Trends in Parasitology

CellPress
REVIEWS

Review

Giardia Cysteine Proteases: The Teeth behind the Smile

Thibault Allain,[1] Elena Fekete,[1,2] and Andre G. Buret[1,*]

***Giardia duodenalis* is one of the most prevalent human enteropathogens and a major cause of diarrheal disease worldwide. Cysteine proteases (CPs) have been identified as major virulence factors in protozoan parasites, playing important roles in disease pathogenesis and in parasitic life cycles. *G. duodenalis* exhibits high proteolytic activity, and CPs play significant roles in giardiasis. *Giardia* CPs are directly involved in intestinal epithelial junctional complex disruption, intestinal epithelial cell apoptosis, and degradation of host immune factors, including chemokines and immunoglobulins. *Giardia* CPs have also been implicated in mucus depletion and microbiota dysbiosis induced by the parasite. This review discusses the most recent advances in characterization of *Giardia* Assemblage A and B CPs, including cathepsin B (catB)-like proteases.**

Highlights

Increasing interest has recently been directed towards parasite CPs, and more specifically catBs and catLs, as virulence factors and targets for therapeutic intervention.

Giardia CP activity is implicated in intestinal barrier dysfunction, mucus depletion and microbiota biofilm alteration during infection.

Recent proteomic profile analysis of *Giardia* trophozoites in axenic cultures and upon attachment to intestinal epithelial cells has suggested a role for CPs in *Giardia* virulence and host–pathogen interactions.

Biochemical and structural characterization of the most highly secreted *Giardia* CPs (i.e., CP2, CP3, CP16160) has prompted further interest in the role of these CPs during infection.

Giardia CPs contribute to the protective role of *Giardia* during concurrent infections with attaching–effacing bacterial enteropathogens by inducing bacterial killing and reducing inflammation in the intestine.

Interactions of *Giardia* with the Intestinal Tissue Barrier: A Role for CPs?

During the acute phase of infection, *Giardia* actively interacts with the intestinal surface, causing epithelial alterations, mucus layer disruptions, and gut microbiota dysbiosis. These effects occur at the site of infection and beyond, and result at least in part from the actions of *Giardia* **excretory and secretory products** (**ESPs**) (see Glossary). In recent years, research discoveries have highlighted important roles for *Giardia* ESPs, such as **cysteine proteases** (**CPs**), in *Giardia* pathogenesis. While the use of inhibitors has made it possible to directly correlate *Giardia* CP activity with tissue barrier alterations, the recent use of recombinant CPs and their characterization represents an important step forward in our understanding of the pathophysiological processes of giardiasis. In particular, the secreted **cathepsin B** (**catB**)-like **Assemblage** A cysteine proteases **CP2** (*syn.* giardipain-1, CP14019), CP3 (*syn.* CP16779), and CP16160, and their Assemblage B counterparts, play a central role in the pathogenic effects of *Giardia* [1–3] (Table 1). Moving forward through this review, CPs (e.g., CP2, CP3) are named in respect to the chronology of discovery [4,5]. The text will first discuss the characteristics and specificity of *Giardia* and other protozoan CPs, and then describe their interactions with host tissue barriers.

CPs: Structure, Activity, and Mode of Action

CPs (EC. 3.4.22) are enzymes containing a cysteine thiol residue in their catalytic center [6]. Most CPs synthesized by parasites are classified into two clans: clan CA, which includes papain-like and calpain-like peptidases, and clan CD [6,7]. The clan CA proteases, which encompass 84% of parasitic CPs, are subdivided into 24 families based on their primary sequence and tertiary structure [8]. Within the clan CA proteases, the family C1 (e.g., C1A), representing 45% of protozoan peptidases, is divided into two cysteine cathepsin subfamilies, catB-like (EC. 3.4.22.1) and **cathepsin L**-like (**catL**-like) (EC. 3.4.22.15) enzymes based on **pro-domain** sequences and length [8–10]. The cysteine cathepsins, including those found in *Giardia* sp., belong to clan CA and family C1. The role of *Giardia* sp. serine proteases and aspartic proteases, which have also been identified in *Giardia* cultures *in vitro*, will not be discussed in this review [11]. The structure, **active site** properties, and mechanism of catalysis for parasitic and *Giardia* CPs are presented in Box 1, and *Giardia* CP structural specificities are presented in Box 2.

[1]Department of Biological Sciences and Host–Parasite Interactions Program, University of Calgary, Calgary, Canada
[2]Co-first author

*Correspondence:
aburet@ucalgary.ca (A.G. Buret).

Protease S1/S1′ and S2/S2′ substrate-binding subsites are responsible for substrate selectivity, binding to P domain counterparts on the target peptide [6]. As with most clan CA CPs, *Giardia* cathepsins have a strong preference for large and hydrophobic residues at the S2 subsite and little selectivity at the S1′ subsite. Family C1 cathepsins can cleave a broad variety of substrates such as extracellular matrix (ECM) components, including collagen, elastins, and proteoglycans [7]. Characterization of substrate specificity confirmed that *Giardia* CPs can degrade gelatin, collagen, albumin, and azocasein [12]. A recent report showed that *Giardia* CPs exclusively display specific cleavage activity against the NH-terminal peptide of substrates [13]. *Giardia* clan CA CPs can directly degrade mucins (MUC2), immunoglobulins, chemokines, the bacterial biofilm extracellular matrix of the gut microbiota, and more (degradation of other host factors is discussed further in this review). The substrate specificity towards the S2 subsite allows for broad-spectrum inhibition of multiple protease activities by inhibitors such as trans-epoxysuccinyl-L-leucylamido (4-guanidino)butane (E-64). This contrasts with clan CD enzymes, which are much more specific at the S1′ subsite and therefore cannot be inhibited by E-64. In the active site, the nucleophilic cysteine residue of clan CA CPs is already ionized prior to substrate binding. As a result, most family C1 CPs are functional at slightly acidic pH [4,7]. Analysis of kinetic properties of recombinant *Giardia* catB-like proteases against the family C1 CP substrate ZFR-AMC and the catB-specific substrate ZRR-AMC showed an optimal pH between 5.5 (CP2) and 6 (CP3, CP16160) for both substrates at 37°C, confirming in class standards [2].

