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Functional analysis of tick salivary serine and cysteine protease inhibitors

Ph.D. Thesis

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Annotation:

The proposed thesis focuses on the characterization of two protease inhibitors present in tick saliva. More specifically, the thesis presents immunomodulatory properties, biochemical specificity and structure of a cysteine protease inhibitor named Iristatin. Another characterized protein, IRS-8, comes from a serpin family (serine protease inhibitors) and inhibits blood coagulation and complement system in the host. Furthermore, the thesis provides a literature overview and discussion of tick salivary molecules in the context of tick-host-pathogen interaction, vaccination potential and medicine potential. Two review manuscripts, which are part of this thesis summarize the effects of tick saliva and protease inhibitors on host immune mechanisms.

Declaration

I hereby declare that this thesis is based on my own work and all other sources of information have been acknowledged. I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my PhD thesis, in full to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages. Further, I agree to the electronic publication of the comments of my supervisor and thesis opponents and the record of the proceedings and results of the thesis defense in accordance with aforementioned Act No. 111/1998. I also agree to the comparison of the text of my thesis with the Theses.cz thesis database operated by the National Registry of University Theses and a plagiarism detection system.

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List of papers and author's contribution

The thesis is based on the following papers (listed chronologically):

1. **Kotal, J.**, Langhansova, H., Lieskovska, J., Andersen, J. F., Francischetti, I. M., Chavakis, T., Kopecky, J., Pedra, J. H., Kotsyfakis, M., and Chmelar, J. (2015) Modulation of host immunity by tick saliva. *Journal of proteomics* **128**, 58-68; (IF: 3.509)
   - Jan Kotál is the first author, searched the literature, wrote the manuscript, prepared tables and figures.

2. Chmelar, J., **Kotal, J.**, Langhansova, H., and Kotsyfakis, M. (2017) Protease Inhibitors in Tick Saliva: The Role of Serpins and Cystatins in Tick-host-Pathogen Interaction. *Frontiers in cellular and infection microbiology* **7**, 216; (IF: 4.123)
   - Jan Kotál is the shared first-second author, searched the literature, wrote the manuscript and prepared tables.

3. **Kotal, J.**, Stergiou, N., Busa, M., Chlastakova, A., Berankova, Z., Rezacova, P., Langhansova, H., Schwarz, A., Calvo, E., Kopecky, J., Mares, M., Schmitt, E., Chmelar, J., and Kotsyfakis, M. (2019) The structure and function of Iristatin, a novel immunosuppressive tick salivary cystatin. *Cellular and molecular life sciences : CMLS* **76**, 2003-2013; (IF: 6.496)
   - Jan Kotál is the first author, designed and performed experiments, performed the analyses and wrote the manuscript.

4. **Kotal, J.**, Polderdijk, S. G. I, Langhansova, H., Ederova, M., Martins, L. A., Berankova, Z., Kotsyfakis, M., Huntington, J. A., and Chmelar, J. (In preparation) Tick Ixodes ricinus serpin 8 (IRS-8) inhibits coagulation and complement in the host.
   - Jan Kotál is the first author, designed and performed experiments, performed the analyses and wrote the manuscript.

Table of Contents

1. Introduction

1.1. Ticks as ectoparasites and disease vectors

Parasitism is an extreme form of symbiotic relationship between species, where the parasite is adapted to exploit the host´s organism, causes harm to the host and benefits from the mutual relationship[1]. Eukaryotic parasites have evolved six main strategies that differ in the way of host exploitation. Parasitoids grow inside the host and kill it when they complete their development, parasitic castrators suppress host reproduction to use the resources for their own reproduction, trophically transmitted parasites are transmitted by their host being eaten by the definitive host, directly transmitted parasites spread by contact among hosts, vector transmitted parasite require two different species – a host and a vector that transmits the parasite and finally micropredators use a variable number of species or hosts to feed on[2]. Ticks are blood-sucking ectoparasites of mammals, birds and reptiles and therefore belong to the micropredators group. Furthermore, they serve as vectors for a broad variety of vector transmitted parasites[2].

Phylogenetically, ticks belong to the class Arachnida, order Ixodida and can be further divided into three families – Ixodidae, Argasidae and Nuttalliellidae[3].

The smallest tick family, Nuttalliellidae, has only one known representative *Nuttalliella namaqua* living in the south of Africa. Nuttalliellidae represent the most basal tick lineage[4, 5].

Argasid ticks are also known as “soft ticks”, because they lack a scutum. Thus far, around 185 soft tick species have been described[6]. Unlike hard ticks, the soft ticks feed repeatedly in nymphal and adult stage, because of limited amount of blood they can ingest. Accordingly, feeding on the host is much shorter, from a few minutes up to two hours. Nymphal stage usually involves 3-6 instars, based on blood meal size and other factors. Both adult males and females feed on blood and females can lay eggs after each blood meal[4]. Medically important soft ticks belong mainly to *Ornithidoros* and *Argas* genera.

Ixodidae, also known as “hard ticks” due to sclerotized dorsal shield (scutum) covering the entire back of unfed individuals, represent the most abundant tick family with more than 700 species[6]. Hard ticks remain attached to their host

for days or even weeks; however only once in each life stage (larva, nymph, adult)[4, 7]. Most important hard ticks belong to genera *Amblyomma*, *Dermacentor*, *Haemaphysalis*, *Hyalomma*, *Ixodes* and *Rhipicephalus*.

Our model organism, tick *Ixodes ricinus* belongs to Ixodidae family. *I. ricinus* is a European tick found also in the neighbouring parts of northern Africa and the Middle East[8]. *I. ricinus* undergoes a life cycle that involves 3 stages, each of which needs a specific host to feed on. Six-legged larvae hatch from eggs, feed mainly on small rodents and moult into nymphs. The eight-legged nymphs feed on mid-sized animals for 3-5 days and they moult into an adult after they drop off the host. Adult males do not feed on blood, but they can be found on hosts seeking females in order to reproduce. Adult females feed mainly on bigger mammals like deer, cattle, or dogs for 7-10 days. Their feeding course can be divided into two main phases. During the 6 days long slow feeding phase, ticks only imbibe a limited amount of blood while modulating the feeding pool and growing an excess of cuticle. Rapid engorgement phase starts 1-2 days before tick detachment from the host. In this phase, ticks ingest most of blood and drop off the host[9]. Tick females only finish feeding successfully, if they are mated and fertilized by a male. Afterwards they lay between 500 and 2000 eggs. New larvae hatch during late spring or fall and usually start feeding during the following season[4]. In nature, this whole generation cycle takes between 2 and 6 years, depending on meal availability and location.

Ticks serve as vectors for a variety of diseases. The tick-borne pathogens consist mainly of viruses and bacteria, but include also protozoa, fungi or helminths[10].

More than 160 viruses[11] have been associated with tick transmission, comprising at least 12 virus genera[12]. The tick *I. ricinus* is a major vector for the tick borne encephalitis virus (TBEV)[13]. Tick borne encephalitis is caused mainly by three subtypes of the virus, European, Siberian and Far-Eastern subtype. The subtypes differ in severity of symptoms that a patient develops and the disease can be prevented by vaccination[14].

Although ticks can transmit a broad range of bacteria, *Anaplasma*, *Rickettsia* and *Borrelia* genera have been studied most intensely due to their direct impact on human health[15, 16]. The most important bacterial disease, transmitted by *I. ricinus*, is Lyme disease. In Europe, the causative agent is *Borrelia burgdorferi sensu lato*[17], which comprises around 20 different

genospecies. Among these, *Borrelia afzelii* and *Borrelia garinii* infections cause the Lyme disease symptoms[18]. In North America, the same disease is caused mostly by a single strain *Borrelia burgdorferi sensu stricto*[18]. Lyme disease can cause dermatological, neurological, rheumatological, or cardiac problems and can be treated with antibiotics. At this moment, there is no effective vaccine against Lyme disease in the market, although promising results have recently been achieved with adjuvanted OspA-ferritin nanoparticles in a mouse model[19]. *Anaplasma phagocytophilum*, causing granulocytic anaplasmosis or *Rickettsia helvetica* and *Rickettsia monacenis*, agents of spotted fever, belong among other bacteria transmitted by *I. ricinus*[20].

Protozoans represent another group of tick-borne pathogens, mainly with a veterinary importance. *Theileria* genus infects cattle, as well as wild animals, is transmitted by *Rhipicephalus*, *Amblyomma*, *Haemaphysalis* or *Hyalomma* ticks[21] and causes agricultural losses. Similarly, *Babesia bovis* transmitted by *Rhipicephalus* ticks is considered to be one of the species with greatest economic impact in cattle industry[15]. *I. ricinus* is also the vector of three *Piroplasmida* pathogens that have been reported to infect humans – *Babesia microti*, *Babesia venatorum* and *Babesia divergens*[20].

1.2. The role of tick saliva in feeding and pathogen transmission

For successful and long feeding on their hosts, tick adapted to modulate host immune reaction and hemostasis by secreting saliva into their feeding cavity (Fig. 1). It is important for the tick to suppress blood clotting and immune reaction in the skin wound caused by hypostome insertion in order to avoid tick rejection by the host[22]. A complex cocktail of salivary components suppresses host responses at the feeding site that may disrupt blood flow, cause pain and itching or lead to edema formation[22]. The mechanical skin injury immediately triggers coagulation, vasoconstriction and platelet aggregation – mechanisms of hemostasis to prevent blood loss. The coagulation cascade results in the production of fibrin that supports platelets during the process of thrombus formation. Vasoconstriction leads to slower blood flow and decreased bleeding and therefore facilitates the formation of thrombus[23].

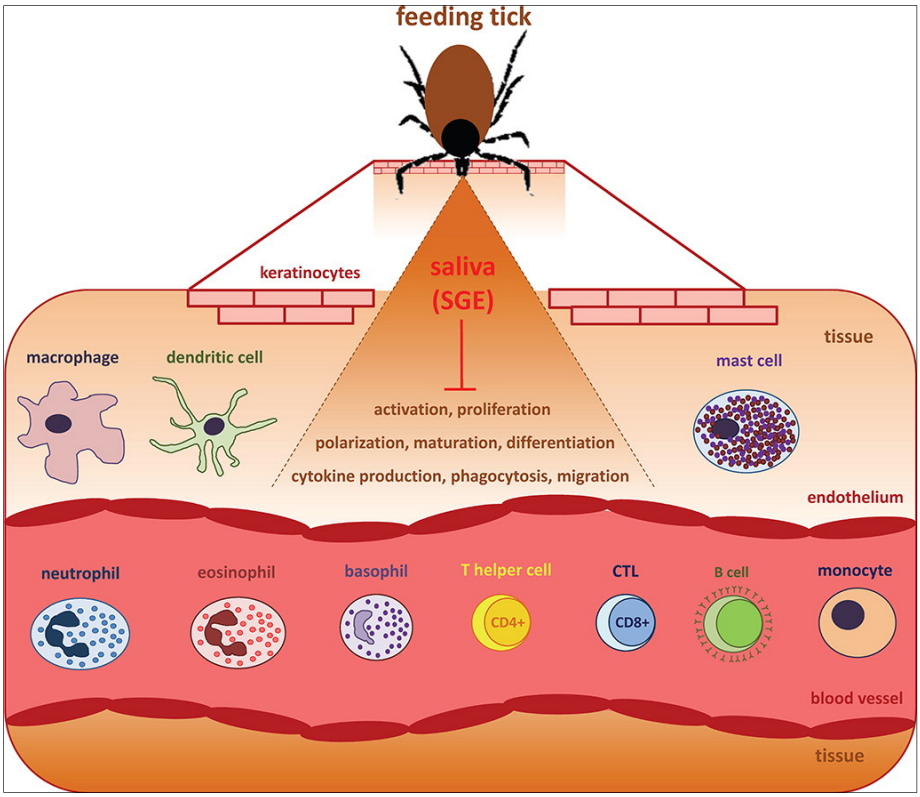

Figure 1: Schematic overview of saliva effects on populations of cells involved in anti-tick immunity. Figure shows the processes that are inhibited in respective cell populations. SGE: salivary gland extract, CTL: cytotoxic T lymphocyte. From: Kotál et al., 2015, J. Proteomics[22].

Blood coagulation is a proteolytic cascade driven by serine proteases that leads to thrombin activation and subsequent production of fibrin clot, where platelet aggregation and thrombus formation occur. Coagulation can be initiated via two pathways, the extrinsic and intrinsic, both of which result in the activation of thrombin, which then cleaves fibrinogen to fibrin, the primary component of the clot[24]. The extrinsic pathway starts with a blood vessel injury and the formation of complex between activated factor VII (fVIIa) and tissue factor (TF). TF/fVIIa complex then activates factor X (fX), either directly or via activation of factor IX (fIX), which in turn activates fX. The intrinsic pathway is triggered by the activation of factor XII (fXII) by a protease kallikrein. Activated fXII (fXIIa) activates factor XI (fXI), which next activates fIX and results in the activation of fX. Following that, a

common pathway ends the coagulation process regardless of the initiation. Activated fX (fXa) forms a complex with factor V (fV) which then cleaves prothrombin to thrombin and converts fibrinogen to fibrin[24, 25].

Since blood coagulation would be harmful for a feeding tick, their saliva contains anti-coagulatory molecules. The largest group of tick anti-coagulatory molecules are serine protease inhibitors that target thrombin, fXa and other related proteases. The main two protease inhibitor families involved are serpins and Kunitz domain inhibitors, followed by trypsin inhibitor-like proteins and Kazal domain inhibitors [26]. The effect of tick salivary anticoagulants is summarized in Figure 2.

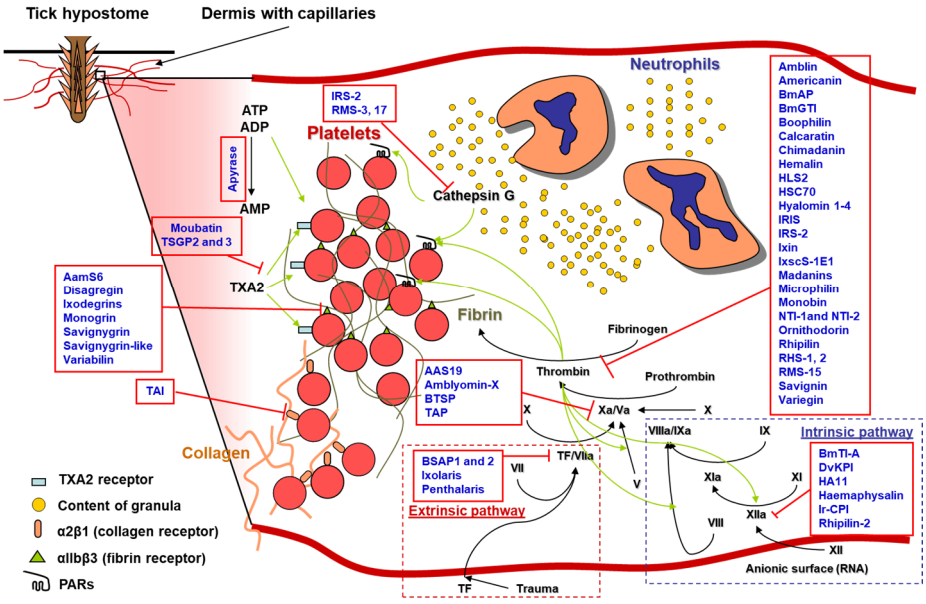

Figure 2: Summary of tick anti-hemostatic molecules and their molecular targets. Roman numerals in the coagulation cascade refer to coagulation enzymes and factors. TXA2: thromboxane A2; TF: tissue factor; AamS6: *Amblyomma maculatum* serine protease inhibitor 6; TSGP2, 3: Tick salivary gland protein 2, 3; IRS-2: *Ixodes ricinus* serpin 2; RMS-3, 15, 17: *Rhipicephalus microplus* serpin 3, 15, 17; AAS19: *Amblyomma americanum* serpin 19; BTSP: basic tail secretory protein; TAP: tick anticoagulant peptide; BSAP1, 2: $BaSO_4$-adsorbing protein 1, 2; BmAP: *R. microplus* anticoagulant protein; BmGTI: *R. microplus* gut thrombin inhibitor; HLS2: *Haemaphysalis longicornis* serpin-2; HSC70: heat shock protein 70; IRIS: *I. ricinus* immunosuppressor; IxscS-1E1: *Ixodes scapularis* serpin 1E1; NTI1, 2: nymphal thrombin inhibitor 1, 2; RHS-1, 2: *Rhipicephalus haemaphysaloides* serpin 1, 2; BmTI-A: *R. microplus* trypsin inhibitor A; DvKPI: *Dermacentor variabilis* Kunitz-

type serine protease inhibitor; HA11: *Hyalomma asiaticum* 11kDa protein; Ir-CPI: *I. ricinus* contact phase inhibitor. Adapted from: Chmelař et al., 2012, J. Proteomics[23].

Similarly to blood coagulation, the complement cascade of immune system is also based on serine proteases. Complement represents fast and robust defense mechanism as a part of innate immunity. Its role is in opsonization of infectious agents and their lysis, while activating other immune mechanisms[27, 28]. Complement can be activated by three pathways – classical pathway is initiated by antigen-antibody complexes, alternative pathway is triggered when the C3b protein directly binds a microbe and a lectin pathway needs a binding of a lectin to specific carbohydrates on pathogen surface[28]. All three pathways result in cleavage of C3 by C3 convertases to C3a and C3b fragments. C3b can consequently either enter a positive feedback loop to amplify complement response, opsonize pathogen cells to label them for phagocytosis or bind other subunits to form a C5 convertase. C5 convertase cleaves C5 to C5a and C5b fragments. C5b initiates the attack complex formation which leads to lysis of a target cell. C3a and C5a subunits have a role in promoting inflammation[27].
Complement is inhibited by ticks mainly at the level of C3 convertase activity by *Ixodes* species[29, 30] or by blocking C5 from being accessed by C5 convertase in the case of *Ornithodoros moubata*[31].

Consequently, both cellular and humoral immune responses are triggered as a result of the intrusion of the tick mouthparts. Resident skin cells like Langerhans and dendritic cells, keratinocytes, mast cells or macrophages are immediately exposed to tick hypostome and saliva. Release of pro-inflammatory cytokines and chemokines like interleukin-8, tumor necrosis factor, and interleukin-1β recruits other immune cells like basophils or neutrophils to the site of injury. Activation of basophils and mast cells leads to histamine release, itching and a possible removal of the tick by scratching off the host[22]. In case of repeated tick infestation, adaptive immunity also plays a role by activating B and T cells and producing specific antibodies. Antibodies can further sensitize histamine release cells or trigger the activation of complement through the classical pathway[32].

Tick saliva disrupts hemostatic processes at different levels. Serine protease inhibitors inactivate proteolytic cascade facilitated by serine proteases leading to thrombin generation and subsequent cleavage of fibrinogen to fibrin and blood clot formation. Platelet aggregation and vasodilatation are also affected, leading further to uninterrupted blood flow to tick´s feeding cavity[23].

Modulation of host immunity by tick saliva affects many different cell populations involved in both innate and adaptive immunity. Macrophages exposed to tick saliva produce less nitric oxide, which is an important signaling and anti-parasitic molecule. Furthermore, macrophages have decreased ability of phagocytosis and cytokine production. A similar effect was described also for cytokine production, migration, proliferation, maturation and phagocytosis by dendritic cells. Granulocytes showed impaired recruitment and adhesion, phagocytosis, degranulation, histamine release and production of reactive oxygen species. Furthermore, tick saliva blocks lymphocytes proliferation, expression of their surface molecules and polarizes their cytokine production towards Th2[22].

A weak immune response at the feeding site caused by tick salivary molecules is not beneficial only for the tick, but facilitates host infection by tick-borne pathogens. An enhancing effect of tick saliva was observed for transmission of viruses[33], bacteria[34] or apicomplexans[35].

A more detailed description of host immunomodulation by tick saliva can be found in a review "**Manuscript 1**"[22] which is a part of this thesis.

1.3. Proteases and their inhibitors

Proteases (or peptidases) are a family of enzymes able to hydrolyze peptide bonds[36]. Similarly to nucleases, proteases can be divided into two subtypes based on their mode of action. Exopeptidases cleave one or few amino acids at the C- or N-terminus of a peptide chain. Carboxypeptidases cut at the C-terminus, while aminopeptidases at the N-terminus. Endopeptidases cleave an amino acid chain within the sequence and are usually very selective for a particular amino acid sequence. They can be further divided into cysteine-, serine-, aspartic-, threonine-, glutamic- and metallo-peptidases according to their catalytic mechanism[37]. Amino acids at the catalytic site facilitate the hydrolysis of the peptide bond. Cysteine, serine, threonine and glutamic acid at the active site or their corresponding proteases initiate the nucleophilic

attack at the substrate molecule. Aspartic proteases and metalloproteases activate a molecule of water to initiate the attack[36, 38, 39].

Protease inhibitors block the activity of proteases. Their nature can vary from small molecules to peptides and proteins. Naturally occurring protease inhibitors are mostly of peptidic or protein origin, while inhibitors used for medical purposes are often small synthetic molecules [39]. Peptidic/protein inhibitors can be classified either by their mechanism of action or by type of protease they target. According to the MEROPS database, there are currently 106 families grouped according to similarities in their primary sequence and 40 clans classified according to similarities in tertiary structures[37]. Protease inhibitors can also be divided into two groups according to reversibility of inhibition. Reversible inhibitors bind to target protease by non-covalent interactions and can be removed by dilution, changes in ionic strength or by a stronger ligand. We recognize three subtypes of reversible inhibitors, classified by their binding to the protease or protease/substrate complex – competitive, uncompetitive and non-competitive inhibitors. On the contrary, irreversible inhibitors permanently inactivate the protease, often by formation of a covalent bond either within protease active site or the inhibitor covalently binds directly the protease[39].

Tick saliva is a rich source of various types of protease inhibitors. They can be divided according to specificity into cysteine and serine protease inhibitors and according to structure to most abundant Kunitz domain inhibitors, followed by cystatins, serpins and less abundant families[26, 40, 41]. While tick salivary cystatins are mostly connected with host immunomodulation and Kunitz domain inhibitors with the regulation of hemostasis, serpins possess both of these features[26]. The scope of this thesis are tick cystatins and serpins, which will be discussed further in more detail.

1.3.1. Cystatins – cysteine protease inhibitors

Cystatins form a family of reversible, competitive, tight binding inhibitors of legumain and papain-like cysteine proteases[42, 43]. According to MEROPS, cystatins (family I25) are represented by three subfamilies, namely type 1 stefins (I25A), type 2 and 3 kininogens (I25B) and type 4 fetuins (I25C)[37]. For illustration, structure of a selected member of each subfamily is shown in Figure 3. Stefins are usually localized intracellularly in the cytosol and the nucleus due to lack of signal peptide and they do not have disulfide bonds and

glycosylations. They are relatively small proteins – about 100 amino acids long with molecular weight ~11 kDa and one cystatin domain[43]. Similarly, type 2 cystatins also have a single cystatin domain with N-terminal glycine and QXVXG sequence in their loop 1. Compared to stefins, type 2 cystatins also possess a PW dipeptide motif in loop 2. Type 2 cystatins have a signal peptide, are exported out of the cell and are thus present in most biological fluids[39,40]. They have two disulfide bridges near their C-terminal, ~11-14 kDa, and they are generally not glycosylated[44]. Kininogens are larger multi-domain molecules that possess three cystatin-like domains, disulfide bonds and glycosylations and are of multifunctional nature. Kininogens are involved in the regulation of coagulation, inflammation or inhibition of papain-like proteases[43]. The fourth subfamily, fetuins are secreted glycoproteins with two cystatin domains that lack the inhibitory activity towards cysteine proteases[45]. They can either serve as metalloprotease inhibitors or carrier plasma proteins[46, 47]. Cystatins in mammals inhibit usually cathepsins that are involved in protein degradation in lysosomes, antigen processing or apoptosis. Consequently, they regulate immune mechanisms such as cytokine production, antigen presentation, phagocytosis, and inflammasome formation[48-50].

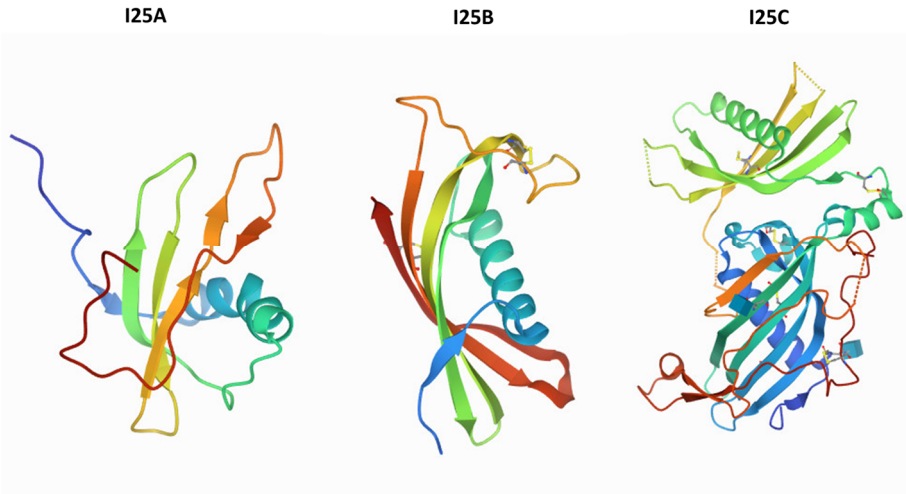

Figure 3: Crystal structures of selected representatives of each cystatin subfamily. I25A: Human cystatin A[51], I25B: Human cystatin D[52], I25C: Mouse fetuin B[53]. Crystal structures were gained from Protein Data Bank (https://www.rcsb.org/).

1.3.1.1. Tick cystatins

To date, only type 1 and 2 cystatins have been characterized in ticks. Since type 1 cystatins are not secreted, they are mostly associated with regulation of intracellular blood digestion in tick midgut and in developmental processes. Secreted type 2 cystatins are present not only in tick midgut as digestion regulators, but also in salivary glands and subsequently tick saliva[26, 54]. Secreted cystatins represent a majority among characterized cystatin transcripts across tick species, implying their importance in immunomodulation[55].

Tick midgut cystatins are mostly associated with regulation of peptidases involved in blood digestion and heme detoxification[54]. Blood digestion occurs in digestive cells in tick midgut. Degradation of albumin and hemoglobin, the two most abundant blood proteins, is initiated by legumain and cathepsin D. Action of these two aspartic peptidases is further supported by cathepsin L. Degradation to single amino acids and dipeptides is facilitated by cathepsins B and C[9]. Tick cystatins are capable of inhibiting most of the involved proteases and maintain a balance between too rapid proteolysis and availability of nutrients for the tick. Example of type 1 midgut tick cystatin is Rmcystatin-1b from *Rhipicephalus microplus* which inhibits cathepsins B and L, papain and *B. bovis* cysteine peptidase[56]. Another example is Hlcyst-1 from *Haemaphysalis longicornis*, an inhibitor of cathepsin L, papain and the tick´s own cathepsin L-like HlCPL-A protease and consequently also hemoglobinolysis[57, 58]. Tick midgut type 2 cystatins can be represented by Om-cystatin 1 from a soft tick *O. moubata[59]*, RHcyst-2 from *Rhipicephalus haemaphysaloides*[60] or JpIocys2a from *Ixodes ovatus*[61]. They inhibit cathepsins B, C, L and other proteases and fulfil the same regulatory purpose as midgut type 1 cystatins[26].

Tick salivary cystatins are solely of the secreted type 2. Some of them like HISC-1 from *H. longicornis* are expressed predominantly in tick salivary glands[62], other cystatins like Om-cystatin 2 (OmC2) can also be found in other tick tissues[59]. Their importance lies in the suppression and regulation of host immune response mechanisms, namely antigen processing and presentation, inflammation, cell proliferation or cell signaling.

OmC2 possesses inhibitory activity against cathepsins B, C, and H and papain[59]. At the tick-host interface, OmC2 inhibited the secretion of pro-inflammatory cytokines by dendritic cells and reduced T cells proliferation.

Feeding of *O. moubata* nymphs on OmC2 immunized mice resulted in increased tick mortality[63].

Two salivary cystatins from *Ixodes scapularis*, Sialostatin L and L2, have been thoroughly characterized at biochemical, immunomodulatory and pathogen transmission level[64-67]. Sialostatin L is a potent inhibitor of cathepsin L with weaker affinity also to other cysteine cathepsins C, S, V, X and papain[66]. It suppressed the proliferation of T cells, migration of neutrophils to inflammation site and the production of cytokines by mast cells, dendritic cells and lymphocytes[68]. Its immunosuppressive potential was successfully tested in experimental models of asthma[69] and autoimmune encephalomyelitis[68]. Sialostatin L2 inhibited cathepsins L, C, S and V and the maturation of caspase-1[65, 70]. It further suppressed cytokine production by macrophages and dendritic cells. Feeding of ticks on rabbit immunized with sialostatin L2 led to higher tick mortality and prolonged feeding time[64]. Sialostatin L2 was also positively correlated with *Borrelia* establishment in host skin. Mice co-injected with *Borrelia* and sialostatin L showed significantly higher spirochetes load than control group with only *Borrelia* injection[71].

Characterization of an *I. ricinus* salivary cystatin named Iristatin is a part of this thesis as "**Manuscript 3**"[72]. Briefly, Iristatin is a type 2 cystatin upregulated in salivary glands of *I. ricinus* during the feeding. It suppressed mammalian cathepsin C with a similar affinity as sialostatins and weakly inhibited also cathepsin L. Iristatin inhibited antigen induced CD4+ T cells proliferation and leukocyte recruitment to inflammation site. Furthermore, it displayed a broad spectrum of effects on cytokines production by various immune cell types.

1.3.2. Serpins – serine protease inhibitors

Serpins form the largest family of protease inhibitors in nature and they are present in all organisms, from a viruses or prokaryotes to vertebrates or plants [73, 74]. Most serpins are serine protease inhibitors, but some serpins also possess other functions like cysteine protease inhibitors, blood pressure regulators, chaperons or hormone transporters[75, 76]. Inhibitory serpins are classified in the I4 family of MEROPS database[37]. Serpins are irreversible inhibitors with a unique and highly conserved tertiary structure, which is important for their so called suicide mechanism of inhibition [77, 78].

Similarly to other serine protease inhibitors, serpin structure contains a reactive center loop (RCL) to interact with the active site of target proteases. Most serine protease inhibitors have a short and rigid loop and act as tight binding reversible inhibitors. On contrary, serpin RCL is around 20-24 amino acids long, flexible and resembles a substrate loop. The rest of serpin structure consists of a C-terminal b-barrel domain and an N-terminal helical domain. The RCL is located near the C-terminus[73]. A serpin molecule can exist in different conformations. Their native fold (Fig. 4A), in which serpins have inhibitory activity, is not the most stable. Upon proteolytical cleavage within the RCL, the RCL moves to the opposite end of the protein and is incorporated into the serpin β-sheet A, forming an extra antiparallel β-strand (Fig. 4C). This can also happen spontaneously without RCL cleavage to form a latent conformation. With the RCL incorporated into the β-sheet A, the serpin molecule is in its hyperstable form and is not capable of further protease inhibition[79, 80]. The ability to rapidly and stably incorporate RCL distinguishes inhibitory serpins from the non-inhibitory ones. The mechanism of protease inhibition involves creating a covalent bond between serpin RCL and protease active site, incorporation of RCL into serpin β-sheet A and subsequent change of the relative position of protease with respect to the serpin molecule[81]. The RCL serves as a bait for the protease and its amino acid sequence determines serpin's inhibitory specificity[82]. After successful docking at protease active site, serpin RCL is cleaved and a covalent bond is formed between serpin RCL and serine from the protease catalytic triad. Normally, the acyl–enzyme intermediate would be dissociated by a water molecule, both cleaved parts of substrate would be released, and protease returned to active state. However, before the protease can be regenerated, the covalently bound serpin dramatically changes conformation and traps the protease in an inactive intermediate form of a serpin-protease complex[81]. Due to formation of the covalent bond, serpins work in a suicidal mechanism, in which a single serpin molecule permanently inactivates a single protease molecule and can only be used once. The serpin mechanism of protease inhibition is shown in Figure 4. Formation of the covalent bond can further create a disorder in protease structure resulting in loss of ability to bind ions or exosite ligands[83-85].

