Tsetse GmmSRPN10 Has Anti-complement Activity and Is Important for Successful Establishment of Trypanosome Infections in the Fly Midgut

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Abstract
The complement cascade in mammalian blood can damage the alimentary tract of haematophagous arthropods. As such, these animals have evolved their own repertoire of complement-inactivating factors, which are inadvertently exploited by blood-borne pathogens to escape complement lysis. Unlike the bloodstream stages, the procyclic (insect) stage of Trypanosoma brucei is highly susceptible to complement killing, which is puzzling considering that a tsetse takes a bloodmeal every 2–4 days. In this study, we identified four tsetse (Glossina morsitans morsitans) serine protease inhibitors (serpins) from a midgut expressed sequence tag (EST) library (GmmSRPN3, GmmSRPN5, GmmSRPN9 and GmmSRPN10) and investigated their role in modulating the establishment of a T. brucei infection in the midgut. Although not having evolved in a common blood-feeding ancestor, all four serpins have an active site sharing remarkable homology with the human complement C1-inhibitor serpin, SerpinG1. RNAi knockdown of individual GmmSRPN9 and GmmSRPN10 genes resulted in a significant decreased rate of infection by procyclic form T. brucei. Furthermore, recombinant GmmSRPN10 was both able to inhibit the activity of human complement-cascade serine proteases, C1s and Factor D, and to protect the in vitro killing of procyclic trypanosomes when incubated with complement-activated human serum. Thus, the secretion of serpins, which may be part of a bloodmeal complement inactivation system in tsetse, is used by procyclic trypanosomes to evade an influx of fresh trypanolytic complement with each bloodmeal. This highlights another facet of the complicated relationship between T. brucei and its tsetse vector, where the parasite takes advantage of tsetse physiology to further its chances of propagation and transmission.

Introduction
Mammalian blood constitutes a challenging source of nutrition, yet a multitude of arthropods have evolved to exploit this survival niche [1]. Besides developing physical and behavioural adaptations to evade their hosts, haematophagous arthropods have also adapted physiologically to ingest a food source that is relatively poor in vitamins and spiked with a cocktail of immunity factors. This cocktail also contains factors involved in the complement cascade, which is activated and mediated by a series of serine proteases [2]. Activated by either the classical, alternative or lectin pathways, the cascade leads to the formation of pores across the plasma membrane of targeted cells. These pores ultimately cause the disruption of cellular compartmentalisation and lead to cell lysis.

Complement-induced cytolysis is detrimental to both blood-borne pathogens and blood-feeding arthropods alike. Unicellular parasites are rapidly lysed upon exposure [3,4], while blood-feeding arthropods can acquire gut damage as a consequence of ingesting a bloodmeal containing active complement [5,6]. As the complement cascade is activated and mediated by a series of serine proteases [7], haematophagous arthropods have evolved molecular counter-measures against complement-mediated damage by secreting serine protease inhibitors (serpins) to suppress cascade activation [8–10]. Serpins can have multiple functions within arthropods, ranging from immunity to development [11–14], including inactivation of serum complement as a means to prevent damage to the alimentary canal of haematophagous arthropods [8,15]. As haematophagy is frequently associated with pathogen transmission, it is unsurprising that transmitted pathogens can hijack the anti-complement defences of their arthropod vector to better improve their chances of survival [9,16].

Trypanosoma brucei, a causative agent of African trypanosomiasis, has an alternating life cycle between mammalian hosts and the tsetse fly. T. brucei can only be transmitted to other vertebrate hosts by the infective bite of a tsetse. To accomplish transmission, the parasite must first establish an infection in the fly midgut before it can attempt a long and complex migratory process.
Blood feeding arthropods are exploited by blood borne parasites as vectors of transmission. Trypanosoma brucei, a salivarian trypanosome species, must survive, migrate and differentiate in the tsetse until they become mature, mammalian-infective forms within the fly salivary glands. This constitutes a significant challenge to trypanosomes as the major parasite form colonising the tsetse midgut is sensitive to lysis by blood complement, which is introduced into the tsetse gut whenever the fly feeds. In this study, we show that *T. brucei* may avoid being eliminated by bloodmeal complement by benefitting from a complement-inhibiting enzyme secreted by the fly itself. We showed that this serine protease inhibitor (serpin) enzyme, Serpin10, can inactivate triggers of the complement cascade, protect tsetse-infective trypanosomes from complement lysis, and is important for trypanosome establishment in the tsetse midgut. Taken together, we propose that GmmSRPN10 may be part of a repertoire of complement-inhibiting proteins secreted by tsetse that are utilised by *T. brucei* to evade complement lysis in the tsetse midgut.

