University of South Bohemia

Faculty of Science

The role of tick salivary serpins in the modulation of host's immune system

Habilitation thesis

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Annotation:

Tick saliva is a rich source of pharmacoactive molecules, especially proteins. High-throughput approaches, such as transcriptomics and proteomics have been used for their identification since the beginning of the 21st century. Lists of salivary proteins were being used as a basis for detailed characterizations of individual proteins. This thesis focuses on both aspects of the research of tick salivary secretion. The description of the first transcriptome of European Lyme disease vector *Ixodes ricinus* is followed by a series of functional studies focused on one group of protease inhibitors found in tick saliva – serpins. The knowledge obtained from high-throughput approach and the functional studies on tick serpins was also reviewed in several reviews that are included as a part of this thesis as well.

Acknowledgement:

I would like to thank to all my colleagues and students from the Department of Medical Biology at the Faculty of Science, University of South Bohemia, for their help, support and collaboration as well as for their friendship and for creating healthy and friendly working environment. Namely, I would like to thank to Prof. Jan Kopecký for his supervision during my student's years and for turning my attention to tick serpins that remained in my scientific focus until now. Further, I want to thank all my collaborators and co-authors for enabling the publication of submitted articles.

My gratitude also goes to the Faculty of Science itself, because it has always been my only alma mater, providing me with everything I needed for my professional development, whether it was education or work position.

Last, but the greatest thanks belong to my wife Radka for walking the way with me and to my children for making our life more complete and meaningful.

Contents

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Reviews

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2. **Chmelař J**, Kotál J, Kopecký J, Pedra JH, Kotsyfakis M. All For One and One For All on the Tick-Host Battlefield. Trends Parasitol. 2016 May;32(5):368-77. **IF=10,528, 56 citations**
3. **Chmelař J**, Kotál J, Karim S, Kopacek P, Francischetti IM, Pedra JH, Kotsyfakis M. Sialomes and Mialomes: A Systems-Biology View of Tick Tissues and Tick-Host Interactions. Trends Parasitol. 2016 Mar;32(3):242-54. **IF=10,528, 72 citations**
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SUMMARY OF THE THESIS

Ticks are obligatory blood-feeding parasites that are of medical importance due to their ability to transmit wide range of pathogens. There are two major families – *Ixodidae* and *Argasidae*, which differ in their lifestyles. This thesis focuses on the first group, because *Ixodidae*, also called hard ticks, are of greater interest from the immunological point of view. Due to their prolonged feeding course that can take up to ten days, hard ticks developed mechanisms to alter all types of host immune responses in order to finish their blood meal, including the alteration of adaptive immunity. These mechanisms of immunomodulation or immunosuppression are entirely dependent on the composition of tick saliva. During the feeding process, ticks alternate between sucking blood and secreting saliva into the wound. The saliva was found to be extremely efficient in altering, inhibiting and modulating every branch of host's immune system, as reviewed in **review 1** [1]. The tick saliva consists of hundreds, in case of hard ticks even thousands of different proteins with diverse pharmacological activities that are responsible for efficient alteration of host's defense mechanisms. There are proteins inhibiting plasma coagulation and platelet aggregation, other groups have anti-complement function, many salivary components inhibit the activation and development of inflammatory response and some proteins are able to inhibit T cell proliferation, survival and differentiation into proinflammatory Th1 subpopulation. An interesting and important aspect of tick salivary proteins is their redundancy and pluripotency. Redundancy means that each protein can be substituted to certain level by another one, even from different protein family. On the other hand, pluripotent protein can affect more pathways or processes at once. This is directly connected with another unique feature of ticks – the presence of multigene protein families. These families are the result of multiple gene duplication and their subsequent evolution, leading to more or less abundant groups consisting of members with similar or different biological activities. This phenomenon was described in the **review 2** [2].

At the beginning of 21st century, there was a big boom of high-throughput approach to the study of tick salivary secretion. The first transcriptomes from tick salivary glands (a.k.a. sialomes) were done by Sanger sequencing and could not cover full range of salivary proteins, but main framework was set. The first transcriptome of European *Ixodes ricinus* was published in 2008 in **article 1** [3] and it defined major salivary protein families for this species. In 2016, systems biology approach, i.e. the combination of transcriptomics and proteomics in tick research was reviewed in **review 3** [4]. Out of many salivary protein groups, inhibitors of proteases form the most abundant and diverse group. In general, protease inhibitors are regulatory molecules that usually keep an equilibrium of proteolysis driven physiological processes, such as coagulation of plasma in vertebrates or hemolymph in arthropods, protein activation and digestion, complement or platelet aggregation and others. Proteases play an important role in tick physiology as well, as they are responsible for blood proteins degradation and blood digestion. Blood digestion occurs mostly intracellularly in the midgut cells, where hemoglobin and other blood proteins are digested proteolytically to single amino acids. Protease inhibitors control all these processes and also protect from deleterious proteolysis that could damage the tissues. There are several protein families expressed in tick salivary glands that are involved in the inhibition of host proteases. When tick protease inhibitors are secreted into the host via saliva, they attack the enzyme-inhibitor equilibrium in the host towards state that is less hostile for the tick. For example, many serine protease inhibitors target proteases of coagulation cascade, resulting in impaired plasma clotting, other inhibitors can target neutrophil proteases, which results in an anti-inflammatory activity. The role of tick secreted protease inhibitors was summarized in several reviews, including **review 4** [5] focused on serpins and cystatins and **review 5** [6], which was focused solely on serpins. In our research group, we focused mainly on serpins and to lesser extent to cystatins. The first research paper on tick serpins that was published by an author of this thesis was **article 2** [7], which was, together with article

1, a basis for author's Ph.D. thesis. In addition to the presentation of anti-inflammatory and anti-platelet activities of a serpin named IRS-2 (Ixodes ricinus serpin-2, recently renamed to Iripin-2), it presented the first serpin crystal structure of parasitic origin. Anti-inflammatory function of IRS-2 was further dissected and one possible mechanism of action was disclosed in **article 3** [8]. Functional characterization of other serpins from *Ixodes ricinus* continued in the following years thanks to the funding from two research grants, awarded by Grant Agency of the Czech Republic, resulting in several original research articles on serpins, written by Ph.D. students either under the supervision of the author or from other collaborating groups. The serpins characterized were Iripin-3 (**article 4**) [9], Iripin-5 (**article 5**) [10] and Iripin-8 (**article 6**) [11]. In this habilitation thesis, a general introduction of tick salivary secretion will be presented as well as the general introduction of serpins. The description of tick serpins and their role in tick-host interaction, as published in submitted articles and reviews will be discussed in more details. The thesis is based on articles that combine the use of high-throughput approach to secreted salivary protein identification and detailed functional characterization of individual proteins, i.e. serpins.

INTRODUCTION

1. TICKS AND TICK-HOST INTERACTION

Ticks are obligatory blood-feeding ectoparasites, belonging to the order Ixodida (phylum Arthropoda, class Arachnida, subclass Acari), which is further subdivided to three families with different life cycles and worldwide distribution – *Ixodidae*, also called hard ticks, *Argasidae* – soft ticks and an obscure group *Nuttalliellidae* with only one African species *Nuttalliella namaqua.* Ticks have global distribution and are of medical importance due to their ability to transmit numerous pathogens. Many of these pathogens can cause serious disease in humans; the most well-known are viral tick borne encephalitis and bacterial borreliosis caused by a spirochete of the genus *Borrelia*. Further diseases are for instance bacterial tularemia (*Francisella tularensis*) and anaplasmosis (*Anaplasma marginale*) or protozoan babesiosis (*Babesia spp.*) and theileriosis (*Theileria spp.*) [12], caused by parasites from phylum Apicomplexa.

Despite all ticks feed on blood, there is big difference in their feeding strategies. While *Ixodidae* feed on a single host for up to ten days until full engorgement and repletion, *Argasidae* usually feed for less than one hour in order to complete the blood meal and to be able to proceed with their development into next instar [13]. Since blood is the only food ticks can utilize, they need to figure out, how to obtain the blood from a living host without being interrupted and/or rejected. When tick penetrates the skin by its hypostome, a wide range of host defense mechanisms is activated, leading to a complex immune response. This could be serious obstacle for a tick, therefore ticks developed multiple mechanisms, by which host defense mechanisms can be blocked, modulated or evaded. It is not only the injury itself that activates host defenses, but also an infection with transmitted pathogens and/or concurrent secondary infection with bacteria from the environment that can stimulate host immune system. Complex immune response against tick feeding comprises of hemostasis, innate immunity, such as complement and inflammation and adaptive immunity with the activation of both T and B cell. The adaptive immunity is important especially in the defense against hard ticks because of their long term feeding and continuous stimulation of host immune system by multiple antigens. There are several excellent reviews summarizing host immune response to tick feeding and tick response to host defense in details [14-18].

2. HOST RESPONSE TO THE TICK FEEDING

A tick bite is recognized by the host as an injury that activates **hemostasis** in order to prevent blood loss and **inflammation** to eliminate the danger and close the wound. It also triggers **complement** to promote inflammatory reaction and kill the bacteria and **specific immunity** in order to raise proper and precise reaction that could lead to tick rejection and the build-up of immune memory.

2.1. HEMOSTASIS

Hemostasis is physiological reaction that prevents blood loss caused by an injury in vertebrates. It consists of three processes that operate in concert - plasma coagulation, platelet aggregation and vasoconstriction. Hemostasis as a complex process has been studied thoroughly and is described in many medical textbooks [19]. Each of these mechanisms can be triggered by several ways. Briefly, the coagulation is driven by a proteolytic cascade with two ways of activation – exogenous (or extrinsic)

and endogenous (or intrinsic), both with several amplification and regulatory points and regulatory mechanisms. Exogenous or extrinsic pathway of coagulation begins with the injury of the blood vessel and the activation of tissue factor (TF) by an exposure to blood, which is followed by the formation of TF/factor VIIa (FVIIa) complex. TF/FVIIa activates factor X (FX) to factor Xa, which finally activates thrombin. Thrombin (Factor II) cleaves fibrinogen to fibrin, the building block of the clot [19]. Moreover, thrombin can activate protease-activated receptors on immune cells, leading to inflammatory response [20]. Intrinsic pathway begins with the auto-activation of FXII (also called Hageman factor) by its contact with polyanionic surfaces; therefore, this pathway is regarded as contact phase. This way of activation leads also to the induction of kinin-kallikrein system, which is involved in the amplification of inflammation (see 2.2.). The second part of hemostasis is platelet aggregation, which is dependent on the cell-cell interactions between platelets and endothelial cells and among platelets themselves. Activated platelets form a thrombotic plug in coordination with fibrin fibres. It leads also to the production of proinflammatory mediators, thus being the link between hemostasis and innate immunity [21]. Moreover, injury induces the release of several vasoconstrictors, i.e. biogenic amines and endothelins from endothelial cells, resident mast cells and activated platelets, leading to the constriction of blood vessel and diminishing of the blood loss.

2.2. INFLAMMATION AND COMPLEMENT

Tick bite is recognized as an injury by vertebrate immune system, therefore defensive and repair mechanisms are activated. Moreover, tick feeding is usually accompanied by the infection, which is either direct by tick-borne pathogens or indirect by concomitant microbes from the surface of the tick mouthparts or host skin. Injury and infection is opposed by an immediate local acute inflammatory reaction. In general, acute inflammation is described by five so called cardinal signs - calor (heat), tumor (swelling), rubor (redness) and dolor (pain). Fifth sign of inflammation is the loss of function (function laesa), which results from a tissue damage and fibrotization [22]. Heat and redness are the results of vasodilatation and increased blood flow; swelling is caused by increased vasopermeability and subsequent accumulation of plasma fluid and proteins in extravascular space and by the extravasation of leukocytes, such as neutrophils and monocytes, from blood vessel to the site of inflammation. Many inflammatory mediators like bradykinin, serotonin, histamine or complement products C3a and C5a are released and cause pain, itching and further chemoattraction of leukocytes from the blood. Pain, itching and swelling have direct impact on tick feeding success, because the first two usually lead to tick removal by the host and swelling can disconnect the tick from blood flow and eventually lead to tick rejection. The mechanisms of innate immunity that are involved in tick x host interaction are described in several reviews [14, 16, 18, 23-26]. Briefly, after skin damage caused by tick mouthparts, factor XII of intrinsic coagulation pathway activates the clotting cascade, as well as the fibrinolytic and kinin-kallikrein system. Mediators of these systems induce pain, vasodilatation and vascular permeability, neutrophil chemotaxis and complement activation via plasmin. Due to vessel damage, platelets are activated by extravascular collagen and by thrombin via protease activated receptors (PARs), leading to platelet activation and the release of more inflammation mediators [21]. Injured cells produce DAMPs (danger associated molecular patterns), which activate neutrophils and other leukocytes. Activated neutrophils degranulate and release antibacterial peptides, chemokines and also proteolytic enzymes, namely serine proteases cathepsin G, elastase and protease 3. These proteases are responsible for tissue remodeling and also for further amplification of the inflammatory response – for example cathepsin G (similarly to thrombin) activates the cells, including platelets, via PARs, in case of cathepsin G, it is PAR-4 [27, 28]. Activation of neutrophils is accompanied by the

generation of pain-inducing prostaglandins. In addition to neutrophils, mast cells and basophils are involved in the inflammatory response to tick bite. They are activated by anaphylatoxins - C5a and C3a compounds of complement, leading to massive degranulation of mast cells and basophils and to the release of important inflammatory mediators such as histamine, serotonin and serine proteases [29]. Basophils play a crucial role in tick rejection during repeated infestation and major part of biogenic amines is produced by this cell population [30-32]. Histamine and serotonin act as vasodilators, notorious due to their involvement in allergic reaction, in which they cause typical symptoms, such as hay fever, tissue edemas and redness of the skin [33]. Other products secreted by mast cells are proteases like mast cell chymase, which can attract other immune cells like eosinophils and neutrophils by processing various chemokines and receptors [34]. Moreover, chymase was shown to degrade tick anticoagulants and thus fight against tick infestation [35]. Proinflammatory cytokines TNF, IL-1β and IL-6 are released from activated macrophages and are involved in the amplification of inflammation. Because of bacterial presence in the site of injury, the lectin and the alternative pathway of the complement are activated and large quantities of anaphylatoxins C5a and C3a are produced. This leads to further attraction of granulocytes and monocytes to the site of injury. All cells involved in the inflammation communicate via the secretion of various cytokines and other mediators that influence their function so that the inflammatory reaction is precisely orchestrated as described in many reviews [14, 18, 26].

2.3. Adaptive immunity

Since hard ticks feed for prolonged period, host immune system has enough time to develop specific response, including the activation of B and T cells. Cellular branch of adaptive immunity is driven by T cells that can differentiate into several subpopulations, either pro- or anti-inflammatory [26]. Major proinflammatory response is orchestrated by Th1 cells that produce especially Interferon-γ and interleukin IL-12, the cytokines that activate effector cells of inflammation such as macrophages. In response, macrophages produce TNF and IL-1b. Another proinflammatory branch of cellular immunity is Th17, which is characterized by the production of Interleukin-17 by Th17 cells and several other immune cells [36]. This cytokine has strong proinflammatory activity, as it stimulates neutrophils activation and infiltration, the production of TNF and nitric oxide by macrophages or the production of antimicrobial peptides. On the other hand, the polarization towards Th2 response is regarded as anti-inflammatory and is characterized by the production of interleukins IL-4, IL-5, IL-6 or IL-13. These cytokines activate Th2-response-specific cells, such as eosinophils. There is also an anti-inflammatory reaction dependent on regulatory T cells [37, 38].

3. Tick saliva in tick-host interaction

Tick bite induces hemostasis, innate immunity and proinflammatory Th1 response. All these responses are deleterious for the tick and they aim for tick rejection, wound closure and the elimination of the infection. During hundreds millions years of co-evolution between ticks and their hosts, ticks have developed wide range of countermeasures that enable them to stay attached and unnoticed on the host. It was discovered that this ability depends on the saliva, produced by ticks into the wound [1, 15, 16, 18, 24]. Researchers started to be interested in tick saliva in eighties of the 20. century, when they realized that tick is not simple “crawling syringe” for pathogen transmission, but that it can efficiently alter every host’s defense mechanism [39-41]. Many works were published on the effects of tick saliva or salivary gland extracts (SGE) on host defenses. These were shown to affect hemostasis and all relevant branches of innate and adaptive immunity (Figure 1). In brief, ixodid tick salivary secretion

can alter wound healing related processes such as coagulation, platelet aggregation and vasoconstriction, innate immunity related responses that include inflammation, complement cascade, macrophage ability to phagocytose and also natural killer cells activity. Acquired immunity is modulated too, so that instead of an efficient Th1 response to tick feeding, less harmful Th2 response is elicited. The outcomes of the research on tick saliva and SGE were summarized in **review 1** [1]. In the following section, the progress in the identification of pharmacologically active salivary constituents is discussed.

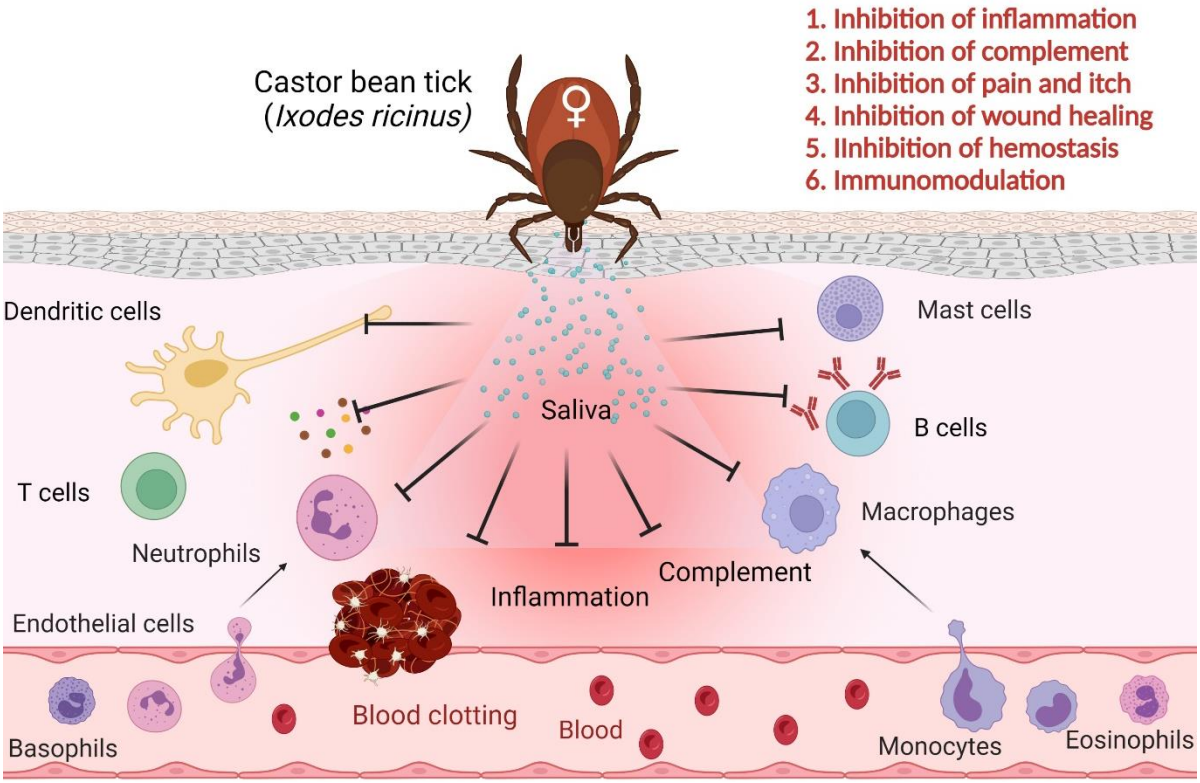


Figure 1. Effects of tick saliva on the host. Ticks can alter and impair all relevant host's defense mechanisms, ranging from hemostasis to adaptive immunity. (Created by BioRender)

4. THE COMPOSITION OF TICK SALIVA

4.1. MULTIGENE FAMILIES – REDUNDANCY AND PLURIPOTENCY

With the development of methods of molecular biology and thanks to transcriptomic approach to the analysis of salivary secretion, large number of transcripts encoding for potentially pharmacoactive proteins was discovered. Moreover, systems biology has been used in ticks to obtain broader picture of gene expression in specific physiological states or developmental stages and to compare these states. The journey of using high-throughput approach in tick research and the summary of transcriptomic works carried out until 2016 were described in **Review 3** [4]. It was established already after first few sialomes (transcriptomes from salivary glands) were published that ticks dispose of rich armamentarium of pharmacoactive molecules that are produced by salivary glands and are secreted into the feeding lesion via saliva [18]. These molecules are predominantly proteins that usually cluster in the **multigene families** - the groups of proteins that consist of tens or hundreds members, more or

less similar to each other [42]. Individual members can display either the same or different activities. This also holds true, when distinct protein families are compared. There is a lot of experimental evidence that tick salivary proteins display high level of redundancy and pluripotency. It means that two or more different proteins can have the same function (redundancy) and one protein can display more activities (pluripotency). As hypothesized and discussed in **review 2** [2], the combination of these two phenomena makes tick saliva a potent weapon against host's immune system and have several interesting outcomes for both the tick and the host. First, it enables the tick to proceed in the alteration of host's defenses even after some salivary proteins are targeted and blocked by antibodies and other immune mechanisms (Figure 2A). Second, the sequential secretion of members of the same multigene family over time of feeding can maintain the immunomodulation while evading strong immune response by using functionally identical proteins, however, with different antigenic profile, i.e. different epitopes. This would lead to the activation of different B cell clones and the production of different antibodies that would not be able to sufficiently respond to tick feeding (Figure 2B).

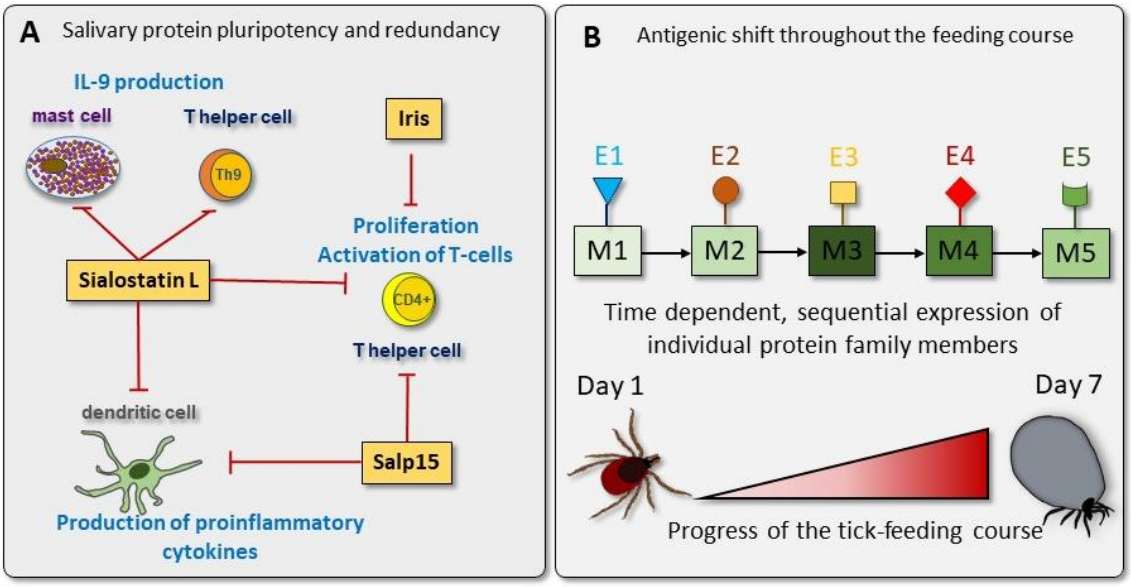


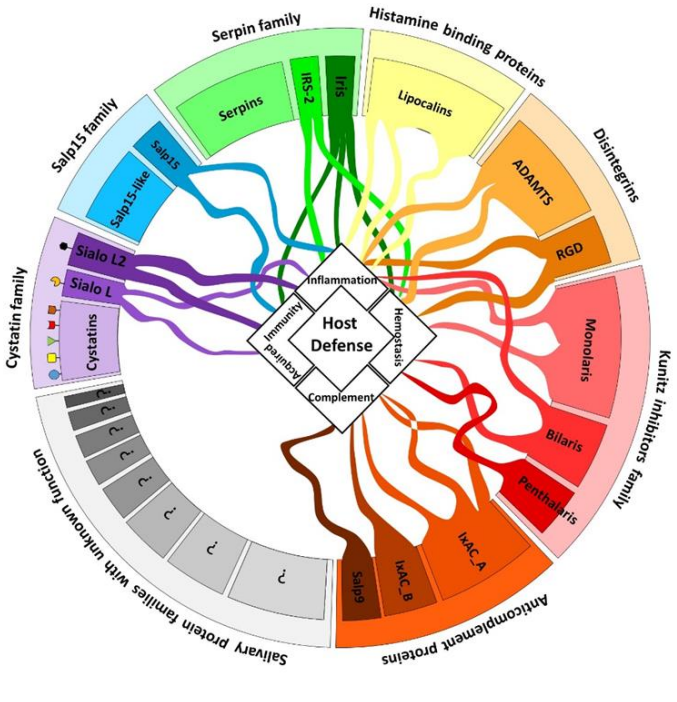
Figure 2. Redundancy and pluripotency of tick salivary proteins. A) The same protein can display different effects on the host (pluripotency) and one function can be carried out by more than one protein (redundancy). B) Members of one multigene family (M1-M5) can be sequentially expressed during feeding and present different epitopes (E1-E5), while keeping the same immunomodulatory function. Adapted from Chmelar et al., 2016 [2].

4.2. FAMILIES OF TICK SECRETED SALIVARY PROTEINS

The pioneer works that employed high-throughput approach for the identification and mass annotation of proteins, expressed in tick salivary glands were conducted by José Ribeiro and Jesus Valenzuela in NIAID/NIH using *Ixodes scapularis* as a model tick [43]. The transcriptome of salivary glands was named sialome [44] and since then many sialomes not only from different tick species, but also from other blood-feeding ectoparasites were published. At the beginning, classical Sanger sequencing was used and in-house developed bioinformatics pipelines were employed for sequence analyses. The first sialome of *Ixodes ricinus* was prepared, annotated and published by our group in the collaboration with Prof. Valenzuela and Ribeiro (**article 1**) [3]. Main tick salivary protein groups were defined. Among the most abundant, there were lipocalins with histamine binding and anti-complement activities, protease inhibitors with mostly anti-hemostatic and anti-inflammatory functions and several unique, tick specific families with various functions, including the modulation of

adaptive immunity. Figure 3 shows an overview of some of tick multigene families that are expressed in tick salivary glands and are responsible for the modulation of host's defense mechanisms. Some of them belong to well described protein families, such as lipocalins, disintegrins or protease inhibitors, such as serpins, cystatins and Kunitz domain inhibitors with variable number of Kunitz domains. Other protein groups are unique for ticks, some of them even for specific genus, such as Salp15 group (Salivary protein 15) that is unique for *Ixodes spp.* [45]. In addition to well characterized protein groups, either generally distributed throughout different organisms or tick-specific, there is a lot of transcripts, coding for completely unknown proteins with unknown functions (Figure 3B). Large portion of transcripts in ticks seem like it does not encode any protein at all. Recent works show that micro RNAs can play important role in tick-host interaction as well and that such big amount of transcript RNA can code for micro RNA [46, 47].

A



B

Name of the family	Members
Secreted unknown	4237
Lipocalin	685
Kunitz containing peptides	612
Salp15	539
Basic tail	468
Metalloproteases	330
Ixodes 10 kDa peptide family	264
8.9 kDa family	145
Prokineticin	145
5.3 kDa family	97
Isac anticomplement family	86
Cytotoxin DAP-36 like	73
18.3 kDa	73
TIL domain peptides	65
Microplusin	22
Ixodes 14 kda family	20
Serpins	18
Ixodegrins	17
Defensins	13
Cystatins	10

Figure 3. An overview of major tick salivary protein families. A) Major tick protein families with disclosed role in tick-host interaction. Adapted from Chmelar et al., 2016 [2]. B) Number of unique members of protein family, according to the data from previously published data [48, 49].

As shown by many transcriptomic and proteomic studies that were conducted in past ten years, different tick species rely on different set of proteins in the host defenses alteration. When a phylogenetic analysis is performed on a certain multigene family, the result usually points at a rapid evolution, which is species specific, i.e. each tick genus or even species choose different member of the family, which undergoes the process of gene duplication and subsequent mutagenesis. This process is most likely exempted from an evolutionary pressure due to surplus of protein family members with necessary function [42, 50]. Therefore, members with novel functions can appear. This was shown already in **article 1** [3] on the example of collagen-like secreted proteins (Figure 4).

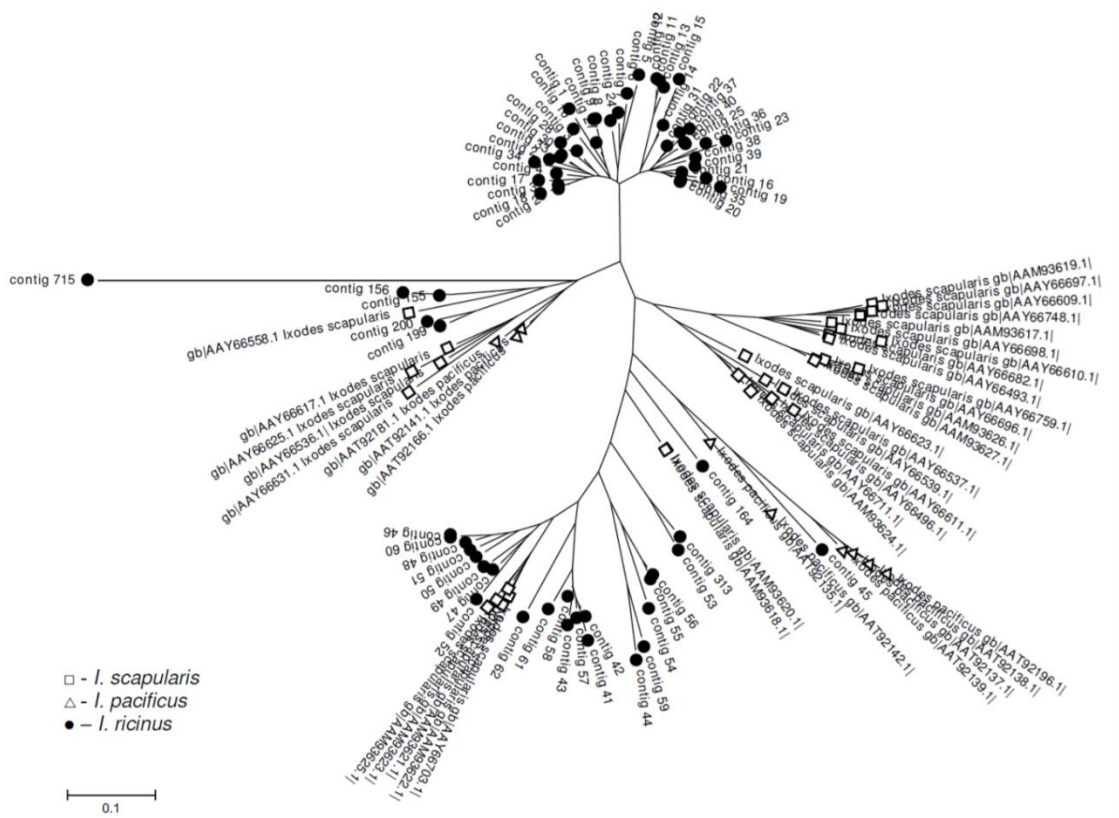


Figure 4. The phylogenetic tree of collagen-like secreted protein multigene family. These proteins seem to be unique for the genus *Ixodes* and show the rapid evolution of these families in the frame of single species. Taken from Chmelar et al., 2008 [3].

4.3. INHIBITORS OF PROTEASES IN TICK SALIVARY SECRETION

In general, protease inhibitors are very important regulators of numerous physiological processes. They can inhibit proteases involved in intracellular digestion or in proteolytically activated cascades of enzymes, such as coagulation, complement or phenoloxidase system in arthropods. They maintain proper amount of activated proteases in both normal and pathological states and thus regulate key homeostatic mechanisms. Protease inhibitors belong to several functional groups – inhibitors of serine proteases, cysteine proteases, aspartic proteases and metalloproteases [51]. These groups consist of several protein families that differ in their structure and mode of inhibition. Specific case is α-2-macroglobulin inhibitor with protease entrapping mechanism of action and wide specificity across all types of proteases, which is also involved in immune processes [52]. Protease inhibitors are catalogued in MEROPS database [51]. Tick salivary secretion contains predominantly serine and cysteine protease inhibitors (see following chapters). No inhibitors of metalloproteases are found in tick saliva. This is because ticks themselves use salivary metalloproteases to adjust the environment of feeding site and thus facilitate tick feeding [53]. Unlike mosquitoes that suck blood directly from the vessels (capillary feeders), ticks are pool feeders. This means that they first need to create a feeding lesion or a cavity, from which they can suck the blood. Metalloproteases are the main enzymes to degrade extracellular matrix, which helps to form such feeding cavity. Therefore, it is not in tick's intent to block them.