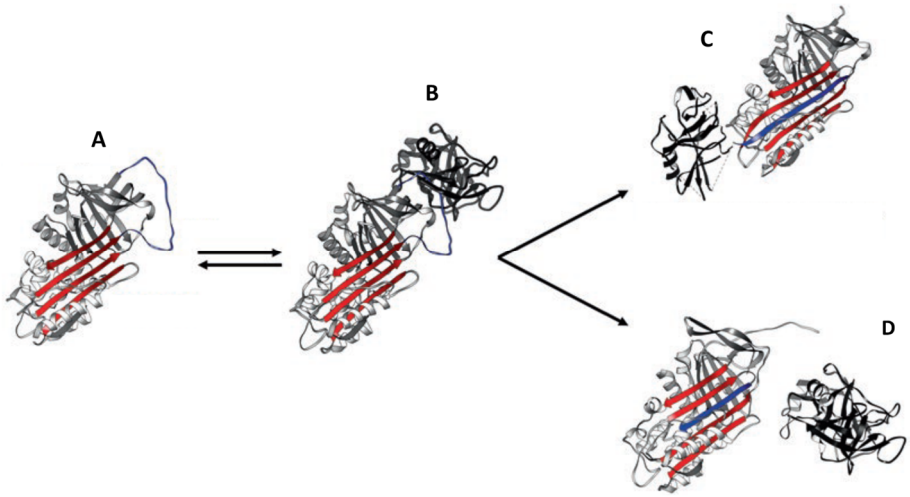

Figure 4: Schematic representation of serpin inhibitory mechanism. A: Alpha-1-antitrypsin in native state. RCL is shown in blue, beta sheets are red. B: Michealis complex between alpha-1-antitrypsin and trypsin (in black). C: Covalent complex between serpin and inactivated protease with RCL incorporated among beta sheets. D: Proteolysis of serpin was completed by trypsin, resulting in serpin inactivation and RCL incorporation, where trypsin remains active. Adapted from Lucas et al., 2018, Methods Mol. Biol.[86].

1.3.2.1. Tick serpins

In humans, 36 serpins have been identified, and most of them regulate important processes such as blood coagulation, fibrinolysis, tumorigenesis or inflammation[73, 87]. Mice possess 60 functional serpin genes, many of them are orthologous to human serpins with a similar role and some of them expanded into paralogous multiple genes[87].

Arthropod serpins have mainly hemostatic and immunological roles. They regulate hemolymph coagulation and its toll pathway or activation of phenoloxidase system in insects. Serpins from blood feeding arthropods evolved another set of functions – modulation of host immunity and hemostasis[88]. Indeed, more than 20 serpins from tick salivary glands have been functionally characterized with effects on coagulation and immunity[26]. However, the total number of tick serpins is significantly higher as seen in numerous transcriptomic studies[26]. In *I. ricinus*, at least 36 serpins were found, out of which only three have been characterized at biochemical, immunomodulatory, anti-coagulatory or anti tick vaccine level[26].

Iris (*I. ricinus* immunosuppressor) is the first described, and until now, the best characterized serpin from *I. ricinus*[89]. Iris transcription is induced

during feeding in tick salivary glands. Recombinant Iris inhibited factor Xa and thrombin from coagulation cascade, fibrinolysis and further interferes with hemostasis by preventing platelet adhesion[90]. Iris displayed also interesting immunomodulatory features. Its secretion to host body inhibits leukocyte and pancreatic elastase, thus suppressing inflammation[90]. It further inhibited the proliferation of T cells and splenocytes and altered pro-inflammatory cytokine levels produced by peripheral blood mononuclear cells[89]. This anti-inflammatory activity can be partially explained by Iris binding to monocytes/macrophages and suppression of tumor necrosis factor secretion[91]. Notably, not all immunomodulatory activities are dependent on Iris anti-protease activity, some involve the exosites in helices D and E[91].

IRS-2 (*I. ricinus* serpin-2) showed specificity against mast cell chymase and cathepsin G, two proteases involved in inflammatory responses and weakly against coagulation protease thrombin. In contrast to Iris, anti-inflammatory properties of IRS-2 are dependent on its inhibitory properties [92]. Inhibition of cathepsin G and thrombin contributed to the blocking of platelet aggregation by IRS-2. It further inhibited neutrophil influx to inflamed tissue and edema formation in mouse paw edema assay[92]. IRS-2 also modulated Th17 T cells maturation and differentiation by inhibiting interleukin-6 production by stimulated dendritic cells[93].

Characterization of IRS-8, a novel salivary *I. ricinus* serpin is a part of this thesis as "**Manuscript 4**". Briefly, IRS-8 is a serpin detected in tick salivary glands by RT-PCR and in the saliva by Western blot. IRS-8 is an inhibitor of a broad range of serine proteases, mostly those involved in blood coagulation and indeed, it inhibited the intrinsic pathway of coagulation. Specificity to different proteases can be explained at the structural level, where IRS-8 has an unusually long RCL. RNA interference of IRS-8 in ticks resulted in prolonged feeding time.

A more detailed list and description of tick salivary protease inhibitors, their specificities and effects on host immunity and hemostasis can be found in a review "**Manuscript 2**"[26] as a part of this thesis.

1.4. Protease inhibitors in other blood feeding parasites

Studying protease inhibitors and other proteins involved in mechanisms of tick-host interactions can also be beneficial in the context of other blood feeding parasites. Salivary components of other blood feeding arthropods like

mosquitoes[94-97], tsetse[98-100] and sand flies[101, 102] or triatomines[103-105] have similar effect on host immune system, hemostasis and pathogen transmission as tick saliva. We can benefit from finding similarities between molecules form different genera of parasites, apply knowledge obtained on one species on another or seek for new mechanisms based on similarities of blood feeding life style.

A salivary serpin named alboserpin from two mosquito species *Aedes aegypti*[106, 107] and *Aedes albopictus*[108] has been characterized as an fXa inhibitor and as an anticoagulant. Unlike other serpins, the mode of action of alboserpin is reversible and working at a 1:1 stoichiometry with fXa. However, saliva of mosquitoes is in general poor on serpins and they rely mainly on other protease inhibitors classes to prevent blood coagulation, as proven by transcriptomic studies[53, 109, 110]. *Rhodnius prolixus*, a member of triatomine bugs, uses a Kazal type inhibitor rhodniin[111], which inhibits thrombin with high affinity. Another triatomine, *Dipetalogaster maximus* inhibits thrombin by dipetalogastin[112], a homologous protein to rhodniin. Thus, triatomines rely mainly on inhibitors with multiple Kazal domains[113]. The tsetse fly *Glossina morsitans morsitans* uses the tsetse thrombin inhibitor (TTI)[114, 115] to suppress blood coagulation and thrombin induced platelet aggregation. TTI represents a unique class of protease inhibitors. Sand flies use different strategies and employ different protein families to stop host blood coagulation. Ayadualin, an Arg-Gly-Asp (RGD) peptide from *Lutzomyia ayacuchensis* inhibits the activation of coagulation factor XIIa and platelet aggregation[116]. Lundep (Lutzomyia NET destroying protein) from *Lutzomyia longipalpis* is an endonuclease involved mainly in destroying neutrophil extracellular traps, but also inhibiting the intrinsic coagulation pathway by blocking fXIIa activation[117]. Lufaxin from the same fly is a tight fXa inhibitor with antithrombotic and anti-inflammatory activities[118]. Anticoagulatory properties of blood feeding parasites were reported also in other phyla than arthropods. The leech *Hirudo medicinalis* secretes hirudin, the strongest known natural anticoagulant[119]. Even vampire bats have mechanisms against blood clotting as shown by desmolaris, an fXIa inhibitor from *Desmodus rotundus*[120].

Notably, the number of publications describing immunomodulatory effects of saliva from other blood feeding parasites than ticks, is rather low. This is probably not caused by lack of scientific interest, but mainly by the need of

the individual parasite. Ticks, which feed for days, need to overcome various host immune mechanisms. Other parasites feed on host blood for significantly shorter times so they focus mainly on ensuring continuous blood flow.

1.5. Vaccination potential of tick salivary and midgut molecules

Research of tick proteins has been pushed forward by two ultimate goals. The first one is the identification of a suitable antigen, or cocktail of antigens, that could be used for animal and human vaccination[16, 121]. The second goal is a description of tick immunomodulatory molecules and their use in human medicine[122, 123].

Vaccination is considered as a tick control strategy complementing the use of acaricides or biological methods like the use of entomopathogenic fungi[16]. Targeting tick antigens in a quest to find an "anti-tick" or pathogen transmission blocking vaccine is an alternative to targeting individual tick borne pathogens one by one[124]. The purpose of such vaccine is to elicit a strong immune response in a vaccinated individual and thus prevent or complicate tick feeding and ideally also pathogen transmission[16]. However, the development of such a vaccine has been problematic, because we still do not fully understand mechanisms of tick rejection. Moreover ticks employ wide range of pharmacoactive molecules that display high level of redundancy[125]. Therefore, when one protein is blocked by a vaccine, several others can substitute its role in evading host defense.

One strategy of anti-tick vaccine development is targeting antigens in tick midgut, where the vaccine would affect tick feeding and blood digestion. An example of such candidate is protein Bm86 from *R. microplus*, which is able to reduce local tick infestation by interfering with *R. microplus* life cycle[126] and is being used for cattle[127, 128]. However, vaccination with Bm86 homologues from *I. ricinus* did not bring any effect on tick fitness[129]. Another vaccine targeting tick midgut antigens is based on a protein named TROSPA (Tick receptor for outer surface protein A), which disrupts *B. burgdorferi* migration within the tick and therefore its capacity as a vector[130]. However, the applicability of TROSPA as a vaccine against all *Borrelia* species has not been evidenced to date. Big hope was raised by recently described vaccine based on OspA-ferritin nanoparticles blocks *Borrelia* colonization in host and is based on bacterial recombinant proteins[19].

In addition to gut antigens, targeting tick salivary proteins that play a crucial role in tick feeding has been proven to be an alternative vaccination approach. Many studies have shown that repeated exposure to ticks raises antibodies in the host and leads to various degrees of tick resistance and/or interruption of *Borrelia* transmission[131-133]. Expression of a salivary protein from *I. scapularis*, Salp 15, is induced in the tick when infected by *B. burgdorferi*. Salp 15 binds to *Borrelia* surface and protects the spirochetes from host immune system[134]. Apart from direct interaction with *Borrelia*, Salp 15 is also a potent immunosuppressor[135]. The importance of Salp15 was evidenced by RNA interference in ticks as well as by vaccination of mice[136]. The vaccination efficiency was further enhanced by combining Salp15 and *B. burgdorferi* outer surface proteins OspA/OspC, thus offering a vaccine based on recombinant proteins from both the vector and pathogen[136]. Another vaccine candidate, TLSPI (Tick mannose-binding lectin inhibitor) is a complement inhibitor and RNA interference of TLSPI or its use as a vaccine led to lower *Borrelia* loads in the skin. TLSPI was found in *I. scapularis* and *I. ricinus* and was upregulated in *I. scapularis* by *B. burgdorferi* colonization[100,101].

Although many research teams have been focusing on identifying target molecules to develop anti-tick or pathogen transmission vaccines, primary goal has still not been reached. Focusing on biological processes involved in tick-host, tick-pathogen and host-pathogen interactions with the help of various omics studies should shed light on these complex interactions and result in our better understanding and tick control mechanisms[124].

1.6. Medical potential of tick salivary molecules

Studying tick molecules for their potential use in medicine is a progressive approach and brings another perspective to the field. Ticks and their hosts have been co-evolving for 120 million years[137]. During this period ticks specialized in manipulation of host defense mechanisms in highly efficient and specific ways. Studying tick salivary (and not only) molecules therefore allows us to benefit from results of a long, nature driven experiment.

The “Drugs from bugs” pipeline is an alternative to more traditional drug discovery approaches such as high-throughput screening of compound libraries[138] or target-oriented molecular design[139, 140]. This approach is becoming more attractive with the development of mass spectrometry

technology and a possibility of identifying individual compounds within active fractions of organism extract[141]. Another approach combines omics studies, reverse genetics and testing the activity of recombinant proteins[142]. Parasitic proteins have evolved to specifically target host defense mechanisms and therefore represent a collection of potential new potential drugs, which is only superficially understood. One of the best examples of a drug isolated from a parasite is hirudin from the leech, *Hirudo medicinalis*, which is the strongest known natural anticoagulant[143].

Having that in mind, tick saliva represents a unique source of novel drugs, among which only very small portion has been identified up to date[122].

Coversin (also named OmCI or EV576) is an *O. moubata* salivary lipocalin, structurally related to histamine binding proteins and is one of the most successful tick salivary molecules in the field of applied pharmacology. Coversin is a complement inhibitor that possesses a unique specificity for C5 component of complement cascade, while leaving C3 intact[31, 144, 145]. While processes connected to C5 activation are blocked, the opsonization effect of C3b component remains intact and immune reaction is not compromised [146]. Coversin's specificity towards C5 has been employed in a model of the peripheral nervous system autoimmune disease – the Guillain-Barré syndrome. Mice treated with Coversin showed deposition of only C3c, but not a membrane attack complex formation, synaptic and neuromuscular damage or high level of asynchronous acetylcholine release[147]. Because of its lipocalin nature, Coversin also binds pro-inflammatory leukotriene B4, which activates leukocyte adhesion and can be used as leukotriene/hydroxyeicosanoid binding molecule[148]. The therapeutical effect of Coversin has been tested in an experimental model of myasthenia gravis, in which neurotransmission is impaired by postsynaptic membrane lysis by terminal products of complement cascade[149]. Mice treated with Coversin showed lower complement activity in serum, lower serum cytotoxicity and reduced deposition of C9 complement unit at the neuromuscular junction[150]. A possible use of Coversin as a treatment has been patented for serious viral diseases including Zika, influenza A, avian influenza H5N1, swine flu H1N1 or SARS coronavirus (patent number WO 2011/083317). The use of Coversin has also been suggested against other diseases like myasthenia gravis[149], heart infarction[151], paroxysmal nocturnal hemoglobinuria[152], thrombotic microangiopathy[153],

sepsis[154] and others. Coversin is currently under phase II / phase III of clinical trials by Akari Therapeutics company.

Ixodes ricinus contact phase inhibitor (Ir-CPI) is a representative of an anti-coagulatory tick molecule. IrCPI is a Kunitz type inhibitor that is able to reduce thrombus formation and prolong activated partial thromboplastin time via the inhibition of fXIa, fXIIa and kallikrein. In experiments, it further reduced the risk of thromboembolism, while keeping blood coagulation unaffected[155]. Recently, Ir-CPI was described to prevent clotting in catheter and arteriovenous shunt animal models with the same effectivity as heparin, but without promotion of bleeding[156]. The beneficial effect of Ir-CPI is attributed to its relatively strict specificity to fXIa and fXIIa. These findings can lead to an effective prevention of medical device-associated clotting in humans like cardiopulmonary bypass or mechanical heart valves[157]. Due to its effectivity, Ir-CPI is now in phase I of clinical trials by Bioxodes company.

Evasins are chemokine binding immunomodulatory molecules present in saliva of *Rhipicephalus sanguineus*. Chemokines play a role in cell infiltration to an inflammation site[158]. Evasin-1 and Evasin-4 bind members of CC chemokines, while Evasin-3 is specific for CXC chemokines[159, 160]. Evasin-1 binds specifically CCL3, CCL4 and CCL19. It is able to inhibit neutrophils, T cells and macrophages migration and production of inflammatory cytokines which can be used in treatment of pulmonary fibrosis[161] or graft versus host disease[162]. Evasin-4 is able to interact with at least 18 chemokines, showing broader variety in CCL interaction[163]. Moreover, it inhibits eosinophil recruitment[164] and post-infarction myocardial injury and remodeling[165]. Similarly to Evasin-1, Evasin-3 is also an inhibitor of myocardial reperfusion [166] and neutrophil recruitment [159], which was associated with reduced atherosclerotic vulnerability for ischemic stroke [167] or neutrophil-mediated inflammation in mouse acute pancreatitis [168]. However, in contrast to Coversin or Ir-CPI, clinical trials with evasins have never been initiated.

While most of the medicinally useful molecules come from tick saliva, an *I. scapularis* antifreeze glycoprotein (IAFGP) plays a role in tick survival in a cold environment[169]. Its medicinal potential lies in the alteration of bacterial biofilm formation and the reduction of biofilm development on implanted catheters[170]. IAFGP combined with antibiotics increased their

effectivity even against resistant bacteria like methicillin-resistant *Staphylococcus aureus*[171].

The reason, why the ratio of tick molecules at clinical trials to tick molecules with published pharmacological activity is relatively low, is not very clear. One explanation can be the pharmaceutical need of designing highly specific drugs with minimum side effects. Ticks have, however, different needs. The role of their salivary components is to suppress blood coagulation or inflammation as a whole process[22]. Therefore many tick salivary proteins show pluripotency, the ability to affect several mechanisms[125]. Following this hypothesis, the success of a complement inhibitor Coversin can be linked to its specificity to C5 inhibition, leaving other members of the complement cascade unaffected[31]. Similarly Ir-CPI only inhibits fXIa and fXIIa from blood coagulation cascade[155], while most other tick salivary proteins target among others thrombin[23]. On the other hand, broad spectrum immunomodulators seem to be more interesting as vaccine candidates than novel drug design. As an example, two immunosuppressive cystatins Sialostatins L1 and L2 can be mentioned, which were patented (patent number WO 2009/017689) for detection and prevention of tick infestation and pathogen transmission, and not for use as drugs.

Protein engineering provides an opportunity to enhance protein-based drug activity, increase specificity or reduce side effects. This approach has been used to modify leech hirudin to increase its potency and ensure high specificity, resulting in bivalirudin[172], commercially presented as Hirulog[173]. Likewise, tick proteins have been subjected to bioengineering. The half-life of Coversin in plasma has been extended more than 50 times by its conjugation to a 600 amino acids long polypeptide consisting of Pro, Ala and Ser, resulting in PASylated Coversin[152]. This modification led also to reduced background hemolysis of erythrocytes in a paroxysmal nocturnal hemoglobinuria model. Another approach was used for Evasin-4, a tick salivary lipocalin. The authors attempted to increase its plasma half-life by fusing Evasin-4 to Fc fragments of human antibodies[174]. However, in this case the modification did not have the expected enhancing effect.

Apart from modifying proteins by adding antibody fragments, PAS, polyethylene glycol (PEG) or knowledge-based mutagenesis, “humanization” of tick molecules is an alternative possibility aiming to reduce protein

immunogenicity and extend molecule’s half-life. Tick proteins, similarly to any molecules of foreign origin, are recognized by the host immune system and antibody response is elicited. Grafting the active site of a tick molecule on a similar molecule originating from its host could reduce the immune response. Although protein humanization has been performed in other organisms, it has not been successfully reported for tick proteins yet[122].

2. Aims and objectives

This thesis aims to:

I. Review current literature about the role of tick saliva and salivary protease inhibitors on host defense mechanisms.

II. Experimentally characterize chosen candidates form cystatin and serpin family present in tick saliva and investigate their role in tick-host-pathogen interaction.

3. Manuscripts

The following section provides full text of Manuscripts 1-4; each with a short introduction.

3.1. Manuscript 1:

Kotál, J., Langhansová, H., Lieskovská, J., Andersen, J. F., Francischetti, I. M., Chavakis, T., Kopecký, J., Pedra, J. H., Kotsyfakis, M., and Chmelař, J. (2015) Modulation of host immunity by tick saliva. Journal of proteomics 128, 58-68

A review Manuscript 1 summarizes the modulatory effects of tick saliva on host immune system. As an adaptation to blood feeding, ticks secrete a complex mixture of salivary components to minimize their host's response. Although nowadays the research trend is shifted more towards characterization of single saliva components, studying the complex effects of tick saliva can help us to design experiments, or discuss our results achieved with single molecules in a bigger context. The publication provides an introduction about the importance of studying ticks and advances in understanding tick saliva composition aided by transcriptomic and proteomic studies.

The main focus of the publication is how tick saliva affects various immune cells populations. We summarized the effect of saliva or salivary gland extract on antigen presenting cells, granulocytes, mast cells and natural killer cells, all representing the innate immune system. A section of the publication also describes the saliva effect on cell of adaptive immunity – B and T lymphocytes.

Jan Kotál is the first author, searched the literature, wrote the manuscript, prepared tables and figures.

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Review Article

Modulation of host immunity by tick saliva

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ABSTRACT

Next generation sequencing and proteomics have helped to comprehensively characterize gene expression in tick salivary glands at both the transcriptome and the proteome level. Functional data are, however, lacking. Given that tick salivary secretions are critical to the success of the tick transmission lifecycle and, as a consequence, for host colonization by the pathogens they spread, we thoroughly review here the literature on the known interactions between tick saliva (or tick salivary gland extracts) and the innate and adaptive vertebrate immune system. The information is intended to serve as a reference for functional characterization of the numerous genes and proteins expressed in tick salivary glands with an ultimate goal to develop novel vector and pathogen control strategies.

Significance: We overview all the known interactions of tick saliva with the vertebrate immune system. The provided information is important, given the recent developments in high-throughput transcriptomic and proteomic analysis of gene expression in tick salivary glands, since it may serve as a guideline for the functional characterization of the numerous newly-discovered genes expressed in tick salivary glands.

Contents

List of abbreviations: Akt, protein kinase B; BMDMs, bone marrow-derived macrophages; BSA, bovine serum albumin; cAMP, cyclic adenosine monophosphate; CCL, chemokine (C–C motif) ligand; CCR, C–C motif receptor; CD, cluster of differentiation; ConA, concanavalin A; CTL, cytotoxic T lymphocytes; CXCL, chemokine (C–X–C motif) ligand; DC, dendritic cell; ERK, extracellular signal-regulated kinase; IDO, indoleamine 2,3 deoxygenase; IFN, interferon; Ig, immunoglobulin; IL, interleukin; IRAK, interleukin-1 receptor-associated kinase; LC, Langerhans cell; LFA-1, leukocyte function-associated antigen-1; LPS, lipopolysaccharide; MC, mast cell; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; NET, neutrophil extracellular trap; NF-κB, nuclear factor kappa light chain-enhancer of activated B cells; NK, natural killer; NO, nitric oxide; PBL, peripheral blood leukocytes; PGE_2, prostaglandin E_2; PI3k, phosphatidylinositol-3 kinase; PMNs, polymorphonuclear lymphocytes; RANTES, regulated upon activation, normal T cell expressed and secreted; ROS, reactive oxygen species; SGE, salivary gland extract; STAT, signal transducer and activator of transcription; sTNFRI, soluble TNF receptor I; TGF, transforming growth factor; Th, helper T cell; TLR, toll-like receptor; TNF, tumor necrosis factor; VLA-4, very late activation-4.

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1. Introduction

Ticks are obligatory blood-feeding arthropods that belong to the subclass *Acari*, order Ixodida, and three families: *Ixodidae* (hard ticks), *Argasidae* (soft ticks), and Nuttallielidae. Soft ticks feed repeatedly for minutes to hours, while hard ticks usually stay attached to their hosts and feed for several days or even weeks, but only once in each life stage [1,2]. The amount of blood ingested is species and life-stage specific, with females of some tick species increasing their volume up to 200 times by the end of blood feeding [3].

Ticks are important vectors that transmit a wide range of pathogens. The most common tick-borne pathogens are viruses and bacteria, but fungi, protozoa, and helminths can also be transmitted [4]. Clinically and epidemiologically, the most important tick-borne diseases are: tick-borne encephalitis (TBE), caused by the TBE virus; Lyme disease, caused by spirochetes belonging to the *Borrelia burgdorferi* sensu lato complex in Europe and *B. burgdorferi* sensu stricto in the USA; tick-borne spotted fever, caused by *Rickettsia* spp.; anaplasmosis, caused by *Anaplasma* spp.; and babesiosis, caused by *Babesia* spp. protozoa [5,6]. Pathogens have different life cycles, but the transmission usually begins with a tick biting an infected vertebrate host and pathogen uptake by the tick in the blood meal. Pathogens, e.g. *Borrelia* spp. spirochetes then stay in the midgut and wait until next feeding, which triggers their proliferation and migration through the midgut wall to hemocoel and, ultimately, to the salivary glands. Moreover, spirochetes interact with some midgut and salivary components that induce *Borrelia* proliferation or increase their infectious potential [7]. When the tick bites its next vertebrate host, pathogens are transmitted via tick saliva. In some tick species the pathogens are transmitted transovarially from the female to laid eggs, thus keeping the level of prevalence in the tick population [8]. Tick saliva has been shown to facilitate pathogen transfer to the vertebrate host by virtue of its pharmacological properties, including modulation of the vertebrate immune system [9–11]. Moreover, tick saliva contains toxins belonging to families also found in venomous animals, such as spiders or snakes, and that can induce paralysis and other toxicoses [12].

To secure uninterrupted blood uptake, ticks suppress and evade the complex physiological host immune and homeostatic responses that are raised against them. Hemostasis, which includes coagulation, vasoconstriction, and platelet aggregation, is the first innate host defense mechanism against the mechanical injury caused by intrusion of tick mouthparts into the host skin. This early vertebrate host response further includes complement activation and inflammation, with the host inflammatory response including, among other factors, rapid leukocyte infiltration after skin injury [13]. Keratinocytes, endothelial cells, and resident leukocytes such as mast cells, dendritic cells, and macrophages make immediate contact with tick saliva or the tick hypostome and are activated. Pro-inflammatory chemokines and cytokines including interleukin-8 (IL-8), tumor necrosis factor (TNF), and IL-1β are released to recruit neutrophils and other inflammatory cells to the area of tick infestation [14]. Following tick feeding, there is activation of both the cellular and humoral branches of vertebrate adaptive immunity [15]. Activated memory T and B cells (in the case of secondary infestation) amplify the host inflammatory response to ticks by releasing specific cytokines and producing antibodies that target tick salivary or mouthpart-derived antigens to activate complement or sensitize mast cells and basophils [9,14,15]. The strength and specificity of the host immune response and its effect on tick physiology depend on the host and tick species, the host's health, and its genotype [16]. The same is true for tick defense mechanisms, since both tick salivary components and host immune mechanisms have been co-evolving. As a result, the tick–host interaction can be considered an "arms race" between the new defense mechanisms developed by the host and the evasion strategies developed by ticks [17]. As an adaptation to blood feeding, ticks secrete a complex mixture of immunomodulatory substances in their saliva that suppress both innate and adaptive host immune responses that can cause pain, itch, blood flow disruption in the tick feeding cavity, or even direct damage to the tick, thereby subverting tick rejection and death [18–20]. Despite the specificity of tick salivary component targets, there is also redundancy at the molecular, cellular, and functional level [9,13]. The richness and diversity of tick salivary compounds have been established in several transcriptomic studies over the last 15 years and, more recently, by next generation sequencing (NGS) studies.

The rapid developments in NGS and proteomics are reflected in the recent progress made in tick research, in which several transcriptomic and proteomic studies have been published over the last few years. These studies represent a rich data source that provides the basis for functional studies and investigation of gene expression dynamics during tick feeding and different physiological states. For instance, significant differences in the salivary proteome of partially and fully engorged female *Rhipicephalus* (*Boophilus*) *microplus* ticks have been described [21]. More recently, a transcriptomic study described over 800 immuno-proteins in *Amblyomma americanum* saliva during 24–48 h of feeding [22]. A transcriptomic analysis of *Dermacentor andersoni* salivary glands resulted in over 500 singletons and 200 clusters in which a number of sequences with similarity to mammalian genes associated with immune response regulation, tumor suppression, and wound healing were identified [23]. By combining transcriptomic and proteomic approaches, nearly 700 proteins were identified in *D. andersoni* saliva after 2 and 5 days of feeding, from which 157 were postulated to be involved in immunomodulation and blood feeding [24]. Schwarz and colleagues performed a comprehensive study of *Ixodes ricinus* salivary and midgut transcriptomes and proteomes and found that the transcriptomic and proteomic dynamics did not 100% overlap in different tick tissues [25]. A recent study by Kotsyfakis and colleagues characterized transcriptional dynamics in the *I. ricinus* female and nymph salivary glands and midguts at various feeding time points [26], and established that some gene families show stage- and time-specific expression, possibly via epigenetic control. In addition, the genes encoding secreted proteins exhibited a high mutation rate, possibly representing a mechanism of antigenic variation, and analysis of the midgut transcriptome revealed several novel enzymes, transporters, and antimicrobial peptides [26]. A transcriptomic analysis of *Amblyomma maculatum* salivary glands revealed almost 3500 contigs with a secretory function [27]. Another sialome (salivary gland transcriptome) of *Amblyomma* ticks was published by Garcia and colleagues [28]: the authors analyzed samples from *Amblyomma triste* nymphs and females, *Amblyomma cajennese* females, and *Amblyomma parvum* females and focused on putative transcripts encoding anticoagulants, immunosuppressants, and anti-inflammatory molecules. A further study characterized *A. americanum* nymph and adult proteomes and compared the data with other *Amblyomma* species [29]. A *Rhipicephalus pulchellus* tick sialome study revealed differences between males and females [30], with the sequences identified used for a preliminary proteomic study to identify 460 male and over 2000 female proteins. A sialomic study was also performed in *Haemaphysalis flava* that revealed tens of thousands of genes, some of which were putative secreted salivary proteins thought to be involved with blood feeding and ingestion [31].

A *Rhipicephalus sanguineus* salivary proteome showed recycling of host proteins and their secretion back into the host [32]. Lewis and colleagues used a transcriptomic approach to characterize immunogenic *Ixodes scapularis* salivary proteins present after 24 h of feeding [33]; these appeared to be involved in tick feeding even before the majority of pathogens could be transmitted.

In addition to the analysis of secreted tick salivary proteins, tick-feeding lesions on the host have been analyzed by high-throughput and histological methods. Recently, the feeding lesion of *D. andersoni* was described in detail together with microarray analysis of host gene expression dynamics, thereby characterizing the inflammatory infiltrate at the feeding site and the changes occurring in the epidermal and dermal compartments near the tick [34,35]. The skin lesions examined from rats infested by *Ornithodoros brasiliensis* showed edema, muscle degeneration, and hemorrhage [36], with the rats themselves presenting with a bleeding tendency and signs of toxicosis. *O. brasiliensis* salivary gland homogenates delayed wound healing and had anti-proliferative or even cytotoxic activity on cultured epithelial cells [37]. An analysis of skin-draining lymph nodes in goats repeatedly infested with *A. cajennese* nymphs revealed an increased number of antigen presenting cells (APCs) such as B lymphocytes, macrophages, and dendritic cells [38]. A skin lesion from a human infested with female *Amblyomma testudinarium* was characterized by an inflammatory infiltrate and an eosinophilic cement in the center of the lesion [39]. Feeding lesions from rabbits injected with salivary gland extract (SGE) collected from *R. sanguineus* ticks after 2, 4, and 6 days of feeding showed signs of inflammation, especially at day four [40], suggesting that molecules present in *R. sanguineus* SGE have high immunogenicity and that immune reaction raised against SGE is stronger than the immunomodulatory action of *R. sanguineus* salivary effectors.

Such high-throughput studies in both ticks and hosts and complemented with histological information and detailed characterization of salivary components have made a valuable contribution to our knowledge of the dynamic processes occurring at the tick–host interface. However, experiments with saliva or SGE highlight the complexity of host modulation by the tick in vivo. Characterizing individual salivary components can help link specific pathophysiological events to particular molecules to provide a complete picture of tick–host interactions. In this review, we focus on the immunomodulatory actions of whole tick saliva or salivary gland extracts (SGE) rather than the effects of the individual salivary components, since these are reviewed elsewhere [13,41,42].

2. The role of tick saliva in modulating host hemostasis and complement

Ticks have developed various mechanisms to counteract the hemostatic responses of the host so that they can successfully feed on blood for many days [13,19]. Serine proteases are key players in host hemostasis and, therefore, are specifically targeted by the wide range of serine protease inhibitors present in tick saliva. The net result is that the physiological balance between host proteases and endogenous anti-proteases is impaired. Tick salivary secretions also contain vasodilators, platelet activation inhibitors, and coagulation modulators, as reviewed elsewhere [14,43,44].

Complement is a cascade of proteolytically-activated components that eventually leads to the creation of pores in the walls of microbes, leading to their destruction. There are three main complement activation pathways: classical, alternative, and lectin; the central reaction in all pathways is the conversion of complement component C3 to C3a and C3b [45,46]. The inhibition of the host alternative complement pathway is crucial for tick feeding and, indeed, the saliva of several *Ixodes* species inhibits this pathway [47,48]. In an in vitro study, the ability of tick saliva to counteract complement activity varied according to the animal species source of serum, with specificity shown towards the most common hosts for each *Ixodes* species [49]. Several anti-complement molecules have been identified to date; however, a detailed description is beyond the scope of this review. Further information about the role of complement in tick–host interactions can be found in the reviews by Schroeder and colleagues [50] or Wikel [14].

3. Innate immunity and tick saliva

Innate immune responses against tick feeding involve the activation of resident immune cells that initiate and promote the local inflammatory response as a reaction to skin damage. The resident leukocytes are macrophages, Langerhans cells (LCs), mast cells, or innate lymphoid cells, and pro-inflammatory mediators are also released by endothelial cells and keratinocytes [51]. These mediators and complement components are chemotactic for circulating inflammatory cells including neutrophils and monocytes.

4. Interaction of macrophages and monocytes with tick saliva

Macrophages are APCs as well as cytokine and chemokine producers [52]. They can be further divided into two different subpopulations: (i) bone marrow-derived hematopoietic macrophages, which circulate as monocytes and, after extravasation at the site of inflammation, differentiate into pro-inflammatory [53] or alternatively-activated macrophages [54] and (ii) tissue-resident macrophages of yolk sac origin that are found in many organs including the skin; the latter tend to be more immune-modulatory [55]. These macrophage subpopulations differ with respect to cytokine production, receptor expression, and their overall effect on any subsequent immune response [54,56,57].

