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A novel type 1 cystatin involved in the regulation of *Rhipicephalus microplus* midgut cysteine proteases

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Abstract

Rhipicephalus microplus is a cattle ectoparasite found in tropical and subtropical regions around the world with great impact on livestock production. *R. microplus* can also harbor pathogens, such as *Babesia* sp. and *Anaplasma* sp. which further compromise cattle production. Blood meal acquisition and digestion are key steps for tick development. In ticks, digestion takes place inside midgut cells and is mediated by aspartic and cysteine peptidases and, therefore, regulated by their inhibitors. Cystatins are a family of cysteine peptidases inhibitors found in several organisms and have been associated in ticks with blood acquisition, blood digestion, modulation of host immune response and tick immunity. In this work, we characterized a novel *R. microplus* type 1 cystatin, named Rmcystatin-1b. The inhibitor transcripts were found to be highly expressed in the midgut of partially and fully engorged females and they appear to be modulated at different days post-detachment. Purified recombinant Rmcystatin-1b displayed inhibitory activity towards typical cysteine peptidases with high affinity. Moreover, rRmcystatin-1b was able to inhibit native *R. microplus* cysteine peptidases and RNAi-mediated knockdown of the cystatin transcripts resulted in increased proteolytic activity. Moreover, rRmcystatin-1b was able to interfere with *B. bovis* growth *in vitro*. Taken together our data strongly suggest that Rmcystatin-1b is a regulator of blood digestion in *R. microplus* midgut.

1. Introduction

The *R. microplus* is one of the most relevant ectoparasites in the tropical and subtropical regions of the globe due to its impact on livestock production (Grisi et al., 2014; Jongejan et al., 2004). Moreover, *R. microplus* also acts as a vector of the etiologic agents of anaplasmosis and babesiosis (de Castro et al., 1997), which can further impair cattle production. Tick control relies mainly on acaricides, substances with several drawbacks, such as environmental contamination and selection of resistant tick

populations (Angus, 1996; Uilenberg, 1996), urging the need for the development of alternative control methods.

Blood meal acquisition and digestion are key steps in the tick life cycle and, in ticks, digestion begins when red blood cells are disrupted in the midgut lumen by a yet-to-be-discovered proteolytic event (Soneshine, 2013). Then, proteins are transported to or taken up by midgut cells (Lara et al., 2005) where they are processed by a multi-enzymatic cascade composed of aspartic and cysteine peptidases (Horn et al., 2009; Sojka et al., 2013; Sojka et al., 2016). Therefore, it has been proposed that aspartic and cysteine peptidase inhibitors can act as regulators of the digestion process. Not surprisingly, in *R. microplus,* cysteine and aspartic peptidases have been previously identified and implicated in blood digestion (Clara et al., 2011; Cruz et al., 2010) as well as cystatins (Cardoso et al., 2017).

Cysteine peptidase inhibitors are classified into the cystatin superfamily based on their primary features (Barret et al., 1985; Rawlings et al., 2018). In the past years, several type 2 cystatins from ticks were identified and associated with different physiological processes, such as blood feeding (Karim et al., 2005; Yamaji et al., 2009), parasite-host relantionship (Kotsyfakis et al., 2006; Salat et al., 2010), blood digestion (Cardoso et al., 2017; Yamaji et al., 2010) and immune response (Lu et al., 2014; Zhou et al., 2006). On the other hand, only a handful of type 1 cystatins have been characterized. Bmcystatin was the first type 1 cystatin described in *R. microplus* and was shown to be located in different tick tissues. Since the recombinant Bmcystatin was able to inhibit VTDCE (vitellin degrading cysteine endopeptidase), it was proposed that Bmcystatin plays a role during tick embryogenesis (Lima et al., 2006). In *Haemaphysalis longicornis* tick*,* a type-1 cystatin (Hlcys-1) identified in midgut cells was able to inhibit *H. longicornis* cathepsin L-like A (HlCPL-A) (Yamaji et al., 2010), indicating a possible role in protein digestion control.

Taking into account the lack of knowledge about type-1 cystatins and their role in tick physiological processes, in this work, we describe and characterize the second type 1 cystatin from *R. microplus*, that we have named Rmcystatin-1b.

2. Materials and methods

2.1. Bioinformatics analysis: A *R. microplus* transcriptome (SRA accession numbers: SRX484287, SRX484284, SRX484280 and SRX484277) was assembled and annotated as described (Karim et al., 2011). The putative Rmcystatin-1b DNA sequence was submitted to a domain analysis with PFAM (https://pfam.xfam.org/) and detection of a putative signal peptide was carried out with SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) (Cruz et al., 2017). The theoretical molecular weight and isoelectric point were estimated using the Compute pI/MW tool (https://web.expasy.org/compute_pi/) (Wilkins et al., 1999). An amino acid alignment was performed with Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) (Sievers et al., 2011) and edited with BioEdit software (Hall 1999). An unrooted phylogenetic tree was constructed with MEGA X (Kumar et al., 2018) using the cystatins sequences from *Ixodes scapularis* (AAY66685.1, AAM93646.1, AAY66864.1, EEC02341.1, EEC07265.1, EEC07262.1, KX513947, KX513948 and XP_029841129.1), *H. longicornis* (ABZ89553.1, ABC94582.1, ABZ89554.1 and BAI59105.1), *R. microplus* (ABG36931.1, AIX97454.1, KC816580.1 and KC816581), *Dermacentor variabilis* (ACF35512.1) and Rmcystatin-1b. The phylogenetic tree was constructed using the Neighbor-joining method based on the alignment of 155 residues (gaps included) from the cystatin sequences with 500 bootstrap replicates.

