Parasitology Research (2019) 118:3377–3386
https://doi.org/10.1007/s00436-019-06487-4

HELMINTHOLOGY - ORIGINAL PAPER

Secreted cathepsin L-like peptidases are involved in the degradation of trapped antibodies on the surface of *Echinostoma caproni*

Alba Cortés[1] · Libor Mikeš[2] · Carla Muñoz-Antolí[1] · María Álvarez-Izquierdo[1] · J. Guillermo Esteban[1] · Petr Horák[2] · Rafael Toledo[1]

Received: 25 June 2019 / Accepted: 25 September 2019 / Published online: 12 November 2019

Abstract

Antibody trapping is a recently described strategy for immune evasion observed in the intestinal trematode *Echinostoma caproni*, which may aid to avoiding the host humoral response, thus facilitating parasite survival in the presence of high levels of local-specific antibodies. Parasite-derived peptidases carry out the degradation of trapped antibodies, being essential for this mechanism. Herein, we show that cathepsin-like cysteine endopeptidases are active in the excretory/secretory products (ESPs) of *E. caproni* and play an important role in the context of antibody trapping. Cysteine endopeptidase activity was detected in the ESPs of *E. caproni* adults. The affinity probe DCG-04 distinguished a cysteine peptidase band in ESPs, which was specifically recognized by an anti-cathepsin L heterologous antibody. The same antibody localized this protein in the gut and syncytial tegument of adult worms. Studies with cultured parasites showed that in vivo-bound antibodies are removed from the parasite surface in the absence of peptidase inhibitors, while addition of cathepsin L inhibitor prevented their degradation. These results indicate that cathepsin L-like peptidases are involved in the degradation of surface-trapped antibodies and suggest that cysteine peptidases are not only crucial for tissue-invading trematodes, but they can be equally relevant at the parasite-host interface in gut-dwelling flukes.

Keywords Echinostoma caproni · Trematode · Cathepsin · Cysteine peptidase · Antibody · Immune evasion

Introduction

Around 100 different trematode species are able to parasitize humans, colonizing several niches inside the human body, such as blood, liver, lungs, and intestine. In spite of many differences in biology and epidemiology, parasitic trematodes share several similarities, e.g., they normally cause long-lasting infections in the definitive host. Although pharmacological treatment is generally effective, infected people are typically concentrated in areas of high endemicity where reinfections are common, leading to accumulative lesions in target organs and development of chronic pathology (Sripa et al. 2010; Colley et al. 2014). In this scenario, there is a necessity for developing new interventions against these parasitic infections (Sripa et al. 2010; Colley et al. 2014). For this purpose, a better understanding of parasite biology and host-parasite relationships is required, and the use of suitable experimental models proved to be a valuable strategy (Harris 2011).

Echinostoma caproni (Trematoda: Echinostomatidae) is an intestinal trematode without tissue migration in the definitive host, which has been broadly employed as a model for studies on host-parasite relationships (Toledo and Fried 2005). In the duodenum, metacercariae excyst and juvenile worms migrate to the ileum, where they attach to the intestinal mucosa by the ventral sucker (Toledo et al. 2009). By using the *E. caproni*-mouse system, we have recently described a mechanism employed by this trematode that may assist in the evasion of

Section Editor: Christoph G. Grevelding

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00436-019-06487-4) contains supplementary material, which is available to authorized users.

✉ Rafael Toledo
rafael.toledo@uv.es

[1] Área de Parasitología, Departamento de Farmacia y Tecnología Farmacéutica y Parasitología, Facultad de Farmacia, Universitat de València, Avda. Vicent Andrés Estellés s/n, 46100 Burjassot, Valencia, Spain

[2] Department of Parasitology, Faculty of Science, Charles University, Viničná 7, 128 44 Prague 2, Czech Republic

the antibody-mediated immunity. This mechanism consists in the entrapment of surface-bound antibodies within a layer of excretory/secretory products (ESPs) and subsequent degradation of trapped antibodies by parasite-derived peptidases (Cortés et al. 2017).

Cysteine cathepsins are papain-like peptidases used by helminth parasites in crucial interactions with the host and are essential for parasite viability and growth (Robinson et al. 2008a), which makes them key targets for developing new anthelmintic drugs, vaccines, and diagnostic tools (reviewed by Sajid and McKerrow 2002). Available knowledge of trematode cathepsins comes from studies on species displaying (at least partial) tissue migration in the definitive host, such as blood flukes (*Schistosoma* spp.), liver flukes (*Clonorchis* spp., *Opisthorchis* spp., and *Fasciola* spp.), and lung flukes of the genus *Paragonimus* (Stack et al. 2011). Conversely, these peptidases in intestinal trematodes have been generally overlooked. The aim of the present study was to carry out the first indication that *E. caproni* cathepsins play a role in the evasion of host immunity via the degradation of trapped antibodies.

Materials and methods

Animals and infection procedures

Five male CD1 mice were infected with 75 metacercariae of *E. caproni* by gastric gavage. The strain of *E. caproni* and the infection procedure have been described previously (Fujino and Fried 1993). Briefly, encysted metacercariae of *E. caproni* were removed from kidneys and pericardial cavity of experimentally infected *Biomphalaria glabrata* snails and used for infection. All animals were necropsied at 4 weeks post-infection and the parasites were employed for immunofluorescence, immunohistochemistry, and to obtain ESPs. Five additional mice were infected as described above and employed to examine whether cathepsin B and/or L were responsible for degradation of trapped antibodies. Mice were maintained under conventional conditions with food and water ad libitum. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals. In particular, this study has been approved by the Ethical Committee on Animal Welfare and Experimentation of the Universitat de València (Ref No. A18348501775). Protocols adhered to Spanish (Real Decreto 53/2013) and European (2010/63/UE) regulations.

Collection of ESPs

E. caproni ESPs were obtained by incubation of the worms in RPMI GlutaMAX (Gibco, Life Technologies) culture medium. Adult flukes were removed from the intestines of infected mice, carefully washed in PBS, and maintained at concentrations of 20 worms/ml for 12 h at 37 °C in culture medium containing 100 U penicillin and 100 mg/ml streptomycin (Cortés et al. 2016). After incubation, the medium was collected and clarified by centrifugation at 15,000×*g* for 30 min at 4 °C. ESPs were lyophilized in the Hetosicc CD 4 lyophilizer at − 52 ± 3 °C and 30 mBa pressure until complete drying (24 h) and stored at − 20 °C. Before use, lyophilized ESPs were rehydrated in distilled water and filtered two times through an Amicon Ultra-4 membrane (cut-off 10 kDa) by centrifugation at 7500×*g* for 10 min at 4 °C. Finally, ESPs were washed in 25 mM/50 mM citrate/phosphate buffer (CPB, pH 5.5) and centrifuged under the conditions described above. Protein concentration was measured using Quant-iT Protein Assay Kit (Invitrogen).