Numerous interactions have been identified between macrophages, tick saliva or SGE, and pathogens, suggesting that they play a major role in host defenses against ticks and tick-borne infectious agents. The effects of saliva or SGE on macrophages are summarized in Fig. 1.

I. ricinus SGE inhibited superoxide and nitric oxide (NO) production by *Borrelia afzelii*-activated macrophages, which led to the inhibition of *Borrelia* killing in a murine host [58]. *I. ricinus* SGE also reduced phagocytosis of *B. afzelii* spirochetes by murine macrophages and inhibited IFN-γ- and *B. afzelii*-stimulated TNF production by macrophages [59]. It was recently shown that *I. ricinus* saliva could induce the production of monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein 2 (MIP-2) in splenocytes [60]. MCP-1 attracts

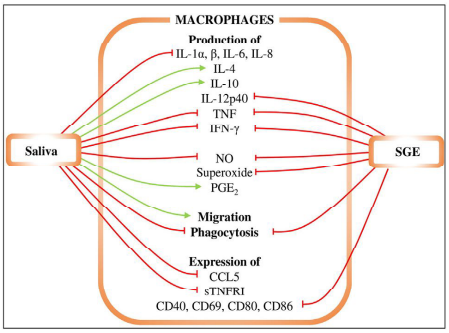

Fig. 1. The effects of saliva and SGE on macrophages. Red lines represent inhibition, green lines enhancement. Tick saliva inhibits production of IL-1α, IL-1β, IL-6, IL-8, TNF, IFN-γ, NO, superoxide, and CCL5, as well as expression of sTNFRI and phagocytosis. Tick saliva increases production of IL-4, IL-10, and PGE_2 and macrophage migration. Tick SGE inhibits production of IL-12p40, TNF, IFN-γ, and NO, expression of CD40, CD69, CD80, and CD86, and phagocytosis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

monocytes, and MIP-2 is a chemokine secreted by monocytes and macrophages that is chemotactic for neutrophils.

Similarly, *I. scapularis* saliva inhibited TNF, IL-1β, IL-6, and IL-12p40 production by murine bone marrow-derived macrophages (BMDMs) after stimulation with lipopolysaccharide (LPS) or *Anaplasma phagocytophilum*. It was further reported to inhibit IL-8 secretion by human peripheral blood mononuclear cells (PBMCs) after TNF stimulation [61] and NO synthesis upon LPS stimulation [62].

Incubation with SGE isolated from *R. microplus*, a tick of veterinary importance, resulted in diminished expression of the co-stimulatory molecules CD80, CD86, CD40, and CD69 on the surface of bovine macrophages after 24 h of LPS stimulation, which was accompanied by a decrease in TNF, IFN-γ, and IL-12 production [63]. Conversely, CD86 expression was increased in the murine macrophage cell line RAW 264.7 in response to *R. microplus* SGE and LPS but not SGE alone. Furthermore, SGE had no effect on CD40 and CD80 expression [64]. However, both bovine primary macrophages and murine macrophage cell line displayed an increase of CD86 expression after 6 h incubation with LPS and SGE. [64]. These partially contradictory observations may be attributed to the host specific response. The difference may also originate from altered signaling in immortalized cell line, as CD86 upregulation was shown to be at least partially dependent on the ERK1/2 pathway and may, therefore, promote polarization of the immune response towards a less pro-inflammatory Th2 profile (see below) [64]. In another study, *R. sanguineus* saliva diminished NO production by IFN-γ-activated macrophages and thus impaired *Trypanosoma cruzi* killing. The authors suggested that decreased NO production was due to a saliva-induced cytokine imbalance, leading to decreased NO synthase activity [65]. Similar to the results with primary macrophages, SGE from *Rhipicephalus appendiculatus* affected cytokine production by the murine macrophage cell line JA-4. SGE from *R. appendiculatus* inhibited the transcription of IL-1α, IL-10, and TNF after macrophage stimulation with LPS. NO production was also lower, in accordance with the similar effect observed with *I. ricinus* saliva [58,66].

Dermatocentor variabilis (Table 1) saliva has been shown to impair phagocytosis and alter gene expression in the murine macrophage cell line IC-21, as well as increase basal and platelet-derived growth factor (PDGF)-stimulated macrophage migration and the expression of the Th2-specific cytokines IL-4 and IL-10 [67].

The tick salivary component prostaglandin E_2 (PGE_2) subverted macrophage secretion of pro-inflammatory mediators and was able to recruit fibroblasts to heal tick-bite wound [68]. In addition to PGE_2 from tick saliva, the saliva of *D. variabilis* upregulated PGE_2 secretion in IC-21 murine peritoneal macrophages and reduced secretion of the pro-inflammatory mediators CCL5, TNF, and soluble TNF receptor I (sTNFRI) via a PGE_2-dependent mechanism mediated by cAMP [68].

In summary, the tick saliva of various tick species inhibits the pro-inflammatory activities of macrophages, supporting a major role for macrophages in anti-tick defenses.

5. Dendritic cells and tick saliva

Dendritic cells (DCs) are APCs and are part of the innate immune system. After immature (unstimulated) DCs recognize and phagocytose pathogens, they mature and migrate to draining lymph nodes where they present antigens derived from the processed pathogen to CD4+ T cells, which subsequently launch an adaptive immune response. Thus, DCs initiate host adaptive immunity via presentation of pathogenic antigens. Two DC states exist: an immature form present in skin or mucosae and a mature form in lymphoid tissues. Langerhans cells (LCs) are a specialized resident cell type found in the vertebrate skin. Similar to macrophages, LCs have two origins and share many properties with macrophages [69]; therefore, they are sometimes considered to be a subtype of tissue macrophage [57]. Immature DCs primarily have an antigen uptake and presenting function, while mature DCs effectively stimulate T cells but have limited phagocytic activity. Several studies suggest that there are interactions between tick saliva and DCs [70–72]. For a review of the interactions between DCs, tick saliva, and *Borrelia*, see [73].

Oliveira and colleagues studied the effect of *R. saguineus* saliva on DC migration and function, and found that tick saliva reduced immature DC migration towards macrophage inflammatory proteins MIP-1α and MIP-1β but not MIP-3β [74]. Tick saliva also inhibited the chemokine RANTES by reducing expression of its surface receptor CCR5 [74]. DC maturation was impaired via toll-like receptor (TLR) signaling [75]. However, the inhibition of migration was limited to immature DCs. DC maturation and differentiation was inhibited in the presence of *A. cajennese* saliva [76]; in this study, the DCs showed reduced expression of CCR5 and CCR7 and, therefore, diminished migration towards the corresponding chemokines. Furthermore, tick saliva polarized cytokine production towards a Th2 phenotype. The authors suggested that most of the observed effects were due to the presence of PGE_2 in tick saliva [76]. *I. scapularis* saliva has displayed various effects on bone marrow-derived DCs: it inhibited TNF and IL-12 production upon stimulation of different TLRs, in particular TLR-2, TLR-4, or TLR-9 [77], and the DC's ability to stimulate antigen-specific CD4+ proliferation and IL-2 production was also suppressed [77]. LC-deficient mice induced Th1 responses after *I. scapularis* infestation, demonstrating the requirement for LCs in attenuating tick-mediated Th1 responses in regional lymph nodes [78].

CD40 or TLR3, 7, and 9 ligation impaired DC maturation, and *I. ricinus* saliva inhibited DC migration in vivo and antigen presentation [79]. *I. ricinus* saliva has also been shown to impair Th1 and Th17 polarization in DCs [79] and activation of specific CD4+ T lymphocyte subsets by *Borrelia*-exposed DCs [80]. In the latter study, *I. ricinus* saliva decreased DC phagocytosis of *B. afzelii*. Interestingly, *I. ricinus* saliva inhibited DC production of both Th1 cytokines (TNF and IL-6) and the Th2 cytokine IL-10 after 48 h (but not 24 h) of incubation with *B. afzelii* [80]. *I. ricinus* saliva also impaired DC maturation and production of TNF and IL-6 in response to infection with TBE virus [81]. Lieskovská and Kopecky studied the signaling pathways activated in DCs via TLR-2 ligand and *B. afzelii* in the presence of tick saliva [82]; upon both types of activation, the NF-κB and phosphatidylinositol-3-kinase (PI3K)/Akt pathways were inhibited by *I. ricinus* saliva. When activated by *Borrelia* spirochetes, TNF levels decreased in DCs due to selective suppression of ERK1/2, Akt, and NF-κB as a result of tick saliva mimicking the native inhibitors. Tick saliva also attenuated IFN-β production, and IFN-β triggered signal transducer and activator of transcription-1 (STAT-1) activation [83]. A summary of the known interactions between DCs and tick saliva is shown in Fig. 2.

6. Mast cells and tick saliva

Mast cells serve as sentinel cells and reside in many tissues. They are divided into two main types based on the presence of mast cell-specific proteases: connective tissue mast cells, which produce both tryptase and chymase (MC_{TC}), and mucosal mast cells, which produce only tryptase (MC_T) [84]; skin mast cells are of the first type. Upon exposure to pathogens or other stimuli, activated mast cells degranulate and release a variety of pre-stored mediators including vasoactive compounds, serine proteases, histamine, and cytokines. Activated mast cells also secrete newly synthesized mediators to recruit more inflammatory cells [85].

The immunological importance of mast cells in tick–host interactions remains unclear. Mast cell numbers increase after secondary or subsequent tick infestations, but remain unchanged during primary tick infestations [86–88]. The number of degranulated mast cells is also significantly higher after repeated tick infestations. Mast cell-deficient mice have been shown to develop some resistance to *D. variabilis* after repeated exposure, similar to wild type mice [89]. On the other hand, mast cell-deficient mice were not resistant to *Haemaphysalis longicornis*, with tick resistance re-established after mast cell injection [90,91]. Such

Table 1
The effects of tick saliva, SGE, or feeding on immune cell populations.

Tick	Saliva/SGE/Feeding	Effect	Reference
Macrophages			
Dermatocentor variabilis	Saliva	Impaired phagocytosis and altered gene expression, stimulation of migration	[67]
		Stimulation of PGE_2 production, inhibition of cytokine production	[68]
Ixodes ricinus	SGE	Inhibition of superoxide and NO production	[58]
		Inhibition of phagocytosis and TNF production	[59]
Ixodes scapularis	Saliva	Inhibition of cytokine production	[61]
		Inhibition of NO production	[62]
Rhipicephalus appendiculatus	SGE	Inhibition of cytokine and NO production	[66]
Rhipicephalus microplus	SGE	Altered surface molecule expression, inhibition of cytokine production	[63,64]
Rhipicephalus sanguineus	Saliva	Inhibition of NO production	[65]
Dendritic cells			
Amblyomma cajennese	Saliva	Inhibited maturation and differentiation; reduced migration due to decreased expression of receptors; polarization towards Th2 cytokines	[76]
I. ricinus	Saliva	Inhibited maturation, migration and antigen presentation; blocked Th1 and Th17 polarization	[79]
		Inhibited proliferation, phagocytosis and cytokine production	[80]
		Impaired maturation and cytokine production	[81]
		Inhibition of signaling pathways	[82,83]
I. scapularis	Saliva	Inhibition of proliferation and cytokine production	[77]
R. sanguineus	Saliva	Reduced migration, maturation and cytokine production	[74,75]
Basophils			
Amblyomma cajennense	Feeding	Increased amount of basophils in feeding cavity	[121]
Amblyomma dubitatu	Feeding	Increased amount of basophils in feeding cavity	[121]
Eosinophils			
Soft and hard ticks	Feeding	Increased amount of eosinophils in feeding cavity	[36,88,120–122]
Hard ticks	SGE	Inhibition of attraction to the feeding site	[123,124]
I. ricinus	Saliva	Basophil activation via MCP-1 released from splenocytes	[60]
Neutrophils			
Soft and hard ticks	SGE	Anti-IL-8 activity	[123,130]
Amblyomma americanum	SGE	Altered dynamics of chemokine activity	[125]
I. ricinus	Saliva	Decrease in ROS production	[132]
I. scapularis	Saliva	Inhibition of granule release, infiltration, phagocytosis	[133]
		Reduced adhesion of polymorphonuclear leukocytes	[134]
R. appendiculatus	SGE	Altered cytokines mRNA production by peripheral blood leukocytes	[170]
R. microplus	SGE	Inhibition of phagocytosis	[135]
Lymphocytes			
Soft and hard ticks	Saliva, SGE	Polarization of the immune response towards Th2 via cytokines	[66,71,139,159,161, 162,171,172]
Amblyomma variegatum	SGE	Inhibition of lymphocyte proliferation	[142]
Dermacentor andersoni	SGE	Reduced T cells proliferation	[149,150]
		Reduced Th1 cytokine production	[173,174]
	Saliva, SGE, feeding	Inhibition of integrin expression	[163]
	SGE, feeding	Increased IL-4 and IL-10 levels	[164]
Haemaphysalis bispinosa	Feeding	Reduction in T lymphocyte count and proliferation, increased CD4+/CD8+ ratio	[153]
Hyalomma anatolicum anatolicum	Feeding	Reduction in T lymphocyte count and proliferation, increased CD4+/CD8+ ratio, increase in circulating B lymphocyte count	[153]
I. ricinus	SGE	Inhibition of lymphocyte proliferation	[142]
		Suppression of B cell proliferation, inhibition of IL-10 production, reduction of markers on the surface of T and B cells	[143]
	Saliva	Inhibition of T cell proliferation	[144]
		Induction of Th2 differentiation of CD4+ T cells via dendritic cells	[71]
	Feeding	Increased CD4+/CD8+ ratio	[147]
		Inhibited proliferation and responsiveness	[145]
		Reduced amount of specific Ig against antigen, no change in total Ig amount	[148,157]
I. scapularis	Saliva	Inhibition of IL-2 production by T cells, inhibition of splenic T cell proliferation	[62,140,141]
	Feeding	Inhibition of Th17 immunity, priming of a mixed Th1/Th2 response during secondary infestation	[35]
	SGE, feeding	Increased IL-4 levels	[165]
R. appendiculatus	SGE	Inhibition of lymphocyte proliferation	[142]
R. microplus	Feeding	Decreased T and B lymphocyte percentage among PBLs	[151]
	Saliva	Decreased PBL responsiveness to phytohemagglutinin	[151]
		Inhibition of the blastogenic response of mononuclear cells	[175]
R. sanguineus	Feeding	Suppressed response to mitogens	[152]
	Saliva	Suppressed response to mitogens	[152]
	SGE	Suppressed Ig production by PBL	[156]
NK cells			
A. variegatum	SGE	Decreased NK cell activity	[168]
Dermatocentor reticulatus	SGE	Decreased NK cell activity	[167]
Haemaphysalis inermis	SGE	Decreased NK cell activity	[168]
I. ricinus	SGE	Suppression of NK cell cytotoxicity	[169]

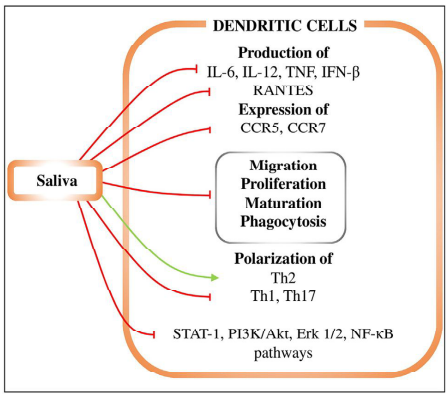

Fig. 2. The effects of saliva on dendritic cells. Red lines represent inhibition, green lines enhancement. Tick saliva inhibits production of IL-6, IL-12, TNF, IFN-β, and RANTES cytokines. It also inhibits expression of CCR5 and CCR7, DC migration, proliferation, maturation, and phagocytosis, and STAT-1, PI3K/Akt, Erk1/2, and NF-κB signaling pathways. The saliva induces Th2 polarization while suppressing Th1 and Th17 differentiation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

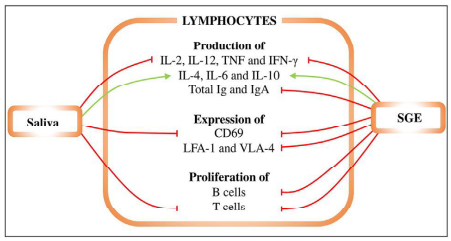

Fig. 4. The effects of saliva and SGE on B and T lymphocytes. Red lines represent inhibition, green lines enhancement. Tick saliva inhibits T cell proliferation, CD69 expression, and production of IL-2, IL-12, TNF, and IFN-γ by lymphocytes. In contrast, it increases production of IL-4, IL-6, and IL-10. Tick SGE has the same effects as tick saliva and, furthermore, suppresses LFA-1 and VLA-4 expression, proliferation of B cells, and total Ig and IgA production. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

differences might be due to species-specific host responses or other unknown factors. Highly tick-resistant zebuine cattle breeds have more dermal mast cells than taurine breeds [92]. F2 crossbreeds of these two cattle were resistant to *R. microplus* infestation, and infestation with *R. micrpoplus* larvae induced significant increases in dermal mast cell numbers. Mast cells are major producers of the inflammatory mediator histamine, and ticks can affect histamine actions by either binding histamine via histamine-binding lipocalins [93,94] or by promoting its secretion via histamine release factor [95], further evidence of the ambiguous role for mast cells in tick feeding responses. One explanation for histamine binding followed by its release can be explained by the need to suppress inflammatory responses at the early stage of feeding, followed by an increased need for vascular permeability during the rapid engorgement phase of tick feeding.

7. Granulocytes and tick saliva

Granulocytes are bone marrow-derived myeloid leukocytes that contain granules in their cytoplasm. The granulocyte group consists of three major cell types: basophils, eosinophils, and neutrophils [96].

8. Basophils and tick saliva

Basophils are IgE-activated granulocytes that, unlike tissue-resident mast cells, circulate in the blood. They play a critical role in the IgE-mediated development of chronic allergic reactions and inflammation [97,98], and they can also promote polarization towards Th2 responses by IgE-independent antigen presentation in mice [99,100]. Basophils are recruited to a tick-feeding site and accumulate in the host skin during second and consequent (but not primary) tick infestation, where they act as important tick rejection factors [101,102]. After migration to the site of injury, basophils degranulate and release mediators such as histamine to reject ticks in a host reaction known as cutaneous basophil hypersensitivity [103]. Similar to mast cells, histamine release from basophils can be mediated by tick histamine release factor binding [95]. Several studies have confirmed the role of basophils in acquired immunity against ticks in mice [102,104,105]. Basophils expressing the immunoglobulin Fc receptor were found to be responsible for antibody-mediated acquisition of *H. longicornis* resistance [102], with selective basophil ablation by diphtheria toxin leading to loss of resistance to *H. longicornis* feeding in subsequent tick infestations [102]. Basophils appear to play a non-redundant role in antibody-mediated acquired immunity against ticks [102].

As noted above, *I. ricinus* saliva increased MCP-1 production by stimulated splenocytes [60]. MCP-1 is a potent basophil activator that triggers their degranulation and histamine release [106].

Basophils can cause cutaneous basophilia, a mechanism of tick resistance [104,105]. The susceptibility or resistance of cattle to ticks (*R. microplus*) was associated with the number of basophils at the feeding site, with skin biopsies from tick-resistant breeds contain significantly more basophils than biopsies from susceptible breeds [107–109].

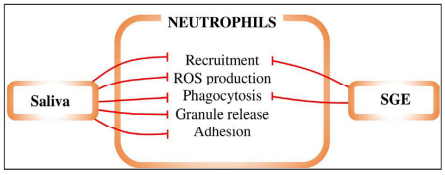

Fig. 3. The effects of saliva and SGE on neutrophils. Red lines represent inhibition, green lines enhancement. Tick saliva inhibits neutrophil recruitment, phagocytosis, adhesion, granule release, and production of ROS. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

9. Eosinophils and tick saliva

Eosinophils are mainly present in mucosal areas in contact with the external environment such as the gut or lung mucosae. Their circulating levels are relatively low in healthy organisms, but increase during allergic reactions or parasitic infections [46]. Eosinophils produce cytokines, chemokines, and other mediators, some of which (e.g., indoleamine 2,3 deoxygenase; IDO) induce apoptosis and inhibit T cell proliferation [110,111]. Eosinophils are also rich in granules that contain cytotoxic effectors such as eosinophil peroxidase, eosinophil cationic protein, eosinophil-derived neurotoxin, or major basic protein, which can cause mast cell (and probably also basophil) degranulation [112]. Finally, eosinophils are an important source of inflammatory and tissue repair-related molecules such as the transforming growth factors TGF-α and TGF-β1 and the extracellular matrix glycoprotein tenascin [113,114].

Repeated exposure to both soft and hard tick species raised eosinophil levels at the feeding site in many host species including cattle [115,116], dogs [117], guinea pigs [118,119], rabbits [86], mice [88],

woolless lambs [120], rats [36], capybaras [121], and even anteaters and armadillos [122]. The relationship between eosinophil number and tick resistance is not clear. Similar to mast cells, the susceptibility or resistance to ticks in cattle was associated with the number of eosinophils (and basophils) at the feeding site. Cattle breeds with more eosinophils (*Bos taurus indicus*, Nelores breed) appeared to be more resistant to *R. microplus* feeding than the *B. taurus taurus* Holstein breed with fewer eosinophils [107]. In contrast, the tick count on Nguni and Bonsmara cattle was positively correlated with the eosinophil count in skin biopsies from tick feeding sites, while the correlation was negative in the case of mast cells and basophils [109].

Ticks inhibit the chemokine-mediated attraction of eosinophils to tick feeding sites. SGE from many tick species blocked CCL3, CCL5, or CCL11 (eotaxin) eosinophil chemoattractant activity [123–126].

10. Neutrophils and tick saliva

Neutrophils are granulocytes with both phagocytosis and degranulation roles. They are highly motile cells and they have a relatively short lifespan. Neutrophils play an important role in the early stages of vertebrate immune homeostasis, such as during acute inflammation, but they also play a role in some chronic inflammatory diseases. Neutrophils are generally activated by pathogens and secrete effectors and mediators that promote inflammation by recruiting other leukocytes, and they also directly kill pathogens by releasing their granules [46,127]. They can also phagocytose and kill pathogens intracellularly [127].

Tick saliva modulates a local cutaneous immune response at the tick feeding site almost immediately after tick attachment, as shown by gene expression analysis of skin biopsies taken at several time points after the initiation of *I. scapularis* nymph feeding [128]. The expression of neutrophil-specific chemokines (CXCL1 and 5) was induced as early as 12 h after tick attachment to the host [128]. Neutrophil abundance in the skin was high during the first tick infestation compared to other cell types but decreased during subsequent tick infestations of the same host [120,129]. It is unknown whether the absence of neutrophils affects resistance of the host to ticks.

Saliva or SGE from soft and hard ticks have been shown to attenuate neutrophil functions such as recruitment by interfering with the neutrophil chemoattractants CXCL8 (IL-8) or CCL3 [123,124,126,130,131]. In one study, *I. ricinus* saliva significantly decreased neutrophil reactive oxygen species (ROS) production [132]. In contrast, the formation of neutrophil extracellular traps (NETs), which are formed by extrusion of neutrophil DNA and can retain and kill bacteria, was not affected by saliva [132]. *I. scapularis* (published as *Ixodes dammini*) saliva inhibited granule release and neutrophil infiltration and had an inhibitory effect on neutrophil phagocytosis of *B. burgdorferi* [133]. *I. scapularis* saliva also reduced polymorphonuclear leukocyte (PMN) adhesion by down-regulating β2-integrin expression and signaling, which decreased pro-inflammatory functions of PMNs [134]. Finally, SGE from *R. microplus* inhibited neutrophil phagocytic activity in cattle [135]. These data show that tick saliva inhibits several pro-inflammatory neutrophil properties that are deleterious to tick feeding but does not affect antibacterial NET formation, suggesting that tick salivary activity is specific. The effects of tick saliva and SGE on neutrophils are illustrated in Fig. 3.

11. T and B lymphocytes and tick saliva

Adaptive immunity relies on a wide range of antigen receptors (with varying antigen recognition specificities), which are clonally distributed in two types of lymphocytes: T cells and B cells. The induction of a specific immune response is only possible when a foreign antigen is recognized by the corresponding receptor. This first recognition signal is consolidated by the interaction of co-stimulatory molecules on T or B cells with those on APCs — such as dendritic cells or macrophages — that belong to the innate immune system. In this way, links are made between the cell populations that play dominant roles in the two branches of vertebrate immunity [136].

T cells are produced in the bone marrow from lymphoid progenitors and differentiate in the thymus. Mature T cells then migrate to the peripheral lymphoid tissues; they also circulate throughout the body [46]. Two major T cell subpopulations are recognized based on the co-receptor molecule expressed at the cell surface: CD4+ (T helper cells) and CD8+ T cells (which develop into cytotoxic T lymphocytes, CTLs). According to the secreted cytokine profile, CD4+ T helper cells can be further divided into several subpopulations that have different roles in immune responses [137], with Th1 and Th2 populations the most thoroughly studied in tick–host interactions thus far. Th1 populations are associated with host cellular and inflammatory responses, and Th2 populations with host humoral responses against ticks [138,139]. Fig. 4 illustrates how tick saliva and SGE influence T and B cell functions.

In 1985, *I. scapularis* (*dammini*) tick saliva was shown to inhibit IL-2 production by T lymphocytes, with PGE_2 proposed to be responsible for this effect [140]. Urioste and colleagues confirmed diminished IL-2 levels in the presence of *I. scapularis* saliva, and showed profoundly inhibited splenic T cell proliferation in response to stimulation with concanavalin A (ConA) or phytohemagglutinin in the presence of saliva [62]; however, they disproved the PGE_2 hypothesis, providing evidence that IL-2 is in fact inhibited by a proteinaceous salivary component. Later, in 2001, an unknown salivary component from *I. scapularis* was reported to bind IL-2 and inhibit T lymphocyte proliferation [141].

The inhibition of lymphocyte proliferation by SGE has also been reported in other tick species such as *I. ricinus*, *Amblyomma variegatum*, and *R. appendiculatus*, with species- and sex-specific differences shown for the effects of tick salivary gland antigens on human lymphocyte proliferation [142]. *I. ricinus* SGE suppressed isolated B cell proliferation and IL-10 production in response to LPS. CD69 activation marker expression on both activated T and B cells was also reduced [143]. *I. ricinus* saliva inhibited splenic T cell proliferation in response to ConA, and both SGE and saliva reduced the responsiveness of T cells draining to lymph nodes and sensitized splenic T cells [144]. The same observation was made with naïve splenic T cells [145]. T lymphocytes from mice infested 9 days previously with *I. ricinus* nymphs displayed suppressed responses to ConA stimulation compared to cells from naïve mice [145]. In contrast, the lymph node cell response to LPS was increased in infested mice compared to naïve mice [145]. The authors attributed the observed effect to increased B lymphocyte numbers or activity [145]. On the other hand, soluble salivary gland antigens derived from female *I. ricinus* ticks stimulated lymph node T cells from mice infested with *I. ricinus* larvae or nymphs, but not those infested with *Amblyomma hebraeum* nymphs [146]. A 65 kDa protein fraction (IrSG65) isolated from the salivary glands of partially fed *I. ricinus* females induced specific T cell proliferation in lymph node cells obtained from mice infested with *I. ricinus* nymphs [146]. Feeding of *I. ricinus* nymphs on BALB/c mice revealed that CD4+ T cells were more abundant than CD8+ cells [147], which changed from 2:1 upon primary tick infestation to 7:1 in tertiary tick infestation. The ratio of CD3+ and CD4+ T lymphocytes was identical in *I. ricinus* infested and control mice [148].

D. andersoni SGE reduced ConA-induced proliferation of T cells [149,150]. *R. microplus* feeding on cattle decreased the T lymphocyte percentage in peripheral blood lymphocytes (PBLs) [151], with the B lymphocyte percentage only lowering after repeated heavy infestations [151]. *R. microplus* saliva also suppressed PBL responsiveness to phytohemagglutinin [151]. *R. sanguineus* feeding on dogs suppressed ConA, phytohemagglutinin, and pokeweed mitogen-induced lymphocyte responses [152]. In the same study, SGE also suppressed all mitogen-stimulated blastogenic responses of lymphocytes from healthy dogs in vitro. Feeding of the *Haemaphysalis bispinosa* and *Hyalomma anatolicum anatolicum* ticks on sheep resulted in reduced circulating T lymphocyte counts as tick feeding progressed [153]. The authors showed that depletion of CD8+ populations and increased CD4+ T cell levels accounted for the observed effects [153]. Feeding of these two ticks also suppressed in vitro

proliferation of T cells isolated from the tick-infested animals [153]. The CD4+/CD8+ and B/T lymphocyte ratios were increased in all sheep during infestation with either *H. bispinosa* and *H. anatolicum anatolicum* [153]. Interestingly, reduced CD4/CD8 T cell ratios were observed in skin biopsies taken at primary and secondary infestation with *H. anatolicum anatolicum* ticks on sheep compared to healthy skin biopsies [154].

B cells also originate from lymphoid progenitors in the bone marrow [46]. Their further differentiation involves migration from the bloodstream to the spleen, where they develop into mature B cells. Mature B cells circulate between the spleen and lymph nodes. The role of B cells lies in the surface expression and secretion of immunoglobulins upon activation [155]. In immunity against ticks, B cells produce specific antibodies against tick salivary and gut antigens.

Both primary and secondary infestations of sheep with *H. anatolicum anatolicum* ticks caused a significant increase in circulating B lymphocytes over several days [153]. In dogs, *R. sanguineus* SGE was shown to suppress total immunoglobulin and IgA (but not IgM) production by PBLs in vitro upon activation with LPS or pokeweed mitogen [156]. It has also been observed that anti-BSA IgG and IgM levels decreased in mice immunized with BSA during *I. ricinus* feeding [148]. However, anti-BSA IgG and IgM production was not decreased when BSA was injected prior to tick infestation. Interestingly, this study did not demonstrate a shift towards the Th2-type immune response when anti-BSA IgG1 and IgG2a antibody levels were compared between mice groups [148]. It was later shown that total IgG and IgM antibody levels were not reduced in animal sera by tick infestation, anti-BSA antibody production was not delayed, and memory cell formation did not appear to be inhibited by tick saliva [157]. Tick saliva did not affect memory B cell production of either anti-BSA IgG or IgM [157].

Experiments with tick saliva or SGE have shown polarization of the immune response from Th1 to Th2 branches by suppression of Th1 and upregulation of Th2 cytokines in both mice and humans. This polarization leads to an attenuated inflammatory response, which is beneficial for tick survival and feeding [15,158]. Briefly, saliva or SGE inhibited secretion of IL-2, IL-12, TNF, and IFN-γ. In contrast, IL-4, IL-6, and IL-10 secretion was stimulated [66,139,159]. IL-10-specific neutralizing antibodies abrogated the suppressive effect of *I. ricinus* SGE on IFN-γ production [160]. IL-1α secretion was inhibited in JA-4 macrophage cells exposed to *R. appendiculatus* SGE [66]. In contrast, and in spite of their pro-inflammatory properties, IL-1α and IL-1β production was increased by Th1 lymphocytes and splenocytes after treatment with *I. ricinus* SGE [161,162]. This can be explained by the fact that IL-1 can also act as a co-stimulator for Th2 lymphocyte proliferation. One of the mechanisms described for the action of *I. ricinus* saliva involves a negative effect on DCs, which then prime naive CD4+ T cells to induce Th2 cell differentiation in vitro and in vivo [71].

Feeding of *D. andersoni* decreased expression of two integrins, leukocyte function-associated antigen-1 (LFA-1) and very late activation-4 (VLA-4), by lymphocytes [163]. The same effect was achieved by exposing tick-naïve mouse lymphocytes to both *D. andersoni* saliva and SGE [163]. Infestation with *D. andersoni* nymphs or intradermal administration of female or male tick SGE increased IL-4 and IL-10 transcript levels in the draining lymph nodes and skin of the host [164]. Intracellular IL-4 levels were significantly increased in CD4+ T cells [164], and increased IL-4 levels were also observed during *I. scapularis* nymph feeding or by intradermal application of SGE [165]. Primary *I. scapularis* infestation on mice was characterized by late induction of an innate immune response and by inhibition of pro-inflammatory Th17 immunity. During secondary tick infestation, a mixed Th1/Th2 response was elicited [35].

Ticks have evolved in various ways to circumvent adaptive immunity. Their saliva inhibits lymphocyte proliferation to reduce immune responses. Furthermore, ticks actively direct the immune response towards the Th2 arm that favors their feeding. The immunosuppressive properties of tick saliva also include inhibition of antibody production by B cells that could damage tick mouthparts and activate other cells or complement. The effects of tick saliva and SGE on lymphocytes are illustrated in Fig. 4.

12. Natural killer cells and tick saliva

Despite their lymphoid origin, natural killer (NK) cells are part of the innate immune system [46]. Their main function is microbial or tumor cell killing and the regulation of endothelial cell, dendritic cell, and macrophage interactions with T lymphocytes [166]. SGE from female *Dermatocentor reticulatus* ticks that fed for 3–6 days on mice decreased human NK cell activity, while SGE from unfed or 1 day-fed ticks had no effect. Weaker activity was reported for SGE from *A. variegatum* and *Haemaphysalis inermis* ticks [167,168], and NK cell cytotoxicity was suppressed after treatment with *I. ricinus* SGE [169].