**Methods**

**Generation of phylogenetic tree**

A list of insect serpins from GenBank (NCBI) was compiled from insect species with sequenced genomes [25]: *Aedes aegypti*, *Anopheles gambiae*, *Apis mellifera*, *Bombyx mori*, *Drosophila melanogaster* and *Tribolium castaneum*. COBALT (NCBI) was used to generate an initial alignment of extracted serpin sequences. Clusters of related serpins were then further aligned and analysed using CLC sequence viewer using the neighbour-joining algorithm with the bootstrap replicate value set for 100.

**Generation of dsRNA**

PCR amplification of the genes for knockdown experiments (GmmSRPN3, GmmSRPN5, GmmSRPN9, GmmSRPN10) used pEGFP-N1 (CLONTECH Laboratories, USA) as the nonspecific dsRNA control, were carried with the following primer pairs with a preceding T7 RNA polymerase promoter sequence (underlined) for use with the MEGAscript RNA transcription kit (Ambion) using plasmid template from a tsetse midgut EST library `< annotated>` [24]:

**GmmSRPN3** <Gmm-2356>

Forward: TAATACGACTCATACTATAGGGCTGGGGGAG-CCCTTGTACAG
Reverse: TAATACGACTCATACTATAGGGCTACGCGAG

**GmmSRPN5** <Gmm-3352>

Forward: TAATACGACTCATACTATAGGGGAGGGCGAAGAAG
Reverse: TAATACGACTCATACTAGGGTTCCCCTTGCGACAAATTAGAGCA

**GmmSRPN9** <Gmm-72e08>

Forward: TAATACGACTCATACTATAGGGGAGGGCGAAGAAG
Reverse: TAATACGACTCATACTATAGGGGAGGGCGAAGAAG

**GmmSRPN10** <Gmm-3334>

Forward: TAATACGACTCATACTATAGGGGAGGGCGAAGAAG
Reverse: TAATACGACTCATACTATAGGGGAGGGCGAAGAAG

**Cloning vector pEGFP-N1**

Forward: TAATACGACTCATACTATAGGGGAGGGCGAAGAAG
Reverse: TAATACGACTCATACTATAGGGGAGGGCGAAGAAG

**Scoring of infection rates in dsRNA knockdown flies**

The *Glossina morsitans morsitans* colony (LSTM) was reared at 24–27 °C, 68–78% humidity and maintained by membrane feeding on defibrinated horse blood (TCS Biosciences). Teneral flies were fed dsRNA-spiked blood at a concentration of 330 µg/ml (~10 µg dsRNA ingested per fly as previously reported [28]. Flies fed on dsRNA were infected in the second bloodmeal (48 h post dsRNA feed) with *T. brucei* TSW 196 BSFs and dissected 10 days post infection. The midguts were analysed by microscopy for the presence of trypanosomes and tissue samples were flash frozen.

**Infection of tsetse with *T. brucei***

For infection experiments utilising horse serum (HS), HS was extracted from each batch of defibrinated blood by centrifugation and frozen in aliquots to prevent loss of complement activity from repeated freeze thawing. Unfed (teneral) flies were infected with TSW 196 BSFs suspended in serum (untreated or treated either with HI or CVF) 48 h post emergence and maintained on the same serum feed until dissection 10 days post-infection.

**Results**

Bloodstream form (BSF) *Trypanosoma brucei brucei* strain MSUS/C1/78/TSW 196 [26] was transformed into procyclic culture forms (PFs) by suspension in DTM medium with a final cystaconitate (Sigma) concentration of 3 mM and cultured at 26°C [27]. Transformed trypanosomes were initially subcultured and maintained in a 1:1 mix of DTM and SDM-79 media (Gibco); but subsequently cultured with SDM-79.

**Discussion**

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in liquid nitrogen and stored at −80°C until required for RT-PCR or qPCR analyses and Western blotting analysis.

Determination of global serpin transcript levels by semi-quantitative RT-PCR

Total RNA from flash frozen samples was extracted using Trizol (Invitrogen) and standardised to a concentration of 25 ng/µl. cDNA was generated using the AccessQuick RT-PCR kit (Promega) according to the manufacturer’s recommendations and subsequent PCR was carried out with primers used to generate dsDNA, as well as primers for tsetse gyceraldehyde-3-phosphate dehydrogenase (GAPDH):

GAPDH

Forward: TAAAATGGGTGGATGCTGAGAGTC
Reverse: CTACGATGAAATTAAAGGCAAAGT

RT-PCR products were visualised on a 1% (w/v) agarose gel and transcript levels were expressed as percentage against the band intensity (SynGene) for GAPDH controls.

Recombinant expression of His::GmmSRPN10

The coding region for GmmSRPN10 was amplified from the G. m. morsitans midgut EST library clone [25] using PCR (New England Biolabs, UK) according to the manufacturer’s recommendations with primers having PmlI and SacI restriction enzyme overhangs (underlined).