Fluorometric assay of cathepsin activity in ESPs

Cathepsin L and B activities were monitored in the presence of the fluorogenic (aminomethylcoumarin (AMC)) peptide substrate Z-Phe-Arg-AMC (FR, Bachem) in a kinetic cycle (Kašný et al. 2007). Assays were carried out in duplicates in 96-well flat bottom black plates (Nunc) at excitation and emission wavelengths of 355 and 460 nm, respectively (Infinite M200 fluorometer, Tecan). Each well contained 5 μg of *E. caproni* ESPs, 50 μM Z-FR-AMC, and 2 mM DTT in CPB. The specificity of peptidase activity was investigated by preincubation of ESPs with either iCL or CA-074 at 10 μM concentrations.

SDS-PAGE and ligand blotting with DCG-04

Blotting with biotinylated DCG-04 ligand (10) was performed as described by Mikeš and Man (2003), with some modifications. Fifteen micrograms of *E. caproni* ESPs were incubated for 20 min at room temperature (RT) with 5 μM DCG-04 in CPB containing 1 mM DTT. To confirm binding specificity, a negative control was prepared by preincubating 15 μg of ESPs with 50 μM E-64 for 10 min before the addition of DCG-04. Samples were electrophoresed under denaturating and reducing conditions (SDS-PAGE) in 10% polyacrylamide precast gels using MiniProtean 3 apparatus (Bio-Rad). Proteins were electrotransferred from onto a PVDF membrane in the Trans-Blot Turbo Transfer Starter System (Bio-Rad) for 12 min at 1.3 A. Strips of the membrane were blocked in 5% skimmed milk dissolved in 20 mM Tris-buffered saline containing 0.05% Tween-20 (TBS-T) for 1 h. Then the membrane was washed three times in TBS-T, incubated in 2.5 μg/ml streptavidin-horseradish peroxidase (HRP) (Sigma-Aldrich) in TBS-T for 1 h, and washed again before being developed using the Opti-4CN Substrate Kit (Bio-Rad).

Western blot with cathepsin L antibodies

For western blotting, 15 μg of ESPs were loaded per well of 10% polyacrylamide precast gel, electrophoresed, and then transferred to PVDF membrane as described above. After blocking in 5% milk in TBS-T, membrane strips were incubated with three heterologous antibodies for 1 h, each diluted to 1:100 in TBS-T. The antibodies were: (i) mouse polyclonal anti-cathepsin L of *Diplostomun pseudospathaceum* (produced by the Prague group) (Mikeš and Man 2003); (ii) rat polyclonal anti-cathepsin L of *Fascioloides magna* (kindly provided by Dr. Martin Kašný, Charles University and Masaryk University) (unpublished); and (iii) sheep polyclonal anti-cathepsin L of *Fasciola hepatica* (kindly provided by Prof. John P. Dalton, Queen's University Belfast) (Piacenza et al. 1999). Negative controls were prepared similarly, using sera of non-immunized mouse, rat, and sheep. After washing in TBS-T, the membranes were incubated for 1 h with appropriate HRP-conjugated secondary antibodies (i.e., rabbit anti-mouse IgG (Sigma-Aldrich), goat anti-rat IgG (Alpha Diagnostic Intl. Inc.), and donkey anti-sheep IgG (Sigma-Aldrich)) diluted 1:1000 in TBS-T. All incubations were performed at RT under gentle agitation. After final washes, the membranes were developed using the Opti-4CN Substrate Kit (Bio-Rad).

Immunohistochemical localization of cathepsin L

Paraformaldehyde-fixed adults were embedded in paraffin and cut into 4 μm sections. Parasite sections were deparaffinized and rehydrated before blocking with 5% BSA in TBS-T at RT for 1 h 30 min. Sheep polyclonal anti-cathepsin L of *F. hepatica*, diluted 1:50 in TBS, and was applied for 1 h 30 min as a primary antibody. Sections were washed three times with TBS before incubating with donkey anti-sheep IgG-FITC (Sigma-Aldrich) diluted 1:200 in TBS, for 1 h at RT in the dark. Finally, sections were washed in TBS and mounted for visualization in an Olympus BX51 fluorescent microscope. Controls with non-immune sera were employed to set image acquisition parameters.

Incubation of parasites with peptidase inhibitors

To study the participation of cysteine peptidases in the degradation of surface-bound antibodies, two *E. caproni* adults collected from the ileum of each infected mouse were incubated in vitro, in either the presence or absence of selected peptidase inhibitors. Incubations lasted for 3 h at 37 °C in RPMI GlutaMAX culture medium (Gibco, Life Technologies), supplemented with 100 U penicillin, 100 mg/ml streptomycin, and one of the following peptidase inhibitors: (i) E-64 [*N*-trans-(epoxysuccinyl)-L-leucine 4-guanidinobutylamide], an irreversible broad-spectrum inhibitor of cysteine peptidases; (ii) iCL (Arg-Lys-Leu-Leu-Trp-NH2), a reversible inhibitor of cathepsin L; (iii) CA-074 [*N*-(L-3-*trans*-propylcarbamoyloxirane-2-carbonyl)-Ile-Pro-OH], an irreversible inhibitor of cathepsin B; or (iv) the peptidase inhibitor cocktail (PIC) cOmplete-mini, EDTA-free (Roche). E-64, iCL, and CA-074 (all from Sigma-Aldrich) were applied at concentrations of 100 μM, while PIC was diluted according to manufacturer's instructions. Additionally, two worms from each mouse were incubated for 3 h in an inhibitor-free medium, and another two were fixed right after collection from the intestine (i.e., non-incubated) and used as baseline controls for staining. The parasites were fixed by immersion in 4% paraformaldehyde and analyzed by double indirect immunofluorescence. This experiment was repeated twice; first using E-64 and PIC, and second employing all individual inhibitors described above.

Determination of in vitro activity of cathepsins against mouse immunoglobulins

To obtain direct evidence on the ability of ESP and cathepsins to degrade mouse antibodies, immunoglobulins from naïve mice were purified from sera using Nab Protein L Aspin Kit (Thermo Scientific) according to the manufacturer's protocol. Eluted immunoglobulins were then incubated with ESP (ESP: Ig 25 μg/15 μg) in absence or presence of each of 100 μM of the peptidase inhibitors (E-64, iCL, CA-074, and PIC) at 37° for 3 h. Thereafter, the products were subjected to SDS-PAGE and western blotting as described above. After washing in TBS-T, the membranes were incubated for 1 h with appropriate HRP-conjugated secondary antibodies (rabbit anti-mouse IgG (Sigma-Aldrich) and goat anti-mouse IgA (Nordic Mubio)). All incubations were performed at RT under gentle agitation. After final washes, the membranes were developed using the Opti-4CN Substrate Kit (Bio-Rad).