Role of CPs during Giardiasis and Other Protozoan Infections

CatB- and catL-like proteases are critical for the life cycles of multiple protozoan parasites, as well as for the establishment of infection via host immune evasion, and in some cases invasion of tissue barriers. CPs play a key role in the pathophysiology of various enteric and systemic protozoan diseases, including African sleeping sickness, Chagas' disease, leishmaniasis, malaria, and amebiasis. Known mechanisms of pathogenesis for catB- and catL-like proteases are discussed in Boxes 3 and 4, respectively. CatB- and catL-like proteases have been identified as major virulence factors, and they have been investigated for decades as targets for therapeutic intervention.

Giardia CP Activity in Axenic Cultures

To date, 26 to 27 clan CA cysteine protease genes have been identified in the *Giardia* genome, including 9 catB-like proteases [5,14,15]; however, the most recent version of *G. duodenalis* WB genome displays 21 CP genes, including 8 catB genes (https://giardiadb.org/). Transcriptomic analysis of axenic cultures of the *G. duodenalis* isolate WB shows that several catB-like, catC-like, and catL-like CPs are putatively expressed [4]. In the trophozoite stage, catB genes such as *cp2* (*syn. glcp2*), *cp3* (*syn. glcp3*), coding for CP2 and CP3, respectively, as well as *eaa40365* and *eaa37074*, coding for CP16468 and CP16160, respectively, are the most highly expressed in Assemblage A isolates [5]. Noticeably, those genes are dramatically overexpressed in encysting trophozoites along with catC-like protease genes (*escp*, *eaa37165*) and catL-like genes (*eaa137680*, *eaa14983*, *eaa16380*), consistent with the key role played by *Giardia* CPs in the process of encystation [5]. In particular, *cp2* is the most highly expressed CP gene in both vegetative and encysting stages [5]. Additionally, iron uptake has been associated with gene expression regulation and function of CPs in *Trichomonas vaginalis*, suggesting that other factors may regulate CP activity in protozoans, and therefore their virulence [16].

The overall intracellular CP activity (i.e., catB and catL) is higher in Assemblage A isolates (isolates WB, NF) when compared to Assemblage B (isolate GSM) [17,18]; however, further research is needed to confirm and expand on these differences between Assemblages. Proteome analysis of axenic trophozoite cultures revealed that *Giardia* expresses a total of 16 catB-like proteases [15].

Glossary

Active site: an enzyme domain where substrates bind and undergo enzymatic reactions.
Apical junctional complex (AJC): a complex that forms between mammalian cells; it is composed of tight junctions (claudins, ZO-1, occludin, JAM-1) and desmosomal proteins. It regulates cell polarity and intercellular adhesion, and is critical for barrier function.
Assemblage: genotype-based classification of *Giardia*. *Giardia duodenalis* is classified into eight Assemblages (A through H). Assemblages A (e.g., NF, WB, Portland-1, S2, etc.) and B (e.g., GSM) are infectious to humans.
Attaching and effacing (A/E) pathogens: pathogenic organisms that attach to the host intestinal epithelium and produce lesions characterized by destruction of brush border microvilli.
Catalytic dyad or triad: a pair of amino acid residues within an enzyme's active site that mediate catalysis. In some cases, the catalytic dyad is stabilized by a third amino acid residue to form a triad.
Cathepsin B (catB): papain-like cysteine proteases expressed in mammalian lysosomes and many parasites. Most catB proteases have an occluding loop that confers exopeptidase activity in addition to typical endopeptidase activity.
Cathepsin L (catL): a papain-like cysteine protease with mainly **endopeptidase activity**. It is expressed in mammalian lysosomes and in many parasites.
CP2 (*syn.* giardipain-1, CP14019): the most expressed and secreted *G. duodenalis* catB-like protease in Assemblage A (isolate WB), which is partially conserved in Assemblage B isolates.
Cyst wall proteins (CWPs): proteins that make up the wall encasing *Giardia* cysts.
Cysteine protease (CP): a proteolytic enzyme in which the active site contains a cysteine residue.
Encystation-specific vesicles (ESVs): secretory microvesicles released during, and involved in, *Giardia* encystation.
Endopeptidase activity: catalysis of nonterminal peptide bonds within a polypeptide chain.
Excretory and secretory products (ESPs): products produced in a cell and

Seven catB-like and two catL-like proteases were found to be secreted in an Assemblage A isolate (WB), whereas an Assemblage B isolate secretes five catB-like and two catL-like proteases [19]. Noticeably, catB-like proteases are among the most abundant secreted proteins both at early (2 h) and later (6 h) time points in *in vitro* trophozoite cultures. CP2 and CP3 are the most abundant catB-like proteases in Assemblage A (WB isolate), and they represent the second and sixth most abundant secreted proteins overall in accordance with previous transcriptomic data [5,19]. Interestingly, CP2 and CP3 protein-coding genes are paralogs, and share 79.58% identity. Their counterparts in Assemblage B (GSM isolate), GL50581_78 (CP2-like, 78% identity) and GL50581_2946 are also abundantly secreted [19]. Moreover, *G. duodenalis* WB CP2 and CP3, and *G. duodenalis* GSM GL50581_78 and GL50581_2946 catB-like protein-coding genes are orthologs, and hence may have originated from a single gene of a last common ancestor [19].

Another proteomic analysis performed on axenic trophozoite cultures indicated that Assemblage A (WB) GL50803_16468 and its Assemblage B (GSM) ortholog GL50581_438 as well as Assemblage A (WB) GL50803_8742 and its Assemblage B (GSM) ortholog GL50581_2036 were the most abundantly secreted catB-like proteases [15]. In this study, 11 secreted catBs were detected, among which 6 belong to orthologous groups [15]. One explanation for the differences between studies may come from *in vitro* culture conditions as *Giardia* trophozoites were cultured in nonsupplemented DMEM in the latter report, whereas the previous study used RPMI supplemented with *Giardia*'s essential nutrients (i.e., L-arginine, L-cysteine, ascorbic acid). Also worthy of notice, the latter study collected secreted proteins after 45 min incubation, while the other report was based on proteins collected after 2 and 6 h [15,19].