On the other hand, serine proteases drive the coagulation and complement cascades and are responsible for several ways of inflammatory activation (e.g. thrombin, plasmin, kallikrein, etc.). Cysteine proteases, such as cathepsins, are involved in intracellular protein digestion, but also in the

activation of inflammasome and the production of proinflammatory cytokines IL-1β and IL-18 [54]. Above mentioned processes are deleterious for tick feeding as they either inhibit the blood flow, cause pain or itching and/or lead to the activation and amplification of immune response to the tick feeding. Therefore, ticks use inhibitors of serine and cysteine proteases to inhibit or modulate processes of hemostasis and both innate and adaptive immunity. Only inhibitors of serine proteases will be discussed here in greater details, while inhibitors of cysteine proteases will be only briefly mentioned as they are related with this thesis only as a part of one review article (**review 4**).

4.3.1. Tick inhibitors of cysteine proteases

Inhibitors of cysteine proteases are named cystatins, which belong to common protein family sharing similar tertiary structure and sequence homology. Cystatins include four types – Stefins, type 2 cystatins, kininogens and fetuins [55]. Tick cystatins fall mostly into the second type, which is secreted and therefore can be part of tick salivary or mid-gut secretion [56]. The functions of tick salivary cystatins were characterized as anti-inflammatory, because they affect processes in several immune cell types responsible for inflammatory reaction. For instance, salivary cystatins from *I. scapularis* Sialostatin L and L2 displayed specific type of redundancy, as they inhibited proinflammatory functions of dendritic cells (DC) by targeting two parts of the same activation pathway. While Sialostatin L inhibited the production of cytokine interferon-β, Sialostatin L2 inhibited directly JAK/STAT phosphorylation (Figure 5). Both activities lead to the inhibition of the production of DC-specific cytokines and chemokines [2, 57].

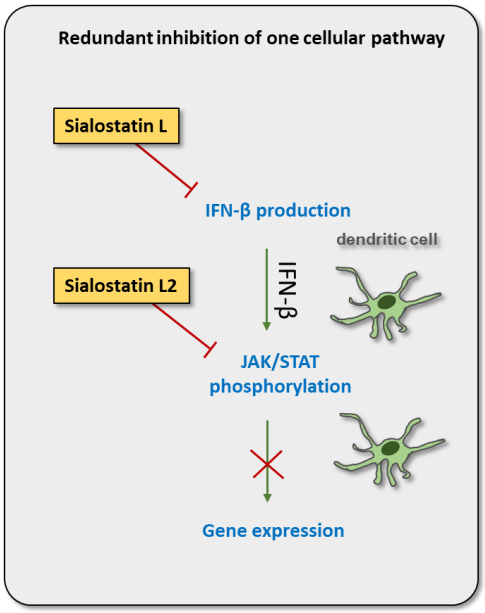


Figure 5. Redundant inhibition of different modules of the interferon-β (IFN-β) signaling pathway by two members of the same multigene family. SialostatinL, unlike sialostatinL2, inhibits IFN-β production by DCs. SialostatinL2, however, inhibits the phosphorylation of STAT-3 in the JAK/STAT signaling pathway and subsequent expression of several genes downstream from IFN-β-dependent DC activation.

In addition to the inhibition of DCs, sialostatins inhibited the proliferation of CD4+ T cells, prevented experimental asthma in mice and inhibited inflammatory infiltration in mouse paw edema model [57-59]. More cystatins were characterized from other tick species with described roles in the regulation of hemoglobin degradation in tick midgut or in the inhibition of host immune responses. Knock-down experiments with RNA interference in ticks lead to higher mortality, reduced weight gain and impaired egg laying, highlighting the importance of cystatins in tick physiology and tick-host interaction [57]. Despite the primary focus of the author of this thesis on serpins, he participated in *I. ricinus* cystatins studies [60, 61] and summarized the up-to-date knowledge of tick cystatins role in tick-host interaction in the **review 4** [5].

4.3.2. Tick inhibitors of serine proteases

Serine protease inhibitors belong into several protein families with distinct molecular weights, structures and modes of action. Major families contain one of the following domains – Kunitz, Bowman-Birk, Kazal or serpin domain [62]. Many more serine protease inhibitors of peptidic nature can be found in the organisms, but for the purpose of this study, we will mention briefly only Kunitz domain inhibitors and further chapters will focus on serpins, as they are in the center of this thesis aim.

Kunitz domain-containing inhibitors are the most abundant and diverse group of protease inhibitors in ticks [63]. They seem to be involved mostly in anti-hemostatic activities. They can inhibit thrombin and trypsin-like proteases of coagulation cascade, such as Factor Xa, IXa, VII and others [64]. Interestingly, single Kunitz domain proteins were also described as components of venoms. These proteins are able to block ion channels and thus block the transmission of neuronal and/or neuromuscular signals [65]. One of the first described tick inhibitors from this family contained two Kunitz domains and was named Ixolaris [66] and the names of other groups were composed of the number of domains and the suffix "–laris". As such, there is single domain monolaris group, two domain bilaris, five domain penthalaris and so on. Inhibitors with 1-5 Kunitz domains were found in tick transcriptomes [49, 63]. Kunitz inhibitors have rigid structure with defined number of disulphide bridges, which makes them suitable scaffold for protein engineering, by which already novel drugable artificial peptides were constructed [67]. The second intensively studied group of tick serine protease inhibitors are **serpins**, to which the rest of this introduction is dedicated.

5. SERPINS

5.1. EVOLUTION OF SERPINS

Serpins are the most diverse group of protease inhibitors with several thousands members across whole realm of life, including viruses and prokaryota [68]. Recent comprehensive phylogenetic study used 6000 unique serpin sequences to reconstruct the evolution of serpin superfamily and to visualize their sequence similarity by aligning and comparing tertiary structures [69]. The study showed an interesting pattern in structure similarity – there is a central hub of presumably intracellular housekeeping serpins with highly conserved structures, which belong to all kingdoms of life and then there are rays of less similar serpins, clustering into groups according to the species of their origin (Figure 6A). This is an important notion, showing the functional versatility of serpins, which derives from their unique structural and mechanical properties (see next chapter). It seems that the conserved structure of hub serpins is a result of convergent evolution rather than of the horizontal gene transfer, which was also one of the hypotheses that tried to explain high similarity of prokaryote and eukaryote serpins [69]. The idea of a central hub serpins containing conserved and indispensable regulators of intracellular proteolysis and peripheral, mostly secreted serpins with diverse functions and faster evolution is supported by a more detailed analysis of tick serpins (unpublished data). In this analysis, approximately 150 full length serpins from all available tick transcriptomes were gathered, aligned, adjusted for a phylogenetic analysis (signal peptides and hypervariable reactive center loops were removed) and analysed by a minimal evolution model in MEGA X software [70]. Resulting tree, as shown in simplified version in figure 6B, shows several well supported clades that consist of secreted serpins from prostriate (green, genus *Ixodes)*, metastriate (blue, genera *Ammblyoma, Dermacentor, Rhipicephalus*) or both tick groups (yellow). The last clade (violet color) contains serpins from all species that bear no classical signal peptide and can be considered as intracellular. This clade resembles the central hub from the work of Spence and colleagues (Figure 6A) [69].

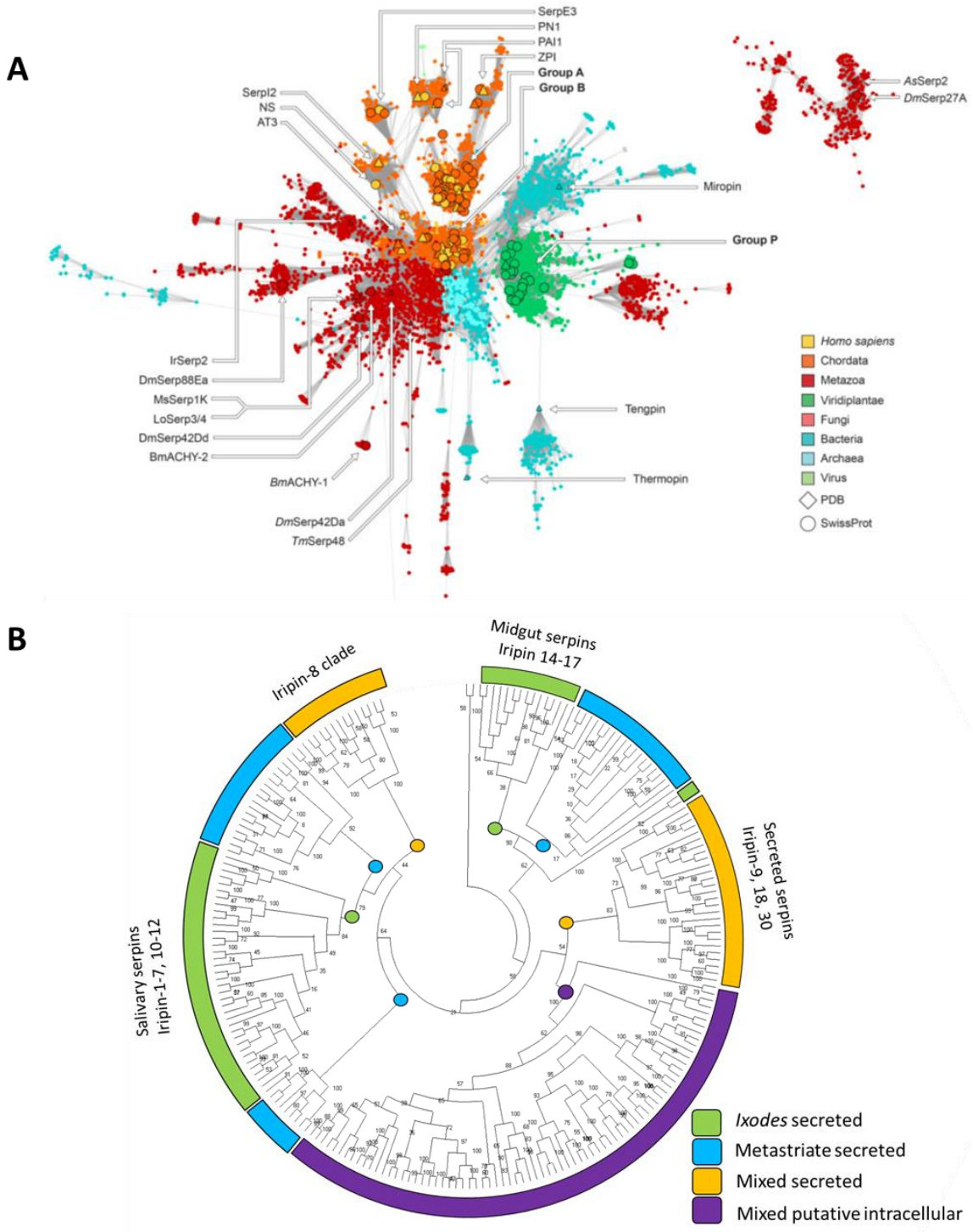


Figure 6. Phylogenetic analyses of serpins. A) Structural comparison of serpins from all organisms. The analysis of more than 6000 sequences shows a central hub of intracellular, presumably housekeeping, serpins with highly conserved tertiary structure and "rays" of less similar serpins, which cluster according to the species of their origin. Adapted from Spence et al., 2021 [69] B) Phylogenetic analysis of tick serpins. The clades are color coded as follows: Green – secreted serpins from the genus *Ixodes*, Blue – secreted serpins from metastriate genera *Rhipicephalus, Dermacentor* and *Ammblyoma*, yellow – secreted serpins from both and ticks, violet – intracellular serpins from all tick species. These serpins belong to the central hub of the figure 6A. Analysis was done in MEGA X software by using minimum evolution algorithm and 500 replication bootstrap to support the clades. (unpublished data).

The tree in the figure 6B shows another two interesting things. The secreted serpins cluster according to their organ of origin, which was disclosed after RT-qPCR analysis of serpin genes expression in adult female *I. ricinus* ticks was conducted (Figure 7, unpublished data).

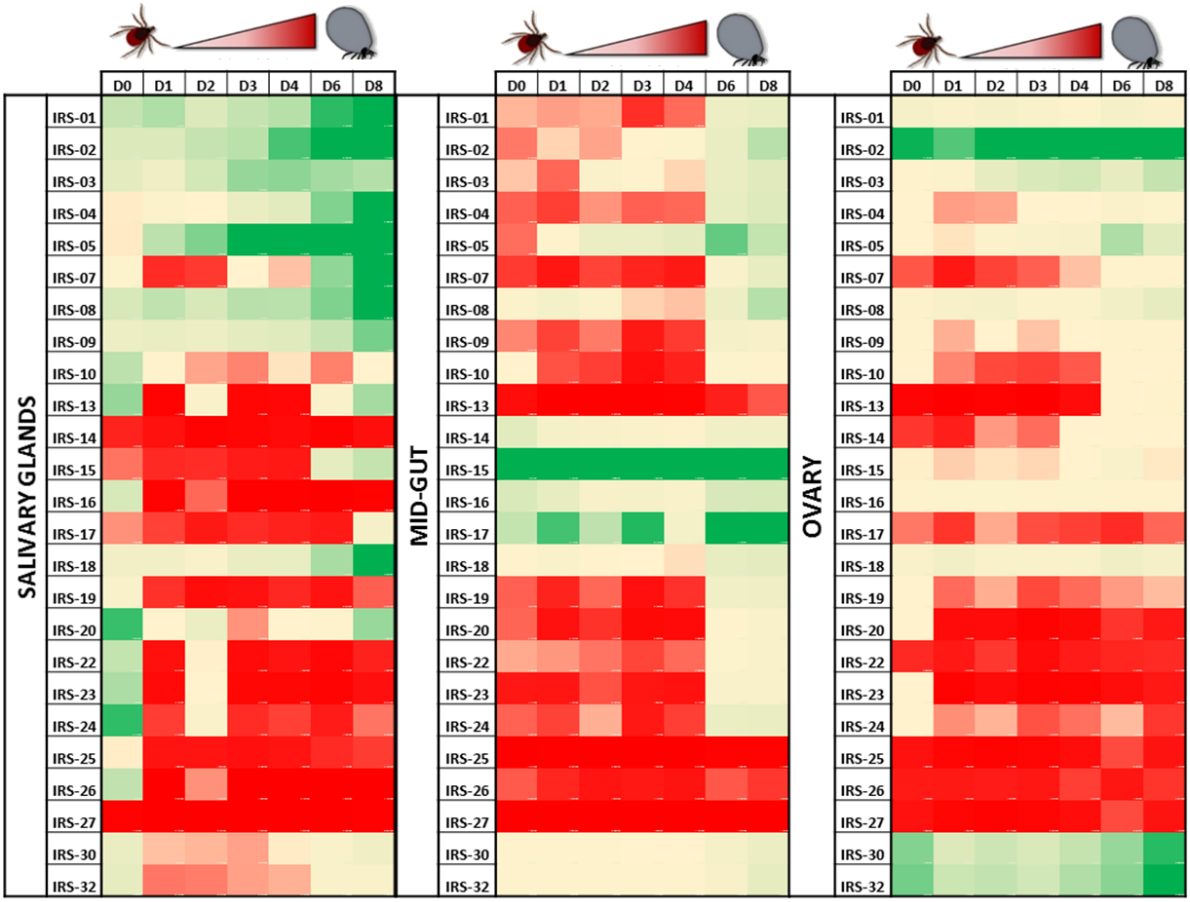


Figure 7. The expression of serpin genes in tick organs during the feeding course. D0-D8 are the days of feeding. The color scale ranges from lowest expression (red) through moderate expression (yellow) to highest expression (green). The range is common for all three organs, so that the expression levels can be compared among them (unpublished data).

When the expression patterns of *I. ricinus* from figure 7 are compared with the tree in the figure 6B, we can see that most of serpins that were upregulated predominantly in the salivary glands during feeding (IRS/Iripin[1]-1-9) belong to a single clade, as well as mid-gut serpins IRS/Iripin-14-17. Thus, the evolution of serpins reflects not only the evolution of the species, but also their function either in the blood digestion or in the host immunomodulation. There is, however, one exception – serpin Iripin-8. This secreted serpin, which displays inhibitory activity against wide range of coagulation proteases and was detected in tick saliva, seems to be conserved across all tick species. It forms a separate clade in the tree (Figure 6B) and unlike other tick serpins, even unusually long RCL is 100% identical in all species. This could point at its importance in tick physiology. Considering its inhibitory activity, Iripin-8 can play a role in the coagulation cascade of tick hemolymph, but its presence in the saliva suggests its role in host anti-hemostatic activities as well. Iripin-8 will be discussed later as it was described in **article 6** [11].

[1] Please note that the name Iripin (Ixodes ricinus serpin) is equivalent to older name IRS (Ixodes ricinus serpin), which is no longer used in order to avoid confusion with identical abbreviation used for insulin receptor substrate.

5.2. STRUCTURE AND MECHANISM OF ACTION OF SERPINS

Serpins are unique among protease inhibitors thanks to their mode of action. Serpins typically contain 350–400 amino acid residues with an average molecular weight between 40–60 kDa. Even despite low sequence homology, serpins share a highly conserved tertiary core structure that usually consists of three β-sheets (A, B, C), eight to nine α-helices, and a variable reactive center loop (RCL) which is a flexible loop of approximately 20 amino acid residues located at the top of the serpin molecule and reaching out of the core. [71]. Serpins inhibit proteases via a unique suicide substrate-like mechanism, which results in the inactivation and degradation of not only the protease but also the serpin [72]. Initially, recognition of the P1 site of the serpin RCL by the protease leads to the creation of a non-covalent Michaelis-Menten-like complex. The protease then cleaves the scissile bond between the P1 and P1' residues of the RCL and forms a covalent bond with the serpin. Finally, the cleaved RCL inserts into the center of the β-sheet A to form an extra strand, and the covalently bound protease is translocated to the opposite end of the serpin molecule [71, 72]. The mechanism of serpin inhibition is described in figure 8.

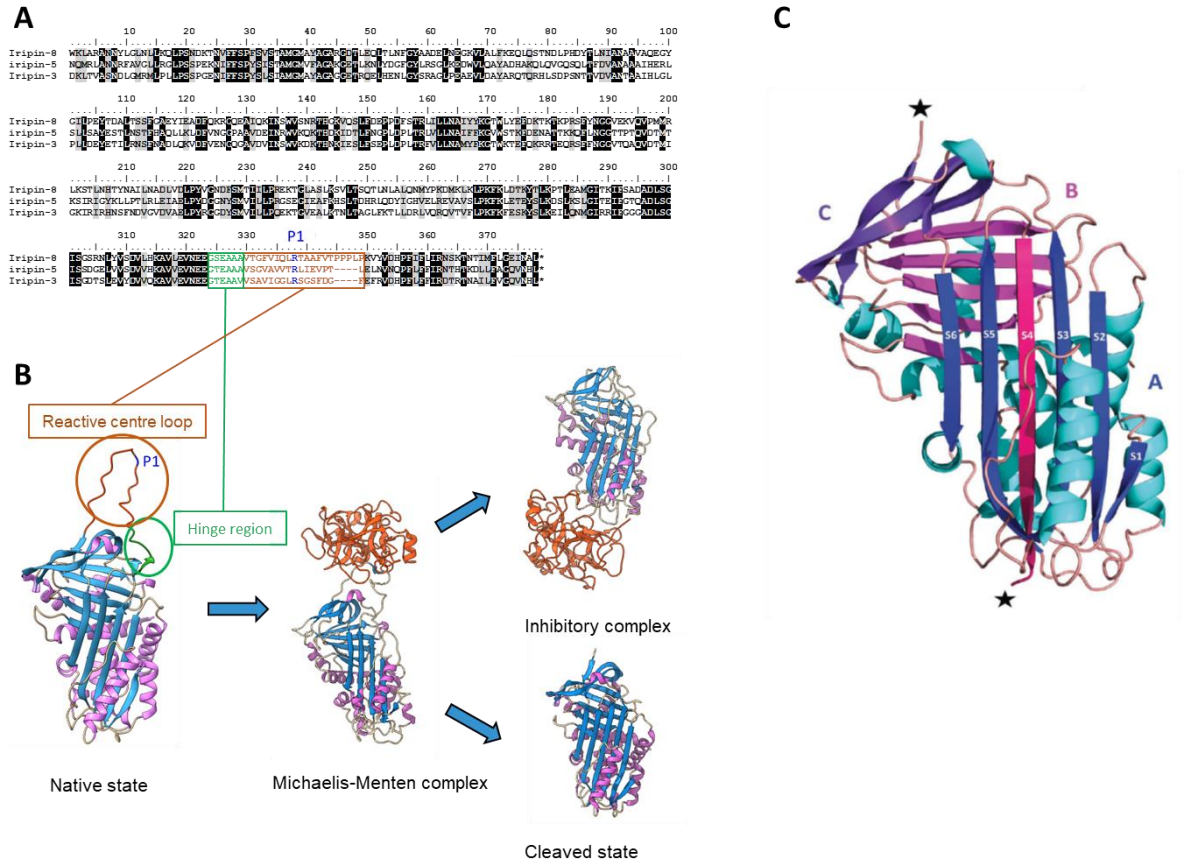


Figure 8. The description of serpin structure and its mechanism of inhibition (from Abbas et al., 2022, **review 5** [6]). A) An alignment of three *I. ricinus* serpins. Highlighted are hinge region, responsible for inhibitory potential (green) and RCL (brown) with P1 site (blue), responsible for serpin specificity. B) Four most common states of serpins during the conformational transition from native, metastable stressed state, through Michaelis-Menten complex to either covalent inhibitory complex or cleaved, stable state. Both final conformations have RCL inserted in the β-sheet A. C) Tertiary structure of stable cleaved state of Iripin-5 with highlighted β-sheets A, B, C and inserted RCL as an additional strand (pink).

The combination of conserved core structure with variable RCL resembles the composition of immunoglobulins that bear both conservative and hypervariable parts to bind to various antigens and specific immune cells. The principle itself reminds of a mousetrap, where both the mechanism and the

bait are necessary to catch the mouse (or protease in our analogy)[73]. This is exploited by *Manduca sexta*, in which multiple serpins are produced by an alternative splicing between the coding sequence for core structure and DNA for RCL [74]. Some serpins display non-inhibitory functions, however some of these functions rely on the flexibility of RCL as well. For example, the hormone release mechanisms in thyroxin binding globulin (TBG) and transcortin (binding cortisol) are also dependent on the thermoresponsive dynamics of serpin conformational changes [75, 76]. The ability of serpins to change their conformation in response to temperature changes makes them useful as sensors. Unfortunately, this serpin ability is not understood well yet.

5.3. SERPINS IN VERTEBRATES

The functions of serpins vary significantly from the inhibition of endogenous serine and rarely cysteine proteases to serving as storage proteins. In vertebrates, serpins play crucial role in the regulation of key physiological pathways and thus, serpins can be considered as regulators of homeostasis [77]. There are 36 serpins in human, 7 of which are non-inhibitory and 14 are intracellular [78]. One of the most abundant protein in human plasma is α-1-antitrypsin (A1AT) with the concentration 2-4 g/l of plasma. A1AT prevents tissues and vessels from the damage caused by neutrophilic proteases, especially elastase. The deficiency or mutation in A1AT leads to severe complications. Specific Pittsburgh mutation of P1 site (M358R) changes the specificity of A1AT from elastase to thrombin, thus leading to bleeding disorders [79]. Diseases related to serpins deficiencies or mutations are called serpinopathies [80]. Among other important vertebrate serpins, there is antithrombin or heparin cofactor II, both regulators of thrombin with lethal phenotype in knockout mice [81, 82]. The non-inhibitory serpin angiotensinogen is the precursor for oligopeptidic hormone angiotensin, which is produced by an enzyme renin. Its deficiency in mice leads to hypertension [83]. Another non-inhibitory serpin PEDF (pigment epithelium derived factor) is a strong inducer of angiogenesis. Serpins regulate also complement cascade and are thus involved in the regulation of immune responses. It is not purpose of this work to describe the function of all vertebrate serpins, but there are several excellent reviews, where the reader can find a summary of human serpin functions [77, 84]. For this work, it is important to mention that since endogenous vertebrate serpins are of such importance, the dysbalance, caused by an injection of tick salivary serpins into the wound can impair defense mechanisms and thus facilitate tick feeding. Serine proteases are involved in the regulation of the most important physiological processes in vertebrates, but also in the progression of some pathological states. Therefore it is intriguing idea that exogenous parasitic serpins specialized on vertebrate immunomodulation can be used for the development of novel pharmaceuticals to treat protease-dependent pathologies (Figure 9).

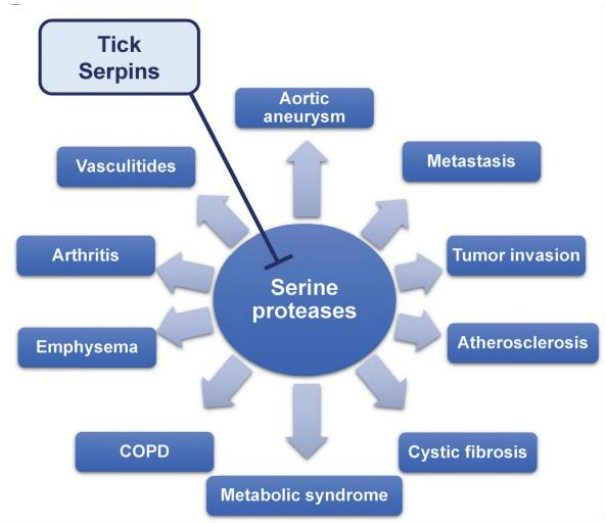


Figure 9. Diseases, in which serine proteases usually play negative role. Tick serpins could be used for the development of novel drugs to treat these diseases. Adapted from Chmelar et al., 2017, **review 4 [5]**.

5.4. SERPINS IN INVERTEBRATES

In invertebrates, serpins have also many functions, both inside and outside the cells. The research on *Drosophila* showed that serpins are involved in the regulation of embryonal development, apoptosis or phenoloxidase in the Toll pathway, which is responsible for defense response in arthropods [85, 86].

Serpins were also described as the regulators of hemolymph coagulation cascade [87]. In addition to keeping homeostasis in arthropods, serpins play key role in blood-feeding parasites. Serpins were detected in the proteome of tick mid-gut, probably contributing to the blood digestion process. More important for the purpose of this work is the role of serpins that are secreted via the saliva into the vertebrate host.

5.5. SERPINS IN TICK PHYSIOLOGY AND TICK-HOST INTERACTION

In ticks, serpins have functions in their own physiology and were shown to be possess multiple functions in the tick-host interaction. The knowledge about tick serpins and their functions in ticks and their hosts was reviewed in several reviews, including **review 4** and **review 5** [5, 6, 64]. To date, around 30 serpins from various tick species were characterized functionally. Salivary serpins were found to possess anti-hemostatic, anti-inflammatory and immunomodulatory properties. They can inhibit neutrophil and mast cell proteases and thus impair the key role of these cells in the development of inflammation. Some serpins were shown to inhibit leukocyte migration, which is an important process of immune response [7, 10]. By inhibiting different members of coagulation cascade and platelet aggregation, serpins display significant anti-hemostatic activity, which contributes to keeping blood flow to the feeding cavity. In the following sections, only serpins from *I. ricinus* will be described, as most of them were characterized by the team of the author of this thesis.

5.6. SERPINS FROM IXODES RICINUS

To date, five *I. ricinus* serpins were characterized in the scientific literature and additional two are about to be published in 2022. One and first of these was described by a Belgian group of Edmond Godfroid [88], the rest of the work was done by the team of author of this thesis in collaboration with other groups [7-11]. The pipeline of studying tick serpins in our group consists of bioinformatics analysis, RT-qPCR analysis of serpin expression in different organs and stages, molecular cloning of serpin transcript and its production in the recombinant and LPS-free form in some protein expression system. The recombinant protein is then used for functional analyses, such as enzymatic assays to find serpins' inhibitory specificities and immunological and biological assays to find their functions. Moreover, serpins usually crystallize very well, therefore we perform also structural analyses. We published the first structure of parasitic serpin in 2011 [7], which was highlighted in the front page of the Blood journal and since then, in collaboration with the group of Prof. Ivana Smatanová-Kutá and Prof. James Huntington, we solved additional 5 *I. ricinus* serpin structures, of which 3 were published so far (Figure 10). In addition to experiments with recombinant proteins, we were performing also RNA interference knock-downs of serpins in ticks in order to find, whether they are indispensable for ticks and could be therefore candidates for an anti-tick vaccine. In further chapters, individual *I. ricinus* serpins will be introduced.

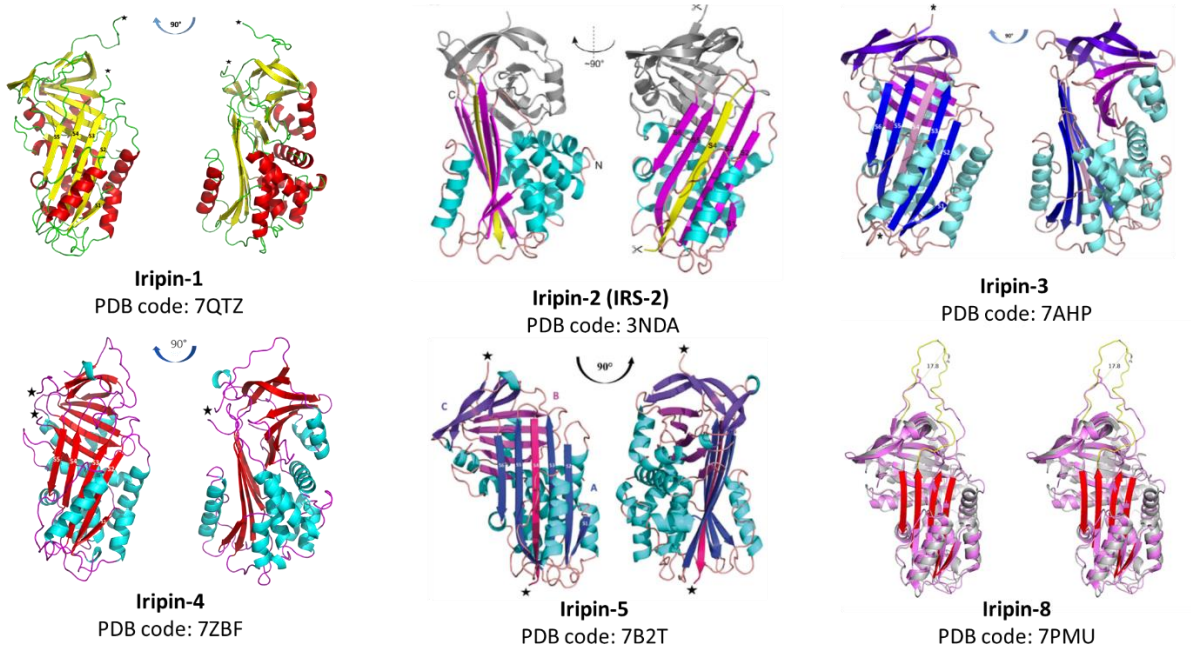


Figure 10. Structures of 6 serpins from *I. ricinus* solved by protein crystallography. [7, 9-11]. Structures of Iripin-1 and 4 were not published yet.

5.6.1. IRIS

The first tick serpin was described in *I. ricinus* and was named IRIS (Ixodes ricinus immunosuppressor) [88]. The original article, however, did not describe its sequence and did not mention that it is a serpin. It was selected as one of the upregulated transcripts in the salivary glands, as assessed by the comparison of cDNA libraries prepared from fed and unfed tick females [89]. IRIS displayed immunomodulatory activities, as it inhibited the proliferation of CD4+ T cells and the production of several proinflammatory cytokines [88]. IRIS seemed to be very good candidate for SAT factor, which is term for a salivary compound that facilitates the transmission of tick-borne pathogens. An abbreviation SAT stood originally for “saliva activated transmission”, but later was corrected to “saliva assisted transmission”, as it became clear that the transmission is not fully dependent on the saliva, but it is definitely facilitated by it. This work inspired the author of this thesis to follow the role of serpins in the tick-host interaction. However, the research on IRIS continued and several follow-up studies were published. IRIS was found to be not only immunomodulator, but also potent anti-hemostatic and anti-inflammatory serpin. Interestingly, the anti-inflammatory activity was shown to be independent on inhibitory properties of IRIS, which targeted predominantly elastase [90-92].

