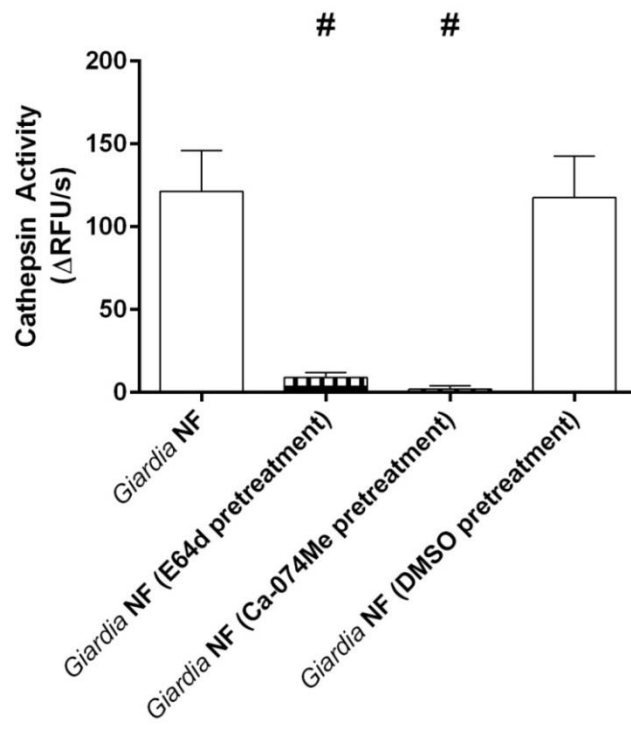


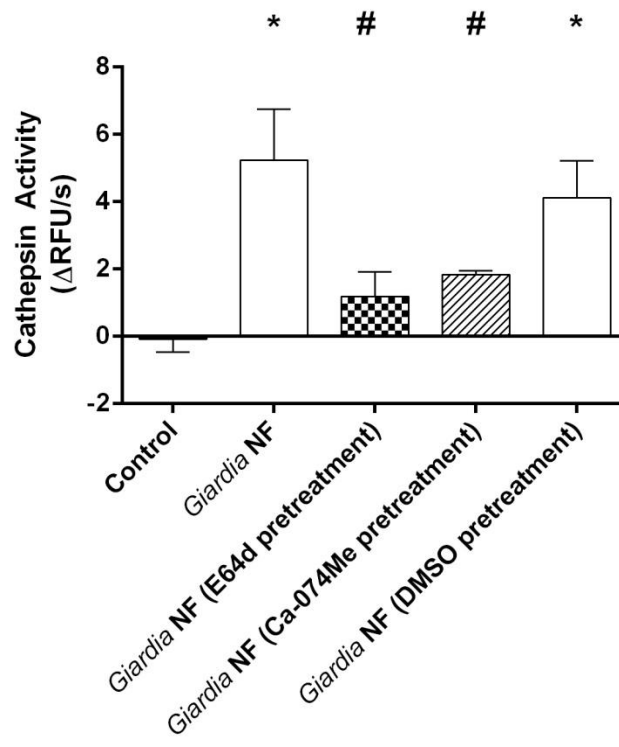
Figure 9. Pre-treatment with E64d or Ca074Me inhibits cathepsin B/L activity both within *G. duodenalis* NF trophozoites and the released activity after a 24 hour incubation with Caco-2 monolayers.

G. duodenalis isolate NF trophozoites were pre-treated with E64d (10 μ M) or Ca074Me (10 μ M) vehicle control, DMSO, prior to co-incubation with Caco-2 monolayers for 24 hours. Trophozoites were isolated from the co-culture and their lysates were assessed for cathepsin B/L activity using ZFR-AMC as the substrate (A). Supernatants from the co-culture were also collected and assessed for cathepsin B/L activity using ZFR-AMC as the substrate (B). *p<0.05 vs Control cells; #p<0.05 vs *Giardia* NF. Data are mean +/- SEM, n=3.

A)



B)



3.2 The role of *G. duodenalis* cathepsin-like cysteine proteases in pathophysiology

The findings illustrated above demonstrated that *G. duodenalis* trophozoites from either Assemblage A (isolates NF and S2) or Assemblage B (isolate GS/M) contain and release cathepsin-like cysteine proteases. However, the role that these proteases play in the pathophysiology of *G. duodenalis* infections is still unknown. Previous findings have demonstrated that *G. duodenalis* results in increased rates of apoptosis and disruption of tight junctional proteins, including ZO-1, occludin, and claudin-1. Whether *G. duodenalis* parasitic products are involved in the initiation of pathophysiology remains to be uncovered. Considering that these proteases are released and are present in the trophozoites (and possibly on the surface of the trophozoite), and similar proteases are necessary for pathogenesis of other protozoan parasites, we hypothesized a role for one or more of these proteases in its pathophysiology.

3.2.1 G. duodenalis isolate NF trophozoites cause a breakdown of ZO-1, but not of claudin-1 or occludin

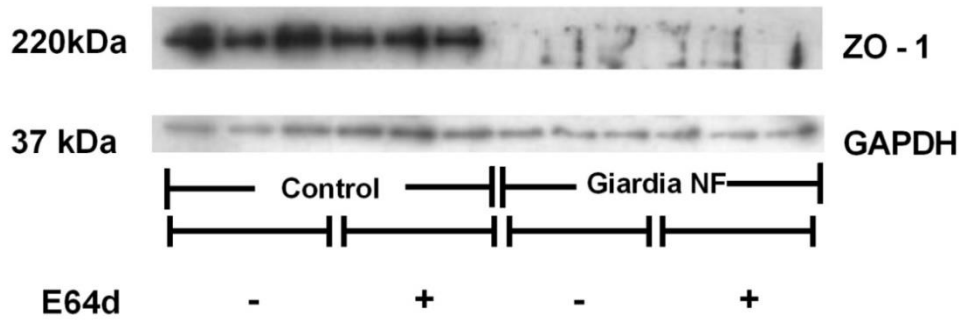
3.2.1.1 *G. duodenalis* NF trophozoites cause a loss of the tight junctional Zonula Occludens (ZO)-1 independent of *G. duodenalis* cathepsin-like cysteine proteases

Previously, it has been established that *G. duodenalis* trophozoites disrupt ZO-1 in a myosin light chain kinase (MLCK)-dependent manner, and consequently causes increases in permeability and a loss of the epithelial barrier function [19]. After a 24 hour incubation with *G. duodenalis* trophozoites, full length ZO-1 protein in Caco-2 monolayers was degraded (Figure 10). Addition of E64d (10uM) (Figure 10A) or Ca-074Me (10uM) (Figure 10B) to the co-culture of NF trophozoites and Caco-2 monolayers

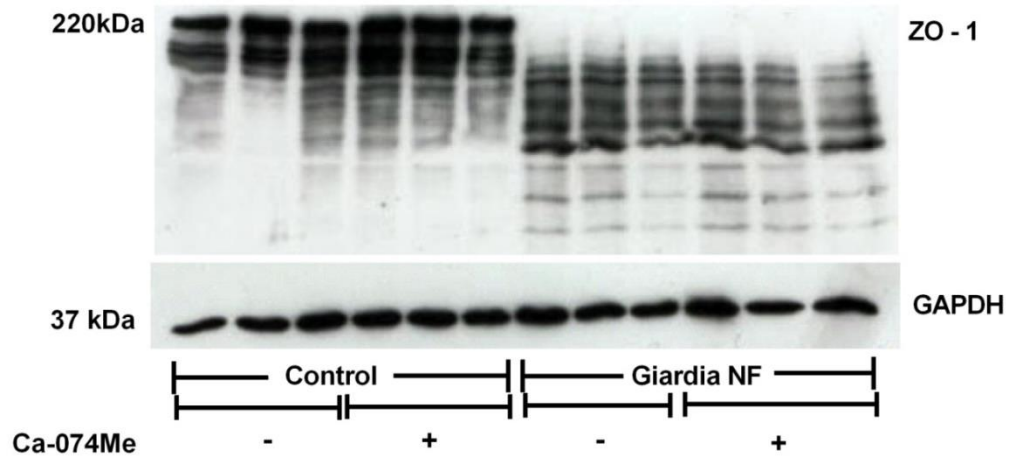
Figure 10. Addition of E64d or Ca074Me does not inhibit *G. duodenalis* NF trophozoite-induced ZO-1 degradation in Caco-2 monolayers after a 24 hour incubation.

G. duodenalis isolate NF trophozoites were co-incubated with Caco-2 monolayers in the presence of E64d (10 μ M) or Ca074Me (10 μ M) for 24 hours. Representative Western blot shows that live *G. duodenalis* NF trophozoites induced ZO-1 degradation in Caco-2 monolayers, and this was not prevented by the addition of E64d to the incubation (A). Representative Western blot shows that *G. duodenalis* NF trophozoite-induced ZO-1 breakdown in Caco-2 monolayers was also not prevented by an addition of Ca074Me (B). n=3.

A)



B)



did not prevent the degradation of ZO-1. *G. duodenalis* NF trophozoites that were pre-treated with E64d (10uM) for 3 hours prior to co-incubation with Caco-2 monolayers also cause a loss of the full length ZO-1 in Caco-2 monolayers after 2 (Figure 11) and 24 hours (Figure 12). ZO-1 is degraded in the presence of live *G. duodenalis* trophozoites independent of Clan CA cysteine proteases.

3.2.1.2 *G. duodenalis* NF trophozoites do not cause any changes in protein levels of transmembrane tight junctional proteins, Occludin or Claudin-1

Having assessed *G. duodenalis* NF trophozoites' effects on a critical intracellular tight junctional protein, ZO-1, we evaluated whether *G. duodenalis* NF has similar effects on transmembrane tight junctional proteins, occludin and claudin-1 that span the intercellular space between two adjacent enterocytes. *G. duodenalis* NF trophozoites were co-incubated with Caco-2 monolayers for 24 hours. No differences were observed in the Caco-2 monolayer occludin levels after this co-incubation (Figure 13A). Similarly, no differences were noticed in claudin-1 levels (Figure 13B). A pre-treatment of NF trophozoites did not have an effect on either occludin or claudin-1 protein levels (Figure 13A,B). Occludin and claudin-1 in Caco-2 cells are not broken down after 24 hours.

3.2.1.3 *G. duodenalis* NF trophozoites do not induce protein level changes of the cytoskeletal protein, F-actin

Previous work in our lab has demonstrated that F-actin is rearranged in Caco-2 monolayers in the presence of *G. duodenalis* as analysed through immunocytochemistry. However, immunoblotting analysis of Caco-2 monolayers that were co-incubated with *G. duodenalis* for 24 hours showed that F-actin protein is not affected biochemically (Figure 14).

Figure 11. Pre-treatment with E64d does not inhibit *G. duodenalis* NF trophozoite-induced ZO-1 degradation in Caco-2 monolayers after a 2 hour incubation.

G. duodenalis isolate NF trophozoites were pre-treated with E64d (10 μ M) or vehicle control, DMSO, prior to co-incubation with Caco-2 monolayers for 2 hours. Representative Western blot shows that live *G. duodenalis* NF trophozoites induced ZO-1 degradation in Caco-2 monolayers and this was not prevented by an E64d pre-treatment (A). Densitometry was performed to compare full length ZO-1 levels vs loading control GAPDH (B). * $p < 0.05$ vs Control cells. Data are mean \pm SEM, $n = 3$.

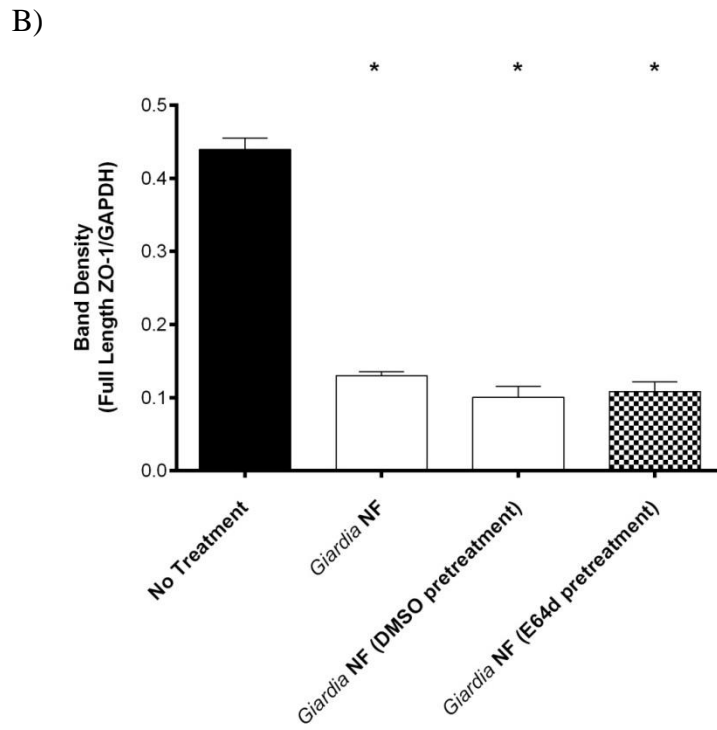
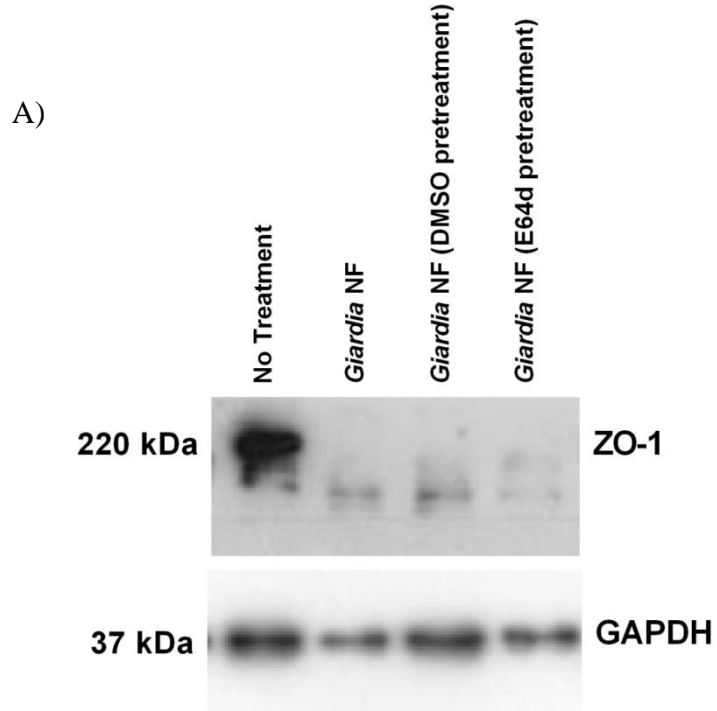


Figure 12. Pre-treatment with E64d does not inhibit *G. duodenalis* NF trophozoite-induced ZO-1 degradation in Caco-2 monolayers after a 24 hour incubation.

