Published in final edited form as:

Int J Parasitol. 2015 August ; 45(0): 613–627. doi:10.1016/j.ijpara.2015.03.009.

Conserved *Amblyomma americanum* tick Serpin19, an inhibitor of blood clotting factors Xa and XIa, trypsin and plasmin, has anti-haemostatic functions

Tae Kwon Kim^{a,1}, Lucas Tirloni^{a,b,1}, Zeljko Radulovic^a, Lauren Lewis^a, Mariam Bakshi^a, Creston Hill^a, Itabajara da Silva Vaz Jr.^{b,c}, Carlos Logullo^d, Carlos Termignoni^{b,e}, and Albert Mulenga^{a,*}

^aDepartment of Veterinary Pathobiology, College of Veterinary Medicine, Texas A&M University, College Station, TX, USA

^bCentro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

^cFaculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

^dLaboratório de Sanidade Animal, Laboratório de Química e Função de Proteínas e Peptídeos and Unidade de Experimentação Animal, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes, RJ, Brazil

^eDepartamento de Bioquímica, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

Abstract

Tick saliva serine protease inhibitors (serpins) facilitate tick blood meal feeding through inhibition of protease mediators of host defense pathways. We previously identified a highly conserved *Amblyomma americanum* serpin (AAS) 19 that is characterized by its reactive center loop being 100% conserved in ixodid ticks. In this study, biochemical characterization reveals that the ubiquitously transcribed AAS19 is an anti-coagulant protein, inhibiting the activity of five of the eight serine protease blood clotting factors. *Pichia pastoris*-expressed recombinant (r) AAS19 inhibits the enzyme activity of trypsin, plasmin and blood clotting factors (f) Xa and XIa, with stoichiometry of inhibition estimated at 5.1, 9.4, 23.8 and 28, respectively. Similar to typical inhibitory serpins, rAAS19 forms irreversible complexes with trypsin, fXa and fXIa. At a higher molar excess of rAAS19, fXIIa is inhibited by 82.5%, and thrombin (fIIa), fIXa, chymotrypsin and tryptase are inhibited moderately by 14 – 29%. In anti-hemostatic functional assays, rAAS19 inhibits thrombin but not ADP and cathepsin G activated platelet aggregation, delays clotting in recalcification and thrombin time assays by up to 250 s, and up to 40 s in the activated partial thromboplastin time assay. Given AAS19 high cross-tick species conservation, and specific

*Corresponding author. Department of Veterinary Pathobiology, College of Veterinary Medicine, Texas A&M University, 422 Raymond Stotzer Parkway, College Station, TX 77845 USA. Tel.: +1 979 458 4300. amulenga@cvm.tamu.edu.

¹These authors contributed equally.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

reactivity of rAAS19 with antibodies to *A. americanum* tick saliva proteins, we conclude that rAAS19 is a potential candidate for development of a universal tick vaccine.

Graphical abstract

Keywords

Amblyomma americanum; Tick anti-haemostatic functions; Tick conserved protein; Tick saliva serpin

1. Introduction

Ticks and tick borne diseases (TBDs) have a global veterinary and public health impact that in livestock production amounts to large monetary losses worldwide (Jongejan and Uilenberg, 2004; Grisi et al., 2014). For many years, ticks and TBDs were mostly a veterinary problem. However, following the description of *Borrelia burgdorferi*, the causative agent of Lyme disease, in the 1980s (Burgdorfer et al., 1982), the impact of human TBDs has increased. Recently, 17 human TBD agents were listed in a study advocating for One Health solutions (Dantas-Torres et al., 2012). Long considered a nuisance tick (Childs and Paddock, 2003), *A. americanum* is the principal vector of *Ehrlichia chaffeensis* and *Ehrlichia ewingii*, the causative agents of human monocytic ehrlichiosis (Anderson et al., 1993; Wolf et al., 2000). It is also associated with epidemiology of *Francisella tularensis* (Taylor et al., 1991); a yet to be described causative agent of the southern rash illness (STARI) (James et al., 2001); and a newly identified heartland virus (Savage et al., 2013). In veterinary health, *A. americanum* transmits *Theileria cervi*, a parasite of white tailed deer (Laird et al., 1988), and heavy infestations caused mortality in white tailed deer fawns (Yabsley et al., 2005) as well as production losses in cattle (Barnard, 1985, 1990).

Ticks feed by disrupting host tissue and sucking up blood that pools in the feeding lesion, which provokes host defense responses including pain, hemostasis (to limit blood loss),

inflammation, complement activation (to protect against invading microbial organisms) and tissue repair responses (to heal the feeding lesion) (Francischetti et al., 2009). To date, many proteins derived from tick saliva have been identified which have a direct role at the feeding site, allowing the tick to successfully acquire its blood meal (Steen et al., 2006; Maritz-Olivier et al., 2007). Following the blood meal acquisition, it is also necessary that blood remains fluid for subsequent digestion, in which different tick proteins have been described to act as anti-coagulant molecules in the tick midgut (Ricci et al., 2007; Anderson et al., 2008; Liao et al., 2009). Serine proteases mediate some host defense pathways to tick feeding and are controlled in some pathways by inhibitors belonging to the serine protease inhibitors (serpins) family (Gettins, 2002; Huntington, 2006; Rau et al., 2007). From this perspective, it is proposed that ticks inject serpins into the host to mediate evasion of host defenses and thus they could be suitable targets for tick vaccines (Mulenga et al., 2001). Several tick serpin-encoding cDNAs have been cloned and characterized, including serpins of *A. americanum* (Mulenga et al., 2007; Porter et al., 2015), *Amblyomma maculatum* (Karim et al., 2011), *Ixodes scapularis* (Ribeiro et al., 2006; Mulenga et al., 2009), *Ixodes ricinus* (Leboulle et al., 2002; Prevot et al., 2006; Chmelar et al., 2011), *Rhipicephalus microplus* (Rodriguez-Valle et al., 2012; Tirloni et al., 2014b), *Rhipicephalus appendiculatus* (Mulenga et al., 2003a), *Rhipicephalus haemaphysaloides* (Yu et al., 2013), and *Haemaphysalis longicornis* (Sugino et al., 2003; Imamura et al., 2005, 2006). As of January 2015, more than 200 tick serpin-encoding cDNAs were available in public databases (Porter et al., 2015).

The concept that ticks utilize serpins to evade host defense mechanisms assumes that ticks inject inhibitory serpins into the host during feeding. Indeed, the presence of serpins in tick saliva was well demonstrated through saliva proteomic studies in *Dermacentor andersoni* (Mudenda et al., 2014) and *R. microplus* (Tirloni et al., 2014a) as well as being inferred from transcriptional analysis of salivary glands from *A. americanum* (Mulenga et al., 2007; Porter et al., 2015), *A. maculatum* (Karim et al., 2011), *Amblyomma triste*, *Amblyomma parvum*, *Amblyomma cajennense* (Garcia et al., 2014), *Amblyomma variegatum* (Ribeiro et al., 2011), *Hyalomma marginatum rufipes* (Francischetti et al., 2011), *I. scapularis* (Valenzuela et al., 2002; Ribeiro et al., 2006; Mulenga et al., 2009), *I. ricinus* (Leboulle et al., 2002; Schwarz et al., 2013, 2014), *R. appendiculatus* (Mulenga et al., 2003a, b), *R. microplus* (Tirloni et al., 2014b), *R. haemaphysaloides* (Yu et al., 2013), *H. longicornis* (Sugino et al., 2003; Imamura et al., 2005), and *Antricola delacruzi* (Ribeiro et al., 2012). Accordingly, inhibitory tick serpins have been found and characterized in tick saliva, including *A. americanum* salivary serpin (AAS) 6 (Mulenga et al., 2007; Chalaire et al., 2011), a cross-class inhibitor of papain and trypsin-like proteases able to inhibit blood clotting and complement activation (Mulenga et al., 2013). A blood meal-induced salivary *I. scapularis* serpin has been shown to act upon thrombin and platelet aggregation (Ibelli et al., 2014). In related studies, an inhibitor of pro-inflammation proteases, elastase, cathepsin G and chymase was found in *I. ricinus* (Prevot et al., 2006, 2009; Chmelar et al., 2011). Similarly, *I. ricinus* serpin Iris2 inhibited inflammation by inhibiting cathepsin G and chymase (Chmelar et al., 2011). *Rhipicephalus haemaphysaloides* has two serpins which are able to inhibit chymotrypsin (Yu et al., 2013). In another study, Rodriguez-Valle et al. (2012) reported a characterization of *R. microplus* serpin-3, an inhibitor of trypsin and

thrombin that is recognized by naturally tick-infested bovine serum, and antibodies against an epitope of this protein impairs tick fertility. Indeed, tick vaccine efficacy studies showed tick-feeding efficiency is reduced when *H. longicornis* (Sugino et al., 2003; Imamura et al., 2005), *R. appendiculatus* (Imamura et al., 2006, 2008), *R. microplus* (Jittapalapong et al., 2010), and *I. ricinus* (Prevot et al., 2007) serpins are used as antigen. The goal of the present study was to characterize the role(s) of *A. americanum* tick serpin-19 (AAS19) in tick feeding success. This study demonstrates that AAS19 is a potential target for development of a universal tick vaccine that is effective against more than one tick species.

2. Materials and methods

2.1. Ethics statement

All animal work was conducted and approved according to the Texas A&M University, USA, Institutional Animal Care and Use Committee (AUP 2011–0207).

2.2. Tick feeding, dissections, total RNA extractions and cDNA synthesis

Amblyomma americanum ticks were purchased from the tick laboratory at Oklahoma State University (Stillwater, OK, USA). Routinely, ticks were fed on rabbits according to animal use protocols approved by the Texas A & M University Institutional Animal Care and Use Committee. Feeding was performed as previously described (Mulenga et al., 2013; Kim et al., 2014a). *Amblyomma americanum* ticks were restricted to feed on the outer part of the ear of New Zealand rabbits with orthopedic stockinets glued with Kamar Adhesive (Kamar Products Inc., Zionsville, IN, USA). Six male ticks were pre-fed for 3 days prior to introducing 15 female ticks in each of the ear stockinets (total of 30 female ticks per rabbit).

Ticks were collected and dissected as previously described (Mulenga et al., 2013). Five ticks were manually detached every 24 h for 5 days (24 – 120 h). Within the first hour of detachment, tick mouthparts were inspected to remove remnant tissue and washed in RNase inhibitor diethylpyrocarbonate (DEPC)-treated water to prepare for dissection. Dissected tick organs, salivary glands (SG), midgut (MG), ovary (OV), synganglion (SYN), Malpighian tubules (MT) and carcass (CA, the remnants after removal of other organs) were placed in RNA Later (Life Technologies, Carlsbad, CA, USA) or 1 mL of Trizol total RNA extraction reagent (Life Technologies) and stored at –80°C until total RNA extraction.

Total RNA was extracted using the Trizol reagent according to the manufacturer's instructions (Life Technologies) and re-suspended in DEPC-treated water. Total RNA was quantified using a UV-VIS Spectrophotometer DU-640B (Beckman Coulter, Brea, CA, USA) or the Infinite M200 Pro plate reader (Tecan, Männedorf, Switzerland). Up to 1 µg of total RNA was used to synthesize cDNA using the Verso cDNA Synthesis Kit following the manufacturer's instructions (Thermo Scientific, Waltham, MA, USA).