5.6.2. IRS-2

By using the sequence of IRIS, a radioactively labeled DNA probe was prepared and phage cDNA library from *I. ricinus* salivary glands was screened in order to see, whether there are similar molecules. Indeed, several novel serpins were discovered that were later named as Iripin-1, 2, 3, 4, and 8. Four of them were published in **article 2** [7].

IRS-2 extensively inhibited edema formation and the influx of neutrophils into the inflamed tissue and it primarily inhibited cathepsin G and chymase, while in higher molar excess, it affected thrombin activity as well. The inhibitory specificity was explained using the crystal structure, determined at a resolution of 1.8 Å (PDB code 3NDA). Moreover IRS-2 inhibited cathepsin G-induced and thrombin-induced platelet aggregation [7]. Later, the mechanism of activity was partially disclosed in **article 3**,

where it was shown that IRS-2 affects the differentiation of proinflammatory Th17 subset of CD4+ T cells [8].

5.6.3. Iripin-3

Iripin-3 was found to be a strong inhibitor of two serine proteases kallikrein and matriptase. In an *in vitro* setup, Iripin-3 was capable of modulating the adaptive immune response as evidenced by reduced survival of mouse splenocytes, impaired proliferation of $CD4^+$ T lymphocytes, suppression of the T helper type 1 immune response, and induction of regulatory T cell differentiation. This was the first observation of such effects for an individual tick salivary protein, because Th1/Th2 immunomodulation was previously observed only for whole saliva or salivary gland extracts [1]. Apart from altering acquired immunity, Iripin-3 also inhibited the production of interleukin-6 by lipopolysaccharide-stimulated bone marrow-derived macrophages and the extrinsic blood coagulation pathway. This is the first observation of this effect for a tick serpin. In addition to its functional characterization, we presented the crystal structure of cleaved Iripin-3 at 1.95 Å resolution (PDB code 7AHP). Iripin-3 proved to be a pluripotent salivary serpin with immunomodulatory and anti-hemostatic properties that could facilitate tick feeding via the suppression of host anti-tick defenses. Above mentioned effects were described in submitted **article 4** [9]. However, physiological relevance of Iripin-3 activities observed *in vitro* needs to be supported by appropriate *in vivo* experiments.

5.6.4. Iripin-5

Iripin-5 is the most highly expressed *I. ricinus* salivary serpin. It acts as a modulator of host defense mechanisms by impairing neutrophil migration, suppressing nitric oxide production by macrophages and altering complement functions, as described in an **article 5** [10]. In addition to functional data, this work's primary aim was to describe the dynamics of the interactions between Iripin-5 and target proteases. Work was based on crystal structure of Iripin-5 in a relaxed state, a typical and thermodynamically the most stable state of serpins and docking models with target proteases. The study provided also a comparative study with other *I. ricinus* serpins with different functions. These results showed that despite very similar 3D structure, the surface electrostatic potential differed a lot, pointing at the role of allosteric interaction in determining the serpin activity. The structure was deposited in the RCSB database under the PDB code 7B2T.

5.6.5. Iripin-8

Similarly to Iripin-3 and 5, Iripin-8 displayed blood-meal induced expression, which is highest in engorged nymphs and in the salivary glands of fully fed adult females. Iripin-8 inhibited proteases involved in the coagulation and blocked intrinsic pathway of coagulation cascade *in vitro*. Moreover, Iripin-8 inhibited the lysis of erythrocytes by complement and RNA interference in tick nymphs resulted in delayed feeding time. In collaboration with Prof. James Huntington from the Cambridge University, we resolved the crystal structure of Iripin-8 at 1.89 Å resolution. The structure revealed unusually long and rigid reactive center loop that is surprisingly conserved among several tick species. The P1 Arg residue is held in place, far from the serpin body by a conserved poly-Pro element on the P' side. The conservation of the RCL of Iripin-8 is intriguing and deserves more attention, as well as its unusual shape. Moreover, cavities that are able to bind small molecules, such as PEG from the crystallization buffer, were found in Iripin-8, including one deep cavity. This finding can have important functional relevance. Iripin-8 was the first crystal structure of tick serpin in native state and was deposited in RCSB database under the PDB code 7PMU. To conclude, Iripin-8 is a tick serpin with conserved reactive center loop that has strong anti-hemostatic features and may interfere with host innate immunity. These findings were published in **article 6** [11].

5.6.6. Iripin-1 and iripin-4

Data on these two salivary serpins were not published yet, but the manuscripts were already submitted to impacted journals. Iripin-1 inhibited primarily trypsin and further exhibited weaker inhibitory activity against kallikrein, matriptase, and plasmin. Iripin-1 significantly attenuated the migration of neutrophils and monocytes to inflamed peritoneal cavities in the mouse model of acute peritonitis despite enhancing the production of some proinflammatory chemokines. Presumably, the Iripin-1-mediated inhibition of neutrophil and monocyte recruitment might be a result of reduced activity of proteases that can facilitate cell migration and a decrease in the expression of cell surface molecules. These molecules are involved in the firm adhesion of leukocytes to endothelial cells and can affect their subsequent transmigration through the blood vessel wall into extravascular space. Finally, the tertiary structure of native Iripin-1 at 2.10 Å resolution was determined by employing the X-ray crystallography technique (PDB code 7QTZ). In conclusion, *I. ricinus* ticks could utilize Iripin-1 to modulate the host's inflammatory response and to increase blood flow to the feeding cavity via MCP-1-triggered histamine release from mast cells and basophils. Thus, Iripin-1 is a salivary anti-inflammatory serpin that contributes to tick immunomodulation of the host.

We do not know much about the function of Iripin-4, but we succeeded in the crystallization of both native and cleaved form (PDB codes 7ZBF and 7ZAS). The native structure was solved at 2.3 Å resolution and the structure of cleaved conformation at 2.0 Å resolution. Furthermore, structural changes during reactive-center loop transition from native to cleaved conformation were observed. In addition to this finding, we confirmed that the main substrate-recognition site for the inhibitory mechanism is represented by glutamate in the position 341. The presence of glutamate instead of typical arginine at the P1 recognition site for all structurally described *I. ricinus* serpins (7B2T, 7PMU and 7AHP) except tyrosine in IRS-2 P1 site (3NDA) would explain no protease inhibition of tested trypsin-like or chymotrypsin-like proteases. The only inhibition observed was against T cell protease granzyme B. Further research on Iripin-4 should focus on thorough functional analysis, as it function can be very different from other tick serpins.

CONCLUSION

This thesis is based on 6 original research articles and 5 reviews. It focuses on tick salivary serpins and their involvement in the host immunomodulation, but presents also broader picture of tick-host interaction and the research of tick salivary secretion in general. The thesis shows, how the high-throughput approach is employed in this research and how it can be combined efficiently with reverse genetic approach, in which the gene is known and its function is being disclosed by various experiments with recombinant protein. The research of tick salivary serpins presents them as pluripotent immunomodulators, anti-hemostatic and anti-inflammatory effectors and inhibitors of various host proteases and defense mechanisms.

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SUBMITTED ORIGINAL RESEARCH ARTICLES AND REVIEWS

Article 1

Insight into the sialome of the castor bean tick, Ixodes ricinus

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In this transcriptomic work, a foundation for further study of *I. ricinus* salivary secretion was built. Total of four cDNA libraries were produced from adult female ticks in different stages of the feeding cycle (unfed, one, four and seven days after attachment). 576 clones, randomely picked from phage libraries, were sequenced for each library, resulting in 2304 of sequences, out of which 1881 were considered as high quality and were further analysed by in house custom cDNA library annotation tools, developed by Prof. José Ribeiro in NIAID/NIH. Total of 1274 clusters, i.e. unique sequences were identified and annotated. It was found that there is significant upregulation in the expression of secreted proteins. These proteins were clustered in multigene families with tens of individual members. Major groups were described, including Basic tail secreted proteins with anti-platelet and anti-coagulant functions, Kunitz-domain protease inhibitors, Salp-15 group, lipocalins and other groups that were later functionally characterized. On the family of collagen like secreted proteins (CLSP), we showed sequential secretion of individual members of the same multigene family. This inspired an idea of salivary proteins redundancy and an evasion from host immune system by switching antigens, which was later described in review 2.

This work was the first high-throughput study performed on *Ixodes ricinus,* a European vector of medically important pathogens. Obtained transcriptomic data and cDNA libraries were later used for more detailed studies on individual proteins or multigene families, as the sequences served as templates for designing primers to clone full length transcripts. Whole study was performed before next generation sequencing was introduced, therefore by Sanger sequencing. This resulted in lower number of longer sequences.

Author's contribution:

Author designed and performed all experiments, analyzed the data and wrote a manuscript

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Research article

Insight into the sialome of the castor bean tick, *Ixodes ricinus*

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Abstract

Background: In recent years, there have been several sialome projects revealing transcripts expressed in the salivary glands of ticks, which are important vectors of several human diseases. Here, we focused on the sialome of the European vector of Lyme disease, *Ixodes ricinus*.

Results: In the attempt to describe expressed genes and their dynamics throughout the feeding period, we constructed cDNA libraries from four different feeding stages of *Ixodes ricinus* females: unfed, 24 hours after attachment, four (partially fed) and seven days (fully engorged) after attachment. Approximately 600 randomly selected clones from each cDNA library were sequenced and analyzed. From a total 2304 sequenced clones, 1881 sequences forming 1274 clusters underwent subsequent functional analysis using customized bioinformatics software. Clusters were sorted according to their predicted function and quantitative comparison among the four libraries was made. We found several groups of over-expressed genes associated with feeding that posses a secretion signal and may be involved in tick attachment, feeding or evading the host immune system. Many transcripts clustered into families of related genes with stage-specific expression. Comparison to *Ixodes scapularis* and *I. pacificus* transcripts was made.

Conclusion: In addition to a large number of homologues of the known transcripts, we obtained several novel predicted protein sequences. Our work contributes to the growing list of proteins associated with tick feeding and sheds more light on the dynamics of the gene expression during tick feeding. Additionally, our results corroborate previous evidence of gene duplication in the evolution of ticks.

Background

Hard ticks (family Ixodidae) are well known ecto-parasites of vertebrates, with worldwide distribution and high medical importance due to their extraordinary ability to transmit various disease agents. Among hard ticks, the genus *Ixodes* is one of the most important vectors of human diseases. Lyme borreliosis, human granulocytic anaplasmosis (ehrlichiosis) and babesiosis are the main diseases transmitted by *I. scapularis* and *I. pacificus* in North America. In addition to these diseases, *Ixodes ricinus* in Europe and *Ixodes persulcatus* in Asia transmit tick-borne encephalitis (TBE). An increase of TBE-virus (Flavi-

viridae) prevalence among *I. ricinus* ticks in middle Europe has been recorded during the last few decades and the findings of *I. ricinus* at higher altitudes (above 1000 m) suggest that it is spreading to new areas, which correlates with the spread of human TBE cases [1]. Ticks and tick-borne diseases are becoming a more important health issue and detailed knowledge of the interactions among the tick, host and pathogen is crucial for understanding the mechanisms of pathogen transmission. The knowledge of saliva components is the basis for further understanding of these interactions.

Tick saliva is a powerful mixture of hundreds of different proteins and other pharmacologically active molecules. The effects of tick saliva or SGE (salivary gland extract) on the host are well described in several reviews [2-4]. Hard ticks require an array of molecules to evade the host haemostatic and immune systems and for successful completion of feeding, which usually lasts for 7–9 days for *I. ricinus*. Tick saliva is able to inhibit all three components of the host haemostatic system: blood coagulation, platelet aggregation and vasoconstriction [3,5]. The innate immune response is altered by the impaired activation of complement, resulting in a decrease in chemokine production and subsequent inhibition of inflammation. Acquired immunity is affected as well; *in vitro* experiments showed the inhibition of T-cell proliferation after incubation with *Ixodes ricinus* saliva [6]. Salivary gland extract alters the production of many cytokines by immunologically responsive cells leading to immunosuppression [7]. The changes in the expression profile of different cytokines indicate a polarization from Th1 toward the Th2 branch of the immune response, which could be a disease-determining factor for tick-borne pathogens [8]. During the late eighties, it was discovered that tick saliva is able to facilitate the transmission of viruses to the host and to another co-feeding tick [9]. This phenomenon was called saliva activated transmission (SAT) and has since been revealed for additional pathogens such as *Borrelia spp*. [10].

During the past decade, there has been great progress toward the description of particular molecules responsible for the host immunomodulation. It was shown that tick saliva contains various protease inhibitors with anticoagulant activity [11-13], as well as anti-inflammatory and immunosuppressive activity [14,15]. Anti-inflammatory activity is a result of several pharmacologically active molecules, including prostaglandins [16,17], apyrase [18] and histamine-binding proteins belonging to the lipocalin family [19,20]. Additionally, immunosuppressive protein Salp15 was identified from *Ixodes* scapularis [21]. Moreover, Salp15 was identified as saliva activated transmission (SAT) factor that facilitates the establishment of *Borrelia burgdorferi* in the mammalian host [22]. Among other proteins, tick saliva contains a metalloprotease with fibrinolytic activity, involved in extracellular matrix remodeling [23], a group of anti-complement proteins [24,25] and an IL-2-binding protein [26]. Many of these proteins were discovered using a high-throughput approach [27] that opened a vast new area for tick research and helped reveal a large number of novel proteins with completely unknown function, as well as many homologous proteins. From this point of view, tick saliva is a rich source of medical compounds. As an example, the immunosuppressive tick salivary protein Salp 15 was shown to suppress the development of experimental asthma in mice [28] and, as an immunosuppressor, Salp 15 is believed to become useful during allogenic transplantation [29].

This work augments the existing knowledge of tick sialomes. High-throughput studies of tick salivary glands have been completed for several ticks, such as American species *I. scapularis*, *I. pacificus* and *Dermacentor andersoni* [30-33] or tropical tick *Amblyomma variegatum* [34]. This is the first high-throughput work on the European tick *Ixodes ricinus*, which is the most important disease vector in Europe. In this study, four cDNA libraries from salivary glands were constructed and 576 EST were sequenced and analyzed per library, resulting in a total of 2304 EST. The four libraries cover the main phases of an adult female tick feeding period: 1) unfed tick, 2) early phase of feeding (24 hours after attachment), 3) middle phase of feeding (4 days after attachment) and 4) late phase of feeding-engorged tick (7 days after attachment).

Although many proteins that we identified were already known from *I. scapularis* and *I. pacificus*, several novel putative proteins were discovered. Moreover, analysis of mRNAs expressed at four points in the blood meal enables us to gain insight into the dynamics of tick feeding, and indirectly sheds light on the evolution of tick genes and genome composition.

Results

Characterization of data

Four different salivary gland cDNA libraries from *I. ricinus* females were analyzed in this work. The following feeding stages were used for mRNA isolation: Unfed (IRUF), 24 hours after attachment (IR24H), four days (IR4D) and seven days (IR7D) after attachment to the host. From the total of 2304 sequenced EST, 1881 were considered of high quality (less than 5% of undetermined base calls, phred quality ≥ 20, at least 80 bases not including poly A). The length of analyzed sequences (after removal of vector sequence) ranged between 84 and 1253 bp with average length of 503 bp. The sequences were clustered and aligned resulting in 1274 clusters comprising 268 contigs with two or more sequences and 1006 singletons. The

high proportion of singletons is due to stringent analysis conditions rather than under-sampling; however, a larger dataset would be beneficial for statistic analysis. BLAST search was done for the consensus of each contig or singleton against the set of databases (NR, GO, KOG, CDD, Pfam, Smart, ACARI, rRNA and mitochondrial). The bioinformatic analysis was combined into a single Excel table where the clusters were manually annotated and sorted into functionally related groups based on the BLAST results from the various databases. Out of 1881 sequenced EST, approximately 30% (550) were identified as housekeeping genes due to a functional prediction and/or intracellular localization, and 32% (583) of ESTs contained a predicted signal for secretion from the cell suggesting their function in saliva (group of secreted genes). Several ESTs were 5' truncated, which restricted prediction by the SignalP server, yet had high similarity to secreted proteins on GenBank and were grouped with secreted genes. Forty-nine ESTs compiled in 37 clusters (3%) contained proteins that are conserved among different organisms but lack any functional prediction and were named 'unknown conserved'. The last largest group comprises 658 unknown transcripts (35%) without a predicted signal peptide and with no match to any database. There are three main reasons for such a high amount of unknowns and all of them probably participate in the resulting number: 1) Sequences contain only 5' untranslated region, 2) Truncated cDNA was cloned into the vector and only the part of 3' non-coding region was sequenced, and 3) Tick salivary glands contain many unknown and unique proteins with no similarity throughout all organisms. The group of unknowns was sorted for illustration between two groups, in accordance with the length of the open-reading frame; the threshold for the division was decided at the length 100 bp. Putative proteins similar to reverse transcriptases or transposases were classified as a single group. Although the protocol for cDNA library construction is designed for elimination of other than messenger-type of RNA, several ribosomal RNAs were sequenced, which is due to AT-rich profile of such transcripts. Main data characteristics are summarized and visualized in Figure 1.

Distribution of transcripts among libraries

All libraries contain a similar number of high-quality sequences ranging from 432 for IR7D to 492 for IR4D. Figure 2 shows the distribution of the main groups among all libraries. The distribution of different gene types varies markedly. There is a large increase in the expression of secreted proteins between IRUF and IR24H libraries, while in IR4D and IR7D the ratio of secreted proteins decreases. This trend results from the distribution of the three most abundant families of secreted proteins: Collagen-Like Secreted Proteins (CLSP), Basic Tail Secreted Proteins (BTSP) and proteins containing Kunitz domains (Kunitz). These three groups represent only 1.7% of all transcripts in IRUF, but 37.6% in IR24H, 20.6% in IR4D and 17.1% in IR7D library. We can assume that during the early phase of feeding, the tick utilizes a large amount of energy for the production of proteins that enable it to feed successfully despite the host defense system. During the first few days after attachment, it is mainly innate defense

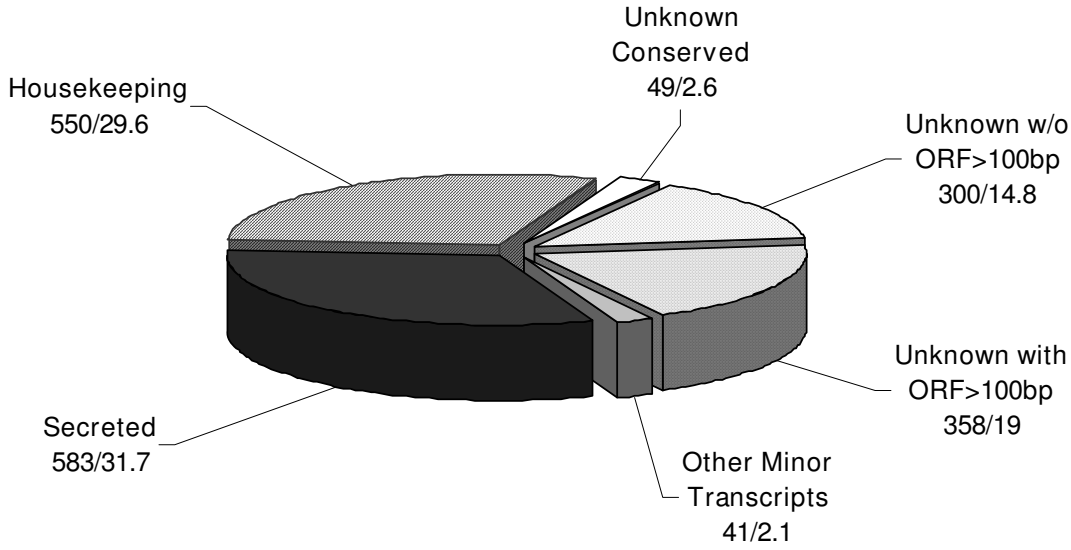

Figure 1

Representation of the main transcript types. Obtained ESTs were sorted into 6 main groups. Their representation in the obtained dataset is shown in Figure 1. Values represent EST number/% of EST.

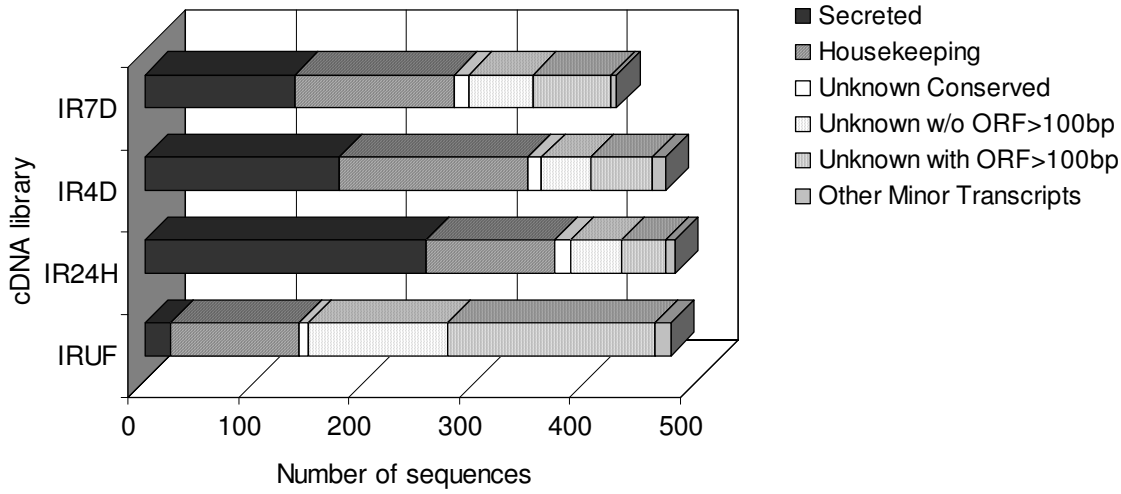

Figure 2
Transcript distribution throughout each analyzed cDNA library. IRUF – *Ixodes ricinus* unfed, IR24H – *I. ricinus* 24 hours after attachment, IR4D – *I. ricinus* 4 d after attachment, IR7D – *I. ricinus* 7 d after attachment.

that the tick has to fight against in a naïve host, and all three groups mentioned above could be involved in anti-haemostatic activity. During the later phases of feeding, the tick starts to digest and process the ingested meal while the site of attachment is already full of pharmacologically active molecules. The slight increase in production of housekeeping genes in the last two stages of feeding is in accordance with the need for higher metabolic activity during rapid growth of the salivary glands. However, at least two groups of secreted proteins appear to be produced in later phases of feeding. Histamine-binding proteins (HBP) and 4.9 kDa proteins are significantly overrepresented in the IR7D library (Table 3). The function of the 4.9 kDa group is completely unknown but HBP are known for their anti-inflammatory activity.

Quite interestingly, 66.8% (323 out of 483 EST) of the unknown proteins are present in the IRUF library. The reason could be the lack of data from unfed tick salivary glands as only cDNA libraries from unfed *Argas monolakensis* and *Amblyomma americanum* were published to date in the GenBank. The lack of a signal peptide in full-length clones suggests housekeeping function of these peptides. In order to identify proteins up-regulated by feeding, a comparison of the four libraries was conducted. Only 38 contigs out of 1274 contain five or more ESTs, the threshold for statistical evaluation (Table 1); therefore, we conducted statistical analysis on both individual contigs and whole classes of peptides. While housekeeping genes were sorted into functional categories, classification of secreted proteins was based on their primary structure similarity because exact function is not usually known. The distribution and statistic evaluation of both housekeeping and secreted proteins are shown in Table 2 (housekeeping) and Table 3 (secreted). Accession numbers for the GenBank EST database in the following sections always represent the largest clone of each mentioned contig. All additional sequences, assembled contigs and other data from the analysis can be downloaded [see Additional file 1].

Housekeeping genes

The group of housekeeping genes was divided among more detailed functional subgroups. The subgroups comprise a large number of diverse proteins with diverse intracellular functions (Table 2). As expected, proteins involved in energetic and nutrient metabolism, such as compounds of the respiratory chain, various ATPase subunits or enzymes participating in the metabolism of carbohydrates, amino acids and lipids, are well represented in the dataset, comprising 217 out of a total 1881 ESTs (11.5%). Their expression increases during feeding, probably due to higher metabolic requirements during digestion of the blood meal (Table 2). For the sake of simplification six proteins were compiled together in a group labeled metabolism. The group (metabolism) includes proteins with various metabolic roles. There are two proteins involved in defense and detoxification. Two ESTs coding for dopachrome-tautomerase, the key enzyme in the process of melanization [35] were detected in the IR4D library (Contig 259, EY199700). Another protein, possibly involved in detoxification, is a peptide similar to nicotinamide N-methyltransferase and is represented by only one EST in IR7D library (Contig 946,

Table 1: Contigs with more than 5 ESTs and their distribution among the four libraries

				IRUF			IR24H			IR4D			IR7D		
						p			*p*			*p*			*p*
Contig	**Comments**	**Class**	**EST**	**obs**	**exp**	**IRUF/IR24H**	**obs**	**exp**	**IR24H/IR4D**	**obs**	**exp**	**IR4D/IR7D**	**obs**	**exp**	**ALL**
83	cytochrome oxidase 3	h/meten	23	1	5.91	**0.004**	1	6.02	**0.001**	12	5.80	**0.002**	9	5.28	**0.000**
78	cytochrome oxidase subunit 1	h/meten	22	0	5.65	**0.004**	2	5.75	0.060	8	5.54	**0.001**	12	5.05	**0.000**
88	cytochrome c oxidase subunit II	h/meten	17	6	4.37	0.070	1	4.45	0.101	4	4.28	0.285	6	3.90	0.215
89	ATP synthase F0 subunit 6	h/meten	16	1	4.11	0.062	2	4.19	0.241	5	4.03	**0.021**	8	3.67	**0.031**
41	collagen-like secreted protein	s/CLSP	14	0	3.59	**0.000**	10	3.66	**0.000**	1	3.53	0.177	3	3.22	**0.001**
106	elongation factor-1alpha	h/ps	14	3	3.59	0.719	4	3.66	0.184	6	3.53	0.071	1	3.22	0.332
46	collagen-like secreted protein	s/CLSP	12	0	3.08	**0.000**	11	3.14	**0.000**	1	3.02	**0.043**	0	2.76	**0.000**
87	16S mitochondrial RNA	rRNA	12	3	3.08	0.519	2	3.14	0.192	5	3.02	0.221	2	2.76	0.588
117	Monolaris II group	s/Kunitz	11	0	2.82	**0.000**	11	2.88	**0.000**	0	2.77	**0.021**	0	2.53	**0.000**
49	collagen-like secreted protein	s/CLSP	11	0	2.82	**0.036**	5	2.88	0.208	3	2.77	0.743	3	2.53	0.210
116	putative 19 kDa secreted protein	s/19	10	0	2.57	**0.000**	10	2.62	**0.000**	0	2.52	**0.028**	0	2.30	**0.000**
66	basic tail secreted protein	s/BTSP	10	0	2.57	**0.029**	5	2.62	0.131	2	2.52	0.570	3	2.30	0.165
56	collagen-like secreted protein	s/CLSP	8	0	2.05	**0.042**	0	2.09	**0.000**	8	2.02	**0.000**	0	1.84	**0.000**
42	collagen-like secreted protein	s/CLSP	8	0	2.05	**0.042**	0	2.09	0.148	2	2.02	**0.002**	6	1.84	**0.003**
23	collagen-like secreted protein	s/CLSP	7	0	1.80	0.057	0	1.83	0.058	0	1.76	**0.000**	7	1.61	**0.000**
48	collagen-like secreted protein	s/CLSP	7	0	1.80	**0.000**	7	1.83	**0.000**	0	1.76	0.066	0	1.61	**0.000**
63	basic tail secreted protein	s/BTSP	7	0	1.80	**0.001**	6	1.83	**0.002**	1	1.76	0.164	0	1.61	**0.004**
68	basic tail secreted protein	s/BTSP	7	0	1.80	**0.001**	6	1.83	**0.002**	1	1.76	0.164	0	1.61	**0.004**
118	cytochrome b	h/meten	7	0	1.80	0.178	2	1.83	0.182	0	1.76	**0.003**	5	1.61	**0.013**
85	16S mitochondrial RNA	rRNA	7	1	1.80	0.294	3	1.83	0.204	3	1.76	0.116	0	1.61	0.310
130	60S ribosomal protein L22	h/psrp	7	3	1.80	0.365	2	1.83	0.182	0	1.76	0.173	2	1.61	0.442
14	collagen-like secreted protein	s/CLSP	6	0	1.54	0.078	0	1.57	**0.000**	6	1.51	**0.000**	0	1.38	**0.000**
134	60S ribosomal protein L3	h/psrp	6	6	1.54	**0.000**	0	1.57	0.079	0	1.51	0.089	0	1.38	**0.001**
13	collagen-like secreted protein	s/CLSP	6	0	1.54	**0.003**	5	1.57	**0.003**	0	1.51	0.204	1	1.38	**0.014**
5	collagen-like secreted protein	s/CLSP	6	0	1.54	0.078	0	1.57	0.189	2	1.51	**0.023**	4	1.38	**0.041**
133	40s ribosomal protein S27	h/psrp	6	4	1.54	**0.019**	0	1.57	0.189	2	1.51	0.215	0	1.38	0.070
159	5.3 kDa secreted protein	s/4.9	5	0	1.28	0.107	0	1.31	0.109	0	1.26	**0.000**	5	1.15	**0.001**
64	basic tail secreted protein	s/BTSP	5	0	1.28	**0.001**	5	1.31	**0.001**	0	1.26	0.121	0	1.15	**0.003**
7	collagen-like secreted protein	s/CLSP	5	0	1.28	**0.001**	5	1.31	**0.001**	0	1.26	0.121	0	1.15	**0.003**
137	NADH dehydrogenase 3	h/meten	5	0	1.28	0.244	1	1.31	0.248	0	1.26	**0.004**	4	1.15	**0.021**
24	collagen-like secreted protein	s/CLSP	5	0	1.28	0.244	1	1.31	**0.014**	4	1.26	**0.008**	0	1.15	**0.037**
158	basic tail secreted protein	s/BTSP	5	2	1.28	0.191	0	1.31	0.054	3	1.26	0.059	0	1.15	0.153
50	collagen-like secreted protein	s/CLSP	5	0	1.28	0.199	2	1.31	0.096	3	1.26	0.059	0	1.15	0.157
142	Monolaris II group	s/Kunitz	5	0	1.28	0.199	2	1.31	0.096	3	1.26	0.059	0	1.15	0.157
157	60S ribosomal protein L7A	h/psrp	5	3	1.28	0.058	0	1.31	0.187	2	1.26	0.208	0	1.15	0.158
154	60S ribosomal protein L31	h/psrp	5	3	1.28	0.124	1	1.31	0.248	0	1.26	0.258	1	1.15	0.301
151	40S ribosomal protein S12	h/psrp	5	2	1.28	0.492	1	1.31	0.476	2	1.26	0.208	0	1.15	0.560
131	unknown	uk	5	2	1.28	0.382	2	1.31	0.202	0	1.26	0.258	1	1.15	0.562

P values show significance of expression difference between two libraries; *P* – ALL shows which contig has random and non-random distribution. Significant values ($P \leq 0.05$) are marked in bold. Obs – observed, Exp – expected, h/– housekeeping, s/– secreted, 19 – 19 kDa protein, 4.9 – 4.9 kDa protein, BTSP – basic tail secreted protein, CLSP – collagen-like secreted protein, Kunitz – Kunitz domain containing protein, meten – energy metabolism, ps – proteosynthesis, psrp – proteosynthesis ribosomal protein, rRNA – ribosomal RNA, uk – unknown.