G. duodenalis isolate NF trophozoites were pre-treated with E64d (10 μ M) or vehicle control, DMSO, prior to co-incubation with Caco-2 monolayers for 24 hours. Representative Western blot shows that live *G. duodenalis* NF trophozoites induced ZO-1 degradation in Caco-2 monolayers and this was not prevented by an E64d pre-treatment (A). Densitometry was performed to compare full length ZO-1 levels vs loading control GAPDH (B). *p<0.05 vs Control cells. Data are mean +/- SEM, n=3.

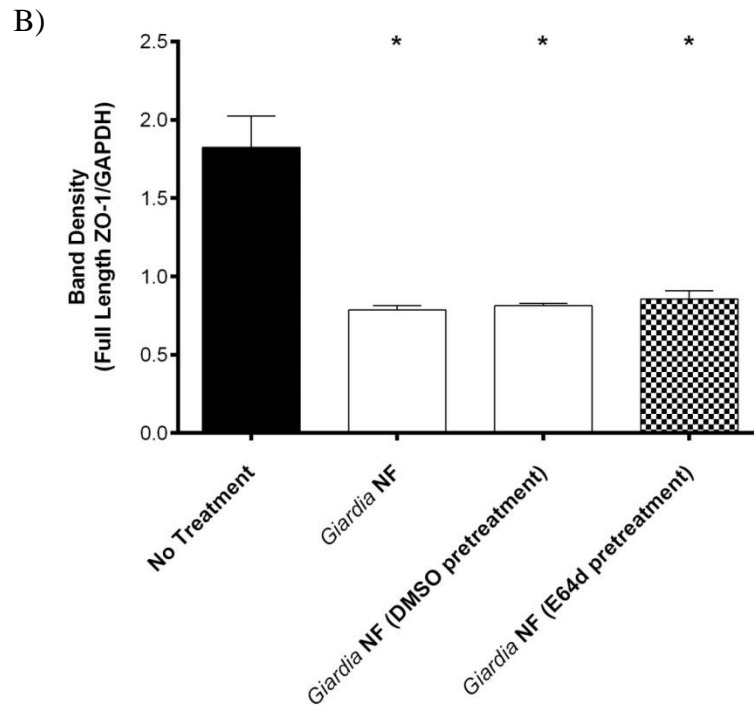
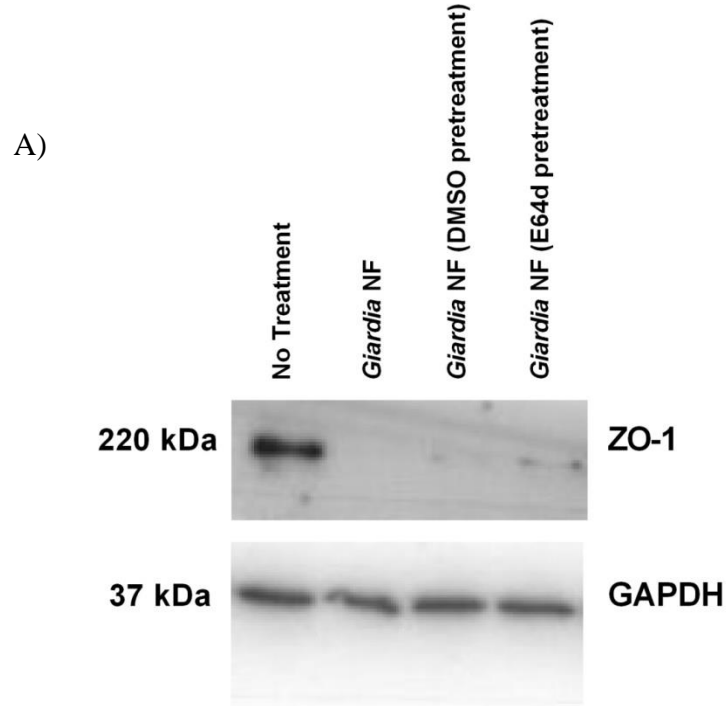


Figure 13. *G. duodenalis* NF trophozoites do not cause a breakdown or protein expression changes in Occludin or Claudin-1 in Caco-2 monolayers after a 24 hour incubation.

G. duodenalis isolate NF trophozoites were pre-treated with E64d (10 μ M) or vehicle control, DMSO, prior to co-incubation with Caco-2 monolayers for 24 hours. Representative Western blot shows that live *G. duodenalis* NF trophozoites do not alter protein expression of occludin in Caco-2 monolayers and this was not affected by an E64d pre-treatment (A). Representative Western blot shows that live *G. duodenalis* NF trophozoites do not alter protein expression Claudin-1 in Caco-2 monolayers and this was not affected by an E64d pre-treatment (B). n=3.

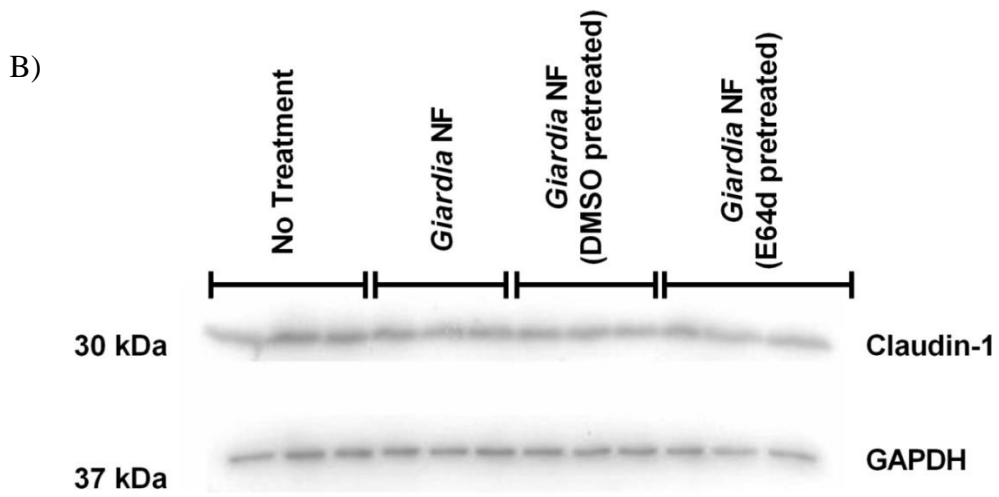
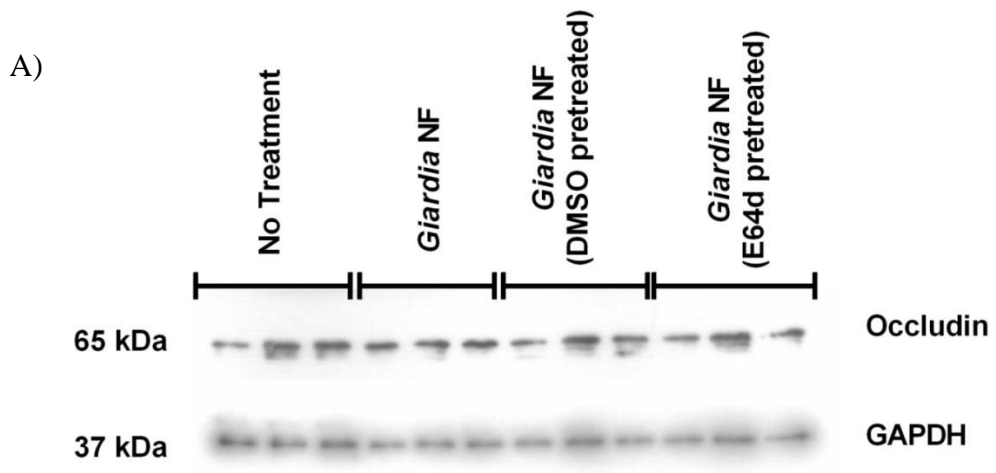
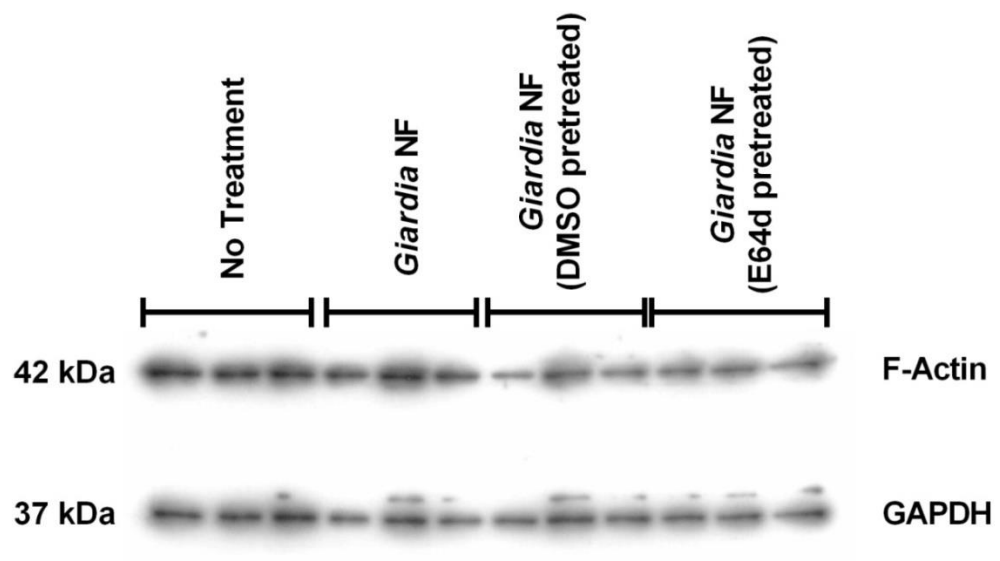


Figure 14. *G. duodenalis* NF trophozoites do not break down the cytoskeletal F-actin protein in Caco-2 monolayers after a 24 hour incubation.

G. duodenalis isolate NF trophozoites were pre-treated with E64d (10 μ M) or vehicle control, DMSO, prior to co-incubation with Caco-2 monolayers for 24 hours. Representative Western blot shows that live *G. duodenalis* NF trophozoites do not alter protein expression of F-actin in Caco-2 monolayers and this was not affected by an E64d pre-treatment; n=3.



3.2.2 *G. duodenalis* NF cathepsin-like cysteine proteases are responsible for villin breakdown

It has previously been shown that *G. duodenalis* disrupts actin filaments in an MLCK dependent manner; however, this parasite's effects on other cytoskeletal proteins of IEC's have yet to be described. As such, we further assessed *G. duodenalis* isolate NF's effects on another cytoskeletal protein with regulatory roles in the intestinal epithelium, villin. Previous findings have shown that another protozoan parasite, *Entamoeba histolytica*, proteolytically degrades villin using cysteine proteases [81]. We hypothesized that *G. duodenalis* cathepsin-like cysteine proteases degrade and disrupt villin.