Double indirect immunofluorescence

Double immunofluorescence was performed on non-permeabilized worms as described by Cortés et al. (2017). In brief, paraformaldehyde-fixed adults were blocked in 5% BSA for 1 h before incubating them for 1 h 30 min in a mixture of two primary antibodies: (i) rabbit sera against *E. caproni*-ESPs (Marcilla et al. 2012) and (ii) goat anti-mouse IgG (Nordic) or goat anti-mouse IgA (Jackson ImmunoResearch) both conjugated with HRP and diluted 1:50 in PBS. After gentle washing, adults were incubated for an additional hour with two secondary antibodies simultaneously: (i) goat anti-rabbit IgG or goat anti-rabbit IgA conjugated with Alexa Fluor 647 and (ii) goat anti-HRP conjugated with FITC (all from Jackson ImmunoResearch), each diluted 1:250 in PBS. Finally, adults were gently washed in PBS before analysis. Negative controls were processed

similarly, excluding the incubation with primary antibodies, and were used to set image acquisition parameters. Fluorescent staining was visualized by laser scanning confocal microscopy on ten specimens (two from each mouse) in each experimental group. Images were analysed using FV10-ASW 4.2 and Imaris software.

Degradation of surface-bound antibodies during in vitro culture was quantified using ImageJ software to calculate the percentage of image area covered by each fluorescent tag, i.e., FITC or Alexa Fluor 647 (Cortés et al. 2017). Confocal micrographs (× 400) were stacked to create Z projections that were converted into binary (black and white) images. Raw integrated density (RawIntDen), which is the sum of the values of all pixels in the image, was measured and used to calculate the percentage of area covered by the fluorescent tag (% AC) according to the following formula, in which 255 is the density value of a positive (tagged) pixel in the binary image and areas are expressed in pixels:

$$\%\mathrm{AC} = \frac{\mathrm{RawIntDen}/255}{\mathrm{Total\ area}} \times 100$$

Statistical analysis

Shapiro-Wilk test was applied to test normality of % AC data sets and statistical differences were assessed either by one-way ANOVA or Kruskal-Wallis tests depending on whether the data followed normal or non-normal distributions, respectively. Bonferroni's and Dunn's multiple comparison tests were applied as post hoc analyses, and statistical significance was considered when $p < 0.05$.

Results

E. caproni ESPs display cysteine peptidase activity

Cathepsin L and B activities in *E. caproni* ESPs were assayed with the fluorogenic peptide substrate Z-FR-AMC in a pH range between 4.0 and 6.0. Comparison of the speed of hydrolysis of Z-FR-AMC at different pH values within the linear range of the reaction (46 min) is shown in Fig. 1a. The highest enzymatic activity was reached at pH 5.0 (Fig. 1a). Inhibition assays confirmed the specificity of the enzymatic activity and showed that cathepsin L activity was higher than that of cathepsin B (Fig. 1). Cathepsin L inhibitor caused over 98% inhibition of the peptidase activity at every pH assayed. CA-074 also caused effective inhibition, over 91%, although with this inhibitor the percentage of inhibition of the peptidase activity decreased progressively as the pH of the medium was increased (Fig. 1b).

Detection of cathepsin L-like peptidases in *E. caproni* ESPs

In order to detect cysteine cathepsin-like peptidases in the ESPs of *E. caproni* adults, the biotinylated probe DCG-04 was employed, which binds covalently to the active site of these enzymes (Greenbaum et al. 2000). Blotting with DCG-04 distinguished a single protein band of ≈ 28 kDa in the electrophoresed ESPs, while the E-64-preincubated control did not show any reaction (Fig. 2a). The same band was specifically recognized on western blot using a heterologous antibody against cathepsin L of *F. hepatica* (Fig. 2b) but not by antibodies prepared against cathepsins L of other trematode species, such as *D. pseudospathaceum* and *F. magna* (data not shown). This could be related to a greater similarity between the proteins of *F. hepatica* and *E. caproni* and the epitopes recognized by the antibody.

Immunolocalization of cathepsin L-like peptidase in *E. caproni* adults

The anti-*F. hepatica* cathepsin L antibody was employed to localize cathepsin L-like peptidase in the tissues of adult *E. caproni*. Specific staining was detected in the gut epithelium and syncytial tegument of the worms with a significant weaker staining in the surrounding tissues (Fig. 3; Online resource 1).

Cathepsin L-like peptidases of *E. caproni* ESPs are able to in vitro degrade mouse immunoglobulins

To determine the ability of *E. caproni* ESPs to in vitro degrade antibodies, purified mouse immunoglobulins were incubated with ESP. The results obtained show that both mouse IgG and IgA became degraded in the presence of *E. caproni* ESPs (Fig. 4). To evaluate whether cathepsin L and/or B presents in the ESPs of *E. caproni* were implicated in the in vitro degradation of immunoglobulins, mouse immunoglobulins were incubated with ESPs in the presence of broad spectrum or specific protease inhibitors (Fig. 4). Significant degradation only was observed in the presence of the inhibitor of cathepsin B (CA-074), suggesting that mouse immunoglobulins were mainly degraded by cathepsin L-like peptidases.

Cathepsin L-like peptidases are involved in the in vivo degradation of ESPs-trapped on worm surface

In order to investigate whether cysteine peptidases have a role in the degradation of surface-bound antibodies, *E. caproni* adults were incubated in culture medium supplemented with E-64. During the incubation periods, adult worms did not seem affected by the inhibitors maintaining their normal movement. During culture, in vivo-bound surface antibodies