2.2. Total RNA and protein extraction from tick tissues: Partially and fully fed *R. microplus* females obtained from a laboratory colony (Porto Alegre strain, Porto Alegre, Brazil) were reared on Hereford cattle (*Bos taurus taurus*) brought from a naturally tick-free area and maintained in insulated pens (Reck et al., 2009). Ticks were washed with 70% ethanol and ultrapure water and dissected. Midgut, ovary, salivary glands and hemocytes were collected and suspended in 0.5 mL of Trizol reagent (Invitrogen, CA, USA). Total RNA extraction was performed using manufacturer's instructions and cDNA was prepared using the Improm-II Reverse Transcription System (Promega, WI, USA). A segment of tick midgut was also suspended in 0.5 mL of PBS, disrupted and centrifuged (10 min, 12000 x g at 4°C); the supernatant was filtered through 0.22 µm and stored at -20°C.

2.3. DNA amplification and cloning: Rmcystatin-1b full-length ORF was amplified by PCR using 100 µM dNTPs, 1.5 mM $MgCl_2$, 5 U Taq DNA polymerase (Sinapse, SP, BR), 25 pmol of each primer (Rmcystatin-1b.Nco.FW and Rmcystatin-1b.*Bam*HI.RV - Suppl. Table 1) and 1 µL of a midgut cDNA preparation from fully engorged *R. microplus* females. The forward and reverse primers contained terminal *Nco*I and *Bam*HI restriction sites, respectively. Reactions were submitted to 94°C – 10 min, followed by 25 cycles of 94°C – 30 s, 55°C – 60 s, 72°C – 60 s and a final extension at 72°C for 10 min. PCR products were observed in a 1% agarose gel and purified with QIAEXII extraction kit (QIAGEN, Hilden, DE). A purified Rmcystatin-1b amplicon and pET14b were digested with *Nco*I (Fermentas, Vilnius, LT) and *Bam*HI (Fermentas, Vilnius, LT) overnight at 37°C, purified with QIAEXII kit (QIAGEN, Hilden, DE) and subjected to ligation with T4 ligase (Promega, WI, USA) overnight at 16°C. Finally, the ligation product was used in the transformation of *Escherichia coli* DH5α and the positive clones were confirmed by colony PCR using T7 promoter and terminator primers and sequenced using a Big Dye Terminator cycle sequencing kit (Applied Biosystems, Warrington, UK) in an ABI Prism 3130 automated sequencer (Applied Biosystems, Warrington, UK).

2.4. Expression and purification: Rmcystatin-1b expression was carried out in *E. coli* BL21 pLysS strain induced with 1 mM IPTG at 37°C. After 16 h of induction, cells were collected by centrifugation (10 min, 3000 x g at 4°C) and suspended in 100 mL of 50 mM Tris-HCl pH 8.0. Bacterial lysis was carried out using a *French pressure cell press* system (Thermo, MA, USA); the bacterial suspension was submitted three times to a 2000 psi pressure. After lysis the sample was centrifuged (10 min, 12000 x g at 4°C) and the supernatant collected and submitted to ionic exchange chromatography with a HiPrep Q resin. The resin was previously equilibrated with 50 mM Tris-HCl pH 8.0 and protein elution was carried out with a linear gradient of NaCl (0 to 1 M) in 50 mM Tris-HCl pH 8.0. Eluted fractions that presented inhibitory activity towards human cathepsin L (item 2.7) were pooled, dialyzed against 50 mM Tris-HCl pH 8.0 and submitted to size exclusion chromatography using a Superdex 75 resin. The purified protein was observed by 15% SDS-PAGE.

2.5. Rmcystatin-1b primary structure analysis by mass spectrometry: Approximately 40 µg of rRmcystatin-1b were precipitated with chloroform/methanol (Wessel and Flugge, 1984). The pellet was resuspended in 100 mM Tris HCl pH 7.0 containing 8 M urea, disulfide bonds were reduced in 5 mM dithiothreitol (DTT) for 20 min at 37 C and then cysteines were alkylated in 25 mM iodoacetamide (IAM) for 20 min at room temperature in the dark. Urea was diluted to 2 M with 100 mM Tris HCl pH 7.0, trypsin was added at a mass ratio of 1:100 (trypsin/protein) and the sample was incubated overnight at 37°C. Formic acid was added to finish the reaction (5% v/v, final concentration). Peptides were separated on an in-house made 20 cm reverse-phase (5 µm ODSAQ C18, Yamamura Chemical Lab, Japan) using a nanoUPLC (nanoLC Ultra 1D plus, Eksigent, USA) connected to a LTQ-XL Orbitrap Discovery hybrid instrument (Thermo Fisher Scientific) through a nanoelectrospray ion source (Thermo Fisher Scientific). The flow rate was set to 300 nL min^{-1} in a 60 minutes reverse-phase gradient. The mass spectrometer was operated in a data-dependent mode, with full MS1 scan collected in the Orbitrap, with m/z range of 400-1600 at 30,000 resolution. The eight most abundant ions per scan were selected to CID MS2 in the ion trap. Raw data were searched against a non-redundant database containing forward and reverse *E. coli* BL21 pLysS proteome and Rmcystatin-1b sequence using Comet (Eng et al., 2013) through PatternLab for Proteomics platform (Carvalho et al., 2016). The validity of the peptide-spectra matches (PSMs) generated was assessed using Patternlab´s module SEPro (Carvalho et al., 2016) with a false discovery rate of 1% based on the number of decoys.