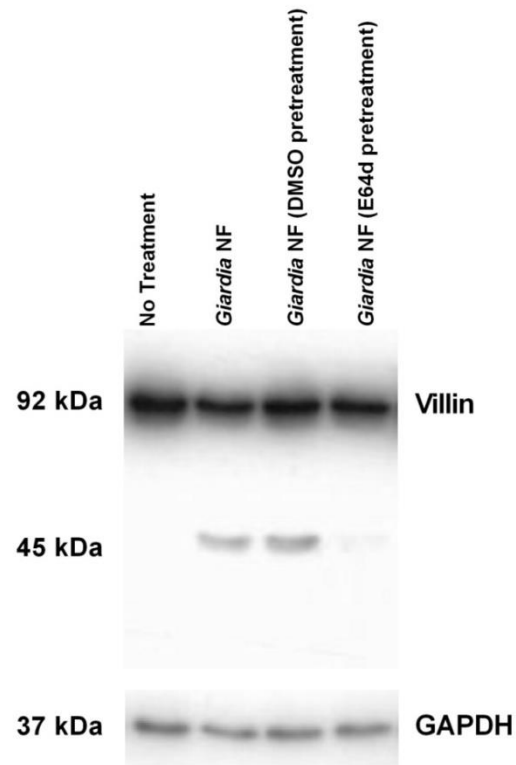
3.2.2.1 Co-incubation of *G. duodenalis* NF trophozoites results in villin degradation, and is prevented by a pre-treatment of trophozoites with E64d

Caco-2 monolayers were co-incubated for 2 or 24 hours with *G. duodenalis* NF that had previously been treated with E64d. Upon co-incubation with *G. duodenalis* NF trophozoites, Caco-2 monolayer lysates display the presence of a cleaved band at about 45kDa. Intriguingly, the total amount of full length villin (90kDa) was not altered. A pre-treatment of *G. duodenalis* NF trophozoites with E64d for 3 hours prevented the cleavage of villin both at 2 hour (Figure 15) and 24 hour (Figure 16) time points. Pre-treating *G. duodenalis* NF trophozoites with DMSO prior to the co-incubation also caused the presence of the 45kDa cleavage band of villin in Caco-2 cells at 2 hours (Figure 15) and 24 hours (Figure 16). *G. duodenalis* Clan CA cysteine proteases are able to cause villin cleavage in Caco-2 cells after a 2 or 24 hour incubation.

Figure 15. *G. duodenalis* NF trophozoites cause a cleavage of villin after a 2 hour incubation with Caco-2 monolayers, which is prevented by a pre-treatment of trophozoites with E64d.

G. duodenalis isolate NF trophozoites were pre-treated with E64d (10 μ M) or vehicle control, DMSO, prior to co-incubation with Caco-2 monolayers for 2 hours. Representative Western blot shows that live *G. duodenalis* NF trophozoites induced villin cleavage without affecting full length villin protein levels in Caco-2 monolayers and the cleavage was prevented by an E64d pre-treatment (A). Densitometry was performed to compare protein levels of cleaved villin vs loading control, GAPDH (B). *p<0.05 vs No Treatment, #p<0.05 vs *Giardia* NF. Data are mean +/- SEM, n=3.

A)



B)

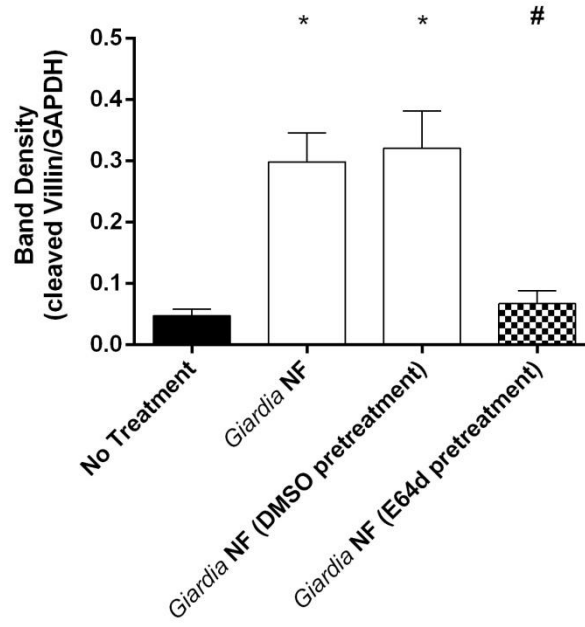
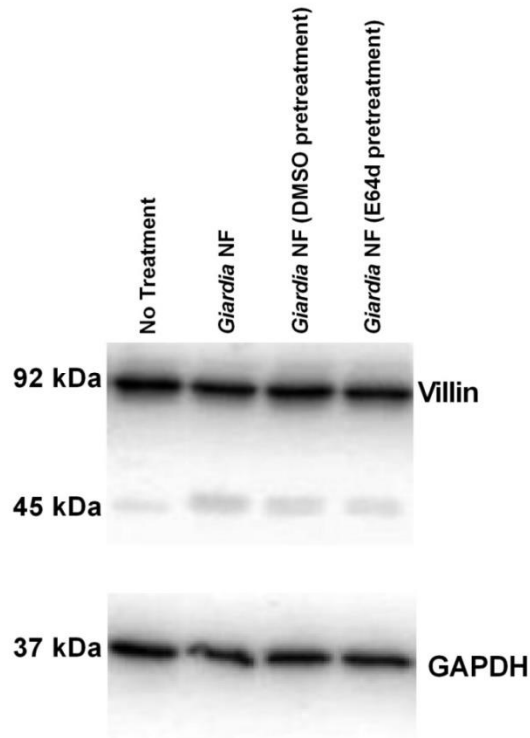


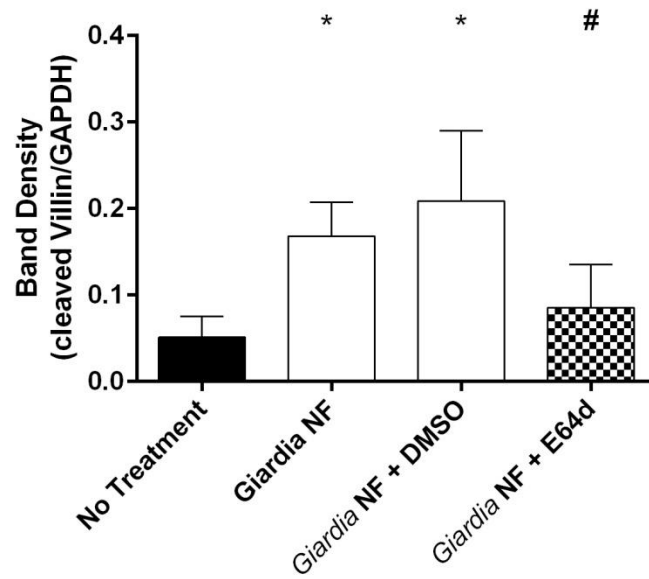
Figure 16. *G. duodenalis* NF trophozoites cause a cleavage of villin after a 24 hour incubation with Caco-2 monolayers, which is prevented by a pre-treatment of trophozoites with E64d.

G. duodenalis isolate NF trophozoites were pre-treated with E64d (10 μ M) or vehicle control, DMSO, prior to co-incubation with Caco-2 monolayers for 24 hours. Representative Western blot shows that live *G. duodenalis* NF trophozoites induced villin cleavage without affecting full length villin protein levels in Caco-2 monolayers and the cleavage was prevented by an E64d pre-treatment (A). Densitometry was performed to compare protein levels of cleaved villin vs loading control, GAPDH (B). *p<0.05 vs No Treatment; #p<0.05 vs *Giardia* NF. Data are mean +/- SEM, n=3.

A)



B)



3.2.2.2 *G. duodenalis* NF-induced villin breakdown requires direct contact with trophozoites

To determine if secreted products that could pass through a pore size of 0.4µm were sufficient to cause a villin break down, we separated NF trophozoites from the Caco-2 monolayers using 0.4µm Transwells and co-incubated for 24 hours. Monolayers that were separated from trophozoites using the Transwells did not have villin breakdown seen when trophozoites were able to make contact with monolayers (Figure 17). *G. duodenalis* trophozoites need to have direct interaction with Caco-2 monolayers for villin cleavage to occur.

3.2.2.3 Co-incubation of lysed *G. duodenalis* NF trophozoites and lysed Caco-2 monolayers results in villin degradation, and is prevented by E64d

To determine whether host cells need to be alive and host signalling pathways are needed for the aforementioned cleavage of villin, we co-incubated *G. duodenalis* trophozoite lysates with Caco-2 monolayer lysates for 2 hours. Similar to the live co-incubation in section 3.2.1.1, we observed a cleavage band at 45kDa without a reduction in overall levels of full length villin in Caco-2 lysates that were treated with *Giardia* lysates (Figure 18). Intriguingly, however, we noticed the presence of a smaller cleavage band at about 37kDa. When E64d at an excess concentration of 200µM was added, there was no breakdown of villin. Ca-074Me (200µM) did not prevent the presence of the cleavage bands (Figure 18). *G. duodenalis* trophozoite lysates also induced a Clan CA cysteine protease-dependent cleavage of villin when co-incubated with Caco-2 lysates.

Figure 17. *G. duodenalis* NF trophozoites cause contact dependent cleavage of villin in Caco-2 cells after a 24 hour co-incubation.

G. duodenalis isolate NF trophozoites were co-incubated directly with Caco-2 monolayers or separated from Caco-2 monolayers via 0.4um Transwells for 24 hours.

Representative Western blot shows that live *G. duodenalis* NF trophozoites induced villin cleavage, which was prevented by separating trophozoites from Caco-2 monolayers (A).

Densitometry was performed to compare protein levels of cleaved villin vs loading control, GAPDH (B). *p<0.05 vs No Treatment; #p<0.05 vs *Giardia* NF. Data are mean +/- SEM, n=3.

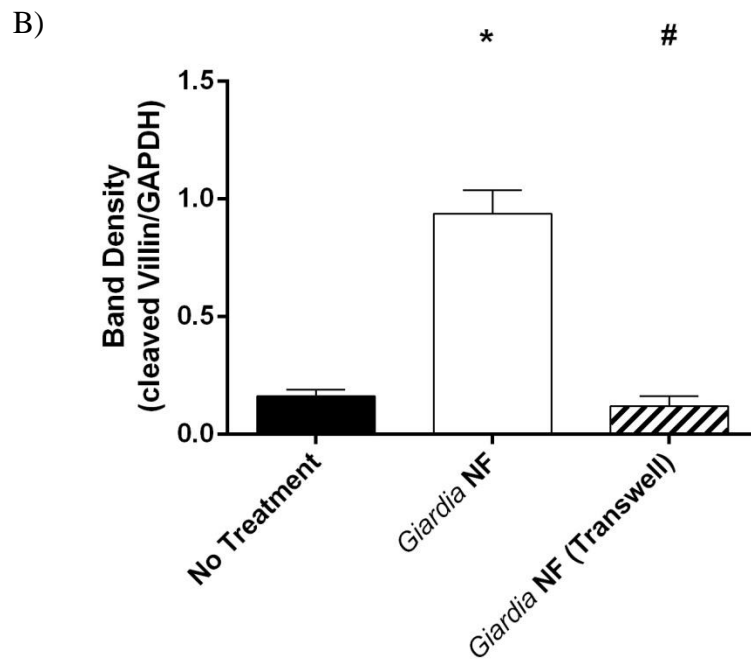
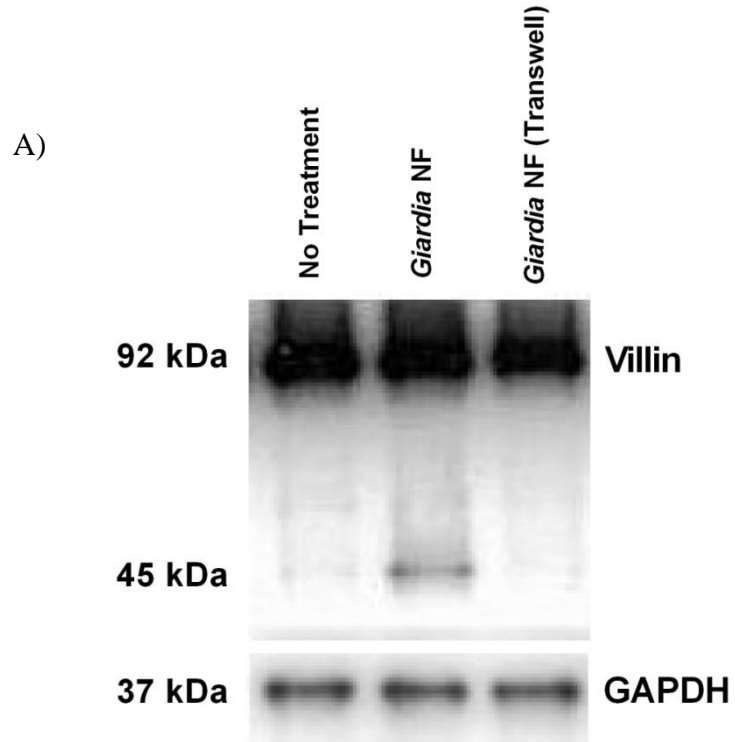
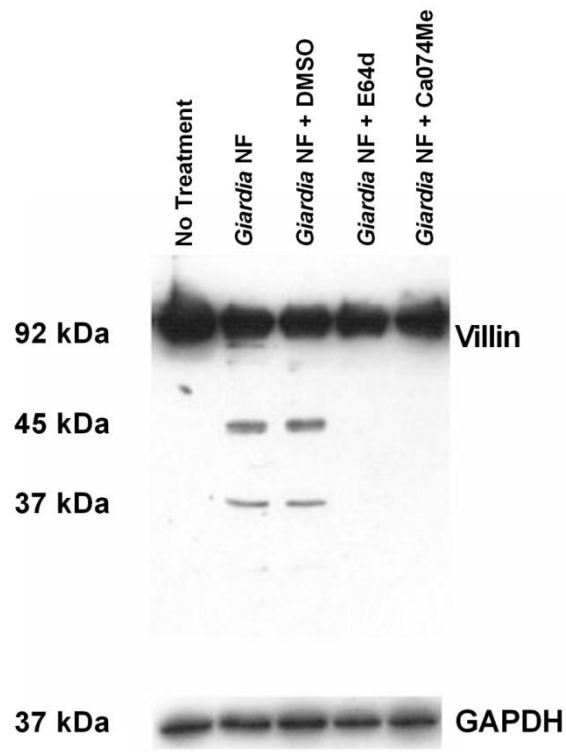