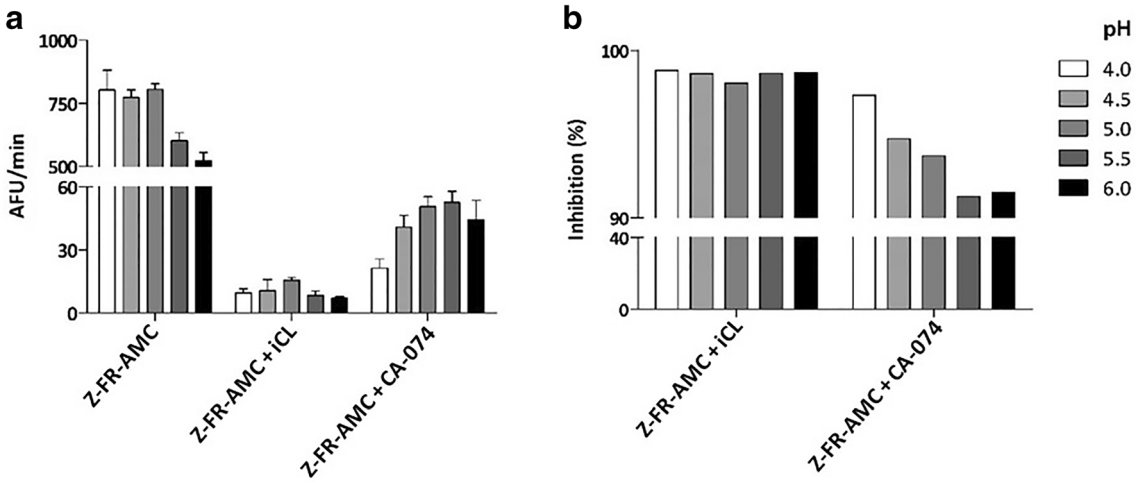

Fig. 1 Cysteine peptidase activity in the excretory/secretory products of *Echinostoma caproni*. **a** pH profile of cysteine peptidase activity against fluorigenic substrate Z-FR-AMC, expressed as arbitrary fluorescence units (AFU) released per minute within the linear range of the reaction. **b** Percentage of inhibition of cathepsin activity by cathepsin L and cathepsin B selective inhibitors (iCL and CA-074, respectively)

became entrapped within a layer of ESPs that covered the parasite (Online resource 2). Whilst in the absence of peptidase inhibitors most of surface-bound antibodies disappeared following 3 h culture, addition of either E-64 or PIC to the culture medium prevented the degradation of surface-trapped IgG, thus confirming that ESPs-entrapped antibodies are degraded by parasite-derived peptidases and, in particular, by cysteine peptidases (Online resources 2 and 3).

In order to determine whether cathepsin L and/or B were specifically involved in the degradation of the ESPs-trapped IgG, *E. caproni* adults were cultured in the presence of either broad-spectrum or specific inhibitors. In accordance to our first experiment (Online resource 3), a significant decrease in anti-IgG staining were observed on the surface of worms cultured for 3 h in the absence of peptidase inhibitors but not on worms incubated with broad-spectrum inhibitors, i.e., PIC or E-64. Moreover, the addition of iCL to the culture medium prevented loss of surface-bound antibodies in vitro. On the other hand, the signal of in vivo-bound IgG were significantly decreased in the presence of CA-074, and the percentage of area covered by the anti-IgG antibody following 3 h culture was similar to that in worms incubated without peptidase inhibitors (Fig. 5). This experiment was also performed using anti-mouse IgA to determine the in vivo activity of cathepsins against mouse IgA. The results were almost identical than those obtained for IgG (Online resource 4).

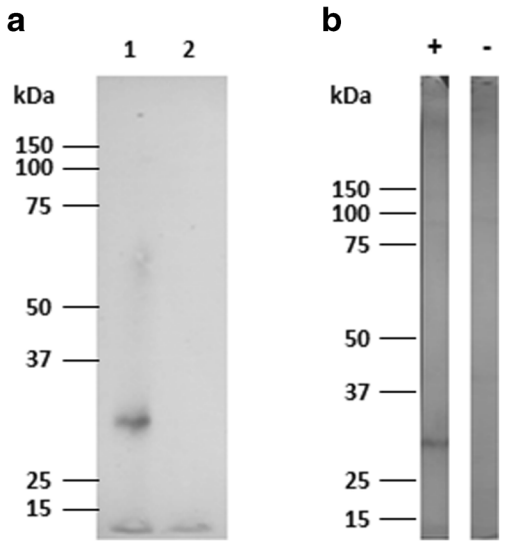

Fig. 2 Detection of *Echinostoma caproni* cathepsin L-like peptidases in excretory/secretory products (ESPs). **a** Blotting with cysteine peptidase specific probe DCG-04 (1), and specificity control using E-64 pretreated ESPs (2). **b** Western blot detection of cathepsin L-like by anti-*Fasciola hepatica* cathepsin L antibody (+) and sheep control serum (−)

Discussion

Parasitic organisms have developed a number of strategies to manipulate and evade the host immune system, thereby increasing their survival and transmission (Schmid-Hempel 2008). The intestinal trematode *E. caproni* is well adapted to establish and grow in an environment with high levels of locally produced IgG and IgA (Toledo et al. 2005; Sotillo et al. 2007). Through studies with this trematode, our group has recently described a novel mechanism of parasite immune evasion, which may serve to minimize the deleterious effects of the antibody-mediated attack (Cortés et al. 2017). This evasion mechanism, which we named antibody trapping, consists in continuous entrapment of surface-bound antibodies by the constantly released ESPs, thus enabling antibody degradation by parasite-derived peptidases (Cortés et al. 2017).

Pathogen-derived peptidases are well-known virulence factors that play key roles in the colonization of host niches and the pathogenesis of infections (reviewed by Robinson et al. (2008a)). In particular, cysteine peptidases are highly

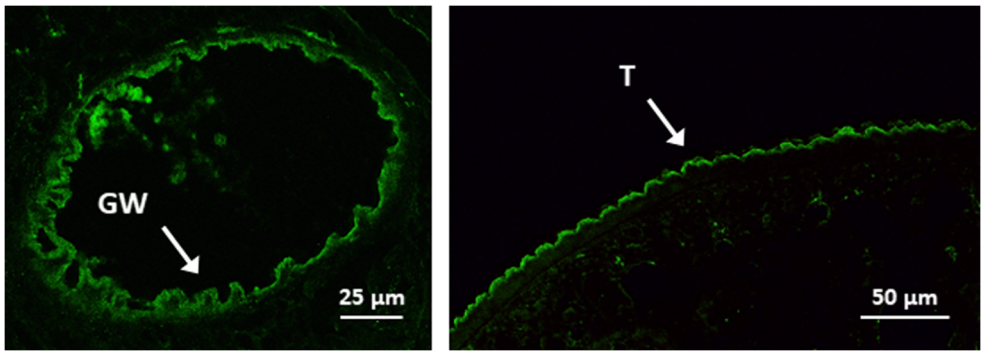

Fig. 3 Immunohistochemical localization of cathepsin L-like in *Echinostoma caproni* adult worms. GW, gut wall; T, tegument

represented in the transcriptome of trematode parasites, such as *F. hepatica* (McVeigh et al. 2012), *F. magna* (Cantacessi et al. 2012), *Clonorchis sinensis* (Yoo et al. 2011), or *Paragonimus westermani* (Kim et al. 2006). In *E. caproni*, the peptidase protein domain C1A was found among the ten most represented domains in the putative proteins annotated in a transcriptomic analysis of the adult stage (Garg et al. 2013). Clan CA, family C1 of the papain superfamily (CA1 peptidases) includes cathepsins B, C, F, and L, which have great relevance in the relationships between trematode parasites and their hosts (Stack et al. 2011). In addition to the evidence at mRNA level (Garg et al. 2013), the presence of cysteine cathepsin-like peptidases has been previously reported in the ESPs of *E. caproni* (Cortés et al. 2016). Herein, however, we show for the first time that both cathepsins L and B are active in these ESPs and provide evidence of a functional role for cathepsin L-like on worm surface.