Forward primer: TTTCAAGTGATGCGATTTTTAATT-TACA;
Reverse primer: TTTGAGCTCTTAAGCGTCTGGTGCGG-TTAAC;

Poly-A tailing was performed using reagents from the NEB PCR kit and ligated into a pGEM-T Easy holding vector (Promega). Holding vectors were transformed into E. coli XL-1 cells. Plasmid extraction from cultured XL-1 cells plus holding vector was performed using a miniprep kit (Qiagen). Extracted plasmids were subjected to PmlI and SacI restriction enzyme (Promega) digest and the digestion products run on a 1% (w/v) agarose gel. The agarose gel bands corresponding to GmmSRPN10 coding region with cut restriction sites were excised from the gel and purified using the PureLink Gel Extraction Kit (Invitrogen). Gel purified bands were ligated into the pET-45b expression vector (Novagen), which allows for a His-tagged expression product. The purified bands were ligated into the pET-45b expression vector and purified using the PureLink Gel Extraction Kit (Invitrogen). Gel purified bands were ligated into the pET-45b expression vector (Novagen), which allows for a His-tagged expression product. The expression construct was transformed into an E. coli Rosetta-gami (DE3)pLysS expression line (donated by Mark Paine, LSTM).

Expression and purification of His::GmmSRPN10

Transformed Rosetta-gami cells were grown in ampicillin LB (50 µg/L) at 37°C to an OD600 of 0.5–0.8. Expression was induced by isopropyl-1-thio-D-galactopyranoside at a final concentration of 1 mM. Expression continued for 24 h at 30°C with agitation. Cells were harvested by centrifugation at 3,500 x g for 15 min at 4°C and reconstituted in PBS. Harvested cells were sonicated on ice at 30 s intervals (with intermittent cooling periods of 30 s). Cellular debris were removed by centrifugation at 3,500 g for 15 min at 4°C and the supernatant was purified with a Ni-NTA agarose column (Qiagen) by selectively binding the His-tagged recombinant protein at an imidazole concentration of 20 mM and a NaCl concentration of 0.25 mM. Bound protein was first washed with wash buffer (50 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole) and then eluted off the column with elution buffer (50 mM sodium phosphate, pH 8.0, 250 mM imidazole). The identity of the recombinant protein was confirmed using SDS-PAGE and mass spectrometry after in-gel trypsinisation and LC-MS/MS analysis (at the University of Dundee Post-Genomics Facility).

Confirmation of inhibitory activity of purified His::Serpin10 was carried out by assaysing its inhibition of bovine pancreatic trypsin (Sigma) activity in the presence of Na-benzoyl-L-arginine ethyl ester (BAEE) substrate according to manufacturer’s protocol (Sigma) with slight modifications. Briefly, 100 BAEE units of trypsin was incubated with His::Serpin10 for 10 min at room temperature prior to addition of BAEE. The absorbance (253 nm) of the reaction mix was then continuously recorded for 5 min or until saturation of the absorbance value. Trypsin activity was subsequently calculated from the maximum linear rate of the increase in absorbance (253 nm).

Trypanosome lysis assays

Complement assays were performed at 26°C for 30 min in 200 µL volumes with 25% (v/v) concentration of horse serum (HS) containing 10³/ml PF trypanosomes. Assays were stopped with 300 µL of cold SDM-79 medium. Surviving trypanosomes were counted on a Neubauer haemocytometer. The % survival was normalised against PFs incubated with heat-inactivated (HI) serum (56°C for 1 h), with the exception of assays where serum was inactivated using His::GmmSRPN10. In these experiments, the % survival was normalised against PFs exposed to serum pre-treated with heat denatured (100°C for 10 min) His::GmmSRPN10. The complement assay was also used to determine the degree of complement inactivation caused by cobra venom factor (Quidell) or bovine trypsin (Sigma).

Generation of GmmSRPN10 antiserum for western blotting

In silico prediction of target peptide antigenicity, solubility and specificity was done using SMMTrfP [29] and a high scoring epitope (QTKDDFWISSEESVQLEYM) was chosen for its specificity to GmmSRPN10 (GMOY012007) via reciprocal BLAST with Vectorbase [30]. The key antigenic region of the epitope (underlined) was predicted using an online resource by the Universidad Complutense de Madrid utilising the Kolaskar and Tongaonkar method [31]. The final peptide sequence (KDDFWISSEESVQLEY) was used to immunise rabbits (Eurogencell) to generate antiserum specific for GmmSRPN10 (α-GmmSRPN10). The polyclonal rabbit antiserum was subsequently affinity purified for IgG using a Nab Spin Kit (Fisher Scientific UK Ltd) to remove cross-reactivity with blood fed midgut samples. Western blots using anti-GmmSRPN10 at 1:100 dilution and goat α-rabbit HRP-conjugated secondary antibody (Thermo Scientific) at 1:25,000 dilution were developed using SuperSignal West Dura (Pierce) substrate and Carestream® Kodak BioMax Light film (Sigma). All blot membranes were subsequently stained with 0.2% (w/v) nigrosin in PBS (Sigma) to confirm equal protein loading.