2.6. RT-qPCR: Rmcystatin-1b mRNA levels were quantified in different tissues of partially and fully engorged *R. microplus* females by RT-qPCR. Reactions were prepared with 6 µL of SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK), 1 µL of the cDNA preparation diluted 5 times and 200 nM of each primer (Suppl. Table 1) in a final volume of 12 µL. Reactions were carried out in a Step-One Plus equipment (Applied Biosystems, Warrington, UK), with 40 cycles (95°C - 1 min, 60°C - 1 min and 72°C - 1 min). The relative quantification was determined by the $2^{-\Delta\Delta Ct}$ method (Livak and Schimttgen, 2011) and the elongation factor 1α gene was used as endogenous control (Nijhof et al., 2009). Relative quantification of Rmcystatin-1b transcripts in cDNA from partially engorged ticks was performed in relation to the salivary glands preparations,

while in fully engorged, in relation to hemocytes. Rmcystatin-1b transcripts in the midgut at different time points were compared to partially engorged ticks (PF).

2.7. Determination of the inhibitory constant (Ki): Human cathepsin L (EC 3.4.22.15), cathepsin B (EC 3.4.22.1) and BmCL1 previously prepared in our laboratory as described (Clara et al., 2011), papain (EC.3.4.22.1) (Calbiochem, USA) and recombinant *B. bovis* cysteine peptidase (XP_001612131) (Lu et al., unpublished data) were incubated in 50 mM sodium acetate buffer pH 5.0 containing DTT (1 mM) for 10 min at 37°C and different concentrations of rRmcystatin-1b were added. Proteolytic activity was monitored by fluorescence (380 nm excitation/ 460 nm emission) after addition of fluorogenic substrate Z-FR-AMC (4 mM) for assays with cathepsin L, papain, BmCL1 and *B. bovis* cysteine peptidase or Z-RR-AMC (4 mM) for cathepsin B, for 30 min at 37°C. The inhibitory constants were determined by fitting the non-linear regression model according to the Morrison's equation (Morrison, 1969) using the Grafit 5.0.11 software (Erithacus Software Limited).

2.8. Proteolytic activity of *R. microplus* midgut: Crude protein extracts from midgut (10 µg) of *R. microplus* obtained at 24, 48, 72, 96, 120 and 144 h post detachment (hpd) were incubated in 50 mM sodium acetate buffer pH 5.0 containing DTT (1 mM) for 10 min at 37°C, following the addition of substrates Z-FR-AMC (4 mM) or Z-RR-AMC (4 mM). Proteolytic activity was monitored by fluorescence (380 nm excitation/ 460 nm emission) for 15 min at 37°C. The proteolytic activity of midguts from 48 and 120 hpd was also evaluated in the presence of different inhibitors (E64, CA-074, rRmcystatin-1b, EDTA and APMSF). The initial rate was determined by linear regression of fluorescence (RFU) per time (min) and the residual activity by the ratio between the proteolytic activity in the presence of the inhibitor and the control reaction (without inhibitor).

2.9. Rmcystatin-1b knock-down by RNA interference (RNAi): Rmcystatin-1b and GFP double strand RNA (dsRmcys1b and dsGFP) were synthesized with the T7 Ribomax RNAi System kit (Promega, WI, USA) following the manufacturer's instructions and 10 µg of dsRNA were injected in the hemolymph of ticks 72 hpd. After 24 h of dsRNA injection, the midguts of 6 ticks were individually dissected and stored in Trizol reagent (Invitrogen, CA, USA) and PBS for RT-qPCR and enzyme activity test, respectively. Confirmation of

Rmcystatin-1b knock-down was carried out by RT-qPCR as described in item 2.6 and proteolytic activity from the midgut of dsRNA-injected ticks was measured as described in 2.8.

2.10. Effect of rRmcystatin-1b on *B. bovis* growth in *in vitro* culture: *B. bovis* S2P strain *in vitro* cultures were maintained in microaerophilous stationary phase (MASP) as described (Levy and Ristic, 1980) with a 10% hematocrit. When the percentage of infected red blood cells (RBC) reached 2%, parasites were transferred to a 96 well-plate containing complete culture medium including 40% bovine serum, 5% RBC to a final percentage of parasitized RBC of 0.2% and supplemented with 15 µL of BSA or rRmcystatin-1b (25 µM) in 15 µL. Every 24 h the medium was replaced and 5 µL of packed erythrocytes were collected from the bottom of each well and smeared onto glass slides, which were then Giemsa-stained and observed by light microscopy. The percentage of infected RBC was determined 24, 48 and 72 h by counting 3000 cells per slide. The experiment was conducted in triplicate wells for each time point.

2.11. Statistical analysis: Rmcystatin-1b RT-qPCR results in different tick tissues, as well as the modulation of Rmcystatin-1b transcripts in the midgut at different time points, were analyzed with one-way ANOVA followed by Bonferroni's multiple comparison test. All RT-qPCR tests were performed using the ΔCt data as described in (Yuan et al., 2006). Confirmation of Rmcystatin-1b knock-down by RT-qPCR and proteolytic activity of dsRmcystatin-1b and dsGFP injected ticks were tested with Mann-Whitney test. Percentages of *B. bovis*-infected RBC in *in vitro* culture at 72 h were tested with Mann-Whitney test. The significant level for statistical differences was set at $p < 0.05$ and all statistical tests were performed using the R statistical computing tool (http://www.R-project.org).