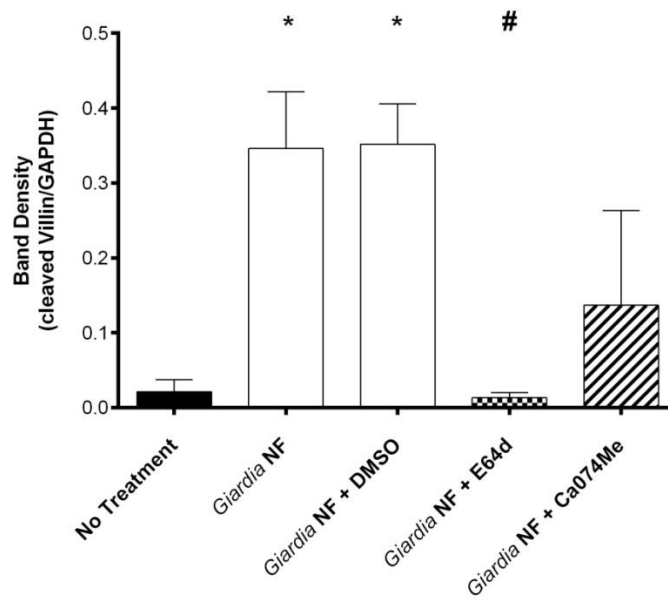
Figure 18. Co-incubation of lysed *G. duodenalis* NF trophozoites and lysed Caco-2 cells results in cleavage of villin, and is prevented by E64d.

G. duodenalis isolate NF trophozoites were lysed with Caco-2 monolayers and incubated in the presence of E64d (200 μ M) or Ca074Me (200 μ M) or the vehicle control, DMSO, for 2 hours. Representative Western blot shows that *G. duodenalis* NF lysates induced villin cleavage, which was prevented by E64d but not by Ca074Me (A). Densitometry was performed to compare protein levels of cleaved villin (45kDa band) vs loading control, GAPDH (B). *p<0.05 vs No Treatment; #p<0.05 vs *Giardia* NF. Data are mean +/- SEM, n=3.

A)



B)



3.2.1.5 *G. duodenalis*-induced villin breakdown is prevented by ML-9 in a time dependent manner

A previous study has shown that *G. duodenalis* disrupts F-actin and ZO-1 in an MLCK dependent manner after a 2 hour incubation in SCBN cells, suggesting that MLCK is active in enterocytes during an infection with *G. duodenalis* [120]. As such, it was relevant to assess the role of MLCK using a selective inhibitor, ML-9 (40 μ M), in the cleavage of villin by *G. duodenalis*. At an earlier time point of 2 hours, co-incubation of *G. duodenalis* NF trophozoites and Caco-2 monolayers in the presence of ML-9 did not prevent villin cleavage (Figure 19). Conversely, after a 24 hour incubation of *G. duodenalis* NF trophozoites with Caco-2 monolayers in the presence of ML-9, villin cleavage was prevented (Figure 20). ML-9 alone did not have an effect on villin cleavage at either time point (Figures. 19, 20). ML-9 prevents *G. duodenalis*-induced villin cleavage in a time-dependent manner.

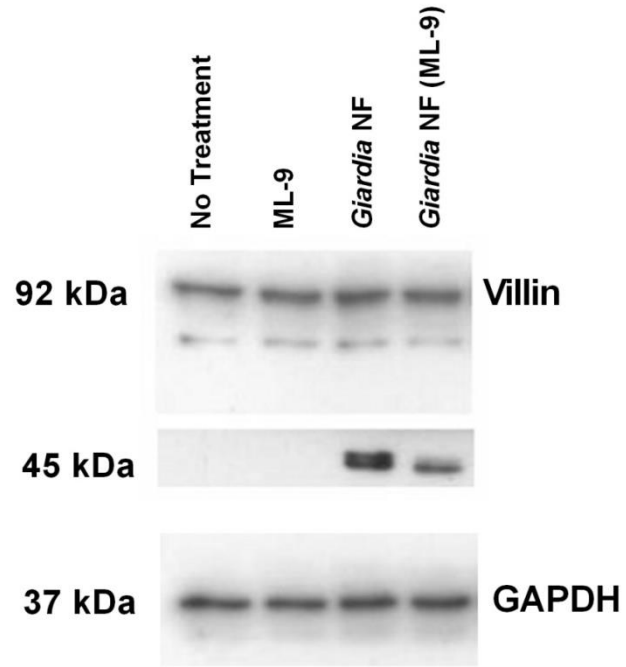
3.2.2.6 *G. duodenalis*-induced villin breakdown is not prevented by a caspase-3 inhibitor, Z-DEVD-FMK

Previous findings have shown that *G. duodenalis* trophozoites cause increased rates of apoptosis and ZO-1 disruption in SCBN enterocytes in a caspase-3 dependent manner [27]. Therefore, with *G. duodenalis* trophozoites also causing a breakdown of villin, we wondered if this was a direct effect or if the enterocyte caspase-3 cleaves villin. As such, Caco-2 monolayers were briefly treated with Z-DEVD-FMK (50 μ M), a caspase-3 selective inhibitor, followed by a co-incubation with *G. duodenalis* NF trophozoites to assess the role of caspase-3 in villin breakdown. Caco-2 monolayers pretreated with Z-DEVD-FMK still exhibited a villin cleavage band of approximately

Figure 19. *G. duodenalis* NF trophozoites-induced villin cleavage in Caco-2 cells is not prevented by ML-9 after a 2 hour co-incubation.

G. duodenalis isolate NF trophozoites were incubated with Caco-2 monolayers for 2 hours in the presence or absence of ML-9 (40 μ M), an MLCK inhibitor. The membrane was cut just above 50kDa prior to development to prevent overexposure for full length villin. Representative Western blot shows that live *G. duodenalis* NF trophozoites induced villin cleavage without affecting full length villin protein levels in Caco-2 monolayers and the cleavage was not prevented by ML-9 (A). The inhibitor alone had no effect on villin cleavage (A). Densitometry was performed to compare protein levels of cleaved villin vs loading control, GAPDH (B). *p<0.05 vs No Treatment. Data are mean +/- SEM, n=3.

A)



B)

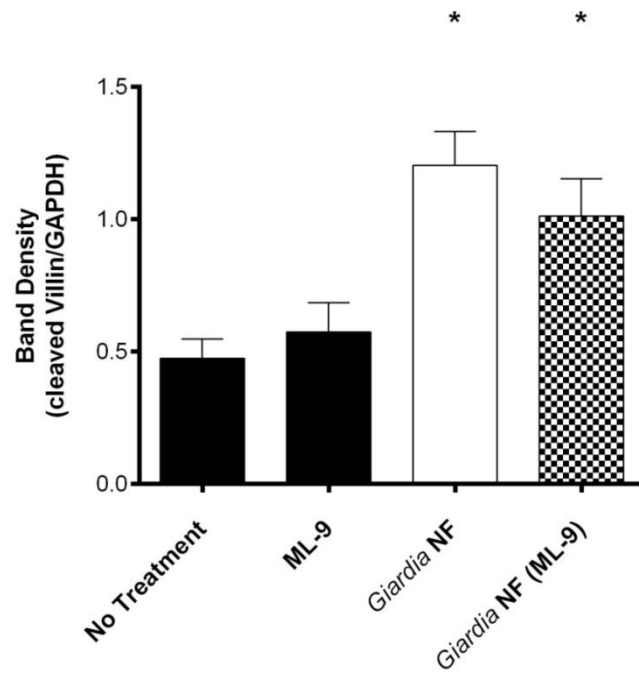
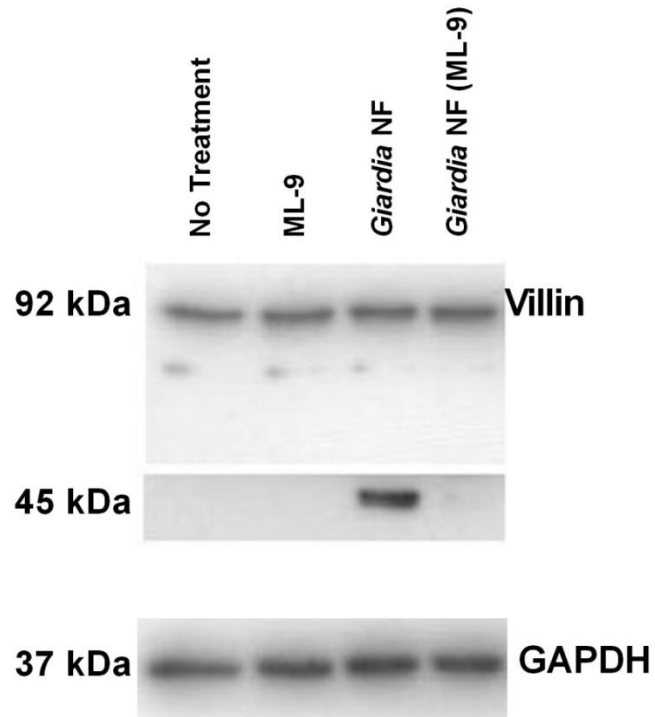


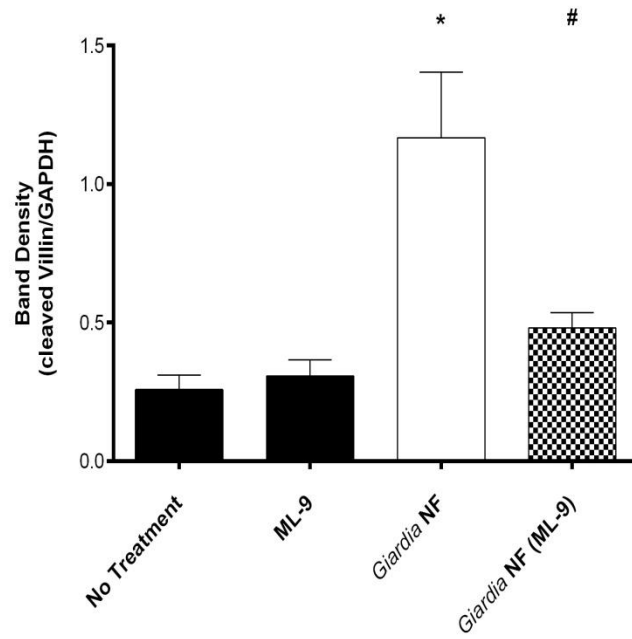
Figure 20. *G. duodenalis* NF trophozoites-induced villin cleavage in Caco-2 cells is prevented by ML-9 after a 24 hour co-incubation.

G. duodenalis isolate NF trophozoites were incubated with Caco-2 monolayers for 24 hours in the presence or absence of ML-9 (40 μ M), an MLCK inhibitor. The membrane was cut just above 50kDa prior to development to prevent overexposure for full length villin. Representative Western blot shows that live *G. duodenalis* NF trophozoites induced villin cleavage without affecting full length villin protein levels in Caco-2 monolayers and the cleavage was prevented by ML-9 (A). The inhibitor alone had no effect on villin cleavage (A). Densitometry was performed to compare protein levels of cleaved villin vs loading control, GAPDH (B). *p<0.05 vs No Treatment; #p<0.05 vs *Giardia* NF. Data are mean +/- SEM, n=3.

A)



B)



45kDa in length after being co-incubated with *G. duodenalis* NF trophozoites for 2 hours (Figure 21). Z-DEVD-FMK did not prevent villin cleavage after a 24 hour co-incubation between Caco-2 cells and *G. duodenalis* NF trophozoites (Figure 22). The inhibitor alone did not have an effect on the appearance of the 45 kDa cleavage band for villin at either time point (Figures 21, 22). While previous research has shown ZO-1 disruption by *G. duodenalis* to be caspase-3 dependent, villin breakdown was not inhibited by Z-DEVD-FMK.