2.3. Structural alignment, amino acid motif scanning and comparative modeling

To gain an insight into the relationship of AAS19 (GenBank accession number: **GAYW01000076**; Porter et al., 2015) protein from the *A. americanum* tertiary structure compared with its homologs in other tick species, amino acid sequences from UniProt in

other tick species (*Rhipicephalus pulchellus* (**L7LRY7**), *I. ricinus* (**V5IHU3**), *A. maculatum* (**G3ML49** and **G3ML50**), *R. microplus* (**V9VP22**), *Ix. scapularis* (**B7QJF1**), *A. triste* (**A0A023GPF9**) and *A. cajenense* (**A0A023FM57**)) were subjected to structure-based ClustalW alignment using the AAS19 tertiary structure as a template. The alignment sequences were subsequently viewed using GeneDoc software (<http://www.nrbsc.org/gfx/genedoc/ebinet.htm>). Additionally, the AAS19 amino acid sequence was manually inspected for annotated glycosaminoglycan (GAG) binding motifs as previously reviewed (Hileman et al., 1998). To determine potential N- or O-linked glycosylation sites, AAS19 amino acid was scanned using NetNGlyc 1.0 and NetOGlyc 4.0 servers (www.cbs.dtu.dk).

The three-dimensional (3-D) structure of AAS19 was predicted using a comparative modeling approach. The serpin protein C inhibitor structure (2OL2) (Li and Huntington, 2008) was retrieved from the Protein Data Bank (PDB) (<http://www.rcsb.org>) and used as a molecular template for AAS19 modeling based on 30% and 53% sequence identity and similarity, respectively. Sequence alignments were generated using the ClustalW algorithm (Larkin et al., 2007) and used as input in the Modeller 9v14 program (Webb and Sali, 2014). Models generated were evaluated using QMEAN4 and PROCHECK to estimate model reliability and predict quality (Morris et al., 1992; Benkert et al., 2008). The electrostatic potential of AAS19 and protein C inhibitor (2OL2, positive control template) was calculated using two approaches. First, the Adaptive Poisson-Boltzmann Solver (APBS) was used and protonation states were assigned using the parameters for solvation energy (PARSE) force field for each structure by PDB2PQR (Unni et al., 2011). Execution of APBS and visualization of resulting electrostatic potentials were performed by using the Visual Molecular Dynamics (VMD) program (Humphrey et al., 1996) at ± 5 -kT/e of positive and negative contour fields. In the second approach, electrostatic potential was computed using APBS and visualized in the SWISS PDB viewer (Guex and Peitsch, 1997) (<http://www.expasy.org/spdbv/>) set to default parameters.

2.4. Temporal and spatial quantitative reverse transcription (qRT)-PCR transcription analyses of AAS19

Transcription analysis was done using a two-step quantitative (q) reverse transcription (RT)-PCR using the Applied Biosystems 7300 Real Time PCR System (Life Technologies) as described (Kim et al., 2014a). AAS19-specific qRT-PCR forward ($5'$ GACAAGACGCACGGCAAAA $3'$) and reverse ($5'$ GAAGTCCGGCGGCTCAT $3'$) primers were used to determine transcript abundance in triplicate pools of cDNA of dissected SG, MG and remnant tissues as CA of unfed, 24 and 48 h fed ($n = 15$ ticks per pool), 72 and 96 h fed ($n = 10$ ticks per pool) and 120 h fed ticks ($n = 5$ ticks). Cycling conditions were as follows: stage one at 50°C for 2 min, stage two at 95°C for 10 min, and stage three contained two steps with 40 cycles of 95°C for 15 s and 60°C for 1 min. Reaction volumes, in triplicate, contained 10-fold diluted cDNAs that were originally synthesized from 1 μg of total RNA, 350 nM of forward and reverse AAS19 primers, and 2X SYBR Green Master Mix (Life Technologies). For an internal reference control, forward ($5'$ GGCGCCGAGGTGAAGAA $3'$) and reverse ($5'$ CCTTGCCGTCCACCTTGAT $3'$) primers targeting the 40S ribosomal protein S4 (RPS4; accession number **GAGD01011247.1**), which is stably expressed in *I. scapularis* during feeding (Koci et al., 2013), was used.

Relative quantification (RQ) of AAS19 transcript was determined using the comparative C_T (2^{-C_T}) method as previously described (Livak and Schmittgen, 2001) and adapted in Kim et al. (2014a). The data was presented as the percent mean (M) transcript abundance $\pm$ S.E.M. per tissue type.

2.5. Expression and affinity purification of recombinant AAS19

Recombinant (r) AAS19 protein was expressed using the *Pichia pastoris* and pPICZ α plasmid expression system (Life Technologies) as described previously (Mulenga et al., 2013; Kim et al., 2014b). A mature AAS19 protein open reading frame (Porter et al., 2015) was sub-cloned into pPICZ α *Eco*RI and *Not*I restriction sites using forward (5'GAATTCGACAGAGCCCGACGAAGATGGC3') and reverse (5'GCGGCCGCGAGGGCGTTAATTTTCGCCAG3') primers with added restriction enzyme sites in bold. The pPICZ α -AAS19 expression plasmid was linearized with *Pme*I and electroporated into the *Pichia pastoris* X-33 strain (Life Technologies) using an ECM600 electroporator (BTX Harvard Apparatus Inc., Holliston, MA, USA) with parameters set to 1.5 kV, 25 μ F and 186 Ω . Transformed colonies were selected on Yeast Extract Peptone Dextrose Medium with Sorbitol (YPDS) agar plates with zeocin (100 μ g/ μ l), and methanol utilization on Minimal Methanol (MM) agar plates, both incubated at 28°C. Positive transformants were inoculated in buffered glycerol-complex medium (BMGY) and grown overnight at 28°C with shaking (230–250 rpm). Subsequently, the cells were used to inoculate buffered methanol-complex medium (BMMY) to A_{600} of 1 after which protein expression was induced by adding methanol to 0.5% final concentration every 24 h for 5 days. rAAS19 in spent culture media was precipitated by ammonium sulfate saturation (525 g/L of media) with stirring overnight at 4°C. The precipitate was pelleted at 11,200 g for 1 h at 4°C and re-suspended in, and dialyzed against, 20 mM Tris-HCl buffer pH 7.4. To verify expression of rAAS19, western blotting analysis was performed using the horseradish peroxidase (HRP)-labeled antibody to the C-terminal hexahistidine tag (Life Technologies) diluted to 1:5000 in 5% blocking buffer (5% skim milk powder in PBS with Tween-20). The positive signal was detected using a metal-enhanced DAB chromogenic substrate kit (Thermo Scientific). Subsequently, rAAS19 was affinity purified under native conditions using Hi-Trap Chelating HP Columns (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). Affinity purified putative rAAS19 was dialyzed against 20 mM Tris-HCl buffer pH 7.4 for downstream assays. To verify purity and background contamination, affinity purified rAAS19 was resolved on a 10% SDS-PAGE gel and silver stained. Samples with the least background were selected and concentrated by either ammonium sulfate precipitation or by centrifugation using MicroSep Centrifugal Concentration Devices (Pall Corporation, Port Washington, NY, USA).

2.6. N- and O- linked deglycosylation assay

Preliminary amino acid sequence analyses predicted N- and O-linked glycosylation sites in AAS19. To determine whether rAAS19 was N-glycosylated and/or O-glycosylated, 5 μ g of affinity purified rAAS19 were treated with protein deglycosylation enzyme mix according to the manufacturer's instructions (New England Biolabs, Ipswich, MA, USA). Deglycosylation was verified by western blotting analysis using the antibody to the C-

terminal hexahistidine tag (Life Technologies) and the positive signal detected using HRP chromogenic substrate (Thermo Scientific).

2.7. Determining whether native AAS19 is injected into the host during tick feeding

To determine whether native AAS19 is injected into the host during feeding, glycosylated and deglycosylated affinity-purified rAAS19 was subjected to routine western blotting analyses using antibodies to replete-fed *A. americanum* tick saliva proteins. The antibodies to replete-fed tick saliva proteins were produced as previously described (Mulenga et al., 2013).

2.8. Protease inhibitor (PI) profiling

Inhibitory activity of rAAS19 was tested against a panel of 16 mammalian serine proteases related to host defense pathways against tick feeding. Mammalian proteases (per reaction) tested were: bovine thrombin (43 U), pancreatic porcine elastase (21.6 nM), pancreatic bovine trypsin (24.6 nM), pancreatic bovine α -chymotrypsin (96 nM), pancreatic porcine kallikrein (33 U), human chymase (10 U), human tryptase (10 U), human plasmin (10 nM) (Sigma-Aldrich, St. Louis, MO, USA), human neutrophil cathepsin G (166 nM) (Enzo Life Sciences Inc., Farmingdale, NY, USA), human factor (f) XIa (3.68 nM), bovine fIXa (306 nM), human fXIIa (7.6 nM), human t-PA (32 nM), human u-PA (47.2 nM) (Molecular Innovations, Inc., Novi, MI, USA), fXa (5.8 nM) (New England Biolabs) and proteinase-3 (68 U) (EMD Millipore, Billerica, MA, USA). Substrates were used at 0.20 mM final concentration and purchased from Sigma-Aldrich: N α -benzoyl-DL-Arg-pNA for tryptase, N-Succinyl-Ala-Ala-Pro-Phe-pNA for chymase, cathepsin G and chymotrypsin, N-Benzoyl-Phe-Val-Arg-pNA for thrombin and trypsin, N-Succinyl-Ala-Ala-Ala-p-nitroanilide for elastase. The following substrates were purchased from Diapharma Inc. (Philadelphia, PA, USA): Bz-Ile-Glu(γ -OR)-Gly-Arg-pNA for fXa, H-D-Val-Leu-Lys-pNA for plasmin, and H-D-Pro-Phe-Arg-pNA for kallikrein, fXIa and fXIIa. The substrate CH₃SO₂-D-CHG-Gly-Arg-pNA was purchased from Molecular Innovations and used for fIXa, u-PA and t-PA. The substrate N-Methoxysuccinyl-Ala-Ala-Pro-Val-pNA was purchased from Enzo Life Sciences and used for proteinase-3.

Reagents were mixed at room temperature in triplicate. One μ M of rAAS19 was pre-incubated with amounts of the enzyme listed above for 15 min at 37°C in 20 mM Tris-HCl, 150 mM NaCl, BSA 0.1%, pH 7.4 buffer. The corresponding substrate for each enzyme was added in a 100 μ L final reaction volume and substrate hydrolysis was measured at A_{405nm} every 11 s for 30 min at 30°C using the Infinite M200 Pro plate reader (Tecan). Acquired A_{405nm} data were subjected to one-phase decay analysis in Prism 6 software (GraphPad Software, La Jolla, CA, USA) to determine plateau values as proxies for initial velocity of substrate hydrolysis ($V_{\max}$) or residual enzyme activity. The percent enzyme activity inhibition level was determined using the formula: $100 - V_{\max}(V_i)/V_{\max}(V_0) \times 100$ where $V_{\max}(V_i)$ = activity in presence of, and $V_{\max}(V_0)$ = activity in absence of rAAS19. Data are presented as mean $\pm$ S.E.M. of triplicate readings.

2.9. Stoichiometry of inhibition (SI)

We determined stoichiometry of inhibition (SI) indices against five proteases (trypsin, plasmin, fXa, fXIa and fXIIa) that were inhibited by more than 80% in the PI profiling assay described in Section 2.8. Different molar ratios (serpin:protease) of rAAS19 were pre-incubated for 1 h with a constant concentration of trypsin (10 nM), fXa (5 nM), fXIa (5 nM) and plasmin (10 nM). The residual enzyme activity was measured using colorimetric substrates specific for each enzyme as described in Section 2.8. The data were plotted as the residual activity (V_i/V_0) versus the inhibitor to enzyme molar ratio. SI, or the molar ratio of rAAS19 to protease when enzyme activity is completely inhibited, was determined by fitting data onto the linear regression line in PRISM.

2.10. rAAS19 and protease complex formation

In varying molar ratios, affinity purified rAAS19 was incubated with trypsin, plasmin, fXa and fXIa, in Tris-HCl reaction buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.4) for 1 h at 37°C. Denaturing sample buffer was added to the reaction mix and incubated at 99.9°C for 5 min in a thermocycler. Samples were subjected to SDS-PAGE electrophoresis on a 12.5% acrylamide gel and stained with Coomassie brilliant blue using routine protocols.