EY200443). This enzyme is engaged in N-methylation of nicotinamide and other pyridines to form pyridinium ions. Its activity is important for biotransformation of many drugs and xenobiotic compounds in humans and other mammals. Its function in ticks or other arthropods is not clear; moreover, there is no previous report of a nicotinamid N-methyltransferase in any arthropod species. Although the cDNA is truncated, the e-value (2.10^{-5}) is convincing and BLASTp search detected a methyltransferase domain; thus we can assume that the 33% identity and 57% similarity between contig 946 and human nicotinamide N-methyltransferase (NP_006160) strongly suggest the homology between tick and human proteins. Other proteins with various metabolic activities included

Table 2: Distribution of groups of housekeeping genes among the four libraries

			IRUF			IR24H			IR4D			IR7D		
					p			*p*			*p*			
Name of group	**Contigs**	**EST**	**Obs**	**Exp**	**IRUF/IR24H**	**Obs**	**Exp**	**IR24/IR4D**	**Obs**	**Exp**	**IR4D/IR7D**	**Obs**	**Exp**	**ALL**
Metabolism energy	72	184	19	47.25	**0.000**	28	48.13	**0.000**	60	46.37	**0.000**	77	42.26	**0.000**
Protein synthesis – ribosomal proteins	82	166	49	42.63	0.293	46	43.42	0.240	49	41.83	**0.005**	22	38.12	**0.018**
Protein modification and degradation	32	36	5	9.24	0.069	13	9.42	0.222	8	9.07	0.484	10	8.27	0.275
Protein synthesis	11	27	8	6.93	0.381	5	7.06	0.252	9	6.80	0.332	5	6.20	0.630
Transcription mechanism	21	24	9	6.16	0.238	7	6.28	0.378	4	6.05	0.292	4	5.51	0.470
Signal transduction	20	20	8	5.14	**0.025**	1	5.23	0.058	6	5.04	0.640	5	4.59	0.151
Metabolism carbohydrates	14	17	1	4.37	**0.047**	2	4.45	**0.001**	11	4.28	**0.001**	3	3.90	**0.002**
Cytoskeleton related proteins	10	14	4	3.59	0.782	4	3.66	0.405	2	3.53	0.356	4	3.22	0.816
Intracellular trafficking mechanism	12	12	1	3.08	0.113	5	3.14	**0.045**	6	3.02	**0.017**	0	2.76	**0.041**
Nuclear structure related proteins	10	12	4	3.08	0.475	4	3.14	0.207	1	3.02	0.241	3	2.76	0.594
Metabolism	6	8	2	2.05	0.450	1	2.09	0.450	2	2.02	0.391	3	1.84	0.726
Metabolism – aminoacids	6	8	2	2.05	0.148	0	2.09	**0.044**	4	2.02	0.161	2	1.84	0.253
Metabolism – lipids	7	8	2	2.05	0.941	2	2.09	0.473	1	2.02	0.264	3	1.84	0.739
Cell Cycle	4	5	0	1.28	0.107	0	1.31	0.187	2	1.26	0.064	3	1.15	0.110
Metabolism – nucleic acids	5	5	1	1.28	0.242	0	1.31	0.054	3	1.26	0.120	1	1.15	0.284
Metabolism – ionts	4	4	1	1.03	0.306	0	1.05	**0.026**	3	1.01	**0.028**	0	0.92	0.116

P values show significance of expression difference between two libraries; *P* – ALL shows which contig has random and non-random distribution. Significant values are ($P \leq 0.05$) are marked in bold. Obs – observed, Exp – expected.

in this group are those involved in coenzyme transport and metabolism or synthesis of secondary metabolites (Contig 323, EY200796; 543, EY199519; 815, EY200185; and 825, EY200205).

The group of proteins involved in post-translational modification and protein degradation contains various heat-shock proteins, proteases, subunits of proteasome machinery and glycosyltransferases, among others. Proteosynthetic proteins involved in translation, including ribosomal proteins, represent 10.3% of the total ESTs (n = 193 EST). There is a significant decrease in expression of ribosomal proteins between IR4D and IR7D. This suggests that there is a decrease in the production of salivary proteins during the final phase before detachment, which is supported by the putative expression profile of secreted proteins (Figure 2).

The group of cytoskeletal proteins is represented by actin, actin-related proteins, myosin, dynein, alpha tubulin, collagen precursor and a protein similar to microtubule-binding protein called translationally-controlled tumor protein (TCTP). TCTP functions in chromosome partitioning during the cell division [36], but it also stimulates the release of histamine by basophils [37]. Homologues of TCTP were found in other ixodid ticks and are referred to as histamine release factors (HRF). One of them was detected in the saliva of *Dermacentor variabilis*, despite the lack of a putative signal peptide. In the same work recombinant tick TCTP/HRF proved its histamine releasing features [38]. The *I. ricinus* TCTP/HRF transcript is truncated at the 5' region, but its high homology with *I. scapularis* (e-value = 7e-86), which lacks a signal peptide, suggests that the signal peptide is not present in *I. ricinus* TCTP/HRF as well. TCTP/HRF seems to be expressed constitutively and independently during feeding [39]. In light of the fact that the only transcript found in our library originates from unfed ticks (Contig 999, EY200552) and that TCTP/HRF proteins are well conserved among different organisms, we can assume that the main function of tick TCTP/HRF homologue is a function of tick physiology although the presence of TCTP/HRF in *D. variabilis* saliva suggests some function in the host.

Proteins associated with the transport and metabolism of ions create a single group with four singletons. The group contains manganese superoxide dismutase (Contig 660, EY199845), CutA1 divalent ion tolerance protein homologue (Contig 744, EY200030), ferritin (Contig 590, EY199666) and protein similar to Rhodanese-related sulfur transferase (Contig 1131, EY200876). Superoxide dismutase catalyses the dismutation of superoxide into oxygen and hydrogen peroxide and functions as an important defense against superoxide radicals [40]. CutA1 divalent ion tolerance protein is found throughout all organisms and is involved in tolerance to divalent ions such as copper or iron ions. This is the first reported CutA1 from the ticks, where it may be important in tolerance to iron ions from the blood meal. Another protein connected to iron transport and deposition is ferritin, which is expressed in all tick tissues [41]. Ferritin is a cytosolic protein usually composed of 24 subunits and is involved in the storage of ferric ions. It was shown that *Ornithodoros moubata* ferritin is expressed constitutively

and independently upon feeding in the midgut [42]. *Ixodes ricinus* salivary ferritin is represented by only one EST in IR4D library (Contig 590). Proteins involved in signal transduction and intracellular trafficking create two functionally related groups where proteins associated with G proteins, receptors, ion channels associated proteins and various transporters, among others, can be found. Contigs 294 (EY200035), 295 (EY200423), 299 (EY200084) and 802 (EY200163) were found in a group of cell cycle proteins and are most likely involved in the cell division cycle, however concrete functions remain unknown.

Reverse transcriptase-like proteins

Besides housekeeping and secreted salivary proteins there are several transcripts similar to reverse transcriptases (RT) or reverse transcriptase-like proteins from other arthropods, mainly insects. It is interesting that 10 out of 11 ESTs similar to RT were found in the unfed tick library and only one originates from IR7D library, as though such mechanisms are suppressed during feeding. Additionally, eight ESTs are similar to RT only as reverse complement. The presence of anti-sense transcripts has been previously reported in eukaryotes and their regulative function on the transcription process has been proposed [43-45]. One clone (Contig 1006, EY200559) shows 51% similarity to a transposase from *Danio rerio* (CAK05416), and contig 255 (EY199575) displays 50% similarity to a mariner-like transposase (2124399A). The presence of these genes suggests an ongoing process of transposition in the tick genome. This mechanism could be responsible for the high duplication rate of some multigenic families. Another explanation is a lysogenic viral origin of these proteins.

Putative secreted genes

The group of secreted genes includes many of the transcripts with predicted signal peptide and truncated transcripts with high similarity to secreted proteins from other tick species. Secreted proteins (both predicted and determined by BLAST) represent 32% of all ESTs. From the distribution among the libraries, it is obvious that the number of secreted protein transcripts is dramatically increased by feeding. There is an 11-fold increase in secreted molecules in the IR24H library compared to the IRUF library. During the last two stages of feeding the proportion of secreted proteins decreases while the number of housekeeping genes, mainly involved in energy and nutrient metabolism, is slightly higher (Figure 2). Table 3 shows the most abundant secreted groups with more than 5 ESTs and their distribution among the libraries.

Collagen-like secreted proteins (CLSP)

The most abundant group in the combined library dataset comprises 209 ESTs within 71 contigs. Collagen-like secreted proteins are significantly upregulated by feeding, as only one contig with two ESTs was found in the IRUF library. The CLSP are small proline- and glycine-rich peptides with mature molecular mass ranging from 4.5 to 5.5 kDa in *I. ricinus*. In *I. pacificus* even smaller peptides (3.6 kDa) were found [31]. The name of the group refers to the high ratio of proline and glycine amino acid residues found in collagen. The presence of several X-Pro-Gly motifs points on the possibility of proline hydroxylation to hydroxyproline, which is responsible for collagen fibers production [46]. Hypothetically, CLSP may be able to create polypeptide chains at the site of attachment and function as the attachment glue or could affect the function of collagen in the interactions among cells, ligands and matrix [31,32]. Comparison of CLSP from *I. ricinus* with homologues from *I. scapularis* and *I. pacificus* reveals four

Table 3: Distribution of the most abundant secreted genes (≥ 5 EST) among the four libraries

			IRUF			IR24H			IR4D			IR7D		
Name of group	Contigs	EST	obs	exp	IRUF/IR24H	*p* obs	exp	IR24H/IR4D	*p* obs	exp	IR4D/IR7D	*p* obs	exp	ALL
CLSP	71	209	2	53.67	**0.000**	104	54.67	**0.000**	62	52.67	0.102	41	48.00	**0.000**
BTSP	46	95	6	24.39	**0.000**	48	24.85	**0.000**	17	23.94	0.135	24	21.82	**0.000**
Kunitz domain	30	61	0	15.66	**0.000**	33	15.96	**0.000**	19	15.37	0.104	9	14.01	**0.000**
18.7 kDa	11	20	2	5.14	**0.001**	12	5.23	**0.003**	6	5.04	**0.029**	0	4.59	**0.001**
Metalloproteases	13	18	3	4.62	0.276	3	4.71	0.409	4	4.54	0.055	8	4.13	0.185
WC proteins	15	18	0	4.62	**0.031**	5	4.71	0.244	7	4.54	0.140	6	4.13	0.075
Ixodegrins	8	15	0	3.85	**0.000**	10	3.92	**0.002**	4	3.78	0.186	1	3.44	**0.002**
19 kDa	4	14	0	3.59	**0.000**	13	3.66	**0.000**	1	3.53	**0.025**	0	3.22	**0.000**
HBP	10	10	0	2.57	0.059	1	2.62	0.172	4	2.52	**0.044**	5	2.30	0.054
ISAC-like	7	7	0	1.80	0.178	2	1.83	0.348	3	1.76	0.327	2	1.61	0.431
Prokineticin domain	7	7	0	1.80	0.111	3	1.83	0.378	2	1.76	0.721	2	1.61	0.455
Salp15-like	7	7	0	1.80	**0.037**	4	1.83	0.089	1	1.76	0.514	2	1.61	0.194
6.78 kDa	4	6	0	1.54	0.198	2	1.57	0.208	3	1.51	0.210	1	1.38	0.360
4.9 kDa	1	5	0	1.28	0.107	0	1.31	0.109	0	1.26	**0.000**	5	1.15	**0.001**

P values shows significance of expression difference between two libraries; *P* – ALL shows which contig has random and non-random distribution. Significant values are ($P \leq 0.05$) are marked in bold. Obs – observed, Exp – expected.

major clades, one of which is exclusively found in *I. ricinus*, one is exclusively found in *I. scapularis* and the two other clades include transcripts from all three *Ixodes* species (Figure 3). The distribution of ESTs from this multigenic group among libraries implies the sequential expression of paralogs during the feeding process. Table 4 illustrates the temporal distribution of the CLSP clusters with more than 5 ESTs, their best match to the Acari database and distribution among the libraries. Some genes are expressed throughout all feeding stages after attachment while other genes appear to be expressed strictly within a specific feeding phase. There are two distinct major groups of CLSP that differ mainly in the length of C terminal region and create two distinct clades on the phylogram as shown in Figures 3 and 4. These groups likely originated from multiplication of two homologs. Others transcripts, scattered throughout the tree in Figure 3 diverged before the speciation of genus *Ixodes*. Alignment of CLSP contigs with ≥ 5 ESTs, their relationship and the library of contig origin are shown in Figure 4. The pattern of gene expression shows different timing for very similar peptides from the same clade. These proteins possibly play the same role in the tick or the host during different feeding periods. One possibility could be an antigenic shift in the most abundant proteins. *Ixodes ricinus* is a long term blood feeder; therefore, a specific immune response is raised against the tick by the host. The humoral branch of specific immune response is based on the elaboration of specific antibodies by B lymphocytes. The production of antibodies is activated by tick saliva that contains many different antigens. It is possible that antibodies raised against CLSP expressed during the early stages of feeding can be ineffective against CLSP that are produced later.

Mechanisms of antigenic variability are well described for protozoan parasites and spirochetes [47], yet only one example has been reported for metazoan parasites thus far [48]. Antigenic variability is usually associated with an increased genome size and lower genome complexity, often due to recombination events [47]. This is the case of the parasitic protozoa in which the genome is much larger compared with their free-living relatives. Hard ticks possess almost twice as large genome as soft ticks and the complexity of hard tick genome appears to be much lower compared with soft ticks [49]. It is unknown if multigenicity and high recombination rates in hard ticks are related to antigenic variation as stated above or if it is the need for fast production of large amounts of immunoactive and other feeding-associated peptides. This question undoubtedly deserves further investigation.

Basic tail secreted proteins (BTSP)

Secreted proteins rich for lysine residues at the C terminus create the second most abundant peptide family with 95 ESTs in 46 contigs (5.1%). This group is well represented in both *I. scapularis* and *I. pacificus* salivary transcriptomes [31,32]. Anticoagulant Salp14 (AAY66785), which was found in *I. scapularis* and has been shown to inhibit factor Xa [13], is a member of the BTSP family suggesting an anticoagulant role for the whole family. The anticoagulation function of the BTSP family is also supported by the fact that positively-charged proteins can interfere with negatively-charged membranes of activated platelets [31]. The timing of protein expression of the BTSP related ESTs is similar to that of the CLSP family; up-regulation by feeding and then subsequent decrease between the IR24H and IR4D time points (Table 3). The phylogram in Figure 5 shows all BTSP sequences from the three *Ixodes* species. We can see a pattern similar to the CLSP group where each species creates its own clade. This suggests very fast gene duplication after speciation.

Table 4: The distribution of Collagen-Like Secreted Proteins (CLSP) with more than 5 EST among the libraries

Contig	Best match to ACARI db [gb accession nr.]	e-value	EST	IRUF	IR24H	IR4D	IR7D	*p*
5	CLSP11 [AAT92166.1]	3E-026	6	0	0	2	4	**0.041**
7	Putative secreted protein [AAY66696.1]	4E-024	5	0	5	0	0	**0.003**
13	CLSP11 [AAT92166.1]	8E-028	6	0	5	0	1	**0.014**
14	CLSP11 [AAT92166.1]	8E-028	6	0	0	6	0	**0.000**
23	CLSP11 [AAT92166.1]	9E-026	7	0	0	0	7	**0.000**
24	CLSP11 [AAT92166.1]	2E-027	5	0	1	4	0	**0.037**
41	Putative secreted protein [AAM93621.1]	3E-028	14	0	10	1	3	**0.001**
42	Putative secreted protein [AAM93621.1]	2E-029	8	0	0	2	6	**0.003**
46	Putative secreted protein [AAM93621.1]	2E-031	12	0	11	1	0	**0.000**
48	Putative secreted protein [AAM93621.1]	1E-031	7	0	7	0	0	**0.000**
49	Putative secreted protein [AAM93622.1]	2E-030	11	0	5	3	3	0.210
50	Putative secreted protein [AAM93622.1]	2E-031	5	0	2	3	0	0.157
56	CLSP1 [AAT92135.1]	1E-028	8	0	0	8	0	**0.000**

Difference in expression is represented by *P* value, significant difference is marked in bold.

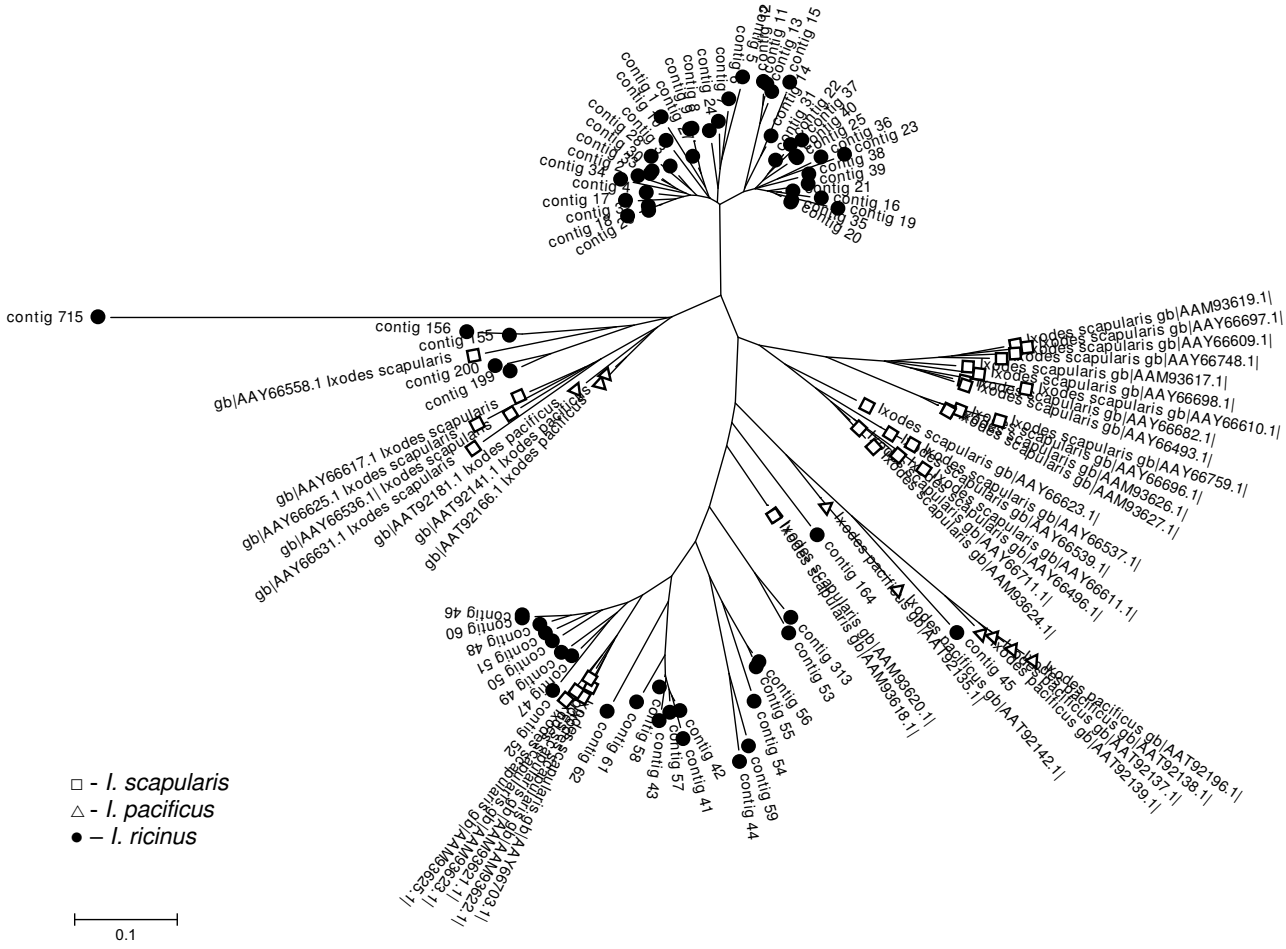

Figure 3
Colagen-like secreted proteins (CLSP). Unrooted tree of CLSP family based on mature protein sequences and created by NJ algorithm. Only full-length sequences are included.

Peptides containing Kunitz domain

Kunitz-type domains are present mainly in inhibitors of trypsin and trypsin-like serine proteinases as chymotrypsin, kallikrein or plasmin. Another type of Kunitz-domain peptides lacking inhibitory properties are toxins from snake venom called dendrotoxins [50]. We found 31 clusters comprising 62 sequences of Kunitz domain-containing peptides expressed only in the stages after attachment. The expression of Kunitz proteins is highest during the first day after attachment (34 sequences) and then decreases throughout the next stages to 11 ESTs on the seventh day after attachment. Among the contigs containing Kunitz sequences, contig 117 was the most abundant with 11 ESTs found in the IR24H library.

Twenty six contigs contain a single Kunitz-domain peptide with the general cysteine framework Xn-**C**-X8-**C**-X(16/18)-**C**-X5-**C**-X12-**C**-X3-**C**-Xn, similar or identical to monolaris II, according to nomenclature used by Francischetti et al. [31]. Within the monolaris II group, there are 11 very similar contigs containing a conserved SMGRL motif in the signal peptide cleavage site. An identical motif is also present in *I. scapularis* homologous group, suggesting common ancestral gene for both *I. ricinus* and *I. scapularis* groups. The phylogram in Figure 6 contains only full-length monolaris II sequences. Sequences from *I. ricinus* are scattered among sequences from *I. scapularis* and *I. pacificus* creating one large group (containing the SMGRL motif) that is common for *I. scapularis* and *I. ricinus*. The subgroup containing the SMGRL motif displays higher polymorphism than other Kunitz peptides. Contigs 771 (EY200101), 792 (EY200139) and 302 (EY200141) show similarity to tissue factor pathway inhibitor (TFPI) and contain Xn-**C**-X(7/9)-**C**-X15-**C**-X(5/7)-**C**-X12-**C**-X3-**C**-Xn framework, which resembles the monolaris III subgroup. Contig 400 contains two Kunitz

```
                  10        20        30        40        50
          ....|....|....|....|....|....|....|....|....|....|.
contig_7  QLYGSSTPCPGGPGQPCNSG-P-GGQGPSNPP--NQPGSRDPQAPGSSRK
contig_5  DLHGSTTPCPGGPGQPCNSG-P-GGQGPSEQP--RQPGTHSPQAPGSSSK
contig_13 QLYGSSTPCPGGPGEPCDSA-P-GPQGPSNPS--HQPGTHGPEAPGSSRK
contig_14 DSYGSSTPCPGGPGEPCDSA-P-GPQGPSNPP--HQPGTRSPQAPGSSRS
contig_23 DSYGNSTPCTGGPGQPCDSA-P-GPQGPSNQP--QQPGTHSPHAPGSSSK
contig_24 DSYGSSTPCPNGPGQPCNSG-P-GGQGPSEQP--RQPGTRNPEAPGSSRK
contig_41 DTYGSPTPCPGAPGQPCGNGNPPGAPAPSSGPNVHQPQDTSP--PG--RK
contig_42 ETYGSPTPCPGAPGQPCGNGNPPGAPAPGHPPNVHPPQNTSP--PG--RK
contig_46 TSWGSSTPCPGAPGEPCDNGRPSKPSAPGSGPNIRQPQNTSP--PG--RK
contig_48 SSYGSSTPCPGAPGEPCDNGRPSKPSAPGSGPNVHQPQNTSP--PG--RK
contig_49 TSYGSSTPCPGAPGESCDNGQPSQPSAPGSGPNIHPPPNTSP--PG--RK
contig_50 ESWGSSTPCPGAPGEPCDNGRPSQPSAPGSGPNVQQPPNTSP--PG--RK
contig_56 ETWGSSTPCPSPPGEPCGNGNPPGGPVQGSQPSPQPPLDTSP--PG--RK
```

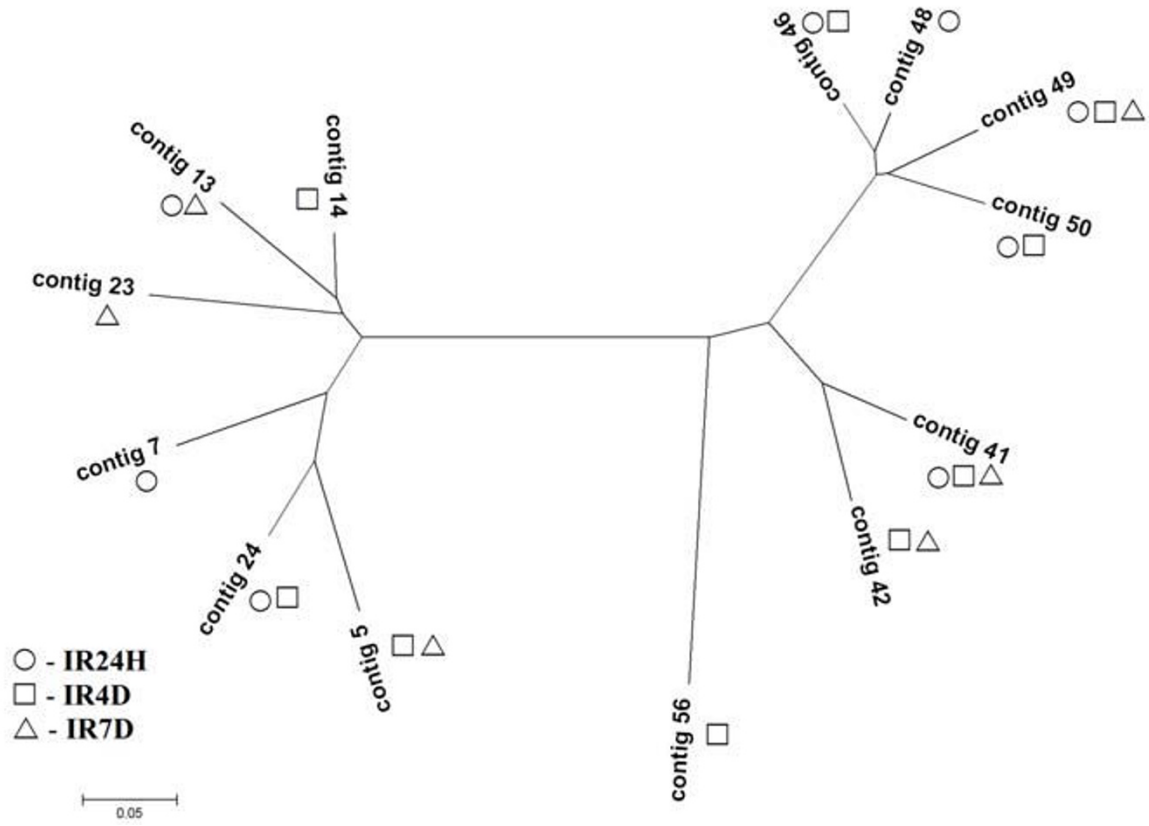

Figure 4

Collagen-like secreted proteins with ≥ 5 EST; alignment and expression profile. Alignment of CLSP contigs with more than 5 EST and graphical visualization of their representation in the libraries. Exact EST number of each contig is stated in Table 4.

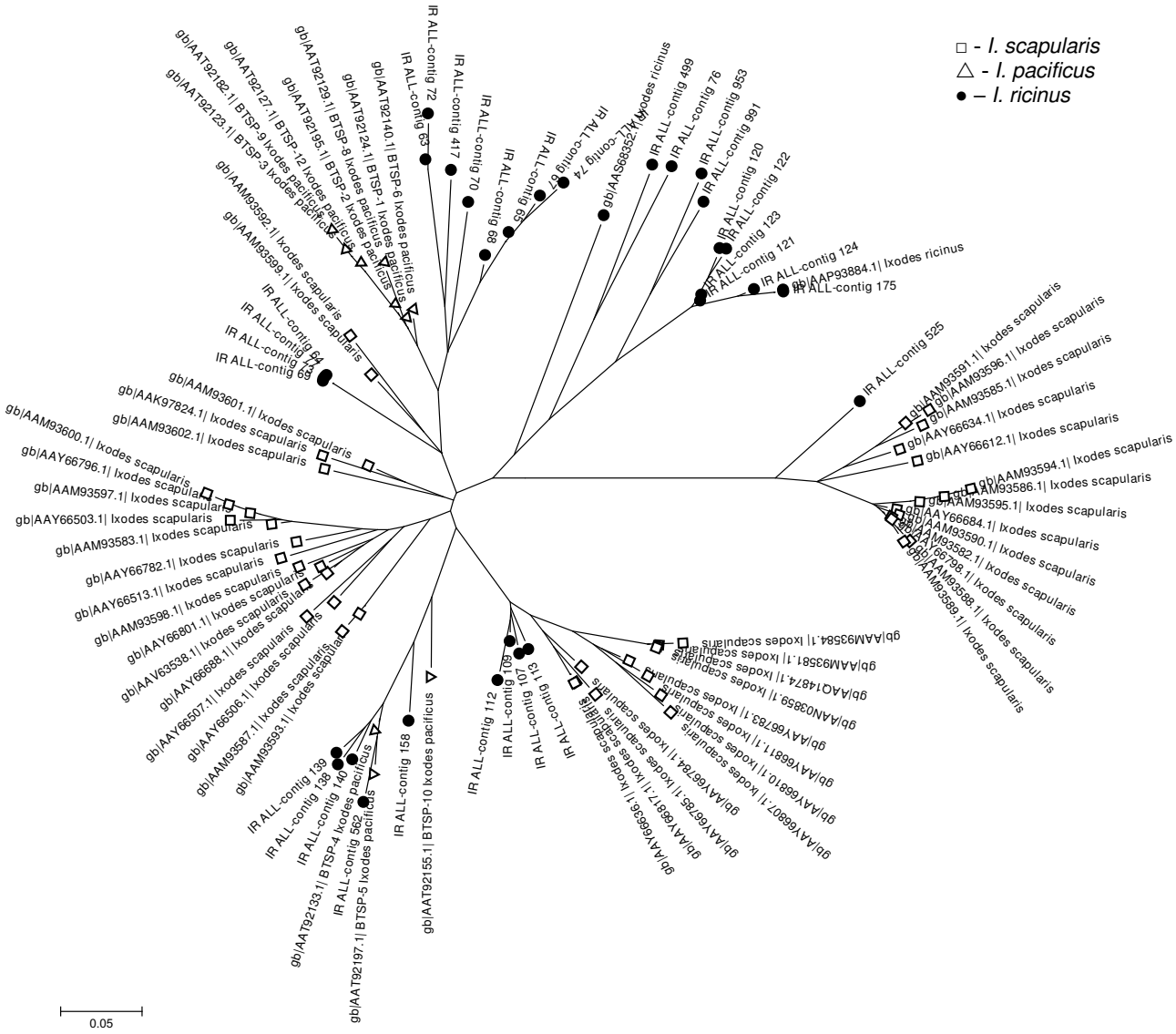

Figure 5
Basic tail secreted proteins (BTSP). Unrooted tree of BTSP family based on protein sequences and created by NJ algorithm. Only full-length sequences are included.

domains and shows high similarity to *I. pacificus* Ixolaris-2 (AAT92212). One EST (contig 807, EY200173) is related to the five-domain (penthalaris) Kunitz proteins. Although it is not a full-length clone, similarity to penthalaris group is evident (81% identity with *I. scapularis* sequence, AAY66743; e-value = 4e-86). The rate of gene duplication appears to be slower in the Kunitz-domain groups compared with the BTSP and CLSP groups, in that each species chose two or three genes for rapid multiplication. We can presume that the Kunitz-domain group diverged before speciation of the genus *Ixodes*.

Kunitz domain-containing proteins function as inhibitors of serine proteases. Serine proteases can act as inhibitors of the blood clotting and coagulation systems. It has been shown that Ixolaris and Penthalaris inhibit the tissue factor pathway of blood clotting [11,12]. Monolaris subgroups can also play a role in anti-clotting and anti-coagulation activity, but such activity has not been proved thus far [32]. Another function could be deduced from the similarity with snake venom dendrotoxins that function as K^+ channel blockers [50].