3. Results

3.1. Primary structure analysis: The complete Rmcystatin-1b nucleotide sequence was identified in a *R. microplus* transcriptome (SRA SRX484287, SRX484284, SRX484280 and SRX484277) and comprises an ORF of 303 nucleotides (Suppl. Fig. 1). Domain analysis of Rmcystatin-1b deduced amino acid sequence revealed the presence of a cystatin domain from G^{8} – Q^{87} and alignment with other type 1 cystatins showed the conserved motifs of the cystatin super-family, the Gly residue in the N-terminal portion and the QxVxG motif (Fig. 1A). Rmcystatin-1b did not present the C-terminal PW residues neither a putative signal peptide; rendering a mature protein with a molecular weight of 11.1 kDa and a theoretical pI of 6.27. Phylogenetic analyses of tick cystatins clustered Rmcystatin-1b with other type 1 cystatins, closely related to the midgut cystatin 1 from *H. longicornis* (Hlcys-1) (Fig. 1B).

3.2. Rmcystatin-1b transcription profile in *R. microplus* tissues: Rmcystatin-1b transcripts were mainly located in the midgut of partially and fully engorged females. In partially engorged ticks, Rmcystatin-1b transcription in the midgut was 65 times higher in relation to salivary glands (Fig. 2A), while in fully engorged ticks, 40 times higher in relation to hemocytes (Fig. 2B). Rmcystatin-1b transcripts were also modulated in the midgut at different hours post-detachment (hpd). Down-regulation was observed between 24 and 72 hpd, followed by up-regulation between 96 and 144 hpd (Fig. 2C). No modulation was found in ovary or salivary glands (Suppl. Fig. 3).

3.3. Expression, purification and characterization of rRmcystatin-1b: Recombinant Rmcystatin-1b was obtained in soluble form in *E. coli* BL21 pLysS strain (Fig. 3C – lane 3). After two purification steps, ionic exchange (Fig. 3A) and size exclusion chromatography (Fig. 3B), a major 12 kDa protein band was observed (Fig. 3C – lane 5) and confirmed to be rRmcystatin-1b by mass spectrometry analysis (Suppl. Table 2 and Suppl. Fig. 2). Purified rRmcystatin-1b presented inhibitory activity towards different cysteine peptidases (table 1), including a recombinant *B. bovis* cysteine peptidase (XP_001612131) (Ki = 7.48 nM), although the highest affinity was observed for the *Boophilus microplus* cathepsin L-like protease (BmCL1) (Ki = 0.013 nM).

3.4. Effect of rRmcystatin-1b in *R. microplus* midgut proteolysis: Proteolytic activity of *R. microplus* midgut homogenates at different time points was measured with two substrates, and two proteolytic peaks, at 48 hpd and at 120 hpd, were observed (Fig. 4A). The proteolytic activities at 48 and 120 hpd were completely inhibited in the presence of E-64 (Fig. 4B). rRmcystatin-1b was able to induce a partial inhibition, similar to the selective cathepsin B inhibitor CA-074 (Fig. 4C and D), although the highest degree of inhibition was observed in the presence of rRmcystatin-1b at 48 hpd. No inhibition was observed in the presence of other peptidase inhibitors such as APMSF and EDTA (Fig. 4E and F)

3.5. Effect of Rmcystatin-1b gene knock-down on the midgut proteolytic activity: RT-qPCR of dsRmcystatin-1b-injected *R. microplus* revealed a reduction of 50% in Rmcystatin-1b transcripts in relation to dsGFP injected ones (Fig. 5A). Moreover, dsRmcystatin-1b-injected ticks presented higher proteolytic activity towards Z-FR-AMC (Fig. 5B), while no difference was observed towards Z-RR-AMC (Fig. 5C), however this activity was already low in the 48 hpd midgut (Fig. 4A).

3.6 Effect of rRmcystatin-1b in *B. bovis* culture: The relevance of apicomplexan cysteine peptidases for parasite survival and proliferation has already been demonstrated (Okubo et al. 2007; Carletti et al. 2016). Therefore, we decided to evaluate the possible inhibitory effect of different concentrations of rRmcystatin-1b on *B. bovis* growth, in which 50% reduction in the percentage of infected RBC was observed when compared to control-treated cultures at 72 h (Fig. 6A). The effect of rRmcystatin-1b (25 µM) was then studied at different time points (24, 48 and 72 h), and significant reductions in infected RBC percentages were observed from 48 h onwards (Fig. 6B).