qPCR analyses of GmmSRPN10 mRNA from tsetse midgut tissue

cDNA was prepared from Trizol-extracted total RNA using AccuScript Reverse Transcriptase (Agilent Technologies) or with
SuperScript III Reverse Transcriptase (Life Technologies). qPCR was performed using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies) and primers specific for GmmSRPN10, tsetse β-tubulin and tsetse α-tubulin, using a Strategene Mx3005P real time PCR detection system (Agilent Technologies). Analysis for fold change in dsGmmSRPN10-fed tsetse compared to dsEGFP-fed tsetse was carried out using the Pfaffl method [32] with β-tubulin as the reference gene for GmmSpn10 and β-tubulin. Primer sequences for β-tubulin were taken from [33], while the primers for α-tubulin and GmmSRPN10 are:

GmmSRPN10 Forward – TACCTTCCAATTCGGACCAG
GmmSRPN10 Reverse – CCTGAATACGCAAACCTCGT

Statistical analyses
Non-parametric t-test and one way ANOVA were carried out with GraphPad Prism5, with P<0.05 considered significant and P<0.001 considered highly significant. All error bars presented in figures represent standard deviation.

Results
Four putative serpins were identified from the tsetse midgut EST library
As an annotated genome was not available at the commencement of this research, the G. m. morsitans EST library was used to identify four putative serpins (Table 1) from midgut tissue [24]. An alignment of the active site of these serpins with that of the human complement cascade inhibitor SerpinG1 using Clustal-W (Fig. 1A) showed key residues were conserved [34] within the reactive centre loop (RCL). As the RCL constitutes the active site of serpins, we postulated that these four serpins may have complement cascade inhibiting properties associated with a haemoprophagous diet.

A phylogenetic tree (Fig. 1B) was generated from neighbour-joining alignments [35] for serpins selected from fully-sequenced arthropod genomes. To determine if these serpins were associated with blood-feeding, we included representatives of blood-feeding arthropods (Aedes aegypti and Anopheles gambiae) and non-blood feeders (Drosophila melanogaster and Tribolium castaneum). This did not result in a clear distinction between serpins evolved as a consequence of haemoprophagacy. To determine if adaptation to blood feeding directly applies evolutionary pressure to the RCL of insect serpins, phylogenetic trees were also generated from a subset of these representative serpins using either full length protein sequence (S1A Fig.) or RCL sequence (S1B Fig.). Likewise there was no clear clustering of serpins associated with adaptation to haemoprophagacy in either analysis. This is perhaps due to the high degree of conservation displayed across serpin proteins [36,37].

Knockdown of tsetse serpins decreases trypanosome infection rate
We reasoned that should these tsetse serpins have complement inhibiting properties, knocking them down in tsetse may be detrimental to PF trypanosomes establishing in the midgut. Gene knockdown in tsetse can be achieved by either injection or feeding of dsRNA [28]. To prevent activation of fly immunity by puncturing its cuticle, the feeding route was chosen. Feeding dsRNA led to consistent knockdown of the target gene compared to control flies fed with dsRNA targeting EGFP (S2 Fig.). Monitoring of gene knockdown was carried out on the transcript level by semi-quantitative RT-PCR, which allowed for a rapid overview of gene knockdown and its associated infection phenotype.

There was variability in band intensity from our RT-PCR experiments and we have therefore highlighted changes of statistical significance from 3 biological replicates (S1 Table) in Fig. 2A. Using this criteria, a degree of cross-reactivity was observed with certain gene targets. There was co-suppression in the case of GmmSRPN9 knockdown, but also up-regulation when GmmSRPN3 was targeted. The co-knockdowns observed with dsGmmSRPN9 was unlikely to be due to non-specific targeting of the generated dsRNA as there was no continuously homologous regions of the expected dsRNA sequences with non-target serpins (S2 Table). This, coupled with the up-regulation observed with GmmSRPN9 when GmmSRPN3 was targeted suggests that this phenomenon may be due to a physiological response by the tsetse.

Regardless of which serpin was targeted, gene knockdown led to a decrease in trypanosome infection rate within the tsetse midgut (Fig. 2B). This was on average half the infection rate expected of a second bloodmeal infection [36]. In this preliminary experiment, the decrease in infection rate was significant when either GmmSRPN10 or GmmSRPN9 were targeted. As illustrated by the co-repression and up-regulation events going on with certain serpin knockdowns, we concluded that it would be difficult to determine gene function via knockdown alone. Furthermore, serpins involved in insect innate immunity may also confer infection phenotypes upon trypanosome elimination [39]. Therefore, we next attempted to express recombinant serpins to further elucidate their function with in vitro biochemical assays.