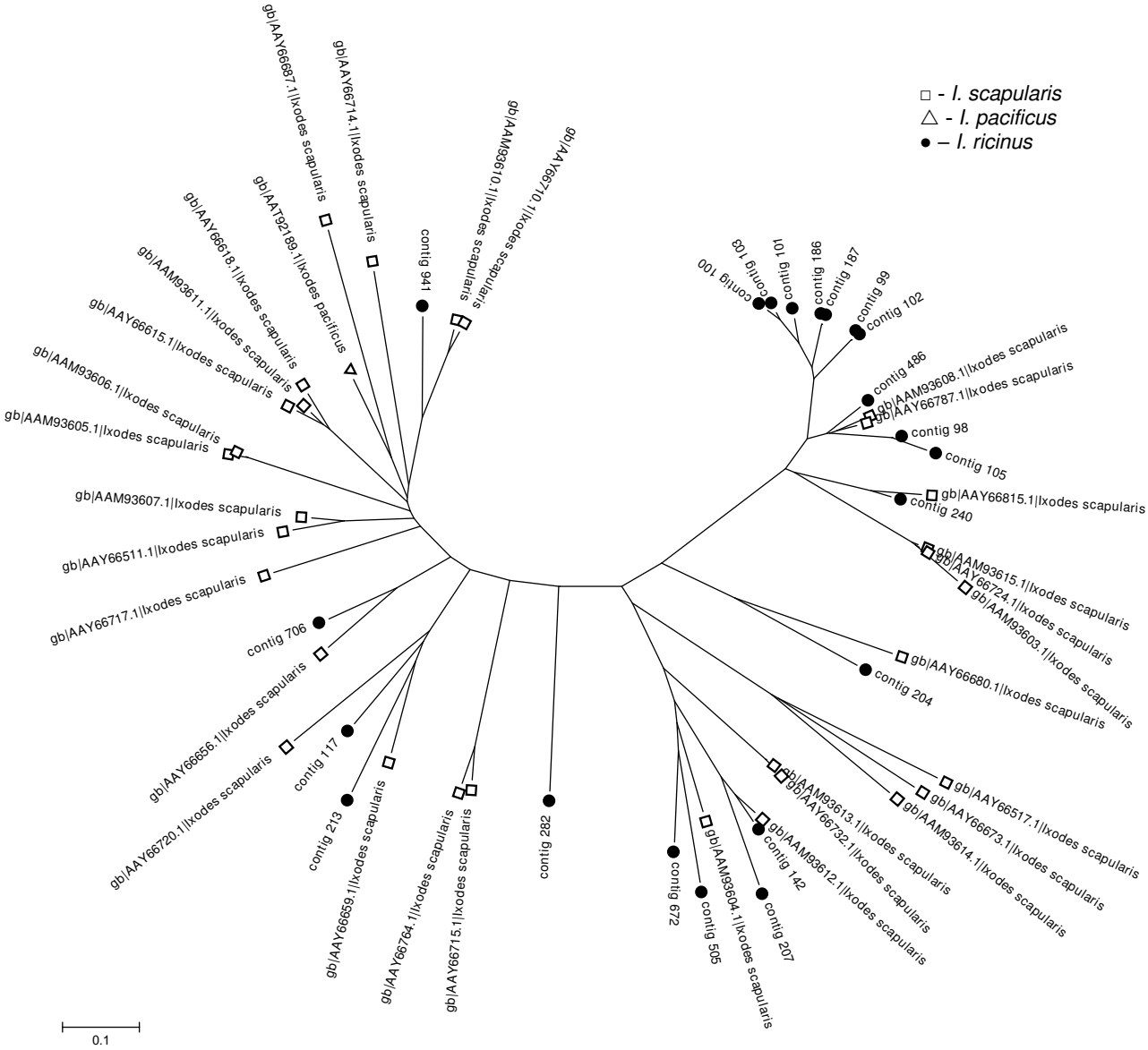

Figure 6
Kunitz domain-containing peptides from monolaris II group. Unrooted tree of all full-length monolaris II peptides from *Ixodes ricinus* and related sequences from *I. scapularis* and *I. pacificus*. The tree is based on protein sequences and created by NJ algorithm.

18.7 kDa group

Fifteen clusters containing 34 ESTs showed similarity to an 18.7 kDa group reported previously from both *I. scapularis* and *I. pacificus* [31,32]. The overexpression of 18.7 kDa ESTs in the IR24H library (Table 3) suggests the role of this group during the early phase of feeding. The most abundant cluster (Contig116, EY200914) contains 10 ESTs from the IR24H library. The 15 clusters create three main clades together with *I. scapularis* and *I. pacificus* sequences (Figure 7). The mature peptides from all the three *Ixodes* species contain 12 conserved cysteines. Four of the *I. ricinus* contigs (Contig 115, EY199897; 116, EY199123; 389, EY199138; 415, EY199200) contain the same insertion with two additional cysteines as three *I. pacificus* and one *I. scapularis* sequences found on GenBank (for accession numbers see Figure 7). Contigs 115, 116 and 389 contain another insertion of six identical amino acids (YFDSHS), which appears to be unique for *I. ricinus* among all *Ixodes* species. Two contigs (Contig 450, EY199271; 477, EY199343) cluster together with three

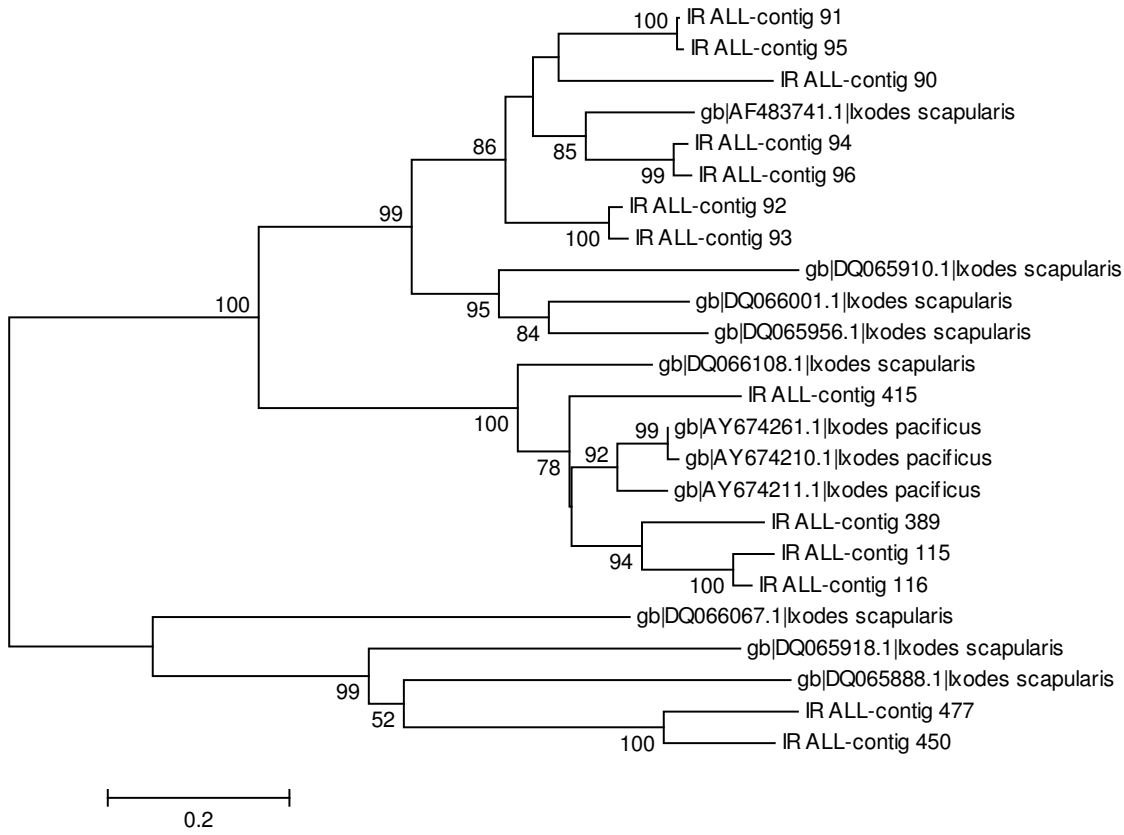

Figure 7
18.7 kDa group of secreted proteins. Cladogram of 18.7 protein group includes all *I. scapularis* and *I. pacificus* sequences obtained form the GenBank. Numbers represent bootstrap support of each clade with value above 50% (1000 rep.).

sequences from *I. scapularis* (for accession numbers see Figure 7) creating a single clade, which appears to have a common ancestral gene. The remaining sequences form the last and largest clade in Figure 5.

The 18.7 kDa group is comprised of at least three different polymorphic genes that share a conserved cysteine framework and few domains that suggest the same tertiary structure. The tertiary structure could be more important for protein function than the individual amino acid composition and conservation. Although we know nothing about the function of the 18.7 kDa family, the early production after attachment is typical for proteins used against blood coagulation and platelet aggregation.

Peptides containing Arg-Gly-Asp (RGD) domain

Integrins are cell receptors responsible for cell adhesion by recognition of an RGD motif on several extracellular matrix proteins such as fibrinogen, vitronectin, collagen or Von Willebrand factor [51]. RGD-containing proteins promote cell adhesion when insolubilized in the matrix, and inhibit cell-cell associations when they are soluble. Such soluble proteins are called disintegrins and were first reported from snake venom [52]. Tick peptides containing an RGD domain were shown to inhibit platelet aggregation by targeting GPIIbIIIa receptor and integrin αIIbβ3 [53-55]. Both *I. pacificus* and *I. scapularis* produce several types of RGD motif-containing proteins that are related to snake venoms [31,32]. Peptides with homology to disintegrins were also found in *I. ricinus*. Two non-homologous groups containing RGD motif were found and may act as disintegrins. The first group (Ixodegrins) is related to Ixodegrins described previously [31] and a snake venom component dendroaspin. Proteins in the second group are similar to prokineticin.

The alignment in Figure 8 compares Ixodegrins from *I. ricinus*, *I. pacificus* and *I. scapularis*. Contig 934 (EY200415) shows high similarity to peptides found in other Ixodes ticks. The remaining *I. ricinus* contigs contain two insertions unique for *I. ricinus* and show high similarity to each other, suggesting another example of multigenic family. Contigs 146 (EY199187) and 147 (EY199165) contain an Arg-Ala-Asp (RAD) domain rather

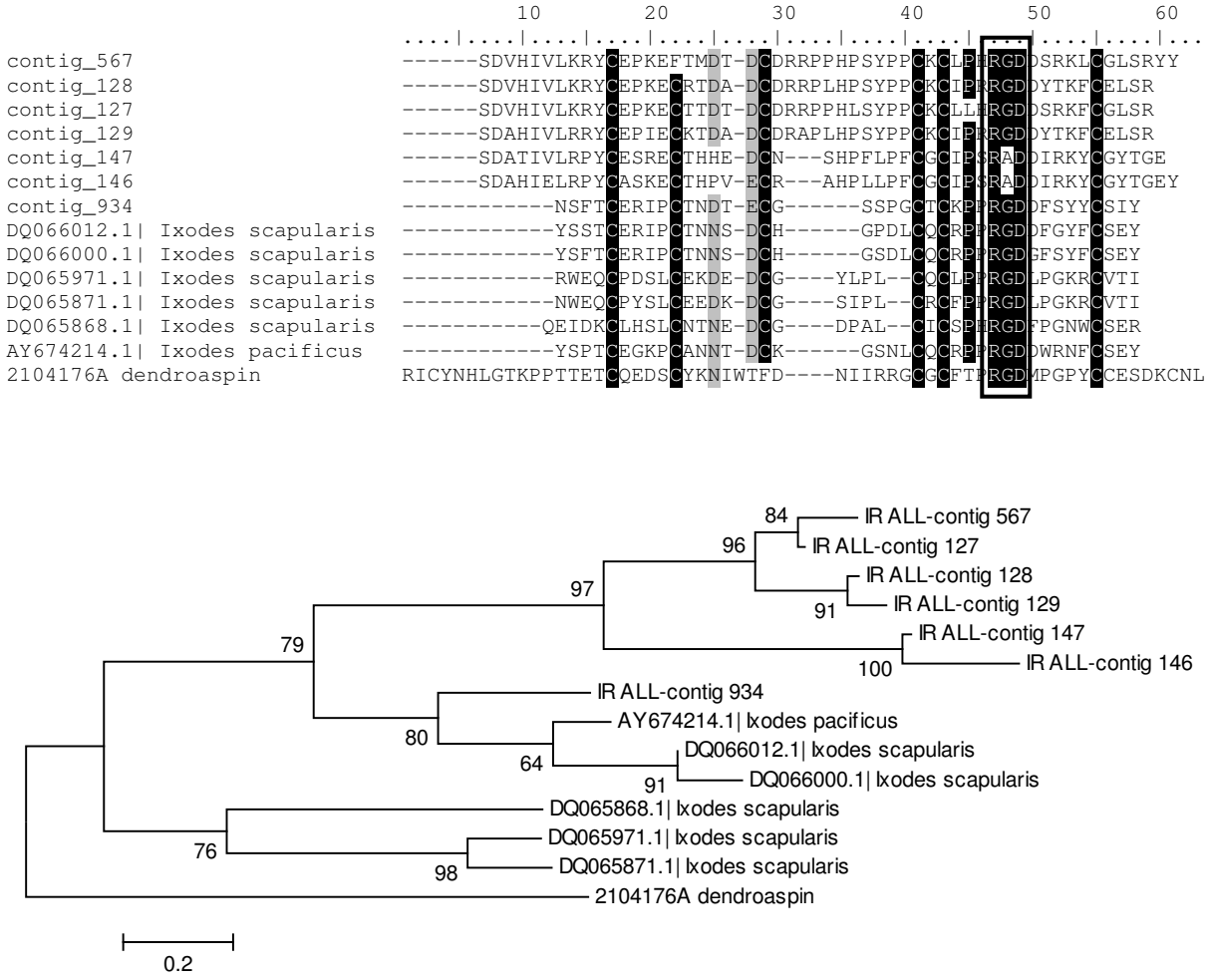

Figure 8

Ixodegrins: Alignment and phylogram. RGD motif, typical for disintegrins is marked in black rectangle. The tree was constructed by NJ method and numbers represent bootstrap support with value above 50% (1000 rep.). Dendroaspin isolated from *Dendroaspis jamesoni* was used as an outgroup.

than a RGD. It was shown that a change from RGD to RAD can block the binding and thus disintegrin activity of the peptide [56]. Ixodegrins are strongly over-expressed 24 hours after attachment and then their expression decreases (Table 3).

Peptides in the second group contain prokineticin (PK) domain and the group contains two subgroups related to Ixodegrin-2A (AAY66752) that differ in molecular mass. The alignment and phylogram of both subgroups is shown in Figure 9. The first subgroup includes acidic peptides with a mature molecular mass of 6.4 kDa and a conserved cysteine framework **C**-X5-**C**-X4-**C**-**C**-X(9–13)-**C**-X9-**C**. These peptides have no RGD motif; however, they contain one or even two XGD motifs in the loops between the cysteines. The second subgroup of PK domain-containing peptides includes acidic peptides with a molecular mass of 9.3 kDa and contain four additional cysteines at the C-terminus of 6.4 kDa subgroup creating the framework **C**-X5-**C**-X4-**C**-**C**-X(9–13)-**C**-X9-**C-X13-C-P-C-X(4–5)-C-X(4–7)-C**. The 9.3 kDa subgroup shows 36% identity and 50% similarity to astakin, a prokineticin domain-containing peptide isolated from *Pacifastacus leniusculus* (Q56R11) and 31% identity and 50% similarity to a prokineticin from *Bos taurus* (NP_001029190). Astakin was shown to be an important hematopoietic cytokine in invertebrates [57] and prokineticin can induce intestinal contraction in mammals [58]. Prokineticin domain proteins were originally identified as non-toxic peptides from black mamba (*Dendroaspis polylepis*) venom and later isolated from skin secretion of the frog *Bombina variegata* [59]. Prokineticin domain peptides appear to be involved

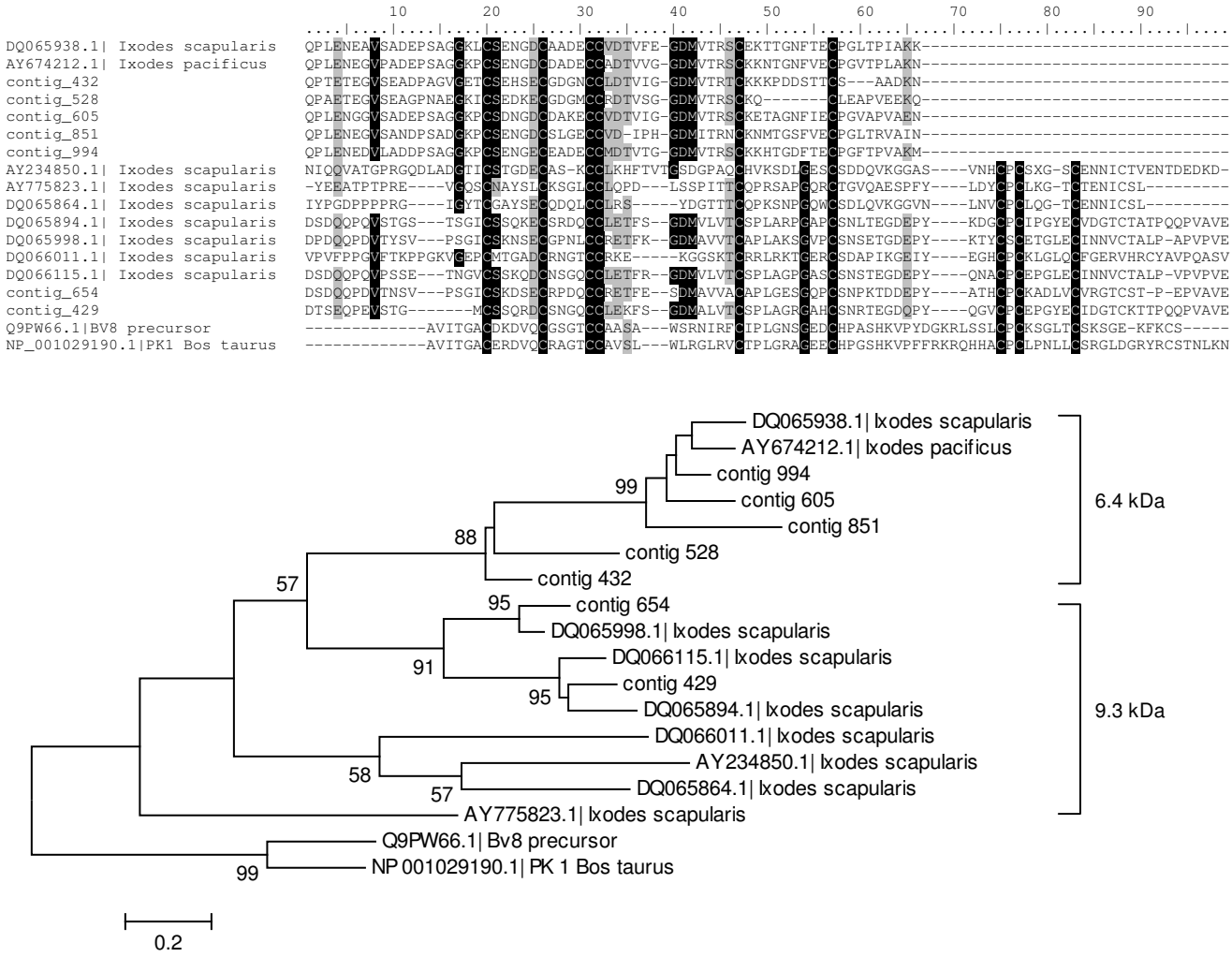

Figure 9

Prokineticin domain-containing peptides. Alignment and phylogram of both 6.4 and 9.3 kDa groups of prokineticin domain-containing proteins from the three Ixodes species. *Bombina variegata* protein Bv8 and prokineticin 1 from *Bos taurus* were used as an outgroup. The tree was constructed by NJ method and numbers represent bootstrap support with value above 50% (1000 rep.).

in various biological processes such as control of circadian rhythm, differentiation of endothelial cells in steroidogenic glands and promotion of angiogenesis in endocrine tissues [60]. Although RGD motifs were found in *I. pacificus* and *I. scapularis* suggesting their disintegrin function, no RGD motif was found in *I. ricinus* sequences from the 9.3 kDa group. The lack of RGD implies a different function of the 9.3 kDa subgroup in *I. ricinus*. Prokineticin domain-containing peptides from both 6.4 and 9.3 kDa appear to be expressed equally during all three stages after attachment. The function of 6.4 kDa and 9.3 kDa subgroups remains to be tested.

Trp-Cys (WC) containing proteins

A group of proteins containing a Trp-Cys (WC) doublet has been found previously in the sialome of *I. scapularis* [32] and appeared to be unique for that species. We can now provide evidence that this group is common for at least *I. scapularis* and *I. ricinus*. Most of the WC-containing proteins share a similar seven-cysteine framework **C**-X11-**C**-X(13–15)-**C**-X3-**C**-X(10–14)-**C**-X(6–7)-**C**-X(22–26)-**C** and several very conserved residues that could be important for proper folding or activity of the mature protein. The most significant conserved residue other than cysteine is the tryptophan residue before the last cysteine residue. It has been proposed that the WC doublet can create a hydrogen bond resulting in a bend of the peptide chain.

Fifteen contigs consisting of 18 ESTs with homology to WC containing proteins were identified in all cDNA libraries except IRUF. The expression of WC peptides is increased after attachment and the distribution of ESTs is equal among the three post-attachment libraries (Table 3). The function of this group remains unknown.

Histamine-binding proteins (HBP)

Ten genes coding for the proteins from the HBP lipocalin family were found in the IR24H, IR4D and IR7D libraries. The function and evolution of these proteins were described in detail elsewhere [20,61,62]. Briefly, tick lipocalins are able to bind histamine in a binding cavity resulting in the inhibition of inflammation, as histamine is a very potent mediator of the inflammatory response [20]. All *I. ricinus* sequences are similar to other *I. scapularis* and *I. pacificus* HBPs except contig 972, which shows only low similarity to other tick HBP (e-value = 5E-005). Additionally, BLASTp analysis found no HBP domain within the sequence, suggesting a potentially different function yet similar ancestral origin with other HBP coding genes. Histamine-binding proteins may only be expressed during the later phases of feeding as no sequences were captured in the unfed library and only one in IR24H.

Metalloproteases

Thirteen clusters consisting of 18 ESTs showed similarity to metalloproteases. All sequences were truncated; no full-length clones have been obtained. Contigs 218 (EY200183), 401 (EY199170) and 841 (EY200242) have extremely high identities (e-values = 1E-158, 1E-121 and 1E-97) with both *I. scapularis* and *I. pacificus* sequences, suggesting homology to coding genes. Notably, there is no feeding-induced associated overrepresentation of metalloproteases sequences (Table 3)

The main function of tick metalloproteases is probably related to anti-clotting activity at the site of attachment. Proteolytic metal-dependent activity toward fibrin(ogen), fibronectin and gelatin has been proved for *I. scapularis* saliva [23]; however, genes coding for metalloproteases have been found in 6 ixodid and 2 argasid species thus far, suggesting the presence of metalloproteases in saliva of more tick species.

Rare expressed genes similar to known proteins

α-2 macroglobulin

Alpha 2 macroglobulin is a protease inhibitor with a wide range of specificity [63]. The mechanism of inhibition is based on the capturing of the protease inside the large molecule of α-2 macroglobulin. This inhibitor has been detected in the hemolymph of *I. ricinus* and *Ornithodoros moubata* and possibly functions in tick innate immune defense against some pathogens [64]. Contig 680 contained one EST (EY199886) coding for a peptide with 34% identity and 55% similarity to a receptor region of α-2 macroglobulin previously found in *I. scapularis* (AAM93645) and *I. ricinus* (unpublished). The finding of another α-2 macroglobulin domain provides evidence that there are at least two different α-2 macroglobulin homologues in *I. ricinus*.

Calreticulin

Calreticulin is a protein highly conserved throughout all animals and usually functions in calcium storage. We have found only one clone in the IR4D library (Contig 614, EY199726) with 96% identity to *I. scapularis* calreticulin (AY271305) at the nucleotide level. It has been proposed that tick calreticulin is secreted into the host and possesses an immunoactive function [65].

Defensins

Defensins are small antimicrobial peptides common for both vertebrates and invertebrates. Defensins are important in defense against various microbial pathogens including *Borrelia spp*. [66]. We found two different defensins in the IR7D library (Contig 921, EY200392 and 969, EY200491). Contig 921 is a homologue of preprodefensin 1 and 2 isolated from *I. ricinus* (ABC88432, AAP94724) suggesting polymorphism in this gene. Contig 969 shows the highest similarity to Varisin A1 from *D. variabilis* (AAO24323).

Neuropeptide-like protein (NPL) with GYG repeats

Peptides with GYG repeats were found in *Caenorhabditis elegans* and show high antimicrobial activity against certain microbial organisms [67]. Three homologues (Contig 279, 494, 935) of NPL proteins from *I. pacificus* (AAT92111, AAT92131) have been identified in IR24H, IR4D and IR7D libraries. NPL proteins could act as antimicrobial peptides at the site of attachment [30] or they may be secreted into the hemolymph and be involved in tick humoral antimicrobial defense.

Carboxypeptidase inhibitor precursor

Contig 929 (EY200408) found in IR7D library showed 66% similarity (e-value = 1E-22) to a carboxypeptidase inhibitor from *Rhipicephalus bursa* salivary glands (AAW72225). The *R. bursa* carboxypeptidase inhibitor is a potent anticoagulant which accelerates fibrinolysis in blood clots [68]. This putative *I. ricinus* carboxypeptidase inhibitor adds to the growing family of tick anticoagulants.

Ixoderin B

Ixoderins are tick lectins related to ficolins, which are responsible for complement activation, and to arthropod lectins, which act as plasma agglutination activators. In tick plasma, lectins also play a role in antimicrobial activ-

ity, potentially against transmitted pathogens [69]. There are at least two ixoderin families in *I. ricinus*: Ixoderin A, which is expressed in all tick tissues and Ixoderin B expressed only in salivary glands [70]. Contig 617 (EY199735) is homologous to Ixoderin B (AAV41827) which implies the multigenicity of ixoderin B group. As ixoderins are related to lectins, they can be involved in tick innate immunity, but specific expression of Ixoderin B in salivary glands suggests also some immunomodulatory function in the host.

Keratinocyte associated protein 2-like protein (KAP2-like)

One transcript (Contig 624, EY199752) coding for KAP2-like protein was found in the IR4D library. KAP2-like proteins have been identified in almost all major groups of eukaryotes according to a tBLASTn search against the EST database of all organisms. Nothing is known about the function of KAP-2 protein, but it probably possesses important housekeeping functions as it is highly conserved among phylogenetically distant organisms.

Phospholipase A2 (PA2)

PA2 activity was found in *Ammblyoma americanum* saliva [71] and the genes homologous to the secreted type of PA2 were identified in other ixodid ticks, according to tBLASTn search against EST database. This suggests that PA2 activity in saliva is common for hard ticks. One clone (Contig 1024, EY200582) was found in the IRUF library and showed approximately 40% identity and 60% similarity to PA2 from various organisms.

Pitituary tumor transforming protein 1 interacting protein – like

Contig 982 (EY200521) displayed high similarity (45% identity, 64% similarity, e-value = 2E-30) to PTTG-1-IP from *Gallus gallus* (XP_422649). PTTG-1-IP is a protein that binds PTTG, a protein with multiple regulative functions in mitosis, gene expression, cell transformation and also angiogenesis [72]. This is the first report of PTTG-1-IP from invertebrates. The exact function of PTTG and PTTG-1-IP is not clear either for mammals or invertebrates.

Other minor secreted proteins

Many tick salivary proteins with a predicted signal peptide are novel peptides with no similarity among other organisms other than ticks or they are unique for *I. ricinus*. These proteins are named after the mature predicted molecular mass [see Additional file 1]. Many peptides are very small with molecular weight below 5 or 3 kDa. This may be due to sequencing artifacts creating false stop codons; however, small peptides can be functional in ticks. For example, there is one thrombin inhibitor isolated from the hard tick *Boophilus microplus* called microphilin with a molecular mass of about 2 kDa [73].

Conclusion

The work presented here highlights several major themes concerning tick salivary proteins. There is an enormous overrepresentation of secreted protein transcripts after attachment and a subsequent shift to the production of molecules associated with energetic metabolism. This is probably a result of increased metabolic rate associated with blood ingestion and digestion. The difference in the production of mRNAs coding for secreted proteins between unfed ticks and ticks 24 hours after attachment is remarkable. An increase of 11 fold shows that hard ticks possess mechanisms allowing a rapid switch in physiology. While many secreted proteins appear to be induced after attachment, they are probably important in the feeding process. Protein production is an energetically demanding process so these proteins should have some function either in ticks or in the host. Similar abundance of secreted proteins among the three *Ixodes* species gives us an idea of which proteins could be crucial for successful feeding. The most abundant protein groups (i.e., CLSP, BTSP, Kunitz-domain or 18.7 kDa groups) are common for all three species and should be the first to be tested for any activity in the host. On the other hand, some proteins that have been tested for activity by other research groups do not belong into any of the most abundant groups. For example homologs of Salp15, the only salivary protein proven to be a SAT factor (peptide facilitating pathogen transmission) were found in only seven ESTs. The same EST number was found for the anti-complement proteins related to ISAC and IRAC isolated from *I. scapularis* and *I. ricinus*. Therefore, rare transcripts should also be evaluated for functionality. Hypothetically, abundant and strongly feeding-induced peptides can be aimed against innate defense mechanisms already active in the early phase of feeding. The less abundant proteins could possess more specific immunomodulatory function and could also participate in the SAT effect.

The most abundant protein families display interesting expression profiles throughout the feeding period and show temporal control of expression of very similar molecules. Feeding phase-specific expression, as was shown in the CLSP family, is an interesting phenomenon with several possible explanations: 1) It can be a result of gene arrangement on the chromosome without any further importance; 2) There can be sequential production of functionally identical peptides with different antigenicity and finally; 3) Similar peptides can have different functions in different stages of feeding. The second explanation is very tempting, because it would be a completely new aspect of the tick-host interaction, which is common among parasitic microorganisms, but very rare among metazoan. However, some points will need to be elucidated. We must know first whether the candidate proteins for possible antigenic variation are antigenic. If these mol-

ecules are antigenic they would then need to be checked for cross-reactivity by antibody recognition.

As there is a wide range of *I. ricinus* hosts, we can discuss the possibility of expression profile differences among ticks that were fed on different hosts. It was shown that different feeding conditions, including different hosts, result in protein profile changes of the saliva [74]. Ticks used in our work were fed on guinea-pigs, which are not natural host of *I. ricinus*; however, it is widely used laboratory model in tick research. Although there is no direct evidence of differences between sialomes of ticks fed on different animals, we assume that the data obtained with ticks fed on guinea-pigs can be applied to other hosts. Future research may be needed to test the possible effects of host influences on the tick sialome.

There is a high ratio of unknown transcripts in unfed ticks. To date, only one salivary gland library from unfed Ixodid ticks (*A. americanum*) was published in the GenBank. The lack of sequences from comparable feeding stage could be the reason for such high proportion of unknown transcripts in unfed library. As the main function of tick salivary glands during the starving phase is osmoregulation [75], some of unknown proteins could be involved in the process of water balance regulation.

The presence of multigenic, highly polymorphic families supports the theory of gene conversion and duplication in tick genome evolution. Comparison of the three phylogenetically close species shows an unusually high rate of gene duplication in many multigenic families. The cause of such fast gene multiplication in hard ticks is still not known; although, there is suspicion for the role of transposition in this process. It is notable that a number of organisms that use multigenic proteins, mainly for antigenic variation, are pathogens transmitted by hard ticks. It would be worthwhile to know whether there is any relationship between tick and transmitted pathogen multigenicity.

There are several abundantly secreted and blood meal-associated proteins that are probably very important for successful feeding. All of the most abundant proteins appear to be multigenic with an amount ranging from several to tens of paralogs. There is also a high probability of polymorphism and the number of homologs is probably higher than the number found in this work. Now, we have large databases of putative proteins expressed in tick salivary glands, but for the most part, the proper function is unknown. For some proteins a prediction based on sequence homology can be made; however, it is still unclear whether similar proteins from one family possess the same function as the homologs. On the whole, there are many salivary proteins that need to be tested for their function. It appears that a high-throughput approach would be highly beneficial for functional screening of tick salivary proteins. Additionally, the contribution of new sequences into the public databases will bring benefits for all the scientific community interested in tick and vector research or comparative phylogenetic studies.

As we unfold more peculiarities about tick genetics, thanks to the sialome research, we can see that ticks are interesting – not only for study of parasite-host interaction or searching for novel pharmacoactive molecules, but they prove to be an important model organism for disclosing some mechanisms of gene and genome evolution.

Methods

Ticks and tissue isolation

Ixodes ricinus adult females were obtained from the colonies maintained at the Institute of Parasitology of Czech Academy of Sciences in Ceske Budejovice. Pathogen-free ticks were fed on the guinea pigs complying with Act No. 207/2004 Coll. and approval AVCR 51/2005 given by the committee of Czech Academy of Sciences. Approximately 10 pairs of salivary glands were dissected from both unfed and fed adult ticks. Dissections were done in phosphate buffered saline (PBS); glands from each stage were washed in sterile ice-cold PBS, pooled together into a single tube and stored in RNAlater (Ambion, USA) until mRNA isolation.