13. Conclusions

Tick saliva clearly contains numerous different pharmacologically-active molecules that affect various immune cell populations and facilitate tick feeding. In this "systems biology" era, the effects of tick saliva described in this review can help in the design of experiments to discover specific salivary molecules that account for those effects. Although molecular biology and biochemical methods such as transcriptome and proteome analyses have provided excellent information about the genes expressed in the salivary glands of different tick species, the number of identified and functionally characterized salivary molecules remains limited. Ultimately, the goal is to fully uncover the complexity of how ticks modulate the host immune system so that this information can be used to pioneer the development of novel control strategies for ticks and tick-borne diseases and aid drug discovery.

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Conflicts of interest

The authors declare that no conflicts of interest exist.

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3.2. Manuscript 2:

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A review Manuscript 2 summarizes the effects of tick salivary protease inhibitors form cystatin and serpin families on host immune system and blood coagulation. Protease inhibitors represent important constituents of tick saliva and fulfill various purposes in host body against defense mechanisms. We review the role of tick salivary cystatins (cysteine protease inhibitors) as regulators of host immunity, and serpins (serine protease inhibitors) as immunosuppressors and anticoagulants. Both these protein families are vital for tick feeding which has been proven experimentally by RNA interference. Next, we discuss a potential use of tick salivary protease inhibitors as new drugs; mostly for treatment of autoimmune or immunity related disorders.

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REVIEW
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Protease Inhibitors in Tick Saliva: The Role of Serpins and Cystatins in Tick-host-Pathogen Interaction

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The publication of the first tick sialome (salivary gland transcriptome) heralded a new era of research of tick protease inhibitors, which represent important constituents of the proteins secreted via tick saliva into the host. Three major groups of protease inhibitors are secreted into saliva: Kunitz inhibitors, serpins, and cystatins. Kunitz inhibitors are anti-hemostatic agents and tens of proteins with one or more Kunitz domains are known to block host coagulation and/or platelet aggregation. Serpins and cystatins are also anti-hemostatic effectors, but intriguingly, from the translational perspective, also act as pluripotent modulators of the host immune system. Here we focus especially on this latter aspect of protease inhibition by ticks and describe the current knowledge and data on secreted salivary serpins and cystatins and their role in tick-host-pathogen interaction triad. We also discuss the potential therapeutic use of tick protease inhibitors.

Keywords: tick-host interaction, immunomodulation, protease inhibitors, serpins, cystatins

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SERPINS AND CYSTATINS AS HOMEOSTATIC REGULATORS

Proteases (also proteinases or peptidases) are ubiquitous enzymes that cleave proteins to smaller peptides and amino acids. Proteases participate in a range of physiological processes including extracellular digestion, protein degradation, and tissue development (Rawlings and Salvesen, 2013). Relevant to this review, however, is the fact that many proteases, in particular highly substrate-specific endopeptidases, mediate defense and homeostatic processes in both vertebrates and invertebrates. Proteolytic pathways rely on the precise and tightly regulated activation and inhibition of these endopeptidases. As a result of this evolutionary need, many crucial pathophysiological processes are regulated via proteolytic cascades, with notable examples being coagulation of plasma (or haemolymph in arthropods), bacterial wall perforation with complement, or melanization in arthropods (Amara et al., 2008; Tang, 2009; Gulley et al., 2013). Each step involves proteolytic activation of another downstream protease, and all proteases in such cascades usually have their own endogenous inhibitors that balance the system. The role of arthropod protease inhibitors in the defense is supported by the fact that the expression of serpins and cystatins in *Ixodes scapularis* nymphs was attenuated upon infection with *Anaplasma phagocytophilum*, as seen in the transcriptomic data (Ayllon et al., 2015). On the other hand, the expression of protease inhibitors in salivary glands and midguts of adult females differed among individual inhibitors, i.e., some cystatins and serpins were upregulated upon the infection and vice versa (Ayllon et al., 2015). Similar data were collected from *Ixodes ricinus* infected with *Bartonella henselae* (Liu et al., 2014). Therefore, precise involvement of every individual inhibitor in tick infection would have to be evaluated experimentally.

Other intracellular and extracellular processes, such as cytokine activation, phagocytosis, intracellular signaling, and antigen processing, are also dependent on proteolysis (Muller et al., 2012). Serpins and cystatins are the two main superfamilies of endogenous serine and cysteine protease inhibitors involved in the regulation of these processes. It is therefore unsurprising that both groups of inhibitors are well represented in parasites and are important in their interactions with hosts (Schwarz et al., 2012; Meekins et al., 2017). In order to obtain a blood meal, ticks secrete hundreds of different pharmacoactive molecules into the host via their saliva. These molecules have anti-hemostatic, anti-inflammatory, anti-complement and immunomodulatory properties and their function is to overcome or evade host defense mechanisms including immune response (Brossard and Wikel, 2004; Chmelar et al., 2012). Moreover, tick saliva and also several salivary compounds were found to facilitate and enhance the establishment of tick-borne pathogens in the host (Anguita et al., 2002; Pal et al., 2004; Kazimirova and Stibraniova, 2013; Wikel, 2013). Inhibitors of proteases represent the most prominent protein families in tick salivary secretion that are responsible for alteration of many different host defense pathways.

SERINE PROTEASE INHIBITORS IN TICKS

Four groups of serine protease inhibitors have been described in ticks: Kunitz domain inhibitors, Kazal domain inhibitors, trypsin inhibitor-like cysteine rich domain (TIL) inhibitors, and serpins. Inhibitors with 1–7 Kunitz domains mostly act as anti-hemostatic proteins and form a large multigenic family of secreted salivary proteins in ticks that have probably played a crucial role in the development of tick hematophagy (Corral-Rodriguez et al., 2009; Dai et al., 2012; Schwarz et al., 2014). Moreover, single Kunitz-domain inhibitors in other organisms are involved in ion channel blockade and may play a similar role in ticks (Frazao et al., 2012; Valdes and Moal, 2014). Kazal domain inhibitors are described in hematophagous insects such as mosquitoes and triatomine bugs (Rimphanitchayakit and Tassanakajon, 2010), but they are only rarely reported in ticks, in which their function is still unknown (Zhou et al., 2006a; Mulenga et al., 2007a, 2008). TIL-domain inhibitors represent an interesting group of small inhibitors with a conserved 5-disulphide bridge structure that were first reported in *Apis melifera* (Bania et al., 1999) and have also been detected in ticks (Fogaca et al., 2006; Sasaki et al., 2008). The sequences of over 80 TIL-domain inhibitors have been found in arthropod genomes (Zeng et al., 2014), and the unique features of TIL-domain proteins make them an excellent model for designing novel serine protease inhibitors and antimicrobial peptides (Li et al., 2007).

Serpins

Serpins form the largest superfamily of protease inhibitors, and they are ubiquitously distributed in nature including viruses and prokaryotes. With over 1,500 members, serpins are the most studied protease inhibitors (Law et al., 2006), also helped by their unique and highly intriguing mechanism of inhibition (Whisstock et al., 2010) and the evolutionary changes that turned inhibitory serpins into non-inhibitory proteins with completely different functions (Law et al., 2006; Silverman et al., 2010). For example, there are 29 inhibitory and seven non-inhibitory serpins in humans and 60 functional serpin genes in mice (Heit et al., 2013). Angiotensinogen is a non-inhibitory serpin that is proteolytically activated by renin into several oligopeptides (angiotensins) that regulate vasoconstriction and blood pressure (Lu et al., 2016). Cortisol and thyroxine-binding proteins (human *SERPINA6* and *SERPINA7*) are also notable serpins that act as major transport proteins for glucocorticoids and progesterone (Carrell and Read, 2016). Inhibitory serpins have very diverse functions depending on their specificity, but their importance is highlighted by the serpinopathies—diseases caused by serpin dysfunction or deficiency (Belorgey et al., 2007). Emphysema, cirrhosis, angioedema, hypertension, and even familial dementia are caused at least in part by serpin dysfunction (Kim et al., 1995; Davis et al., 1999; Ekeowa et al., 2009; Huntington and Li, 2009; Lomas et al., 2016).

Arthropod serpins have mostly immunological and hemostatic functions. Serpins have been shown to regulate haemolymph coagulation, are involved in phenoloxidase system activation in insects, and regulate an immune toll pathway in haemolymph (Kanost, 1999; Gulley et al., 2013; Meekins et al., 2017). Furthermore, in bloodfeeding arthropods, serpins can act as modulators of host hemostasis and/or immune responses. Indeed, several insect serpins act as anti-coagulants, anti-complement proteins and immunosuppressors (Stark and James, 1995, 1998; Colinet et al., 2009; Calvo et al., 2011; Ooi et al., 2015). Serpins are abundant in ticks, and one of their functions is to modulate host immune system. Recent advances in this area have been facilitated by the publication of *I. scapularis* genome (Gulia-Nuss et al., 2016) and several next-generation sequencing transcriptome studies that added tens of unique sequences from different tick species to already existing and long list of tick serpins. In 2009, Mulenga and colleagues found 45 serpins in the genome of *I. scapularis* (Mulenga et al., 2009). Two years earlier, the same group described 17 serpins (Lospins) in *Amblyomma americanum* (Mulenga et al., 2007b). This number was, however, substantially broadened by the combination of several approaches up to approximately 120 serpins (Karim and Ribeiro, 2015; Porter et al., 2015, 2017). In the work of Porter and colleagues (Porter et al., 2015), the authors compare homologous serpins across tick species, showing both conserved and species-specific inhibitors. The conservation seems to be higher in serpins with basic or polar uncharged amino acid residues at P1 site (Porter et al., 2015). Other 32 serpin transcripts from the *Amblyomma* genus were found in *Amblyomma maculatum* (Karim et al., 2011) and 50 in *Amblyomma sculptum* (Moreira et al., 2017). Two groups described 18 and 22 serpins in *R. microplus,* respectively (Tirloni et al., 2014b; Rodriguez-Valle et al., 2015) and at least 36 serpins were found in several published trancriptomes from *I. ricinus* (our own unpublished data based on the analysis of transcriptomes) (Schwarz et al., 2013; Kotsyfakis et al., 2015a,b; Perner et al., 2016). Another recent publication described 10 different serpin transcripts in the sialotranscriptome of the tick *Hyalomma excavatum* (Ribeiro et al., 2017). Despite

high number of identified transcripts, only small portion was characterized functionally.

Tick Serpins with Known Function

To date, almost 20 tick serpins from different tick species have been functionally validated by *in vitro* assays, *in vivo* experimental models, vaccination and by RNA interference (RNAi) experiments (**Table 1**). These are detailed below.

AamS6 (A. americanum)

Only two serpins (AamS6 and AAS19) have been characterized thus far in *A. americanum*, despite the overall high number of serpins identified in this tick (Porter et al., 2015). *A. americanum* serpin 6 (AamS6) is upregulated during first 3 days of feeding and is likely to be injected into the host during feeding; however, RNAi did not affect tick feeding ability (Chalaire et al., 2011). Recombinant AamS6 inhibited the serine proteases trypsin, chymotrypsin, elastase, and chymase and the cysteine protease papain in a dose-dependent manner (Chalaire et al., 2011). AamS6 also reduced platelet aggregation and delayed plasma clotting time, suggesting that this serpin facilitates blood feeding by ticks (Mulenga et al., 2013). The complement activation pathway, however, was not affected (Mulenga et al., 2013).

AAS19 (A. americanum)

AAS19 is an anti-coagulant that was shown to inhibit five of the eight serine protease blood clotting factors. AAS19 inhibited thrombin—but not ADP—and cathepsin G-activated platelet aggregation and delayed clotting in re-calcification and thrombin time assays (Kim et al., 2015). AAS19 RNAi halved the blood intake and resulted in morphological deformation of ticks (Kim et al., 2016). In rabbits, immunized with AAS19, tick feeding was faster, but smaller blood volumes were ingested, and tick ability to lay eggs was impaired (Kim et al., 2016).

HLS-1 and 2 (Haemaphysalis longicornis)

Sugino and colleagues isolated a serpin from *H. longicornis* in 2003 (HLS1) (Sugino et al., 2003). Recombinant HLS1 displayed anticoagulant activity, and nymph and adult tick feeding on immunized rabbits resulted in 43.9 and 11.2% tick mortality, respectively. Antibodies raised against tick saliva did not react with recombinant HSL1, suggesting that the serpin was not secreted (Sugino et al., 2003). Moreover, HLS1 expression was detected in the midgut rather than the salivary glands, and HLS1 was therefore considered a concealed antigen, similar to the first commercially used anti-tick vaccine based on the Bm86 tick protein (Willadsen et al., 1995). HLS1 does not contain a signal peptide. Therefore, it is likely that HLS1 is not a secreted protein playing an immunomodulatory or anti-hemostatic role in the host during tick feeding.

A second serpin from *H. longicornis* (HLS2) possesses a signal sequence and seems to be secreted by hemocytes into the haemolymph but not by the salivary glands or midgut (Imamura et al., 2005). HLS2 prolonged the coagulation time in a dose-dependent manner (Imamura et al., 2005), and rabbit vaccination with HLS2 resulted in greater immunization than with HLS1 and almost 50% mortality of feeding nymphs and adults (Imamura et al., 2005). This might be explained by better accessibility and inactivation of extracellular HLS2 in the haemolymph by antibodies from the ingested blood of immunized animals.

Ipis-1 (Ixodes persulcatus)

To date, Ipis-1 is the only characterized salivary serpin from tick *I. persulcatus* (Toyomane et al., 2016). Ipis-1 transcripts were detected only in salivary glands of ticks at same level throughout all phases of feeding. It significantly reduced IFN-γ production and the proliferation of bovine PBMC cells after ConA stimulation. Authors suggest that Ipis-1 could inhibit T cells function by direct interaction with this cell population (Toyomane et al., 2016).

Iris (I. ricinus)

The first tick serpin to be described that had an effect on host defense mechanisms was named Iris (*Ixodes ricinus* immunosuppressor) (Leboulle et al., 2002a,b). Iris displayed several notable and important features. First, Iris was noted to inhibit T cell and splenocyte proliferation and altered peripheral blood mononuclear cell (PBMC)-derived cytokine levels (Leboulle et al., 2002a). Second, Iris showed anti-hemostatic properties including suppression of coagulation and fibrinolysis (Prevot et al., 2006). Finally, Iris was shown to bind to monocytes/macrophages and suppress the secretion of TNF (Prevot et al., 2009). Interestingly, these activities were independent on the protease inhibitory function of Iris. Of note, Iris, together with HLS1 and several other proteins, belongs to a group of serpins in *Ixodes* spp. that have methionine and cysteine in their reactive center loop (RCL) and lack a signaling peptide, suggesting intracellular rather than extracellular function. However, Iris has been detected in tick saliva using a polyclonal serum raised against recombinant protein (Leboulle et al., 2002a; Prevot et al., 2007), and vaccination of rabbits with recombinant Iris increased the mortality of feeding ticks and lowered weight after engorgement (Prevot et al., 2007). This contradictory observation might be explained by cross-reactivity with another secreted serpin or by the action of another, non-classical secretory mechanism (Nickel, 2003). Nevertheless, Iris represents a pleiotropic protein that affects multiple processes simultaneously via independent mechanisms.

IRS-2 (I. ricinus)

IRS-2 (*Ixodes ricinus* serpin-2) was the second serpin to be characterized in *I. ricinus*. IRS-2 has tryptophan in its P1 site, confirmed by its resolved crystal structure (Kovarova et al., 2010; Chmelar et al., 2011). IRS-2 displayed inhibitory specificity against mast cell chymase and cathepsin G, two proteases involved in inflammatory responses (Chmelar et al., 2011), with its anti-inflammatory function evidenced by *in vivo* paw edema experiments, in which IRS-2 significantly decreased paw swelling and neutrophil recruitment in treated animals (Chmelar et al., 2011). Moreover, IRS-2 inhibited the production of proinflammatory cytokine IL-6 in dendritic cells (DC) and impaired IL-6-dependent JAK/STAT3 signaling in T-helper (Th) cells, inhibiting the maturation of proinflammatory Th17 lymphocytes (Palenikova et al., 2015). IRS-2 also inhibited

TABLE 1 | Tick serpins with known function.

Serpin	Tick species	Secreted	Effect (where known)	Tissue/stage	Target enzyme	References
AamS6	*A. americanum*	Yes	Reduced platelet aggregation and delayed plasma clotting time	SG, MG, OVA, CA	Plasmin, papain, elastase, chymase	Chalaire et al., 2011; Mulenga et al., 2013
AAS19	*A. americanum*	Yes	Anti-coagulant protein, delayed clotting in recalcification and thrombin time assays		Trypsin, plasmin, fIXa, fXa, fXIa, fXIIa, thrombin, tryptase, chymotrypsin	Kim et al., 2015; Porter et al., 2015
			RNAi led to smaller blood meals and deformed ticks	SG, MG, OVA, SYN, CA, MT		Kim et al., 2016
			Feeding on immunized rabbits led to smaller blood meals and disrupted egg laying			
HLS1	*H. longicornis*	No	Feeding on immunized animals increased tick mortality rate	MG		Sugino et al., 2003
HLS2	*H. longicornis*	No	Prolonged coagulation. Immunization of rabbits increased tick mortality	LY		Imamura et al., 2005
Ipis-1	*I. persulcatus*	Yes	Inhibited proliferation and IFN-γ production of bovine PBMCs	SG		Toyomane et al., 2016
Iris	*I. ricinus*	Yes	Disrupted blood coagulation and fibrinolysis	SG, saliva	Elastase, thrombin, t-PA, fXa, trypsin	Prevot et al., 2006
			Suppressed T cell and splenocyte proliferation			Leboulle et al., 2002a
			Altered cytokine secretion by PBMC			Prevot et al., 2009
			Bound monocytes/macrophages and inhibited TNF secretion			Prevot et al., 2007
			Vaccination resulted in higher mortality and lower engorgement			
IRS-2	*I. ricinus*	Yes	Inflammation inhibitor, bound mast cell protease-4, blocked induced platelet aggregation	SG, OVA, MG	Cathepsin G, chymase, thrombin, trypsin, a-chymotrypsin	Chmelar et al., 2011
			Inhibited Th17 differentiation by reduced production of IL-6 in DC			Palenikova et al., 2015
IxscS-1E1	*I. scapularis*	Yes	Inhibited platelet aggregation and plasma clotting	SG, MG	Thrombin, trypsin, cathepsin G, fXa	Mulenga et al., 2009; Ibelli et al., 2014
RAS-1, 2	*R. appendiculatus*	No	Feeding on immunized animals increased tick mortality rate			Imamura et al., 2006
RAS-3, 4	*R. appendiculatus*	Yes	Feeding on immunized animals increased tick mortality rate and delayed *Theleria* infection			Imamura et al., 2008
RHS-1	*R. haemaphysaloides*	Yes	Anticoagulation activity, RNAi disrupted tick feeding	SG	Chymotrypsin, thrombin, fXa	Yu et al., 2013
RHS-2	*R. haemaphysaloides*	No	RNAi disrupted tick feeding	MG	Chymotrypsin, thrombin, fXa	Yu et al., 2013
RmS-3	*R. microplus*	Yes	Reduced platelet aggregation Feeding of ticks with RMS-3 antibodies impaired reproduction	SG, MG, CA	Chymotrypsin, cathepsin G, elastase, chymase	Tirloni et al., 2014b, 2016; Rodriguez-Valle et al., 2012, 2015
RmS-6	*R. microplus*	Yes		SG, MG, OVA, CA	Trypsin, plasmin, fXa, fXIa, chymotrypsin	Tirloni et al., 2014b, 2016
RmS-15	*R. microplus*	Yes	Delayed plasma clotting	SG, OVA, CA	Thrombin	Tirloni et al., 2014b; Xu et al., 2016
RmS-17	*R. microplus*	Yes	Delayed plasma clotting, reduced platelet aggregation	SG, MG, OVA, CA	Trypsin, plasmin, cythepsin G, chymotrypsin, fXIa	Tirloni et al., 2014b, 2016
rSerpin	*R. microplus*	Yes	Feeding on immunized animals increased feeding time and tick mortality and reduced tick engorgement and egg mass	SG		Kaewhom et al., 2007; Jittapalapong et al., 2010

SG, salivary glands; MG, midgut; OVA, ovaries; FB, fat body; HE, hemocytes; MAL, Malpighian tubules; DC, dendritic cells.

platelet aggregation induced by cathepsin G but not other inducers such as collagen or arachidonic acid derivatives (Chmelar et al., 2011).

IxscS-1E1 (I. scapularis)
A blood meal-induced salivary serpin IxscS-1E1 from *I. scapularis* has been shown to trap thrombin and trypsin in SDS- and heat-stable complexes, reduce their activity and inhibit the activities of cathepsin G and factor Xa, although protease/inhibitor complexes were not detected (Ibelli et al., 2014). Furthermore, IxscS-1E1 inhibited adenosine diphosphate- and thrombin-activated platelet aggregation and delayed plasma clotting time, suggesting an anti-hemostatic role (Ibelli et al., 2014). IxscS-1E1 had no effect on the classical complement activation pathway (Ibelli et al., 2014).

RAS-1, 2, 3, 4 (Rhipicephalus appendiculatus)
Four serpin cDNAs, two putatively secreted (RAS-3 and RAS-4) and two putatively intracellular (RAS-1 and RAS-2), were identified in and isolated from the salivary glands of *R. appendiculatus* (Mulenga et al., 2003). Although RAS-1 and RAS-2 are expressed in the salivary glands, antibodies against them were not found at the bite site as determined by the reactivity of anti-tick saliva sera to recombinant RAS-1 and RAS-2 (Imamura et al., 2006). This finding is, however, consistent with their predicted intracellular location (Imamura et al., 2006). Vaccination of cattle with a RAS-1/RAS-2 cocktail resulted in a 61.4% reduction in nymph engorgement rate and a 28 and 43% increase in mortality rate in female and male adult ticks, respectively (Imamura et al., 2006). Similar results were obtained when cattle were vaccinated with a mixture of two secreted serpins RAS-3 and RAS-4 and a 36-kDa immunodominant cement protein RIM36 (Imamura et al., 2008): immunization resulted in 40% mortality rate for *R. appendiculatus* ticks and almost 50% for *Theileria parva*-infected female ticks (Imamura et al., 2008). However, no significant protective effect against infection with *T. parva* was observed in spite of a 1–2 day delay in the detection of pathogens in the host peripheral blood after immunization (Imamura et al., 2008).

RHS-1 and 2 (Rhipicephalus haemaphysaloides)
Two serpins (RHS-1 and RHS-2) have been identified and characterized from *R. haemaphysaloides* (Yu et al., 2013), both of which were expressed in the salivary glands and midguts of ticks fed for 4 days. Both inhibited chymotrypsin, and RHS-1 also inhibited thrombin (Yu et al., 2013). Consistent with their inhibitory activity, only RHS-1 exhibited anticoagulation activity based on the activated partial thrombin time assay (Yu et al., 2013). Only RHS-1 seems to be secreted into the saliva and the host, as only RHS-1 was detected by serum from rabbits that were exposed to ticks and only RHS-1 possesses a signal peptide sequence (Yu et al., 2013). Nevertheless, RNAi of both serpins negatively affected the attachment rate after 24 h and decreased the engorgement rate (Yu et al., 2013).

RmS-3, 6, 15, 17 (R. microplus)
Serpin RmS-3 from *R. microplus* displayed anti-elastase and anti-chymotrypsin inhibitory activities (Rodriguez-Valle et al., 2015). Tirloni and colleagues subsequently confirmed this specificity (albeit with much lower inhibitory activity), tested more proteases, and found the highest inhibitory activity against chymase and cathepsin G (Tirloni et al., 2016). RmS-3 is likely to be secreted into the saliva and the host as evidenced by differential antibody responses of tick-resistant and tick-susceptible cattle (Rodriguez-Valle et al., 2012). RmS-3 is expressed in nymphs and in the salivary glands of adult ticks, data on RmS-3 transcription in ovaries differ between the two studies (Tirloni et al., 2014b; Rodriguez-Valle et al., 2015). Capillary feeding of ticks with a RmS-3 antibody reduced tick reproductive capacity (Rodriguez-Valle et al., 2012, 2015).

In addition to RmS-3, three other recombinant *R. microplus* serpins were produced for enzymatic and functional characterization (Tirloni et al., 2014a,b; Xu et al., 2016). RmS-6 inhibited factor Xa, factor XIa and plasmin, suggesting an anticoagulant function, while RmS-17 showed weaker inhibitory activity against chymotrypsin, cathepsin G, trypsin, and plasmin (Tirloni et al., 2016). Both RmS-3 and RmS-17 inhibited cathepsin G-induced platelet aggregation. Interestingly, RmS-3, -6, and -17 from *R. microplus* were recognized by antibodies raised by the saliva of *A. americanum*, *I. scapularis*, and *Rhipicephalus sanguineus*, suggesting a potential use for these proteins as an universal tick vaccine (Tirloni et al., 2016) but also highlighting the pitfall of false-positive detection of serpins in tick saliva. RmS-15 was identified as a thrombin inhibitor and, together with RmS-17, delayed plasma clotting in a re-calcification time assay (Tirloni et al., 2016; Xu et al., 2016). Moreover, RmS-15 is an immunogen, as the infestation of cattle with *R. microplus* resulted in increased anti-RmS-15 IgG titers (Xu et al., 2016).

rSerpin (R. microplus)
Rabbits immunized with putatively secreted serpin (rSerpin) from *R. microplus* (Kaewhom et al., 2007) led to extended feeding time, an 83% reduction in adult engorgement, 67% mortality of engorged females and a 34% reduction in egg mass weight (Jittapalapong et al., 2010).

Cystatins

Cystatins form a superfamily of tight-binding reversible inhibitors of papain-like cysteine proteases and legumains and, similar to serpins, they are present in all organisms including prokaryotes (Kordis and Turk, 2009). Cystatins regulate many physiological processes including immunity-related mechanisms such as antigen presentation, phagocytosis, and cytokine expression (Zavasnik-Bergant, 2008). There are four cystatin subgroups: type 1 (stefins), type 2, type 3 (kininogens), and type 4 cystatins (fetuins) (Rawlings and Barrett, 1990). Cystatins' target proteases are usually lysosomal cathepsins involved in protein degradation, but they also target those involved in degradation of antigens presented via MHCII to lymphocytes or in the activation of caspase 1 and thus inflammasome regulation (Jin and Flavell, 2010; Turk et al., 2012).

Cystatins with Known Function

Similarly to serpins, there are around 20 tick cystatins described in the literature and only type 1 and type 2 cystatins have thus

far been reported in ticks. While stefins lack a secretory signal and are most likely involved in the intracellular digestion of hemoglobin or in developmental processes, type 2 cystatins are secreted and expressed in both the midgut and salivary glands (Schwarz et al., 2012). Tick cystatins either regulate hemoglobin digestion, which is driven by cathepsins (Horn et al., 2009), or they can be secreted as immunomodulators into the host with saliva. The majority (84%) of tick cystatin transcripts that are conserved across tick species, belong to the extracellular group, suggesting predominantly immunomodulatory role (Ibelli et al., 2013) Tick cystatins with experimentally validated functions are listed in **Table 2** and detailed below.

Bmcystatin (R. microplus)
Bmcystatin from *R. microplus* is specifically expressed in the salivary glands, ovaries, and fat bodies. Bmcystatin did not inhibit papain but inhibited human cathepsin L and tick vitellin-degrading cysteine endopeptidase (VDTCE), suggesting a role in regulating tick embryogenesis (Lima et al., 2006).

BrBmcys2a, b, c, d, e, (R. microplus)
In addition to Bmcystatin, another five cystatins (BrBmcys2a, b, c, d, e) were identified in the cattle tick *R. microplus*. Their expression differs among various developmental stages and tissues, but since their presence has only been assessed by immunodetection methods, cross reactivity between antibodies is possible and has indeed been reported (Imamura et al., 2013). This study also examined the inhibitory specificity of two cystatins: while BrBmcys2b targeted cathepsins B, C, and L, BrBmcys2c only inhibited cathepsins C and L (Parizi et al., 2015). Antibodies raised against recombinant proteins detected BrBmcys2b in all tick tissues, while anti-BrBmcys2c serum only recognized the protein in the gut from partially engorged females and in the ovaries, salivary glands, and fat bodies from fully engorged females (Parizi et al., 2015). The expression patterns suggest rather homeostatic function of these cystatins in ticks than immunomodulatory activity in the host (Imamura et al., 2013).

Cystatin (A. americanum)
One cystatin was detected in the salivary glands and midguts of unfed and partially fed *A. americanum* ticks (Karim et al., 2005). RNAi of this cystatin led to a 90 and 50% reduction in transcript abundance in the early and late phases of feeding, respectively. RNAi knockdown decreased tick body weight, killed ticks during feeding, and disrupted feeding to full engorgement. Rabbits pre-exposed to dsRNA-injected ticks were re-exposed to naïve ticks, which led to detachment of 34% ticks after 1 day and over 50% mortality of attached ticks (Karim et al., 2005). No such effect was observed in the control group, in which rabbits were pre-exposed to normal ticks. Such a strong immune response indicates an important immunomodulatory function for silenced cystatin that impairs responses to salivary antigens and leads to an overall less intense immune reaction (Karim et al., 2005).

HISC-1 (H. longicornis)
HISC-1 is a type 2 cystatin detected in *H. longicornis* (Yamaji et al., 2009b). It is found mainly in the acinar cells of the tick salivary glands and is therefore likely to be secreted into the host. The number of transcripts was found to be approximately 5-fold higher in the salivary glands than in the midgut, with strong upregulation in early phase of blood feeding and with a pattern suggestive of importance in the feeding process. HISC-1 inhibited cathepsins L and papain but not cathepsin B (Yamaji et al., 2009b).

Hlcyst-1, 2 and 3 (H. longicornis)
While Hlcyst-1 is a type 1 intracellular cystatin with specificity against papain and cathepsin L (Zhou et al., 2009), Hlcyst-2 and Hlcyst-3 are secreted type 2 cystatins (Zhou et al., 2006b, 2010). Hlcyst-2 has been shown to inhibit cathepsin L and cathepsin B, with transcripts found mainly in the midgut and hemocytes of all tick developmental stages. Expression increased with tick development and was induced by blood feeding (Zhou et al., 2006b). Moreover, Hlcyst-2 expression was induced by injecting ticks with LPS or *Babesia gibsoni*, suggesting a role in tick immunity. *In vitro* cultivation of *B. gibsoni* in the presence of Hlcyst-2 significantly inhibited pathogen growth (Zhou et al., 2006b). Hlcyst-1 and Hlcyst-2 also inhibited cysteine protease HlCPL-A with hemoglobinase activity, isolated from *H. longicornis*, which can act as natural target of these cystatins, suggesting an involvement of both the protease and its inhibitors in blood digestion (Yamaji et al., 2009a). Hlcyst-3 inhibited papain and cathepsin L, and its expression was detected preferentially in the midgut (Zhou et al., 2010).

JpIocys2 (Ixodes ovatum)
JpIocys2 was isolated from *I. ovatum* and was shown to modulate the enzymatic activity of cathepsins B, C, and L with cathepsin L as the preferred target (Parizi et al., 2015). Similar to BrBmcys2b and BrBmcys2c, JpIocys2 is considered to be involved in tick homeostasis and egg development.

JpIpcys2a, b, c (I. persulcatus)
Three novel cystatins from *I. persulcatus*, JpIpcys2a, b, and c, have recently been described in terms of sequence and structural analysis and expression profile (Rangel et al., 2017). All three possess a signal peptide and two disulfide bridges in their mature form. Although varying in their tertiary structure, all three *I. persulcatus* cystatins should bind human cathepsin L and papain, based on *in silico* analyses. Transcripts of all three cystatins were detected in almost all tissues (salivary glands, midgut, carcass) and stages (larvae, nymphs, adults) of tick development. The only exception was absence of JpIpcys2c transcripts in unfed larvae. Furthermore, vaccination of hamsters with a structurally similar BrBmcys2c cystatin from *R. microplus* did not show any cross-reactivity and did not lead to impaired *I. persulcatus* feeding or reproduction (Rangel et al., 2017).

Om-cystatin 1 and 2 (Ornithodoros moubata)
Om-cystatin 1 and 2 were described in a soft tick *O. moubata* (Grunclova et al., 2006). While Om-cystatin 1 transcripts were found only in the midguts of unfed ticks, Om-cystatin 2 mRNA was present in all tissues. Transcript levels were rapidly suppressed after tick feeding. Both possessed inhibitory activity against cathepsins B, C, and H and papain (Grunclova et al.,

TABLE 2 | Tick cystatins with known function.