E. caproni ESPs were tested for their activity against Z-FR-AMC, a peptide substrate commonly used to detect cathepsins L and B. A substantial reduction in peptidase activity was observed upon addition of either iCL or CA-074 inhibitors to the reaction medium, although iCL displayed greater inhibitory efficacy at every pH assayed. This result indicates that cathepsin L-like peptidase(s) in the ESPs of adult *E. caproni* work over a broader range of pH than cathepsin(s) B-like. However, it should be taken into consideration that, even though CA-074 is described as a selective inhibitor of cathepsin B, it has been reported to reduce the activity of cathepsins L under reducing conditions (Steverding 2011; Steverding et al. 2012; Jedličková et al. 2018). Therefore, we cannot rule out the possibility that the inhibitory effect observed for CA-074 on the peptidase activity of ESPs was due to the inactivation of cathepsin L. Indeed, blotting with DCG-04 only distinguished a single band in the ESPs, which was specifically recognized by an anti-cathepsin L antibody, thus supporting the notion that cathepsins of L-type are likely the most abundant cysteine endopeptidases in this parasite material.

Localization of cathepsin L-like peptidase in the gut wall and the syncytial tegument of *E. caproni* adults are consistent with previous observations in other trematode species (Collins et al. 2004; Na et al. 2006, 2008; Kang et al. 2010). In particular, the presence of cathepsin L-like peptidase(s) in the tegument and ESPs of *E. caproni* suggests that they can be

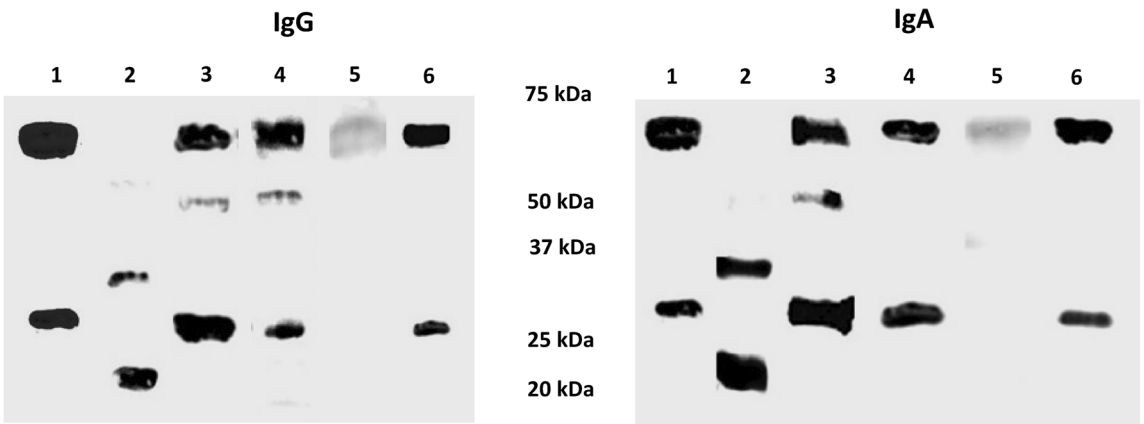

Fig. 4 Western blot determination of the in vitro effect of excretory secretory products (ESPs) of *Echinostoma caproni* and specific peptidase inhibitors on the degradation of mouse IgG (**a**) and IgA (**b**) before incubation (lanes 1) and after 3 h of incubation with ESPs (lanes 2) and ESPs in the presence of protease inhibitors: peptidase inhibitor cocktail (PIC; cOmplete-Mini, EDTA-free) (lanes 3); irreversible, broad-spectrum, cysteine peptidase inhibitor (E-64) (lanes 4); cathepsin B selective inhibitor (CA-074) (lanes 5); and cathepsin L selective inhibitor (iCL) (lanes 6)