4. Discussion

Rmcystatin-1b primary structure presents typical features of the cystatin super family, such as an N-terminal Gly residue and a QxVxG motif (Fig. 1A) (Abrahamson et al., 1987; Bode et al., 1988; Stubbs et al., 1990). Since the inhibitor lacks both a signal peptide and disulfide bridges, it can be classified into the I25A group (Rawlings et al, 2018). Like other type 1 cystatins, Rmcystatin-1b does not possess the C-terminal PW motif (Ibeli et al., 2013; Lima et al., 2006) and the Asn residue relevant for legumain inhibition (Suppl. Fig. 4) (Alvarez-Fernandes et al., 1999). Phylogenetic analysis clustered Rmcystatin-1b with other type 1 cystatins, closely related to the intracellular cystatin from *H. longicornis* (Hlcys-1) confirming their close molecular phylogenetic relationship (Fig. 1B). Hlcys-1 was co-localized in midgut cells with the cysteine peptidase cathepsin L-like (HlCPL-A) and was able to inhibit the hemoglobinolytic activity of HlCPL-A *in vitro*. These data suggest that Hlcys-1 may act as an endogenous inhibitor of HlCPL-A, thus regulating blood digestion in *H. longicornis* (Yamaji et al., 2010; Zhou et al., 2009). (Yamaji et al., 2010; Zhou et al., 2009). A similar biological function might be carried out by Rmcystatin-1b. In addition, Rmcystatin-1b transcripts were found in higher concentrations in the midgut of partially and fully engorged females (Fig. 2A and B), substantiating its possible role during blood digestion. So far only a handful of type 1 cystatins were biochemically described in ticks (Wang et al., 2015; Yamaji et al., 2009; Zhou et al., 2009) and most of them were identified in salivary gland transcriptomes (Anderson et al., 2008; Karim et al., 2015; de Castro et al., 2016; Esteves et al., 2017) suggesting a role in blood acquisition rather than in blood digestion. In *R. microplus* only another type 1 cystatin has been previously described and associated with embryogenesis (Lima et al., 2006).

Although it is well accepted that cysteine peptidases and cystatin interplay can be relevant for tick digestion (Schwarz et al., 2012; Sojka et al., 2013) and several putative enzymes and inhibitors were identified in the tick midgut by transcriptome studies (Anderson et al., 2008; Perner et al., 2016), little is known about the temporal dynamics of this interaction. In *Ixodes ricinus* different classes of enzymes responsible for blood digestion have been identified (Horn et al., 2009) and shown to be active at different days of feeding (Franta et al., 2010). Similarly, screening of *R. microplus* midgut proteolytic activity at different

days post-detachment revealed a distinct behavior towards different substrates (Fig. 4A), in which higher proteolytic activity towards Z-FR-AMC was observed at 48 hpd. Interestingly, Rmcystatin-1b transcripts were found to be down-regulated at 48 hpd and up-regulated later on (Fig. 2C). Together, these data suggest that enzymes with higher affinity for Z-FR-AMC are relevant in the early phase of blood digestion when Rmcystatin-1b concentrations are low and as digestion progresses, Rmcystatin-1b concentration increases, thus regulating such enzymes in a later period.

To test this hypothesis, we evaluated the proteolytic activity of tick midguts at 48 and 120 hpd in the presence of different inhibitors (Fig. 4B - F). Notably, purified rRmcystatin-1b (Fig. 3) was able to inhibit 67% of the proteolytic activity at 48 hpd and 56% at 120 hpd, indicating a higher affinity for enzymes present at the early steps post-detachment. As expected, no inhibitory activity was observed in the presence of APMSF (Fig. 4E) or EDTA (Fig. 4E), confirming that the activity observed is from cysteine peptidases. Rmcystatin-1b gene knock-down at 96 hpd (Fig. 5A) resulted in an increase of proteolytic activity towards Z-FR-AMC (Fig. 5B) but not towards Z-RR-AMC (Fig. 5C), moreover rRmcystatin-1b presented higher inhibitory activity towards cathepsin L-Like peptidases, such as human cathepsin-L (Ki = 0.41 nM) and BmCL1 (Ki = 0.013 nM), rather than cathepsin B (Ki = 6.27 nM) (table 1). Taken together, these data strongly suggest that the Rmcystatin-1b target is, preferentially, cathepsin L-Like peptidases, which have been previously described in the early phases of digestion in *I. Ricinus* (Horn et al., 2009; Sojka et al., 2013).

Previous studies have shown that inhibition of cysteine peptidases from apicomplexan parasites can interfere with parasite survival (Carletti et al., 2016; Okubo et al., 2007). Moreover a type 2 cystatin from *H. longicornis* (Hlcyst-2), found mainly in the midgut of this tick, was able to interfere with *B. bovis* growth *in vitro* (Zhou et al., 2006), demonstrating the possible interaction between cysteine peptidases from parasites with vector cystatins. Since *R. microplus* is a natural *B. bovis* vector, we decided to investigate the possible role of Rmcystatin-1b during parasite-vector interaction. Although rRmcystatin-1b was able to inhibit recombinant *B. bovis* cysteine peptidase (XP_001612131) with high affinity (Ki = 7.48 nM), rRmcystatin-1b was only able to impair

B. bovis growth in high concentrations (Fig. 6A and B), suggesting that XP_001612131 may not be involved in parasite invasion or rRmcystatin-1b is inhibiting unspecifically other *B. bovis* cysteine peptidases. It is important to note that the *B. bovis* experiments performed in this study try to mimic the parasite-vertebrate host interaction, in which the importance of cysteine peptidases has already been shown (Carletti et al., 2016; Okubo et al., 2007). However, in *P. falciparum,* the importance of the cysteine protease falcipain-1 for parasite development in the mosquito midgut was demonstrated (Eksi et al., 2004). Moreover, a cystatin from *H. longicornis* was found to be up-regulated in ticks fed on dogs infected with *B. gibsoni* compared to non-infected ones and the recombinant cystatin was also able to inhibit *B. bovis* growth in culture (Zhou et al., 2006). Taken together, these data suggest a potential role of cysteine peptidases during parasite-vector interaction, in which vector cystatins may play a role during parasite proliferation in order to avoid deleterious effects to the tick (Florin-Christensen and Schnittger, 2009).