Cystatin	Tick species	Secreted	Effect (where known)	Tissue/stage	Target enzyme	References
Bmcystatin	*R. microplus*	No		SG, OVA, FB	Cathepsin L, VDTCE	Lima et al., 2006
BrBmcys2a	*R. microplus*	Yes		MG, OVA, FB		Imamura et al., 2013
BrBmcys2b	*R. microplus*	Yes		MG	Cathepsin B, C, L	Imamura et al., 2013; Parizi et al., 2015
BrBmcys2c	*R. microplus*	Yes		MG	Cathepsin C, L	Imamura et al., 2013; Parizi et al., 2015
BrBmcys2d, e	*R. microplus*	Yes		larvae		Imamura et al., 2013
Cystatin	*A. americanum*	Yes	RNAi caused decreased tick body weight, dying of ticks during feeding or disrupted feeding to the fully engorged state	MG, SG		Karim et al., 2005
HISC-1	*H. longicornis*	Yes		SG	Cathepsin L, papain	Yamaji et al., 2009b
Hlcyst-1	*H. longicornis*	No	Regulated hemoglobin degradation	MG	Cathepsin B, H, L, papain, HlCPL-A	Zhou et al., 2006b, 2009; Yamaji et al., 2009a, 2010
Hlcyst-2	*H. longicornis*	Yes	Regulated hemoglobin degradation, inhibited *Babesia* growth *in vitro*	MG, SG, OVA, HE, FB	Cathepsin L, papain, HlCPL-A	Zhou et al., 2006b; Yamaji et al., 2009a, 2010
Hlcyst-3	*H. longicornis*	Yes		MG, SG, OVA, HE, FB	Cathepsin L, papain	Zhou et al., 2006b, 2010
Jplocys2	*I. ovatum*	Yes		Assumed MG	Cathepsin B, C, L	Parizi et al., 2015
Jplpcys2a, b, c	*I. persulcatus*	Yes		SG, MG / larvae, nymphs, adult	Cathapsin L, papain	Rangel et al., 2017
Om cystatin 1	*O. moubata*	Yes		MG	Cathepsin B, C, H	Grunclova et al., 2006
Om cystatin 2	*O. moubata*	Yes	Inhibited TNF-α and IL-12 production by DC and proliferation of CD4+ T cells, immunization decreased tick feeding success	SG, OVA, MAL, MG	Cathepsin B, C, H, L, S, papain	Grunclova et al., 2006; Kotsyfakis et al., 2010
RHcyst-1	*R. haemaphysaloides*	No	Inhibitors, RNAi of RHcyst-1 impaired tick attachment rate and decreased hatching rate	Egg, larvae	Cathepsin B, C, H, L, S, papain	Wang et al., 2015b
RHcyst-2	*R. haemaphysaloides*	Yes		Egg, adult MG, SG, OVA, FB	Cathepsin B, C, H, L, S, papain	Wang et al., 2015a
Rmcystatin3	*R. microplus*	Yes		FB, HE	Cathepsin B, L, BmCl1	Lu et al., 2014
Sialostatin L	*I. scapularis*	Yes	Inhibited CTL proliferation, anti-inflammatory effects	SG	Cathepsin C, L, V, X, papain	Valenzuela et al., 2002
			Impaired DC maturation and differentiation and T cells proliferation		Binds cathepsin S	Kotsyfakis et al., 2006
			Prevented experimental asthma, inhibited IL-9 production by Th9 cells and mast cells by targeting IRF-4			Sa-Nunes et al., 2009
			Decreased IFN-β production in DC and DC maturation			Horka et al., 2012; Klein et al., 2015
			Attenuated IFN-β-triggered JAK/STAT signaling pathway in dendritic cells			Lieskovska et al., 2015a
						Lieskovska et al., 2015b
Sialostatin L2	*I. scapularis*	Yes	RNAi caused tick mortality, reduced weight and less eggs	SG, MG	Cathepsin C, L, S, V	Kotsyfakis et al., 2007
			Immunization caused decreased feeding ability of nymphs			Kotsyfakis et al., 2008
			Enhanced establishment of *Borrelia* infection			Kotsyfakis et al., 2010
			Inhibited caspase-1 maturation and diminished IL-1β and IL-18 secretion by macrophages during *Anaplasma phagocytophilum* infection			Chen et al., 2014
			Attenuated IFN-β-triggered JAK/STAT signaling in DC and promotes TBEV replication, decreases MIP-a and IP-10 production by DC			Lieskovska et al., 2015a,b

SG, salivary glands; MG, midgut; OVA, ovaries; FB, fat body; HE, hemocytes; MAL, Malpighian tubules; VDTCE, vitellin-degrading cysteine endopeptidases; DC, dendritic cell; TBEV, tick-borne encephalitis virus.

2006). Om-cystatin 2 was further functionally and structurally characterized under the name OmC2 (Salát et al., 2010). OmC2 inhibited the secretion of pro-inflammatory cytokines TNF and IL-12 by DC after LPS stimulation and reduced antigen-specific $CD4^+$ T cell proliferation induced by DC (Salát et al., 2010). Exposing OmC2 immunized mice to *O. moubata* nymphs reduced feeding ability and increased mortality during nymphal development to the next stage. Interestingly, nymphs mortality was positively correlated with higher titers of anti-OmC2 antibodies in the serum (Salát et al., 2010).

RHcyst-1 and RHcyst-2 (R. haemaphysaloides)

Two cystatins have been described in *R. haemaphysaloides*, RHcyst-1 and RHcyst-2. RHcyst-1 is an intracellular type 1 cystatin that inhibited cathepsins L, B, C, H, and S and papain, with strongest affinity to cathepsin S (Wang et al., 2015b). RHcyst-1 was expressed at all developmental stages but was most abundant in tick eggs, and its expression decreased throughout the development. RNAi of RHcyst-1 reduced the attachment rate of adult ticks and decreased hatching rate (Wang et al., 2015b). RHcyst-2 is a secreted type 2 cystatin that inhibited the same cathepsins as RHcyst-1 (Wang et al., 2015a) and was again present at all developmental stages with highest expression in eggs. However, RHcyst-2 expression increased during blood feeding, and RHcyst-2 was secreted to the host during tick feeding according to immunodetection methods (Wang et al., 2015a).

Rmcystatin3 (R. microplus)

Rmcystatin3 inhibited cathepsins L and B and *Boophilus microplus* cathepsin L-1 (BmCl1) (Lu et al., 2014). Bmcystatin3 transcripts were found in tick hemocytes, fat bodies, and salivary glands, but protein was only detected in hemocytes and the fat bodies by western blotting. Infection of ticks with *E. coli* significantly downregulated Bmcystatin3 expression (Lu et al., 2014) but increased efficacy of pathogen clearance, suggesting that Rmcystatin3 may be a negative regulator of tick immune responses, probably by regulating cysteine proteases responsible for the production of antimicrobial effectors in hemocytes (Lu et al., 2014).

Sialostatin L (I. scapularis)

One of the best studied tick cystatins is sialostatin L, a type 2 cystatin detected in *I. scapularis*. Sialostatin L has preferential specificity for cathepsin L; however, cathepsins V, C, X, S, and papain were also inhibited in enzymatic assays (Kotsyfakis et al., 2006). In the same study, sialostatin L inhibited the proliferation of the cytotoxic T lymphocyte cell line CTLL-2, suggesting its effect on adaptive immunity. Moreover, the anti-inflammatory activity of sialostatin L was confirmed in a mouse model of carrageenan-induced paw edema, in which sialostatin L reduced edema and neutrophil myeloperoxidase activity (Kotsyfakis et al., 2006).

Sialostatin L has been shown to inhibit IL-2 and IL-9 production by Th9 lymphocytes (Horka et al., 2012). IL-9 production by Th cells is IL-2 dependent (Schmitt et al., 1994), but the addition of exogenous IL-2 did not rescue IL-9 synthesis, suggesting that mechanisms other than IL-2 reduction may be involved in IL-9 inhibition (Horka et al., 2012). Nevertheless, the impairment of Th9 cells by sialostatin L abrogated the eosinophilia and airway hyperresponsiveness of mice challenged with OVA antigen (Horka et al., 2012). The inhibition of IL-9 production together with reduced expression of IL-1β and IRF4 (interferon regulating factor 4) was also observed in mast cells, with IL-9 production rescued by the application of exogenous IL-1β (Klein et al., 2015). The inhibition of IL-9 was IRF4 or IL-1β dependent, as proven by the fact that IRF4-deficient or IL-1 receptor-deficient mast cells failed to produce IL-9. The transcription factor IRF4 binds to IL-1β and IL-9 promoters, implying that sialostatin L inhibits IL-9 production via its effect on IRF4 (Klein et al., 2015). Furthermore, mice with IRF4 knockdown in mast cells or mice administered with sialostatin L showed a strong reduction in eosinophilia and airway hyperresponsiveness, important symptoms of asthma. Conversely, sialostatin L did not affect mast cell degranulation or IL-6 expression (Klein et al., 2015).

Sialostatin L inhibits cathepsin S, resulting in reduced antigen-specific $CD4^+$ T cell proliferation *in vitro* and *in vivo*; sialostatin L treatment during OVA immunization impaired early T cell expansion of splenocytes in OT-II mice and late recall immune responses by impairing the proliferation of lymph node cells (Sa-Nunes et al., 2009). Sialostatin L also potently prevented symptoms of experimental autoimmune encephalomyelitis in mice accompanied by impaired IFN-γ and IL-17 production and specific T cell proliferation (Sa-Nunes et al., 2009).

In addition to modulating T cells, sialostatin L inhibited DC maturation and reduced the production of IL-12 and TNF by DC (Sa-Nunes et al., 2009). These effects on DC can also be attributed to anti-cathepsin S activity, as cathepsin S plays a role in an invariant chain processing (Pierre and Mellman, 1998) and its inhibition thus leads to poor antigen presentation by DC (Sa-Nunes et al., 2009). Similar to another *I. scapularis* cystatin Sialostatin L2 (Lieskovska et al., 2015b), sialostatin L attenuated IFN-β-triggered JAK/STAT signaling in DC (Lieskovska et al., 2015a). However, unlike Sialostatin L2, it did not suppress expression of the IP-10 chemokine or IRF-7, suggesting that these two cystatins can produce the same phenotype by impairing different pathways in the same cell (Chmelar et al., 2016). It also decreased IFN-β production in DC activated by either *Borrelia* or TLR-7 ligand (Lieskovska et al., 2015a).

Sialostatin L2 (I. scapularis)

Sialostatin L2 is an *I. scapularis* cystatin similar in sequence to sialostatin L but with different anti-protease potency, antigenicity, and expression pattern. Unlike sialostatin L, sialostatin L2 transcripts accumulate in the salivary glands during tick feeding (Kotsyfakis et al., 2007). Its target proteases are cathepsins L, V, S, and C with preferential affinity for cathepsins L and V (Kotsyfakis et al., 2007). Sialostatin L2 was shown to inhibit inflammasome formation during infection with *A. phagocytophilum* (Chen et al., 2014) via sialostatin L2-driven inhibition of caspase-1 maturation, leading to diminished IL-1β and IL-18 secretion by macrophages after stimulation with *A. phagocytophilum* (Chen et al., 2014). However, the mechanism was not due to direct caspase-1 or cathepsin L inhibition, but was

instead dependent on reactive oxygen species (ROS) production by NADPH oxidase that was affected by the Loop2 domain of the cystatin (Chen et al., 2014). As mentioned above, sialostatin L2 interfered with JAK/STAT signaling in DC (Lieskovska et al., 2015b), attenuating STAT phosphorylation upon IFN-β treatment and thus inhibiting the IFN-β stimulated IP-10 and IRF7 chemokine genes (Lieskovska et al., 2015b). No interference with the IFN-β receptor was observed, so the downstream components of the pathway were most likely affected. Moreover, this activity enhanced the replication of tick borne encephalitis virus in DC (Lieskovska et al., 2015b). Sialostatin L2 decreased the production of specific DC chemokines MIP-1α and IP-10 in response to *Borrelia* (Lieskovska et al., 2015a). Upon LTA/TLR2 stimulation of DC, sialostatin L2 attenuated Erk1/2 phosphorylation, inhibited the PI3K pathway by reducing Akt phosphorylation, and also reduced NF-κB phosphorylation. Impaired Erk1/2 phosphorylation was the only effect observed for sialostatin L2 after stimulation of DC with *Borrelia* spirochetes (Lieskovska et al., 2015a).

The role of sialostatin L2 in *Borrelia* transmission and tick feeding has also been addressed. RNAi of sialostatin L2 led to 40% mortality in tick feeding, reduced tick size, and reduced the number of eggs by about 70% (Kotsyfakis et al., 2007). Similar effects were seen when *I. scapularis* nymphs were exposed to guinea pigs immunized with sialostatin L2 (Kotsyfakis et al., 2008). The rejection rate of nymphs fed on immunized animals was three times higher compared to controls, and the time needed to finish a blood meal was prolonged by approximately 1 day (Kotsyfakis et al., 2008). Moreover, IgG isolated from immunized animals reduced sialostatin L2 inhibitory activity against cathepsin L (Kotsyfakis et al., 2008). Of note, sialostatin L2 has been referred to as a "silent antigen," meaning that corresponding antibodies cannot be found in naïve animals exposed to ticks despite an increased titer of specific antibodies in animals pre-immunized with recombinant protein. This can be explained by the amount of sialostatin L2 injected via the saliva into the host being too small to elicit a response (Kotsyfakis et al., 2008). Sialostatin L2 has also been shown to play an important role in *Borrelia* infection (Kotsyfakis et al., 2010). The skin of mice simultaneously injected with *Borrelia* and sialostatin L2 contained six-times more spirochetes than controls. Sialostatin L2 does not appear to bind spirochetes directly and had no effect on *Borrelia* growth *in vitro*, so the mechanism of *Borrelia* growth boost in skin remains unknown (Kotsyfakis et al., 2010).

PROTEASE INHIBITORS AT THE TICK-HOST INTERFACE

Tick cystatins and serpins can obviously affect many intracellular pathways and thus impair the functions of host immune cells. Moreover, they can also interfere with extracellular proteolysis, thereby inhibiting hemostasis (**Figure 1**). These activities take place at the site of attachment, where they cause local immunosuppression and inhibition of blood clotting. Of note, different inhibitors can cause similar phenotypes by targeting different pathways or even different components of the same pathway. Their actions are therefore redundant. Conversely, more than one effect is usually observed for a single inhibitor. Such concept of redundancy and pluripotency is probably a strategy developed by ticks during long-term co-evolution with their hosts (Chmelar et al., 2016). There is no doubt that salivary secretion at the tick-host interface is beneficial for the tick and deleterious for the host. From this perspective, tick inhibitors represent an important and interesting research field for the development of anti-tick vaccines and tick control strategies.

As shown on vaccination experiments, tick serpins and cystatins can contribute to the establishment of pathogens in the host (Imamura et al., 2008; Kotsyfakis et al., 2010). Such role of serpins is in accordance with observed positive effect of activated plasminogen activation system (PAS) with upregulated serpin PAI-2 on the establishment of *Borrelia burgdorferi* infection. The facilitation of infection resulted from direct enhancement of *Borrelia* dissemination and from the inhibition of inflammatory infiltration to the site of exposure (Haile et al., 2006). *Borrelia recurrentis* was shown to bind host serpin—C1 inhibitor—on its surface and thus inhibit complement activation (Grosskinsky et al., 2010). On contrary, mammalian cystatins were shown as regulators of cysteine proteases like cathepsin S and L, which contribute to the establishment of several viral infections (Kopitar-Jerala, 2012). Thus, the involvement of cystatins in the establishment of microbial and viral infection is not clear and cannot be easily addressed without experimental evidence.

TICK PROTEASE INHIBITORS AS NOVEL DRUGS

Cystatins

The inhibition of target proteases with tick-derived inhibitors can, however, be beneficial in different scenarios. Almost all the mammalian serine and cysteine proteases that are targets of tick inhibitors described in this review play important roles in various human diseases and pathologies. For a long time, the functions of lysosomal cysteine cathepsins (B, C, F, H, K, L, O, S, V, X, and W) were thought to be strictly limited to intracellular protein degradation and cellular metabolism. Recently, many cathepsins have been shown to be involved in multiple pathological processes. For example, increased serum levels of cathepsin L are associated with metastatic stage of different cancer types and poor patient prognosis (Tumminello et al., 1996; Chen et al., 2011). Tumor cells can produce high amounts of cathepsin L, leading to high serum level, which is considered as blood marker of cancer (Denhardt et al., 1987). High concentration of cathepsin L in tumor and its vicinity leads to extracellular matrix degradation, higher tumor invasiveness, and several cancer-related health complications (Sudhan and Siemann, 2015). Other cysteine cathepsins may also participate in tumor invasion and metastasis (Kuester et al., 2008; Tan et al., 2013), so cystatins are considered possible effectors that could block the deleterious activity of cysteine cathepsins in cancer (Cox, 2009; Hap et al., 2011). Cysteine cathepsins also contribute to neurodegenerative disorders such as Alzheimer's, Parkinson's, and Huntington's disease and amyotrophic lateral

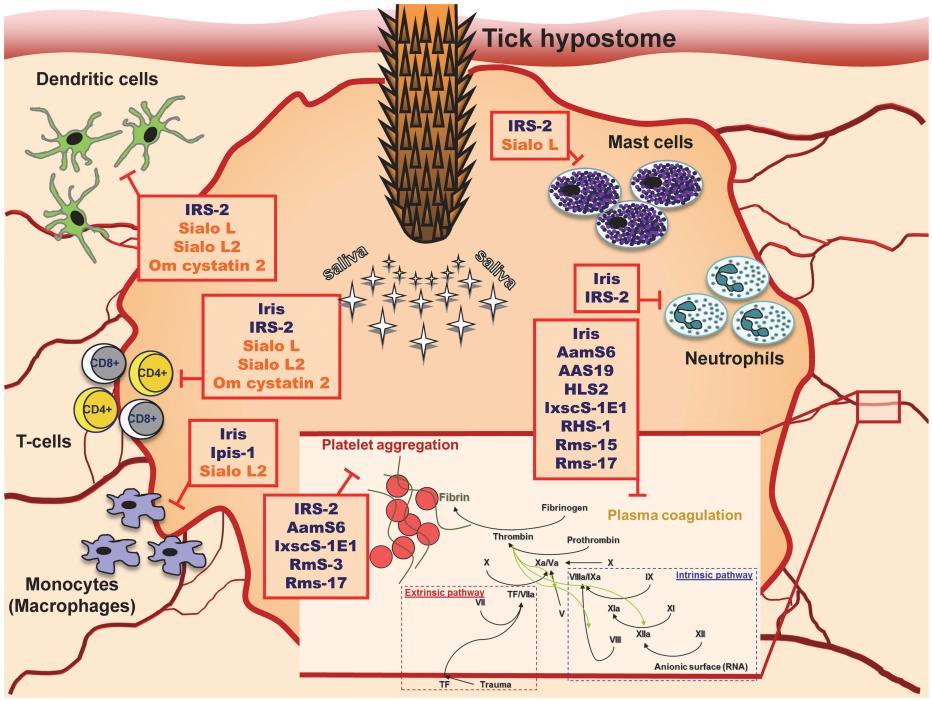

FIGURE 1 | Tick serpins and cystatins and their targets at the site of tick attachment.

sclerosis (**Figure 2A**; Pislar and Kos, 2014). The leakage of lysosomal cathepsins induces neuronal apoptosis and can also increase the inflammatory milieu in the central nervous system (Pislar and Kos, 2014). Cysteine cathepsins are also implicated in the pathogenesis of psoriasis (Kawada et al., 1997), muscular dystrophy (Takeda et al., 1992), abdominal aortic aneurysm and atherosclerosis (Liu et al., 2006), osteoporosis and rheumatoid arthritis (Yasuda et al., 2005), and acute pancreatitis (Halangk et al., 2000). Relatively recent data are accumulating to suggest that cysteine cathepsins are promising therapeutic targets (Kos et al., 2014; Sudhan and Siemann, 2015). The wide spectrum of tick cystatins with varying specificities provides an opportunity to take advantage of this rich source of natural cathepsin inhibitors.

Serpins

Serine proteases are best known as the building blocks of proteolytic cascades in the blood such as coagulation (**Figure 1**) or complement activation. The portfolio of their activities, however, is much wider. Neutrophils, mast cells, natural killer cells, and cytotoxic T cells all produce serine proteases responsible for extracellular matrix remodeling, microbe killing, cytokine activation, signaling via protease-activated receptors (PARs), or chemoattraction of leukocytes. As regulators of many processes, serine proteases often contribute to disease pathologies. Some diseases, in which serine proteases are implicated, are shown in **Figure 2B**. Signaling via PARs and the activation of coagulation in the tumor microenvironment link coagulation proteases with some of the complications seen in cancer (Shi et al., 2004; Han et al., 2011; Lima and Monteiro, 2013). Neutrophil proteases from azurophilic granules, namely cathepsin G, elastase, and protease 3 (PR3), play crucial roles in neutrophil anti-microbial activity and are indispensable for the clearance of some pathogens (Hahn et al., 2011; Steinwede et al., 2012). Many studies have also described neutrophil proteases as important regulators of inflammatory and immune processes (Pham, 2006, 2008), albeit with deleterious effects in some cases. For instance, due to the large amounts of elastin present in the lung connective tissue, lungs are very sensitive to dysregulation and/or increased levels of elastolytic proteases such as neutrophil elastase (Sandhaus and Turino,

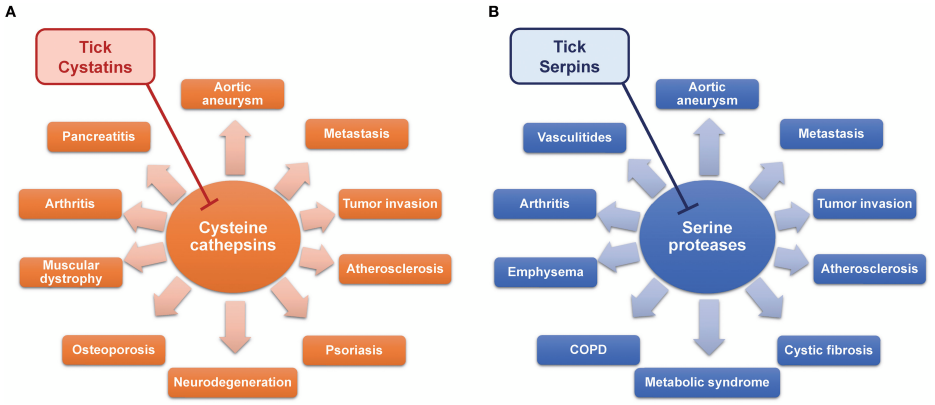

FIGURE 2 | The therapeutic potential of tick cystatins (A) and serpins **(B)** by inhibiting cysteine and serine proteases in various diseases.

2013), which results in several lung diseases. Elastase and cathepsin G facilitate the spreading of metastases to the lungs due to the degradation of antitumorigenic factor thrombospondin-1 (El Rayes et al., 2015). Furthermore, neutrophil proteases have been implicated in the pathogenesis of cystic fibrosis (Twigg et al., 2015; Wagner et al., 2016), chronic obstructive pulmonary disease (COPD) (Shapiro, 2002; Owen, 2008), and emphysema (Ekeowa et al., 2009). In anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitides such as Wegener's granulomatosis, neutrophils are activated by auto-antibodies against PR3 (Niles et al., 1989), leading to the production of neutrophil extracellular traps (NETs) containing PR3 and to necrosis (Kessenbrock et al., 2009). Cathepsin G is chemotactic for monocytes in rheumatoid arthritis (Miyata et al., 2007), and the inhibition of neutrophil elastase improved some of the symptoms of this disease (Di Cesare Mannelli et al., 2016). Interestingly, obesity and metabolic syndrome also seem to be affected by neutrophil proteases (Talukdar et al., 2012; Mansuy-Aubert et al., 2013). Mast cells are another significant source of several serine proteases, mainly chymases and tryptases, which are involved in extracellular matrix remodeling, chemoattraction of neutrophils, and protein processing and activation (Pejler et al., 2010). Mast cell chymase and tryptase have been shown to be involved in the pathogenesis of abdominal aortic aneurysm (Sun et al., 2009; Zhang et al., 2011) and atherosclerosis (Sun et al., 2007; Bot et al., 2015).

Due to these diverse and clinically relevant effects of serine proteases, their potential use as therapeutic targets is being thoroughly discussed by scientific community (Guay et al., 2006; Quinn et al., 2010; Caughey, 2016). Tick salivary glands express a large number of serine protease inhibitors with different specificities that could be used as novel drugs against malfunctioning proteases.

CONCLUDING REMARKS

Novel pharmacoactive compounds are being developed either by artificial synthesis or by isolating potential candidates from various organisms including parasites (Cherniack, 2011). For instance, hirudin (a thrombin inhibitor from leeches) and its congener bivalrudin have been useful in the treatment of blood coagulation disorders (Kennedy et al., 2012). Ticks are parasites that have evolved multiple ways to evade or manipulate host immune and hemostatic systems (Chmelar et al., 2012). Tick saliva contains hundreds of proteins not only with anti-hemostatic features (Maritz-Olivier et al., 2007) but also with anti-complement, anti-inflammatory, and immunomodulatory effects on the host (Kazimirova and Stibraniova, 2013).

As discussed in this review, salivary cystatins and serpins display such features and their functions have been studied thoroughly. Moreover, both superfamilies are represented in the vertebrate host and the functions of their members are often known. Therefore, we can predict at least to some degree, which processes or pathways will be targeted by tick proteins. An important advantage of cystatins and serpins is their functional specificity; for example, sialostatins L and L2 cause similar phenotypes (inhibition of IFN-β signaling) either by inhibiting the IFN-β production (sialostatin L) or by inhibiting STAT3 phosphorylation downstream from IFN-β (sialostatin L2) (Lieskovska et al., 2015a,b). The possibility of targeting specific processes is crucial for the development of "patient-tailored" immunotherapeutic strategies (Scherer et al., 2010; Stephenson et al., 2016). Furthermore, tick cystatins and serpins are not the only families in ticks that deserve attention, since there are many tick-specific proteins secreted into the saliva of unknown function. Characterizing ticks using the transcriptomic approach has created a broad field and data

repository, which we can search for novel drugs and potential therapeutics.

AUTHOR CONTRIBUTIONS

JC and JK wrote the manuscript, JK prepared the tables, JC prepared the figures, HL and MK edited and revised the manuscript.

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3.3. Manuscript 3:

Kotál, J., Stergiou, N., Buša, M., Chlastáková, A., Beránková, Z., Řezáčová, P., Langhansová, H., Schwarz, A., Calvo, E., Kopecký, J., Mareš, M., Schmitt, E., Chmelař, J., and Kotsyfakis, M. (2019) The structure and function of Iristatin, a novel immunosuppressive tick salivary cystatin. Cellular and molecular life sciences : CMLS 76, 2003-2013

A research Manuscript 3 focuses on characterization of an immunomodulatory cystatin from *I. ricinus* saliva. Tick saliva is rich in protease inhibitors that regulate host immune response to tick feeding. We present a novel secreted tick salivary cystatin, that we named Iristatin. Iristatin transcripts are upregulated in tick salivary glands during tick feeding. Recombinant Iristatin is an inhibitor of cathepsins C and L, while it did not affect other tested cysteine or serine proteases. More importantly, Iristatin showed a broad spectrum of immunomodulatory effects. It suppressed production of pro-inflammatory cytokines IFN-γ and IL-2 by stimulated Th1 cells, anti-inflammatory cytokine IL-4 by Th2 cells, IL-2 and IL-9 by Th9 cells and IL-9 and IL-6 production by mast cells. Iristatin further inhibited CD4+ T cells proliferation and leukocyte recruitment. Finally, we show a crystal structure of Iristatin. Iristatin is a potent immunosuppressor and its characterization can help us better understand the tick-host interaction.

Jan Kotál is the first author, designed and performed experiments, performed the analyses and wrote the manuscript.

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ORIGINAL ARTICLE

The structure and function of Iristatin, a novel immunosuppressive tick salivary cystatin

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Abstract
To successfully feed, ticks inject pharmacoactive molecules into the vertebrate host including cystatin cysteine protease inhibitors. However, the molecular and cellular events modulated by tick saliva remain largely unknown. Here, we describe and characterize a novel immunomodulatory cystatin, Iristatin, which is upregulated in the salivary glands of feeding *Ixodes ricinus* ticks. We present the crystal structure of Iristatin at 1.76 Å resolution. Purified recombinant Iristatin inhibited the proteolytic activity of cathepsins L and C and diminished IL-2, IL-4, IL-9, and IFN-γ production by different T-cell populations, IL-6 and IL-9 production by mast cells, and nitric oxide production by macrophages. Furthermore, Iristatin inhibited OVA antigen-induced $CD4^+$ T-cell proliferation and leukocyte recruitment in vivo and in vitro. Our results indicate that Iristatin affects wide range of anti-tick immune responses in the vertebrate host and may be exploitable as an immunotherapeutic.

Keywords Cathepsin · Crystal structure · Immune responses · *Ixodes ricinus* · Saliva

Introduction

Ticks are obligatory ectoparasites that feed on the blood of their vertebrate hosts. Hard ticks (family Ixodidae) feed continuously for days to weeks during each life stage, so must overcome specific host antigen-specific immune responses, non-specific innate responses, and hemostasis to successfully finish their blood meal [1]. The hard tick *Ixodes ricinus* is an important arthropod disease vector of several pathogens in Europe [1]. To counteract vertebrate host anti-tick responses, *I. ricinus* secretes saliva rich in biomolecules that facilitate tick feeding and pathogen transmission [2].

Tick saliva affects blood coagulation, complement activation, and immune reaction in terms of immune cell recruitment, cytokine production, and cell maturation [2]. It also facilitates the transmission of *Borrelia*, *Anaplasma*, *Rickettsia*, and various viruses to the vertebrate host [2]. Protease inhibitors are an important group of tick salivary effectors that are divided according to specificity into serine and cysteine protease inhibitors, and according to structure into Kunitz domain inhibitors, serpins, cystatins, and other less abundant families [1]. Kunitz inhibitors are thought to be mainly anti-hemostatic, serpins both anti-hemostatic and

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immunomodulatory, and cystatins mainly anti-inflammatory and immunosuppressive [1, 3].

Cystatins are tight binding, reversible legumain, and papain-like cysteine protease inhibitors [4]. Cystatins are subdivided into three subfamilies according to the MEROPS nomenclature: I25A (type 1, stefins), I25B (type 2 and type 3, kininogens), and I25C (type 4, fetuins) [5]. Only type 1 and type 2 cystatins have, thus, far been identified in ticks [6]. Type 1 cystatins lack a signaling peptide for secretion and are, therefore, thought to regulate intracellular blood digestion in the tick midgut, while type 2 cystatins are secreted and expressed in both tick salivary glands and the midgut, and are, therefore, thought to play pleiotropic roles in both ticks and vertebrate hosts [6]. For example, sialostatin L, a cystatin identified in the hard tick *Ixodes scapularis*, inhibits cathepsins C, L, S, V, X, and papain, modulates cytokine production by lymphocytes, dendritic cells, and mast cells, and impairs T-cell proliferation [7, 8]. A similar cystatin in *I. scapularis*, sialostatin L2, inhibits cathepsins C, L, S, and V [9], diminishes IL-1β and IL-18 secretion by macrophages, and inhibits caspase-1 maturation [10]. Furthermore, both sialostatins alter dendritic cell signaling [11], and inhibition of sialostatin by RNA interference and immunization of guinea pigs impairs tick feeding [9, 12, 13]. Therefore, salivary cystatins may be useful targets for anti-tick vaccines.

However, until the first *I. ricinus* genome was released [14], tick genomic and proteomic studies have been hampered by a lack of full genomic sequences. Here, we report the structural and functional characterization of a novel type 2 cystatin in the hard tick *I. ricinus*, which we name Iristatin. We present the crystal structure of Iristatin, which inhibits the vertebrate cathepsins C and L. Furthermore, we report the anti-inflammatory and immunomodulatory activities of Iristatin. Rather than being target specific, Iristatin appears to affect many immune mechanisms and is a broad-spectrum immunosuppressor that may be useful in the treatment of immune-mediated diseases.

Materials and methods

Quantitative real-time PCR

Female *I. ricinus* ticks were fed on rabbits for 1, 2, 4, 6, or 7 days. Tick salivary glands were dissected, and total RNA isolated and transcribed to cDNA for quantitative analysis of Iristatin by qRT-PCR. Expression profiles were normalized to ferritin mRNA, the levels of which are independent of blood feeding [15]. Detailed methods can be found in the supplement, and the primer and probe sequences are in Supplementary Table S3.

Crystallization, data collection, and structure determination

Screening for crystallization conditions was performed using the JCSG-plus kit (Molecular Dimensions Ltd., Newmarket, UK) and the sitting drop vapor diffusion technique. Preliminary crystals were obtained in 0.1 M Bis–Tris, pH 5.5, 1 M ammonium sulfate, 1% PEG 3350. Optimal Iristatin crystals were prepared at 18 °C using the hanging drop vapor diffusion technique in 15-well NeXtal plates (Qiagen, Hilden, Germany). Experiments, crystal parameters, data collection statistics, and structure determination are detailed in the Supplementary Methods and in Supplementary Table S2.