Synthesis of cDNA libraries and sequencing

Messenger RNA was isolated using Micro Fast Track mRNA isolation kit (Invitrogen, USA) according to the manufacturer protocol. Precipitated, washed and dried mRNA was diluted into 4 μl of DEPC-treated H_2O and 3 μl of mRNA were used for first strand cDNA synthesis. The construction of cDNA libraries was done using the SMART cDNA library construction kit (BD Clontech, USA) according to the protocol provided by manufacturer, with some modifications. In order to determine optimal number of cycles, two identical amplification reactions were prepared. After 10^{th} amplification cycle the first one was stored on the ice, while the second one was used for the PCR cycles number optimization by removing 3 μl samples from the reaction every two cycles until cycle number 22. The samples were checked by visualization on an agarose gel. The optimal number of cycles with visible and equally represented products was used for the first amplification reaction (e.g. When 18 cycles were optimal, 8 additional cycles were used). The amplified DNA was treated with proteinase K, which was subsequently washed away by several washings with ultrapure water using Microcon YM-100 (Millipore). After Sfi I digestion and fractionation using a Chroma Spin-400 column (BD Clontech, USA) the fractions were checked using agarose gel and pooled into three tubes in a size-dependent man-

ner (large, medium and small PCR products). Each pooled cDNA was washed with ultrapure water and concentrated to 4–7 μl using Microcon YM-100 column (Millipore). Three microliters from each tube were used for the ligation into the λTripleEx2 vector. The ligation reaction was packed into the phages using the Gigapack III Gold Packaging extract (Stratagene). Three libraries (large, medium, small) were constructed for each feeding phase resulting in total of 12 libraries. Each un-amplified library was plated onto LB agar plates aiming for approximately 300 clones per one 150 mm plate. Randomly selected clones were picked into the 96-well plate with 75 μl of water per well. Two plates were picked per library resulting in 6 plates for each feeding phase and 24 plates (2304 clones) for the experiment. Polymerase chain reaction with vector-specific primers (PT2F1 5'- AAGTACTCTAGCAATTGTGAGC -3' and PT2R1 5'- CTCTTCGCTATTACGCCAGCTG - 3') was done on selected clones. Cleaned PCR products were used as template for cycle-sequencing reaction using BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and forward primer PT2F3 primer 5' - CTCGGGAAGCGCGCCATTGT - 3'. Samples were directly sequenced on an ABI 96 capillary DNA sequencer (Applied Biosystems), or stored at -80°C.

Bioinformatics

A detailed description of the bioinformatic treatment of the data appears elsewhere [33,76]. For this analysis, EST trace files were analyzed using a customized program based on the Phred algorithm [77]. Resulting sequences with average quality score greater than 20 were retained for subsequent analysis. Primer and vector sequences were removed from the 5' and 3' ends of the ESTs. The resultant sequences were grouped into clusters using a customized program (Cluster 6) based on identity (95% identity, 64 word size) and aligned into contiguous sequences (contigs) using the CAP3 sequence assembly program [78]. BLAST searches of individual contigs and singletons using executable programs obtained from the NCBI FTP site as previously described [76,79] were conducted against the non-redundant (NR) protein database of the NCBI, the gene ontology (GO) fasta subset [43], the conserved domains database (CDD) of NCBI [80] which contains the KOG [81], Pfam [82] and Smart databases [83] and to custom-downloaded databases containing ACARI (a subset containing mite and tick sequences), mitochondrial and rRNA nucleotide sequences available from NCBI. Peptides were submitted to the SignalP server as previously described [76,84] to detect signal peptides indicative of secretion. The individual cDNA libraries were directly compared with each other using a customized program (Count Libraries) that assesses the individual contribution of each individual library to the combined contig of interest. This analysis indicates putative proteins that may be over- or under-represented at a given time point. Chi-square analysis was conducted on contigs that contained more than 5 ESTs.

Authors' contributions

JC carried out the conception and design of the work, tissue preparation, RNA isolation, cDNA libraries construction, preparation of samples for sequencing, obtained data interpretation and manuscript preparation. JMA carried out bioinformatic treatment of raw sequence data and participated in manuscript revision. JM participated in sequencing the libraries. RCJ participated in statistical evaluation of data and manuscript revision. JGV participated in the design of the work, manuscript revision and final approval of the version and JK carried out the conception and design of the work, participated in manuscript revision and approval of the final version.

Additional material

Additional file 1

Supplemental table. Zip file contains MS Office Excel table with links to text files, containing additional information on all ESTs.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2164-9-233-S1.zip]

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Article 2

A tick salivary protein targets cathepsin G and chymase and inhibits host inflammation and platelet aggregation

Chmelar J, Oliveira CJ, Rezacova P, Francischetti IM, Kovarova Z, Pejler G, Kopacek P, Ribeiro JM, Mares M, Kopecky J, Kotsyfakis M.

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The first paper we published on *I. ricinus* serpins described serpin named IRS-2 (Ixodes ricinus serpin-2). We succeeded in the production of large quantity of LPS free recombinant protein and were therefore able to perform thorough analysis that included *in vivo* experiments on mice and structural study.

IRS-2 is a strong inhibitor of mast cell chymase and cathepsin-G. Both proteases are involved in the activation and amplification of inflammatory response, therefore we predicted some anti-inflammatory activity. Indeed, IRS-2 inhibited the recruitment of neutrophils to the site of inflammation in the mouse model of paw edema induced by an injection of 2% solution of carrageenan in saline. The edema itself was also diminished. In addition, IRS-2 inhibited the aggregation of platelets induced by cathepsin G, showing also anti-hemostatic potential. Finally, we solved the crystal structure of IRS-2 in cleaved, stable state. This confirmed tyrosine in P1 site of the serpin.

The significance of this article lies in the fact that the study was published in highly impacted journal and therefore it brought tick serpins into spotlight of tick research. It was also the first publication of parasitic serpin crystal structure, which later served as a template for further structural studies on tick serpins.

Author's contribution:

Author designed the experiments, performed RT-qPCR, produced recombinant protein and performed experiments with mast cells and mast cell proteases. He analyzed the data and wrote the manuscript.

A tick salivary protein targets cathepsin G and chymase and inhibits host inflammation and platelet aggregation

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Platelet aggregation and acute inflammation are key processes in vertebrate defense to a skin injury. Recent studies uncovered the mediation of 2 serine proteases, cathepsin G and chymase, in both mechanisms. Working with a mouse model of acute inflammation, we revealed that an exogenous salivary protein of *Ixodes ricinus*, the vector of Lyme disease pathogens in Europe, extensively inhibits edema formation and influx of neutrophils in the inflamed tissue. We named this tick salivary gland secreted effector as *I ricinus* serpin-2 (IRS-2), and we show that it primarily inhibits cathepsin G and chymase, while in higher molar excess, it affects thrombin activity as well. The inhibitory specificity was explained using the crystal structure, determined at a resolution of 1.8 Å. Moreover, we disclosed the ability of IRS-2 to inhibit cathepsin G-induced and thrombin-induced platelet aggregation. For the first time, an ectoparasite protein is shown to exhibit such pharmacological effects and target specificity. The stringent specificity and biological activities of IRS-2 combined with the knowledge of its structure can be the basis for the development of future pharmaceutical applications. (*Blood*. 2011; 117(2):736-744)

Introduction

The first response of a vertebrate organism to a skin injury involves blood coagulation, vasoconstriction, platelet aggregation, and innate immune mechanisms, such as inflammation and complement activation, that have an important antibacterial role. Upon a tick bite, these mechanisms are activated as a first line of defense and are dependent, among others, on serine protease cascades (in blood coagulation and complement activation) or on the proteolytic activation of bioactive peptides, such as chemokines, vasoconstrictors, or protease-activated receptors (PARs). Previous studies demonstrated that these serine protease–dependent processes are regulated by endogenous inhibitors. To prevent pathophysiological conditions, proteases and inhibitors must be in balance; in the case of an exogenous inhibitor (eg, of parasitic origin) infiltrating the system, this balance will be impaired.

This phenomenon is also observed in the saliva of hematophagous arthropod ectoparasites, including ticks.[1] The saliva of ixodid ticks plays a crucial role at the tick-host interface not only because it contains numerous proteins that affect many host nonspecific defensive mechanisms, such as inflammation, blood coagulation, and platelet aggregation, but also because it can target acquired immunity.[2] Activation of these host physiological mechanisms immediately after a tick bite would be detrimental for tick feeding success, and therefore it is vital for the tick to overcome them. Investigation of the underlying mechanism, although time consuming, revealed salivary constituents with pharmacologic activities targeting vertebrate host defense, including molecules with anti-inflammatory potential.[3,4] This process was accelerated by recent transcript-sequencing projects on tick salivary glands (ie, sialotranscriptomics), where numerous salivary protein candidates were identified that may account for the pharmacologic properties of tick saliva.[5-7]

Of interest, a number of genes encoding for potential serine protease inhibitors were identified in *Ixodes spp* salivary glands, including serpins, the largest, most diverse family of protease inhibitors. The mode of action of serpins is unique and depends on their folding and the primary structure of specific conserved domains. The literature dealing with the structural and functional properties of serpins and the evolution of this protein family is reviewed elsewhere.[8,9] More than 60 serpins were identified at the sequence level in ixodid ticks, largely due to the completed *I scapularis* genome[10-14]; however, only 1 tick serpin originating from *I ricinus*, the vector of Lyme disease pathogens in Europe, has been functionally characterized.[14] The serpin was named *I ricinus* immunosuppressant (IRIS), and it targets the serine protease, elastase. Despite not bearing a classical secretion signal, it can affect vertebrate hemostasis apart from vertebrate immunity.[15] Recently, Prevot and colleagues showed the involvement of an exosite domain in the anti-inflammatory activity of IRIS. Surprisingly, unlike its antihemostatic activity, the anti-inflammatory properties of IRIS are independent of its inhibitory nature.[16] Thus, the inhibitory function of IRIS was not directly shown to be responsible for its observed immunomodulatory properties, so the answer—whether salivary serpins can mimic the function of vertebrate regulators and thus immunomodulate the host—

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remained undisclosed. The same is true for the structural basis of IRIS function. Apart from IRIS, no other serpin from *I ricinus* has been described to date.

Here, we present the functional characterization of a novel inhibitory serpin from the saliva of the tick, *I ricinus*, that we named *I ricinus* serpin-2 (IRS-2). It bears a clear secretion signal and inhibits edema formation and neutrophil influx in the inflamed tissues in a mouse model of acute inflammation. The protein targets primarily 2 proinflammatory serine proteases, cathepsin G and mast cell chymase, and, in higher molar excess, thrombin. Because of its inhibitory activity, IRS-2 blocks cathepsin G– and thrombin-induced platelet aggregation, thus playing a dual role during tick feeding, as it can interfere with both inflammation and wound healing. We also determined a high-resolution crystal structure of IRS-2 that provides structural insight into the observed inhibitory specificity. This is the first report of the crystal structure of a serpin isolated from a parasitic organism. Moreover, such a mechanism in which the parasite uses inhibition of cathepsin G and chymase to overcome the host defense system, has not been shown for any blood-feeding arthropod salivary constituent to date.

Methods

Unless otherwise indicated, standard procedures were followed,[17] and experiments were performed at room temperature (25 ± 1°C). All water used was of 18-MΩ quality produced by a MilliQ apparatus (Millipore). If not otherwise stated, all reagents were purchased from Sigma-Aldrich, and all cells were cultured at 37°C under an atmosphere of 5% CO_2. The procedures of gene cloning, sequence analysis, real-time polymerase chain reaction (PCR), protein expression, and biochemical methods are detailed in supplemental Methods (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

Paw edema assay

Female C57BL/6 mice (6-8 weeks old) were used. The mice were purchased from The Jackson Laboratory and maintained in the National Institute of Allergy and Infectious Diseases (NIAID) Animal Care Facility under pathogen-free conditions in temperature-controlled rooms, receiving water and food ad libitum. All treatments were performed in accordance with the National Institutes of Health [NIH]'s *Guide for the Care and Use of Laboratory Animals* , and the animal study protocols were approved by the Division of Intramural Research/NIAID Animal Care and Use Committee. The carrageenan-induced hind-paw inflammation model was used to investigate the potential anti-inflammatory role of IRS-2. Before each injection, the basal footpad thickness of each mouse was recorded using a caliper (Mitutoyo America Corp). Subsequently, 40 μL of carrageenan (2% in saline) was administered by intraplantar injection in each footpad in the absence or presence of different concentrations of lipopolysaccharide-free IRS-2. As a control, each group of mice received the same volume of saline (vehicle) in the presence of IRS-2 only. The same experimental design and concentrations were used for chymostatin, a generic protease inhibitor, and indomethacin, a nonsteroid anti-inflammatory compound. Each effector was coadministered with carrageenan in the mouse footpad. As an index of edema formation, paw thickness (in millimeters) was measured at 4 and 24 hours after injection, and myeloperoxidase (MPO) activity was measured to estimate the potential effect of IRS-2 on neutrophil migration. For details, please see supplemental Methods.

Isolation and cultivation of PCMCs

Animal experiments were performed in accordance with protocols approved by the local ethical committee. Peritoneal cells were obtained from 6-8-week-old C57BL/6 mice by lavage of the peritoneal cavity with ice-cold phosphate-buffered saline (PBS), washed in fresh PBS, and resuspended in growth medium. The growth medium was Dulbecco modified Eagle medium with GlutaMAX (Gibco), 10% of conditioned media from Chinese hamster ovary cells transfected with mouse stem cell factor (a gift from Dr Marc Daeron), 10% fetal calf serum, 1% penicillin-streptomycin (Invitrogen), 1% minimum essential medium with nonessential amino acids (Gibco), and 50μM mercaptoethanol. Nonadherent cells (mainly peritoneal cell–derived mast cells [PCMCs]) were collected and resuspended in fresh medium to a concentration 10^5 cells/mL every third day. After 6 weeks of cultivation, the enriched PCMCs were used for experiments.

Preparation of washed human platelets and platelet aggregation assays

Platelet-rich plasma was obtained by plateletpheresis from medication-free, adult, healthy platelet donors at the Department of Transfusion Medicine/NIH blood bank under the direction of Dr S. Leitmann as described elsewhere.[18] Briefly, after the addition of 0.2 U/mL apyrase, platelet-rich plasma was centrifuged at 1100 × *g* for 15 minutes and washed twice by centrifugation in Tyrode buffer (137mM NaCl, 27mM KCl, 12mM $NaHCO_3$, 0.42mM NaH_2PO_4, 1mM $MgCl_2$, 5.55mM glucose, 0.25% bovine serum albumin; pH 7.4). Platelets were resuspended in apyrase-free Tyrode buffer and adjusted to a concentration of 200 000-400 000 platelets/μL. Washed human platelets (300 μL) were placed in a Chrono-Log Lumi-aggregometer (Chrono-Log Corp) and stirred at 1200 rpm at 37°C for 1 minute, followed by the addition of reagents, as indicated in the figure legends. In all experiments, 1.5μM IRS-2 was preincubated for 15 minutes with the various effectors tested, and the mixture was added to platelets.

Crystallization and data collection

Details of the crystallization procedure and diffraction data collection are reported elsewhere.[19] Crystals of IRS-2 were prepared at 20°C using the hanging-drop, vapor-diffusion technique. The crystallization drop consisted of 2 μL of the IRS-2 solution (3.5 mg/mL in 20mM HEPES [N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid]; pH 7.2) and 1 μL of the reservoir solution (75mM MES [2-(N-morpholino)ethanesulfonic acid], pH 6.5, 9% [wt/vol] polyethylene glycol 20 000). For data collection, crystals were soaked in reservoir solution supplemented with 20% (vol/vol) polyethylene glycol 400 and flash-cooled in liquid nitrogen. Diffraction data were collected at 100 K using the X12 EMBL beamline (DESY) and processed using the HKL-2000 suite of programs.[20] Crystals exhibited the symmetry of space group $P4_3$ and contained 2 molecules in the asymmetric unit. Crystal parameters and data collection statistics are given in supplemental Table 1.

Structure determination

The structure of IRS-2 was solved by molecular replacement using the MoLrep 9.2 program.[21] The search model was derived from the structure of the equine leukocyte elastase inhibitor in R-state conformation (Protein Data Bank [PDB] code 1HLE).[22] Model refinement was carried out using the program REFMAC 5.3[23] from the CCP4 package.[24] Manual building was done using Coot.[25] Tight noncrystallographic symmetry restraints were applied during initial refinement; in later stages, the restraints were loosened as guided by the behavior of R_{free}. The final steps included translation, libration, and screw refinement.[26] The quality of the final models was validated with MoLProbity server.[27] Final refinement statistics are given in supplemental Table 1. Figures showing structural representations were prepared with the program PyMOL 0.99 (DeLano Scientific).[28] The DALI server was used to search for structural homologs.[29] Ramachandran plot statistics were determined by PROCHECK.[30]

Results

Cloning of serpins from *I ricinus*

The sequences of numerous serine protease inhibitors from the serpin superfamily have been identified from various ixodid tick

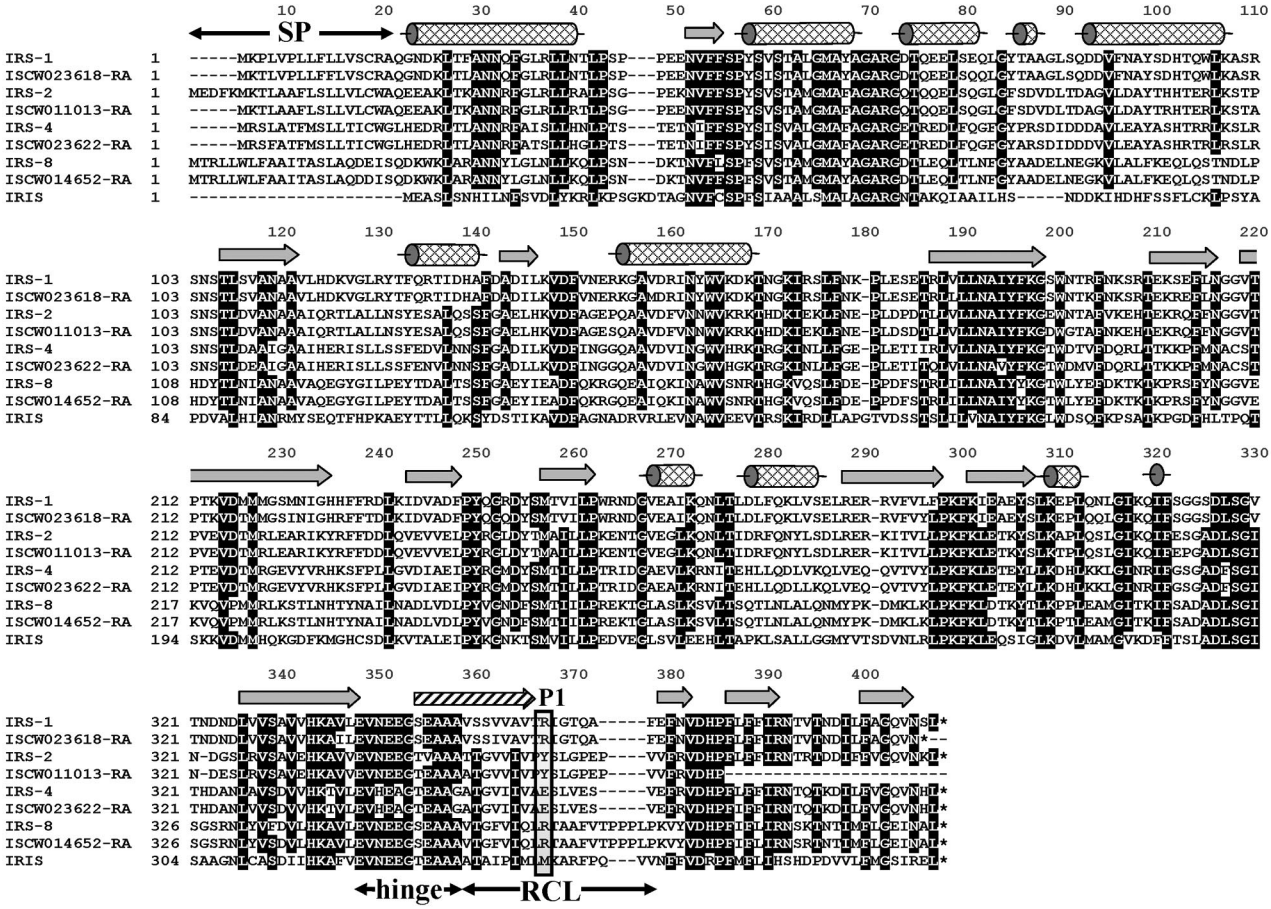

Figure 1. Alignment of the 4 full-length *I ricinus* serpin clones with their orthologs from *I scapularis* obtained from the completed *I scapularis* genome. All serpins are compared with IRIS, the only functionally characterized tick serpin so far. Unlike IRIS, IRS-2 has a clear signal peptide (residues 1-21). The secondary structure elements are indicated according to the IRS-2 crystal structure: α-helixes (cylinders), β-strands (arrows); the hatched arrow represents the region that undergoes a conformational change from a loop to a β-strand after proteolytic cleavage of RCL. SP, signal peptide; hinge, hinge region, an important determinant of serpin inhibitory potential; RCL, reactive center loop; P1 (rectangle), predicted residue behind which the target protease cleaves the RCL.

species, and it is also known that the family is represented in *I ricinus* salivary glands.[14] However, the knowledge of *I ricinus* salivary serpins, and the function of tick serpins in general, is limited to IRIS, a serpin that does not follow a classical secretion pathway from the salivary glands. Therefore, with the aim to identify classically secreted *I ricinus* serpins (ie, those containing a clear signal peptide), we designed degenerate primers based on the 2 most conserved domains (NAIYFKG and PFLFFI) found in the alignment of 10 arthropod serpins, for which sequence data were available at that time. The obtained PCR-amplified fragment was radioactively labeled and used for the screening of a cDNA library prepared from salivary glands isolated from adult female ticks after 5 days of attachment to the host to find as many serpin clones as possible. Four different full-length serpin cDNA clones that contained signal peptide, namely, IRS-1, -2, -4, and -8 (National Center for Biotechnology Information accession numbers DQ915842, DQ915843, DQ915844, and DQ915845), were identified. Orthologs for all 4 genes can be found in the genome of *I scapularis* (http://iscapularis.vectorbase.org/index.php), the vector of Lyme disease pathogens in the Eastern and Central parts of the United States. Amino-acid identity between the corresponding serpin orthologs varies from 94.9% for IRS-4 to 98.8% for IRS-8. The alignment in Figure 1 compares the 4 discovered serpins with the homologous proteins from *I scapularis* and with IRIS.[14] All proteins, including IRIS, display putative inhibitory features in the serpin hinge region, and all proteins except IRIS bear a clear signal peptide, suggesting a classical secretory mechanism from the salivary glands. Based on their amino acid (aa) sequence and on the preliminary prediction of the P1 position of each serpin, we proceeded with further analysis of IRS-2. More specifically, the predicted P2-P2′ sequence of the IRS-2–reactive center loop is Pro-Tyr-Ser-Leu with the tyrosine at the P1 position, suggesting antichymotrypsin, rather than antitrypsin, specificity. Mature IRS-2 is a 376-aa protein, weakly acidic with pI 5.53; the predicted molecular weight was 41.9 kDa. The unprocessed protein contains a 21-aa predicted signal peptide, according to the SignalP 3.0 server program.

IRS-2 expression is up-regulated after tick attachment

Next, we determined the expression profile of IRS-2 in different tick tissues throughout the feeding period of adult ticks using quantitative real-time PCR (Figure 2). IRS-2 displayed 36 times higher mRNA expression in tick salivary glands at day 6 after tick attachment to the host, compared with salivary glands derived from unfed ticks. Already, at day 2 after attachment, the expression was 9-fold higher than in unfed ticks, suggesting a role in the tick salivary glands, even in the early stage of tick feeding. The increase of IRS-2 gene expression was also notable in tick ovaries, with a 4-fold increase at day 6 after attachment. Finally, IRS-2 transcript abundance fluctuated in the tick midgut as feeding progressed.

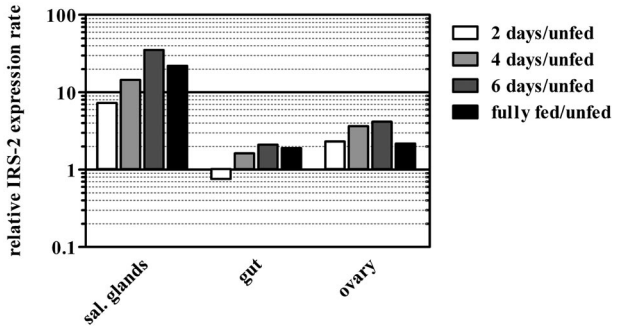

Figure 2. Expression of IRS-2 is up-regulated after tick attachment to the host. IRS-2 transcripts accumulate rapidly in salivary glands after the attachment of an adult *I ricinus* female, less rapidly in its ovaries, and they remain almost unchanged in its midgut. Each bar in the graph represents the ratio between IRS-2 transcript abundance in a certain tick tissue and a given tick feeding stage, compared with the corresponding tissue from unfed ticks (ie, the black bar in the salivary glands part of the graph shows that the transcript abundance of IRS-2 mRNA is 20× higher in the salivary glands of fully fed adult female ticks than in the salivary glands of unfed females). The scale in the y-axis is logarithmic.

IRS-2 inhibits acute inflammation

Induction of IRS-2 transcription in the salivary glands upon blood feeding and the known anti-inflammatory properties of tick saliva led us to investigate whether IRS-2 could contribute to the immunomodulatory activity of tick saliva. For this purpose, we produced recombinant IRS-2 in a bacterial expression system and proceeded with lipopolysaccharide decontamination. Subsequently, we assessed its effect in a mouse model of acute inflammation. It is well described that when carrageenan is inoculated in the mouse footpad, it induces an acute inflammatory response characterized by edema formation in the paw accompanied by neutrophil influx.[31] To investigate whether IRS-2 could modulate carrageenan-induced inflammation, carrageenan was administered in the mouse footpads in the presence or absence of IRS-2. When carrageenan was injected in the presence of IRS-2, a dose-dependent inhibition of edema formation was observed (Figure 3 A). More specifically, in the presence of 1 mg/kg of IRS-2, the thickness of the mouse paw was similar to that of negative controls; that is, PBS-injected mice (data not shown) or mice injected with IRS-2 only (Figure 3A). The decrease in edema formation reached 67.2% ($P < .001$) at 4 hours after injection, compared with positive control (carrageenan-injected footpads). This was not 100% because there was a background increase in paw thickness even when injecting vehicle alone, since the inoculum (40 μL of liquid) could not be completely absorbed by the footpad within 4 hours. The inhibition observed after 4 hours of carrageenan injection with IRS-2 at the concentration 0.3 mg/kg was 23.4%, compared with positive control, and it was not statistically significant (Figure 3A). At 24 hours after injection, there were no differences in edema formation in any of the experimental groups of mice. Next, we analyzed carrageenan-induced recruitment of neutrophils in the footpads by measuring tissue MPO activity. The experimental design was the same as in the paw edema experiment. MPO activity in the tissue was evaluated at 4 hours after injection, the time point at which edema peaks. Statistically significant inhibition ($P < .05$) of MPO activity (34.3%) was observed even when 0.3 mg/kg of IRS-2 was coadministered with carrageenan. Inhibition reached 71.6% when 1 mg/kg of IRS-2 was coadministered with carrageenan ($P < .001$; Figure 3B). Finally, we normalized the detected MPO activity for the number of neutrophils per milligram of inflamed tissue, and we further evaluated the effect of chymostatin (a generic inhibitor of primarily chymotrypsin-like serine proteases) and indomethacin (a nonsteroidal anti-inflammatory compound) on MPO activity/neutrophil migration. Both effectors displayed similar inhibitory effect with IRS-2 on neutrophil migration (Figure 3C).

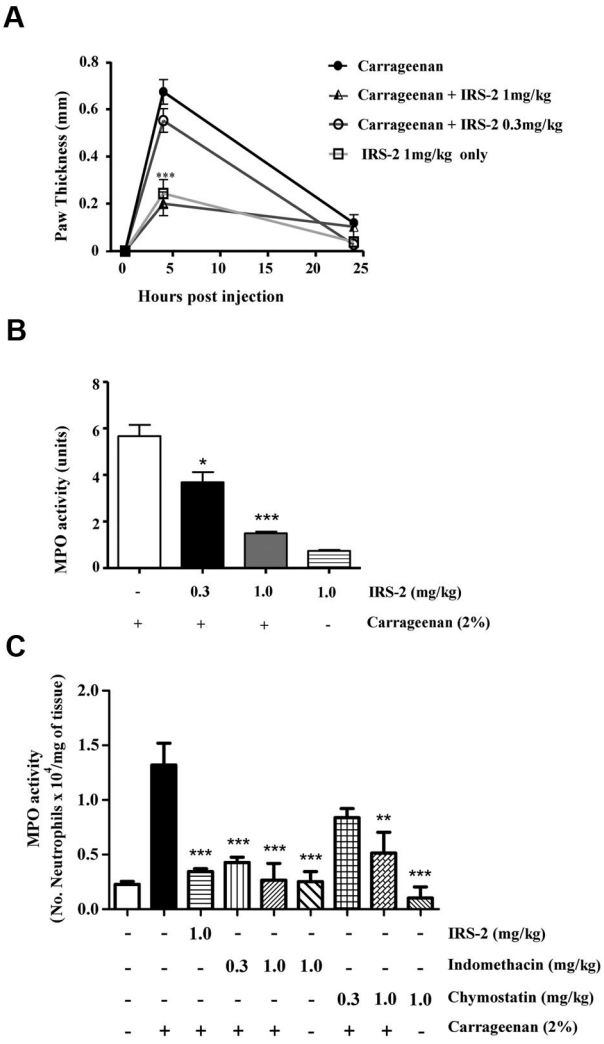

Figure 3. IRS-2 inhibits carrageenan-induced acute inflammation. Mice received carrageenan injections intraplantary in either the absence or presence of 0.3 or 1 mg/kg IRS-2. (A) Edema formation was evaluated at 4 and 24 hours after injection (abscissa) as the increase in paw thickness (in millimeters). (B) Neutrophil recruitment in inflamed footpads was evaluated by measuring tissue myeloperoxidase activity, expressed as units of activity/g of tissue (ordinate). Bar 1 (numbering left to right), activity detected when only carrageenan was administered to mice; bars 2 and 3, effect of coadministration of carrageenan with 0.3 and 1 mg of IRS-2 per 1 kg of body weight, respectively. Bar 4, IRS-2 injected without carrageenan. (C) Mice received injections of saline (-), carrageenan, carrageenan plus IRS-2 (1 mg/kg), indomethacin (0.3 and 1 mg/kg) or chymostatin (0.3 and 1 mg/kg). At 4 hours after injection, the hind paws were collected for MPO analysis and the amount of neutrophils per milligram of tissue was estimated by comparison with purified neutrophils. Asterisks represent statistically significant differences in MPO activity (*$P < .05$; **$P < .01$; and ***$P < .001$), compared with groups injected with carrageenan only (1-way analysis of variance followed by Tukey post-hoc test; n:4 in each group).

IRS-2 specifically targets cathepsin G and chymase

Given the observed anti-inflammatory effect of IRS-2, we proceeded to a more detailed analysis of the IRS-2 mechanism of action. Considering that serpins are potential inhibitors of serine proteases that play a role in inflammation, and that the bioinformatic analysis of IRS-2 reactive center loop (RCL) suggests an

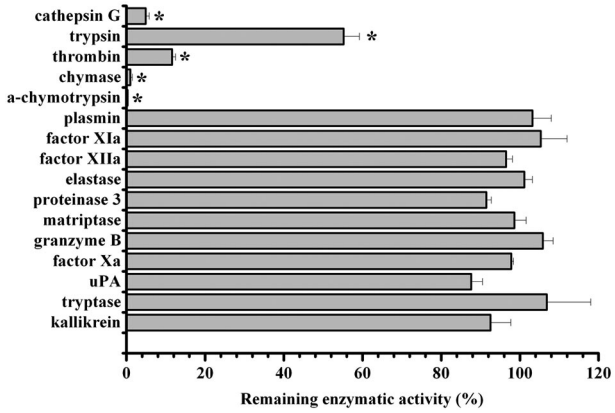

Figure 4. Inhibitory specificity of IRS-2. IRS-2 (400nM) was tested against 16 different serine proteases in triplicates. The enzyme concentration is stated in supplemental Table 2. Bars represent the mean remaining enzymatic activity in the presence of IRS-2, while error bars represent the SEM. Enzymes with an asterisk were inhibited with a statistical significance (*t* test; $P < .05$).

inhibitory specificity against chymotrypsin-like, rather than trypsin-like, serine proteases, we tested the protein for inhibitory activity against a panel of pure recombinant human serine proteases. Recombinant IRS-2 was tested against an array of 14 different physiologically relevant serine proteases, in addition to α-chymotrypsin and trypsin, the 2 archetypes for chymotrypsin- and trypsin-like serine proteases. As shown in Figure 4, IRS-2 inhibited 2 trypsin-like (trypsin and thrombin) and 3 chymotrypsin-like (α-chymotrypsin, cathepsin G, and mast cell chymase) proteases significantly ($P < .05$), with higher activity against the latter group. IRS-2 did not inhibit a series of serine proteases related with the coagulation or inflammation, such as plasmin, factor Xa, factor XIa, or elastase and proteinase 3, suggesting a stringent specificity (Figure 4). Table 1 and Figure 5A-B summarize the concentration-dependence of IRS-2 inhibition for all 5 targeted enzymes. Table 1 further describes the binding characteristics for thrombin, cathepsin G, and chymase, 3 physiologically relevant proteases. For further details on the related biochemical analysis, please see supplemental Results and supplemental Figures 1 and 2.