Active complement in horse serum is lethal to T. brucei PFs
HS is trypanocidal to trypanosome PFs. This activity can be inhibited by pre-treating the serum with heat inactivation (HI) or cobra venom factor (CVF). CVF exhausts the complement cascade by an overdrive mechanism where the cascade components are consumed and activated in the absence of an activating antigen [40,41]. Complement-inactivated HS prevents lysis of trypanosomes in vitro (Fig. 3A & B), demonstrating that the complement cascade is the lethal component in HS. When complement-inactivated HS was used for tsetse infection experiments, groups where T. brucei infection and subsequent feeding were carried out using complement-inactivated HS had an increase in T. brucei infection rate (Fig. 3C & D). This increase was statistically significant (P = 0.01) when the HS was inactivated by HI but not by CVF. Taken together, these data suggest that the complement component of HS is lethal towards T. brucei PFs, a phenomenon that remains relevant in the context of infection.

Recombinant GmmSRPN10 has complement-inhibiting activity
To facilitate a better understanding of the function of midgut serpins in G. m. morsitans, we attempted to express recombinant proteins using a bacterial expression system with the pET-45b expression vector. Addition of a histidine tag (His-tag) on the N-terminus of the recombinant proteins permitted purification by a nickel-affinity column without interfering with the RCL on the C-terminus. All attempts to express recombinant proteins using BL21(DE3) failed, perhaps due to the extent of rare eukaryote-specific codons present (S3 Table). However, recombinant expression was possible for GmmSRPN10 using a Rosetta-gami E. coli cell line as this bacterial strain allows for expression of proteins with rare eukaryotic codons.

Elution of His::GmmSRPN10 from a nickel column produced two proteins of similar apparent molecular mass (~40 kDa and ~37 kDa) as determined by Coomassie staining of SDS-PAGE.
The relative abundance of the two eluted proteins was dependent on the temperature at which expression was carried out, with a higher proportion of the higher molecular weight band produced when the expression temperature was set at 30°C compared to when expression was carried out at 37°C. Identification of excised bands by trypsin digest followed with mass spectrometry revealed the 40 kDa band to be His::GmmSRPN10 while the 37 kDa product was truncated His::GmmSRPN10 lacking the C-terminus RCL (S3A Fig.). This indicates that the stability of His::GmmSRPN10 is either temperature sensitive or susceptible to bacterial heat shock proteins.

His::GmmSRPN10 inhibits the trypanocidal activity of HS in a concentration dependent manner (Fig. 4B), suggesting that His::GmmSRPN10 inactivates the complement cascade in HS. Increasing concentrations of His::GmmSRPN10 were able to inhibit the activity of bovine trypsin (S3B Fig.), thus we reasoned that the RCL of His::GmmSRPN10 was functional and may be targeting complement cascade-specific serine proteases.
Tsetse GmmSRPN10 Prevents PF Lysis in Tsetse Midgut

**Table 1. Investigated tsetse midgut serpins.**

| VectorBase genome designation | PubMed EST designation | Number of amino acids |
|-------------------------------|------------------------|-----------------------|
| GMMY ID annotation GMOY ID    | Serpin annotation ABC25072 | 406                   |
| Serpin 1                      | ABC25075               | 421                   |
| Serpin 5                      | ABC25079               | 437                   |
| Serpin 6                      | ABC25076               | 240                   |
| Serpin 10                     | AFG28184               | 376                   |

Four putative serpins were initially identified from a tsetse midgut EST library. Subsequent sequencing and annotation of the genome has now assigned these serpins with GMOY IDs in the Invertebrate Vectors of Human Pathogens database, VectorBase (https://www.vectorbase.org).

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We next tested the inhibitory activity of His::GmmSRPN10 against two commercially available human complement cascade serine proteases, recombinant (r) C1s and Factor D. C1s activates the classical complement pathway and Factor D activates the alternative pathway. rFactor D was chosen for this analysis as complement lysis of *T. brucei in vitro* is activated via the alternative pathway [22]. rC1s was also included as we reasoned that a serpin that inhibits complement cascade serine proteases may not be pathway specific. His::GmmSRPN10 blocked the activity of both rC1s and rFactor D in a concentration dependent manner (Fig. 4C & D) compared to a commercial trypsin inhibitor at comparable concentrations. Taken together, these data suggests that GmmSRPN10 is an inhibitory serpin that inactivates complement killing of PF trypanosomes within the tsetse midgut.

**GmmSRPN10 is a secreted protein important for PF survival in the tsetse midgut**

For GmmSRPN10 to inactivate bloodmeal complement cascade serine proteases, it should be secreted into the midgut lumen. Peptide sequence analysis using SignalP 3.0 [42] predicted that GmmSRPN10 lacked an N-terminal signal peptide associated with the secretory pathway, while SecretomeP 2.0 [43] prediction suggests that GmmSRPN10 has sequence elements indicative of non-classically secreted proteins (S4 Fig.). As such, we questioned if GmmSRPN10 could be secreted via the non-classical pathway without a leader sequence [44,45]. To determine if GmmSRPN10 localises to the midgut lumen, rabbit α-GmmSRPN10 antisera was raised by immunising rabbits with a short, immunogenic peptide specific to GmmSRPN10 (S5A Fig.). The antisera recognised and bound to His::GmmSRPN10 in a concentration dependent manner (S5B Fig.). Probing of harvested tsetse midguts with IgG-enriched antiserum identified GmmSRPN10 (migrating at approximately 40 kDa) both in midgut tissues and contents washed from the midgut lumen (Fig. 5A, S6 Fig.). This suggests that GmmSRPN10, as it is secreted into the midgut lumen, comes into contact with an ingested bloodmeal.