Enzyme assays

Iristatin inhibition constants against various proteases were determined by measuring the loss of enzymatic activity in the presence of increasing Iristatin concentrations, the corresponding enzyme and a fluorogenic substrate. The enzymes tested were: human liver cathepsin B (BiomolGmBH, Hamburg, Germany); human recombinant cathepsins C, L, S (Calbiochem, Merck Millipore, Burlington, MA, USA); human cathepsin G (Molecular Innovations Inc., Novi, MI, USA); and human factor Xa (Calbiochem). *I. ricinus* legumain IrAE [16] was kindly provided by Daniel Sojka, Ph.D. Experimental details are provided in the Supplementary Methods.

Measurement of cytokine production

T cells were differentiated as follows. For Th1 T cells, naïve $CD4^+$ T cells from BALB/c mice were stimulated with anti-CD3/CD28 (4 μg/ml each) under Th1-skewing conditions (IL-12, anti-IL-4). For Th2 cells, naïve $CD4^+$ T cells were stimulated with anti-CD3/CD28 (4 μg/ml each) under Th2-skewing conditions (IFN-γ, anti-IL-4). For Th9 cells, naïve $CD4^+$ T cells from BALB/c mice were stimulated with anti-CD3/CD28 (4 μg/ml each) under Th9-skewing conditions (IL-4, TGF-β, anti-IFN-γ). Fully differentiated cells were then re-stimulated solely with plate-bound CD3 mAb for 48 h in the presence or absence of 6 μM LPS-free Iristatin. IL-2, IL-4, IL-9, and IFN-γ production were determined by Ready-SET-Go! ELISA (eBioscience, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Bone marrow-derived mast cells (BMMCs) were stimulated with ionomycin (Iono, 0.75 μM) in the presence or absence of 6 μM Iristatin. IL-4, IL-6, and IL-9 production was determined by ELISA after 48 h of stimulation.

For viability screening, naïve $CD4^+$ T cells from BALB/c mice were stimulated with anti-CD3/CD28 (4 μg/ml each) in the presence or absence of different concentrations of LPS-free Iristatin (6, 3, 1.5, and 0.75 μM) under Th9-skewing conditions for 72 h. T cells were stained with a fixable viability dye and cell viability determined by flow cytometry.

Nitric oxide (NO) measurement

Macrophages of the PMJ2-R cell line were preincubated for 4 h with 3 and 6 μM Iristatin and then stimulated by adding LPS to final concentration 100 ng/ml and IFN-γ to final concentration 5 ng/ml. NO concentration was assessed 24 h after stimulation with modified Griess reagent (Sigma-Aldrich, St. Louis, MO, USA).

OVA antigen-induced proliferation of $CD4^+$ splenocytes

OT-II mouse spleens were disintegrated through a 70 μm cell strainer to obtain a single-cell suspension, and red blood cells were removed using RBC lysis buffer (eBioscience). Splenocytes were then stained with eFluor™ 670 cell proliferation dye (eBioscience). Stained splenocytes were seeded in a 96-well plate (5×10^5 cells in 200 μl complete RPMI) and preincubated for 2 h in the presence or absence of 3 μM Iristatin. Cells were then stimulated using ovalbumin (OVA) peptide 323–339 (100 ng/ml; Sigma-Aldrich), and splenocytes were incubated for 72 h at 37 °C in 5% CO_2. Cells were then stained with FITC-labeled anti-CD4 antibody and propidium iodide and analyzed by flow cytometry on a BD FACSCanto™ II using BD FACSDiva™ Software v. 6.1.3.

Thioglycollate-induced peritonitis

Female C57BL/6N mice were purchased from Velaz (Prague, Czech Republic). Mice were housed in individually ventilated cages maintained in a 12 h light/dark cycle and given a standard pellet diet and water ad libitum. All animals were used at 8–12 weeks of age. All experiments were approved by the local ethical committee and the Ministry of Education and Sports in accordance with law 246/1992 Sb (ethical approval number MSMT-19085/2015-3).

Control group mice were injected intraperitoneally (i.p.) with saline (10 ml/kg of body weight) and, 1 h later, acute peritonitis was induced by i.p. injection of 200 μl 3% sterile, fully oxidized Difco™ thioglycollate medium (BD Biosciences, Franklin Lakes, NJ, USA). Mice in the experimental group were first injected i.p. with Iristatin (2 mg/kg of body weight in saline). One hour later, mice were treated i.p. with Iristatin (2 mg/kg of body weight) together with 200 μl of 3% thioglycollate medium.

Four hours after thioglycollate medium injection, mice were killed by cervical dislocation, and peritoneal cavities were washed with 10 ml cold PBS to harvest cells. Red blood cells were lysed with RBC lysis buffer (eBioscience). Collected peritoneal cells were counted using a hemocytometer and light microscope. The percentage of live myeloid cells ($CD11b^+$), neutrophils ($CD11b^+Ly\text{-}6g^+$), monocytes ($CD11b^+Ly\text{-}6c^+$), and eosinophils ($CD11b^+Siglec\text{-}F^+$) was assessed by flow cytometry (see Supplementary Methods for details). Absolute cell counts were obtained by combining flow cytometry data with cell counting under a light microscope.

Neutrophil in vitro migration assay

Neutrophils were obtained from the bone marrow of C57BL/6J mice by magnetic separation using a Neutrophil Isolation Kit (Miltenyi Biotec). Isolated neutrophils were preincubated in the RPMI medium-containing 0.5% BSA in the presence or absence of 3 μM Iristatin for 1 h at 37 °C and 5% CO_2. Cells were than seeded into the upper inserts of 3 μm pore Corning® Transwell® chambers (24-well format; Sigma-Aldrich, St. Louis, MO, USA) and allowed to migrate towards 1 μM fMLP in RPMI with 0.5% BSA in the lower chamber. After incubation for 1 h at 37 °C and 5% CO_2, cell migration was determined by counting cells in the lower chamber using a hemocytometer.

Statistical analysis

All experiments were performed in biological triplicates. Data are presented as mean ± standard error of mean (SEM) in all graphs. Student's *t* test or one-way ANOVA was used to calculate statistical differences between two or more groups, respectively. Statistically significant results were marked: $*P \le 0.05$; $**P \le 0.01$; $***P \le 0.001$; $****P \le 0.0001$.

Results

Expression and functional and structural analyses of Iristatin, a novel tick cystatin

Consistent with the other proteins that play important roles in the tick lifecycle [9], Iristatin mRNA expression increased significantly over time in the salivary glands of *I. ricinus* ticks fed on rabbits: 15–20-fold over the first 1–4 days; 50-fold between days 4 and 6, and 80-fold by day 7, when compared with unfed ticks (Supplementary Figure S1). Iristatin (GenBank accession number KY348759) BLAST search revealed three genomic contigs (Genbank accession numbers JXMZ02144755.1; JXMZ02161024.1, and JXMZ02194599.1) [17] with 92.6%, 90.3%, and 96.4%

nucleotide identity, respectively, each representing a unique exon of a single gene. Furthermore, the search in available *I. ricinus* transcriptomes revealed 98.8% identity of Iristatin nucleotide sequence with its best match (Genbank accession number GFVZ01039973.1). Sequence analysis showed that Iristatin belongs to the cystatin superfamily [7–9, 13], specifically to a clade-containing only tick cystatins from the genus *Ixodes* (Fig. 1a). For structural and functional analyses, Iristatin was overexpressed in a prokaryotic system [7] to produce > 95% pure recombinant protein with 119 amino acids, molecular weight 13.8 kDa, and a p*I* of 7.67, as predicted (Supplementary Figure S2 and S3).

The crystal structure of Iristatin was determined by molecular replacement using the soft tick *Ornithodoros moubata* cystatin OmC2 structure as a search model and refined using data to 1.76 Å resolution (Supplementary Table S2). The orthorhombic crystal form contained two molecules in the asymmetric unit with a solvent content of 50.8%. All protein residues could be modeled into a well-defined electron density map with the exception of the first two N-terminal residues (Gly1 and Met2) and the last four C-terminal residues (Lys116 to Glu119). The final model consisted of two Iristatin molecules, each containing 114 amino acid residues. The root-mean-square deviation (RMSD) for superposition of the C_α atoms of the two molecules was 0.52 Å, a value within the range observed for different crystal structures of identical proteins. Minor structural changes were localized to loop regions exposed to solvent and/or involved in crystal contacts (residues 1–2, 47–48, 77–80, 95–96, and 111–114).

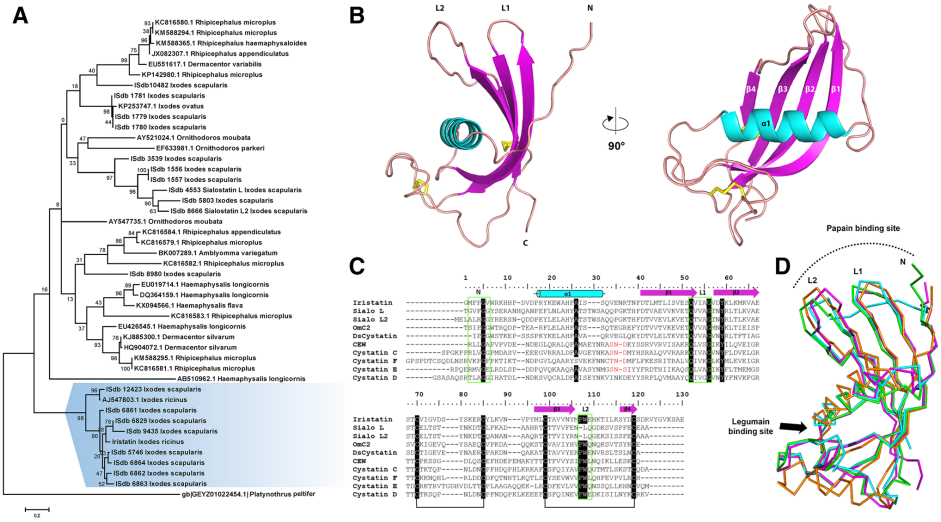

Fig. 1 Crystal structure of Iristatin and its comparison with the other family 2 cystatins. **a** Molecular phylogenetic analysis (maximum-likelihood model) of secreted tick cystatins. Iristatin clusters with the other cystatins in the genus *Ixodes* (highlighted in blue). *Platynothrus* cystatin was used as an outgroup. The tree with the highest log likelihood (−4870, 9711) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. **b** The three-dimensional structure of Iristatin is shown as a cartoon representation colored by secondary structure elements (α1, cyan; β1-4, magenta). The N- and C-termini and two disulfide bridges, Cys64–Cys76 and Cys87–Cys107 (yellow sticks), are indicated. The hairpin loops L1 and L2 and the N-terminus of cystatins are involved in the binding of papain-type peptidases. **c** Structure-based sequence alignment of Iristatin (Iris) with OmC2 (from the soft tick *O. moubata*), sialostatins L and L2 (from the hard tick *I. scapularis*), DsCystatin (from the hard tick *D. silvarum*), chicken egg-white cystatin (CEW), and representative human members of family 2 cystatins (cystatins D, C, E/M, and F). Residues identical to those of Iristatin are shaded black. The secondary structure elements of Iristatin are depicted as in **b** (magenta for strands, cyan for helices). The conserved disulfide bridges are indicated by the connecting black lines. Three regions involved in the interaction of cystatins with papain-type peptidases are boxed in green and labeled; the region size was selected based on predominant binding residues in the available complex structures. The putative legumain-binding site in four cystatins is highlighted in red. Mature sequences (i.e., without signal peptide) were used in the alignment; residue numbering is according to Iristatin. **d** A superposition of Cα traces of Iristatin with three other cystatin structures. The tick salivary cystatins Iristatin (PDB code 5O46), OmC2 (3L0R), and sialostatin L2 (3LH4) are colored magenta, green, and cyan, respectively. Chicken egg-white cystatin (1CEW) is shown in orange. Positions of the binding sites for papain-type peptidases and legumains are indicated

Figure 1b shows the overall structure of Iristatin. The molecule adopts a typical cystatin fold similar to that of the other homologs, characterized by a twisted antiparallel β-sheet wrapped around an α-helix. However, the Iristatin β-sheet is four-stranded instead of five-stranded, lacking the N-terminal β-strand. Iristatin contains two conserved disulfide bridges connecting Cys64 with Cys76 and Cys87 with Cys107. The structure-based sequence alignment demonstrated that Iristatin displays all the characteristics of family 2 cystatins, including disulfide pattern, the Gln-Xaa-Xaa-Xaa-Gly motif, and a secretion signal removed from the mature protein (Fig. 1c) [18].

The closest structural homologs of Iristatin were salivary cystatins OmC2 from the soft tick *O. moubata* and sialostatin L2 from the hard tick *I. scapularis*; the RMSDs for Cα were 1.10 Å and 1.18 Å (without flexible N-termini) and sequence identity was 42% and 36%, respectively (Fig. 1d). Lower structural similarity was found with vertebrate family 2 cystatins, namely human cystatins C, D, F and E/M and chicken egg-white cystatin (RMSDs from 1.53 to 2.21 Å); their sequence identity with Iristatin was between 16 and 24%.

Interaction of family 2 cystatins with papain-type peptidases is mediated by three regions, the N-terminal segment and two hairpin loops L1 and L2, which form a tripartite wedge-shaped edge that binds to the enzyme active site cleft (Fig. 1d). In Iristatin, the first part of the binding site is formed by the N-terminal segment around a conserved Gly5 residue, the orientation suggesting conformational flexibility, as with the other cystatins: Gly5 can function as a hinge that allows the flexible N-terminal segment to adopt an optimal conformation for target enzyme binding. The L1 loop of Iristatin (between β1 and β2) is similar in conformation to the other cystatins, only with an isoleucine instead of valine in the conserved sequence motif Gln–Xaa–Val–Xaa–Gly (Fig. 1c). The L2 loop (between β3 and β4) is characterized in Iristatin and other cystatins, except sialostatins, by the presence of conservative Pro-Trp residues.

We next analyzed which relevant representative proteases recombinant Iristatin inhibited. As predicted by the crystal structure and the absence of the legumain-binding site localized at a critical Asn residue in Iristatin (Fig. 1c, d), there was no activity against legumain. Among papain-type peptidases, cathepsins B and S were not significantly inhibited under given experimental conditions (Supplementary Table S3). Iristatin was active only against two tested enzymes, cathepsins C and L, displaying similar sub-micromolar affinity as the *I. scapularis* cystatins sialostatins L and L2 to cathepsin C but much lower micromolar affinity to cathepsin L (Supplementary Figure S4). We did not observe any effect of Iristatin against two representative serine peptidases—cathepsin G and factor Xa (Supplementary Table S3).

Iristatin affects cytokine production by T cells and mast cells

Tick salivary cystatins are known to inhibit T-cell cytokine production [3]. To elucidate Iristatin's influence on host immunity and inflammation, we activated different immune cell populations and measured the effect of Iristatin on the production of characteristic cytokines for a given subpopulation. As predicted, Iristatin was a potent inhibitor of T-cell-derived cytokines (Fig. 2). In cell cultures, recombinant Iristatin inhibited the production of pro-inflammatory cytokines IFN-γ and IL-2 by polyclonally stimulated [CD3/CD28 monoclonal antibody (mAB)] Th1 cells after 48 h of incubation (Fig. 2a, b). Iristatin also suppressed the production of the anti-inflammatory cytokine IL-4 by Th2 cells (Fig. 2c) and IL-2 and IL-9 by Th9 cells (Fig. 2d, e). The inhibition of IL-9 may be an indirect effect, because IL-9 production is known to be IL-2 dependent [19]. Iristatin had no effect on IL-17 production by Th17 cells (Fig. 2f).

Mast cell numbers are positively correlated with resistance to tick feeding [2]. The only tick cystatin reported to have a direct effect on mast cells is *I. scapularis* sialostatin L, which indirectly reduced IL-9 expression [20]. We stimulated Iristatin pretreated mast cells with ionomycin in cell cultures and measured their cytokine production. While IL-4 production by mast cells was not affected (Fig. 2g), IL-6 levels decreased significantly (Fig. 2h) and IL-9 production was almost blocked by preincubation with 6 μM Iristatin (Fig. 2i).

Iristatin inhibits antigen-specific $CD4^+$ T-cell proliferation and impairs leukocyte recruitment in vitro and in vivo

Saliva, salivary gland extract, or even individual salivary molecules from many tick species inhibit $CD4^+$ T-cell proliferation [1]. Cystatins from *I. scapularis* [7] and *O. moubata* [21] are known inhibitors of T lymphocyte proliferation. Accordingly, we investigated whether Iristatin has similar properties in the OVA antigen-specific $CD4^+$ T-cell proliferation model using splenocytes isolated from OT-II mice. Iristatin significantly decreased dendritic cell-dependent $CD4^+$ T-cell proliferation upon OVA treatment from 88% in PBS-treated controls to 72% in the Iristatin-treated group (Fig. 3a; $p \leq 0.05$) without affecting cell viability (Fig. 3b).

We next investigated whether Iristatin alters inflammatory responses in vivo in a mouse model of thioglycollate-induced peritonitis. Iristatin significantly impaired recruitment of total immune cells to the peritoneum (Fig. 4a) without affecting the proportion of living cells (Fig. 4b), excluding the possibility of Iristatin cytotoxicity. When individual cell populations were examined, Iristatin significantly inhibited the migration of myeloid cells and neutrophils,

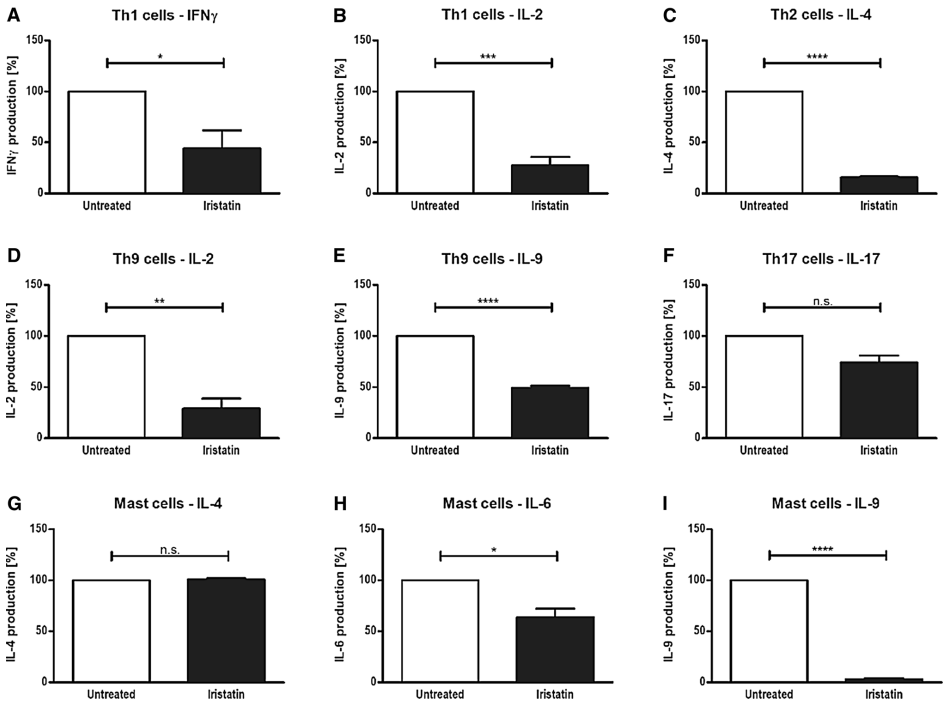

Fig. 2 The effect of Iristatin on T-cell and mast cell cytokine production. **a**–**f** Different subpopulations of T-helper cells were preincubated with 6 μM Iristatin and polyclonally stimulated with a combination of CD3 and CD28 mAbs. Cytokine levels were determined 48 h after stimulation. Th cells activated in the absence of Iristatin were used as controls and set as 100% in all experiments; all other values are expressed as percentages of these controls. Iristatin inhibited IFN-γ (**a**) and IL-2 (**b**) production by Th1 cells. **c** Iristatin strongly reduced IL-4 production by Th2 cells. Iristatin inhibited IL-2 (**d**) and IL-9 (**e**) production by Th9 cells. **f** The inhibition of IL-17 production by Th-17 cells was not significant. **g**–**i** Mast cells were pretreated with Iristatin and stimulated with ionomycin. Cytokine levels were measured 48 h after stimulation. **g** IL-4 production was not affected by Iristatin treatment. **h** 6 μM Iristatin inhibited IL-6 production by mast cells. **i** Iristatin strongly inhibited IL-9 production by mast cells. The mean of three independent experiments (±SEM) is shown in all graphs. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$; $****P \leq 0.0001$; *n.s.* not significant

and showed a trend to decreasing monocyte and eosinophil migration. Consistent with these in vivo findings, the migration of neutrophils pretreated with Iristatin was significantly less than untreated controls towards an fMLP gradient (8.4% vs. 12.1%, $p \leq 0.05$; Fig. 4g).

Finally, macrophages play an important role in the interaction between the host immune system, ticks, and transmitted pathogens. Activated macrophages are crucially involved in immune cell recruitment to sites of inflammation or towards pathogens by secreting signaling molecules such as chemokines or nitric oxide (NO) [22]. Saliva (or salivary gland extracts) from different tick species has been shown to reduce NO production by macrophages [23]. Accordingly, the incubation of monocyte/macrophage PMJ2-R cells with Iristatin reduced in vitro production of NO in a dose-dependent manner to nearly 40% of controls in the presence of 6 μM Iristatin (Fig. 4h), suggesting a considerable suppression of macrophage activation, perhaps, explaining the reduced recruitment of other immune cell types.

Discussion

Hard ticks feed for several days on their vertebrate host. To feed successfully, ticks control and evade the host immune response and maintain blood flow by secreting saliva into the feeding cavity. As *I. scapularis* cystatins are known to be strong immunomodulators [2], we focused on cloning a

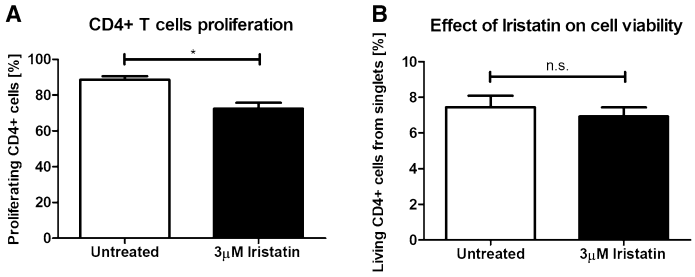

Fig. 3 Iristatin inhibits CD4$^+$ lymphocyte proliferation. **a** Splenocytes from OT-II mice were preincubated with Iristatin and subsequently stimulated with OVA peptide. The percentage of proliferating cells was evaluated after 72 h. Incubation with 3 μM Iristatin decreased the percentage of proliferating cells from 88% in the control group to 72% in the experimental group. The mean of three independent experiments (±SEM) is shown. $*P \leq 0.05$ (two-tailed, unpaired *t* test). **b** Iristatin had no effect on cell viability

cystatin from the closely related tick *I. ricinus*, which we named Iristatin. At the time of this project initiation, *I. ricinus* genome has not yet been sequenced, so the traditional cloning procedures were needed to reveal this first immunomodulatory cystatin from this important disease vector.

The three-dimensional structural analysis of Iristatin provided useful insights into its biochemistry and function. Three conserved cystatin domains mediate their specificity to papain-like proteases [4, 24], including the N-terminal domain and two hairpin loops L1 and L2. Iristatin differed by over 50% in the N-terminal domain sequence but by only one amino acid in hairpin L1 (Ile50 instead of Val) or L2 (Glu99 instead of Gln) compared to the most structurally similar tick cystatin OmC2. This hairpin loop similarity, perhaps, explains why both cystatins inhibit cathepsins C and L. Iristatin showed a major difference in affinity to cathepsin L compared to sialostatins L and L2, in which target specificity is attributed to the lack of a conserved Pro-Trp motif in hairpin L2, at least with regard to the lower affinity to cathepsin B and no increased inhibition of cathepsin L [7, 9, 25]. While the only difference between Iristatin and the sialostatins in hairpin L1 is a Val/Ile substitution, we speculate that the significant difference in cathepsin L affinity can be explained by the different N-termini of these cystatins or in structures outside the conserved domains. Similarly, both Iristatin and DsCystatin [26] inhibited cathepsin L, but differed in their affinity to cathepsins B and C. While both of these cystatins are almost identical in their L1 and L2 hairpin sequences, the difference in their inhibitory specificity probably originates in the N-terminal region or outside the conserved domains. Similar to sialostatin L, we speculate that inhibition of cathepsins C and L by Iristatin impairs the maturation of other proteases from their proenzymes by blocking the cleavage of their N-terminal propeptides by cathepsins C and L [7]. This effect could reduce granzyme activity in cytotoxic T lymphocytes and natural killer cells, tryptase and chymase in mast cells, cathepsin G, proteinase 3, and elastase in neutrophils, or impair the maturation of cathepsins D and B in various cell types [2].

Iristatin suppressed immune responses both in vitro and in vivo. *I. ricinus* saliva which has previously been shown to polarize immune responses towards the Th2 pathway [27], although saliva-driven inhibition of both Th1 and Th2 pathways has been observed in dendritic cells [28]. However, Iristatin appears to have a more general effect on vertebrate immunity. Inhibition of the production of pro-inflammatory cytokines TNF and IL-12 by the tick cystatin OmC2 or IL-1β, IFN-γ, TNF, and IL6 by DsCystatin has been described in dendritic cells [21] and macrophages [26]. Moreover, impaired T-cell production of IFN-γ by sialostatin L has also been reported [8] together with a decrease in IL-17 production, which was not observed with Iristatin. Similar to sialostatin L, Iristatin suppressed IL-2 and IL-9 production by Th9 and mast cells, respectively, suggesting that Iristatin could also have a similar inhibitory effect on experimental asthma [29]. To our knowledge, no secreted tick cystatin has been reported to inhibit both Th1 and Th2 cytokines as much as Iristatin. It has been observed in ticks and other blood-feeding parasites that whole saliva can have general immunosuppressive effects, with individual proteins inhibiting specific elements. Saliva from the *Aedes aegypti* mosquito has been reported to suppress both Th1 and Th2 cytokines [30]. In contrast, a single *A. aegypti* salivary protein, SAAG-4, potently polarized a Th2 immune response by reducing expression of the Th1 cytokine IFN-γ and upregulating the Th2 cytokine IL-4 [31]. Such immunomodulation is not limited to arthropods, as the parasitic trematode *Schistosoma japonicum* also polarized the vertebrate immune response towards Th2 upon infection, with rSjCystatin increasing IL-4 production by splenocytes [32].

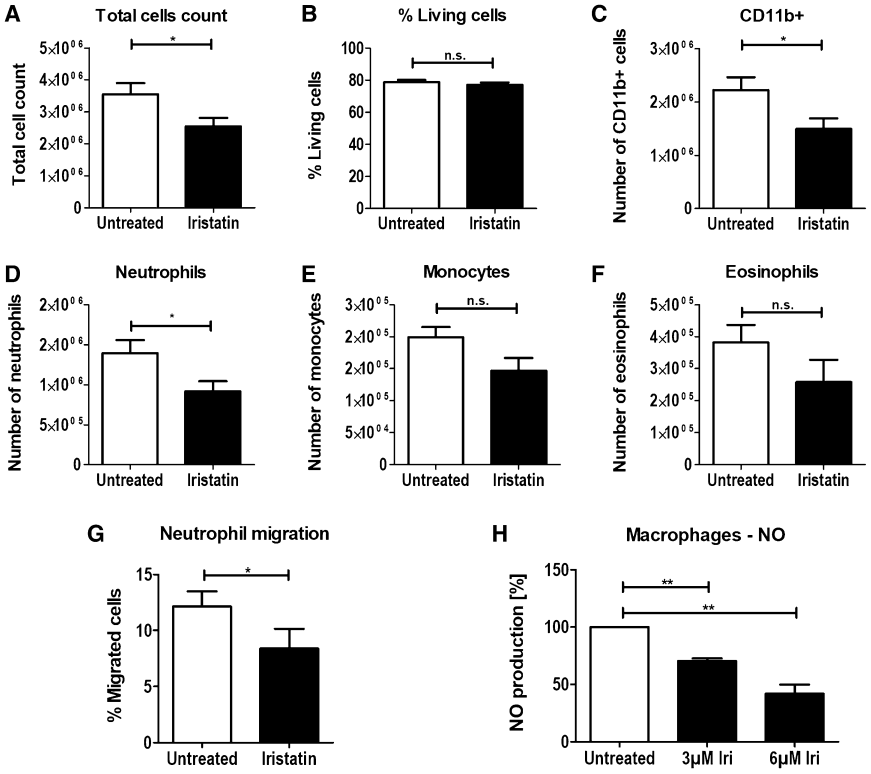

Fig. 4 Iristatin's effect on leukocyte recruitment and migration and macrophage production of NO. **a**–**f** Mice were injected with 3% TGM with saline or Iristatin. After 4 h, peritoneal lavage was performed and infiltrating cells analyzed by flow cytometry. In all figures, mice injected with TGM and saline are marked as Untreated, while all mice injected with TGM and Iristatin (2 mg/kg of mouse weight) are labeled as Iristatin. **a** Total number of cells in the peritoneum in tested mice. **b** Percentage of all living cells in mouse peritoneum. **c**–**f** Total number of all living $CD11b^+$ cells, neutrophils, monocytes, or eosinophils, respectively. **g** Mouse bone marrow neutrophils were preincubated with 3 μM Iristatin and subjected to migration towards fMLP in a Boyden chamber. **h** Iristatin inhibited NO production by PMJ2-R macrophages in a dose-dependent manner. Macrophages were preincubated with 3 and 6 μM Iristatin, stimulated with LPS and IFN-γ, and NO concentration was assessed after 24 h. The mean of three independent experiments (±SEM) is shown. $*P \leq 0.05$; $**P \leq 0.01$; *n.s.* not significant

Macrophages play a crucial role in inflammatory responses through high cytokine and NO production and represent known cystatin targets [26]. Most cystatins tend to increase macrophage NO production [33]. Conversely, rSjCystatin from *S. japonicum* [34] and Iristatin decreased macrophage NO levels. Therefore, Iristatin has a rather unique effect on NO production, which is consistent with the previous studies, showing that *I. ricinus* saliva inhibits NO production [23]. Since NO is an important regulator of many processes in various immune and inflammatory cell types, we propose that its reduction by Iristatin leads to further immunosuppression. NO can be associated with decreased T-cell proliferation [35]. Moreover, the dose-dependent differences in NO-mediated polarization of immune response have been described. Low NO levels selectively enhanced Th1 polarization, while Th1 differentiation was suppressed at higher NO levels [36]. In contrast to these studies, we observed that Iristatin decreased NO production by macrophages and also reduced T-cell proliferation; however, since both experiments were performed separately in vitro, the in vivo milieu may be different. Furthermore, we used whole splenocytes in the proliferation assays, so it is unclear

whether the observed lower $CD4^+$ cell proliferation was a direct or an indirect effect via APCs.

Finally, Iristatin suppressed immune cell recruitment to the site of inflammation. Several parasitic nematode cystatins have been reported to affect inflammatory cell migration while polarizing towards Th2 immune responses and recruiting IL-10-producing macrophages or T cells [37–39]. Conversely, cystatin C inhibited T-cell and monocyte transmigration [40]. Until now, only one tick cystatin, DsCystatin, has been reported to have an impact on immune cell recruitment in a mouse arthritis model [26]. Another tick cystatin, RHcyst-1 from *Rhipicephalus haemaphysaloides*, suppressed tumor cell migration and invasion [41]. We speculate that the Iristatin effect on cell recruitment can partially be mediated by the suppression of macrophage activation [22] as shown by NO inhibition.

In conclusion, Iristatin is a potent immunomodulator of the host immune system. Iristatin is a specific cathepsin C and L inhibitor, as evidenced by both structure and function. Iristatin attenuated both Th1 and Th2 vertebrate host immune responses and inhibited T-cell proliferation and leukocyte recruitment. Our data clearly demonstrate that individual molecules contribute differentially to the overall effect of arthropod saliva on blood feeding. Our model for the action of tick salivary immunomodulators [42] stresses the importance of pluripotency and redundancy in their action; specifically, the function of each tick salivary immunomodulator may overlap with that of another one and the same tick salivary immunomodulator may simultaneously affect different vertebrate immune system functions [42]. We speculate that synergy exists between individual salivary proteins that might increase the activity of tick saliva; however, there is currently no direct evidence for such an effect. Pluripotency and redundancy seem to be essential for potent immunomodulation by tick saliva, and Iristatin displays both of these features. These structural and functional data further increase our understanding of vertebrate host immunomodulation by tick saliva. Furthermore, these properties could potentially be exploited for the development of novel immune-related disease drugs or vaccines.

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Author contributions JKot designed and performed experiments, performed the analyses, and wrote the manuscript; NS, MB, AC, ZB, PŘ, HL, and AS designed and performed experiments; JC and MK designed experiments, performed analyses, and edited the manuscript; MM and ES designed experiments and revised the manuscript; EC and JKop revised the manuscript.