Giardia CP Activity upon Interaction with Intestinal Epithelial Cells

When trophozoites are attached to **intestinal epithelial cells** (**IECs**), CPs are the sixth most abundant class of secreted protein in Assemblage A and B isolates [19]. The most abundant secreted catB-like proteases in Assemblage A (WB) are CP2, CP16160, and CP3, respectively [19]. In Assemblage B isolates, the most abundant secreted catB-like proteases are GL50581_78 (CP2-like), GL50581_438 (CP16468-like), and GL50581_2036 (CP15564-like), respectively [19]. Expression at the mRNA level of *Giardia cp2* and *cp17516* genes are upregulated upon exposure to IECs. However, cathepsin expression does not change at the protein level upon attachment [20–22]. Secreted CP activity is increased upon attachment to IEC for Assemblage A, but not for Assemblage B isolates *in vitro*; the overall intracellular CP activity remains unchanged for both Assemblage A and B upon exposure to IEC [18]. This difference could be explained by the lower metabolic activity of the Assemblage B (GSM) isolate [18]. Conversely, exposure to epithelial (HT-29 cell line) cellular factors induces an upregulation of CP3 (*syn*. CP16779) protein expression (1.58-fold change) in Assemblage A isolates [20]. It is worth mentioning that *Giardia* trophozoites do not induce CP secretion in IECs [18]. Interestingly, *Giardia* CP activity plays a role in adhesion of trophozoites to IECs, as observed in *T. vaginalis* and *Entamoeba hystolytica* [23–25]. Taken together, these observations suggest that *Giardia* CPs are important mediators of the interactions of *Giardia* with IECs.

Intracellular and Extracellular Localization of *Giardia* CPs

During encystation, **cyst wall proteins** (**CWPs**) are grouped at the periphery of trophozoites in **encystation-specific vesicles** (**ESVs**), which subsequently traffic to the plasma membrane. Intracellular GFP-tagged CPs expressed from an episomal vector indicate that CP2 and CP16160 colocalize with CWP2 in ESVs [5], while CP1 (*syn.* CP10217) has been localized in the endoplasmic reticulum [26]. Further investigation revealed that CP2, CP3, and CP16160 also colocalize in cytoplasmic vesicles of the endoplasmic reticulum [2]. *Giardia* excretory–secretory products, such as surface proteins, secreted proteases, and extracellular vesicles appear to play key

released through excretion- or secretion-specific pathways. They include proteins, enzymes, arginine deaminase and ornithine carbamoyltransferase, apoptotic bodies, microvesicles etc.

Exopeptidase activity: catalysis of peptide bonds on the terminal ends of polypeptide chains.

Intestinal epithelial cell (IEC) lines: commonly used in the field to study host–*Giardia* interactions such as Caco-2, IEC-6, and MCLK.

Nitric oxide (NO): a chemical messenger synthesized by host cells in response to parasitic infection. It can mediate microbe killing and damage host cells.

Occluding loop: a 20-residue insertion sequence in some catB-like proteases that allows for exopeptidase activity in addition to typical endopeptidase activity.

Pathobiont: temporarily benign microbes or commensals in humans, animals, and plants that may cause disease under environmental or host-induced stress.

Polymicrobial infections (concomitant infections): infections with multiple species in a single host.

Pro-domain: an enzyme domain that acts as a chaperone for protein folding and an autoinhibitor. Cleavage of the pro-domain converts enzymes to their active form.

Variant-specific surface proteins (VSPs): proteins expressed on a cell surface that play a role in antigenic variation.

Zymogen: the inactive form of an enzyme that must undergo biochemical change to become an active form.

Table 1. *Giardia duodenalis* Cysteine Protease (CP) Genes with Known Pathogenesis Roles from the Reference Strain *G. duodenalis* WB (Assemblage A) as well as Assemblage B (*G. duodenalis* GS and GS_B) Orthologs and Paralogs

G. d. WB (Assemblage A) Gene ID	UniProt entry ID	GenBank accession number	Protein name(s)	Activity	Proposed functions	Assemblage B orthologs and paralogs (*Giardia* DB)	Refs
GL50803_10217	A8BKX9	CAC18646	CP1 CP10217 CAC18545	Cathepsin B-like	Excystation	GSB_155190 (*G. d.* GS_B) GL50581_159 (*G. d.* GS) GL50581_3619 (*G. d.* GS) GSB_153364 (*G. d.* GS_B)	[29]
GL50803_14019	A8BTG7	EAA41050	CP2 Giardipain-1 CP14019 GlCP2	Cathepsin B-like	Excystation Induction of apoptosis Tight junction disruption Encystation Degradation of endocytosed proteins Degradation of tight junction proteins and host chemokines	GSB-14019 (*G. d.* GS_B) GL50581_2946 (*G. d.* GS) GL50581_3635 (*G. d.* GS) GL50581_78 (*G. d.* GS) GSB_153399 (*G. d.* GS_B) GSB_16160 (*G. d.* GS_B) GSB_16779 (*G. d.* GS_B)	[2,3,5,26,29]
GL50803_16779	A8BVM3	EAA37433	CP3 CP16779 GlCP3	Cathepsin B-like	Excystation Degradation of tight junction proteins and host chemokines	GL50581_78 (*G. d.* GS) GSB-16779 (*G. d.* GS_B) GL50581_2946 (*G. d.* GS) GL50581_3635 (*G. d.* GS) GSB-14019 (*G. d.* GS_B) GSB_153399 (*G. d.* GS_B) GSB_16160 (*G. d.* GS_B)	[2,29]
GL50803_22553	A8BCF2	EAA36907	Encystation-specific cysteine protease (ESCP)	Cathepsin C-like	Encystation	GSB_151035 (*G. d.* GS_B)	[28]
GL50803_16160	A8BX07	EAA37074	CP16160	Cathepsin B-like	Degradation and cleavage of host chemokines	GL50581_2946 (*G. d.* GS) GSB_16160 (*G. d.* GS_B) GL50581_3635 (*G. d.* GS) GL50581_3635 (*G. d.* GS) GL50581_78 (*G. d.* GS) GSB_14019 (*G. d.* GS_B) GSB_153399 (*G. d.* GS_B) GSB_16779 (*G. d.* GS_B)	[2]