2.11. Anti-platelet aggregation function of rAAS19

Anti-platelet aggregation function(s) of rAAS19 was determined using platelet rich plasma (PRP) isolated from citrated (acid citrate dextrose) whole bovine blood (WBBL) as previously described (Horn et al., 2000; Berger et al., 2010). To prepare PRP, freshly citrated WBBL was centrifuged at 200 g for 20 min at 18°C. Subsequently, the PRP (top layer) was transferred into a new tube and centrifuged at 800 g for 20 min at 18°C. The pellet containing platelets was washed and diluted with Tyrode buffer, pH 7.4 (137 mM NaCl, 2.7 mM KCl, 12 mM NaHCO₃, 0.42 mM Na₂HPO₄, 1 mM MgCl₂, 0.1% glucose, 0.25% BSA), until $A_{650} = 0.15$. To determine anti-platelet aggregation function, various amounts of rAAS19 (1, 0.5, 0.25 and 0 µM) were pre-incubated for 15 min at 37°C with agonist 10 NIH-U thrombin, 20 µM ADP or 0.7 µM cathepsin G in a 50 µL reaction. Adding 100 µL of pre-warmed PRP triggered platelet aggregation. Vice versa, PRP was pre-incubated with various amounts of rAAS19 prior to addition of the agonist. Platelet aggregation was monitored every 20 s for 30 min at A_{650nm} using the Infinite M200 Pro plate reader (Tecan). In this assay, a higher O.D. was observed in our blank control (platelet only), and increased platelet aggregation was correlated with a reduction in O.D. To determine the percentage of platelet aggregation inhibition, O.D. data was fitted to the one-phase decay equation in PRISM (GraphPad) to determine the plateau O.D. The percent reduction in platelet aggregation was calculated using this $Y = 100 - (\Delta P_n) / \Delta P_C * 100$, where Y = % reduction in platelet aggregation, P_n and P_C = a respective rAAS19-treated and positive control plateau O.D. subtracted from the blank plateau O.D. Data are presented as mean ± S.E.M. of duplicate platelet aggregation assays.

2.12. Anti-blood clotting function of rAAS19

The effect of rAAS19 against blood clotting was assessed using modified recalcification time (RCT), activated partial thromboplastin time (aPTT), thrombin time (TT) and

prothrombin time (PT) to, respectively, measure the effect on the entire blood cascade, the intrinsic, extrinsic and common blood clotting activation pathways, as described (Mulenga et al., 2013). All assays were done in duplicate, with clotting time monitored at $A_{650\text{nm}}$ using the Infinite M200Pro plate reader (Tecan) set to 37°C. In these assays, clot formation was directly proportional to an increase in O.D., and results presented as mean $\pm$ S.E.M.

In the RCT assay, 50 μL of universal coagulation reference human plasma (UCRP) (Thermo Scientific) were pre-incubated with 10, 5, 1.25, 0.625, 0 μM rAAS19 in 90 μL of Tris-HCl reaction buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.4) for 15 min at 37°C. Adding 10 μL of pre-warmed 150 mM calcium chloride (CaCl_2) triggered plasma clotting. Plasma clotting was monitored at every 20 s for 30 min.

In the aPTT assay, various amounts of rAAS19 (indicated above) were pre-incubated for 15 min at 37°C with 50 μL of UCRP diluted to 100 μL with 20 mM Tris-HCl, 150 mM NaCl, pH 7.4. Subsequently, 50 μL of the aPTT reagent (Thermo Scientific) was added to plasma and incubated at 37°C for an additional 5 min to activate the reaction. Addition of 50 μL of 25 mM CaCl_2 to the reaction triggered clotting, and clot formation was monitored every 10 s for 5 min.

In the TT assay, various amounts of rAAS19 (indicated above) were pre-incubated at 37°C for 15 min with 25 μL of the TT reagent (Thermo Scientific) containing CaCl_2 in 50 μL of Tris-HCl reaction buffer with 20 mM Tris-HCl, 150 mM NaCl, pH 7.4. Adding 50 μL of pre-warmed UCRP started plasma clotting and clotting time was monitored every 10 s for 5 min.

In the PT assay various rAAS19 amounts as above were pre-incubated at 37°C for 15 min with 100 μL of PT reagent (Thermo Scientific) containing CaCl_2 diluted up to 150 μL with 20 mM Tris-HCl, 150 mM NaCl, pH 7.4 reaction buffer. Adding 50 μL of the pre-warmed UCRP (Thermo Scientific) triggered plasma clotting and clotting time was monitored every 10 s for 5 min.

2.13. Statistical analysis

Statistical software packages in PRISM version 6 (GraphPad Software Inc.) were used.

3. Results

3.1. AAS19 tertiary structure retains features of a typical serpin

AAS19 amino acid sequence is homologous to serpins from other tick species, being that its functional domain reactive center loop (RCL) is 100% conserved among the majority of ixodid tick species (Porter et al., 2015). In this study we conducted comparative amino acid motif and secondary structure analyses to determine the relationship of AAS19 to its homologues in other tick species (Fig. 1). Comparative modeling using protein C inhibitor tertiary structure (2OL2) as a template showed that AAS19 predicted tertiary structure retains the typical serpin fold formed by nine α -helices, three β sheets (five strands each in βsA and βsB , and three strands in βsC) (Figs. 1, 2) (Gettins, 2002). Amino acid motif scanning analyses showed conservation of the “RGD” motif (positions 40 – 42, Fig. 1),

which is the binding site to integrin GPIIb-IIIa (Srinivasan et al., 2010; Nurden, 2014). Additionally, two N-glycosylation sites at positions 97 – 99 and 198 – 200 in AAS19 and in the majority of its homologues are conserved as noted with “#” symbol below the sequence (Fig. 1). Visual inspection of the alignment in Fig. 1 revealed seven clusters of basic amino acid residues that show similarity to annotated GAG binding sites (BS) (reviewed in Hileman et al., 1998). The seven clusters of basic residues (basic residues in bold, amino acid positions based on structural alignment in Fig. 1) in AAS19 are:

[⁵⁹**KVLALFREQLDASR**⁷²],
 [¹¹⁷**KKGEEAVEKINNWVSDKTHGKIRR**¹⁴⁰],
 [¹⁵¹**RLILLNAVYYKGTWLYEFNKARTKPR**¹⁷⁶],
 [¹⁸⁵**KVLVPMMKMK**¹⁹⁴],
 [²²⁹**RNGLEHLKSVLTTQTLNRAISRMYPKDMKFRMPKLKLDTKYTLK**²⁷²],
 [²⁸¹**KKKIFSADADLSGISGAKNLYVSDVLHK**³⁰¹] and
 [³⁴³**KVYVDHPFIFLIR**³⁵⁵].

To gain further insight regarding the possibility that clusters of basic amino acid residues in AAS19 form basic patches, we calculated surface electrostatic potentials on the AAS19 model (Fig. 2B). This analysis predicts that [K¹³⁷-R¹³⁹-R¹⁴⁰-K¹⁶¹-K²⁶⁴-K²⁶⁸-K³⁰⁸], [K⁸-K²³⁶-R²⁴⁶-R²⁵⁰], [R¹⁷²-K¹⁷⁴-R¹⁷⁶-K¹⁸⁵-K¹⁹²-K¹⁹⁴-K³⁴³], and [K¹¹⁷-K¹¹⁸-R¹⁵¹-K²⁷²-K²⁹⁸], respectively, interact to form putative GAG binding sites (GAGBS) 1 – 4 on the AAS19 surface (Fig. 2B). This methodology was able to recover the GAGBS in PCI, a protein whose GAGBS was previously described (Fig. 2A) (Li and Huntington, 2008).

3.2. AAS19 mRNA is expressed in several tick tissues during feeding

To determine the relationship of AAS19 transcription with the *A. americanum* tick-feeding phase, we determined its spatial and temporal transcription profile. Fig. 3 shows AAS19 mRNA is expressed during blood meal and in unfed ticks. AAS19 is transcribed in SG (Fig. 3A), MG (Fig. 3B) and other tissues (eg. CA) (Fig. 3C). AAS19 transcription in the SG does not change from unfed to 120 fed ticks. Contrarily in MG and CA,, AAS19 transcription increases (RPS4 was used for normalization) with feeding (Fig. 3B, C).

3.3. AAS19 protein is injected into the host during feeding

Fig. 4A and B summarize expression and affinity purification of rAAS19 in *P. pastoris*. To verify expression, daily samples of 1 mL of yeast spent media were concentrated by ammonium sulfate saturation and subjected to western blotting analysis using a specific antibody to the C-terminal histidine tag (as summarized in Fig. 4A). For large-scale expression, ammonium sulfate-precipitated rAAS19 was affinity purified under native conditions and purity checked by SDS-PAGE with silver staining (Fig. 4B). Fractions W2, E1, E2 and E4 in Fig. 4B, the fractions that showed the smallest amount of contaminating protein, were pooled, concentrated and dialyzed in appropriate buffer by spin columns and used for further assays. Fraction E3 was used in western blotting analysis assays summarized in Fig. 4C – E. As shown in Fig. 4C, a 1:50 dilution of rabbit pre-immune

serum did not recognize rAAS19, while a 1:250 dilution of antibodies against saliva proteins of fully engorged *A. americanum* (Fig. 4B), and against the C-terminal histidine tag (Fig. 4E) recognize both glycosylated (lane 1) and de-glycosylated (lane 2) rAAS19. In Fig. 4D and E, there is smearing in lane 1 but not in lane 2. Additionally, there is a downward molecular weight shift in lane 2. This shift in molecular weight and disappearance of smears following de-glycosylation validates that rAAS19 is glycosylated.

3.4. rAAS19 is a broad spectrum inhibitor of trypsin-like proteases

Protease inhibition profiling was done against 16 mammalian proteases that regulate host defenses against tick feeding such as hemostasis, wound healing/tissue remodeling and inflammatory response (Table 1). Molar excess of rAAS19 inhibited the activity of trypsin and trypsin-like proteases associated with hemostasis (Fig. 5). Pre-incubation of 1 μ M rAAS19 inhibited, respectively, approximately 80, 82, 86, 95 and 98% the activity of plasmin (34.8 nM), fXIIa (7.62 nM), fXa (5.81 nM), fXIa (3.68 nM) and trypsin (24.6 nM) (Fig. 5A, B). Additionally, rAAS19 inhibits thrombin (43 U), tryptase (10 U), fXIa (30.6 nM) and chymotrypsin (96 nM) by approximately 13, 16, 20 and 28%, respectively.

To evaluate rAAS19 inhibitory efficiency, we determined the SI of proteases that were inhibited by more than 80% by rAAS19. Fig. 6A, C, E and G show the kinetics of the hydrolysis of specific substrates by these enzymes in the absence (a) and in the presence (b – g) of rAAS19 at various concentrations. These data were fitted to a linear regression, as shown in Fig. 6B, D, F and H. The rAAS19 SI index was estimated from the X-axis intercept ($Y = 0$) for trypsin, plasmin, fXa and fXIa at 5, 9, 23 and 28, respectively. The SI index for fXIIa was estimated at 103 (not shown). The rAAS19 mechanism of action as a typical inhibitory serpin was confirmed by the inability of heating and SDS (Huntington et al., 2000) to dissociate it from trypsin (Fig. 7A), fXa (Fig. 7B) and fXIa (Fig. 7C). Irreversible complex between rAAS19 and the target enzyme was observed only at inhibitor enzyme molar ratios (Fig. 7A lanes 1 – 3, Fig. 7B lanes 1 – 2 and Fig. 7C lanes 1 – 6) where enzyme activity inhibition was observed (Fig. 6A, E, G).