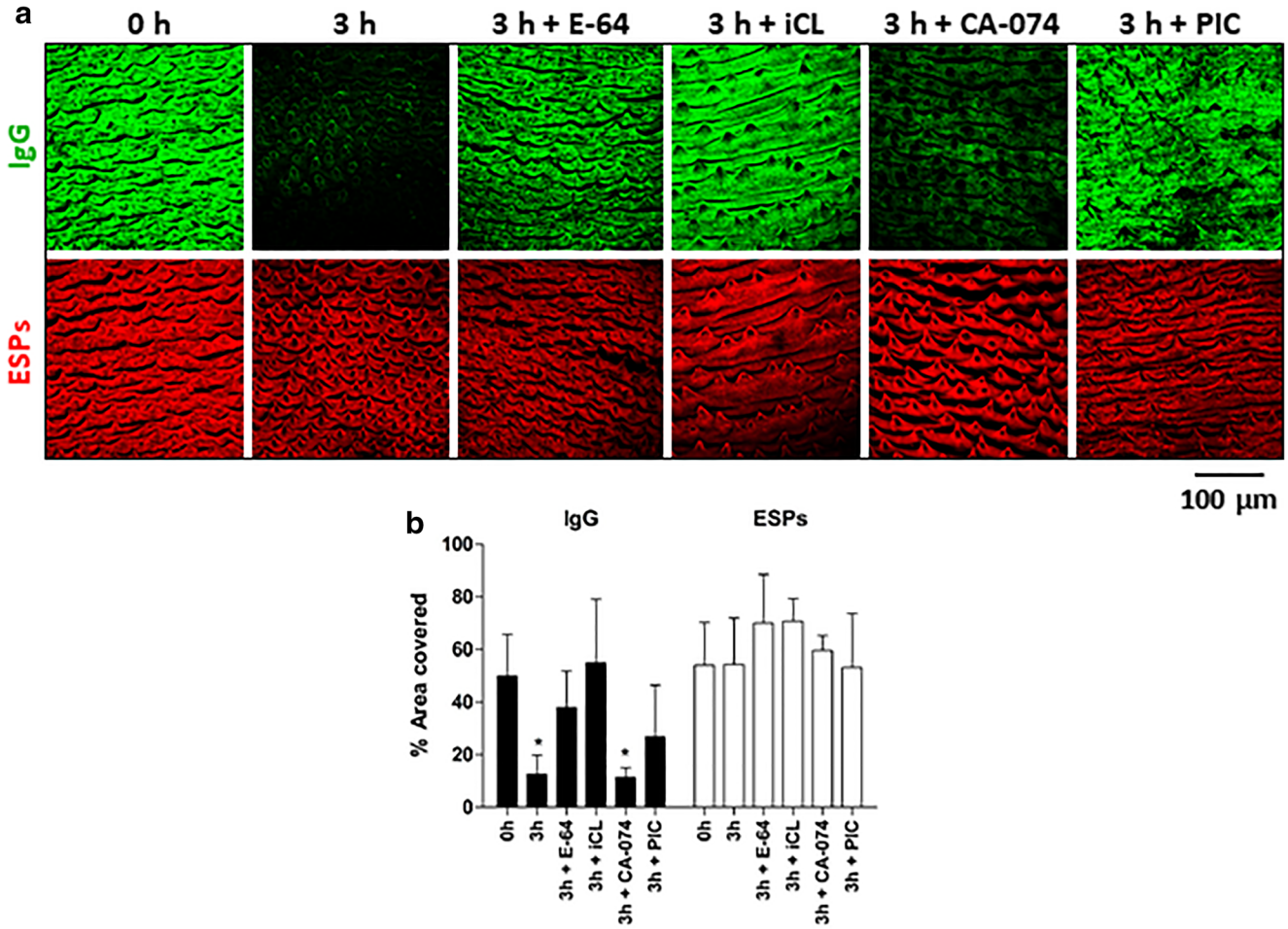

Fig. 5 Effect of specific peptidase inhibitors on the degradation of trapped antibodies. **a** Staining of the surface of *Echinostoma caproni* adults with anti-mouse IgG and anti-excretory/secretory products (ESPs) before (0 h) and after (3 h) in vitro incubation in the presence of peptidase inhibitors. irreversible, broad-spectrum, cysteine peptidase inhibitor (E-64); cathepsin L selective inhibitor (iCL); cathepsin B selective inhibitor (CA-074); peptidase inhibitor cocktail (PIC; cOmplete-Mini, EDTA-free). **b** Quantitation of the percentage of area covered by anti-IgG and anti-ESP staining. Vertical bars show standard deviation and asterisks indicate statistical differences (Kruskal-Wallis and Dunn's post hoc test) in relation to non-incubated worms, i.e., 0 h ($p < 0.05$)

secreted trans-tegumentally to accomplish functions at the host-parasite interface (Na et al. 2006), such as participating in the degradation of ESP-trapped antibodies on the worm surface.

Degradation of host immunoglobulins by pathogen-derived cysteine peptidases had been previously reported (Sajid and McKerrow 2002). Notably, *F. hepatica* secretes cathepsin L-like peptidases, which are able to degrade IgG in vitro (Berasain et al. 2000) and prevent antibody-mediated attachment of eosinophils to newly excysted juveniles (Carmona et al. 1993). However, in contrast with previous studies using either ESP mixtures or purified enzymes (Sajid and McKerrow 2002), our results demonstrate that this cathepsin-mediated evasion mechanism is highly probable to occur in vivo. Although the parasites were maintained in culture, degradation of host antibodies was carried out by peptidases released by live worms, on their own surface. In particular, we show that *E. caproni* secreted cathepsin L-like peptidases were involved in the degradation of mouse IgG and IgA bound to the parasite surface in our experiments. The role of cathepsin B-like peptidases in this process is, however, difficult to ascertain according to our current results. In contrast with E-64 and iCL, addition of cathepsin B selective inhibitor CA-074 to the culture medium failed to prevent from degradation of surface-bound antibodies. It is possible that, as discussed above, cathepsin B-like peptidases are scant, or not even present, in the ESPs of *E. caproni*. Notwithstanding, it is also plausible that this result was influenced by the features of the culture medium.

In order to assess the role of secreted cysteine peptidases in the degradation of trapped antibodies, freshly collected parasites were incubated in RPMI at physiological pH. While trematode cysteine peptidases are generally active through a pH range between 4 and 10 (Kašný et al. 2009), it has been observed that their optimum pH may shift to acid in vitro (Dalton et al. 1994; Dvořák et al. 2005; Kašný et al. 2007).

Herein, fluorometric assay of the cysteine peptidase activity in ESPs showed that CA-074 largely inhibited the enzymatic activity at pH 4.0, but its inhibitory effect decreased progressively as the pH of the medium was increased; this suggests that, if present, cathepsin B-like peptidases are more efficient at acid pH and may explain the results observed with cultured worms.

The exact pH at which antibody degradation occurs in vivo, in the mouse intestine, is difficult estimate. A study showed that the pH of the ileal content of mice is about 5 (McConnell et al. 2008). However, it is known that the mucus layer that covers the gastrointestinal epithelium creates a pH gradient from the lumen to the vicinity of the epithelium, where the pH gets close to 7 (Schade et al. 1994; Phillipson et al. 2002). *E. caproni* adults inhabit the lumen of the ileum but are in close contact with the mucosa, where they attach by the ventral sucker (Toledo et al. 2009); therefore, a pH ranging between 5 and 7 can be expected at the host-parasite interface. Overall, the results presented herein showed that secreted cathepsin L-like peptidases are active through this pH range, thus proving that these enzymes can catalyze the degradation of surface-trapped antibodies in vivo. Furthermore, this evidence shows that shedding of surface-bound antibodies is not a major mechanism of immune evasion in this parasite, as previously thought (Andresen et al. 1989), but, in contrast, bound antibodies are mostly proteolytically degraded on parasite's surface (Cortés et al. 2017). Future studies addressing whether cathepsins are responsible for either partial antibody cleavage (i.e., to Fc and Fab fragments, which is a general capability of papain) or complete digestion will aid to defining the roles of these peptidases in the evasion of antibody-mediated immunity.

Besides local antibodies, other molecules of the host can be a substrate of trematode-derived cathepsins (Dalton et al. 1997; Na et al. 2006; Kašný et al. 2007; Robinson et al. 2008b; Jedličková et al. 2018). In a comparative proteomic study of the secretome of *E. caproni*, Cortés et al. (2016) found that a cathepsin L-like peptidase was significantly over-represented in the ESPs of worms collected from rats, the low-compatible hosts that are able to rapidly expel the parasites a few weeks post-infection, and produce low levels of local-specific antibodies (Sotillo et al. 2007). Over-secretion of this cathepsin L by worms grown in an environment with low levels of antibodies suggests that it may perform additional proteolytic functions at the host-parasite interface (Cortés et al. 2016). For instance, *Entamoeba histolytica* secretes cysteine peptidases that cleave intestinal mucins, thus disrupting the mucus structure and enabling the invasion of the epithelium by the parasite (Moncada et al. 2003; Lidell et al. 2006). *E. caproni* adults are well adapted to subsist in the mouse intestine in the presence of high amounts of mucus, a well-known effector mechanism for the clearance of intestinal worms (Cortés et al. 2015). Further studies will clarify if cathepsins secreted by *E. caproni* may be also involved in parasite resistance against this defense mechanism of the host.