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Data availability The data set supporting the conclusions of this article is available in GenBank, accession number KY348759; PDB code 5O46.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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Supplementary Material

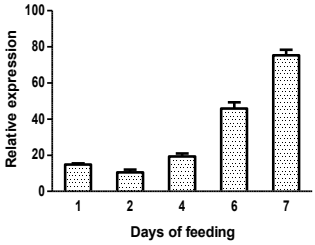

Figure S1 - *Iristatin* transcripts accumulate in *I. ricinus* salivary glands upon tick feeding. Pools of *I. ricinus* salivary glands from female ticks were dissected under RNase-free conditions. cDNA was subsequently prepared as a template for qRT-PCR. Iristatin expression was normalized to the reference gene ferritin and compared to unfed ticks. Values on the y-axis show the fold increase in transcript (unfed = 1). Each bar represents the ratio between the number of *Iristatin* transcripts in salivary glands at a specific tick feeding stage (shown on the x-axis) compared to unfed ticks. The data show an average of three biological replicates (±SEM).

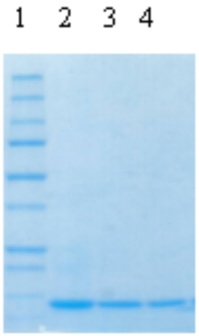

Figure S2 - Iristatin purification. Lane 1: MW standards (250, 150, 100, 75, 50, 37, 25, 20, 15, 10 kDa); Lane 2: Iristatin, load 6 µg; Lane 3: Iristatin, load 3 µg; Lane 4: Iristatin, load 1.5 µg.

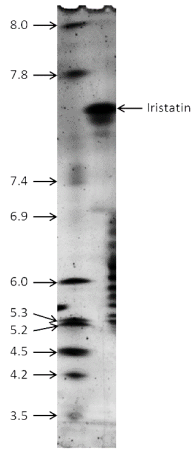

Figure S3 - Iristatin isoelectric focusing. The pI of Iristatin was between 7.6 and 7.7.

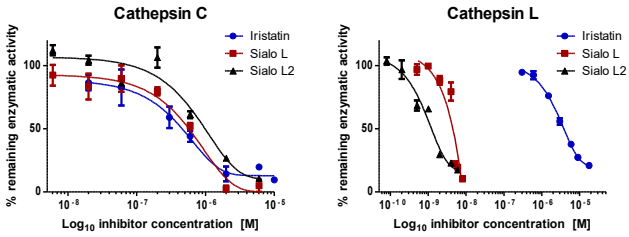

Figure S4 - Iristatin inhibits cathepsins C and L. Iristatin and sialostatins L and L2 differ in affinity for two of their common enzymatic targets cathepsins C and L. The three cystatins were allowed to interact with the same amount of enzyme under the same assay conditions. The resulting reduction in enzymatic activity was plotted against the corresponding inhibitor concentration. Each experiment was performed in triplicate.

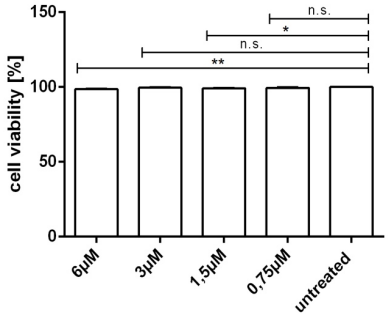

Figure S5 - Iristatin does not alter cell viability. The mean of three independent experiments (±SD) is shown. *≤P 0.05; **≤P 0.01; ***≤P 0.001, ****≤P 0.0001 (two- tailed unpaired t-test). ns=not significant.

Table S1 - Primer and probe sequences.

Amplicon name	Forward primer 5′ - 3′	Reverse Reverse primer 5′ - 3′
Iristatin full clone	ATGAGTATCGTGAAGGCAGCGC	CGGTG TCATTCAGCTGGCTTGA
Iristatin no SigP	ATCATATGTTTCCCGGGGTCTGGAGGAAGCAC	CGGTG ATCTCGAGTCATTCAGCTGACTTCA
Iristatin RT-PCR	CACACTTCGCCATCTCCTCT	GCCTC CATGTAGTTGACCACGGCTGTAC
Iristatin RT-PCR probe	YAK-TCATCAGCGTCGAGTCTCAGGTAATTGC-BBQ	

Table S2 - Crystallography data collection parameters.

Data collection statistics	
Space group	$P2_12_12_1$
Cell parameters (Å)	39.03 76.93 92.12
Number of molecules in AU	2
Wavelength (Å)	0.918
Resolution range (Å)	46.06 - 1.76 (1.82 - 1.76)
Number of unique reflections	27667 (2700)
Redundancy/multiplicity	3.7 (3.7)
Completeness (%)	97.76 (97.90)

R_{merge}[a]	0.0377 (0.79)
Average I/ σ (I)	19.32 (1.39)
Wilson B ($Å^2$)	29.42
Refinement statistics	
Resolution range (Å)	46.06 - 1.76 (1.82 - 1.76)
No. of reflections in working set	27631 (2700)
No. of reflections in test set	1381 (135)
R value (%)[b]	19.38
R_{free} value (%)[c]	25.68
RMSD bond length (Å)	0.016
RMSD angle (□)	1.71
Number of atoms in AU	2196
Number of protein atoms in AU	1922
Number of water molecules in AU	239
Ramachandran plot statistics[d]	
Residues in favored regions (%)	1.71
Residues in allowed regions (%)	97.77

The data in parentheses refer to the highest-resolution shell.

[a] $Rmerge = \Sigma_{hkl}\Sigma_i I_i(hkl) - \langle I(hkl)\rangle|/\Sigma_{hkl}\Sigma_i I_i(hkl)$, where the $I_i(hkl)$ is an individual intensity of the ith observation of reflection hkl and $\langle I(hkl)\rangle$ is the average intensity of reflection hkl with summation over all data.

[b] R-value = $||F_o| - |F_c||/|F_o|$, where F_o and F_c are the observed and calculated structure factors, respectively.

[c] R_{free} is equivalent to R value but is calculated for 5% of the reflections chosen at random and omitted from the refinement process [1].

[d] as determined by PROCHECK ([2]

Table S3 - Effect of Iristatin on protease activity

Protease	**% of remaining enzymatic activity**
Cathepsin B	98.5 ± 7.6
Cathepsin C	0 *

Cathepsin G	103.1 ± 2.8
Cathepsin L	37.7 ± 2.3 *
Cathepsin S	108.7 ± 1.1
Factor Xa	102.0 ± 3.8
Legumain	100.4 ± 1.8

Remaining enzymatic activity of tested enzymes in presence of 6 µM Iristatin. * $p \leq 0.05$

Supplementary Methods

RT-PCR and primer design

Tick salivary glands were dissected on a petri dish under a drop of ice-cold DEPC-treated PBS. Total RNA was isolated from dissected tissue using the NucleoSpinRNA II kit (Macherey-Nagel, Düren, Germany), with quality checked by agarose gel electrophoresis before storing the RNA at -80°C. cDNA preparations were made from 0.5 µg of total RNA from independent biological triplicates using the Transcriptor High-Fidelity cDNA Synthesis Kit (Roche Diagnostics, Risch-Rotkreuz, Switzerland) according to the manufacturer´s instructions. The cDNA served as templates for subsequent quantitative expression analyses of Iristatin transcription by qRT-PCR. Samples were analysed by a LightCycler 480 (Roche) using the LightCycler® 480 Probes Master kit (Roche). Relative expression was calculated using the ΔΔCt method [3]. Expression profiles were normalised to *I. ricinus* ferritin mRNA, levels of which are independent of blood feeding [4]. The primer and probe sequences for Iristatin cloning and RT-PCR are shown in **Table S1**.

Iristatin cloning, expression, refolding, and purification

The full cDNA sequence of the gene encoding Iristatin was amplified using primers designed based on the orthologous *I. scapularis* cystatin genes (**Figure 2**). The exact primer sequences used for the final cloning of Iristatin are presented in **Table S1**. *I. ricinus* cDNA prepared from the salivary glands of female ticks fed for 3 and 6 days on rabbits was used as a template. The Iristatin gene without a signal peptide and with an inserted ATG codon (360 bp) was cloned into a pET-17b vector and transformed into *Escherichia coli* strain BL21(DE3)pLysS for expression. Bacterial cultures in LB medium with 100 mg/ml ampicillin and 34 mg/ml chloramphenicol were grown to an OD600 of 0.8, and protein expression was induced by the addition of isopropyl 1-thio-β-D-galactopyranoside at a final concentration 1 mM. The cultures were harvested after 5 h of fermentation at 37°C. Following washing and recovery of inclusion bodies, DEAE Sepharose FF chromatography was performed under denaturing conditions, followed by three-step refolding by dialysis in 50 mM Tris, pH 8.5, 0.5 mM KCl, 100 mM NaCl, 50 mM betaine, 1 mM EDTA, 10% glycerol, and <0.02% Triton X114. The resulting refolded protein was purified by HiLoad Superdex 200 26/60 gel filtration chromatography and endotoxin removal was performed using a detergent-based method according to [5].

Phylogeny tree calculation

The evolutionary history of Iristatin was inferred using the maximum likelihood model based on the Dayhoff matrix-based model [6] using cDNA sequences without signal peptides. Initial tree(s) for the heuristic search were obtained by applying the neighbor-joining method to a matrix of pairwise distances estimated using a JTT model. There were 112 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [7].

Flow cytometry

Cell suspensions were incubated with rat anti-mouse CD16/CD32 monoclonal antibody (clone 93, eBioscience, Thermo Fisher Scientific, Waltham, MA) for 5 minutes on ice to block Fc gamma II/III receptors. Subsequently, cells were stained with population-specific antibodies for 30 minutes in the dark at 4°C. The following rat anti-mouse monoclonal antibodies was used: CD11b-FITC (clone M1/70, BD Biosciences, Franklin Lakes, NJ), Ly-6G-PE (clone 1A8; BD Biosciences), Ly-6C-APC (clone HK1.4; eBioscience), and Siglec-F-PerCP-Cy^{TM}5.5 (clone E50-2440, BD Pharmingen). Propidium iodide (eBioscience) was added to distinguish living and dead cells. Cell suspensions were analyzed by flow cytometry using BD FACSCanto^{TM} II and BD FACSDiva™ Software v. 6.1.3. Neutrophils were identified as CD11b^{+}Ly-6G^{+} cells, monocytes as CD11b^{+} Ly-6C^{+} cells and eosinophils as CD11b^{+}Siglec-F^{+}.

Enzymatic assays

All assays were performed in triplicate. The mean percentage of remaining enzymatic activity in the presence of various Iristatin concentrations compared to control enzymatic activity (in the absence of Iristatin) was plotted against the concentration of Iristatin used in the assays and in logarithmic scale. Finally, sigmoidal fitting of the data gave an estimate of the IC50 of Iristatin for the various enzymes.

Assay buffers were as follows: 100 mM Na-acetate, 100 mM NaCl, 1 mM EDTA, 0.01% Triton X-100, 100 µg/ml cysteine, pH 5.5 for cathepsins B, L, S; 50 mM Na-acetate, 50 mM NaCl, 5 mM DTT, pH 5.5 for cathepsin C; 20 mM Tris-HCl, pH 8, 200 mM NaCl, 5 mM $CaCl_2$, 0.1% BSA for factor Xa, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.01% Triton X-100 for cathepsin G; 50 mM Na-acetate, 100 mM NaCl, 4 mM DTT, pH 5.2 for legumain.

Enzymes were used at the following final concentration: 10 pM cathepsin B, 500 pM cathepsin C, 10 nM cathepsin G, 33 pM cathepsin L, 350 pM cathepsin S, 800 pM factor Xa.

Substrates were as follows: Z-LR-AMC, 250 µM final concentration for cathepsins B, L; Z-VVR-AMC, 250 µM final concentration for cathepsin S; H-GR-AMC, 250 µM final concentration for cathepsin C; methylsulfonyl-D-cyclohexylalanyl-GR-AMC, 250 µM final concentration for factor Xa; Suc-AAPF-AMC, 250 µM final concentration for cathepsin G; Z-AAN-AMC, 250 µM final concentration for legumain.

The substrate hydrolysis rate was followed in an Infinite® 200 PRO 96-well plate fluorescence reader (Tecan, Männedorf, Switzerland) using 365 nm excitation and 450 nm emission wavelengths with a cutoff at 435 nm.

Crystallization, data collection, and structure determination

The crystallization drop consisted of 1 µl of Iristatin protein solution (5 mg/ml in 10 mM Tris buffer, pH 8.0) and 1.5 µl of the reservoir solution (0.1 M Bis-Tris, pH 5.5, 1 M ammonium sulfate, 1% PEG 3350). Brick-shaped crystals reached their final size of 0.4 × 0.1 × 0.1 mm within 7 days. For data collection, crystals were soaked in reservoir solution supplemented with 12.5% glycerol and flash cooled in liquid nitrogen. Diffraction data were collected at 100 K using the MX-14-1 beamline at BESSY, Hamburg, Germany, and processed using the HKL-3000 suite of programs. Crystals exhibited the symmetry of space group $P2_12_12_1$ and contained two molecules in the asymmetric unit.

The phase problem was solved by molecular replacement using the program Molrep. The search model was derived from the structure of cystatin OmC2 from *O. moubata* (PDB code 3L0R), sharing 41% sequence identity with Iristatin. Model refinement was carried out using the program REFMAC 5.5 from the CCP4 package. Manual building was performed using Coot. Tight non-crystallographic symmetry (NCS) restraints were applied during initial refinement, and, in later stages, NCS restraints were loosened as guided by the behavior of Rfree. The final refinement steps included TLS refinement. The quality of the final model was validated with Molprobity. Final refinement statistics are given in **Table S2**. Figures showing structural representations were prepared with the PyMOL Molecular Graphics System (Schrödinger). Atomic coordinates and structure factors were deposited in the PDB under accession code 5O46.

Isoelectric focusing

Isoelectric focusing (IEF) was performed on precast Criterion™ IEF gels (Bio-Rad, Hercules, MA) and separated by IEF cathode buffer 2 mM lysine (free base), 2 mM arginine (free base) and IEF anode buffer 0.7 mM phosphoric acid at increased voltage modes: 100 V 60 min, 250 V 60 min, 500 V 30 min. IEF markers of pI range 3–10 were used (SERVA 39212.01).

The value of Iristatin pI predicted to be 7.67 (by https://web.expasy.org/protparam) was verified by isoelectric focusing. Iristatin showed a clear band between pI values 7.6 and 7.7 (**Figure S2**)

Iristatin shows no toxic effect on $CD4^+$ cells

Naïve $CD4^+$ T cells from Balb/c mice were stimulated with anti-CD3/CD28 (4 µg/mL) in the presence and absence of different concentrations of LPS-free Iristatin (6, 3, 1.5, and 0.75 µM) under Th9-skewing conditions for 72 h. T cells were stained with a fixable viability dye, and cell viability was determined by flow cytometry (**Figure S5**). The value of the untreated cells is considered 100%; the other values are expressed as percentages.

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3.4. Manuscript 4 (in preparation):

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A research Manuscript 4 characterizes a tick salivary serpin with a role in regulation of host hemostasis. Tick salivary serpins can have anticoagulation or immunosuppressive role. Here we report IRS-8, a serpin from *I. ricinus* saliva. We show in the primary structure of IRS-8 an unusually long reactive center loop. We biochemically characterized IRS-8 as an inhibitor of a variety of serine proteases, mostly those involved in blood coagulation. Subsequently, we have proven the anti-coagulatory effect of IRS-8 in an aPTT assay and anti-complement IRS-8 function in a rabbit erythrocyte lysis assay. IRS-8 RNA interference in ticks led to lower feeding success, but had no effect on *Borrelia* transmission. Although we could not see an effect in pathogen transmission, IRS-8 can still be considered a candidate component of a cocktail anti-tick vaccine.

Jan Kotál is the first author, designed and performed experiments, performed the analyses and wrote the manuscript.

Ixodes ricinus protein IRS-8 inhibits coagulation and complement in the host

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Abstract

Tick saliva is a rich source of anti-hemostatic, anti-inflammatory and immunomodulatory molecules that actively help the tick to finish its blood meal. Moreover, these molecules are believed to facilitate the transmission of tick-borne pathogens. Herein, we present functional and structural characterization of IRS-8, a salivary serpin from the tick *Ixodes ricinus*, a European vector of tick-borne encephalitis and Lyme disease. IRS-8 has a blood-meal induced expression, which is highest in nymphs and salivary glands of adult females, as shown by qRT-PCR profiling of different feeding stages. IRS-8 inhibited proteases involved in the coagulation and blocked intrinsic pathway of coagulation cascade *in vitro*. However, the highest inhibition rate was towards plasmin, a fibrinolytic enzyme responsible also for the regulation of inflammatory response. Moreover, IRS-8 inhibited the lysis of erythrocytes by complement and RNA interference in tick nymphs resulted in delayed feeding time. To conclude, IRS-8 is a tick serpin with conserved reactive centre loop that has strong anti-hemostatic features and may interfere with host innate immunity.

Introduction

Ticks are blood feeding ectoparasites and vectors of human pathogens, including agents of Lyme disease or tick borne encephalitis. *Ixodes ricinus* is a European tick from the family Ixodidae (hard ticks), found also in the northern part of Africa and in the Middle East[1]. *I. ricinus* ticks feed only once in each of the three developmental stages (larva, nymph, imago) and their feeding course can take more than one week in adult females[2].

In order to stay attached to the host for such extended periods of time, ticks counteract host defense mechanisms that would otherwise lead to tick rejection or death.

Insertion of tick mouthparts to host skin causes a mechanical injury that immediately triggers blood coagulation, vasoconstriction and platelet aggregation – mechanisms of hemostasis to prevent blood loss[3]. Consequently, innate immunity is activated as noted by edema formation, inflammation, cell

infiltration and itching at tick feeding site. Long term feeding and/or repeated exposures of the host to ticks activate adaptive immunity as well[4]. As an adaptation to host defense, ticks modulate and suppress host immune responses and hemostasis by secreting a complex cocktail of pharmacoactive substances via their saliva into the host. For further information on this topic, several reviews were published that describe the role of saliva and salivary components on the host[4-8].

Blood coagulation is a cascade driven by serine proteases that leads to the production of fibrin clot and thrombus. Coagulation can be initiated via extrinsic and intrinsic pathway[9]. The extrinsic pathway starts with a blood vessel injury and complex formation between activated factor VII (fVIIa) and tissue factor (TF). TF/fVIIa complex then activates factor X (fX) either directly or via activation of factor IX (fIX) which in turn activates fX. The intrinsic pathway is triggered by the activation of factor XII (fXII) via kallikrein. Activated fXII (fXIIa) activates factor XI (fXI), which next activates fIX and results in the activation of fX, followed by a common pathway that closes the coagulation process by the activation of thrombin (factor II) and the cleavage of fibrinogen to fibrin, the primary component of the clot[9, 10].

Similarly to blood coagulation, the complement cascade is based on serine proteases as well. Complement represents fast and robust defense mechanism against bacterial pathogens that are lysed or opsonized by complement to facilitate their killing by other immune mechanisms[11, 12]. Complement can be activated by three pathways – classical pathway responding to antigen-antibody complexes, alternative pathway is triggered when the C3b protein directly binds to a microbial surface and a lectin pathway that needs a lectin to bind to specific carbohydrates on pathogen surface[12]. All three pathways result in the cleavage of C3 by C3 convertases to C3a and C3b fragments. C3b then triggers a positive feedback loop to amplify complement response and opsonizes pathogen cells for phagocytosis. Together with other complement components, C3b forms C5 convertase that cleaves C5 to C5a and C5b fragments. C5b initiates the membrane attack complex (MAC) formation leading to a lysis of a target cell. C3a and C5a subunits have a role in promoting inflammation[11].

Both processes, coagulation and complement, are detrimental for a feeding tick, therefore their saliva contains many anti-coagulatory and anti-complement molecules, often from the group of protease inhibitors[13-16].

Serpins form the largest and most ubiquitous family of protease inhibitors in nature that can be found in viruses, prokaryotes and eukaryotes[17, 18]. Serpins are irreversible inhibitors with unique inhibitory mechanism and highly conserved tertiary structure[19, 20] which are classified in the I4 family of MEROPS database[21]. Similarly to other serine protease inhibitors, serpin structure contain a reactive centre loop (RCL) that serves as a bait for the protease and its sequence determines serpins inhibitory specificity[22].

Arthropod serpins have mostly homeostatic and immunological functions. They regulate haemolymph coagulation or activation of phenoloxidase system in insects[23]. Serpins from blood feeding arthropods found another use – they can modulate host immunity and hemostasis[23]. Indeed, more than 20 tick salivary serpins have been functionally characterized with described effects on coagulation or immunity[13]. However, according to numerous transcriptomic studies, total number of tick serpins is significantly higher[13]. In *I. ricinus*, at least 36 serpins were found based on transcriptomics data, but only two of them have been characterized at biochemical, immunomodulatory, anti-coagulatory or anti-tick vaccine level to date[13, 24-27].

Interestingly, there is one serpin with fully conserved RCL across various tick species[28]. Homologues of this serpin have been described in *Amblyomma americanum* as AAS19[29], *Rhipicephalus*

haemaphysaloides as RHS8[30], *Rhipicephalus microplus* as RmS-15[31], *I. ricinus* as IRS-8[26] and in other tick species, where the serpins have not been functionally characterized yet.

Herein we present the characterization of IRS-8, a salivary *I. ricinus* serpin with RCL conserved among several tick species. We show its inhibitory specificity for serine proteases involved in coagulation and strong inhibition of intrinsic pathway of coagulation. Moreover, for the first time we report the inhibition of complement by a tick serpin.

Materials and Methods

Ticks and laboratory animals

All animal experiments were carried out in accordance with the Animal Protection Law of the Czech Republic No. 246/1992 Sb., ethics approval No. 34/2018 and protocols approved by the responsible committee of the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences (IP BC CAS). Male and female adult *I. ricinus* ticks were collected by flagging in a forest near the town České Budějovice in the Czech Republic and further kept in 95% humidity chambers with 12 h light/dark cycle at laboratory temperature. Tick nymphs were obtained from the tick rearing facility of the IP BC CAS. C3H/HeN mice were purchased from Velaz (Prague, Czech Republic). Mice were housed in individually ventilated cages with a 12h light/12h dark cycle and used at the age of 6–12 weeks. Laboratory rabbits were purchased from RABBIT CZ a.s. (Trhový Štěpánov, Czech Republic) and housed individually in cages in the animal facility of the Institute of Parasitology. Guinea pigs were bred and housed in cages in the animal facility of the Institute of Parasitology. All mammal animals were given a standard pellet diet and water ad libitum.

Gene expression profiling

I. ricinus nymphs were fed on C3H/HeN mice for 1 day, 2 days and until full engorgement (3-4 days); *I. ricinus* females were fed on guinea pigs for 1, 2, 3, 4, 6 and 8 days. Adult salivary glands, midguts and ovaries, as well as nymph whole bodies were dissected under RNAse-free conditions and total RNA was isolated using TriReagent solution (MRC). cDNA was prepared using 1 µg of total RNA from pools of ticks fed on three different guinea pigs using the Transcriptor First Strand cDNA Synthesis kit (Roche) according to manufacturer´s instructions. The cDNA was subsequently used for the analysis of *IRS-8* expression by qPCR in RotorGene 6000 cycler (Corbett Research) using Fast Start Universal SYBR® Green Master Mix (Roche). *IRS-8* expression profiles were calculated using Livak and Schmittgen mathematical model[32] and normalized to *I. ricinus* elongation factor 1α (ef1; Genbank number GU074829.1). Sequences of primers are shown in Table 1.

Table 1: List of primers

Amplicon name	Forward primer 5´ - 3´	Reverse primer 5´ - 3´	Amplicon length
IRS-8 RT-PCR	GACTCGGTTAATCCTCCTCAAC	ATGGGTACCTGGACCTTCT	123 bp
I.ricinus ef1 RT-PCR	CTGGGTGTGAAGCAGATGAT	GTAGGCAGACACTTCCTTCTG	105 bp
IRS-8 cloning	CACAGAGAACAGATTGGTGGACA AGACGAAATCAGCCAAG	GTCTCCTGAGTTCTAGAGTACTTTA TCAGAGGGCGTTGATCT	1207 bp
IRS-8 RNAi	ACTACCTGGGGCTCAATCTT	CCTGTTGCTAACCCAGTGT	401 bp
Borrelia flagellin	AGCAAATTTAGGTGCTTTCCAA	GCAATCATTGCCATTGCAGA	173 bp
Mouse β-actin	AGAGGGAAATCGTGCGTGAC	CAATAGTGATGACCTGGCCGT	138 bp
Borrelia flagellin probe	TGCTACAACCTCATCTGTCATTGTAGCATCTTTTATTTG		
Mouse β-actin probe	CACTGCCGCATCCTCTTCCTCCC		

RNA silencing and *Borrelia* transmission

Borrelia afzelii-infected *I. ricinus* nymphs were prepared as described earlier[33, 34]. A fragment of *IRS-8* gene was amplified from *I. ricinus* cDNA using primers containing restriction sites for ApaI and XbaI (Table 1; IRS-8 RNAi) and cloned into pll10 vector with two T7 promoters in reverse orientations[35]. Double stranded RNA (dsRNA) of *IRS-8*, and dsRNA of green fluorescent protein (*gfp*) used for control were synthesized using the MEGAscript T7 transcription kit (Ambion) as described previously[36]. The dsRNA (32 nl; 3 μg/μl) was injected into the haemocoel of sterile or infected nymphs using Nanoinject II (Drummond). After 3-day rest in a humid chamber at laboratory temperature, ticks were fed on C3H/HeN mice (15-20 nymphs per mouse) until full engorgement. Two weeks later the mice were sacrificed and the numbers of *Borrelia* spirochetes in ear lobe, urinary bladder, heart tissue and ankle joint were estimated by qPCR[37] and normalized to the number of mouse genomes[38] (primers and probes sequences in Table 1). The level of gene knock-down was checked by qPCR in an independent experiment.

Cloning, expression, purification

The full cDNA sequence of the gene encoding IRS-8 was amplified with primers presented in Table 1 using cDNA prepared from the salivary glands of female *I. ricinus* ticks fed for 3 and 6 days on rabbits as a template. The IRS-8 gene without a signal peptide was cloned into a linearized Champion™ pET SUMO expression vector (Life Technologies) using NEBuilder® HiFi DNA Assembly Master Mix (New England BioLabs) and transformed into *Escherichia coli* strain Rosetta2(DE3) pLysS (Novagen) for expression. Bacterial cultures were fermented in auto-induction TB medium supplemented with 50mg/l kanamycin at 25 °C for 24 hours.

SUMO-tagged IRS-8 was purified from clarified cell lysate using a HisTrap FF column (GE Healthcare) and eluted with 200mM imidazole. After the first purification, His and SUMO tags were cleaved using a SUMO protease (1:100 w/w) overnight at laboratory temperature. Samples were then re-applied to the HisTrap column again to separate tags from the native serpin. This step was followed by Ion Exchange Chromatography using HiTrap Q HP column (GE Healthcare) and by Size Exclusion Chromatography using HiLoad 16/60 Superdex 75 column (GE Healthcare) to ensure sufficient protein purity.

SDS-PAGE of complex formation

IRS-8 and proteases were incubated at 1 μM final concentrations in a buffer corresponding to each protease (please see below) for 1 hour at laboratory temperature. For assay with fVIIa, we added 1 μM tissue factor (TF). Covalent complex formation was then analyzed in a reducing SDS-PAGE followed by silver staining.

Determination of inhibition constants

Second-order rate constants of protease inhibition were measured by a discontinuous method under pseudo first-order conditions, using at least a 20-fold molar excess of serpin over protease. Reactions were incubated at laboratory temperature and were stopped for each time point by the addition of the chromogenic / fluorogenic substrate appropriate for the protease used. The slope of the linear part of fluorescence increase over time gave the residual protease activity at each time point. The apparent (observed) first-order rate constant k_{obs} was calculated from the slope of a plot of the natural log of residual protease activity over time. k_{obs} was measured for 5-6 different serpin concentrations and plotted against serpin concentration. The slope of this linear plot gave the second-order rate constant k_2. For each determination, the standard error is given.

The assay buffer was 20 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, 0.2% BSA, 0.1% PEG 8000, pH 7.4 for thrombin, fXa and fXIa; 20 mM tris, 150 mM NaCl, 5 mM $CaCl_2$, 0.1% PEG 6000, 0.01% Triton X-100, pH 7.5 for activated protein C (APC), fVIIa, fIXa, fXIIa, plasmin and chymotrypsin; 20 mM Tris, 150 mM NaCl, 0.02% Triton X-100, pH 8.5 for kallikrein; 50 mM Tris, 150 mM NaCl, 10 mM $CaCl_2$, 0.05% Brij 35.

Substrates were: 400 µM S2238 (DiaPharma) for thrombin; 400 µM S2222 for fXa (DiaPharma); 400 µM S2366 (DiaPharma) for fXIa; 250 µM Boc-QAR-AMC for fVIIa; 250 µM D-CHA-GR-AMC for fXIIa; 250 µM Boc-VPR-AMC for kallikrein, trypsin and APC; 250 µM D-VLK-AMC for plasmin; 250 µM Boc-G(OBzl)GR-AMC for fIXa.

Final concentration and origin of human proteases was the following: 2 nM thrombin (Haematologic Technologies); 20 nM fVIIa (Haem. Tech.); 20 nM TF (BioLegend); 200 nM fIXa (Haem. Tech.); 5 nM fXa (Haem. Tech.); 2 nM fXIa (Haem. Tech.); 10 nM fXIIa (Molecular Innovations); 8 nM plasma kallikrein (Sigma); 1.25 nM plasmin (Haem. Tech.); 15nM APC (Haem. Tech.); 20 pM trypsin (RnD); chymotrypsin (Merck).

Anti-IRS-8 serum production and Western blot

Serum with antibodies against IRS-8 was produced by immunization of a rabbit with pure recombinant protein as described previously[39]. Tick saliva was collected from ticks fed for 6 days on guinea pigs by pilocarpine induction as described previously[40]. Tick saliva was separated by reducing electrophoresis using NuPAGE™ 4-12% Bis-Tris gels. Proteins were either visualized using Coomassie staining or transferred onto PVDF membrane (Thermo Scientific). Subsequently, membranes were blocked in 5% skimmed milk in Tris-buffered saline (TBS) with 0.1% Tween 20 (TBS T) for 1 hour at laboratory temperature. Membranes were then incubated with rabbit anti-IRS-8 serum diluted in 5% skimmed milk in TBS-T (1:100) overnight at 4 °C. After washing in TBS-T, the membranes were incubated with secondary antibody (goat anti-rabbit) conjugated with horseradish peroxidase (Cell Signaling; 1:2000). The proteins were visualized using enhanced chemiluminescent substrate WesternBrightTM Quantum (Advansta) and detected using CCD image system (Uvitec).

Coagulation assays

All assays were performed at 37 °C using pre-heated reagents (Technoclone). Normal human plasma (Coagulation Control N) was pre-incubated with IRS-8 for 10 min prior to coagulation initiation. All assays were analyzed by Technoclone CEVERON four coagulometer.

For prothrombin time (PT) estimation, 100 µl plasma was pre-incubated with 6 µM IRS-8, followed by addition of 200 µl Technoplastin HIS solution and estimation of fibrin clot formation time. For activated partial thromboplastin time (aPTT), 100 µl plasma was pre-incubated with various concentrations of IRS-8 (94 nM – 6 µM), followed by an addition of 100 µl of Dapttin and incubation for 2 min. Coagulation was triggered by an addition of 100 µl of 25 mM $CaCl_2$ solution. Thrombin time (TT) was measured using 200 µl plasma pre-incubated with 6 µM IRS-8 for 1 min. Fibrin clot formation was initiated by an addition of 200 µl of Thrombin Reagent.

Complement assay

Fresh rabbit erythrocytes were collected in Alsever's solution from rabbit marginal ear artery. Fresh human blood serum was obtained from three healthy individuals. Erythrocytes were washed three times in an excess of PBS and finally diluted to a final 2% suspension (v/v). The assay was performed in a 96-well round bottomed microtiter plate (Nunc, Denmark). Each well contained 100 µl of 50% human serum in PBS premixed with different concentrations of IRS-8 (315 nM – 10 µM). After 10 min incubation at laboratory temperature, 100 µl of erythrocyte suspension was added. Reaction wells were observed individually under a stereomicroscope using oblique illumination and an aluminum pad, and the time needed for erythrocytes lysis was measured. When full lysis was achieved, the reaction mixture turned from opaque to transparent. Negative controls did not contain either IRS-8 or human serum. Additional controls were performed with heat-inactivated serum (56 ºC, 30 min). The experiment was performed in 3 biological replicates.

Immunological assays

Both, CD4+ T cell proliferation assay and neutrophil migration assay we performed following the protocol described by Kotal et al,[41]. Briefly, for CD4+ T cell proliferation assay, splenocytes were isolated form OT-II mice, fluorescently labelled, pre-incubated with serpin for 2 hours and their proliferation was stimulated by addition of OVA peptide. After 72 h, the cells were labelled with anti-CD4 antibody and analyzed by flow cytometry. For migration assay, neutrophils were isolated from mouse bone marrow by using immunomagnetic separation and pre-incubated with serpin for 1 hour. Cells were then seeded in a 5 µm pore Corning® Transwell® chambers and allowed to migrate towards fMLP gradient for 1 hour. Migration rate was determined by cell counting.