3.5. rAAS19 inhibits thrombin-triggered platelet aggregation

PRP was used in platelet aggregation assays using three agonists: thrombin (0.03 U/ μ L), ADP (20 μ M) and cathepsin G (0.7 μ M). rAAS19 inhibited thrombin activated platelet aggregation (Fig. 8). In data shown in Fig. 8A, we did not observe platelet aggregation without addition of the agonist (a). However with platelets activated by thrombin that was pre-incubated with 1 (b), 0.5 (c), 0.25 (d) and 0 μ M (e), rAAS19 showed variable levels of platelet aggregation. Pre-incubating 3U of thrombin with 1 μ M and 0.5 μ M rAAS19, platelet aggregation was reduced by 56% and 27%, respectively, while at 0.25 μ M rAAS19 did not have any effect (Fig. 8B). The rAAS19 did not have any effects on ADP and cathepsin G activated platelet aggregation. On the other hand, pre-incubation of rAAS19 with platelets prior to addition of the agonist did not affect platelet aggregation.

3.6. rAAS19 delays plasma clotting

Plasma clotting time (CT), RCT, activated aPTT and TT were delayed by rAAS19 in a dose-response manner (Fig. 9). However, rAAS19 did not affect PT. In the RCT assay, to

measure the effect of on the entire blood clotting activation system, 0.625, 1.25 and 5 μM (arrowhead R-CT2), and 10 μM (arrowhead R-CT3) rAAS19 dose responsively delayed RCT-CT by ~100 and 250 s compared with CT in the absence of rAAS19 (arrowhead R-CT1, 0 μM) (Fig. 9A). In the aPTT assay to measure the effect on the intrinsic blood clotting activation pathway (Fig. 9B), 0.625 and 1.25 μM rAAS19 did not have any effect (arrowhead A-CT1), while 5 and 10 μM rAAS19 delayed aPTT-CT by ~20 (A-CT2) and 40 (A-CT3) s, respectively. In the TT assay (Fig. 9C) 0.625 μM rAAS19 was not different from the control (arrowhead T-CT1), while 1.25 and 5 μM rAAS19 (arrowhead T-CT2), and 10 μM rAAS19 (arrowhead T-CT3) delayed clotting by ~180 and 250 s, respectively.

4. Discussion

This study was prompted by the characteristic high conservation of AAS19 in all ixodid tick species, for which data are available (Porter et al., 2015). We were particularly interested in AAS19 due to its functional domain RCL being 100% conserved in other ixodid ticks, suggesting this serpin has a role in regulating proteolytic pathways crucial to all ixodid tick species. The observation that AAS19 motifs and secondary structure are conserved in AAS19 homologues in several tick species further suggests this protein has the potential to regulate processes that are important to ixodid ticks.

In proposing that ticks use serpins to evade host defense mechanisms, the assumption is that ticks inject serpins into the host during feeding. Although several serpin cDNAs have been cloned, only a few reports confirm that saliva actually contains serpins (Mulenga et al., 2013; Ibelli et al., 2014; Mudenda et al., 2014; Tirloni et al., 2014a). Thus, observations in this study demonstrating native AAS19 is injected into the host during tick feeding and its activity inhibiting proteases participating in host defenses reinforces the idea that inhibitory serpins have a role in the tick-host relationship. Based on the rAAS19 inhibitory activity against trypsin and trypsin-like proteases including plasmin and blood clotting factors Xa and XIa, we conclude this serpin inhibits proteases involved in host defense mechanisms against tick feeding. Albeit at a low rate, it is notable that rAAS19 also inhibits other blood clotting enzymes including fXIIa, fIIa (thrombin) and fIXa, as well as chymotrypsin and trypsin, further confirming that AAS19 has a broad spectrum of inhibitory functions. Although AAS19 inhibits these enzymes, we are cautious about these enzymes being physiological targets for AAS19 because the SI indices estimated in this study were not near 1:1, as would be expected for an inhibition by serpins (Irving et al., 2000; Silverman et al., 2001). However, we note here that the observed high SI indices are not unique for rAAS19. For instance, a SI index of 10 was estimated for *Drosophila melanogaster* Spn1, which inhibits trypsin activity by 96% (Fullaondo et al., 2011). A high SI index could also be explained by the possibility that AAS19 requires a co-factor to enhance inhibition. Indeed, nearly all serpin acting upon the blood clotting activation cascade and fibrinolysis (antithrombin III, heparin-cofactor II, plasminogen activator inhibitor 1, protein C inhibitor and protease nexin inhibitor) need to bind GAGs in order to accelerate the inhibition rate, which can be up to 10,000-fold higher compared with the unbound native serpin (Rein et al., 2011). Accordingly, Z-protein-dependent inhibitor (ZPI) has been shown to accelerate the inhibitory effect of ZPI upon fXa by 1000-fold (Broze, Jr., 2001; Munoz and Linhardt, 2004). Our demonstration that AAS19 has four putative GAGBSs suggests its activity could

be modulated by heparin or another GAG. Studies to investigate the effects of heparin on rAAS19 function are underway.

From the perspective of tick feeding physiology, the AAS19 inhibition activity upon trypsin, fXa and fXIa could be rationally explained. Trypsin, mostly known for its role as a digestive enzyme (Rawlings and Barrett, 1994), is also associated with inflammation in the skin (Cattaruzza et al., 2014; Meyer-Hoffert et al., 2004), while blood clotting factors Xa and XIa are critical proteases of the blood clotting activation cascade (Monroe and Hoffman, 2006; Roberts et al., 2006). On the other hand, AAS19 inhibition of plasmin can, at a glance, be viewed as contradictory since plasmin is mostly known for its role in digesting blood clots (fibrinolysis) (Angles-Cano, 1994; Angles-Cano et al., 1994), an activity that seems beneficial to tick feeding. Similarly, plasmin degrades and inactivates blood-clotting factors V, VIII, IX and X in vitro, suggesting plasmin has anti-coagulant functions (Hoover-Plow, 2010) and is thus beneficial to tick feeding. On the other hand, plasmin has been reported to participate in several processes such as pro-inflammatory cytokine release (Syrovets et al., 2001), inducing monocyte and dendritic cell chemotaxis (Li et al., 2010), modifying IL-8 and producing a potent attractant of neutrophils (Mortier et al., 2011), tissue remodeling and wound healing (Shen et al., 2012), all of which can negatively impact tick-feeding success. From this perspective, AAS19 inhibition of plasmin seems to contribute to feeding on blood.

Given that AAS19 is injected into the host during tick feeding and due to its inhibitory activity on blood clotting factors, we investigated its action on platelet aggregation and plasma clotting. Platelet aggregation is important to stop bleeding in injured small blood vessels, as observed in tick feeding (Francischetti et al., 2009). Platelet aggregation is activated by multiple agonists including thrombin, collagen, ADP and cathepsin G (Selak et al., 1988; Ohlmann et al., 2000; Brass, 2003). Since thrombin is considered the most efficient platelet aggregation agonist (Furman et al., 1998; Davi and Patrono, 2007), the observation that rAAS19 reduced thrombin-activated platelet aggregation by up to $56 \pm 14\%$ suggests that AAS19 may play a crucial role in tick modulation of platelet function. Thrombin activates platelet aggregation through activation of Protease Activated Receptors (PARs) 1, 3 and 4 (Kahn et al., 1999; Asokanathan et al., 2002), and thus it is plausible that native AAS19 may contribute to binding and inhibiting thrombin at the tick feeding site, preventing the formation of the platelet plug and thus impairing the integrity of the blood clot. The observation that pre-incubating rAAS19 with platelets for 15 min prior to addition of the agonist had minimal effect suggests that the observed rAAS19 anti-platelet aggregation effects were mediated through inhibition of thrombin activity. However, it is interesting to note that the “RGD” motif, which is known to interfere with platelet aggregation binding the integrin GPIIb-IIIa on activated platelets (Varon et al., 1993; Katada et al., 1997) is conserved in AAS19 and its homologues. If this motif was functional, pre-incubation with platelets should interfere with platelet aggregation function.

Platelet aggregation is followed by fibrin clot formation, which reinforces the platelet plug (Clemetson, 2012). Blood clotting can be activated via the extrinsic pathway initiated upon release of tissue factor from injured tissue or via the intrinsic pathway when factors XII, XI, IX and VIII are converted into their active forms. These pathways converge into the common pathway when fXa activates prothrombin to thrombin, which in turn catalyzes

formation of the fibrin clot (Hoffman and Monroe, III, 2001; Hoffman, 2003; Monroe and Hoffman, 2006). The effects of anti-blood clotting agents are routinely addressed by using RCT assays, while effects on the extrinsic, intrinsic and common blood clotting activation pathways are examined using PT, aPTT, and TT assays (Davie et al., 1991), as were done in this study. Eight of the 11 blood clotting factors are serine proteases (Walsh and Ahmad, 2002). Thus, although empirical evidence is needed, it is conceivable that the observed rAAS19 anti-coagulant function could be attributed to its inhibitory activity against five (fIIa, fIXa, fXa, fXIa and fXIIa) of the eight serine protease mediators of the blood clotting activation cascade. Except for fVIII, the other four blood clotting factors (IXa, Xa, XIa, and XIIa) in the intrinsic pathway (aPTT assay) are serine proteases, all of which were inhibited by rAAS19, and this could explain the observation of up to 40 s plasma clotting time delay in the aPTT assay. Similarly, in the TT (common pathway) assay, the observed 250 s delay in plasma clotting time could be explained by rAAS19 inhibitory activity against thrombin. The discrepancy of 250 s delay in the TT assay and ~14% inhibition of thrombin activity in substrate hydrolysis is interesting and raises questions for further investigation. There is a possibility that the observed significant clotting time delay in the TT assay could be explained by a possibility of a yet unknown factor in host serum that potentiates AAS19 anti-thrombin activity. Given that AAS19 has four putative heparin binding sites, it is conceivable that GAGs in serum binding to AAS19 could potentiate its activity against thrombin. Experiments to resolve this question are underway.

We would like to note here that arthropods do have hemolymph-clotting cascades that are also controlled by serpins (Agarwala et al., 1996; Iwanaga et al., 1998; Kanost, 1999). Thus, there is a possibility that native AAS19 could be a well-conserved regulator of the tick's hemolymph clotting in itself and the observed anti-coagulant function in this study could be an artifact. However, the observation in this study that native AAS19 was injected into the host during tick feeding strongly suggests the role of this protein at the tick-feeding site. Alternatively, AAS19 may function both in the tick and at the tick-feeding site. It is important to note here that based on transcript abundance, AAS19 is predominant in the tick MG, which could strongly indicate a role for this protein in the MG. Tick digestion of the blood meal nutrients is intracellular (Lara et al., 2005; Franta et al., 2010), and for this to happen, host blood must remain in the unclotted state. Could it be that AAS19 anti-coagulant function is part of the mechanism in the MG to prevent host blood from clotting before digestion commences?

In conclusion, data in this study contribute towards our understanding of the molecular basis of tick feeding physiology. Data here contribute to the growing list of tick saliva proteins that potentially regulate tick evasion of the mammalian host's defense mechanisms to tick feeding as well as having role(s) in other tick physiological systems. On the basis of AAS19 being highly conserved, coupled with the fact that its RCL is 100% conserved, we conclude that tick physiological processes regulated by AAS19 are evolutionary conserved in all tick species. From the perspective of our long-term interest to find target tick vaccine antigens, AAS19 represents an interesting candidate. Human and animal tick borne diseases are transmitted by different species of ticks, and thus developing universal tick vaccines based on highly conserved proteins such as AAS19 has been advocated (Fragoso et al., 1998;

Mulenga et al., 2001, 2013; de la Fuente and Kocan, 2006; Azhahianambi et al., 2009; Parizi et al., 2012). Work toward validating the tick vaccine efficacy of rAAS19 is warranted.

Acknowledgments

This research was supported by National Institutes of Health, USA grants (AI081093, AI093858, AI074789, AI074789-01A1S1) to AM. LT is a receiver of the CNPq (Brazil) Ciência sem Fronteiras doctoral fellowship program (PVE 211273/2013- 9).