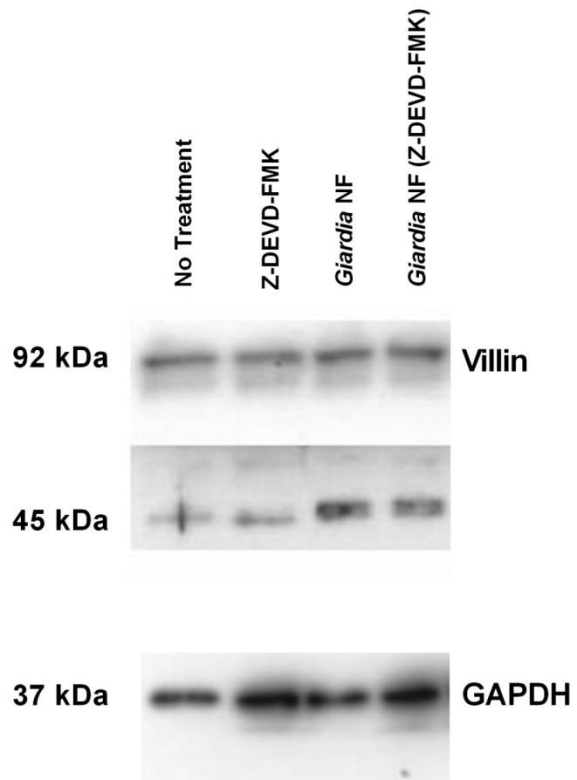
3.2.3 *G. duodenalis* NF cathepsin-like cysteine proteases are responsible for villin disruption

Photomicrographs consistently showed a disruption of villin in the presence of *G. duodenalis* NF at 2 or 24 hours of co-incubation with Caco-2 monolayers (Figures 23, 24, 25, 26). Untreated Caco-2 monolayers showed a “chicken-wire” pattern that was lost or reduced in the *G. duodenalis* NF treated groups at both time points. *G. duodenalis* NF trophozoites induced a flocculation of villin, resulting in a number of globular structures within the cells (Figures 23-26). NF trophozoites pre-treated with E64d were co-incubated with Caco-2 cells, and this prevented the disruption of villin at 2 and 24 hours (Figure 23, 24 respectively). Villin disruption in Caco-2 monolayers was also MLCK-dependent, and was not inhibited by ML-9 at 2 hours (Figure 25) but was reduced, at least in part, after 2 hours of co-incubation with *G. duodenalis* NF trophozoites (Figure 26).

Figure 21. *G. duodenalis* NF trophozoites induced-villin cleavage in Caco-2 cells is not prevented by Z-DEVD-FMK after a 2 hour co-incubation.

G. duodenalis isolate NF trophozoites were incubated with Caco-2 monolayers for 2 hours in the presence or absence of Z-DEVD-FMK (50 μ M), a caspase-3 selective inhibitor. The membrane was cut just above 50kDa prior to development to prevent overexposure for full length villin. Representative Western blot shows that live *G. duodenalis* NF trophozoites induced villin cleavage without affecting full length villin protein levels in Caco-2 monolayers and the cleavage was not prevented by Z-DEVD-FMK (A). The inhibitor alone had no effect on villin cleavage (A). Densitometry was performed to compare protein levels of cleaved villin vs loading control, GAPDH (B). *p<0.05 vs No Treatment. Data are mean +/- SEM, n=3.

A)



B)

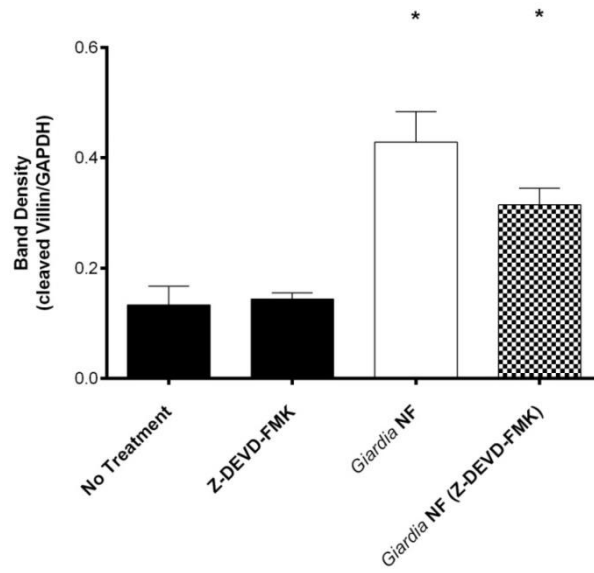
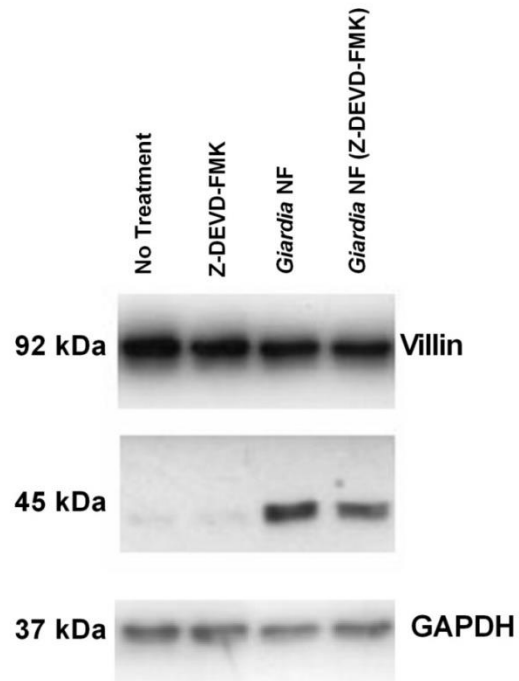


Figure 22. *G. duodenalis* NF trophozoites induced-villin cleavage in Caco-2 cells is not prevented by Z-DEVD-FMK after a 24 hour co-incubation.

G. duodenalis isolate NF trophozoites were incubated with Caco-2 monolayers for 24 hours in the presence or absence of Z-DEVD-FMK (50 μ M), a caspase-3 selective inhibitor. The membrane was cut just above 50kDa prior to development to prevent overexposure for full length villin. Representative Western blot shows that live *G. duodenalis* NF trophozoites-induced villin cleavage without affecting full length villin protein levels in Caco-2 monolayers and the cleavage was not prevented by Z-DEVD-FMK (A). The inhibitor alone had no effect on villin cleavage (A). Densitometry was performed to compare protein levels of cleaved villin vs loading control, GAPDH (B). * $p < 0.05$ vs No Treatment. Data are mean \pm SEM, n=3.

A)



B)

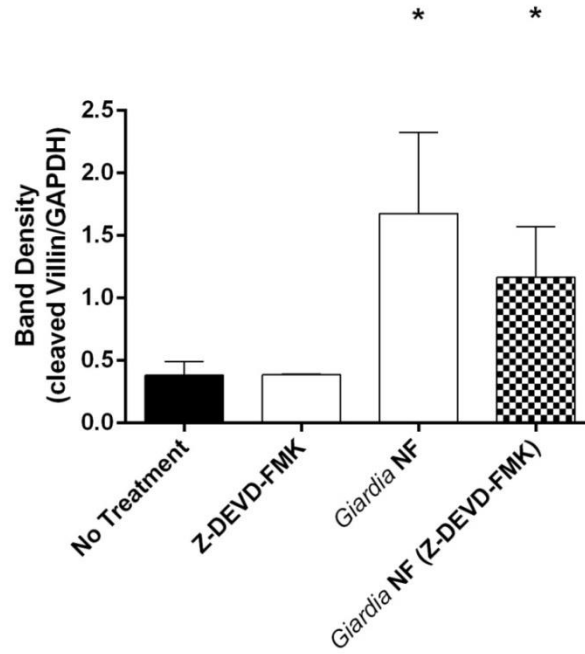


Figure 23. *G. duodenalis* NF trophozoites disrupt villin after a 2 hour incubation with Caco-2 monolayers, which is prevented by a pre-treatment of trophozoites with E64d.

G. duodenalis isolate NF trophozoites were pre-treated with E64d (10 μ M) or vehicle control, DMSO, prior to co-incubation with Caco-2 monolayers for 2 hours. Representative photographs show that live *G. duodenalis* NF trophozoites induced villin disruption in Caco-2 monolayers, which was prevented by an E64d pre-treatment. Micrographs are cropped versions of the full image; gain and exposure were maintained constant throughout. Magnification=400x. n=3.

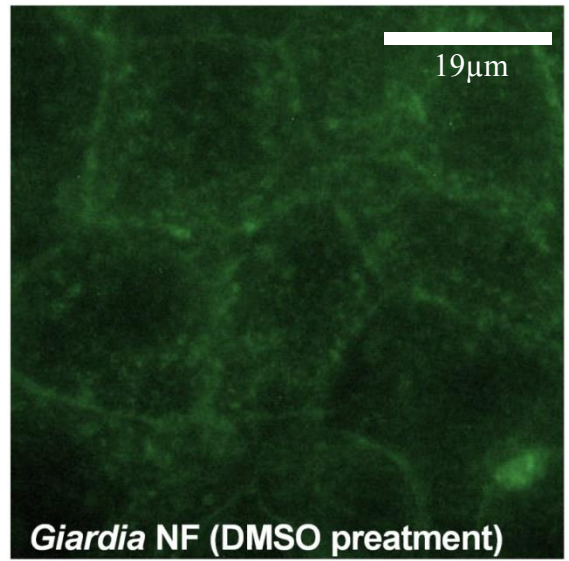
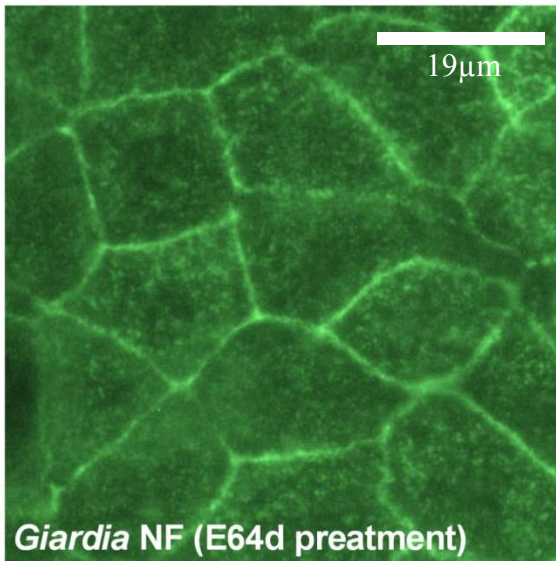
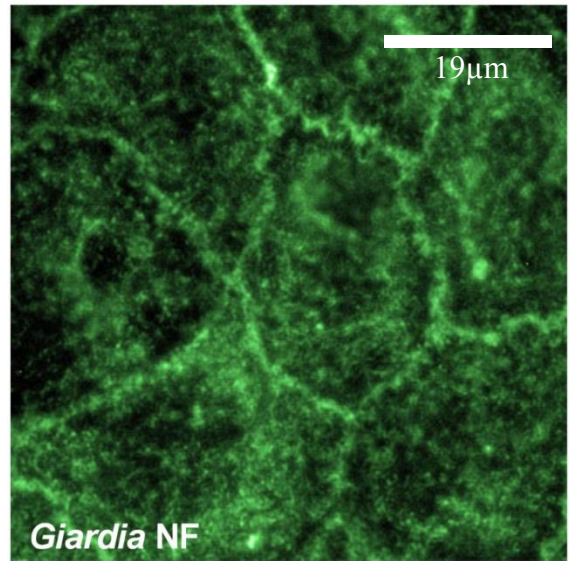
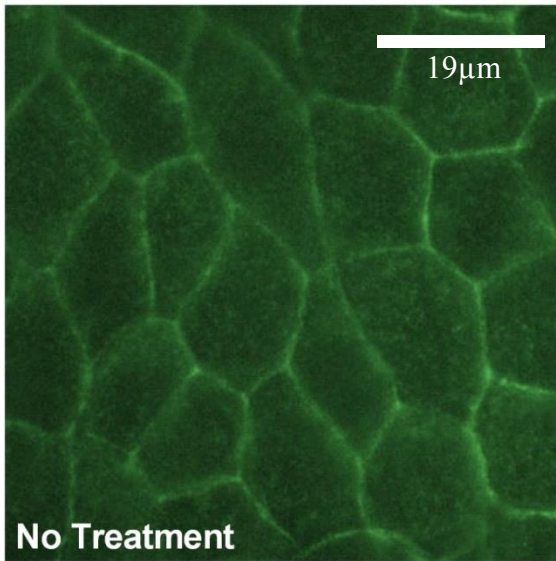


Figure 24. *G. duodenalis* NF trophozoites disrupt villin after a 24 hour incubation with Caco-2 monolayers, which is prevented by a pre-treatment of trophozoites with E64d.

G. duodenalis isolate NF trophozoites were pre-treated with E64d (10 μ M) or vehicle control, DMSO, prior to co-incubation with Caco-2 monolayers for 24 hours.

Representative photographs show that live *G. duodenalis* NF trophozoites induced villin disruption in Caco-2 monolayers, which was prevented by an E64d pre-treatment.

Micrographs are cropped versions of the full image; gain and exposure were maintained constant throughout. Magnification=400x. n=3.