Box 1. Structure, Specificity, and Catalytic Properties of *Giardia* CPs

Most cysteine proteases have two domains, termed left domain (α-helix motif) and right (β-barrel motif), with both domains containing two loops. All CPs have an active **catalytic dyad** site composed of residues of cysteine (Cys-25) and histidine (His-159), spatially conserved and forming an ion pair [7]. Most family C1 cathepsins share the classic Cys–His catalytic dyad; however, this ion pair is stabilized by an asparagine residue (Asn-175) in some CPs, thus forming a **catalytic triad** (papain numbering) [8,10]. The Cys residue is part of the left domain, while the His residue is part of the right domain. The mature domains of cathepsins are 220 (catL) to 260 (catB) amino acids in length, and are highly conserved [10]. Cysteine proteases are synthesized as **zymogens** to prevent uncontrolled proteolytic activity, with pro-domains of variable sizes between catB- and catL-like proteases that block the active site [10,58,59]. The catalytic activation of catB and catL is modulated by glycosaminoglycans through an autoactivation process [60]. Characterization of recombinant *Giardia* catBs showed that CP2 and CP3 proenzymes autocleaved quickly, while *Giardia* CP16160 was autoactivated to its mature form at later time points [2]. Depending on the studies, the molecular weight of *Giardia* catB proenzymes ranges from 33 to 37 kDa, while its mature form is between 25 and 28 kDa [2,3]. In addition to a proenzyme, *Giardia* catB-like proteases have a signal peptide 17–20 amino acids long. The predicted probability of an N terminal signal peptide ranging between 78% and 100% suggests that at least some *Giardia* catBs are secreted [15,61]. Catalysis begins when the nucleophilic Cys residue attacks the carbonyl carbon of the reactive peptide bond, forming a tetrahedral thioester intermediate that is stabilized by an oxyanion hole through a conserved glutamine (Gln-19) [58]. Breakdown of this tetrahedral intermediate causes release of the C terminal portion of the substrate, producing an acyl enzyme. The acyl enzyme is hydrolyzed by a water molecule that enters the active site following release of the C terminal portion of the substrate, forming a second tetrahedral intermediate [7]. This intermediate subsequently breaks down, releasing the N terminal portion of the substrate and a free enzyme.

Box 2. *Giardia* CPs: Out of the Loop?

As with most cysteine proteases, catB- and catL-like proteases display an endopeptidase activity [6,7]. In addition to this, catB-like proteases exhibit dipeptidyl carboxydipeptidase activity [7]. This specific cleavage property is due to the insertion of a peptide loop, 20 amino acids long, in the catalytic domain/pro-domain known as the **occluding loop** in place of a conserved ERFNIN motif [6]. This occluding loop allows the enzyme to bind to histidine residues at the carboxy terminus of peptide substrates [7]. Sequence alignment of several *Giardia* catB-like proteases reveal an absence of the characteristic occluding loop region, resulting in the loss of carboxypeptidase activity [6]. As an early divergent organism, *Giardia* sp. contains the earliest known lineage in the catB-like protease family among protozoa, suggesting that the occluding loop insertion appeared later in evolution [6].

roles in the pathogenesis of *Giardia* infection, and they disrupt host epithelial integrity [19]. Recent studies showed that *Giardia* secretes microvesicles (MVs) that may play a role in innate immune system activation, host attachment, and pathogenicity [27]. Recent proteomic analyses of trophozoites failed to detect CPs in these MVs [27]. More research is needed to assess and compare the proteome of *Giardia* MVs in several isolates from Assemblages A and B.

Roles in Encystation and Excystation Processes

A role for *Giardia* CPs was first reported in the parasite's life cycle. Indeed, inhibition of CP activity prevents the release of cyst wall components to the plasma membrane and therefore blocks encystation [28]. Characterization of clan CA CP transcriptomes for Assemblage A (WB) trophozoites showed that the expression of several CP genes increases during encystation [5]. In particular, CP2 is highly expressed during the trophozoite and encystation stages. During encystation, CWPs such as CPW2 are expressed along with intracellular CP2, which processes CWP2 to initiate its polymerization and subsequent formation of the cyst wall [5,28]. Moreover, CP2 and CP16160 colocalize with CWPs in ESVs. It is worth noting that the proteolytic processing of CWP2, which requires **endopeptidase activity**, was first attributed to the catC-like protease ESCP; however, this role was further attributed to CP2 as ESCP does not exhibit endopeptidase properties [5,28]. *Giardia* CPs also contribute to the excystation process since CP inhibitors block excystation of *Giardia* cysts *in vitro* [29].

Box 3. catB-like Protease Activities in Protozoal Infections

The TbCatB of *Trypanosoma brucei* is activated during the bloodstream stages of the parasite's life cycle, and is crucial for the parasite's survival due to its role in nutrient acquisition [62,63]. RNAi targeting of TbCatB cured mice of experimental *T. brucei* infection [62]. Similarly, inhibition of *cruzain,* the major cysteine protease from *Trypanosoma cruzi*, arrested growth at the intracellular amastigote stage, and protected mice against lethal infection [64].

Cruzain mediates invasion of *T. cruzi* trypomastigotes into host cells, in part via digestion of host extracellular matrix proteins [65]. Cruzain also plays a central role in immune evasion. Cruzain-deficient *T. cruzi* parasites fail to suppress macrophage activation and are subsequently unable to survive and replicate within macrophages [37]. Attenuated virulence within macrophages is also demonstrated for *Leishmania donovani* lacking the catB-like enzyme CPC, and *cpc* mutant parasites are more susceptible to killing by host cells [66]. CPC activates TGF-β1, which drives lesion development, and limits IFN-γ production, hence preventing parasite killing [40]. Inhibition of *L. donovani* catB also leads to proteome remodeling and regulates the parasitic secretome [66]. Mutation or deletion of CPC proteases in *Leishmania mexicana* reduces macrophage infection, and attenuates lesion size in murine infections without affecting parasite multiplication [67].

Entamoeba histolytica catB-like proteases EhCP1, EhCP2, and EhCP5 account for most of the trophozoite CP activity, and are crucial for pathogenicity. Nonpathogenic *Entamoeba dispar* does not express EhCP1 and EhCP5 [68]; however, a pathogenic phenotype can be conferred on nonpathogenic strains via overexpression of EhCP1, EhCP2, and EhCP5 [69,70]. *E. histolytica* CPs cleave MUC2 mucin, to disrupt mucus, and EhCP5 induces mucin hypersecretion from colonic goblet cells [71,72]. Together, these effects facilitate tissue invasion. *E. histolytica* CPs degrade extracellular matrix proteins, disrupt epithelial tight junctions, and are involved in host immune evasion via cleavage and inactivation of secretory IgA and IgG, and inactivation of complement pathways by cleaving C3a and C5a [73,74]. EhCP5 induces IL-1β and IL-8 production by intestinal epithelial cells, promoting inflammatory damage to the intestinal epithelial barrier [75]. Inhibition of CP activity in *E. histolytica* trophozoites reduces the formation of liver abscesses in SCID mice, and reduces inflammation and parasite burden in a cecal model of amebiasis [76].