We next attempted to verify that the trypanosome infection phenotype observed in flies fed with dsGmmSRPN10 was indeed caused by depletion of both the transcript and the protein. Experimental test fed with dsRNA were infected with trypanosomes and subsequently dissected at day 10 post-infection. In conjunction with scoring for the percentage of infected midguts, tissue samples were collected and subjected to qPCR and Western analyses. Using *Glossina* α-tubulin as the reference gene, we determined that GmmSRPN10 transcript abundance was decreased in dsGmmSRPN10-fed midguts compared to controls fed with dsEGFP (Fig. 5B). This decrease in GmmSRPN10 transcript level was significant compared to that of α-tubulin, which remained relatively unchanged (fold change ~1) regardless of dsRNA treatment. Western blotting using α-GmmSRPN10 also confirmed a decrease in protein expression in the same pool of knockdown midguts used for qPCR (Fig. 5C).

The infection rate of trypanosomes in GmmSRPN10-knockdown tsetse fell by one third compared to dsEGFP-fed controls (Fig. 5D). This reduced rate of midgut infections was statistically significant (non-parametric t-test, P = 0.0137) across 8 biological replicates with a total of more than 300 flies dissected for each treatment. As the experimental tsetse are fed with fresh blood once every two days post-infection, this demonstrates a correlation between reduced PF infection in the presence of bloodmeal complement upon GmmSRPN10 knockdown. Together with the *in vitro* assays involving complement cascade serine protease inhibition, these data suggest that one function of GmmSRPN10 is to act as a complement inhibitor in the tsetse midgut, which PF trypanosomes use to evade lysis by the serum complement introduced into the midgut environment with every bloodmeal.

**Discussion**

Blood-borne pathogens typically evolve an intimate life-cycle with haematophagous arthropods that inadvertently become their vectors for transmission. As such, pathogens can often develop extensive interactions with their arthropod vectors where they modify their feeding behaviour or exploit pre-existing biological secretions from the vector to improve their chances of survival and transmission [8,36,46,47]. Here we described how PF trypanosomes benefit from the activity of tsetse serpin, GmmSRPN10, to avoid lysis by bloodmeal complement when in the fly’s midgut.

Phylogenetic analysis suggests that GmmSRPN10, along with three other tsetse serpins, may not have evolved in a common blood feeding ancestor, but may have evolved independently after speciation from the last common ancestor. While all four of the investigated serpins showed causality between gene knockdown and decreased infection of trypanosomes in tsetse, only GmmSRPN10 could be correlated to the inactivation of complement cascade serine proteases by activity assays utilising a recombinant version of the protein. Inactivation of both human rC1s and rFactor D, initiating serine proteases of the classical and alternative pathways, respectively [48], suggests that GmmSRPN10 may have a generic inhibitory function against complement cascade serine proteases. This might be a characteristic of arthropod complement-inhibitory factors, as exhaustive investigation into scabies mite serine proteases (SMB3, SMB4) as well as inactive serine proteases have found them to inhibit all three complement cascade pathways [8,49].
For GmmSRPN10 to function as an inhibitor of bloodmeal complement, it should be secreted into the midgut lumen where it can come into direct contact with the resting bloodmeal. Unlike other documented complement-inhibiting arthropod serpins [8,49], GmmSRPN10 does not have an N-terminal signal peptide to identify it as a protein secreted via the classical pathway. However, the lack of an N-terminal signal sequence is not definitive evidence that a protein is not secreted, as analyses of other biological systems indicate that proteins without an N-terminal signal peptide can be present at comparable, if not higher, levels in the secretome compared to proteins with an N-terminal signal peptide [50,51]. Our Western blots detected GmmSRPN10 in the tsetse midgut lumen in both teneral and non-teneral flies. As teneral flies are newly emerged and have never fed on blood, this suggests that GmmSRPN10 is constitutively secreted into the midgut lumen of emergent tsetse in preparation for feeding.