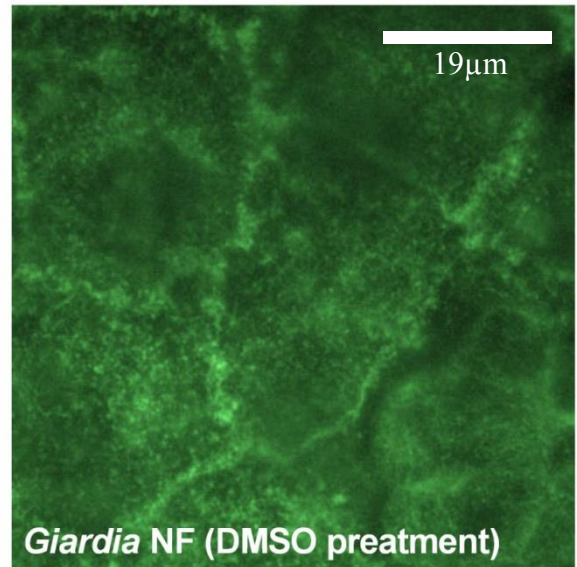
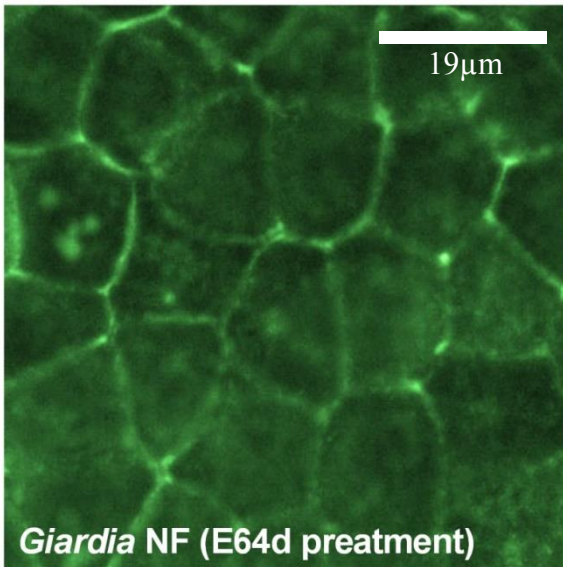
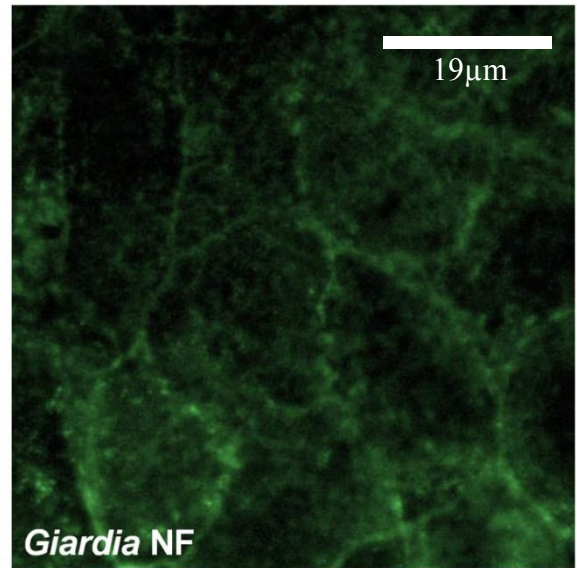
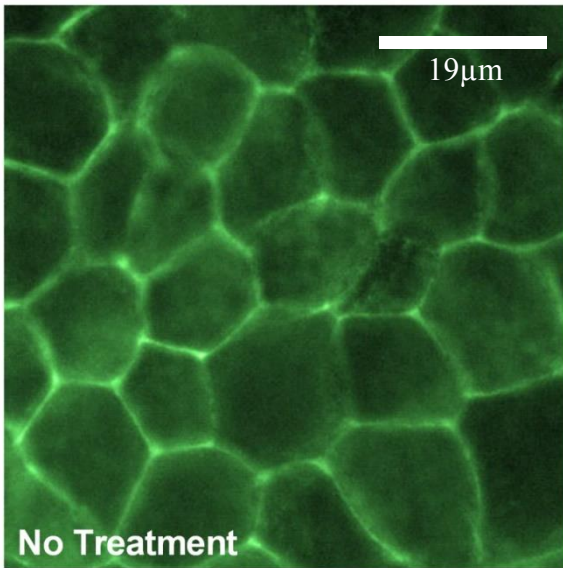


Figure 25. *G. duodenalis* NF trophozoites cause a disruption of villin after a 2 hour incubation with Caco-2 monolayers, which is not inhibited by ML-9.

G. duodenalis isolate NF trophozoites were co-incubated with Caco-2 monolayers for 2 hours. Representative photographs show that live *G. duodenalis* NF trophozoites induced villin disruption in Caco-2 monolayers, which was not prevented by ML-9 (40 μ M) (MLCK inhibitor). ML-9 alone did not affect villin localization in Caco-2 cells.

Micrographs are cropped versions of the full image; gain and exposure were maintained constant throughout. Magnification=400x. n=3.

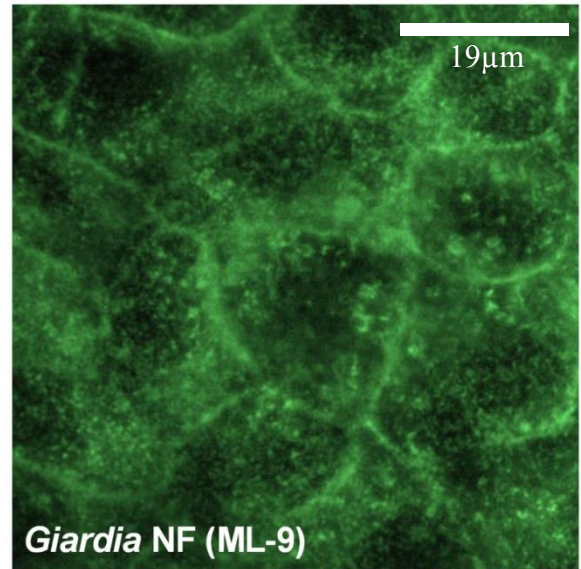
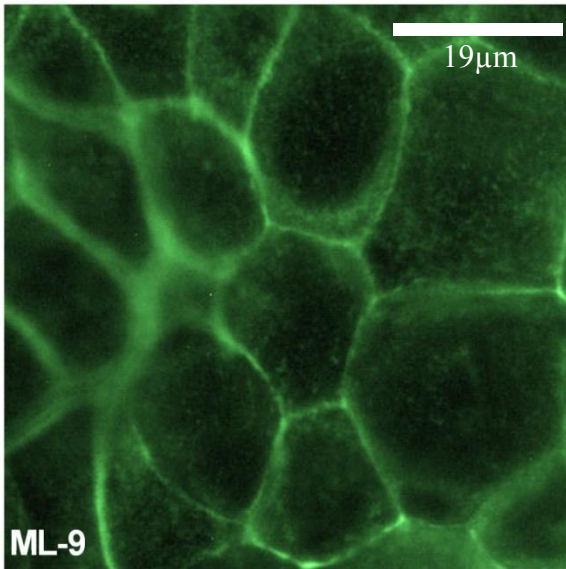
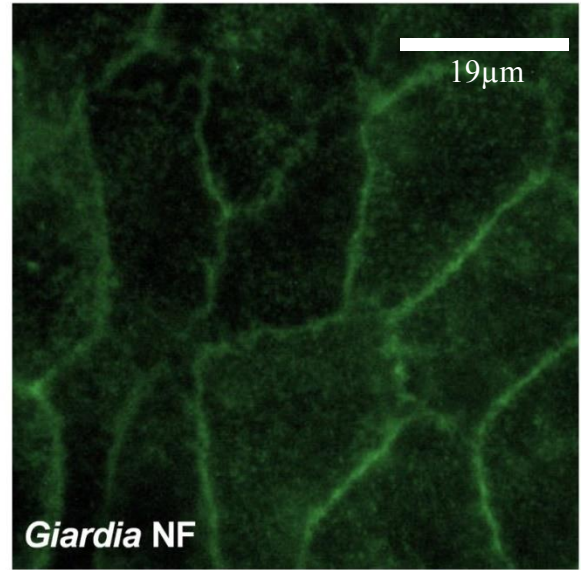
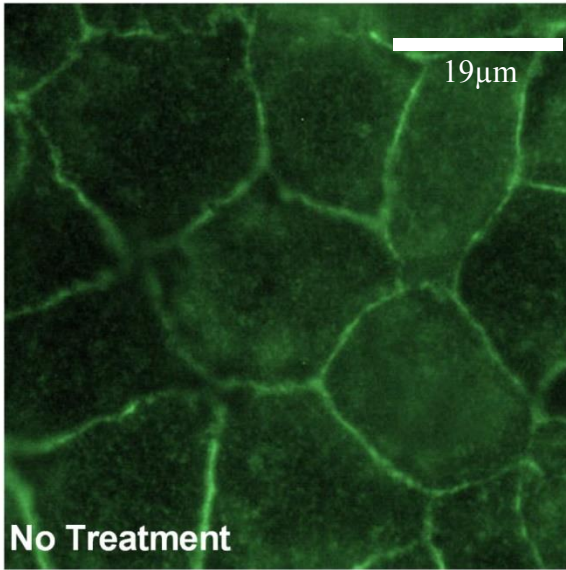
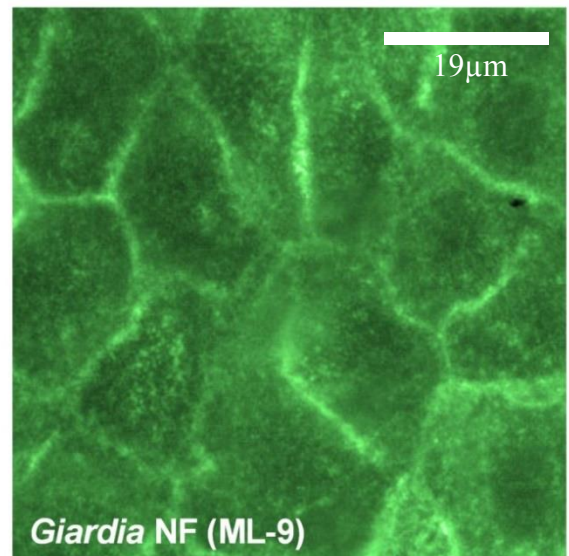
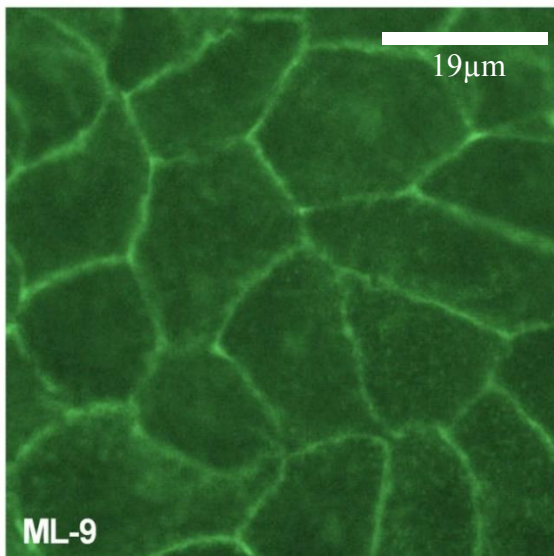
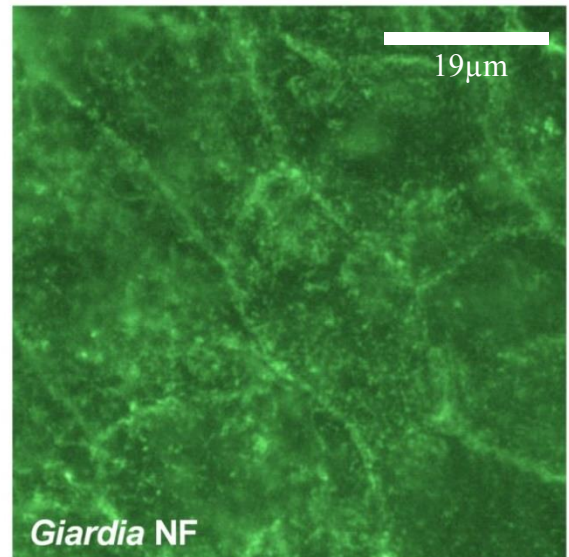
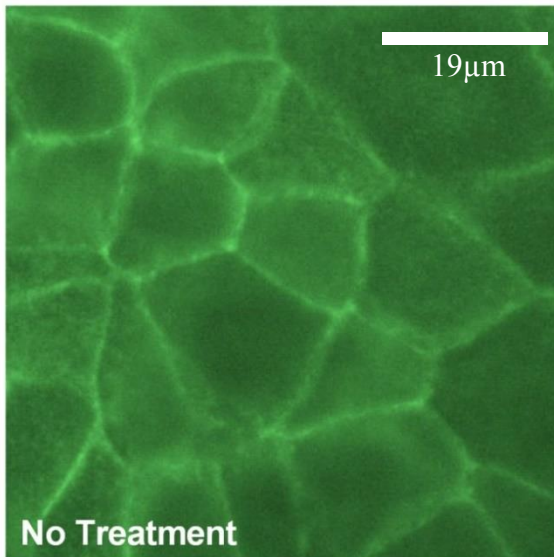


Figure 26. *G. duodenalis* NF trophozoites cause a disruption of villin after a 24 hour incubation with Caco-2 monolayers, which is prevented by ML-9.

G. duodenalis isolate NF trophozoites were co-incubated with Caco-2 monolayers for 24 hours. Representative photographs show that live *G. duodenalis* NF trophozoites induced villin disruption in Caco-2 monolayers, which was in part prevented by ML-9 (40 μ M) (MLCK inhibitor). ML-9 alone did not affect villin localization in Caco-2 cells.

Micrographs are cropped versions of the full image; gain and exposure were maintained constant throughout. Magnification=400x. n=3.