Giardia CPs Disrupt Epithelial Apical Junctional Complexes (AJCs)

Impaired barrier function represents a key pathophysiological process in giardiasis. Several studies reported *Giardia*-induced reduction of IEC transepithelial resistance (TER), a marker of epithelial integrity and permeability [30]. A recent report showed that recombinant CP2 causes a decrease of TER in MDCK epithelial cells in a time- and dose-dependent fashion [3]. Moreover, *Giardia* trophozoites have a direct deleterious effect on epithelial barrier function by disrupting several components of the **AJC**, such as claudin 1, claudin 4, and occludin, as well as a rearrangement of ZO-1 [30,31]. In a recent report, recombinant CP2, CP16160, and CP3, the most highly secreted CPs in Assemblage A (WB), disrupted claudin-4, claudin-1, and occludin in Caco-2 cells, in a dose-dependent manner [2]. Surprisingly, rearrangement of ZO-1 observed upon exposure to *Giardia* trophozoites is not abolished in the presence of the CP inhibitor E-64, suggesting that other parasitic factors are involved [18]. Similarly, degradation of claudin-1 and occludin was observed in IEC-6 and MDCK cell lines exposed to recombinant CP2 [3]. Additionally, CP2 colocalizes with claudin-1 and occludin at the junctional level [3]. Alteration of adherens junction B-catenin and adhesion molecule JAM-1 were also observed to a lesser extent [2]. In contrast, no evidence of direct degradation of adherens junction (AJ) E-cadherins was observed, whereas *Giardia* CPs were able to degrade recombinant E-cadherin in a dose-dependent fashion [2]. Those results are in keeping with previous reports showing disturbance of E-cadherin and B-catenin distribution patterns in *Giardia* trophozoites exposed Caco-2 cells, with no degradation [32]. It was found that E-64 does not inhibit *Giardia* recombinant CPs activity to 100%, an important observation when studying causal effects of CPs [1,2].

Together, findings from these studies show that *Giardia* CPs directly disrupt some components of the host epithelial barrier, while others may be altered via different, CP-independent mechanisms.

Giardia CPs Induce Epithelial Apoptosis and Villin Breakdown

In addition to effects on tight junctional proteins, CP2 causes other types of host epithelial injury [3]. Interestingly, *cp2* gene silencing reduces overall *Giardia* proteolytic activity and abolishes IEC-

Box 4. CatL-like Proteases in Protozoal Infections

As with catB-like proteases, catL-like proteases regulate the proliferation of protozoan parasites. CatL-like falcipains from *Plasmodium falciparum*, the causative agent of malaria, are critical for the malaria parasite life cycle. Indeed, falcipain-1 is active during the merozoite stage of parasite development, and its deletion or inhibition blocks invasion of host erythrocytes [77]. Falcipain-2 and -3 are involved in degradation of host proteins, including hemoglobin [78]. Inhibition of falcipain-2 also inhibits erythrocyte membrane rupture and subsequent release of merozoites into the bloodstream, and hence prevents the completion of the parasite's life cycle [79].

CatL-like proteases CPA and CPB are key virulence factors for *Leishmania mexicana*. CPA is present in a single copy, but CPB proteases are encoded by a tandem gene array whose complexity varies between *Leishmania* species, perhaps accounting in part for species-specific differences in virulence [80]. CPA and CPB are involved in autophagy, and their deletion results in defects in nutrient acquisition and poor parasite survival in macrophages, as well as failure of parasites to differentiate to the amastigote stage within macrophages [81]. Cathepsins are also involved in host immune modulation. Establishment of *L. mexicana* infection requires suppression of Th1 type immune responses in favor of Th2 type responses. Deletion or inhibition of CPB results in Th1 type responses predominating, resulting in failure of *L. mexicana* to sustain virulence and the development of self-healing lesions [80]. Compared to wild-type *L. mexicana*, CPB-deficient mutants induce only small, slow-growing lesions in BALB/c mice, likely due to defects in both parasite growth and immune modulation. CPB proteases also promote expression of the virulence-associated GPI-anchored metalloprotease GP63. Downregulation of expression in the absence of CPB activity results in impaired macrophage infection [82].

TbCatL from *Trypanosoma brucei* is also important for the establishment of infection and promotes crossing of the blood–brain barrier by trypomastigotes via activation of protease-activated receptor 2 (PAR2) [83]. Although inhibition of catL activity in trypomastigotes could not cure mice of an experimental *T. brucei* infection, survival was significantly improved, and transversal of the blood–brain barrier was reduced [62]. Similarly, catL-like protease activity is a key component of the virulence of the vaginal protozoan parasite *Trichomonas vaginalis*. Indeed, TvCP1-4, TvCP12, and TvCP39 display cytotoxic effects, induce host cell apoptosis and cytoskeleton disruption, degrade host immune factors, and cause hemolysis [16,84]. Interestingly, *T. vaginalis* CP activity is mediated, at least in part, by iron uptake [16].

6 cell damage [3]. It is well established that *Giardia* causes epithelial apoptosis through the activation of caspase-3, -8 and -9 [33,34]. Exposure to recombinant CP2 induces the formation of apoptotic bodies in epithelial cells [3]. Phosphatidylserine is increased upon exposure to CP2, along with a time-dependent expression of caspase-3 and subsequent induction of PARP-1 cleavage [3]. In addition, the use of the CP inhibitor E-64 against CP2 prevented induction of apoptosis in IEC-6 cells, confirming the implication of CPs in *Giardia*-induced cell apoptosis. Furthermore, the severity of *Giardia*-induced cell apoptosis is isolate-dependent (NF, S2, WB, Portland-I), suggesting strain differences of CP activity and overall CP profile [33]. Interestingly, we have previously established a role for protease-activated receptors (PARs) in caspase-3-dependent tight junctional disruption, begging the question of whether proteolytic activation of PARs by *Giardia* CPs may contribute to some of these effects [35].

Villin is an epithelial cytoskeletal protein that regulates the integrity of epithelial brush border microvilli. Upon attachment to IECs, *Giardia* induces a degradation of villin (Figure 1A) [18]. Villin breakdown is caused, at least in part, by parasitic CP activity. In addition, coincubation of *Giardia* trophozoite sonicates and Caco-2 lysates results in villin cleavage [18]. This phenomenon is abolished when trophozoites are preincubated with the CP inhibitor E-64. Moreover, the disruption of villin in IECs is mediated by the activation of myosin light-chain kinase (MLCK) in a CP-dependent fashion [45]. The downstream mechanisms by which *Giardia* CPs can activate MCLK have yet to be elucidated [18]. Interestingly, the cleavage of villin is independent of caspase-3 activation. Finally, a $CD4^+$ and $CD8^+$ T cell-dependent proteolysis of villin has been observed in SCID mice following *Giardia* infection, suggesting that the host adaptive immune system also plays a role in villin cleavage [36].