In conclusion, in the present article, it has been proven that secreted cathepsins of L-type are functionally active on the surface of *E. caproni*, where they can participate in immune evasion by degrading host local antibodies. Even though this mechanism has been broadly recognized for tissue-dwelling flukes, to our knowledge, this is the first evidence of it operating on the surface of living parasites and, in particular, of an intestinal species. These results point towards a crucial role of cysteine peptidases in adaptation and niche colonization also by intestine-dwelling trematodes.

Acknowledgments Research at Universitat de València was supported by Ministerio de Economía y Competitividad (Madrid, Spain) (grant number: BFU2016-75639-P); Conselleria d'Educació, Generalitat Valenciana (Valencia, Spain) (grant number: PROMETEO2014-083 Fase II); and from Ministerio de Sanidad y Consumo (Madrid, Spain) (No. RD12/0018/0013, Red de Investigación Cooperativa en Enfermedades Tropicales—RICET, IV National Program of I+D+I 2008-2011, ISCIII—Subdirección General de Redes y Centros de Investigación Cooperativa and FEDER). Research of PH and LM (Charles University) is currently supported by European Regional Development Fund and Ministry of Education, Youth and Sports of the Czech Republic (CZ.02.1.01/0.0/0.0/16_019/0000759) and Institutional Grants (Charles University PROGRES Q43 and UNCE/SCI/012-204072/2018). We would like to express our sincere gratitude to Professor John P. Dalton, Medical Biology Centre, School of Biological Sciences, Queen's University Belfast and Dr. Martin Kašný, Department of Parasitology, Faculty of Science, Charles University (Prague), and Department of Botany and Zoology, Faculty of Science, Masaryk University (Brno) for kindly providing some of the antibodies employed in this study.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Andresen K, Simonsen PE, Andersen BJ, Birch-Andersen A (1989) *Echinostoma caproni* in mice: shedding of antigens from the surface of an intestinal trematode. Int J Parasitol 19:111–118

Berasain P, Carmona C, Frangione B, Dalton JP, Goni F (2000) *Fasciola hepatica*: parasite-secreted proteinases degrade all human IgG subclasses: determination of the specific cleavage sites and identification of the immunoglobulin fragments produced. Exp Parasitol 94: 99–110

Cantacessi C, Mulvenna J, Young ND, Kašný M, Horák P, Aziz A, Hofmann A, Loukas A, Gasser RB (2012) A deep exploration of the transcriptome and "excretory/secretory" proteome of adult *Fascioloides magna*. Mol Cell Proteomics 11:1340–1353

Carmona C, Dowd AJ, Smith AM, Dalton JP (1993) Cathepsin L proteinase secreted by *Fasciola hepatica in vitro* prevents antibody-mediated eosinophil attachment to newly excysted juveniles. Mol Biochem Parasitol 62:9–17

Colley DG, Bustinduy AL, Secor WE, King CH (2014) Human schistosomiasis. Lancet 383:2253–2264

Collins PR, Stack CM, O'Neill SM, Doyle S, Ryan T, Brennan GP, Mousley A, Stewart M, Maule AG, Dalton JP, Donnelly S (2004)

Cathepsin L1, the major protease involved in liver fluke (*Fasciola hepatica*) virulence: propetide cleavage sites and autoactivation of the zymogen secreted from gastrodermal cells. J Biol Chem 279: 17038–17046

Cortés A, Muñoz-Antolí C, Sotillo J, Fried B, Esteban JG, Toledo R (2015) *Echinostoma caproni* (Trematoda): differential in vivo mucin expression and glycosylation in high- and low-compatible hosts. Parasite Immunol 37:32–42

Cortés A, Sotillo J, Muñoz-Antolí C, Trelis M, Esteban JG, Toledo R (2016) Definitive host influences the proteomic profile of excretory/secretory products of the trematode *Echinostoma caproni*. Parasit Vectors 9:185-016-1465-x

Cortés A, Sotillo J, Muñoz-Antolí C, Molina-Durán J, Esteban JG, Toledo R (2017) Antibody trapping: A novel mechanism of parasite immune evasion by the trematode *Echinostoma caproni*. PLoS Negl Trop Dis 11:e0005773

Dalton JP, McKerrow JH, Brindley PJ (1994) Trematode cysteine endopeptidases. In: Barrett AJ, Rawlings ND, Woessner JF (eds) Handbook of proteolytic enzymes, vol 2. Elsevier, Amsterdam, pp 1176–1182

Dalton JP, Clough KA, Jones MK, Brindley PJ (1997) The cysteine proteinases of *Schistosoma mansoni* cercariae. Parasitology 114: 105–112

Dvořák J, Delcroix M, Rossi A, Vopalensky V, Pospisek M, Sedinova M, Mikeš L, Sajid M, Sali A, McKerrow JH, Horák P, Caffrey CR (2005) Multiple cathepsin B isoforms in schistosomula of *Trichobilharzia regenti*: identification, characterisation and putative role in migration and nutrition. Int J Parasitol 35:895–910

Fujino T, Fried B (1993) *Echinostoma caproni* and E. trivolvis alter the binding of glycoconjugates in the intestinal mucosa of C3H mice as determined by lectin histochemistry. J Helminthol 67:179–188

Garg G, Bernal D, Trelis M, Forment J, Ortiz J, Valero ML, Pedrola L, Martínez-Blanch J, Esteban JG, Ranganathan S, Toledo R, Marcilla A (2013) The transcriptome of *Echinostoma caproni* adults: further characterization of the secretome and identification of new potential drug targets. J Proteome 89:202–214

Greenbaum D, Baruch A, Hayrapetian L, Darula Z, Burlingame A, Medzihradszky KF, Bogyo M (2000) Chemical approaches for functionally probing the proteome. Mol Cell Proteomics 1:60–68

Harris NL (2011) Advances in helminth immunology: optimism for future vaccine design? Trends Parasitol 27:288–293

Jedličková L, Dvořákova H, Dvořák J, Kašný M, Ulrychova L, Vorel J, Zarsky V, Mikeš L (2018) Cysteine peptidases of *Eudiplozoon nipponicum*: a broad repertoire of structurally assorted cathepsins L in contrast to the scarcity of cathepsins B in an invasive species of haematophagous monogenean of common carp. Parasit Vectors 11:142-018-2666-2