Statistical analysis

All experiments were performed in 3 biological replicates. Data are presented as mean ± standard error of mean (SEM) in all graphs. Student's t-test or one-way ANOVA were used to calculate statistical differences between two or more groups, respectively. Statistically significant results were marked: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; n.s. not significant.

Results

IRS-8 is predominantly a salivary protein with increasing transcription during tick feeding

Analysis of IRS-8 mRNA expression levels revealed its highest abundance in tick nymphs, where its peak was during the first day of feeding (Fig. 1A). In salivary glands, increased IRS-8 transcription

positively correlates with the length of tick feeding on its host. A similar increasing trend was observed also in tick midgut; however, the total number of IRS-8 transcripts was lower than in salivary glands. The presence of IRS-8 transcripts in ovaries was the lowest from all tested tissues/stages. Next, we performed a Western blot and confirmed IRS-8 presence in tick saliva also at the protein level (Fig. 1B). Based on these results, we proceeded with testing the role of IRS-8 in the impairment of host defense mechanisms as a component of tick saliva, although its activity in other tissues cannot be ruled out.

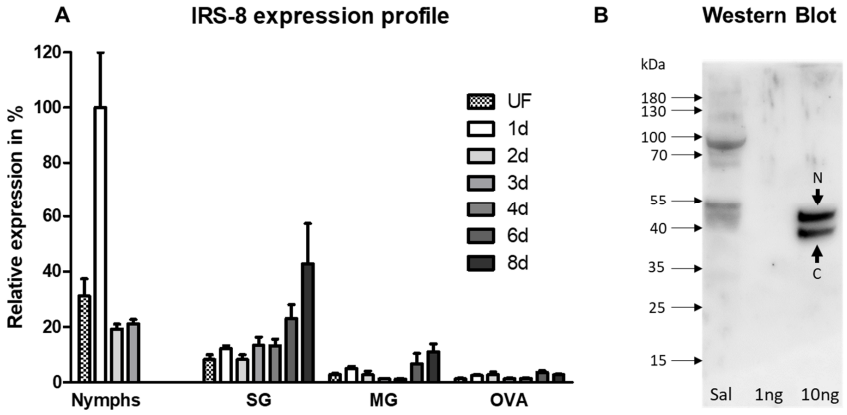

Figure 1: IRS-8 expression in ticks and its presence in tick saliva.

A Pools of *I. ricinus* salivary glands, midguts and ovaries from female ticks and whole bodies form nymphs were dissected under RNase-free conditions. cDNA was subsequently prepared as a template for qRT-PCR. IRS-8 expression was normalized to elongation factor 1α and compared among all values with highest expression set to 100% (y-axis). The data show an average of three biological replicates for adult ticks and six replicates for nymphs (±SEM). SG = Salivary glands; MG = Midgut; OV = Ovaries; UF = Unfed ticks; 1d, 2d, 3d, 4d, 6d, 8d = ticks after 1, 2, 3, 4, 6 or 8 days of feeding. In nymphs, the last column represents fully fed nymphs.

B IRS-8 can be detected in tick saliva by Western blot. Saliva from ticks after 6 days of feeding and IRS-8 recombinant protein were visualized by Western blot using serum from naïve and IRS-8 immunized rabbit. Sal = tick saliva; 1ng, 10ng = IRS-8 recombinant protein at 1 ng and 10 ng load. N: Native IRS-8, C: Cleaved IRS-8

Sequence analysis and production of IRS-8

Full sequence of IRS-8 was obtained using cDNA from tick salivary glands. Following sequencing, we found four amino acid mutation (K10 → E10, L36 → F36, P290 → T290 and F318 → S318) compared to the sequence of IRS-8 published as a supplement of our previous work[26] (Genbank No. DQ915845.1; ABI94058.1). The RCL was identical to other homologous tick serpins[30], with arginine at the P1 position.

IRS-8 was expressed in 2 liters of medium with a yield of 45 mg of protein at >90% purity, as analyzed by pixel density analysis in ImageJ software; where a majority is formed by the native serpin and a

fraction by a serpin cleaved in its RCL (Supplementary Figure 1). Proper folding of IRS-8 was verified by CD spectroscopy (supplementary data) and subsequently by activity assays against serine proteases as shown below. IRS-8 recombinant protein solution was tested for presence of LPS, resulting in 0.038 endotoxin unit/ml, which is below the threshold of pyrogenic effect[42, 43].

IRS-8 inhibits serine proteases involved in coagulation

Based on sequence analysis of IRS-8 and arginine in P1 position, we focused mainly on analyzing its specificity towards serine proteases related to blood coagulation. Considering unique serpin mechanism of inhibition, we analyzed by SDS-PAGE, whether IRS-8 forms covalent complexes with selected proteases. Figure 2 shows complex formation between IRS-8 and 10 out of 11 tested proteases – thrombin, fVIIa, fIXa, fXa, fXIa, fXIIa, plasmin, APC, kallikrein and trypsin. We did not detect any complex formation with chymotrypsin. All inhibited proteases were also able to partially cleave IRS-8 molecule as indicated by a C-terminal fragment and a stronger signal of cleaved serpin molecule. Chymotrypsin cleaved IRS-8 in its RCL completely. Subsequently, inhibition rates of IRS-8 against these proteases were determined and are shown in Table 2. Among tested proteases, plasmin was inhibited significantly stronger than other proteases with *k2* over 200 000 mol^{-1} s^{-1}. Trypsin, kallikrein, fXIa and thrombin were inhibited with *k2* at a range of tens thousands, the rest of proteases with lower *k2*. The *k2* for IRS-8 and fIXa was not estimated due to the need of use too high serpin concentration. Incubation of fXIa with 10 fold excess of IRS-8 for 30, 60 and 90 minutes resulted in 26.7±2.7%, 30±7.6% and 39.9±6.1% decrease in fIXa activity.

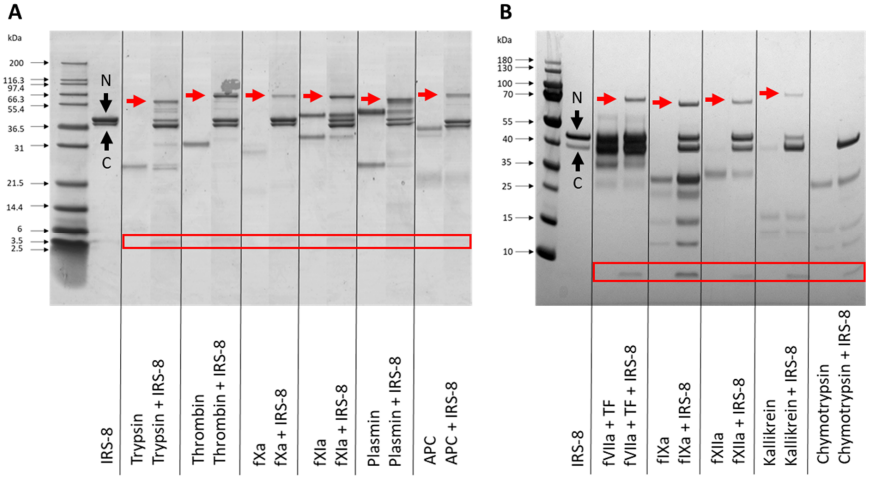

Figure 2: Formation of covalent complexes between IRS-8 and serine proteases

IRS-8 and selected serine proteases were incubated for 1 hour and subsequently analyzed for complex formation by reducing SDS-PAGE. Protein separation differs between A and B due to use of gels with different polyacrylamide content. Gels show the profile of IRS-8 serpin alone; various serine proteases alone; and proteases incubated with IRS-8. Complex formation between fVIIa and IRS-8 was tested at presence of tissue factor at equimolar concentration (TF). Covalent complexes between IRS-8 and

protease are marked with a red arrow. Cleaved IRS-8 C-terminal is marked with a red rectangle. N: Native IRS-8, C: Cleaved IRS-8

Table 2: Inhibition rate of IRS-8 against 9 selected serine proteases

Protease	k_2 [mol^{-1} s^{-1}]	± SE
Plasmin	225064	14183
Trypsin	29447	3508
Kallikrein	16682	1119
fXIa	16328	948
Thrombin	13794	1040
fXIIa	3324	409
fXa	2088	115
APC	523	35
fVIIa + TF	456	35
fIXa	N.A.	N.A.

Anti-complement activity of IRS-8

The complement readily lyses erythrocytes from various mammals, and those from rabbits were found to be the best complement activators[44]. We used human serum and rabbit erythrocytes to test the effect of tick protease inhibitors on the activity of human complement in vitro. Since complement cascade is driven by serine proteases, we tested a potential effect of serpin IRS-8 as a complement regulator. We could see a statistically significant reduction in complement activity against erythrocytes, when incubating human plasma with IRS-8 at concentrations 2.5 µM and higher (Fig. 3A). Erythrocytes were fully lysed after 7.57 ± 0.12 s in the control group.

IRS-8 inhibits intrinsic coagulation pathway

Following the results from *in vitro* inhibition of coagulation proteases by IRS-8, we tested its activity in three coagulation assays. Prothrombin time (PT) assay simulates the extrinsic pathway of coagulation, activated partial thromboplastin time (aPPT) represents the intrinsic pathway and thrombin time (TT) evaluates the final step of clot formation, in which fibrinogen is cleaved to fibrin. IRS-8 had no significant effect on PT and TT, where it increased PT from 15.3 to 16.7 s and TT from 17.6 to 18.4 s, when using 6 µM serpin (not shown). IRS-8 extended aPTT in a dose dependent manner with statistically significant increase already at 375 nM. When using 6 µM IRS-8, aPTT was delayed more than four times from 31.8 ± 0.4 s to 167.9 ± 3.2 s (Fig. 3B).

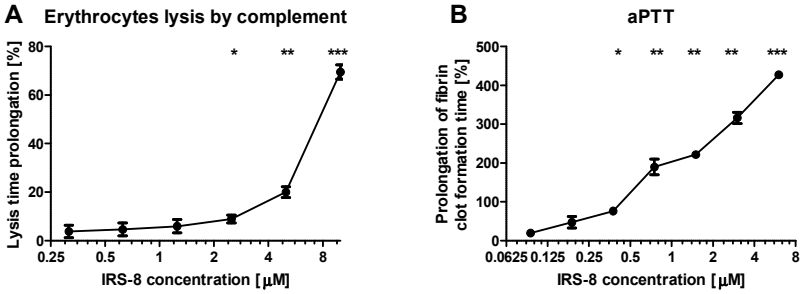

Figure 3: Inhibition of complement and coagulation pathways by IRS-8

A IRS-8 inhibits erythrocytes lysis by human complement. Human plasma was pre-incubated with increasing concentration of IRS-8 (315 nM – 10 μM). After addition of rabbit erythrocytes, their lysis time by complement was estimated.

B IRS-8 inhibits intrinsic coagulation pathway. Human plasma was pre-incubated with increasing concentration of IRS-8 (94 nM – 6 μM). Coagulation was triggered by addition of Dapttin reagent and $CaCl_2$ and clot formation time was measured.

Both graphs show time prolongation in percent compared to a control group without IRS-8, in which there was no time prolongation.

IRS-8 transcription knockdown has an effect on tick feeding but not on *Borrelia* transmission

Based on IRS-8 strongest transcription in tick nymphs (Fig. 1A), we decided to investigate its importance for tick feeding by RNA interference (RNAi) in nymphal stage. Knock-down was successful with 87% efficiency in transcripts down-regulation. Ticks with down-regulated IRS-8 expression showed lower feeding success rate and higher mortality, when 51.0% finished feeding, compared to 94.1% in control group. We have also detected a longer feeding time compared to control nymphs (Fig. 4A). Despite this promising phenotype, we did not observe any effect of IRS-8 RNAi on weight of fully engorged nymphs (Fig. 4B) or on *B. afzelii* transmission from infected nymphs to mice in any of tested mouse tissues (Fig. 4C).

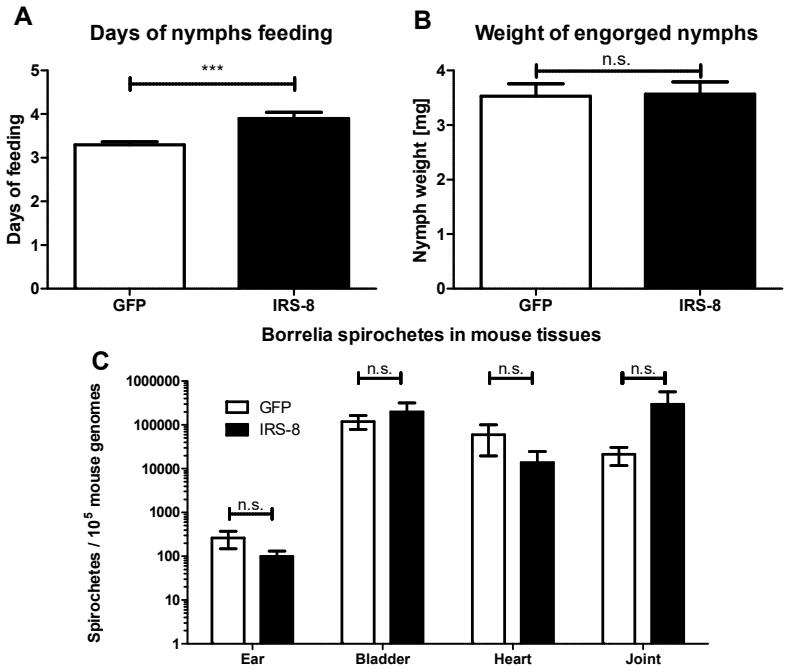

Figure 4: Effect of RNAi on tick fitness and *Borrelia* transmission

A RNAi of IRS-8 had prolonged length of *I. ricinus* nymphs feeding course when compared to the control group (GFP)

B Weight of fully engorged nymphs with IRS-8 knock-down was not different from the control group (GFP).

C Presence of *B. afzelii* spirochetes in mouse tissues after being infested by infected *I. ricinus* nymphs. We did not detect any significant difference between IRS-8 knocked-down and GFP control groups in any of tested tissues.

Role of IRS-8 in modulating host immunity

Next we evaluated a possible role of IRS-8 in modulation of host immune response to tick feeding. We tested the effect of IRS-8 in two assays, where we could see some effect with another *I. ricinus* salivary serpin Iripin-3 (unpublished data) and tick salivary cystatin Iristatin[41]. We investigated properties of IRS-8 in the OVA antigen-specific CD4+ T cell proliferation model using splenocytes isolated from OT-II mice and we tested its effect on neutrophils migration towards their attractant fMLP. However, we did not observe any inhibition by IRS-8 in either of the assays (Supplementary Figure 3).

Discussion

Similarly to other characterized tick salivary serpins[13], IRS-8 can modulate host defense mechanisms and therefore can facilitate tick feeding. Moreover, in accordance with other serpins possessing anti-coagulatory activity[45], IRS-8 has Arg in its P1 position. In combination with its unusually long RCL

and a possibility of P1 variability, this makes IRS-8 a molecule with a potential effect beyond the inhibition of coagulation. We characterized IRS-8 as an inhibitor of at least 10 serine proteases. The inhibition of kallikrein, thrombin, fVIIa, fIXa, fXa, fXIa and fXIIa can be directly correlated to their interference with blood coagulation cascade[46]. The disruption of this process would be highly beneficial for the ticks[3]. Apart from the role of trypsin in meal digestion, it has also been linked to skin inflammation[47, 48]. Therefore, the inhibition of trypsin could be another tick mechanism, how to evade host immune system. From all tested proteases, IRS-8 showed greatest inhibition of plasmin, known for fibrin degradation and clot removal[49], a process that actually should be beneficial for ticks. However, it is not fully understood, whether fibrin clot formation occurs at tick feeding site at all with the excess of anti-coagulatory molecules. Apart from fibrinolysis, plasmin participates also in modulation of several immunological processes. Plasmin interacts with leukocytes, endothelial cells, extracellular matrix or immune system factors[49-51]. Excessive plasmin generation can even lead to pathophysiological inflammatory processes[50]. Considering the pro-inflammatory role of plasmin, its inhibition by tick salivary serpin could be more beneficial for the tick than unimpaired fibrinolysis. Although we did not see any effect of IRS-8 in two immune assays, we cannot exclude the possibility of another immunomodulatory effect of IRS-8.

The anti-complement activity of tick saliva or its protein components has been known for decades and described in numerous publications[14, 15, 52-54]. Although the active molecules originate either from unique tick protein families[55-60] or lipocalins[16], an anti-complement activity has never been reported for tick salivary serpins or even other tick protease inhibitors. Since complement products might directly damage the tick hypostome or initiate a stronger immune response[11], we suggest that the role of IRS-8 is in attenuating these mechanisms. At the same time, impaired complement system cannot effectively fight pathogens entering the wound at the same time as tick saliva[14]. In this context, we wanted to test a potential effect of IRS-8 transcriptional down-regulation on *Borrelia* transmission from ticks to the host. Although we saw some effect of RNA interference (RNAi) on tick fitness, the amount of *Borrelia* in host tissues was not significantly lower. Such a result can be explained by redundancy of tick salivary molecules, as ticks secrete a variety of effectors against the same host defense mechanism and knock-down of one molecule can be supplemented by the activity of others[61]. Furthermore, increased tick mortality after IRS-8 knockdown can be connected to a potential role of IRS-8 within the tick body. As an anti-coagulant, IRS-8 can help to keep the ingested blood in tick midgut in an unclotted state to be later available for intracellular digestion[62-64]. A similar principle has previously been suggested for midgut serpins from various tick species[65]. The function of IRS-8 can also possibly be in hemolymph clotting[66, 67] or reproduction and egg development[68, 69].

In comparison of IRS-8 with other members of a tick serpin group with identical RCL, we were able to confirm the anti-coagulatory property reported also for AAS19[29] and RmS-15[31]. RNAi or IRS-8 led to lower feeding success, while RNAi of AAS19 resulted in decreased blood intake and morphological deformation of ticks[70] and RNAi of RHS8 had effect on body weight, feeding time and vitellogenesis[30]. However, these findings are difficult to correlate due to using different tick species and life stages. Although IRS-8 was detected in tick saliva and should therefore play role in regulation of host defense mechanisms, experiments, focused defining IRS-8 function in other tick tissues can bring interesting results. Similarly to AAS19[70], a possible role of IRS-8 in tick body can be regulation of hemolymph clotting, which is naturally regulated by serpins[65]. IRS-8 can also contribute to maintaining ingested blood in tick midgut in an unclotted state to stay available for intracellular digestion[62, 64].

We conclude that tick serpin IRS-8 is secreted into the host as a component of *I. ricinus* saliva. Its main role is in the modulation of host blood coagulation and complement activity, with a possible function in regulating immunity. Based on the its inhibitory activity, aimed mainly at proteases of coagulation cascade[46], we suggest that IRS-8 inhibits host defense mechanisms against tick feeding.

A more detailed comparative study of tick serpins with conserved RCL, in which IRS-8 belongs, might shed some light on their role in different tick species or reveal a potential in development an anti-tick vaccine.

Acknowledgement

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Authorship

JK: designed and performed experiments, performed the analyses, and wrote the manuscript; SGIP, HL, ME, LAM, ZB: designed and performed experiments; JAH, JC: designed experiments, performed analyses, and edited the manuscript; MK: revised the manuscript.

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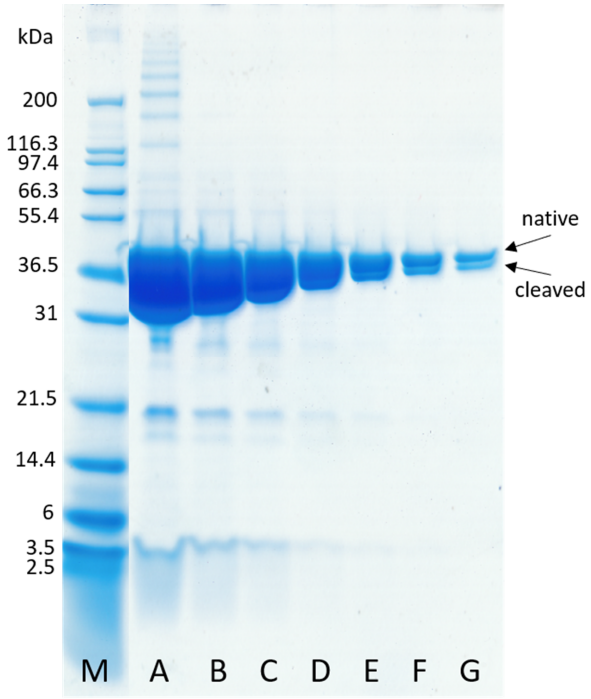
Supplement

Sequence analysis and production of IRS-8 – supplement



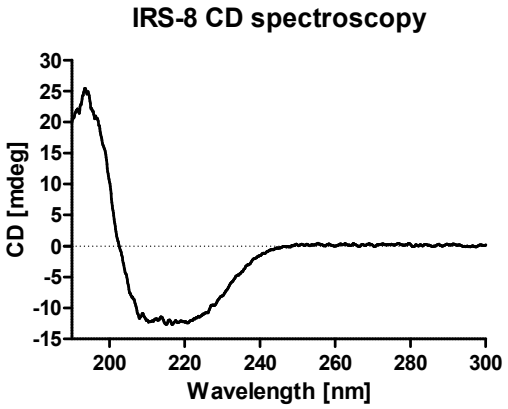
Supplementary Figure 1: Analysis of IRS-8 purity by SDS-PAGE

IRS-8 was analyzed by a reducing SDS-PAGE gel. M: Molecular weight marker, A-G: IRS-8 with load of 50, 25, 12.5, 6.2, 3.1, 1.55, 0.8 µg per well. Arrows show IRS-8 in its native and cleaved state.

Circular dichroism (CD) spectroscopy

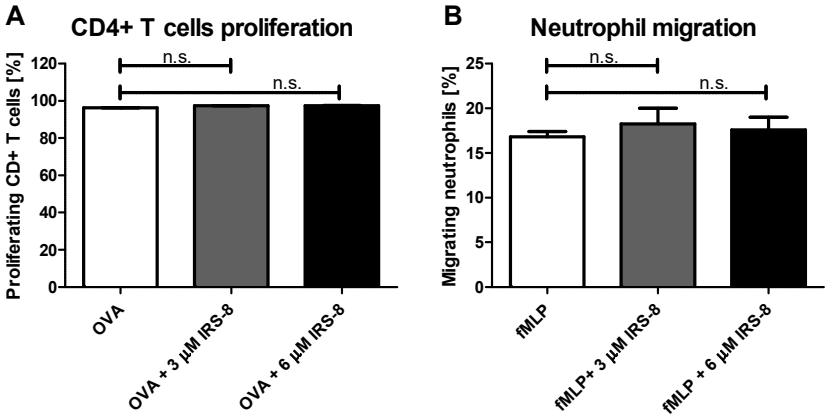
In order to verify a correct fold of IRS-8 recombinant protein, we performed CD spectroscopy analysis. Prior to analysis, buffer of IRS-8 solution was exchanged for 20mM NaH_2PO_4, 150mM NaF, pH 7.4 CD spectra were obtained using JASCO J-810 spectropolarimeter at 22 °C at wavelengths ranging from 190 to 300 nm using a 0.1 mm path-length cuvette.

As shown in Supplementary Figure 2, IRS-8 displays properties typical for serpins secondary structure[71, 72].



Supplementary Figure 2: CD spectrogram of IRS-8

Role of IRS-8 in modulating host immunity – supplement



Supplementary Figure 3: Effect of IRS-8 on T cells proliferation and neutrophil migration

A Splenocytes from OT-II mouse were pre-incubated with IRS-8 and stimulated by OVA peptide. Percentage of proliferating CD4+ T cells was evaluated after 72 hours by flow cytometry.

B Mouse primary bone marrow neutrophils were pre-incubated with IRS-8 and subjected to migration towards fMLP in a Boyden chamber. Figure shows percent of neutrophils that migrated from an insert with 3 µm membrane to compartment with fMLP.

Both experiments were performed in three biological replicates.

4. Conclusion

Despite the effort of many research teams worldwide and several patented drug candidate molecules, the number of tick-related discoveries with a potential to improve human or animal life quality is still relatively low. Except for lower general interest in ticks compared to mosquitoes or trypanosomes, this can also be explained by some specific difficulties of tick research.

The life cycle of *I. ricinus* takes generally several years in nature[4], can be, however, compressed to one year under ideal laboratory conditions. Moreover, adult female ticks feed for over a week[4], which requires an animal facility or use of an elaborate artificial feeding system[175]. Faster results can be achieved using a different species of hard ticks as a model organism, like *R. microplus* with a 5 weeks long life cycle[4]. An advantage in studying soft ticks like *O. moubata* can be in significantly shorter feeding time, taking only 30 minutes using an in vitro system[176] and avoiding the need of feeding on animals.

Tick research also still suffers from lack of modern molecular and genetic tools that are routinely available in model organisms like *Drosophila melanogaster*, *Caenorhabditis elegans* or *Plasmodium*. Currently, the only tool to manipulate gene expression in ticks is at the level of transcripts by RNAi. Applying changes to genomes by Clustered Regularly Interspaced Short Palindromic Repeats / CRISPR associated protein 9 (CRISPR/Cas9) [177], zinc finger nucleases (ZFNs)[178] or transcription activator-like effector nucleases (TALENs)[179] has not been successful in ticks yet[180]. Successful genome editing in Acari subclass was reported in a mite *Tetranychus urticae* using marker-assisted introgression[181]. Although the progress in understanding tick-host-pathogen interaction has been tremendous in the past few decades, there is still a lot left to investigate to fully understand all the complex relationships.

Studying ticks and their molecules can bring interesting results with a potential to be applied in human or veterinary medicine one day. Tick saliva is a rich and still mostly unexplored source of biologically active molecules, which have been evolving for millions of years to modulate the vertebrate immune system in the most effective way. Among these, the protease inhibitors represent a group with a strong medical potential, because many immune or hemostatic responses require proteolytic activity. Research

manuscripts included in this thesis aim to shed more light on the modulation of host immune response by specific salivary components of a tick *I. ricinus*. Presented results contribute to characterization of molecules form tick saliva with immunomodulatory or anti-hemostatic effects. We showed inhibition of specific host defense mechanisms at a molecular level by characterization of a tick salivary cystatin and a serpin and their mode of action. Iristatin - cystatin presented in Manuscript 3, is an immunomodulator suppressing both, Th1 and Th2 branches of immune response. Furthermore it has an effect on inflammation by inhibiting leukocyte migration. Serpin IRS-8 presented in Manuscript 4 is an inhibitor of complement system and intrinsic pathway of blood coagulation. RNA interference experiment with IRS-8 showed a limited potential in IRS-8 use as a vaccine candidate.
Taken together, results from the manuscripts increase our understanding of processes at the tick-host interface. More detailed characterization of above described molecules and describing more candidate molecules will bring deeper insight into the role of protease inhibitors in tick-host-pathogen interaction.

As summarized above, tick saliva has an impact on biological processes in the host, including inflammation, blood coagulation or complement activation. Protease inhibitors present in tick saliva regulate many of these processes and are therefore essential for tick feeding and for pathogen transmission. The therapeutic potential of tick salivary gland molecules is evident from literature and is therefore an interesting topic for research. In a summary, studying ticks and their proteins will not only increase our general knowledge of parasitology, but can result in discoveries beneficial for the mankind.

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6. List of abbreviations

Aam6	*Amblyomma maculatum* serine protease inhibitor 6
AAS19	*Amblyomma americanum* serpin 19
aPTT	Activated partial thromboplastin time
BmAP	*Rhipicephalus microplus* anticoagulant protein
BmGTI	*Rhipicephalus microplus* gut thrombin inhibitor
BmTI-A	*Rhipicephalus microplus* trypsin inhibitor A
BSAP1/2	BaSO4-adsorbing protein 1, 2
BTSP	Basic tail secretory protein
Cas9	CRISPR associated protein 9
CCL3/4/19	CC chemokine ligand 3/4/19
CD4+	Cluster of differentiation 4 positive
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CTL	Cytotoxic T lymphocytes
DvKPI	*Dermacentor variabilis* Kunitz-type serine protease inhibitor
fIX	Factor IX
fV	Factor V
fVIIa	Activated factor VII
fX	Factor X
fXa	Activated factor X
fXIa	Activated factor Xia
fXII	Factor XII
fXIIa	Activated factor XIIa
HA11	*Hyalomma asiaticum* 11kDa protein
HlCPL-A	*Haemaphysalis longicornis* cathepsin L-like protease
Hlcyst-1	*Haemaphysalis longicornis* cystatin 1
HLS2	*Haemaphysalis longicornis* serpin-2
HSC70	Heat shock protein 70
IAFGP	*Ixodes scapularis* antifreeze glycoprotein
IFN-γ	Interferon γ
IL-2/4/6/9	Interleukin 2/4/6/9
Ir-CPI	*Ixodes ricinus* contact phase inhibitor
Iris	*Ixodes ricinus* immunosuppressor

IRS-2	*Ixodes ricinus* serpin 2
IRS-8	*Ixodes ricinus* serpin 8
IxscS-1E1	*Ixodes scapularis* serpin 1E1
NTI1, 2	Nymphal thrombin inhibitor 1, 2
Om-cystatin 1	*Ornithodoros moubata* cystatin 1
OmC2	*Ornithodoros moubata* cystatin 2
OmCI	*Ornithodoros moubata* complement inhibitor
OspA	Outer surface protein A
OspC	Outer surface protein C
PAS	Proline-Alanine-Serine
PEG	Polyethylene glycol
RCL	Reactive centre loop
RGD	Arginine-Glycine-Aspartate
RHcyst-2	*Rhipicephalus haemaphysaloides* cystatin 2
RHS-1, 2	*Rhipicephalus haemaphysaloides* serpin 1, 2
Rmcystatin-1b	*Rhipicephalus microplus* cystatin 1b
RMS-3/15/17	*Rhipicephalus microplus* serpin 3/15/17
RNA	Ribonucleic acid
RNAi	RNA interference
RT-PCR	Real time polymerase chain reaction
SGE	Salivary gland extract
TAP	Tick anticoagulant peptide
TBEV	Tick borne encephalitis virus
TF	Tissue factor
Th1/2/9/17	T helper cells 1/2/9/17
TLSPI	Tick mannose-binding lectin inhibitor
TROSPA	Tick receptor for outer surface protein A
TSGP2/3	Tick salivary gland protein 2/3
TTI	Tsetse thrombin inhibitor
TXA2	Thromboxane

7. Curriculum Vitae

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Education
Doctoral: 2014 – now
Program: Molecular and Cell Biology and Genetics; University of South Bohemia (CZE)

Master: 2011 – 2014
Program: Experimental Biology; University of South Bohemia (CZE)

Bachelor: 2008 – 2011
Program: Biological Chemistry; University of South Bohemia (CZE) and Johannes Kepler University (AUT)

Work experience
PhD student position at Biology Centre of Czech Academy of Science and at Faculty of Science University of South Bohemia; 2014 - now

- Functional analysis of tick salivary serine and cysteine protease inhibitors

PhD internship at University of Cambridge, Department of Haematology, (head prof. James Huntington), Cambridge Institute for Medical Research, United Kingdom; 11 weeks in 2017

- Testing antihemostatic effects of tick serpins

Internship at University Hospital at the Technische Universität Dresden; Laboratory for Vascular Inflammation, (head prof. Triantafyllos Chavakis); Germany; 3 weeks in 2013, 3 weeks in 2014

- Learning host immunomodulatory assays applicable to study effects of tick feeding

Teaching and other activities

- Supervisor or co-supervisor of 3 master and 5 bachelor students
- Teaching Practicals in Immunology at Faculty of Science, University of South Bohemia
- Volunteering in Covid19 diagnostics

List of publications (chronological)

1. Klein, M., Bruhl, T. J., Staudt, V., Reuter, S., Grebe, N., Gerlitzki, B., Hoffmann, M., Bohn, T., Ulges, A., Stergiou, N., de Graaf, J., Lower, M., Taube, C., Becker, M., Hain, T., Dietzen, S., Stassen, M., Huber, M., Lohoff, M., Campos Chagas, A., Andersen, J., **Kotal, J**., Langhansova, H., Kopecky, J., Schild, H., Kotsyfakis, M., Schmitt, E., and Bopp, T. (2015) Tick Salivary Sialostatin L Represses the Initiation of Immune Responses by Targeting IRF4-Dependent Transcription in Murine Mast Cells. Journal of immunology 195, 621-631

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Conference proceedings

- XVI. Interdisciplinary Meeting of Young Biologists, Biochemists and Chemists, 2016, Milovy, Czech Republic; poster
- 34th Winter School on Proteases and Inhibitors, 2017, Tiers, Italy; oral presentation
- GRC - Proteolytic Enzymes and Their Inhibitors, 2018, Barga, Italy; poster
- International Symposium "Adaptations to Hematophagy in Blood-Feeding Parasites", 2018, Greifswald, Germany; oral presentation
- 13th International Symposium on Ticks and Tick-borne Diseases, 2019, Weimar, Germany; poster
- SERPINs 2019, 2019, Sevilla, Spain; poster
- 11th General Meeting of the International Proteolysis Society, 2019, Mariánské Lázně, Czech Republic; poster

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