References

- Agarwala KL, Kawabata S, Miura Y, Kuroki Y, Iwanaga S. Limulus intracellular coagulation inhibitor type 3. Purification, characterization, cDNA cloning, and tissue localization. *J Biol Chem.* 1996; 271:23768–23774. [PubMed: 8798603]
- Algermissen B, Hermes B, Feldmann-Boeddeker I, Bauer F, Henz BM. Mast cell chymase and tryptase during tissue turnover: analysis on in vitro mitogenesis of fibroblasts and keratinocytes and alterations in cutaneous scars. *Exp Dermatol.* 1999a; 8:193–198. [PubMed: 10389636]
- Algermissen B, Laubscher JC, Bauer F, Henz BM. Purification of mast cell proteases from murine skin. *Exp Dermatol.* 1999b; 8:413–418. [PubMed: 10536969]
- Anderson BE, Sims KG, Olson JG, Childs JE, Piesman JF, Happ CM, Maupin GO, Johnson BJ. *Amblyomma americanum*: a potential vector of human ehrlichiosis. *Am J Trop Med Hyg.* 1993; 49:239–244. [PubMed: 8357086]
- Anderson JM, Sonenshine DE, Valenzuela JG. Exploring the mialome of ticks: an annotated catalogue of midgut transcripts from the hard tick, *Dermacentor variabilis* (Acari: Ixodidae). *BMC Genomics.* 2008; 9:552. [PubMed: 19021911]
- Angles-Cano E. Overview on fibrinolysis: plasminogen activation pathways on fibrin and cell surfaces. *Chem Phys Lipids.* 1994; 67–68:353–362.
- Angles-Cano E, Hervio L, Rouy D, Fournier C, Chapman JM, Laplaud M, Koschinsky ML. Effects of lipoprotein(a) on the binding of plasminogen to fibrin and its activation by fibrin-bound tissue-type plasminogen activator. *Chem Phys Lipids.* 1994; 67–68:369–380.
- Asokanathan N, Graham PT, Fink J, Knight DA, Bakker AJ, McWilliam AS, Thompson PJ, Stewart GA. Activation of protease-activated receptor (PAR)-1, PAR-2, and PAR-4 stimulates IL-6, IL-8, and prostaglandin E2 release from human respiratory epithelial cells. *J Immunol.* 2002; 168:3577–3585. [PubMed: 11907122]
- Azhahianambi P, Ray DD, Chaudhuri P, Gupta R, Ghosh S. Vaccine Efficacy of Bm86 Ortholog of *H. a. anatolicum*, rHaa86 Expressed in Prokaryotic Expression System. *J Parasitol Res.* 2009; 1155/2009/165812
- Barnard DR. Injury thresholds and production loss functions for the lone star tick, *Amblyomma americanum* (Acari: Ixodidae), on pastured, preweaner beef cattle, *Bos taurus*. *J Econ Entomol.* 1985; 78:852–855. [PubMed: 4056195]
- Barnard DR. Population growth rates for *Amblyomma americanum* (Acari: Ixodidae) on *Bos indicus*, *B. taurus* and *B. indicus* x *B. taurus* cattle. *Exp Appl Acarol.* 1990; 9:259–265. [PubMed: 2261818]
- Belaouaj A. Neutrophil elastase-mediated killing of bacteria: lessons from targeted mutagenesis. *Microbes Infect.* 2002; 4:1259–1264. [PubMed: 12467768]
- Benkert P, Tosatto SC, Schomburg D. QMEAN: A comprehensive scoring function for model quality assessment. *Proteins.* 2008; 71:261–277. [PubMed: 17932912]
- Berger M, Reck J Jr, Terra RM, Pinto AF, Termignoni C, Guimaraes JA. *Lonomia obliqua* caterpillar envenomation causes platelet hypoaggregation and blood incoagulability in rats. *Toxicon.* 2010; 55:33–44. [PubMed: 19577588]
- Brass LF. Thrombin and platelet activation. *Chest.* 2003; 124:18S–25S. [PubMed: 12970120]
- Broze GJ Jr. Protein Z-dependent regulation of coagulation. *Thromb Haemost.* 2001; 86:8–13. [PubMed: 11487045]

- Burgdorfer W, Barbour AG, Hayes SF, Benach JL, Grunwaldt E, Davis JP. Lyme disease-a tick-borne spirochetosis? Science. 1982; 216:1317–1319. [PubMed: 7043737]
- Carmo AA, Costa BR, Vago JP, de Oliveira LC, Tavares LP, Nogueira CR, Ribeiro AL, Garcia CC, Barbosa AS, Brasil BS, Dusse LM, Barcelos LS, Bonjardim CA, Teixeira MM, Sousa LP. Plasmin induces in vivo monocyte recruitment through protease-activated receptor-1-, MEK/ERK-, and CCR2-mediated signaling. J Immunol. 2014; 193:3654–3663. [PubMed: 25165151]
- Cattaruzza F, Amadesi S, Carlsson JF, Murphy JE, Lyo V, Kirkwood K, Cottrell GS, Bogyo M, Knecht W, Bunnett NW. Serine proteases and protease-activated receptor 2 mediate the proinflammatory and algescic actions of diverse stimulants. Br J Pharmacol. 2014; 171:3814–3826. [PubMed: 24749982]
- Caughey GH. Mast cell tryptases and chymases in inflammation and host defense. Immunol Rev. 2007; 217:141–154. [PubMed: 17498057]
- Chalautre KC, Kim TK, Garcia-Rodriguez H, Mulenga A. *Amblyomma americanum* (L.) (Acari: Ixodidae) tick salivary gland serine protease inhibitor (serpin) 6 is secreted into tick saliva during tick feeding. J Exp Biol. 2011; 214:665–673. [PubMed: 21270316]
- Chao J, Shen B, Gao L, Xia CF, Bledsoe G, Chao L. Tissue kallikrein in cardiovascular, cerebrovascular and renal diseases and skin wound healing. Biol Chem. 2010; 391:345–355. [PubMed: 20180644]
- Childs JE, Paddock CD. The ascendancy of *Amblyomma americanum* as a vector of pathogens affecting humans in the United States. Annu Rev Entomol. 2003; 48:307–337. [PubMed: 12414740]
- Chmelar J, Oliveira CJ, Rezacova P, Francischetti IM, Kovarova Z, Pejler G, Kopacek P, Ribeiro JM, Mares M, Kopecky J, Kotsyfakis M. A tick salivary protein targets cathepsin G and chymase and inhibits host inflammation and platelet aggregation. Blood. 2011; 117:736–744. [PubMed: 20940421]
- Clemetson KJ. Platelets and primary haemostasis. Thromb Res. 2012; 129:220–224. [PubMed: 22178577]
- Collen D. The plasminogen (fibrinolytic) system. Thromb Haemost. 1999; 82:259–270. [PubMed: 10605712]
- Dantas-Torres F, Chomel BB, Otranto D. Ticks and tick-borne diseases: a One Health perspective. Trends Parasitol. 2012; 28:437–446. [PubMed: 22902521]
- Davi G, Patrono C. Platelet activation and atherothrombosis. N Engl J Med. 2007; 357:2482–2494. [PubMed: 18077812]
- Davie EW, Fujikawa K, Kisiel W. The coagulation cascade: initiation, maintenance, and regulation. Biochemistry. 1991; 30:10363–10370. [PubMed: 1931959]
- de la Fuente J, Kocan KM. Strategies for development of vaccines for control of ixodid tick species. Parasite Immunol. 2006; 28:275–283. [PubMed: 16842264]
- Fragoso H, Rad PH, Ortiz M, Rodriguez M, Redondo M, Herrera L, de la Fuente J. Protection against *Boophilus annulatus* infestations in cattle vaccinated with the *B. microplus* Bm86-containing vaccine Gavac off. Vaccine. 1998; 16:1990–1992. [PubMed: 9796055]
- Francischetti IM, Anderson JM, Manoukis N, Pham VM, Ribeiro JM. An insight into the sialotranscriptome and proteome of the coarse bontlegged tick, *Hyalomma marginatum rufipes*. J Proteomics. 2011; 74:2892–2908. [PubMed: 21851864]
- Francischetti IM, Sa-Nunes A, Mans BJ, Santos IM, Ribeiro JM. The role of saliva in tick feeding. Front Biosci. 2009; 14:2051–2088.
- Franta Z, Frantova H, Konvickova J, Horn M, Sojka D, Mares M, Kopacek P. Dynamics of digestive proteolytic system during blood feeding of the hard tick *Ixodes ricinus*. Parasit Vectors. 2010; 3:119. [PubMed: 21156061]
- Fullaondo A, Garcia-Sanchez S, Sanz-Parra A, Recio E, Lee SY, Gubb D. Spn1 regulates the GNB3-dependent Toll signaling pathway in *Drosophila melanogaster*. Mol Cell Biol. 2011; 31:2960–2972. [PubMed: 21576362]
- Furman MI, Liu L, Benoit SE, Becker RC, Barnard MR, Michelson AD. The cleaved peptide of the thrombin receptor is a strong platelet agonist. Proc Natl Acad Sci USA. 1998; 95:3082–3087. [PubMed: 9501219]

- Garcia GR, Gardinassi LG, Ribeiro JM, Anatriello E, Ferreira BR, Moreira HN, Mafra C, Martins MM, Szabo MP, de Miranda-Santos IK, Maruyama SR. The sialotranscriptome of *Amblyomma triste*, *Amblyomma parvum* and *Amblyomma cajennense* ticks, uncovered by 454-based RNA-seq. *Parasit Vectors*. 2014; 7:430. [PubMed: 25201527]
- Gartner TK, Williams DC, Minion FC, Phillips DR. Thrombin-induced platelet aggregation is mediated by a platelet plasma membrane - bound lectin. *Science*. 1978; 200:1281–1283. [PubMed: 663608]
- Gettins PG. Serpin structure, mechanism, and function. *Chem Rev*. 2002; 102:4751–4804. [PubMed: 12475206]
- Grisi L, Leite RC, Martins JR, Barros AT, Andreotti R, Cancado PH, Leon AA, Pereira JB, Villela HS. Reassessment of the potential economic impact of cattle parasites in Brazil. *Rev Bras Parasitol Vet*. 2014; 23:150–156. [PubMed: 25054492]
- Guex N, Peitsch MC. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis*. 1997; 18:2714–2723. [PubMed: 9504803]
- Hallgren J, Pejler G. Biology of mast cell tryptase. An inflammatory mediator. *FEBS J*. 2006; 273:1871–1895. [PubMed: 16640553]
- Hileman RE, Fromm JR, Weiler JM, Linhardt RJ. Glycosaminoglycan-protein interactions: definition of consensus sites in glycosaminoglycan binding proteins. *Bioessays*. 1998; 20:156–167. [PubMed: 9631661]
- Hoffman M. A cell-based model of coagulation and the role of factor VIIa. *Blood Rev*. 2003; 17(Suppl 1):S1–S5. [PubMed: 14697207]
- Hoffman M, Monroe DM III. A cell-based model of hemostasis. *Thromb Haemost*. 2001; 85:958–965. [PubMed: 11434702]
- Hoover-Plow J. Does plasmin have anticoagulant activity? *Vasc Health Risk Manag*. 2010; 6:199–205. [PubMed: 20407627]
- Horn F, Dos Santos PC, Termignoni C. *Boophilus microplus* anticoagulant protein: an antithrombin inhibitor isolated from the cattle tick saliva. *Arch Biochem Biophys*. 2000; 384:68–73. [PubMed: 11147837]
- Humphrey W, Dalke A, Schulten K. VMD: visual molecular dynamics. *J Mol Graph*. 1996; 14:33–38. [PubMed: 8744570]
- Huntington JA. Shape-shifting serpins--advantages of a mobile mechanism. *Trends Biochem Sci*. 2006; 31:427–435. [PubMed: 16820297]
- Huntington JA, Read RJ, Carrell RW. Structure of a serpin-protease complex shows inhibition by deformation. *Nature*. 2000; 407:923–926. [PubMed: 11057674]
- Ibelli AM, Kim TK, Hill CC, Lewis LA, Bakshi M, Miller S, Porter L, Mulenga A. A blood meal-induced *Ixodes scapularis* tick saliva serpin inhibits trypsin and thrombin, and interferes with platelet aggregation and blood clotting. *Int J Parasitol*. 2014; 44:369–379. [PubMed: 24583183]
- Imamura S, da Silva VJI, Sugino M, Ohashi K, Onuma M. A serine protease inhibitor (serpin) from *Haemaphysalis longicornis* as an anti-tick vaccine. *Vaccine*. 2005; 23:1301–1311. [PubMed: 15652673]
- Imamura S, Konnai S, Vaz IS, Yamada S, Nakajima C, Ito Y, Tajima T, Yasuda J, Simuunza M, Onuma M, Ohashi K. Effects of anti-tick cocktail vaccine against *Rhipicephalus appendiculatus*. *Jpn J Vet Res*. 2008; 56:85–98. [PubMed: 18828446]
- Imamura S, Namangala B, Tajima T, Tembo ME, Yasuda J, Ohashi K, Onuma M. Two serine protease inhibitors (serpins) that induce a bovine protective immune response against *Rhipicephalus appendiculatus* ticks. *Vaccine*. 2006; 24:2230–2237. [PubMed: 16314008]
- Irving JA, Pike RN, Lesk AM, Whisstock JC. Phylogeny of the serpin superfamily: implications of patterns of amino acid conservation for structure and function. *Genome Res*. 2000; 10:1845–1864. [PubMed: 11116082]
- Iwanaga S, Kawabata S, Muta T. New types of clotting factors and defense molecules found in horseshoe crab hemolymph: their structures and functions. *J Biochem*. 1998; 123:1–15. [PubMed: 9504402]