In this study, we described the functional characterization of a novel type 1 cystatin from the tick *R. microplus*. Altogether our data suggested the involvement of Rmcystatin-1b in the regulation of cathepsin L-Like peptidases in the later phase of blood digestion, and a possible role in the interaction with the tick-borne hemoparasite *B. bovis*.

Conflict of interest

The authors declare there is no conflict of interest.

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Author's contributions

Conceived and designed the experiments: Lu, S. and Tanaka, A.S.

Performed the experiments: Lu, S., Rocha, L.A., Torquato, R.J.S. and Florin-Christensen, M.

Contributed reagents/materials/analysis tools: Tanaka, A.S., Florin-Christensen, M. and da Silva Vaz, I.

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Critical revision of the article: Tanaka, A.S., Florin-Christensen, M. and da Silva Vaz, I.

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Table 1. Biochemical characterization of *Rhipicephalus microplus* Rmcystatin-1b inhibitory activity.

Enzyme	Ki (nM)
BmCL1	0.013
Papain	0.72
Cathepsin L	0.41
Cathepsin B	6.27
B. bovis cysteine peptidase	7.48

FIGURES CAPTIONS

Figure 1: Rmcystatin-1b primary structure analysis. **(A)** Alignment of amino acid sequence of *Rhipicephalus microplus* Rmcystatin-1b (MH378687) with other type 1 cystatins from ticks; *Dermacentor variabilis* (ACF35512.1), *Rhipicephalus haemaphysaloides* (AIZ78005.1), *Haemaphysalis longicornis* (ABZ89553.1), *R. haemaphysaloides* (AIZ78005.1), *R. microplus* (ABG36931.1) and *Ixodes scapularis* (AAY66864.1). Identical residues are black boxed and the residues relevant for the inhibitory activity are indicated by arrows. **(B)** Phylogenetic analysis of type 1 and 2 cystatins from the ticks *H. longicornis*, *I. scapularis*, *D. variabilis* and *R. microplus*. Rmcystatin-1b is indicated by an arrow.

Figure 2: Localization of *Rhipicephalus microplus* Rmcystatin-1b transcripts in different tick tissues by RT-qPCR. Relative quantification or Rmcystatin-1b transcripts in the cDNA of **(A)** partially engorged ticks was determined in relation to the salivary glands and in **(B)** fully engorged ticks, in relation to hemocyte preparations. **(C)** Modulation of Rmcystatin-1b transcripts in tick midgut at different hours post-detachment (hpd) in relation to partially engorged (PF) ticks. The averages ± SEM of four replicates are shown (* $p < 0.05$).

Figure 3: Expression and purification of *Rhipicephalus microplus* rRmcystatin-1b. **(A)** Ion exchange chromatography with a HiPrep Q resin. Proteins were eluted using a linear gradient of 25 mM Tris-HCl buffer pH 8.0 containing 1.0 M NaCl (buffer B). **(B)** Size exclusion chromatography using a Superdex 75 columm. Arrows indicate the fractions in which inhibitory activity towards human cathepsin L was detected. **(C)** SDS-PAGE 15% of (1) non-induced bacteria, (2) IPTG-induced bacteria, (3) supernatant from bacterial lysate, (4) pooled protein from ion exchange and (5) pooled protein from size exclusion chromatography.

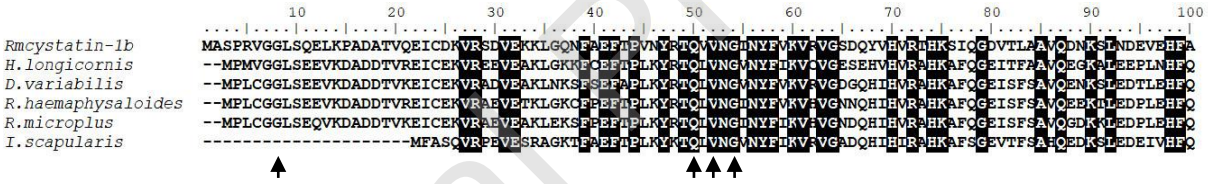
Figure 4: Characterization of proteolytic activity of *Rhipicephalus microplus* midgut. **(A)** Proteolytic activity against typical cysteine peptidases substrates using midguts from different hpd. Proteolytic activity of midgut from 48 and 120 hpd were assayed in the presence of **(B)** E64, **(C)** CA-074, **(D)** rRmcystatin-1b¸ **(E)** APMSF and **(F)** EDTA.

Figure 5: *Rhipicephalus microplus* Rmcystatin-1b gene knock-down by RNA interference. **(A)** Relative quantification of Rmcystatin-1b transcripts in the midgut of ticks at 96 hpd. **(B)** Proteolytic activity of the midguts towards Z-FR-AMC and **(C)** Z-RR-AMC. Mann-Whitney test revealed significant statistical difference with p = 0.0087. Bars represent the averages ± SEM from 6 independent samples.

Figure 6: *Rhipicephalus microplus* Rmcystatin-1b and *Babesia bovis* growth **(A)**: Effect of different concentrations of rRmcystatin-1b in *B. bovis* S2P strain growth *in vitro* after 72 h of treatment and **(B)** parasite growth curve during treatment with rRmcystatin-1b (25 µM), parasitemia was determined at 24, 48 and 72 h by microscopic observation of Giemsa-stained smears. Average ± SEM of three replicates are shown. * $p < 0.05$.