4. DISCUSSION

Giardia duodenalis is a highly infectious, diarrhea-causing parasite that inhabits the small intestinal lumen of a broad range of species, including humans. It has been shown to injure human intestinal epithelial cells both *in vitro* and *in vivo* [26, 27]. *G. duodenalis* is also implicated in causing PI-IBS through mechanisms that remain unclear [58]. There is increased overall proteolytic activity, and higher concentrations of proteases including serine proteases, cysteine proteases, and matrix metalloproteinases (MMPs) in the feces of patients with UC and IBS [5, 21]. The source of such proteases has yet to be established, but the microbiota and host immune proteases have been implicated [133]. The mechanisms whereby these proteases may initiate or exacerbate disease have yet to be elucidated, but their increased profile may correlate with disease activity [4]. While *G. duodenalis* cysteine proteases have largely unknown roles, the work presented here showed that they are able to breakdown and disrupt a crucial cytoskeletal protein.

The goal of this study was to characterize cysteine protease activity in *G. duodenalis*, and to assess its role in pathophysiology. The first objective was to characterize cathepsin-like cysteine proteases in *G. duodenalis* trophozoites during host-parasite interactions using a model of human enterocyte Caco-2 monolayers. This cell line has been used in several studies to study the effects of *G. duodenalis* [34, 46, 92, 137]. The findings from this study indicate that *G. duodenalis* trophozoites contain cathepsin-like cysteine proteases, and that the parasite is able to release these proteases into the extracellular environment. All of the *G. duodenalis* isolates tested herein,

whether belonging to Assemblage A or B, displayed similar amounts of cathepsin-like cysteine protease activity.

Further experiments investigating the pathogenic effects of *G. duodenalis* cysteine proteases found that NF (Assemblage A) trophozoites induce a biochemical breakdown of ZO-1, but this breakdown was not prevented by a pre-treatment or co-treatment of NF trophozoites with the protease inhibitor E64d suggesting that *G. duodenalis* NF may cause this tight junctional injury independently of cathepsin-like cysteine proteases. Experiments also characterized the effects of *G. duodenalis* cathepsin-like cysteine proteases on transmembrane tight junction proteins. No breakdown of occludin or claudin-1 in Caco-2 monolayers in the presence of NF trophozoites was found, which suggests that *G. duodenalis*' effects on these proteins is through other means. Importantly, *G. duodenalis* NF trophozoites disrupt cytoskeletal villin in a cysteine protease- and time- dependent manner. MLCK is involved in villin breakdown at 24 hours post-incubation, but not at the early time point of 2 hours. The work presented in this thesis failed to reveal actin breakdown upon exposure to *G. duodenalis* isolate NF.

4.1 Characterization of cathepsin-like cysteine protease activity during parasite-host interactions

The *G. duodenalis* genome has been shown to contain genes for 23 cathepsin-like cysteine proteases, including cathepsin B-, C-, K/L-like cysteine proteases with unknown functions. The roles that have been established for the parasitic proteases to date are for encystation, and immune-modulation [34, 39]. How such proteases may interact with the host intestinal epithelium has yet to be uncovered. Findings from this study indicate that there are proteases that are housed within the trophozoites and are released into their

extracellular environment regardless of the presence or absence of host monolayers. The *in vitro* model described herein provides a simple yet effective method for studying the presence of proteases, and changes in proteolytic activity during host-parasite interactions. The findings of this study demonstrate that *G. duodenalis* trophozoites from three different isolates, belonging to two different assemblages, display similar amount of proteolytic activity compared to each other, but this activity may be somewhat substrate-specific.

To further help clarify whether *G. duodenalis* trophozoites contain cathepsin-like cysteine proteases, substrates that are selective for cathepsin B/L or only cathepsin B were used. Lysates of trophozoites from isolate NF, S2, or GS/M that were co-incubated with Caco-2 monolayers displayed the same amount of activity against ZFR-AMC or ZRR-AMC, suggesting that they have similar intra-trophozoite protease profile across the isolates. Moreover, this activity was not altered by the presence of Caco-2 monolayers. Interestingly, the proteases within the trophozoites did not cleave the cathepsin B-selective substrate (ZRR-AMC) at the same rate as the cathepsin B/L substrate (ZFR-AMC), suggesting that either the proteases in the trophozoites are more selective for ZFR-AMC, or that there are several proteases in the lysates. A study by David *et al.* assessed the presence of proteases in a newly found *G. duodenalis* isolate in Brazil used gelatin gels to assess the gelatinolytic activity in trophozoite lysates [36]. The study demonstrated that there are several proteases present in this isolate [36]. *G. duodenalis* trophozoites contain cysteine proteases that have the ability to cleave both substrates, but more proteases may be able to cleave one substrate better than the other.

The next objective was to determine if *G. duodenalis* trophozoites release any cathepsin B- or L-like cysteine proteases. In addition to housing these proteases, *G. duodenalis* trophozoites from all three isolates released cathepsin-like cysteine proteases. Whether this release of proteases reflects active secretion or excretion requires further investigation. It was also observed that the amount of proteolytic activity present in the supernatants of assemblage A isolates NF and S2, in the presence of Caco-2 monolayers, was significantly increased compared to that in the absence of monolayers. This suggests that the presence of Caco-2 monolayers may be inducing the release of cathepsin-like proteases from either isolate NF or S2. This is supported by previous findings that established that cathepsin L-like cysteine proteases are upregulated in a transcriptional microarray analysis of *G. duodenalis* WB, another Assemblage A isolate, in the presence of Caco-2 monolayers [109]. In contrast, this was not observed in the *Giardia* isolate GS/M (belonging to Assemblage B), where the amount of proteolytic activity observed was the same in the presence or absence of Caco-2 monolayers. Whether this increase in proteolytic activity being released into the supernatant could be explained by nutrition competition needs to be further investigated. This hypothesis is further supported by a study that showed that *Giardia*'s excretory/secretory products alone may cause a decrease in the absorption of D-glucose and phenylalanine in mice [116]. Intriguingly, this effect was not observed in the processing of ZRR-AMC, suggesting that proteases capable of processing ZFR-AMC are released are produced in larger amounts in the presence of Caco-2 cells whereas protease(s) able to cleave ZRR-AMC could be released constitutively. Understanding if these proteases in isolates NF and S2 are released in larger amounts from the trophozoites in the presence of Caco-2 monolayers or other cell

lines will further our knowledge and may provide further insights in their function and role in pathogenesis.

Another aim of this study was to determine whether *G. duodenalis* cysteine proteases are able to directly enter Caco-2 enterocytes, possibly reflecting the existence of parasitic secretory systems similar to the type 3 secretion system of a gram-negative pathogenic bacteria [30]. *G. duodenalis* is not an invasive parasite; therefore, it may employ mechanisms of transporting its proteases could contribute to the pathogenesis of giardiasis. Caco-2 monolayers were treated with formononetin prior to lysis for the assessment of cathepsin activity to assure the removal of adhered trophozoites. This isoflavone has been shown to be very effective at causing *G. duodenalis* trophozoites to detach from epithelial cells [78]. Results from this study revealed that Caco-2 monolayers did not contain more cathepsin activity upon exposure to *G. duodenalis* trophozoites, using any of the three isolates of the study. We have shown here that while *G. duodenalis* trophozoites house cathepsin-like cysteine proteases and release them into the extracellular environment, the said proteases do not transit into the Caco-2 cells.

G. duodenalis isolate NF was used to study the effect of commercially available inhibitors on its cathepsin-like cysteine proteases. Our findings suggest that *G. duodenalis* cysteine proteases in isolate NF are susceptible to inhibition by a broad spectrum Clan CA cysteine protease inhibitor, E64d, as well as a cathepsin B-selective inhibitor, Ca-074Me. There are questions regarding the specificity and limitations of these inhibitors. However, as of now these serve as limited, yet effective, tools for the initiation of this study, since in the presence of these inhibitors the cysteine protease activity from *G. duodenalis* is eliminated. A complete inhibition of ZFR-AMC

processing by trophozoite lysates was observed when the trophozoites were pre-treated with Ca-074Me or E64d, suggesting that these inhibitors may be working through similar mechanisms of inhibition. It is interesting to note that these proteases were able to process ZFR-AMC better than ZRR-AMC, and yet were susceptible to inhibition by Ca-074Me; this suggests structural differences between parasitic proteases and mammalian proteases, for which these substrates and inhibitors were designed. These inhibitors were not toxic to the trophozoites, suggesting that the lack of activity observed was not due to dying trophozoites. Our work demonstrated that protease inhibition through trophozoites pre-treatment with one of these inhibitors serves as a reliable model for inhibiting cathepsin-like cysteine protease activity in *G. duodenalis*. While these inhibitors block proteolytic activity towards one substrate does not mean that they have the same mode of action. E64d is an epoxysuccinyl irreversible Clan CA cysteine protease inhibitor that works through S-alkylation of the active site cysteine in the protease to open up the epoxide ring. Ca-074Me, also an epoxysuccinyl irreversible inhibitor of cathepsin B, works by targeting the occluding loop of cathepsin B. E64d is attacked at the C2 position of the epoxide ring, whereas Ca-074Me at the C3 position [104]. It is important to remember, however, that these protease inhibitors were developed and studied using the mammalian protease system. The *G. duodenalis* proteases are slightly different and these inhibitors may not have the same specificity towards these proteases as they do against mammalian cathepsins. A limitation of this study was that the empirical half maximal effective concentration (EC50) and half maximal inhibitory concentrations (IC50) were not obtained for each inhibitor, and should be assessed in future work for *G. duodenalis* cysteine proteases as they are isolated as they may be different for each protease.

4.2 Investigating the role of *Giardia* cathepsin-like cysteine proteases in giardiasis.

As mentioned in the previous section, the scientific literature has elaborated the role of parasitic factors, particularly cysteine and other types of proteases, in the initiation of diseases. For example, *Entamoeba histolytica* uses its arsenal of 4 cysteine proteases (called EhCP1, 2, 4, and 5) to carry out a number of disease initiating functions, including their critical roles in invasion and colonization [107]. Indeed, other protozoans also use cysteine proteases in the initiation of disease, including *Leishmania* and *Plasmodium* [88]. In addition, a recent study showed that fecal cysteine proteases are elevated in IBS-C patients, and play a role in the breakdown of tight junctional integrity of the patients' intestinal epithelium [4]. Interestingly, despite knowing that *G. duodenalis* expresses genes for cathepsin-like cysteine proteases, no such protease or virulence factor has been identified for *G. duodenalis*, and there is a gap in our knowledge and understanding regarding what parasitic factors may be involved in the triggering of the giardiasis-associated pathophysiology.

The findings of our work in this thesis indicate that, while *G. duodenalis* cysteine proteases may not be involved in the breakdown of tight junctional proteins such as ZO-1, they may have a particular role in the disruption and breakdown of the cytoskeletal protein, villin. This proteolytic breakdown early after infection is later sustained by the host machinery, when MLCK contributes to villin disruption.

Tight junctions regulate intestinal epithelial permeability, thereby preventing the passage of unwanted luminal contents across the epithelium, while still allowing transport of ions and nutrients. A number of gastrointestinal disorders, including IBD, coeliac disease and bacterial enteritis, are associated with a breakdown in tight junctional barrier

function [12, 97, 147]. Pathogenic virulence factors have been implicated in the disruption of tight junctional proteins, including ZO-1. For example, *Clostridium difficile* toxins A and B disorganize ZO-1 and increase permeability, in cooperation with host pro-inflammatory factors, such as IFN- γ [95, 159]. Previous studies have shown that *G. duodenalis* disrupts ZO-1, claudin-1, and occludin to increase permeability leading to a breakdown in epithelial barrier function [26, 137, 142]. The present study assessed the role of *G. duodenalis* cathepsin-like cysteine proteases in cytoskeletal and tight junctional injury. *G. duodenalis* failed to show any proteolytic effect on ZO-1, claudin-1, and occludin in Caco-2 monolayers. As claudin-1 disruption has been reported in patients with chronic giardiasis [142], our observations may reflect the lack of host immune cell-derived factors in the present study [142]. As discussed earlier, pro-inflammatory cytokines like TNF- α and IFN- γ are both implicated in the loss of permeability seen in IBD patients [143]. Indeed, IFN- γ levels are elevated during giardiasis, pointing to one possible mechanism via which claudin-1 may be disrupted [87]. Other reasons for a lack of claudin-1 breakdown or downregulation in our model could reflect short exposure times and/or isolate-specific effects. Another study assessing the role of trophozoite adhesion in the induction of tight junctional injury used the GS/M isolate as a model for studying giardiasis [64]. This particular study assessed claudin-1 breakdown through immunocytochemistry, suggesting that while there may have been contact-dependent changes to claudin-1 taking place, these changes may have occurred via the action of regulatory proteins, such as MLCK, resulting in their delocalization from the tight junction rather than their degradation.