- James AM, Liveris D, Wormser GP, Schwartz I, Montecalvo MA, Johnson BJ. *Borrelia lonestari* infection after a bite by an *Amblyomma americanum* tick. J Infect Dis. 2001; 183:1810–1814. [PubMed: 11372036]
- Jittapalapong S, Kaewhom P, Pumhom P, Canales M, de la Fuente J, Stich RW. Immunization of rabbits with recombinant serine protease inhibitor reduces the performance of adult female *Rhipicephalus microplus*. Transbound Emerg Dis. 2010; 57:103–106. [PubMed: 20537119]
- Jongejan F, Uilenberg G. The global importance of ticks. Parasitology. 2004; 129:S3–14. [PubMed: 15938502]
- Kahn ML, Nakanishi-Matsui M, Shapiro MJ, Ishihara H, Coughlin SR. Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. J Clin Invest. 1999; 103:879–887. [PubMed: 10079109]
- Kanost MR. Serine proteinase inhibitors in arthropod immunity. Dev Comp Immunol. 1999; 23:291–301. [PubMed: 10426423]
- Karim S, Singh P, Ribeiro JM. A deep insight into the sialotranscriptome of the gulf coast tick, *Amblyomma maculatum*. PLoS One. 2011; 6:e28525. [PubMed: 22216098]
- Katada J, Hayashi Y, Sato Y, Muramatsu M, Takiguchi Y, Harada T, Fujiyoshi T, Uno I. A novel peptide motif for platelet fibrinogen receptor recognition. J Biol Chem. 1997; 272:7720–7726. [PubMed: 9065431]
- Kessenbrock K, Dau T, Jenne DE. Tailor-made inflammation: how neutrophil serine proteases modulate the inflammatory response. J Mol Med. 2011; 89:23–28. [PubMed: 20809089]
- Kessenbrock K, Frohlich L, Sixt M, Lammernann T, Pfister H, Bateman A, Belaouaj A, Ring J, Ollert M, Fassler R, Jenne DE. Proteinase 3 and neutrophil elastase enhance inflammation in mice by inactivating antiinflammatory progranulin. J Clin Invest. 2008; 118:2438–2447. [PubMed: 18568075]
- Kim TK, Curran J, Mulenga A. Dual silencing of long and short *Amblyomma americanum* acidic chitinase forms weakens the tick cement cone stability. J Exp Biol. 2014a; 217:3493–3503. [PubMed: 25189365]
- Kim TK, Ibelli AM, Mulenga A. *Amblyomma americanum* tick calreticulin binds C1q but does not inhibit activation of the classical complement cascade. Ticks Tick Borne Dis. 2014b; 6:91–101. [PubMed: 25454607]
- Koci J, Simo L, Park Y. Validation of internal reference genes for real-time quantitative polymerase chain reaction studies in the tick, *Ixodes scapularis* (Acari: Ixodidae). J Med Entomol. 2013; 50:79–84. [PubMed: 23427655]
- Korkmaz B, Moreau T, Gauthier F. Neutrophil elastase, proteinase 3 and cathepsin G: physicochemical properties, activity and physiopathological functions. Biochimie. 2008; 90:227–242. [PubMed: 18021746]
- Laird JS, Kocan AA, Kocan KM, Presley SM, Hair JA. Susceptibility of *Amblyomma americanum* to natural and experimental infections with *Theileria cervi*. J Wildl Dis. 1988; 24:679–683. [PubMed: 3193564]
- Lara FA, Lins U, Bechara GH, Oliveira PL. Tracing heme in a living cell: hemoglobin degradation and heme traffic in digest cells of the cattle tick *Boophilus microplus*. J Exp Biol. 2005; 208:3093–3101. [PubMed: 16081607]
- Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. Clustal W and Clustal X version 2.0. Bioinformatics. 2007; 23:2947–2948. [PubMed: 17846036]
- Leboulle G, Rochez C, Louahed J, Ruti B, Brossard M, Bollen A, Godfroid E. Isolation of *Ixodes ricinus* salivary gland mRNA encoding factors induced during blood feeding. Am J Trop Med Hyg. 2002; 66:225–233. [PubMed: 12139212]
- Li HJ, Yin H, Yao YY, Shen B, Bader M, Chao L, Chao J. Tissue kallikrein protects against pressure overload-induced cardiac hypertrophy through kinin B2 receptor and glycogen synthase kinase-3beta activation. Cardiovasc Res. 2007; 73:130–142. [PubMed: 17137568]
- Li W, Huntington JA. The heparin binding site of protein C inhibitor is protease-dependent. J Biol Chem. 2008; 283:36039–36045. [PubMed: 18974053]

- Li X, Syrovets T, Genze F, Pitterle K, Oberhuber A, Orend KH, Simmet T. Plasmin triggers chemotaxis of monocyte-derived dendritic cells through an Akt2-dependent pathway and promotes a T-helper type-1 response. *Arterioscler Thromb Vasc Biol.* 2010; 30:582–590. [PubMed: 20056908]
- Li X, Syrovets T, Simmet T. The serine protease plasmin triggers expression of the CC-chemokine ligand 20 in dendritic cells via Akt/NF-kappaB-dependent pathways. *J Biomed Biotechnol.* 2012; 2012:10.1155/2012/186710
- Liao M, Zhou J, Gong H, Boldbaatar D, Shirafuji R, Battur B, Nishikawa Y, Fujisaki K. Hemalin, a thrombin inhibitor isolated from a midgut cDNA library from the hard tick *Haemaphysalis longicornis*. *J Insect Physiol.* 2009; 55:164–173. [PubMed: 19061894]
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-(Delta Delta C(T))} Method. *Methods.* 2001; 25:402–408. [PubMed: 11846609]
- Mak P, Pohl J, Dubin A, Reed MS, Bowers SE, Fallon MT, Shafer WM. The increased bactericidal activity of a fatty acid-modified synthetic antimicrobial peptide of human cathepsin G correlates with its enhanced capacity to interact with model membranes. *Int J Antimicrob Agents.* 2003; 21:13–19. [PubMed: 12507833]
- Mancek-Keber M. Inflammation-mediating proteases: structure, function in (patho) physiology and inhibition. *Protein Pept Lett.* 2014; 21:1209–1229. [PubMed: 25141937]
- Maritz-Olivier C, Stutzer C, Jongejan F, Neitz AW, Gaspar AR. Tick anti-hemostatics: targets for future vaccines and therapeutics. *Trends Parasitol.* 2007; 23:397–407. [PubMed: 17656153]
- Matsunaga Y, Kido H, Kawaji K, Kamoshita K, Katunuma N, Ogura T. Inhibitors of chymotrypsin-like proteases inhibit eosinophil peroxidase release from activated human eosinophils. *Arch Biochem Biophys.* 1994; 312:67–74. [PubMed: 8031148]
- Matsunaga Y, Shono M, Takahashi M, Tsuboi Y, Ogawa K, Yamada T. Regulation of lymphocyte proliferation by eosinophils via chymotrypsin-like protease activity and adhesion molecule interaction. *Br J Pharmacol.* 2000; 130:1539–1546. [PubMed: 10928955]
- Meyer-Hoffert U, Rogalski C, Seifert S, Schmeling G, Wingerts Zahn J, Proksch E, Wiedow O. Trypsin induces epidermal proliferation and inflammation in murine skin. *Exp Dermatol.* 2004; 13:234–241. [PubMed: 15086339]
- Monroe DM, Hoffman M. What does it take to make the perfect clot? *Arterioscler Thromb Vasc Biol.* 2006; 26:41–48. [PubMed: 16254201]
- Morris AL, MacArthur MW, Hutchinson EG, Thornton JM. Stereochemical quality of protein structure coordinates. *Proteins.* 1992; 12:345–364. [PubMed: 1579569]
- Mortier A, Gouwy M, Van DJ, Proost P. Effect of posttranslational processing on the in vitro and in vivo activity of chemokines. *Exp Cell Res.* 2011; 317:642–654. [PubMed: 21146523]
- Mudenda L, Pierle SA, Turse JE, Scoles GA, Purvine SO, Nicora CD, Clauss TR, Ueti MW, Brown WC, Brayton KA. Proteomics informed by transcriptomics identifies novel secreted proteins in *Dermacentor andersoni* saliva. *Int J Parasitol.* 2014; 44:1029–1037. [PubMed: 25110293]
- Mulenga A, Khumthong R, Blandon MA. Molecular and expression analysis of a family of the *Amblyomma americanum* tick Lospins. *J Exp Biol.* 2007; 210:3188–3198. [PubMed: 17766296]
- Mulenga A, Khumthong R, Chalaire KC. *Ixodes scapularis* tick serine proteinase inhibitor (serpin) gene family; annotation and transcriptional analysis. *BMC Genomics.* 2009; 10:217. [PubMed: 19435496]
- Mulenga A, Kim T, Ibelli AM. *Amblyomma americanum* tick saliva serine protease inhibitor 6 is a cross-class inhibitor of serine proteases and papain-like cysteine proteases that delays plasma clotting and inhibits platelet aggregation. *Insect Mol Biol.* 2013; 22:306–319. [PubMed: 23521000]
- Mulenga A, Misao O, Sugimoto C. Three serine proteinases from midguts of the hard tick *Rhipicephalus appendiculatus*; cDNA cloning and preliminary characterization. *Exp Appl Acarol.* 2003a; 29:151–164. [PubMed: 14580067]
- Mulenga A, Sugino M, Nakajim M, Sugimoto C, Onuma M. Tick-Encoded serine proteinase inhibitors (serpins); potential target antigens for tick vaccine development. *J Vet Med Sci.* 2001; 63:1063–1069. [PubMed: 11714020]