Figure 1

A



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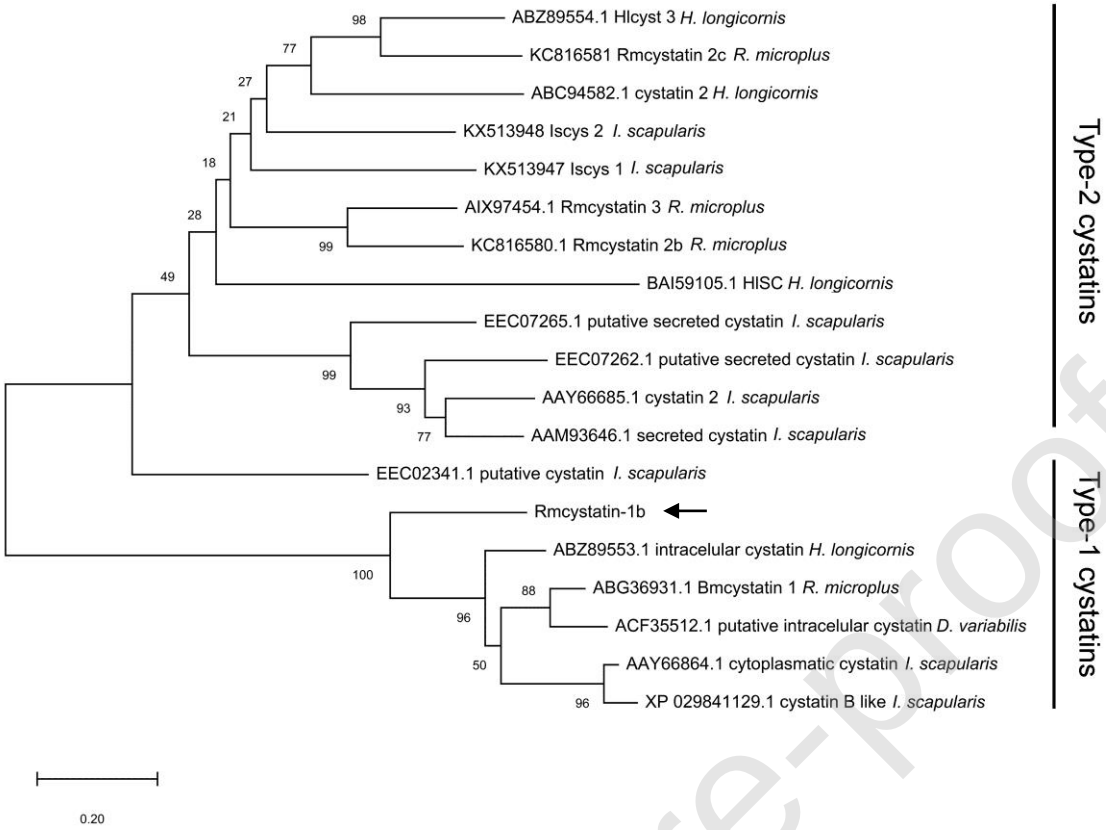
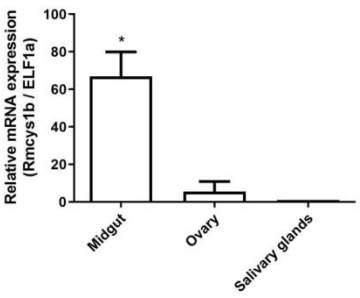
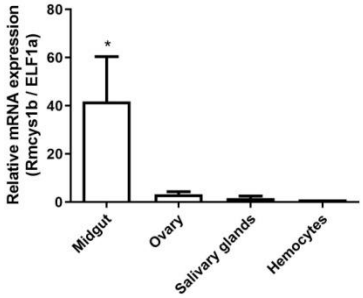