Giardia CPs Cleave Immune Factors and Are Potent Immunomodulators

CPs have been implicated in parasitic immune evasion as well as in the modulation of intestinal inflammatory responses. CPs of several parasites, including *Giardia*, can degrade host effector molecules and chemokines as well as modulate pro- and anti-inflammatory responses [37–41]. The acute phase of giardiasis is characterized by an absence of overt intestinal inflammation [42]. Host immune responses include a modest recruitment of mast cells and eosinophils in the small intestine, the activation of the complement system via the lectin pathway, dendritic-cell-mediated IL-6 secretion, as well as **nitric oxide** (**NO**) secretion by IEC (iNOS [NOS2]), which contribute to parasite clearance [30,43–45]. Furthermore, clearance of *Giardia* parasites relies on adaptive immune responses, including B cell-mediated immunoglobulin A production at the mucosal level and $CD4^+$ T cell activation; however, the detailed mechanisms by which $CD4^+$ T cells act are still unclear [43,45]. As observed in other protozoan parasites, *Giardia* has developed several immune-evasion strategies, such as antigenic variation via **variant-specific surface proteins** (**VSPs**) as well as an inhibition of NO production by IECs by competing for arginine [46,47]. Recent reports have demonstrated that *Giardia* exerts potent immunomodulatory effects by degrading inflammatory mediators, and by modulating their gene expression. For instance, *Giardia* degrades CXCL8, a proinflammatory cytokine and a major neutrophil attractant, and IL-1β, in *Salmonella thyphimurium*-stimulated epithelial cells (Figure 1C) [48]. Pretreatment of *G. duodenalis* (NF isolate) trophozoites with the broad-spectrum CP inhibitor E-64, and the catB-specific inhibitor Ca074Me, abolished these effects [48]. Interestingly, *Giardia* CPs failed to degrade human IL-1β *in vitro* [48]. Recent reports showed that *Giardia* ESPs may also be implicated in disruption of epithelial AJCs and host immune factor degradation [1,2,19]. A broad array of chemokines and cytokines, commonly observed during *Giardia* attachment to IECs, were tested for their cleavage susceptibility to *Giardia* CPs. Results indicate that CP2, CP3, and CP16160 can cleave or degrade several chemokines such as CXCL1, CXCL2, CXCL8, CCL2, and CCL20, while only CP16160 was able to degrade CXCL3 [2]. In the presence of E-64 CP inhibitor,