IRS-2 inhibits mMCP-4

Having dissected the target specificity of IRS-2, and considering that chymase (1 of the 2 targets of IRS-2) is released from mast cells upon acute inflammation, we next investigated whether IRS-2 would bind to mouse (the animal model used for the anti-inflammatory experiments) mast cell protease-4 (mMCP-4). Furthermore, mMCP-4 is the main chymotrypsin-like serine protease produced by connective tissue–type mouse mast cells and is a functional homolog to the human chymase.[32] Therefore, we evaluated the effect of IRS-2 on mMCP-4. Indeed, IRS-2 was found to inhibit mMCP-4 both in vitro and ex vivo. Analogous to the inhibition observed for human chymase, purified mMCP-4 was inhibited by IRS-2 in equimolar concentrations. The estimated inhibitory concentration at half-maximum of IRS-2 against purified mMCP-4 was 5.21nM, when using a 5nM concentration of the enzyme (Figure 5C). Moreover, IRS-2 inhibited the chymotryptic activity present in the suspension of ionomycin-activated PCMCs in a dose-dependent manner (Figure 5C). We also detected the formation of covalent mMCP-4/IRS-2 complexes by Western blot. Notably, mMCP-4/IRS-2 complexes were only detected in association with the cell layer, while no complexes were detected in the conditioned medium. The number of produced complexes was dependent on the concentration of IRS-2 (Figure 5D). More specifically, at 20nM concentration of IRS-2, most of the mMCP-4 remained in the free state, while at 50 and 200nM IRS-2, higher amounts of mMCP-4 were recovered in complex with IRS-2.

IRS-2 inhibits cathepsin G– and thrombin-induced platelet aggregation

Cathepsin G, the second target of IRS-2, plays a role in platelet aggregation, which is crucial in wound-healing processes that are essential for host ability to reject a blood-feeding tick. Therefore, we evaluated the effects of IRS-2 on cathepsin G–induced platelet aggregation, which is mediated by protease-activated receptor 4 (PAR4).[33] Figure 6A shows that cathepsin G–induced shape change and platelet aggregation was completely inhibited by 15-minute incubation with IRS-2 (Figure 6A). In higher excess to the enzyme, IRS-2 inhibited also thrombin-induced platelet aggregation (Figure 6B), which is mediated by PAR1 and PAR4. Collagen-induced (Figure 6C), convulxin-induced[34] (Figure 6D), U46619-induced (Figure 6E), and arachidonic acid-induced (Figure 6F) platelet aggregation remained unaffected, demonstrating the specificity in IRS-2–driven inhibition of platelet aggregation. These results suggest that IRS-2 interferes with platelet aggregation by blocking their activation through PARs, thus implicating IRS-2 as an inhibitor of wound-healing processes.

Structural analysis of IRS-2

Finally, we resolved the crystal structure of IRS-2, which was determined by molecular replacement and refined using data to 1.8 Å resolution. The structure of equine leukocyte elastase inhibitor (PDB code 1HLE) was used as a search model; this homolog had the highest sequence similarity with IRS-2 (35% identity) among the serpin structures available in the PDB. The tetragonal crystal form of IRS-2 contains 2 molecules in the asymmetric unit with solvent content of 45%. The root mean square deviation (RMSD) for superposition of the main-chain atoms of these

Table 1. A tabular representation of IRS-2 inhibition characteristics for the targeted serine proteases

Enzyme	Amount of enzyme used, nM	IRS2 IC_{50}, nM	IC_{50}/enzyme ratio	Inhibitor characteristics
Cathepsin G	12.2	11.5 ± 0.7	0.94	Fast binding tight
Chymase	3.6	4 ± 0.2	1.11	Slow binding tight
Thrombin	0.02	170.3 ± 11	8515	Slow binding classical
α-chymotrypsin	0.038	0.38 ± 0.02	10	N/A
Trypsin	0.25	562.7 ± 27.3	2251	N/A

The amount of enzyme used in the assays as well as the inhibitory concentration at half-maximum (IC_{50}) of IRS-2 for these enzymes is stated in the first and second columns. The third column represents the ratio between IRS-2 IC_{50} and enzyme concentration, and the last column shows the binding characteristics of the inhibitor to the targeted enzymes with the relevance in host physiology (ie, tight or classical inhibitor, fast binding, or slow binding to the enzyme). Because trypsin and α-chymotrypsin were not physiologically relevant to our studies, we did not further study their inhibition characteristics, as depicted in by N/A.

NA indicates not applicable.

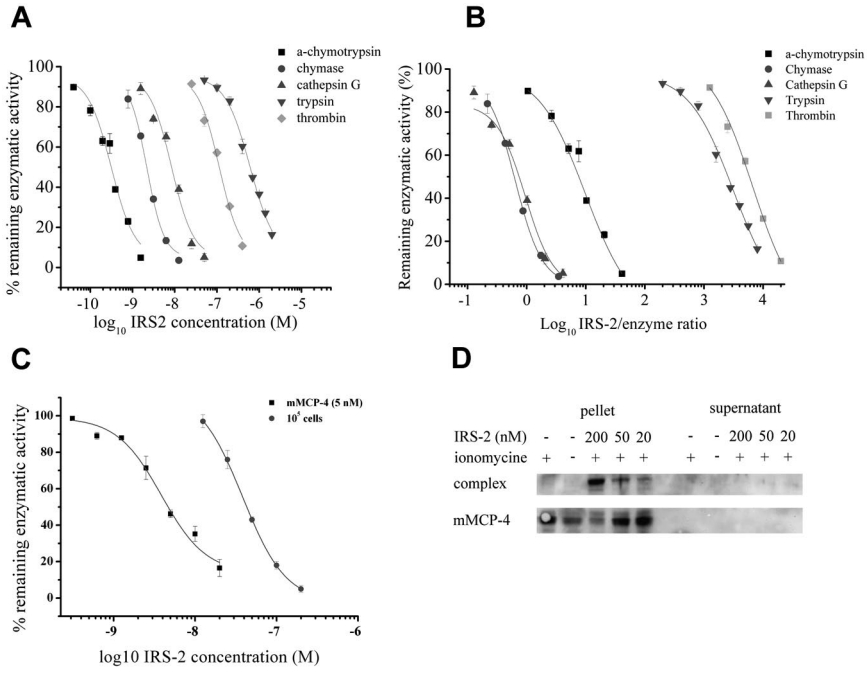

Figure 5. IRS-2 is a serpin with activity against chymotrypsin-like, rather than trypsin-like, serine proteases. (A) Proteases targeted by IRS-2. α-chymotrypsin, chymase, cathepsin G, trypsin, and thrombin are inhibited by IRS-2. The amount of enzyme used is stated in Table 1. The mean remaining enzymatic activity in the presence of various concentrations of IRS-2 is represented, while the error bars represent the SEM in triplicate assays. (B) Inhibition data are normalized by plotting the remaining enzymatic activity (y-axis) against the IRS-2/enzyme ratio (x-axis). (C) Inhibition of purified mMCP-4 (5nM) or present in the suspension of activated mouse PCMCs by IRS-2. The mean remaining enzymatic activity in the presence of various concentrations of IRS-2 is presented (± SEM). (D) Western blot analysis showing covalent complex formation between IRS-2 and mMCP-4 produced by PCMCs. Notably, enzymatic activity and complexes between mMCP-4 and IRS-2 were predominantly cell associated, rather than being present in cell-free supernatants. Cells activated by ionomycin were used as positive control (+), and untreated cells were used as negative control (−). All samples with IRS-2 were activated by ionomycin.

2 molecules is 0.69 Å, a value within the range observed for different crystal structures of identical proteins.[35] Minor structural changes are localized in solvent-exposed loop regions. The N-terminal residue, Met1, represents a cloning artifact that is not part of the native protein sequence. Atomic coordinates of IRS-2 and experimental structure factors have been deposited in the PDB, with the accession code 3NDA.

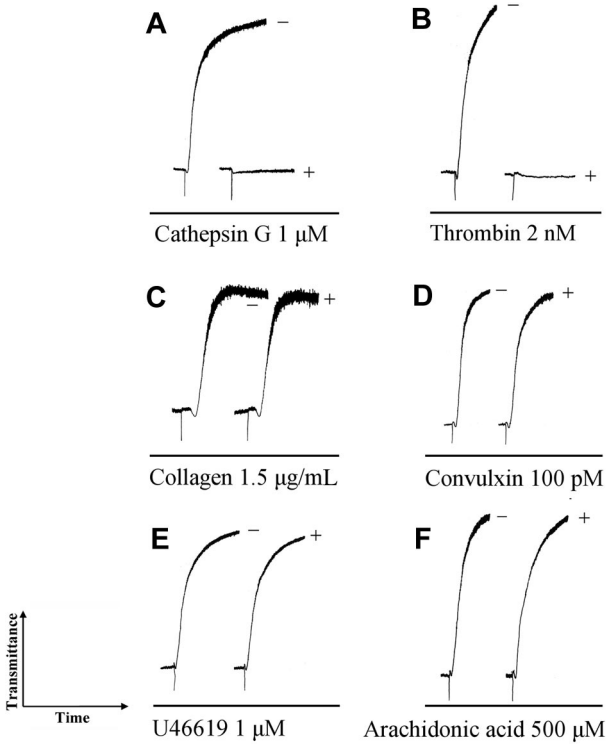

Figure 6. IRS-2 inhibits platelet aggregation induced by cathepsin G. In all experiments, 1.5μM IRS-2 was incubated with the indicated amount of platelet aggregation activator for 15 minutes and the mixture was added to platelets. (−) activator only, (+) activator plus IRS-2.

Figure 7 shows the overall structure of IRS-2. It adopts a typical serpin fold composed of 3 large β-sheets and 9 α-helices. Both molecules in the asymmetric unit adopt a conformation known as the relaxed or R state of the serpins, in which the RCL is cleaved and inserted into the central β-sheet A as a strand, S4 (Figure 7A). Cleavage of RCL occurs during the crystallization process and is catalyzed by traces of contaminating proteases, as demonstrated in the crystallization study.[19] The cleavage site is positioned at residue Tyr341, which represents the P1 substrate residue. In our structure, this residue has a well-defined electron density for its C-terminal carboxyl group. The P1′–P5′ residues of the cleaved RCL are disordered to various extents in the crystal structure; more specifically, residues 342-344 and 342-346 in molecules A and B, respectively, could not be modeled and are missing in the final structure.

A structural comparison of IRS-2 with structures from the serpin superfamily deposited in the PDB identified bovine antithrombin III (PDB code 1ATT) as the closest structural homolog (RMSD approximately 1.4 Å for 369 aligned residues). A lower structural similarity was found to typical representatives of the mammalian serpins, human α-1-antichymotrypsin (PDB code 2ACH, RMSD approximately 1.6 Å for 337 residues), human α-1-antitrypsin (PDB code 9API; RMSD approximately 1.8 Å for 335 residues), and to human corticosteroid-binding globulin and human plasminogen activator inhibitor-1 (PDB codes 2VDX and 3CVM; both RMSD approximately 1.7 Å for 363 residues). All the compared structures are in the R-state conformation; a superposition of selected structures with IRS-2 is presented in Figure 7B.

Discussion

Many *I scapularis* serpins possess Arg at the P1 position,[10] which corresponds to typical cleavage sites for trypsin-like enzymes. This suggests their involvement in modulation or regulation of trypsin-like proteases involved in host-blood coagulation or tick hemolymph, probably with some redundancy in their function. On the

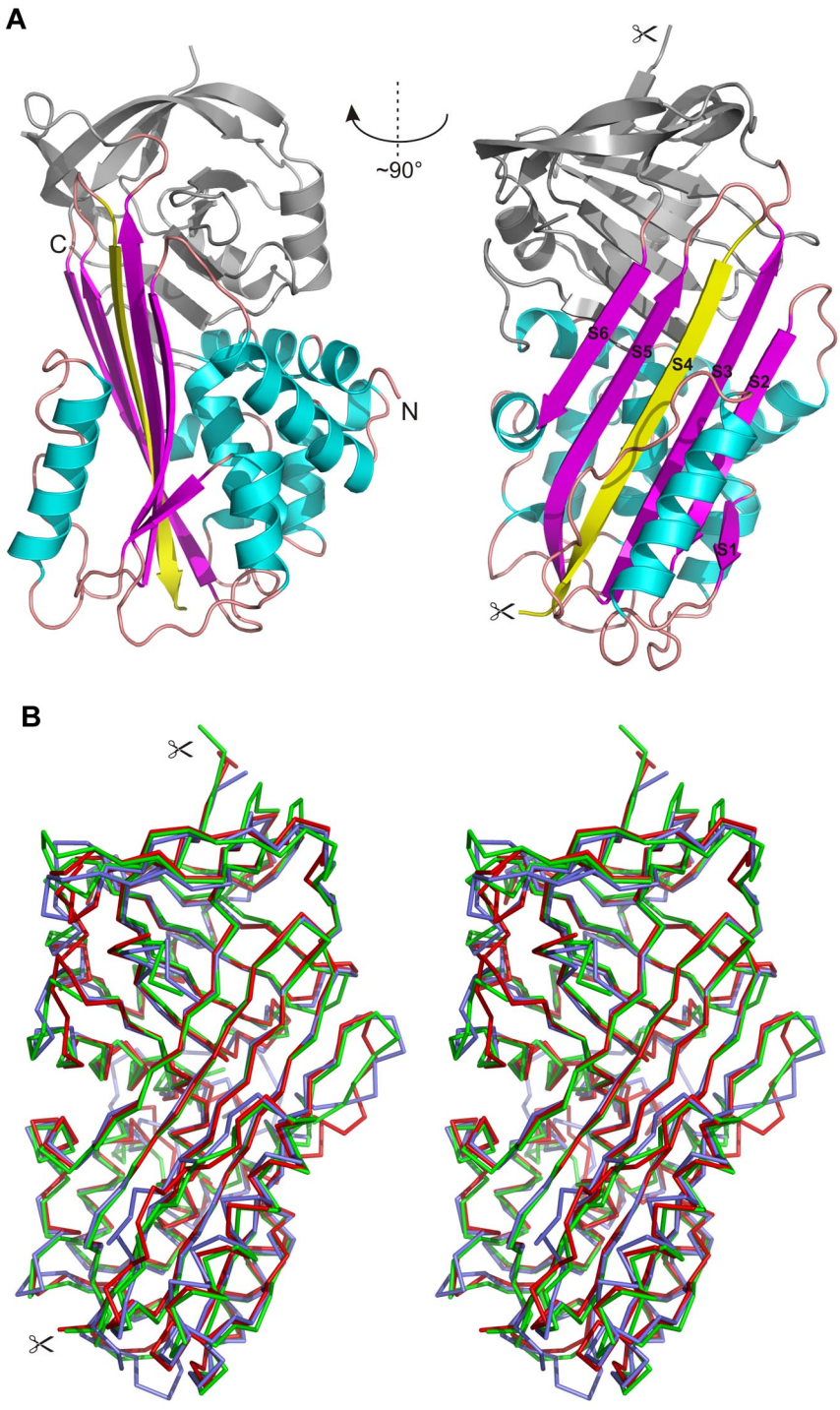

Figure 7. Crystal structure of IRS-2. (A) Overall 3-dimensional structure of IRS-2 in a cartoon representation. The central β-sheet A (magenta) and surrounding helices (cyan) are highlighted. The reactive center loop is cleaved in the relaxed (R-state) conformation of IRS-2 and forms the S4 β-strand (yellow) inserted into the β-sheet A; the termini generated by this proteolytic cleavage are marked by scissors. N- and C-termini of IRS-2 are labeled (N, C). (B) Stereo image showing a superposition of Cα traces of IRS-2 (red) with 2 homologous mammalian serpins in the R-state conformation. Antithrombin III (blue; 1ATT) and α-1-antichymotrypsin (green; 2ACH) display a high level of similarity to IRS-2 with regard to structural homology and inhibitory specificity, respectively.

other hand, only 2 putative serpins that possess the aromatic aa (ie, Trp, Tyr, or Phe) at the P1 position can be found in the *I scapularis* genome, thus having the potential to inhibit chymase and other chymotrypsin-like proteases.[36] According to the nomenclature used by the VectorBase server (http://iscapularis.vectorbase.org), these serpins are encoded by the transcripts, ISCW004156-RA and ISCW011013-RA. The latter transcript is the homolog of IRS-2. Considering the preferential aa residues in the cleavage site for cathepsin G,[37] we believe that IRS-2 is the only known salivary serpin from *I ricinus* that can target both cathepsin G and chymase.

IRS-2 interacts with proteases via a classical "suicide inhibition" mechanism of serpins. It involves cleavage of RCL and formation of S4 β-strand that is inserted in the middle of β-sheet A (Figure 7A). We determined a high-resolution crystal structure of the protease-cleaved IRS-2 that provided an experimental evidence of the Tyr residue at the P1 position. These structural data will be valuable for designing smaller peptides that may mimic IRS-2 inhibitory activities and its pharmacological actions. IRS-2 targets specifically the chymotrypsin-like proteases, cathepsin G and chymase. Both of these proteases are secreted after neutrophil

(cathepsin G) and mast cell (chymase) activation, and they are involved in a whole range of physiological processes associated with the development of an acute inflammatory response and, in particular, in the cross-talk between neutrophils and platelets in the hemostatic process.[38] Cathepsin G is produced mainly by neutrophils, where it is stored in azurophile granules, but it may also be produced in low amounts by mast cells. Cathepsin G is known primarily for its ability to kill engulfed pathogens and for its role in tissue remodeling during inflammation. Furthermore, it is involved in the proteolytic activation of various chemoattractants and hormones as well as in cell signaling by cleaving PAR4, the latter having been identified as an important signaling receptor in inflammation and platelet activation.[39] Cleavage of PAR4 by cathepsin G is responsible for the platelet activation that leads to their aggregation and clot formation. Here, we showed, for the first time, that a tick salivary protein inhibits cathepsin G–induced platelet aggregation, which apparently helps the tick in obtaining its blood meal. In addition, we revealed that in higher molar excess, IRS-2 affects thrombin-induced platelet aggregation as well, further disclosing its multipotential role in inflammation and hemostasis through the modulation of PAR activation.

Besides preventing blood loss after an injury, activated platelets produce several chemokines and their precursors, which are further proteolyticaly processed. In this respect, the role of cathepsin G overlaps with that of chymase. Chymase is produced almost exclusively by mast cells that are resident in mucosal and connective tissues and it processes many substrates, such as angiotensin I, extracellular matrix components, and also several proinflammatory substances, such as interleukin-1β and interleukin-18 precursors.[32] Both cathepsin G and chymase activate connective tissue–activating peptide-III (CTAP-III), which is secreted by activated platelets, into an active neutrophil-activating peptide-2 (NAP-2), the chemokine responsible for further activation of neutrophils and their attraction to the site of injury. It was also shown that chymase is mainly responsible for CTAP-III/NAP-2 conversion, and that activated mast cells displayed a 1000-fold higher conversion rate than activated neutrophils.[40] Other natural substrates for both enzymes are the big endothelins (ETs), the precursors for several vasoconstrictors with different potency. Their cleavage products are 31-aa fragments, denoted Ets (1-31), that, apart from being among the most potent vasoconstrictors, also act as chemoattractants for neutrophils and monocytes.[41] Interestingly, the RCL of IRS-2 strongly resembles the chymase cleavage site in big ETs. The sequence in the P3–P3′ region consists of VPY–SLG for IRS-2 and VPY–GLG for big ETs. We can therefore hypothesize that this is the result of convergent evolution that created almost identical recognition sites that target both cathepsin G and chymase on otherwise unrelated proteins. To our knowledge, this is the first time that a parasitic protein is shown to inhibit host proteases by mimicking host endogenous substrates. Moreover, structural comparison revealed significant structural homology of IRS-2 with α-1-antichymotrypsin, which is supposed to be a natural vertebrate host regulator of cathepsin G and mast cell chymase.

Neutrophil-derived cathepsin G and, in particular, mast cell chymase are present at the early stages of an acute inflammatory response, probably with some level of redundancy and/or cooperation in their functions. By inhibiting both enzymes, IRS-2 can significantly alter a defensive response to tissue destruction caused by a tick bite. Indeed, we demonstrated that IRS-2 inhibits carrageenan-induced acute inflammation and cathepsin G and thrombin-induced platelet aggregation. According to recent findings relating to the biologic function of cathepsin G and mast cell chymase, we can hypothesize that both described effects may be mediated by proteolyticaly activated transducers and receptors during the early steps of inflammatory response that occurs after a tick bite. To explain the observed effects of IRS-2, we suggest that IRS-2 targets neutrophils, platelets, and mast cells at the early steps of their activation by preventing cathepsin G– or thrombin-driven platelet activation, which may lead to reduced CTAP-III release. Subsequently, IRS-2, similar to chymase inhibitors,[40] inhibits the cathepsin G– and chymase-catalyzed conversion of CTAP-III into NAP-2, resulting in impaired extravasation of neutrophils. The direct proof of the suggested, literature-based hypothesis exceeds the purpose of this work.

Here, we describe a mechanism of vertebrate host modulation that is novel not only for a tick salivary component, but to our knowledge for any parasite. Moreover, the dual specificity of IRS-2 for both cathepsin G and chymase is rare even among natural serine protease inhibitors in general. The saliva of *I ricinus* is a complex mixture of many proteins, including protease inhibitors with unknown specificity and function. This work describes a novel salivary protein that is unique among other salivary serpins of *Ixodes spp*—and proteases inhibitors in general—regarding both specificity and the mode of action in the vertebrate host. Therefore, the herein described findings contribute to our understanding of vertebrate physiology and parasite-host interaction, while the herein disclosed structural basis of IRS-2 activity can lead to the development of pharmaceutical applications in the near future.

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Because I.M.B.F. and J.M.C.R. are government employees and this is a government work, the work is in the public domain in the United States. Notwithstanding any other agreements, the NIH reserves the right to provide the work to PubMedCentral for display and use by the public, and PubMedCentral may tag or modify the work consistent with its customary practices. One can establish

Authorship

Contribution: J.C., C.J.O., I.M.B.F., M.M., and M.K. designed and performed experiments, analyzed data, and wrote the manuscript; P.R. and Z.K. designed and performed experiments; and G.P., P.K., J.M.C.R., and J.K. designed experiments and analyzed data.

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Article 3

Ixodes ricinus salivary serpin IRS-2 affects Th17 differentiation via inhibition of the interleukin-6/STAT-3 signaling pathway

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Since IRS-2 proved as an anti-inflammatory protein, we tried to find the mechanism behind. In this study, Jana Páleníková performed a series of assays with recombinant IRS-2 and found that it inhibited Th17 differentiation by affecting the interleukin-6 (IL-6)/STAT-3 signaling pathway. The production of IL-6 was diminished at the level of mRNA production by IRS-2 after activation with Borrelia spirochetes. This lead to attenuated STAT-3 phosphorylation and to impaired Th17 differentiation as was assessed by flow cytometry and ELISA. After IRS-2 treatment the population of Th17 cells decreased and also the production of Th17 specific cytokine IL-17A was significantly diminished. Since Th17 is a proinflammatory subset of CD4+ cells, we could assume that one of the mechanisms, how IRS-2 impair inflammatory response, is in preventing Th cells from differentiation into Th17 subset. Interestingly, the inhibition of IL-6 production was specific, other inflammatory cytokines, such as IL-1β and TNF were not affected.

The significance of this study is in the disclosing of the mechanism how IRS-2 inhibits the inflammatory response.

Author's contribution:

Author produced and provided recombinant IRS-2 for the experiments and participated on writing of the manuscript.

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Ixodes ricinus Salivary Serpin IRS-2 Affects Th17 Differentiation via Inhibition of the Interleukin-6/STAT-3 Signaling Pathway

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Th17 cells constitute a subset of CD4$^+$ T lymphocytes that play a crucial role in protection against extracellular bacteria and fungi. They are also associated with tissue injury in autoimmune and inflammatory diseases. Here, we report that serpin from the tick *Ixodes ricinus*, IRS-2, inhibits Th17 differentiation by impairment of the interleukin-6 (IL-6)/STAT-3 signaling pathway. Following activation, mature dendritic cells produce an array of cytokines, including the pleiotropic cytokine IL-6, which triggers the IL-6 signaling pathway. The major transcription factor activated by IL-6 is STAT-3. We show that IRS-2 selectively inhibits production of IL-6 in dendritic cells stimulated with *Borrelia* spirochetes, which leads to attenuated STAT-3 phosphorylation and finally to impaired Th17 differentiation. The results presented extend the knowledge about the effect of tick salivary serpins on innate immunity cells and their function in driving adaptive immune responses.

Ticks are bloodsucking arthropods, major vectors of human pathogens like *Borrelia burgdorferi* and tick-borne encephalitis virus. Ticks from the family Ixodidae (hard ticks) require several days to fully engorge. During feeding, ixodid ticks remain tightly attached to their host (1, 2). To avoid attack from the host immune system during the feeding period, tick saliva contains two groups of molecules, the first with antihemostatic and the second with immunomodulatory properties. These groups include both proteinaceous and nonprotein molecules (3). One group of immunomodulatory proteins is represented by serine proteinase inhibitors (serpins), a large superfamily of structurally related, but functionally diverse, proteins that control essential proteolytic pathways (4, 5). Recently, three serine protease inhibitors, namely, purified human urinary trypsin inhibitor (UTI) and two synthetic serpins, gabextate mesilate (FOY) and nafamostat mesilate (FUT), which are widely used in treatment of acute inflammatory disorders, such as disseminated intravascular coagulation (DIC), have been shown to attenuate allergic airway inflammation and remodeling in a murine model of chronic asthma. These effects were associated with inhibition of Th2 cytokines (interleukin-4 [IL-4], IL-5, IL-6, and IL-13) and Th17 cell functions. These serpins also inhibited NF-κB activation in lung tissues (6).

Until now, more than 60 serpins have been identified at the sequence level in ixodid ticks, but only two serpins from *Ixodes ricinus* have been further functionally characterized (7–9). The first known *I. ricinus* serpin, Iris (*I. ricinus* immunosuppressor), is known to preferentially target leukocyte elastase. It also interferes with the contact phase coagulation pathway, fibrinolysis, and disrupts platelet adhesion. Moreover, Iris has the ability to modulate both innate and adaptive immunity. It affects T lymphocyte and macrophage responsiveness, and it induces a Th2-type response and inhibits the production of proinflammatory cytokines. Interestingly, it was shown that the anti-inflammatory properties of the protein are independent of its proteolytic activity and are mediated through its exosite domain (10–13).

IRS-2, the second described serpin from *I. ricinus*, targets cathepsin G and chymase. Both enzymes are part of the acute inflammatory response and are produced by activated neutrophils (cathepsin G) and mast cells (chymase). Moreover, IRS-2 is able to inhibit swelling and the migration of neutrophils into the inflamed tissue (14). The effects of IRS-2 on other cells of innate and acquired immunity have not been described so far.

Dendritic cells (DCs) are known as antigen-presenting cells and play a critical role in initiating and modulating the immune response. With their ability to recognize, process, and present antigens on their surfaces and thus activate T lymphocytes, DCs form a unique link between innate and acquired immunity (15, 16). Depending upon the recognized pathogens and other stimuli produced by activated DCs, such as cytokines and chemokines, T lymphocytes differentiate into cytotoxic CD8$^+$ or helper CD4$^+$ cells, which can further differentiate into various subsets (17). The IL-6/STAT-3 signaling pathway leads to differentiation of CD4$^+$ T lymphocytes into the Th17 subset. IL-6, a pleiotropic cytokine produced by dendritic cells in response to invading pathogens, binds to IL-6 receptors on T cells and activates the signaling pathway, leading to phosphorylation of the transcription factor STAT-3, an essential molecule for Th17 differentiation (18, 19). Th17 cells participate in host defense against extracellular bacteria and fungi by mediating the recruitment of neutrophils and macrophages into infected tissues. It is also known that regulation of Th17 cells plays a significant role in the pathogenesis of various inflammatory and autoimmune disorders (20–22). Moreover, it was shown that Th17 cells are involved in

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the development of severe destructive arthritis caused by the Lyme disease spirochete *B. burgdorferi* (23).

The objective of this study was to analyze the effect of a tick salivary serpin on dendritic cells and its consequences for the development of proinflammatory cells, like Th17 lymphocytes.

MATERIALS AND METHODS

Experimental animals. Specific-pathogen-free C57BL/6 mice (6- to 10-week-old females) were purchased from Charles River Laboratories. The animals were maintained under standard conditions in the animal house facility of the Institute of Parasitology, Biology Centre AS CR, České Budějovice. All experiments were performed with permission of the Czech animal ethics committee.

Recombinant IRS-2. Recombinant serpin from *I. ricinus*, IRS-2, was overexpressed in *Escherichia coli* BL21(DE3) pLysS cells. The expressed protein accumulated in inclusion bodies, which were separated. Refolded and concentrated IRS-2 was purified using a standard chromatographic method (fast protein liquid chromatography [FPLC]) (14, 24). Lipopolysaccharide (LPS) contamination was removed by Arvys Proteins Company using the detergent-based method.

Bacteria. *B. burgdorferi sensu stricto* ATCC 35211 isolated from *I. ricinus* was grown in Barbour-Stoenner-Kelly-H (BSK-H) medium (Sigma-Aldrich) supplemented with 6% rabbit serum at 34°C. The number of spirochetes was calculated by dark-field microscopy according to the method of Magnuson et al. (25). The fourth to sixth passages were used in the experiments.

Splenic DC isolation. Isolated mouse spleens were minced with scissors, digested in RPMI containing 0.25 mg/ml Liberase DL (Roche) and 0.2 mg/ml DNase I (Roche) at 37°C for 30 min, and passed through a 70-μm nylon cell strainer (BD Falcon). The dendritic cells were isolated using magnetic beads conjugated with anti-CD11c antibody (Ab) and magnetically activated cell sorting (MACS) column separation following the manufacturer's instructions (Miltenyi Biotec). The purified dendritic cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 50 μM 2-mercaptoethanol, 100 μg/ml penicillin, and 100 U/ml streptomycin (all from Sigma-Aldrich). The purity of the isolated dendritic cells (~90% $CD11c^+$ cells) was determined by subsequent fluorescence-activated cell sorter (FACS) analysis.

$CD4^+$ T cell isolation. The fourth day after subcutaneous infection of mice with 1×10^5 *Borrelia* spirochetes, isolated mouse spleens were passed through a 70-μm nylon cell strainer (BD Falcon), and $CD4^+$ T cells were isolated using magnetic beads conjugated with anti-CD4 Ab and MACS column separation following the manufacturer's instructions (Miltenyi Biotec). Purified $CD4^+$ T cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 50 μM 2-mercaptoethanol, 100 μg/ml penicillin, and 100 U/ml streptomycin (all from Sigma-Aldrich). The purity of the isolated $CD4^+$ T cells (≥90% $CD4^+$ $CD62L^{high}$) was determined by FACS analysis.

Specific activation of $CD4^+$ T lymphocytes. Purified splenic DCs were seeded at 5×10^4 cells per well in 96-well plates and stimulated with *Borrelia* spirochetes (5×10^5 per well) and IRS-2 (6 μM). After 24 h incubation, the medium was removed, and 3×10^5 freshly isolated *Borrelia*-primed $CD4^+$ T lymphocytes in 200 μl of culture medium were added to each well. The T cells were incubated with DCs for 3 days before restimulation with phorbol myristate acetate (PMA) (20 ng/ml) and ionomycin (1 μM) (both Sigma-Aldrich). Cell-free culture supernatants for IL-17 and IL-9 assessment were harvested at 2, 6, 12, 24, and 48 h after restimulation.

To determine the number of IL-17-producing Th cells, *Borrelia*-exposed DCs and *Borrelia*-primed $CD4^+$ T cells were cocultured as described above. On day 5 of coculture, the cells were restimulated with PMA and ionomycin and, after an additional 2 h, treated with monensin (2 μM; eBiosciences). The cells were then incubated for 4 h before staining was performed with anti-IL-17 antibody conjugated with phycoerythrin (PE) (eBioscience).