Figure 2

A B C





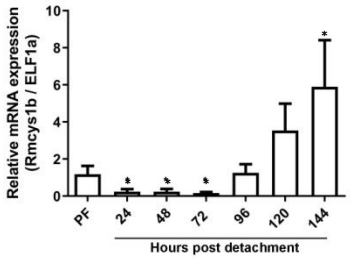


Figure 3

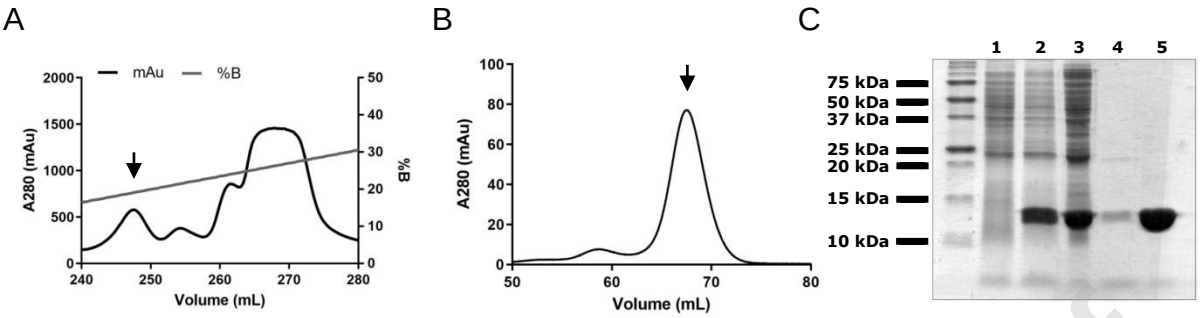


Figure 4

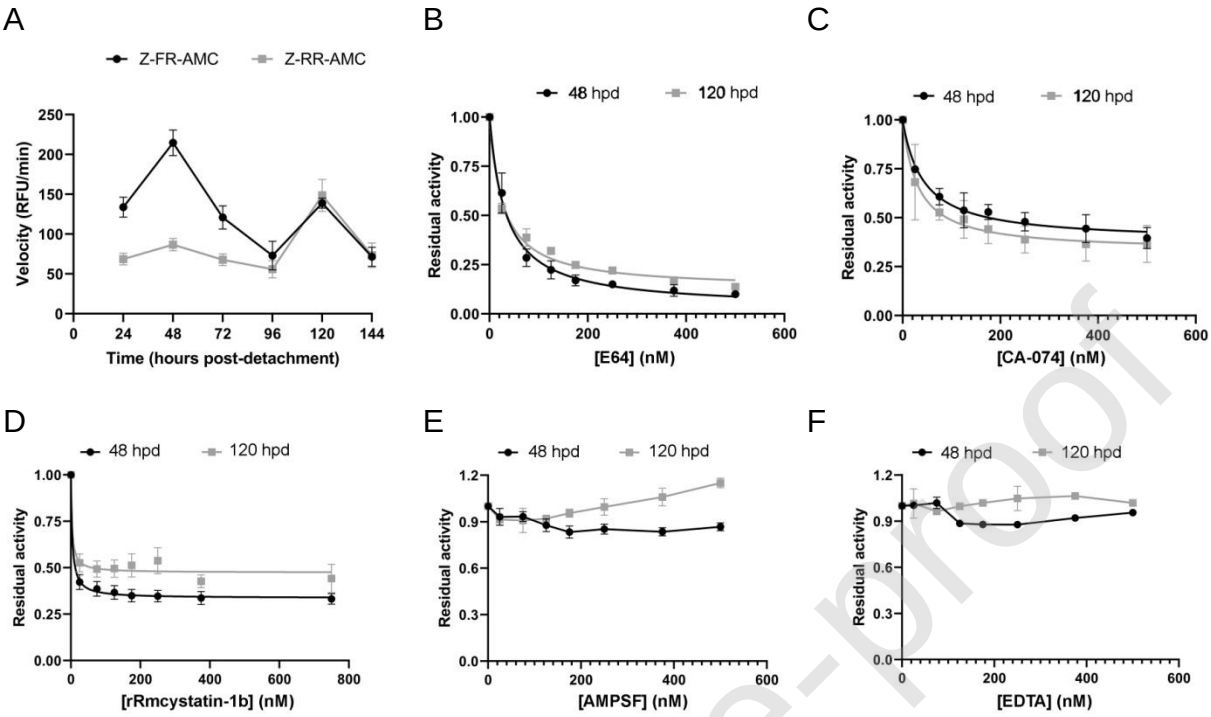


Figure 5

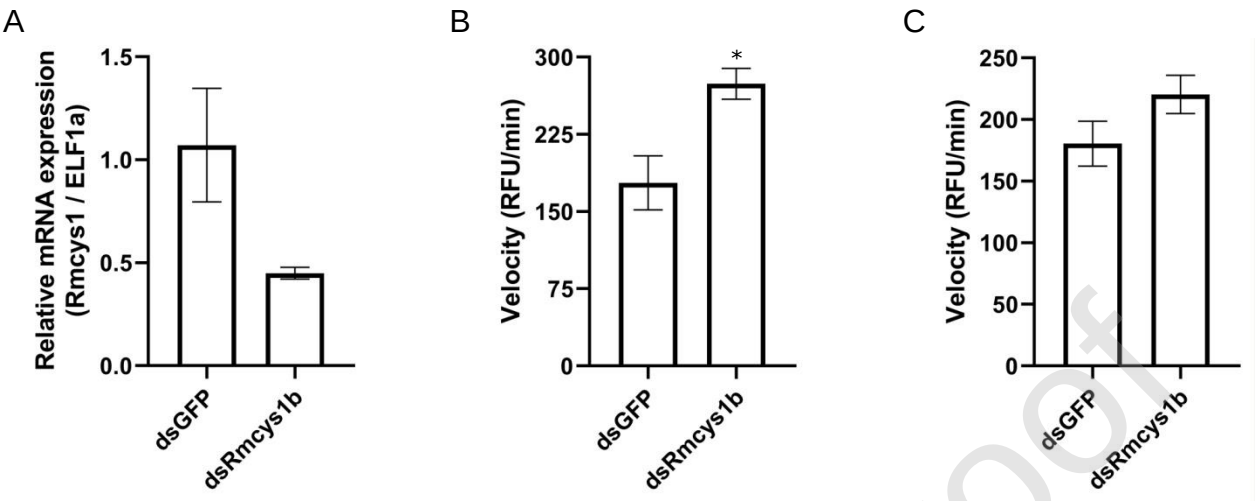
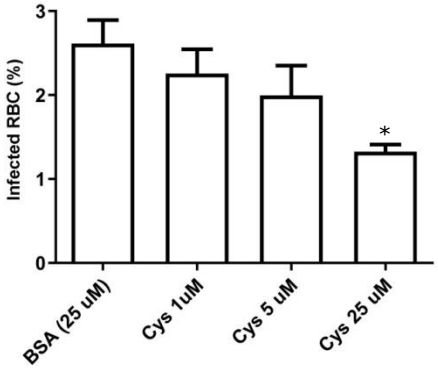


Figure 6

A



B