- Mulenga A, Tsuda A, Onuma M, Sugimoto C. Four serine proteinase inhibitors (serpin) from the brown ear tick, *Rhiphicephalus appendiculatus*; cDNA cloning and preliminary characterization. *Insect Biochem Mol Biol*. 2003b; 33:267–276. [PubMed: 12535684]
- Munoz EM, Linhardt RJ. Heparin-binding domains in vascular biology. *Arterioscler Thromb Vasc Biol*. 2004; 24:1549–1557. [PubMed: 15231514]
- Nurden AT. Platelet membrane glycoproteins: a historical review. *Semin Thromb Hemost*. 2014; 40:577–584. [PubMed: 24967889]
- Ohlmann P, Eckly A, Freund M, Cazenave JP, Offermanns S, Gachet C. ADP induces partial platelet aggregation without shape change and potentiates collagen-induced aggregation in the absence of Galphaq. *Blood*. 2000; 96:2134–2139. [PubMed: 10979958]
- Olszewska-Pazdrak B, Hart-Vantassell A, Carney DH. Thrombin peptide TP508 stimulates rapid nitric oxide production in human endothelial cells. *J Vasc Res*. 2010; 47:203–213. [PubMed: 19893317]
- Parizi LF, Githaka NW, Logullo C, Konnai S, Masuda A, Ohashi K, Da Silva VI Jr. The quest for a universal vaccine against ticks: cross-immunity insights. *Vet J*. 2012; 194:158–165. [PubMed: 22766309]
- Porter L, Radulovic Z, Kim T, Braz GR, Da Silva VI Jr, Mulenga A. Bioinformatic analyses of male and female *Amblyomma americanum* tick expressed serine protease inhibitors (serpins). *Ticks Tick Borne Dis*. 2015; 6:16–30. [PubMed: 25238688]
- Prevot PP, Adam B, Boudjeltia KZ, Brossard M, Lins L, Cauchie P, Brasseur R, Vanhaeverbeek M, Vanhamme L, Godfroid E. Anti-hemostatic effects of a serpin from the saliva of the tick *Ixodes ricinus*. *J Biol Chem*. 2006; 281:26361–26369. [PubMed: 16672226]
- Prevot PP, Beschin A, Lins L, Beaufays J, Grosjean A, Bruys L, Adam B, Brossard M, Brasseur R, Zouaoui BK, Vanhamme L, Godfroid E. Exosites mediate the anti-inflammatory effects of a multifunctional serpin from the saliva of the tick *Ixodes ricinus*. *FEBS J*. 2009; 276:3235–3246. [PubMed: 19438720]
- Prevot PP, Couvreur B, Denis V, Brossard M, Vanhamme L, Godfroid E. Protective immunity against *Ixodes ricinus* induced by a salivary serpin. *Vaccine*. 2007; 25:3284–3292. [PubMed: 17270322]
- Rau JC, Beaulieu LM, Huntington JA, Church FC. Serpins in thrombosis, hemostasis and fibrinolysis. *J Thromb Haemost*. 2007; 5(Suppl 1):102–115. [PubMed: 17635716]
- Rawlings ND, Barrett AJ. Families of serine peptidases. *Methods Enzymol*. 1994; 244:19–61. [PubMed: 7845208]
- Reimers HJ, Kinlough-Rathbone RL, Cazenave JP, Senyi AF, Hirsh J, Packham MA, Mustard JF. In vitro and in vivo functions of thrombin-treated platelets. *Thromb Haemost*. 1976; 35:151–166. [PubMed: 785686]
- Rein CM, Desai UR, Church FC. Serpin-glycosaminoglycan interactions. *Methods Enzymol*. 2011; 501:105–137. [PubMed: 22078533]
- Renesto P, Chignard M. Enhancement of cathepsin G-induced platelet activation by leukocyte elastase: consequence for the neutrophil-mediated platelet activation. *Blood*. 1993; 82:139–144. [PubMed: 8324217]
- Renesto P, Halbwachs-Mecarelli L, Nusbaum P, Lesavre P, Chignard M. Proteinase 3. A neutrophil proteinase with activity on platelets. *J Immunol*. 1994; 152:4612–4617. [PubMed: 8157974]
- Ribeiro JM, Alarcon-Chaidez F, Francischetti IM, Mans BJ, Mather TN, Valenzuela JG, Wikel SK. An annotated catalog of salivary gland transcripts from *Ixodes scapularis* ticks. *Insect Biochem Mol Biol*. 2006; 36:111–129. [PubMed: 16431279]
- Ribeiro JM, Anderson JM, Manoukis NC, Meng Z, Francischetti IM. A further insight into the sialome of the tropical bont tick, *Amblyomma variegatum*. *BMC Genomics*. 2011; 12:136. [PubMed: 21362191]
- Ribeiro JM, Labruna MB, Mans BJ, Maruyama SR, Francischetti IM, Barizon GC, de Miranda Santos IK. The sialotranscriptome of *Antricola delacruzi* female ticks is compatible with non-hematophagous behavior and an alternative source of food. *Insect Biochem Mol Biol*. 2012; 42:332–342. [PubMed: 22306723]
- Ricci CG, Pinto AF, Berger M, Termignoni C. A thrombin inhibitor from the gut of *Boophilus microplus* ticks. *Exp Appl Acarol*. 2007; 42:291–300. [PubMed: 17710557]

- Roberts HR, Hoffman M, Monroe DM. A cell-based model of thrombin generation. *Semin Thromb Hemost.* 2006; 32(Suppl 1):32–38. [PubMed: 16673264]
- Rodriguez-Valle M, Vance M, Moolhuijzen PM, Tao X, Lew-Tabor AE. Differential recognition by tick-resistant cattle of the recombinantly expressed *Rhipicephalus microplus* serine protease inhibitor-3 (RMS-3). *Ticks Tick Borne Dis.* 2012; 3:159–169. [PubMed: 22608113]
- Savage HM, Godsey MS Jr, Lambert A, Panella NA, Burkhalter KL, Harmon JR, Lash RR, Ashley DC, Nicholson WL. First detection of heartland virus (Bunyaviridae: Phlebovirus) from field collected arthropods. *Am J Trop Med Hyg.* 2013; 89:445–452. [PubMed: 23878186]
- Schwarz A, Tenzer S, Hackenberg M, Erhart J, Gerhold-Ay A, Mazur J, Kuharev J, Ribeiro JM, Kotsyfakis M. A systems level analysis reveals transcriptomic and proteomic complexity in *Ixodes ricinus* midgut and salivary glands during early attachment and feeding. *Mol Cell Proteomics.* 2014; 13:2725–2735. [PubMed: 25048707]
- Schwarz A, von Reumont BM, Erhart J, Chagas AC, Ribeiro JM, Kotsyfakis M. De novo *Ixodes ricinus* salivary gland transcriptome analysis using two next-generation sequencing methodologies. *FASEB J.* 2013; 27:4745–4756. [PubMed: 23964076]
- Selak MA, Chignard M, Smith JB. Cathepsin G is a strong platelet agonist released by neutrophils. *Biochem J.* 1988; 251:293–299. [PubMed: 3390156]
- Shen Y, Guo Y, Du C, Wilczynska M, Hellstrom S, Ny T. Mice deficient in urokinase-type plasminogen activator have delayed healing of tympanic membrane perforations. *PLoS One.* 2012; 7:e51303. [PubMed: 23236466]
- Silverman GA, Bird PI, Carrell RW, Church FC, Coughlin PB, Gettins PG, Irving JA, Lomas DA, Luke CJ, Moyer RW, Pemberton PA, Remold-O'Donnell E, Salvesen GS, Travis J, Whisstock JC. The serpins are an expanding superfamily of structurally similar but functionally diverse proteins. Evolution, mechanism of inhibition, novel functions, and a revised nomenclature. *J Biol Chem.* 2001; 276:33293–33296. [PubMed: 11435447]
- Srinivasan R, Marchant RE, Gupta AS. In vitro and in vivo platelet targeting by cyclic RGD-modified liposomes. *J Biomed Mater Res A.* 2010; 93:1004–1015. [PubMed: 19743511]
- Steen NA, Barker SC, Alewood PF. Proteins in the saliva of the Ixodida (ticks): pharmacological features and biological significance. *Toxicon.* 2006; 47:1–20. [PubMed: 16364387]
- Sugino M, Imamura S, Mulenga A, Nakajima M, Tsuda A, Ohashi K, Onuma M. A serine proteinase inhibitor (serpin) from ixodid tick *Haemaphysalis longicornis*; cloning and preliminary assessment of its suitability as a candidate for a tick vaccine. *Vaccine.* 2003; 21:2844–2851. [PubMed: 12798626]
- Syrovets T, Jendrach M, Rohwedder A, Schule A, Simmet T. Plasmin-induced expression of cytokines and tissue factor in human monocytes involves AP-1 and IKKbeta-mediated NF-kappaB activation. *Blood.* 2001; 97:3941–3950. [PubMed: 11389038]
- Taylor JP, Istre GR, McChesney TC, Satalowich FT, Parker RL, McFarland LM. Epidemiologic characteristics of human tularemia in the southwest-central states, 1981–1987. *Am J Epidemiol.* 1991; 133:1032–1038. [PubMed: 2035503]
- Tirloni L, Reck J, Terra RM, Martins JR, Mulenga A, Sherman NE, Fox JW, Yates JR III, Termignoni C, Pinto AF, da Vaz IS Jr. Proteomic analysis of cattle tick *Rhipicephalus (Boophilus) microplus* saliva: a comparison between partially and fully engorged females. *PLoS One.* 2014a; 9:e94831. [PubMed: 24762651]
- Tirloni L, Seixas A, Mulenga A, daVaz IS Jr, Termignoni C. A family of serine protease inhibitors (serpins) in the cattle tick *Rhipicephalus (Boophilus) microplus*. *Exp Parasitol.* 2014b; 137:25–34. [PubMed: 24333790]
- Unni S, Huang Y, Hanson RM, Tobias M, Krishnan S, Li WW, Nielsen JE, Baker NA. Web servers and services for electrostatics calculations with APBS and PDB2PQR. *J Comput Chem.* 2011; 32:1488–1491. [PubMed: 21425296]
- Valenzuela JG, Francischetti IM, Pham VM, Garfield MK, Mather TN, Ribeiro JM. Exploring the sialome of the tick *Ixodes scapularis*. *J Exp Biol.* 2002; 205:2843–2864. [PubMed: 12177149]
- Varon D, Lider O, Dardik R, Shenkman B, Alon R, HersHKoviz R, Kapustina G, Savion N, Martinowitz U, Greenspoon N. Inhibition of integrin-mediated platelet aggregation, fibrinogen-

- binding, and interactions with extracellular matrix by nonpeptidic mimetics of Arg-Gly-Asp. *Thromb Haemost.* 1993; 70:1030–1036. [PubMed: 8165596]
- Walsh KA, Kauffman DL, Kumar KS, Neurath H. On the structure and function of bovine trypsinogen and trypsin. *Proc Natl Acad Sci USA.* 1964; 51:301–308. [PubMed: 14124328]
- Walsh PN, Ahmad SS. Proteases in blood clotting. *Essays Biochem.* 2002; 38:95–111. [PubMed: 12463164]
- Webb B, Sali A. Comparative Protein Structure Modeling Using MODELLER. *Curr Protoc Bioinformatics.* 2014; 47:5. [PubMed: 25199792]
- White MJ, Glenn M, Gomer RH. Trypsin potentiates human fibrocyte differentiation. *PLoS One.* 2013; 8:e70795. [PubMed: 23951012]
- Wolf L, McPherson T, Harrison B, Engber B, Anderson A, Whitt P. Prevalence of *Ehrlichia ewingii* in *Amblyomma americanum* in North Carolina. *J Clin Microbiol.* 2000; 38:2795. [PubMed: 10979750]
- Yabsley MJ, Quick TC, Little SE. Theileriosis in a white-tailed deer (*Odocoileus virginianus*) fawn. *J Wildl Dis.* 2005; 41:806–809. [PubMed: 16456174]
- Yu Y, Cao J, Zhou Y, Zhang H, Zhou J. Isolation and characterization of two novel serpins from the tick *Rhipicephalus haemaphysaloides*. *Ticks Tick Borne Dis.* 2013; 4:297–303. [PubMed: 23601911]

Highlights

- AAS19 protein sequence is highly conserved with other ixodid tick species.
- rAAS19 is a broad spectrum inhibitor of trypsin and trypsin-like proteases.
- rAAS19 prevents platelet aggregation and delays plasma clotting.