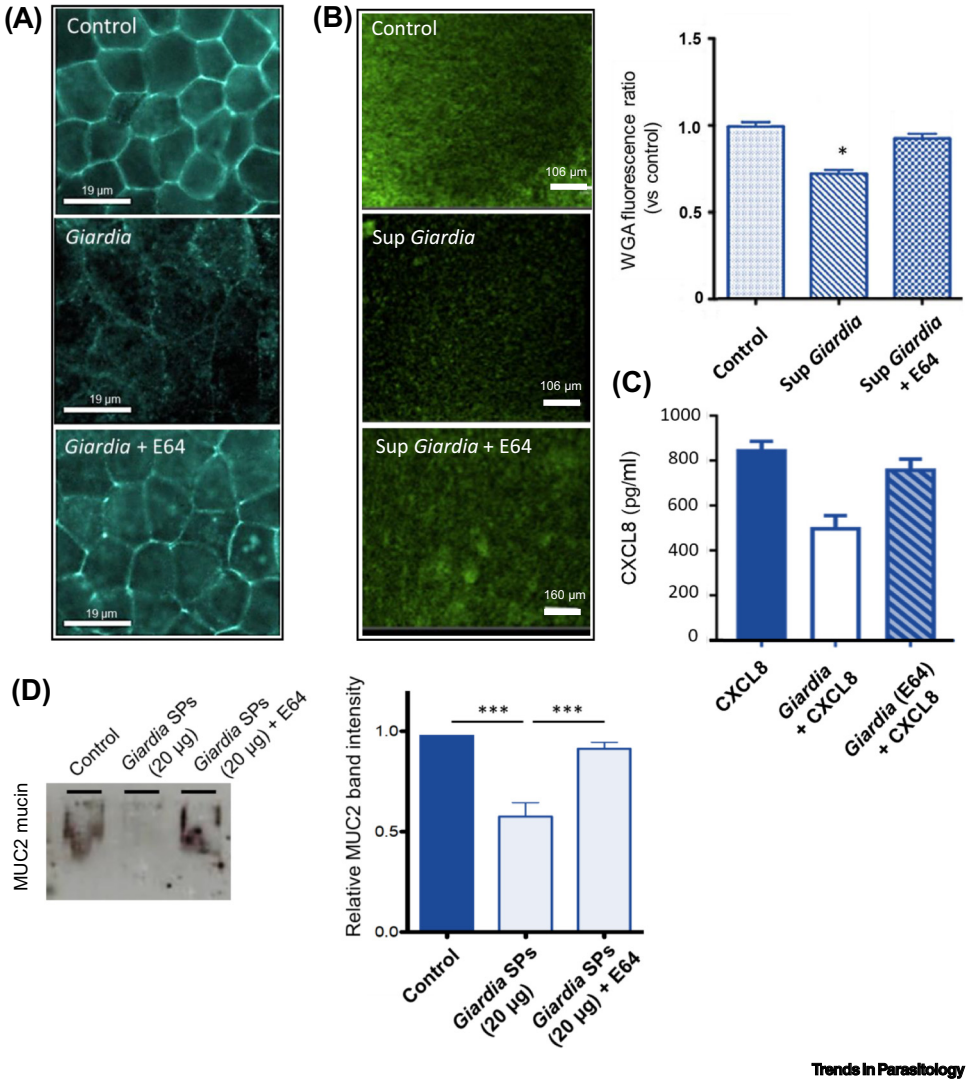

Figure 1. Examples of the Effects of *Giardia* Cysteine Proteases (CPs) on Intestinal Structure and Function.
(A) *G. duodenalis*-induced disruption of epithelial villin, an important element of the structural integrity of brush border microvilli, is prevented when trophozoites are pretreated with a CP inhibitor (E64). Caco-2 cells were exposed to *Giardia* trophozoites, either untreated or pretreated with the CP inhibitor E-64 for 24 h, and cells were stained for immunofluorescence detection of villin (modified from [18]). (B) Degradation of the human gut microbiota biofilm extracellular matrix induced by *G. duodenalis* secretory–excretory products is CP-dependent. Human microbiota biofilms were exposed to *Giardia* spent medium with and without E-64 pretreatment, stained with wheat germ agglutinin (WGA) for the microbiota biofilm extracellular matrix, and prepared for confocal scanning laser microscopy (modified from [17]). (C) Degradation of interleukin-8 (CXCL8) by *G. duodenalis* CPs. Recombinant CXCL8 was added to supernatants collected from Caco-2 cells exposed to *Giardia* trophozoites following pretreatment with E64. CXCL8 levels following coincubation were then measured using ELISA (modified from [47]). (D) Degradation of the major gut mucus component, MUC2 mucin, by *G. duodenalis* secretory–excretory products, is CP-dependent. Purified human MUC2 was exposed to *Giardia* secreted products with or without E-64 CP inhibitor for 3 h and the MUC2 protein level was measured by Western Blot (modified from [56]). Results are mean +/– SEM. *P <0.05, ***P <0.001.

those effects were abolished, thus supporting the hypothesis that *Giardia*-secreted CPs can modulate the host local immune response. Further experiments explored the role of *G. duodenalis* CPs in acting against immunoglobulins and defensins. CP2, CP3, and CP16160 were also able to degrade human IgG, IgA1, and IgA2, which are strongly involved in the host

response to *Giardia* infection, in a dose-dependent manner [1]. Moreover, the role of CPs in cleavage of α- and β-defensins produced by Paneth cells in the small intestine was also investigated. CP2 and CP3 were able to degrade α-HD6 (DEFA6), while CP2, CP16160, and CP3 were able to degrade β-HD1 [1]. In contrast, as indicated below, during coinfections with attaching–effacing enteropathogens, *Giardia* promotes the secretion of β-defensin 2 (HBD-2) and Trefoil factor 3 (TFF3) antimicrobial peptides, and attenuates the disease induced by these pathogens, via mechanisms that remain incompletely understood [80].

Giardia CPs Promote Antimicrobial Peptide (AMP) Secretion in Coinfection with Attaching and Effacing Enteropathogens

Giardia infection often occurs concomitantly with other pathogens, including viral, bacterial, and parasitic organisms, particularly in countries with poor sanitation [49]. Several reports indicate/suggest that young individuals infected by *Giardia* exhibit attenuated enteropathogen-associated symptoms, such as diarrhea and inflammation, suggesting that *Giardia* may play a protective role during at least some **polymicrobial infections** [50–52]. In an *in vivo* model mimicking polymicrobial infection with **attaching and effacing** (**A/E**) **pathogens**, *Giardia muris* attenuates the pathogenesis of *Citrobacter rodentium*-induced colitis, and inhibits weight loss, bacterial translocation, and micro- and macroscopic damage, while enhancing the production of AMPs [53]. Increased expression of AMPs, such as human β-defensin 2 (HBD2) and Trefoil factor 3 (TFF3) in Caco-2 cells during coinfection, is catB-like protease-dependent [53]. In addition, *G. muris* and *G. duodenalis* display direct antibacterial effects against *C. rodentium* and *Escherichia coli* EPEC, respectively [53]. The effects were abolished when *Giardia* trophozoites were pretreated with broad-spectrum CP and catB-like protease inhibitors [53]. These effects may be key to the observations that *Giardia* appears to protect against diarrheal disease in low-income parts of the world where such infections with attaching–effacing enteropathogens are common.

The Intestinal Mucus Barrier Is Altered by *Giardia* CP Activity

The intestinal mucus layers are critical for host innate immunity [54], and their disruption is associated with increased rates of infection and inflammation, and may facilitate parasite attachment to the underlying epithelium [56].

In a variety of parasitic infections, including giardiasis, overcoming the mucus barrier is a prerequisite for establishment of infection. *Giardia* may achieve this through proteolytic degradation of mucus, and by disruption of mucus secretion by intestinal goblet cells. Although *Giardia* exclusively colonizes the upper small intestine, *Giardia* infection is associated with thinning of the colonic mucus layer, implicating contact-independent mechanisms in *Giardia*-induced mucus disruption [55]. *Giardia* CPs can cleave human MUC2, the major secretory mucin in the large and small intestines, which may promote dissolution of the mucus gel by disrupting polymerization (Figure 1D). Additionally, *Giardia* CPs alter mucin gene expression in the mouse jejunum and colon, and in human colonic epithelial cell cultures [55]. These effects were found to be isolate-dependent, with the Assemblage A isolates *Giardia* NF, S2, and WB, but not the Assemblage B isolate GSM inducing increased MUC2 mucin gene expression [55,56]. *Giardia* trophozoites can also induce CP-dependent mucin hypersecretion in human colonic epithelial cells and human colon biopsies, resulting in depletion of intracellular mucin stores [55]. Effects on mucus secretion, unlike those on gene expression, were found to be, in part, dependent on catB activity. Because catB activity does not account for all CP-dependent effects on mucus-producing cells, other CPs, such as catL, may be implicated [55].

Giardia CPs Directly Alter Microbiota Biofilms to Release Pathobionts

In healthy individuals, the gut microbiome consists of mucosal multispecies biofilms, which, when disrupted by enteropathogens or inflammation, may affect gut homeostasis and lead to