Kang JM, Bahk YY, Cho PY, Hong SJ, Kim TS, Sohn WM, Na BK (2010) A family of cathepsin F cysteine proteases of *Clonorchis sinensis* is the major secreted proteins that are expressed in the intestine of the parasite. Mol Biochem Parasitol 170:7–16

Kašný M, Mikeš L, Dalton JP, Mountford AP, Horák P (2007) Comparison of cysteine peptidase activities in *Trichobilharzia regenti* and *Schistosoma mansoni* cercariae. Parasitology 134: 1599–1609

Kašný M, Mikeš L, Hampl V, Dvořák J, Caffrey CR, Dalton JP, Horák P (2009) Chapter 4. Peptidases of trematodes. Adv Parasitol 69:205–297

Kim TS, de Guzman JV, Kong HH, Chung DI (2006) Comparison of gene representation between diploid and triploid *Paragonimus westermani* by expressed sequence tag analyses. J Parasitol 92: 803–816

Lidell ME, Moncada DM, Chadee K, Hansson GC (2006) *Entamoeba histolytica* cysteine proteases cleave the MUC2 mucin in its C-terminal domain and dissolve the protective colonic mucus gel. Proc Natl Acad Sci U S A 103:9298–9303

Marcilla A, Trelis M, Cortés A, Sotillo J, Cantalapiedra F, Mínguez MT, Valero ML, Sánchez del Pino MM, Muñoz-Antolí C, Toledo R, Bernal D (2012) Extracellular vesicles from parasitic helminths contain specific excretory/secretory proteins and are internalized in intestinal host cells. PLoS ONE 7:e45974

McConnell EL, Basit AW, Murdan S (2008) Measurements of rat and mouse gastrointestinal pH, fluid and lymphoid tissue, and implications for in-vivo experiments. J Pharm Pharmacol 60:63–70

McVeigh P, Maule AG, Dalton JP, Robinson MW (2012) *Fasciola hepatica* virulence-associated cysteine peptidases: a systems biology perspective. Microbes Infect 14:301–310

Mikeš L, Man P (2003) Purification and characterization of a saccharide-binding protein from penetration glands of *Diplostomum pseudospathaceum*—a bifunctional molecule with cysteine protease activity. Parasitology 127:69–77

Moncada D, Keller K, Chadee K (2003) *Entamoeba histolytica* cysteine proteinases disrupt the polymeric structure of colonic mucin and alter its protective function. Infect Immun 71:838–844

Na BK, Kim SH, Lee EG, Kim TS, Bae YA, Kang I, Yu JR, Sohn WM, Cho SY, Kong Y (2006) Critical roles for excretory-secretory cysteine proteases during tissue invasion of *Paragonimus westermani* newly excysted metacercariae. Cell Microbiol 8:1034–1046

Na BK, Kang JM, Sohn WM (2008) CsCF-6, a novel cathepsin F-like cysteine protease for nutrient uptake of *Clonorchis sinensis*. Int J Parasitol 38:493–502

Phillipson M, Atuma C, Henriksnas J, Holm L (2002) The importance of mucus layers and bicarbonate transport in preservation of gastric juxtamucosal pH. Am J Physiol Gastrointest Liver Physiol 282: G211–G219

Piacenza L, Acosta D, Basmadjian I, Dalton JP, Carmona C (1999) Vaccination with cathepsin L proteinases and with leucine aminopeptidase induces high levels of protection against fascioliasis in sheep. Infect Immun 67:1954–1961

Robinson MW, Dalton JP, Donnelly S (2008a) Helminth pathogen cathepsin proteases: it's a family affair. Trends Biochem Sci 33:601–608

Robinson MW, Tort JF, Lowther J, Donnelly SM, Wong E, Xu W, Stack CM, Padula M, Herbert B, Dalton JP (2008b) Proteomics and phylogenetic analysis of the cathepsin L protease family of the helminth pathogen *Fasciola hepatica*: expansion of a repertoire of virulence-associated factors. Mol Cell Proteomics 7:1111–1123

Sajid M, McKerrow JH (2002) Cysteine proteases of parasitic organisms. Mol Biochem Parasitol 120:1–21

Schade C, Flemstrom G, Holm L (1994) Hydrogen ion concentration in the mucus layer on top of acid-stimulated and -inhibited rat gastric mucosa. Gastroenterol 107:180–188

Schmid-Hempel P (2008) Parasite immune evasion: a momentous molecular war. Trends Ecol Evol 23:318–326

Sotillo J, Muñoz-Antolí C, Marcilla A, Fried B, Esteban JG, Toledo R (2007) *Echinostoma caproni*: kinetics of IgM, IgA and IgG subclasses in the serum and intestine of experimentally infected rats and mice. Exp Parasitol 116:390–398

Sripa B, Kaewkes S, Intapan PM, Maleewong W, Brindley PJ (2010) Food-borne trematodiases in Southeast Asia epidemiology, pathology, clinical manifestation and control. Adv Parasitol 72:305–350

Stack C, Dalton JP, Robinson MW (2011) The phylogeny, structure and function of trematode cysteine proteases, with particular emphasis on the *Fasciola hepatica* cathepsin L family. Adv Exp Med Biol 712:116–135

Steverding D (2011) The cathepsin B-selective inhibitors CA-074 and CA-074Me inactivate cathepsin L under reducing conditions. Open Enzym Inhib J 4:11–16

Steverding D, Sexton DW, Wang X, Gehrke SS, Wagner GK, Caffrey CR (2012) *Trypanosoma brucei*: chemical evidence that cathepsin L is essential for survival and a relevant drug target. Int J Parasitol 42: 481–488

Toledo R, Fried B (2005) Echinostomes as experimental models for interactions between adult parasites and vertebrate hosts. Trends Parasitol 21:251–254

Toledo R, Espert A, Muñoz-Antolí C, Marcilla A, Fried B, Esteban JG (2005) Kinetics of antibodies and antigens in serum of mice experimentally infected with *Echinostoma caproni* (Trematoda: Echinostomatidae). J Parasitol 91:978–980

Toledo R, Esteban JG, Fried B (2009) Chapter 3. Recent advances in the biology of echinostomes. Adv Parasitol 69:147–204

Yoo WG, Kim DW, Ju JW, Cho PY, Kim TI, Cho SH, Choi SH, Park HS, Kim TS, Hong SJ (2011) Developmental transcriptomic features of the carcinogenic liver fluke, *Clonorchis sinensis*. PLoS Negl Trop Dis 5:e1208