Fig. 1. Structure-based sequence alignment of *Amblyomma americanum* serpin-19 (AAS19; GenBank accession number: **GAYW01000076**) protein sequence with its homologs in other tick species from the Uniprot database: *Rhipicephalus pulchellus* (**L7LRY7**), *Ixodes ricinus* (**V5IHU3**), *Amblyomma maculatum* (**G3ML49** and **G3ML50**), *Rhipicephalus microplus* (**V9VP22**), *Ixodes scapularis* (**B7QJF1**), *Amblyomma triste* (**A0A023GPF9**) and *Amblyomma cajenense* (**A0A023FM57**). Secondary structures, assigned based on AAS19 comparative tertiary structure, are labeled “H” for α -helix and “E” for β -strand. Helices are labeled from “hA” to “hI” and β -strands that constitute β -sheets A – C are labeled “s1 – 6A”, “s1 – 5B” and “s1 – 4C”, respectively. The “RGD” motif is noted by the “&” symbol and the two putative N-glycosylation sites are noted by the “#” symbols below the sequence. The residues that correspond to the basic patches are indicated by an asterisk (*). The high and low conserved residues are highlighted in black and gray, respectively. The 100% conserved reactive center loop (RCL) region is denoted with black broken lines (---) above the sequence.

Fig. 2. Structure and putative glycosaminoglycan-binding sites of *Amblyomma americanum* serpin-19 (AAS19). The AAS19 model was constructed using the coordinates generated with the Modeler 9v14 program and protein C inhibitor (PCI) as template (PDB 2OL2). Calculation of the electrostatic potential surface map was generated using the Adaptive Poisson-Boltzmann Solver (APBS) tool in the Visual Molecular Dynamics (VMD) program. (A) Electrostatic surface potential (indicated by a dark surface for positively, and light surface for negatively, charged regions) and ribbon diagram of the native PCI (template) showing the glycosaminoglycan (GAG)-binding along the helix-H. Residues responsible for GAG-binding activity are shown as sticks and labeled (Li and Huntington, 2008). (B) Electrostatic surface potential and ribbon diagram of the AAS19 model showing putative GAG-binding sites (GAGBS 1 – 4). Clusters of basic amino acid residues potentially involved in formation of GAGBS 1 – 4 [K¹³⁷-R¹³⁹-R¹⁴⁰-K¹⁶¹-K²⁶⁴-K²⁶⁸-K³⁰⁸], [K⁸-K²³⁶-R²⁴⁶-R²⁵⁰], [R¹⁷²-K¹⁷⁴-R¹⁷⁶-K¹⁸⁵-K¹⁹²-K¹⁹⁴-K³⁴³] and [K¹¹⁷-K¹¹⁸-R¹⁵¹-K²⁷²-K²⁹⁸] are shown as sticks and labeled.

Fig. 3.

Temporal and spatial expression analysis of *Amblyomma americanum* serpin-19 (AAS19) mRNA through 120 h post-attachment. AAS19 mRNA relative expression analysis in unfed (UF), 24, 48, 72, 96, and 120 h fed tick dissected tissues: (A) salivary gland, (B) midgut and (C) carcass analyzed by quantitative reverse transcription-PCR. To determine the AAS19 mRNA relative expression (y-axis), data were analyzed using the 2^{-C_t} method comparing with ribosomal protein S4 (RPS4) in three biological replicates of different tissues in relation to time of feeding (x-axis). The lowest expressed time point was used as a calibrator for each tissue.

Fig. 4.

Expression and affinity purification of recombinant (r) *Amblyomma americanum* serpin-19 (AAS19) in *Pichia pastoris*. Construction of expression plasmid, induction and validation of expression levels using the antibody to the C-terminal hexahistidine tag and affinity purification of rAAS19 were performed as described in Section 2.5. (A) Daily expression levels of rAAS19 through 5 days. (B) Affinity purified rAAS19 resolved on a 10% SDS-PAGE and silver staining (L, molecular weight ladder; T, total protein loaded onto column; R, column run through; B, binding buffer wash; W1 and W2, wash buffers; E1 – E4, eluted rAAS19 fractions). Purified rAAS19 fractions used for assays in Figs. 5 – 9 are noted with asterisks (*). Western blotting analysis with (C) 1:50 dilution pre-immune sera, (D) 1:250 dilution immune sera to replete-fed *A. americanum* tick saliva proteins, and (E) antibody to C-terminal hexahistidine tag. Lanes L, 1 and 2 in C, D and E show a molecular weight ladder, glycosylated and de-glycosylated rAAS19.

Fig. 5.

Protease inhibitor function profiling. Enzymes, at concentrations indicated in Section 2.8, were pre-incubated with recombinant *Amblyomma americanum* serpin-19 (rAAS19) (1 μM) at 37°C for 15 min. Subsequently, specific colorimetric substrates were added and hydrolysis monitored at A_{450nm} over 30min at 30°C. The percent enzyme activity inhibition level was determined using the formula: $100 - \frac{V_{max}(V_i)}{V_{max}(V_0)} \times 100$ where $V_{max}(V_i)$ = activity in presence of, and $V_{max}(V_0)$ = activity in absence of, rAAS19. Data are presented as mean ± S.E.M. of triplicate readings.

Fig. 6. Stoichiometry inhibition (SI) assay for *Amblyomma americanum* serpin-19 (AAS19). Residual enzyme activity without (a) and with presence of increasing (b – g) amounts of rAAS19 were pre-incubated for 1 h at 37°C with a constant concentration of trypsin (A,B: 10 nM), plasmin (C,D: 10 nM), factor Xa (E,F: 5 nM), and factor XIa (G,H: 5 nM), resulting in rAAS19 inhibitor : enzyme protease (I:E) molar ratios varying from 0 – 10 (for plasmin and trypsin) or 0 – 20 (for factor Xa and factor XIa). Residual enzymatic activity was measured using specific colorimetric substrate for each enzyme noted in Section 2.9. The data were plotted as enzymatic residual activity (V_i/V_0) versus molar ratio (rAAS19 : enzyme protease). The SI was determined by fitting data onto a linear regression line.

Fig. 7.

SDS-stable complex formation assay. Increasing amounts of recombinant *Amblyomma americanum* serpin-19 (rAAS19) were pre-incubated for 1 h at 37°C with a constant concentration of trypsin (A), factor Xa (B) and factor XIa (C), resulting in molar ratios varying from 0.625:1 to 10:1 (rAAS19: enzyme protease). Samples were resolved on 12.5% SDS-PAGE and Coomassie blue-stained to identify SDS-stable complexes. The rAAS19 : enzyme protease complex formation is denoted with an asterisk.

Fig. 8.

Effect of recombinant *Amblyomma americanum* serpin-19 (rAAS19) on thrombin activated platelet aggregation. (A) The platelet aggregation function assay was done using the platelet rich plasma (PRP) approach described in Section 2.11. Tyrode buffer without thrombin (a), various amounts of rAAS19 with 1 μM (b), 0.5 μM (c), and 0.25 μM (d) rAAS19 and thrombin only (e) were pre-incubated with thrombin (3 U) in a 50 μL reaction for 15 min at 37°C. Platelet aggregation was initiated with the addition of 100 μL of pre-warmed PRP and monitored at intervals of 20 s over 30 min at A_{650nm} . (B) Percent reduction of thrombin-induced platelet aggregation inhibition by rAAS19. In the assay used here, platelet aggregation was directly proportional to reduced O.D. (A_{650nm}). Data are presented as mean $\pm$ S.E.M. of duplicate platelet aggregation assays.

Fig. 9.

The effect of recombinant *Amblyomma americanum* serpin-19 (rAAS19) on plasma clotting time in the recalcification time (RCT), activated partial thromboplastin time (aPTT) and thrombin time (TT) assays. (A) Universal coagulation reference human plasma (UCRP) (50 μL) was incubated with 0 (arrowhead R-CT1), 0.625, 1.25 and 5 (arrowhead R-CT2), and 10 (arrowhead R-CT3) μM rAAS19 in 90 μL of Tris-HCl reaction buffer for 15 min at 37°C followed by the addition of 150 mM CaCl₂ (10 μL) thereafter clotting was measured every 20 s for 30 min. (B) UCRP (50 μL) was incubated with 0, 0.625, 1.25 (arrowhead A-CT1), 5 (arrowhead A-CT2), and 10 (arrowhead A-CT3) μM rAAS19 up to 100 μL of Tris-HCl reaction buffer for 15 min at 37°C, followed by the addition of aPTT reagent and incubation for 5 min at 37°C before the addition of 25 mM CaCl₂ (10 μL); clotting time was monitored every 10 s for 5 min. (C) rAAS19 with 0, 0.625 (arrowhead T-CT1), 1.25 and 5 (arrowhead T-CT2), and 10 (arrowhead T-CT3) μM up to 25 μL of Tris-HCl reaction buffer were incubated with 25 μL of the TT reagent containing CaCl₂ for 15 min at 37°C before addition of 50 μL of pre-warmed UCRP with clotting time monitored every 10 s for 5 min. All assays were done in duplicate, with clotting time monitored at A_{650nm}.

Table 1Proteases used in recombinant *Amblyomma americanum* serpin-19 (rAAS19) protease inhibitor profiling.

Proteases	Biological function	References
Thrombin	Common, intrinsic and extrinsic coagulation pathways; fibrinolysis pathway; platelet activation and aggregation pathways via activation of protease-activated receptors (PARs); wound healing/tissue remodeling; inflammation.	Reimers et al., 1976; Gartner et al., 1978; Davie et al., 1991; Hoffman, 2003; Olszewska-Pazdrak et al., 2010
Trypsin	Digestion; wound healing/tissue remodeling; inflammation and pain via activation of PAR2.	Walsh et al., 1964; White et al., 2013; Cattaruzza et al., 2014
Cathepsin G	Platelet activation; wound healing/tissue remodeling; inflammation; antimicrobial properties.	Renesto and Chignard, 1993; Mak et al., 2003; Korkmaz et al., 2008
Kallikrein	Wound healing/tissue remodeling; inflammation; intrinsic coagulation pathway.	Li et al., 2007; Chao et al., 2010
Elastase	Wound healing/tissue remodeling; inflammation; digestion of elastin; activation of platelets and lymphocytes; antimicrobial properties.	Renesto and Chignard, 1993; Belaouaj, 2002; Korkmaz et al., 2008; Kessenbrock et al., 2008, 2011
Plasmin	Fibrinolysis pathway; inflammation.	Collen, 1999; Li et al., 2012; Carmo et al., 2014; Mancek-Keber, 2014
Factor IXa	Intrinsic coagulation pathway; platelet amyloid precursor protein pathway	Davie et al., 1991; Hoffman, 2003
Factor Xa	Common, intrinsic and extrinsic coagulation pathways.	Davie et al., 1991; Hoffman, 2003
Factor XIa	Intrinsic coagulation pathway; platelet amyloid precursor protein pathway	Davie et al., 1991; Hoffman, 2003
Factor XIIa	Intrinsic coagulation pathway.	Davie et al., 1991; Hoffman, 2003
Tissue-type plasminogen activator (t-PA)	Fibrinolysis pathway; platelet amyloid precursor protein pathway.	Angles-Cano, 1994; Angles-Cano et al., 1994; Collen, 1999
Urokinase plasminogen activator (u-PA)	Fibrinolysis pathway; platelet amyloid precursor protein pathway.	Angles-Cano, 1994; Angles-Cano et al., 1994; Collen, 1999
α -chymotrypsin	Wound healing/tissue remodeling; inflammation; digestion; regulation of proteases: chymotrypsin, cathepsin G, mast cell chymase.	Algermissen et al., 1999a; Matsunaga et al., 1994, 2000; Korkmaz et al., 2008
Chymase	Wound healing/tissue remodeling; inflammation.	Algermissen et al., 1999a, b; Caughey, 2007
Tryptase	Wound healing/tissue remodeling; inflammation.	Algermissen et al., 1999a; Hallgren and Pejler, 2006; Caughey, 2007
Proteinase-3	Wound healing/tissue remodeling; inflammation; platelet activation. Antimicrobial properties.	Renesto et al., 1994; Algermissen et al., 1999a; Kessenbrock et al., 2008; Korkmaz et al., 2008;