Occludin has been observed to be enzymatically degraded by cysteine proteases in IBS-C patients [4]. A recent study of giardiasis in mice also revealed occludin disruption *in vivo* [26]. Future research needs to assess the role of host and microbiota factors in this process, as occludin disruption was not observed *in vitro* in this study. Post-infectious changes to the microbiota could make the commensal microbiota pathogenic that may play a role in the disruption of tight junctions; indeed, tight junctional disruption and particularly occludin cleavage was observed *in vivo* even after *G. duodenalis* had been cleared [26]. Again, isolate specificity may also be involved in damage to occludin as the *Giardia* isolate GS/M was used in the given study [26]. Another study observed occludin phosphorylation and localization to the cytosol in the presence of *G. duodenalis* isolate GS/M in Caco-2 cells after 24 hours, suggesting that in addition to this possibly being an isolate specific effect, occludin undergoes changes that do not necessarily result in its breakdown or downregulation [64].

ZO-1 is a plaque protein that connects the transmembrane proteins, i.e. claudin-1 and occludin, to cytoskeletal F-actin and myosin light chains. ZO-1 disruption is evident during enteric infections, and in response to inflammation [96]. Previous work has demonstrated that ZO-1 is disrupted in the presence of *G. duodenalis* in a caspase-3- and MLCK-dependent manner, contributing to a reduction in intestinal epithelial barrier function [27]. In the present study, ZO-1 was degraded in the presence of live *G. duodenalis* trophozoites as assessed via immunoblotting, suggesting that *G. duodenalis* trophozoites do not only disrupt ZO-1, but also induce a breakdown or downregulation of this important tight junctional protein. Pre-treatment of *G. duodenalis* NF trophozoites with E64d prior to co-incubation with Caco-2 monolayers, as well as co-treatment with

E64d or Ca-074Me, did not prevent this degradation of ZO-1. This suggests that ZO-1 breakdown induced by *G. duodenalis* may occur independently of the parasitic cysteine proteases. While a Clan CA cysteine protease inhibitor (E64d) or a cathepsin B-selective inhibitor (Ca-074Me) did not prevent ZO-1 degradation, it is also possible that there may be other proteases or factors that are not sensitive to these inhibitors that may have been involved in the breakdown of ZO-1. Overall, the results of this study indicate that *G. duodenalis* NF trophozoites do not directly break down the transmembrane proteins occludin or claudin-1, but cause a degradation of the tight junctional plaque protein ZO-1 in a *G. duodenalis* cysteine protease-independent manner. While Western blotting provides a valuable analysis of the tight junctions at the protein level, functional changes to tight junctional proteins by *G. duodenalis* cysteine proteases should be assessed in the future. This could be done via the use of Transwells to assess changes in permeability of the epithelial monolayers.

Within the epithelium, the cytoskeleton plays a very important role in the maintenance, migration, and repair of the epithelium. In addition, the apical cytoskeleton of enterocytes is involved heavily in the structural and functional integrity of microvilli. The cytoskeleton is comprised of F-actin and several of its regulators. The scientific literature has shown that F-actin is a target not only for pathogens, but it is also severely disrupted in inflammatory conditions. Several pathogens, including *C. difficile*, EPEC, and *G. duodenalis*, disrupt F-actin and impair barrier function [95, 102, 137]. *G. duodenalis* also causes a diffuse shortening of the brush border microvilli in a CD8⁺ T cell lymphocyte-dependent manner in mice [121]. This seems to be independent of villous atrophy, however. As *G. duodenalis* disrupts F-actin *in vitro* and since *G.*

duodenalis sonicates are enough to do so, parasitic factors may play a role in this pathophysiological effect. One possible mechanism for the actin disruption observed during *G. duodenalis* infections is through the disruption of an actin-regulatory protein called villin. Villin is a cytoskeletal protein that regulates actin dynamics through binding, capping, severing and bundling actin. This function of villin makes it an important candidate to study during enteric infections such as giardiasis as it could explain the changes to F-actin seen during infections. Indeed, another parasite *Entamoeba histolytica* breaks down villin resulting in a loss of the brush border microvilli as well [81]. The current study demonstrated for the first time that *G. duodenalis* NF trophozoites cleave villin in human Caco-2 monolayers. This change was observed at an earlier time point (2h) as well as at a later time point of 24h, suggesting that this injury is sustained even though it is initiated early on during the infection. Pre-treatment of *G. duodenalis* NF trophozoites with a Clan CA cysteine protease inhibitor (E64d) prevents this cleavage of villin, suggesting that the breakdown of villin at an earlier time point is occurring in a *G. duodenalis* cysteine protease-dependent manner. At a later time point, an E64d pre-treatment also brought the cleavage product down to control levels. A potential caveat is that E64d that was present in the trophozoites may act on Caco-2 cells, even though the trophozoites were washed with PBS prior to co-incubation with Caco-2 monolayers. The role of MLCK, a potent regulator of actin at the tight junctional level, was also assessed and it was observed that inhibiting MLCK with ML-9 did not prevent this cleavage after a 2 hour incubation. Interestingly, an ML-9 treatment of Caco-2 monolayers prior to incubation with *G. duodenalis* trophozoites prevented the presence of the cleavage product of villin after 24 hours. This may suggest a role for MLCK-dependent signaling

for the breakdown of villin. These findings were corroborated by immunocytochemistry, as *G. duodenalis*-induced cytosolic relocalization of villin is prevented by E64d pretreatment or by a co-incubation with ML-9. For additional information from these micrographs, Z-stack analysis needs to be performed in the future to determine the effects of *G. duodenalis* cathepsin-like cysteine proteases on the microvillous villin protein specifically. Studies also assessed the role of caspase-3 through the use of Z-DEVD-FMK, a caspase-3 selective inhibitor, as done in other studies [27]. Caco-2 monolayers treated with Z-DEVD-FMK followed by a co-incubation with *G. duodenalis* trophozoites demonstrated villin cleavage, suggesting that while these changes are MLCK dependent, they are independent of caspase-3. Therefore, this mechanism differs from ZO-1 disruption, which is MLCK- and caspase-3 dependent [27, 120], but independent of *G. duodenalis* cysteine proteases as illustrated here. Further studies need to focus on the interactions of MLCK and villin, as the mechanisms of their apparent interplay have yet to be characterized. As well, previous research demonstrated that *G. duodenalis*-induced tight junctional disruption and increased permeability are MLCK-dependent. Further investigation is required to assess whether villin cleavage during *G. duodenalis* infections may also play a role in this previously established phenomenon in view of the key actin regulatory function of villin. Studies looking at MLCK-villin interaction may provide a better understanding of changes seen in F-actin, and as a result of tight junctional permeability.

Interestingly, the protein levels of the full length villin protein did not change even in the presence of the cleavage band, raising the question of whether the villin in the groups with the cleavage product is still functional and what the role of this cleavage

product is in *G. duodenalis* pathophysiology. Studies need to elucidate whether the cleaved product may have a role in cell survival. Indeed, previous work has demonstrated that villin has other roles than just in actin dynamics, such as acting as a pro-survival protein [148]. A recent study showed that *in vivo*, *G. duodenalis* GS/M trophozoites induce a CD8⁺ and CD4⁺ dependent cleavage of villin in mice [130]. Together with the observations from this *in vitro* study, the findings indicate that *G. duodenalis* cysteine proteases may initiate changes that are then sustained by host machinery and host immunity.

Finally, the findings from this study show that villin breakdown product in Caco-2 monolayers requires direct contact with *G. duodenalis* NF trophozoites. This may indicate that there may be trophozoite surface cysteine proteases that may play a role in the cleavage of villin. Indeed, *Entamoeba histolytica* has surface proteases that play crucial roles in the pathogenesis of amebiasis [63, 68]. Previous findings have suggested that lipid raft-mediated adhesion of trophozoites is required for the induction of *G. duodenalis* pathogenesis, at least in Caco-2 monolayers [64]. It is intriguing to note that villin is found in close association with phospholipids in the plasma membrane, including phosphatidylinositol-4,5 bisphosphate, as well within the microvilli [76]. *G. duodenalis* trophozoites' interactions with the host IEC membranes may be driving villin breakdown in a cysteine protease-dependent manner. Future studies should explore the effects of this villin cleavage due to *G. duodenalis* cysteine proteases on villin's interactions with such phospholipids, as they have important signaling functions.

How *G. duodenalis* membrane proteins, particularly adhesive disc proteins, may contribute to pathogenesis remains unclear. It would also be interesting to determine the

role of these cysteine proteases in trophozoite adhesion to the host intestinal epithelium, and whether this villin breakdown is a way for the parasite to disrupt structural integrity of the microvilli for better adhesion. It has previously been shown that a diffuse brush border shortening is dependent on host immunity, but the findings presented here suggest that the process of this diffuse shortening may also be initiated by *G. duodenalis* trophozoites and their proteases soon after their first contact with the epithelium. Further investigation needs to determine whether the villin cleavage product contains the N-terminus or the C-terminus fragment. Indeed, it was suggested in a recent study that in mice, the *G. duodenalis* isolate GS/M induces a CD8⁺ and CD4⁺ lymphocyte dependent villin cleavage and this results in an accumulation of the COOH-terminal fragment [130]. This fragment was also implicated with a pro-survival function in enterocytes [148]. Hence, villin breakdown may also impair enterocyte differentiation, further contributing to digestive enzyme deficiencies and malabsorption. The present study did not determine whether the cleaved product of villin is pro-survival or pro-apoptosis. However, studies assessing if this villin breakdown may contribute, at least in part, to the increased rates of enterocyte apoptosis induced by *G. duodenalis* are needed.

The most common treatment for giardiasis is metronidazole. This drug is associated with resistance, and reduces the thickness of the mucus layer, making mice more susceptible to pathogen-induced colitis [157]. Understanding the role of the proteases expressed and released by *G. duodenalis* trophozoites may further our knowledge and help develop a better treatment against the parasite. Indeed, parasitic proteases have become important targets for drug development [89]. While broad spectrum protease inhibitors that also act on mammalian proteases may not be viable

treatments, more specific inhibitors of microbial proteases may provide important therapeutic avenues. For instance, if villin cleavage is critical for *G. duodenalis* adhesion, then perhaps inhibiting these proteases may prevent key pathophysiological effects of giardiasis.

4.3 Summary

There were two parts to the work presented herein. The first part of the study demonstrated that *G. duodenalis* trophozoites contain and release cathepsin B/L-like cysteine proteases, independent of the presence of Caco-2 monolayers, and independent of the isolate genotype. Cathepsin B/L-like cysteine proteases from *G. duodenalis* NF, at least, are susceptible to a broad spectrum Clan CA cysteine protease inhibitor, E64d, and to a cathepsin B-selective inhibitor, Ca-074Me. It was also found that *G. duodenalis* trophozoites, independent of isolate genotype release cathepsin activity into the extracellular environment; however, this release is further increased in assemblage A isolates, but not in the assemblage B isolate GS/M, when the parasite is exposed to Caco-2 monolayers. Furthermore, these proteases do not enter Caco-2 cells.

The second part of the study assessing the role of *G. duodenalis* cysteine proteases in pathophysiology made use of cathepsin inhibitors. When *G. duodenalis* trophozoites were pre-treated with the E64d inhibitor prior to co-incubation with Caco-2 monolayers, *G. duodenalis* NF trophozoites failed to induce claudin-1 or occludin breakdown as assessed through immunoblotting; however, *G. duodenalis*-induced ZO-1 degradation was insensitive to cysteine proteases inhibitors. In contrast, *G. duodenalis* trophozoites also disrupted villin in a cysteine protease-dependent manner initially, an

injury which later became MLCK-dependent suggesting a role for *G. duodenalis* proteases in the initiation of disease.

4.4 Future Directions

Preliminary work (not presented) has shown a role for secreted cysteine proteases in the activation of protease-activated receptor 1 (PAR1). Previous research has shown that PAR1 activation is not only involved in the induction of apoptosis in enterocytes but it also causes an activation of MLCK [28]. Based on these preliminary observations and the data presented in this thesis, the following studies are suggested for the future:

1. To determine if PAR1 is activated by *G. duodenalis* secreted or surface cysteine proteases.
2. To determine which cysteine protease from *G. duodenalis* results in villin breakdown.
3. To determine if cysteine protease-dependent villin cleavage is required for the establishment of *G. duodenalis* infections.
4. To determine the effects of villin cleavage. For example, whether the cleaved villin contributes to heightened rates of apoptosis seen during giardiasis.
5. To determine whether *G. duodenalis* cysteine proteases also break down other cytoskeletal proteins, including ezrin and gelsolin. These cytoskeletal proteins are also important in actin bundling and microvilli formation.

5. CONCLUSIONS

Giardia duodenalis is a protozoan parasite that causes zoonotic diarrheal disease all over the world. While the pathophysiology associated with this parasite has been studied, the implications of parasitic products in the initiation of disease are limited. Previous research has highlighted the disruption of key proteins that are involved in the homeostasis of the epithelium layer by *G. duodenalis*, and a better understanding of its parasitic factors was warranted. Our findings reveal that *G. duodenalis* trophozoites house and release cathepsin-like cysteine proteases with pathogenic potential. We found that *G. duodenalis* cysteine protease cause a cleavage of villin, a key cytoskeletal protein, which is sustained at least in part by MLCK over time. These findings further our understanding of the role of proteases in the initiation of disease. Furthermore, we demonstrate that proteases of parasitic origin may have specific functions that may also be time dependent, and their effects may be sustained by host regulatory pathways. As well, it provides an important platform for potential drug development as targeting these proteases may be an important avenue that has not been previously identified.

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