Key Figure

Roles of *Giardia* Cysteine Proteases (CPs) in Host–Microbial Interactions

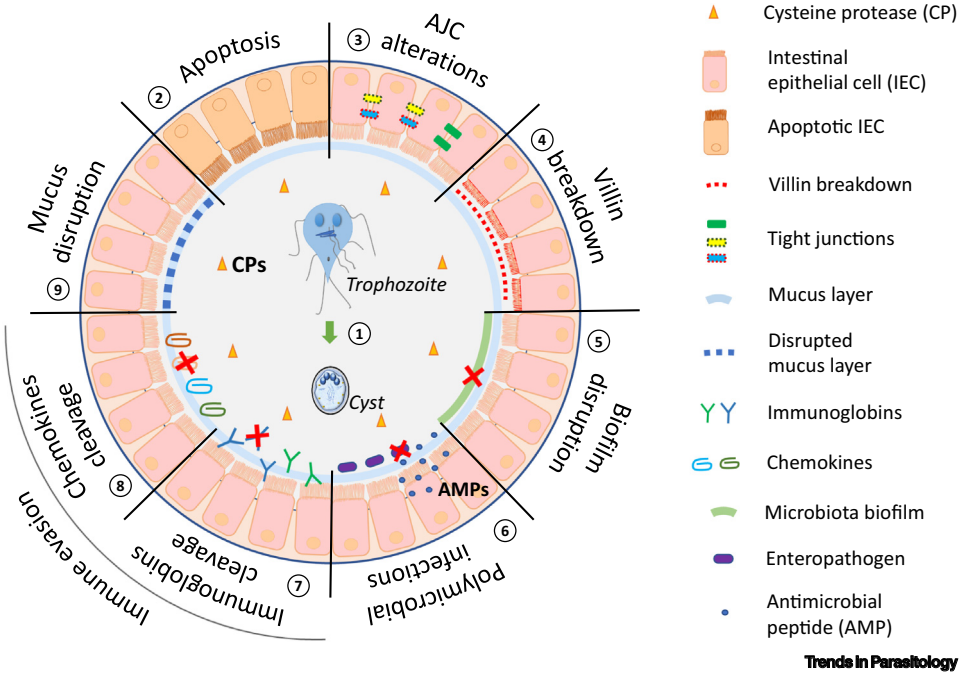

Figure 2. (1) CPs play a role in the process of encystation and trophozoite adhesion. (2) CPs induce cell apoptosis with caspase-3 activation and induction of PARP-1 cleavage. (3) CPs disrupt the epithelial barrier by disrupting apical junctional complexes (AJCs) and increasing permeability. (4) CPs alter epithelial brush border integrity through MCLK-dependent breakdown of intestinal epithelial cell (IEC) villin. (5) *Giardia* CP activity disrupts commensal microbiota biofilms and promotes the release of pathobionts. (6) *Giardia* CPs exert protective effects during concomitant infections via direct antibacterial effects and stimulation of antimicrobial peptides (AMPs). (7,8) *Giardia* CPs induce immunomodulatory effects by degrading host chemokines (CXCL1, CXCL2, CXCL3, CXCL8, CCL2, and CCL20), immunoglobins (IgG, IgA1, and IgA2), and defensins (α-HD6, β-HD1, and TFF3). (9) *Giardia* CPs alter mucus secretion and degrade mucin proteins to disrupt their polymerization.

disease [57]. These mucosal-associated biofilms produce an ECM composed of polysaccharide proteins and DNA which facilitates their adhesion to the mucosa. Recent studies showed that *Giardia* breaks down this ECM and promotes the release of planktonic **pathobionts** (Figure 1B) [17]. The CP activity of *Giardia* trophozoites was, at least in part, responsible for these effects [17]. Indeed, upon exposure to *Giardia* and *Giardia* ESPs, commensal bacteria are dispersed from their mucosal biofilm communities and acquire virulence factors enabling them to translocate across IEC monolayers, disrupt tight junctional ZO-1, and trigger increased chemokine CXCL8 and TLR4 expression in Caco-2, all in a CP-dependent fashion. Hence, *Giardia* CP activity strongly contributes to the release of these newly formed pathobionts [17]. Interestingly, a selective catB inhibitor (Ca-074Me) failed to abolish those effects, suggesting that other CPs might be involved.

Concluding Remarks

In the past 5 years, an ever-increasing number of studies have investigated the role of *Giardia* CPs in the biology of this parasite, as well as in its interactions with the host, as summarized in Figure 2

Outstanding Questions

How would deletion of *Giardia* CPs via CRISPR/cas9 on *Giardia* affect parasite virulence and survival *in vitro* and *in vivo*?

Would host responses be different if infection occurred during infection with *Giardia* CP knockouts?

Are *Giardia* CPs a target for therapeutic intervention? Will chemotherapeutic inhibition of *Giardia* CP activity reduce symptom severity and/or parasite burden during acute and chronic giardiasis?

Specific roles have been identified for *Giardia* CPs in *Giardia* pathogenesis. However, selective catB inhibitors only abolish a subset of these protease-dependent effects in experimental models. What are the roles of catL- and catC-like proteases in *Giardia* pathophysiology?

Protease-activated receptors (PARs) are implicated in a variety of pathophysiological processes (mucus hypersecretion, barrier dysfunction, disrupted gene expression etc.). Does proteolytic activation of PARs by *Giardia* CPs play a role in *Giardia* pathophysiology?

How do host and/or parasite protease inhibitors, such as serpins and cystatins, modulate *Giardia* CP activity and virulence?

Could *in vivo* administration of recombinant *Giardia* CPs mimic symptoms of acute giardiasis and chronic postinfectious complications? Conversely, as *Giardia* CPs are implicated in immunomodulation, could these recombinant CPs exert anti-inflammatory properties?

(Key Figure). Together, the findings show that *Giardia* CPs, and more specifically catB-like protease activity, are critical for *Giardia* pathogenesis, presenting *Giardia* CPs as potentially important targets for therapeutic intervention. In a variety of protozoal infections, including those caused by *Trypanosoma* sp. and *Entamoeba* sp., inhibition of protease activity reduces disease severity. Similar approaches in giardiasis provide an important topic for future investigation (see Outstanding Questions). In addition to contributing to acute symptoms in giardiasis, CP-mediated effects may contribute to chronic postinfectious complications, such as postinfectious irritable bowel syndrome. Further study of how *Giardia* CPs may contribute to long-term disruption of gut homeostasis is warranted. The role of PARs in the pathophysiology of giardiasis represents an important topic for future research. Future experiments utilizing *Giardia* CP knockouts will also be important for further characterization of the role of *Giardia* CPs in host–parasite interactions. The role of host- and *Giardia*-derived CP inhibitors, such as cystatins and serpins, will also be of great interest. Similarly, the use of recombinant *Giardia* CPs will help to clarify the roles of CPs in *Giardia* virulence and pathogenicity. Further investigation should also clarify the importance of *Giardia* catL- and catC-like protease activity. Finally, we hypothesize that parts of the deleterious effects of *Giardia* CPs may in fact represent collateral damage as CP activity is dramatically increased during the trophozoite encystation process.

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