A recent analysis of transcriptome fold changes within tsetse SGs identified GmmSRPN10 (annotated as Serpin 6, GeneBank ID ABC25076) as amongst an exclusive subset of genes up-regulated during chronic trypanosome infection [52]. Although implicated as a possible modulator of tsetse immunity, our knockdown experiments suggest that it is unlikely to fit its suggested role as a positive regulator of fly immunity in the...
midgut environment. A possible reason for up-regulation of GmmSRPN10 in trypanosome-infected SGs may be related to the changes in feeding behaviour exhibited by these flies. Tsetse with infected SG take longer to probe and feed [53], and this may necessitate increased expression of GmmSRPN10. Regardless, expression in the tsetse SG suggests inactivation of complement in the bloodmeal may already begin prior to ingestion and continue in the resting bloodmeal within the tsetse midgut. Although we are currently uncertain how dsRNA feeding affects GmmSRPN10 expression in the SG, gene knockdown by dsRNA feeding has been shown to be specific to genes found in the midgut [28]. Likewise, we are uncertain regarding the knockdown status of GmmSRPN10 in the SG. However our data demonstrates that GmmSRPN10 knockdown on the transcript and protein levels is consistent in the midgut with dsRNA feed, and this leads to a decrease in the rate of infection of *T. brucei* in tsetse.

Collectively, our findings suggest that GmmSRPN10 can inactivate the complement cascade by inhibiting the activity of cascade activators present in the ingested bloodmeal. When establishing PF trypanosomes encounter a fresh bloodmeal, the complement inhibitory activity of GmmSRPN10 appears to confer protection for these trypanosome PFs, as a decrease in GmmSRPN10 lowers the success rate of PF midgut infection. While all tsetse midgut serpins appear to be important for PF midgut survival, direct inhibition of complement cascade serine proteases could only be confirmed with GmmSRPN10. Therefore, we are currently unable to discern if the reduction in midgut PF infection with knockdown of the other three serpins is indeed related to the loss of complement inhibition, or if the impact on PF survival from GmmSRPN10 knockdown alone is moderated by the redundancy of another three serpins of similar function.

While exploitation of complement-inactivating proteins in vectors by the pathogens has previously been described in other pathogen-vector relationships [9,13], it is the first time this phenomenon has been reported in African trypanosomes and tsetse flies. Our experiments confirm the sensitivity of PFs to

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**Fig. 3. HS complement is lethal to PFs.** (A) The lethality of HS can be inactivated by pre-treating the serum with heat. PF mortality decreases significantly when HS is heat inactivated at 50°C for 1 h, and up to 88.5±5.67% survive when the temperature of inactivation was 60°C. (B) PF lysis by HS decreases when HS was pre-treated with CVF to exhaust complement cascade factors. (C) Tsetse infection experiments carried out using heat inactivated serum resulted in a significant (P = 0.01) increase in infected midguts in experimental groups where heat inactivated serum was used for infection and feeding. (D) A similar increase in infection rate was observed when the HS used in experiments was pre-treated with CVF, though this increase was not statistically significant compared to tsetse that were infected and maintained with HS pre-treated with boiled CVF after 4 experimental replicates.

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complement lysis by fresh serum and they likewise reveal that midgut-infecting PFs benefit in a small but significant manner from a complement-deactivating serpin secreted by the fly. It remains unresolved why the tsetse would secrete a serpin that favours a parasitic infection, but investigations into complement-inhibiting molecules secreted by other haematophagous arthropods suggest that this may be part of a strategy to minimise damage to the midgut epithelium or peritrophic matrix by bloodmeal complement during feeding [5,6].

As other trypanosomatids, such as *Trypanosoma cruzi*, have been shown to express innate complement-inactivating factors [54], we cannot discount that similar factors exist within *T. brucei*. One possible complement-evasion mechanism that may be innate to PF *T. brucei* and *T. congolense* involves sequestering sialic acids (SA) from red blood cells, which could consequently mask trypanosomes from activating the complement cascade. *T. brucei* PFs defective in the expression glycosylphosphatidylinositol (GPI)-anchored trans-sialidases, do not survive in the tsetse midgut and this may be due to the inability of these mutant parasites to transfer sialic acids to their cell surface GPI acceptor molecules [55]. However, there is currently no direct evidence suggesting that the transfer of SA or the expression of TS itself is related to a complement evasion strategy.

Furthermore, *T. brucei* crosses the tsetse peritrophic matrix (PM) on at least two occasions during the course of infection in the fly. This occurs once in the posterior midgut to gain access to the ectoperitrophic space located between the PM and the midgut epithelium, and a second time in the anterior midgut upon trypanosome re-entry into the lumen of the alimentary canal [18,56–58]. Why trypanosomes would exhibit this presumably energetically costly behaviour is unknown, but our findings suggest this may be related to the attempts by complement-susceptible PFs to escape fresh bloodmeal content in the midgut lumen. As secreted proteins can be found at higher concentrations in proximity to epithelial tissue (as in the case of mammalian intestinal anti-microbial peptides [59]), we postulate that PM crossing may also be due to PFs seeking regions of high tsetse serpin concentration as further protection against complement lysis.

Fig. 4. Expression of His::GmmSRPN10 is temperature sensitive and the expressed protein inhibits killing of PF trypanosomes by HS *in vitro*. (A) Recombinant expression of His::GmmSRPN10 results in an intact (~40 kDa) and truncated (~37 kDa) protein fraction that were identified using mass spectrometry. Expression at 30 °C enriches the intact fraction. (B) Suppression of trypanocidal activity by His::GmmSRPN10 can be achieved by pre-treating HS with the recombinant protein. (C–D) His::GmmSRPN10 inhibits the activity of rC1s and rFactorD, recombinant human complement cascade serine proteases, in a concentration dependent manner. Inhibition of the complement cascade serine proteases at comparable concentrations were not observed in the controls performed in tandem using a commercially available soybean trypsin inhibitor.

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In conclusion, evasion of bloodmeal complement by T. brucei PFs by taking advantage of tsetse biology illustrates the complicated relationship African trypanosomes share with the tsetse, as well as the innovative evolutionary adaptations in blood borne parasites to achieve cyclical transmission within their insect vectors.

**Supporting Information**

**S1 Fig.** There is no clear evolutionary relationship in the RCL of serpins in haematophageous insects. Phylogenetic trees for representative insect serpins generated in parallel using (A) full length sequence or (B) RCL sequence did not resolve a clear evolutionary relationship of serpins in insects adapted to blood feeding. Bootstrap values are presented and tsetse serpins are denoted with *. (TIF)

**S2 Fig.** Representative gel of RT-PCR results of tsetse serpin transcript levels with dsRNA feeding. Amplicons generated using RT-PCR from total miRNA extracted from the...
midgut tissue of experimental tsetse fed with dsRNA with different gene targets (GmmSRPN3, GmmSRPN10, GmmSRPN5, GmmSRPN9 and EGFP). The relative intensity of bands generated from primers targeting GmmSRPNs were normalised against the band intensity for G3PDH for each dsRNA treatment. The normalised band intensity for each transcript was subsequently presented as a % (S1 Table) of the normalised band intensity of the corresponding band generated from mRNA extracted from EGFP dsRNA-fed tsetse.

S3 Fig. His::GmmSRPN10 is an inhibitory serpin. (A) Mass spectrometry analysis of ~40 kDa and ~37 kDa fractions of recombinant His::GmmSRPN10 confirm the ~40 kDa fraction is the full length protein, while the ~37 kDa protein represents a C-terminal truncation of His::Serpin10 at the reactive centre loop. (B) Recombinant His::GmmSRPN10 can inhibit trypsin activity in a concentration dependent manner.

S4 Fig. SignalP and SecretomeP analysis of GmmSRPN10. (A) SignalP analysis did not detect a signal peptide (S-score) nor a signal peptide cleavage site (C-score) either independently or as a geometric average (Y-score). (B) SecretomeP analysis GmmSRPN10 indicates an NN-score above the threshold of 0.5. In the absence of a signal peptide, this suggests that GmmSRPN10 is secreted in a non-classical manner.

S5 Fig. Generation of antibody against GmmSRPN10. (A) The immunising peptide used to generate a polyclonal α-GmmSRPN10 rabbit antisemum, represents positions 184–199 of the GmmSRPN10 protein sequence. The predicted region with highest immunogenicity is shaded in grey. (B) Western blotting of GmmSRPN10 protein sequence. The predicted region with highest immunogenicity is shaded in grey. (C) Western blotting GmmSRPN10 rabbit antisemum, represents positions 184–199 of GmmSRPN10.

S6 Fig. Western blots showing GmmSRPN10 is detected in tsetse midgut tissue and washed lumen content. Midgut (MG) tissue was collected from teneral (unfed) flies and flies receiving one bloodmeal. Midgut lumen content was washed out with PBS and both collected tissue and lumen content were resolved on 12.5% SDS-PAGE gel prior to blotting with α-GmmSRPN10 rabbit antisemum. (A) Midgut tissue lysate (2, 3, 4, 5 MG equivalents) and (B) midgut lumen content (1 and 2 MG equivalents) were isolated from teneral and fed flies. GmmSRPN10 is present in the MG lumen at comparable levels to MG tissue. PVDF membrane staining with nigrosine is shown to indicate protein loading.

S1 Table. Relative band intensity (%) for GmmSRPN3, GmmSRPN5, GmmSRPN9 and GmmSRPN10 in from RT-PCR amplicon products on midgut RNA extracted from dsRNA treated flies.

S2 Table. BLAST alignment of tsetse serpin dsRNA fragments. Output from multi-alignment BLAST of dsRNA fragments targeting tsetse serpins against all serpin gene sequences. Each dsRNA fragment only has significant alignment against its target gene.

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Author Contributions
Conceived and designed the experiments: CPO MJL AAS. Performed the experiments: CPO LRH DMS. Analyzed the data: CPO LRH DMS MJL AAS. Contributed reagents/materials/analysis tools: MJL AAS. Wrote the paper: CPO MJL AAS.

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