Cytokine measurement. Freshly isolated dendritic cells were seeded at 2×10^5 cells per well on 96-well plates. The next day, the DCs were stimulated with *B. burgdorferi* spirochetes at a multiplicity of infection (MOI) of 10 (2×10^6 per well) in the presence or absence of IRS-2 (6 μM). Cell-free culture supernatants were harvested 2, 9, 12, 24, or 48 h after stimulation and used for detection of IL-1β, IL-6, IL-10, and tumor necrosis factor alpha (TNF-α) with Ready-Set-Go! enzyme-linked immunosorbent assay (ELISA) kits (eBioscience) following the manufacturer's instructions. Cell-free culture supernatants for IL-17 and IL-9 assessment were prepared as described in "Specific activation of $CD4^+$ T lymphocytes" above, and the amount of cytokines was measured with a Ready-Set-Go! ELISA kit (eBioscience) following the manufacturer's instructions. All reactions were performed in triplicate.

RNA extraction, quantitative real-time PCR, and mRNA half-life determination. To assess relative mRNA expression, DCs were seeded at 2×10^6 cells per well in 24-well plates. The next day, the DCs were stimulated with *Borrelia* spirochetes at an MOI of 10 (2×10^7 per well) in the presence or absence of IRS-2 (6 μM) and incubated for 6 or 12 h. RNA was then isolated with the Nucleospin RNA II kit (Macherey-Nagel) following the manufacturer's instructions. The quality and concentration of the isolated RNA were assessed by measurement on a Nanophotometer P-330 (Implen). cDNA was synthesized with the High-Capacity RNA-to-cDNA kit (Applied Biosystems). Real-time PCR analysis was performed with a TaqMan gene expression set (Applied Biosystems) containing primers and probe specific for IL-6 and β-actin using a Rotor Gene 3000 and Rotor-Gene 6.0.19 software (Corbett Research). The relative expression of IL-6 mRNA was determined by the comparative threshold cycle (C_T) method (26), where the mouse β-actin gene was used as a housekeeping gene (Applied Biosystems). All reactions were performed in triplicate.

Immunoblotting. Freshly isolated dendritic cells were seeded at 1×10^6 cells per well in 24-well plates. The next day, the DCs were stimulated with *Borrelia* spirochetes at an MOI of 10 (1×10^7 per well) in the presence or absence of IRS-2 (6 μM). Following stimulation (15, 30, and 60 min for C/EBP, phosphorylated NF-κB [p-NF-κB], p-CREB, p-p-38, and p-ERK1/2 and 6 and 16 h for p-STAT-3), the cells were lysed in a modified RIPA buffer (1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EGTA, 150 mM NaCl, and 50 mM Tris-HCl, pH 7.5) in the presence of protease and phosphatase inhibitors (10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 μg/ml pepstatin, 25 mM NaF, and 2 mM $NaVO_3$). The protein extracts, mixed with Laemmli sample buffer, were separated by SDS-PAGE and transferred to Immobilon-P membranes. Following blocking in Tris-buffered saline (TBS)-containing 5% fat-free milk, the blots were incubated overnight with the antibodies against C/EBP, phospho-STAT-3 (Tyr^{705}), phospho-NF-κB (Ser^{536}), phospho-CREB (Ser^{133}), phospho-p38 (Thr^{180}), and phospho-ERK1/2 (Thr^{202}) (all from Cell Signaling) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) or β-actin (Santa Cruz Biotechnology). The proteins were visualized using enhanced chemiluminescence (Pierce), and their abundances were analyzed using a charge-coupled-device (CCD) imaging system (ChemiDoc MP Imaging System) and Image Lab software v. 4.1 (Bio-Rad).

To assess the level of phosphorylated STAT-3 in T lymphocytes, freshly isolated dendritic cells were seeded at 1. 5×10^5 per well in a 96-well plate. After 6 h, the DCs were stimulated with *Borrelia* spirochetes (1×10^7 per well) in the presence or absence of IRS-2 (6 μM). The next day, cell-free culture supernatants were harvested and added to freshly isolated *Borrelia*-primed $CD4^+$ T lymphocytes (9×10^5 per well). Following stimulation (15 and 30 min), the cells were lysed, and Western blotting was performed as described above with phospho-STAT-3 (Tyr^{705}) and β-actin antibodies.

Flow cytometry. $CD4^+$ cells were prepared and stimulated as described in "Specific activation of $CD4^+$ T lymphocytes" above. After 4 h of restimulation with PMA, ionomycin, and monensin, the cells were harvested (using cold 5 mM EDTA in PBS) and stained with anti-CD4 antibody (conjugated with allophycocyanin [APC]; eBioscience). After wash-

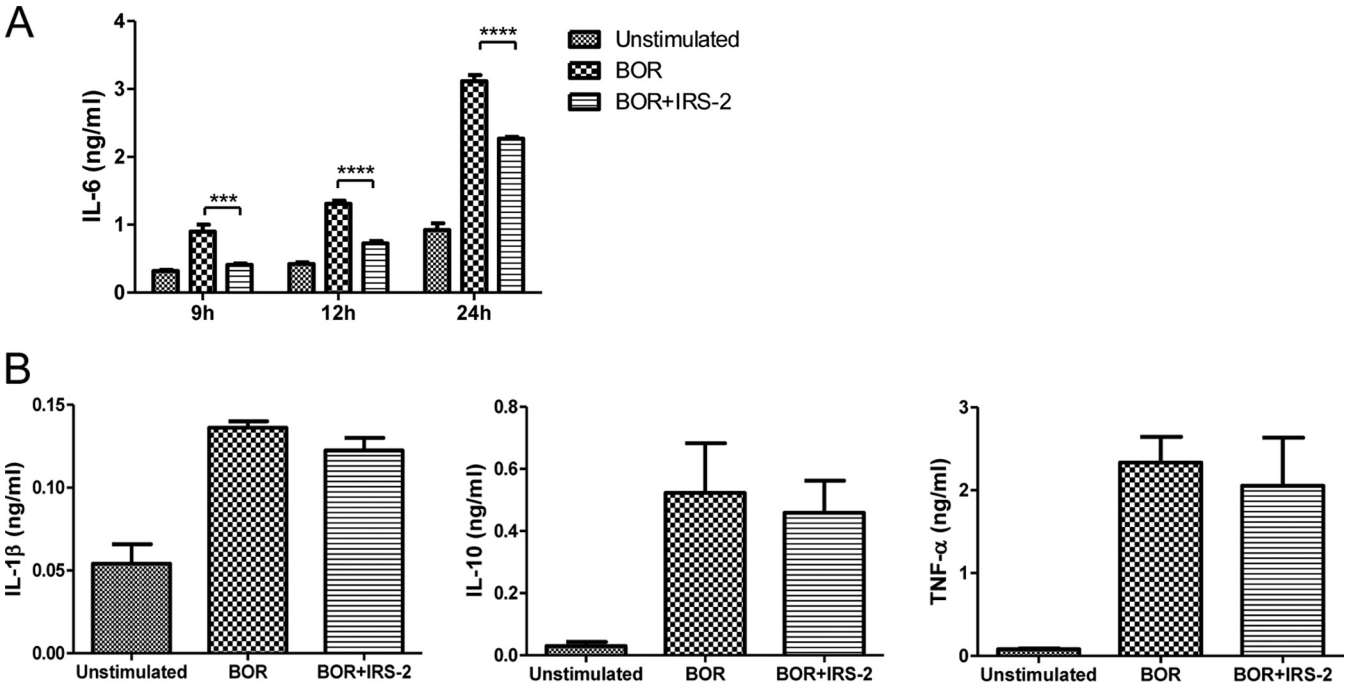

FIG 1 IRS-2 selectively inhibits IL-6 production by DCs. Splenic dendritic cells were stimulated with *Borrelia* spirochetes (BOR) (10 spirochetes per cell) in the presence or absence of IRS-2 (6 μM). (A) Culture supernatants were harvested 9, 12, and 24 h after stimulation, and the amount of IL-6 was determined by ELISA. Two independent experiments were performed, and data from a representative experiment are shown. The data are expressed as the mean cytokine concentrations from three wells plus standard errors of the mean (SEM). *** and ****, effects of IRS-2 on IL-6 production were significant at P values of <0.001 and <0.0001, respectively. (B) Culture supernatants were harvested 24 h (TNF-α) or 48 h (IL-1β and IL-10) after stimulation, and the presence of cytokines was detected by ELISA. Three independent experiments were performed, and the data were pooled. The data are expressed as the mean cytokine concentrations from nine wells plus SEM.

ing, the cells were fixed and permeabilized with a Foxp3/transcription factor staining buffer set (eBioscience) and labeled with anti-IL-17A antibody (conjugated with PE; eBioscience). The prepared cells were resuspended in cold PBS with 1% FCS. Flow cytometry was performed on a FACSCanto II cytometer using FACS Diva software v. 5.0 (BD Biosciences).

Statistical analysis. One-way analysis of variance (ANOVA) followed by a Bonferroni test in GraphPad Prism, version 5.0, was used to compare the differences between control and treated groups. A P value of ≤ 0.05 was considered statistically significant.

RESULTS

IRS-2 selectively inhibits IL-6 production by DCs upon stimulation with *Borrelia* spirochetes. Cytokines produced by activated DCs play a key role in shifting the immune response toward particular Th subsets. To investigate the effects of IRS-2 on the production of different pro- and anti-inflammatory cytokines by DCs, immature DCs were stimulated with *Borrelia* spirochetes in the presence or absence of IRS-2 for 9, 12, 24, or 48 h, and the production of IL-1β, IL-6, IL-10, and TNF-α was measured.

Serpin significantly inhibited the production of IL-6 in DCs (Fig. 1A), whereas the production of other cytokines remained unaltered (Fig. 1B). The same inhibitory effect of IRS-2 on the production of IL-6 was also observed in the PMJ2-R cell line (macrophages) and primary neutrophils (data not shown).

IRS-2 inhibits IL-6 production at the level of mRNA. Gene expression can be regulated by many mechanisms at many stages, including chromatin accessibility, transcription activation, mRNA nuclear export, mRNA decay, and translation. To understand the mechanism of IL-6 decline caused by IRS-2, the expression of the IL-6 gene was measured in DCs activated with *Borrelia* spirochetes in the presence or absence of IRS-2. mRNA specific for IL-6 was determined by quantitative RT-PCR. As shown in Fig. 2, the IL-6 transcript level was slightly increased after as little as 6 h (no significant effect of IRS-2 was observed). However, the mRNA of IL-6 was severely suppressed by IRS-2 at a later time point (12 h). We concluded that a decline in IL-6 production is the result of impaired

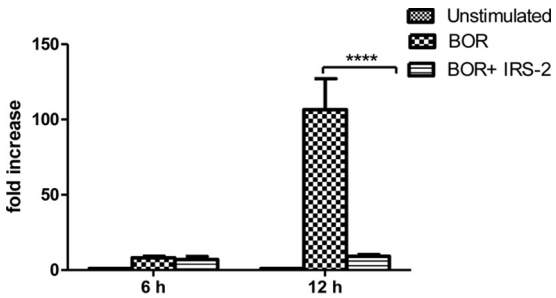

FIG 2 IRS-2 inhibits IL-6 production at the level of mRNA expression. Splenic dendritic cells were stimulated with *Borrelia* spirochetes (10 spirochetes per cell) in the presence or absence of IRS-2 (6 μM). The transcript level of IL-6 was determined by quantitative PCR (qPCR) using specific primers for IL-6. The gene expression of IL-6 was normalized to the β-actin transcript. Two independent experiments were performed, and the data were pooled. The data are expressed as the average fold IL-6 mRNA increase (plus SEM) from six wells compared with the control. ****, the effect of IRS-2 on the relative expression of IL-6 mRNA was significant at a P value of <0.0001.

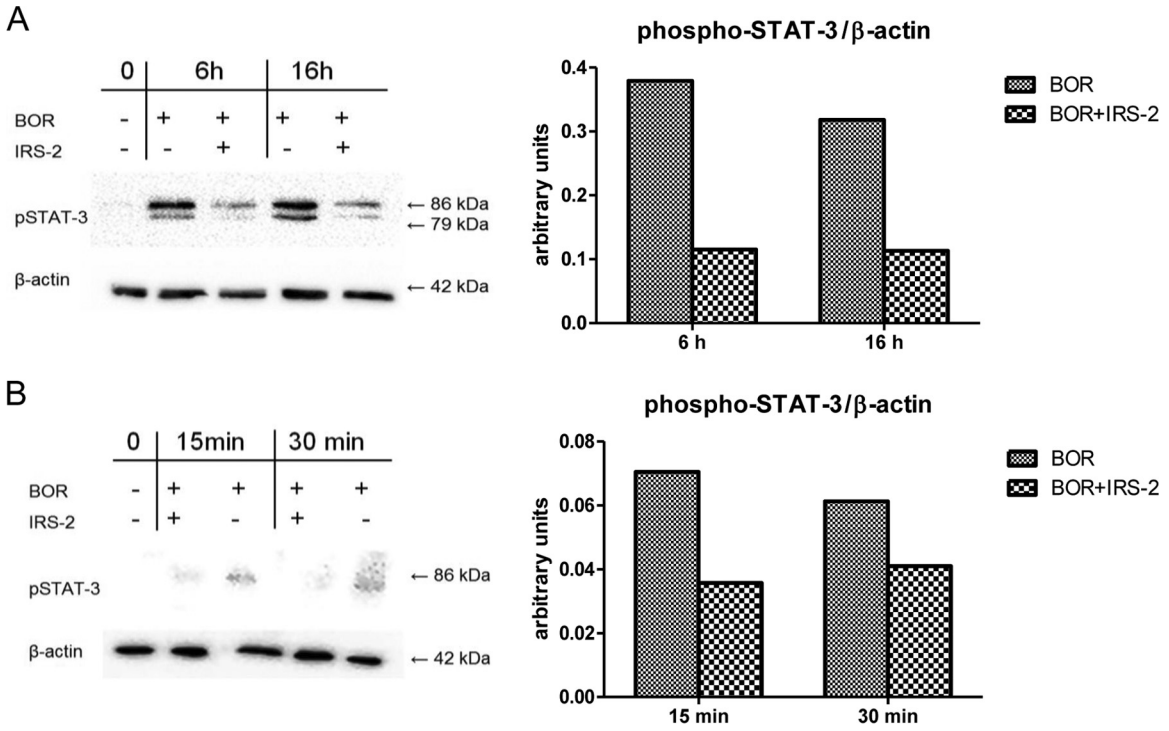

FIG 3 IRS-2 inhibits STAT-3 molecule phosphorylation. (A) Splenic dendritic cells (sDCs) were stimulated with *Borrelia* spirochetes (10 spirochetes per cell) in the presence or absence of IRS-2 (6 μM). Six and 16 h after stimulation, cell lysates were prepared and analyzed by immunoblotting with anti-phospho-STAT-3. The membranes were reprobed with antibody against β-actin. The phosphorylation of STAT-3 was quantified using chemiluminescence and normalized to the β-actin protein level. The two bands represent different isoforms (α and β) of pSTAT-3 that are present in DCs. (B) Freshly isolated *Borrelia*-primed $CD4^+$ T cells were stimulated with 24-h supernatants from sDCs stimulated with *Borrelia* spirochetes (10 spirochetes per cell) in the presence or absence of IRS-2 (6 μM). Fifteen and 30 min after stimulation, cell lysates were prepared and analyzed by immunoblotting with anti-phospho-STAT-3. The membranes were reprobed with antibody against β-actin. The phosphorylation of STAT-3 was quantified using chemiluminescence and normalized to the β-actin protein level.

gene expression in IRS-2-exposed cells after activation with *Borrelia* spirochetes (Fig. 2).

Decreased stability of IL-6 mRNA is often responsible for a decline in IL-6 production. Moreover, inhibition of IL-6 production due to increased IL-6 mRNA decay was observed with another tick salivary protein (27). Therefore, we investigated whether the same mechanism could be responsible for the IRS-2-induced effect. Splenic DCs were stimulated with *Borrelia* spirochetes in the presence or absence of IRS-2. After 9 h, actinomycin D was added to block mRNA synthesis, cells were harvested (after 1 and 2 h), and mRNA decay was determined. The IL-6 mRNA half-life observed in the presence of IRS-2 was comparable to that of control cells stimulated only with *Borrelia* spirochetes (data not shown). This result suggests that the impaired gene expression of IL-6 is not due to impaired stability of IL-6 mRNA.

In our effort to reveal the mechanism of the IRS-2 effect on IL-6, we further tested whether signaling pathways leading to induction of IL-6 are affected by IRS-2.

Gene expression of IL-6 is controlled by several transcription factors and signaling molecules, including NF-κB, C/EBP, CREB, and kinases p38 and ERK1/2 (28–32); therefore, the phosphorylation of these molecules was tested. DCs were stimulated with *Borrelia* in the presence or absence of IRS-2 for 15, 30, and 60 min. After stimulation, cell lysates were prepared and analyzed by immunoblotting. The phosphorylation of none of these signaling molecules was inhibited by IRS-2, so we concluded that induction of the IL-6 gene is intact and does not seem to be responsible for decreased IL-6 transcript expression (data not shown).

IRS-2 impairs Th17 differentiation via inhibition of the IL-6/STAT-3 signaling pathway. It is well known that the major transcription factor activated by IL-6 is STAT-3. STAT-3 phosphorylation is mediated through the association of IL-6 with the IL-6 receptor (IL-6R) and the signal transducer glycoprotein 130 (gp130), followed by subsequent activation of Janus kinases (19). Since the production of IL-6 in DCs was strongly inhibited by IRS-2, we expected that the phosphorylation of the STAT-3 signaling molecule would be decreased. DCs were activated with *Borrelia* spirochetes in the presence or absence of IRS-2, and the level of phosphorylated STAT-3 molecules was determined 6 and 16 h after activation. Indeed, a marked decrease of phospho-STAT-3 was observed (Fig. 3A). *Borrelia*-primed T lymphocytes were activated with supernatants from DCs (stimulated for 24 h with *Borrelia* spirochetes in the presence or absence of IRS-2), and the phosphorylation of STAT-3 in T lymphocytes was also decreased, likely due to diminished production of IL-6 by DCs (Fig. 3B).

The IL-6/STAT-3 signaling pathway is known to be crucial for development of the Th17 subset (18, 20). The main effector cytokines produced by Th17 cells are IL-17 (IL-17A), which is a hallmark of the subpopulation; IL-21; IL-22; and IL-9 (22, 33, 34). We predicted that the inhibition of IL-6/STAT-3 signaling by IRS-2 could lead to impaired Th17 differentiation, and therefore, the number of Th17-producing cells and the amounts of IL-17 and

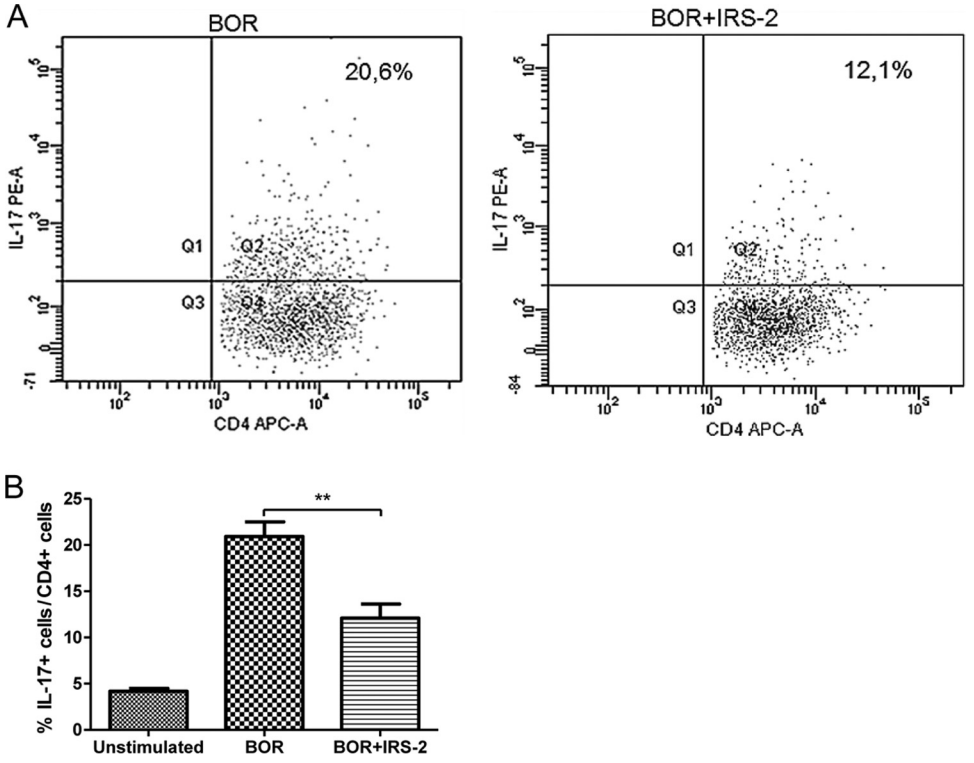

FIG 4 IRS-2 reduces the number of IL-17-producing $CD4^+$ T cells. Splenic dendritic cells were stimulated with *Borrelia* spirochetes (10 spirochetes per cell) in the presence or absence of IRS-2 (6 μM). After 24 h, freshly isolated *Borrelia*-primed $CD4^+$ T cells were added to the cultured DCs, and the cells were cocultured for 5 days. Then, T lymphocytes were restimulated with PMA and ionomycin, treated with monensin, and stained for IL-17. (A) Flow cytometry dot plots of T lymphocytes treated with DCs stimulated with *Borrelia* in the presence or absence of IRS-2. Quadrant 2 (Q2) shows $CD4^+$ $IL\text{-}17^+$ cells. (B) The percentage of IL-17-producing cells was determined in live $CD4^+$ cells. The data are expressed as the mean percentages of $CD4^+$ $IL\text{-}17^+$ cells from triplicate wells plus SEM. **, the effect of IRS-2 on the presence of IL-17-producing cells was significant at a *P* value of <0.01.

IL-9 were determined. Splenic DCs were stimulated with *Borrelia* spirochetes in the presence or absence of IRS-2. After 24 h, freshly isolated *Borrelia*-primed $CD4^+$ T cells were added to the cultured DCs, and the cells were cocultured for the next 5 days. Afterward, the cells were restimulated with PMA and ionomycin. To detect the Th17 subset, intracellular staining for IL-17A was performed, and the cells were analyzed by flow cytometry. To block cellular transport, monensin was added to the restimulated cells. As seen in Fig. 4A and B, the number of IL-17-producing $CD4^+$ T cells was significantly decreased by IRS-2.

To measure the production of IL-17 and IL-9 cytokines, the coculture of DCs and *Borrelia*-primed $CD4^+$ T cells lasted 3 days before restimulation with PMA and ionomycin. The supernatants were then collected at various time points and analyzed. The levels of both IL-17 and IL-9 were significantly decreased in the presence of IRS-2 (Fig. 5A and B). The reduced levels of the measured cytokines, together with the decreased number of IL-17-producing $CD4^+$ T cells, in the presence of IRS-2 clearly indicate that IRS-2 inhibits Th17 differentiation.

DISCUSSION

During coevolution with their hosts, ticks evolved various mechanisms enabling them to avoid the hosts' hemostatic and immune systems and successfully finish their blood meals.

In recent years, attention has been focused on identification and functional characterization of particular tick salivary proteins that are responsible for antihemostatic and immunomodulatory effects (3).

Thanks to this intensive research, many tick salivary substances that have immunomodulatory effects on various immune cell populations have been identified. Among these substances, molecules that can affect DC functions seem to play important roles, since DCs are among the first cells present at the site of inflammation and can further modulate or shift the immune response by driving T cell differentiation.

Here, we describe specific and extensive inhibitory effects of the tick salivary serpin IRS-2 from the hard tick *I. ricinus* on Th17 differentiation mediated by impairment of the IL-6/STAT-3 signaling pathway.

Proteins from the serpin superfamily are involved in fundamental biological processes, such as blood coagulation, complement activation, fibrinolysis, angiogenesis, inflammation, and tumor suppression (35, 36). From this enumeration, it is apparent that tick serpins can be expected to play a role in tick feeding, suppressing both the antihemostatic and immune responses of the host. To date, only two *I. ricinus* serpins have been functionally characterized (12, 14).

We showed that IRS-2 decreased IL-6 at the protein and mRNA levels in spleen dendritic cells activated by *B. burgdorferi*. A decrease by Sapl15, the best-studied tick salivary protein, in the IL-6 level in response to *B. burgdorferi* was also observed. Salp15 binds to DCs via the DC-SIGN receptor, which results in activa-

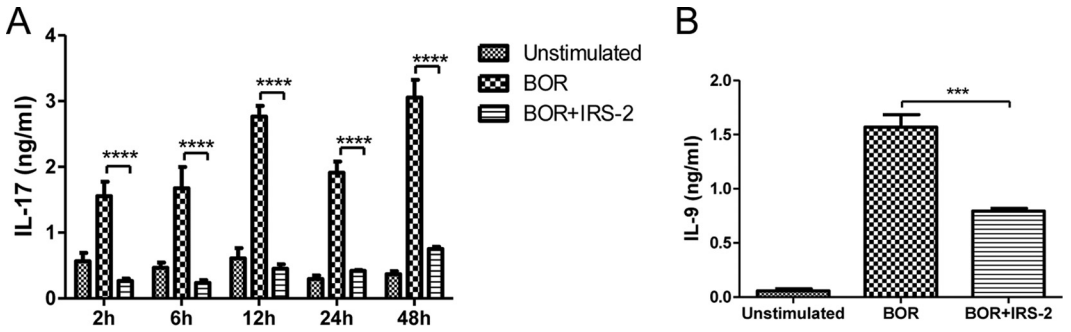

FIG 5 IRS-2 reduces levels of Th17 cytokines. Splenic dendritic cells were stimulated with *Borrelia* spirochetes (10 spirochetes per cell) in the presence or absence of IRS-2 (6 μM). After 24 h, freshly isolated *Borrelia*-primed $CD4^+$ T cells were added to the cultured DCs, and the cells were cocultured for the next 3 days. Afterward, the cells were restimulated with PMA and ionomycin, and the production of cytokines was analyzed at various time points. (A) Cell supernatants for IL-17 assessment were harvested 2, 6, 12, 24, and 48 h after restimulation and analyzed by ELISA. The data are expressed as the mean cytokine concentrations from triplicate wells plus SEM. Two independent experiments were performed, and data from a representative experiment are shown. ****, the effect of IRS-2 on the IL-17 level was significant at a *P* value of <0.0001. (B) IL-9 production was assessed by ELISA 24 h after restimulation. The data are expressed as the mean cytokine concentrations from triplicate wells plus SEM. ***, the effect of IRS-2 on IL-9 production was significant at a *P* value of <0.001.

tion of the serine/threonine kinase Raf-1/mitogen-activated protein kinase (MEK)-dependent signaling pathway and subsequently in a decrease of IL-6 and TNF-α mRNA stability and impaired nucleosome remodeling at the IL-12p35 promoter in human DCs activated with *B. burgdorferi* (27). However, the authors point to the fact that the addition of rabbit polyclonal anti-Salp15 antibodies abrogates the capacity of *I. ricinus* saliva to inhibit IL-12, but not IL-6 and TNF-α, which might be due to the presence of other molecules in tick saliva that are able to block IL-6 and TNF-α. In our study, we proved that one of these molecules, which can be responsible for IL-6 inhibition, is the salivary serpin IRS-2. To reveal the possible mechanism of IRS-2 effects, mRNA for IL-6 was assessed. However, it turned out that, in contrast to Salp15, IRS-2 does not act through impaired stability of IL-6 mRNA. In addition, monitoring of signaling pathways important for induction of IL-6 did not show any defect that led us to the conclusion that gene induction is not impaired. There is a positive-feedback loop in IL-6/STAT-3 signaling (IL-6 binds to IL-6R on a cell and activates phosphorylation of the STAT-3 molecule, which in turn boosts the production of autocrine IL-6), so direct inhibition of STAT-3 phosphorylation by IRS-2 could explain the observed decrease in IL-6 mRNA expression and, subsequently, IL-6 production (37). However, this option was also excluded (data not shown). Thus, we did not reveal the precise mechanism of the IRS-2 effect.

It has been shown that tick saliva and tick salivary proteins, like Salp15, Japanin, and sialostatin L, can modulate the T cell response by modulating DC accessory functions or directly by interaction with $CD4^+$ T cells. It was well demonstrated that tick saliva or salivary gland extract (SGE) diminishes the production of Th1-related cytokines and increases the production of Th2-related cytokines. Salp15 specifically binds to CD4 molecules on the surfaces of $CD4^+$ T (helper) cells, which results in inhibition of T cell receptor-mediated signaling, leading to reduced IL-2 production and impaired T cell proliferation (38). Japanin, a lipocalin from *Rhipicephalus appendiculatus*, specifically reprograms the response of DCs to a wide variety of stimuli *in vitro*, altering their expression of costimulatory and coinhibitory transmembrane molecules and secretion of proinflammatory, anti-inflammatory, and T cell-polarizing cytokines (it blocks LPS-induced secretion of Th17- and Th1-promoting cytokines); it also inhibits the differentiation of DCs from monocytes (39). Recently, Horka et al. showed that cystatin from *I. scapularis*, sialostatin L, which also inhibits several dendritic cell functions, can inhibit IL-9 production by Th9 cells, thus preventing the development of experimental asthma (40). Another cystatin, OmC2 from the soft tick *Ornithodoros moubata*, can also suppress the host adaptive immune response by reducing TNF-α and IL-12 production and the proliferation of antigen-specific $CD4^+$ T cells (41).

In line with these reports, our data show that the serpin IRS-2 is another tick salivary protein that is able to modulate T cell differentiation. We demonstrated that inhibition of *Borrelia*-induced IL-6 production in the presence of IRS-2 in DCs was accompanied by decreased phosphorylation of the STAT-3 signaling molecule, which is essential for the development of Th17 cells. Indeed, the impairment by IRS-2 of Th17 development was observed and was demonstrated by a decreased amount of IL-17 produced and by flow cytometry assessment of intracellular IL-17 in $CD4^+$ T lymphocytes cocultured with activated DCs. Similar results, showing that tick saliva inhibits the Th17 subset, were reported by Skallova and colleagues, who showed that saliva-exposed DCs failed to induce efficient Th1 and Th17 polarization and promoted development of Th2 responses (42). Interestingly, treatment with Salp15, which also inhibits IL-6 production in dendritic cells, was shown to increase the differentiation of Th17 cells *in vivo*, as evidenced by higher IL-17 production from PLP139-151-specific $CD4^+$ T cells isolated from the central nervous system and the periphery (43).

Th17 cells, a quite recently described subpopulation of $CD4^+$ T lymphocytes, can be characterized by production of the hallmark cytokine IL-17. Overproliferation of Th17 cells is connected with many severe autoimmune diseases, like human psoriasis, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, asthma, and some bacterial and fungal infections. However, it is well established that Th17 cells not only play an important role in autoimmunity, but also function in the clearance of specific types of pathogens that require a massive inflammatory response and are not adequately dealt with by Th1 or Th2 immunity. Thus, the Th17 response can be triggered by many bacteria, including *Borrelia* spirochetes (22). Infante-Duarte showed that *B. burgdorferi*

lysate is able to induce massive amounts of IL-17 in T cell cultures and that microbe-induced IL-17 production can mediate infection-induced immunopathology in Lyme disease (44). Involvement of the Th17 subset in the development of severe destructive arthritis in patients with Lyme disease was also demonstrated by Burchill et al. (23). A causative protein, neutrophil-activating protein A (NapA) from *B. burgdorferi*, which is able to stimulate IL-17 production in synovial-fluid-derived T cells and could thus be crucial for the induction and maintenance of Lyme arthritis, was identified (45). Moreover, it is well described that synthetic or human-derived serpins, which are commonly used in the treatment of many autoimmune diseases, are able to decrease Th17 differentiation (6).

All these findings highlight the importance and potential of the *I. ricinus* serpin IRS-2 described here as a prospective molecule in many pharmaceutical applications.

In conclusion, here, we present a newly described ability of the *I. ricinus* salivary serpin IRS-2 to inhibit Th17 differentiation upon *B. burgdorferi* exposure via inhibition of the IL-6/STAT-3 signaling pathway, thus extending the knowledge about the effect of tick salivary serpins on innate immunity cells and their function in driving the adaptive immune response. This paper contributes to the understanding of tick saliva-mediated modulation of the host immune system.

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Article 4

Iripin-3, a New Salivary Protein Isolated From Ixodes ricinus Ticks, Displays Immunomodulatory and Anti-Hemostatic Properties *In Vitro*

Chlastáková A, Kotál J, Beránková Z, Kaščáková B, Martins LA, Langhansová H, Prudnikova T, Ederová M, Kutá Smatanová I, Kotsyfakis M, **Chmelař J**.

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Next functionally characterized serpin from *I. ricinus* was published 10 years after IRS-2. In between, we changed the names of *I. ricinus* serpins from IRS to Iripins due to possible confusion with the abbreviation for insulin receptor substrate (IRS). Iripin-3 inhibited predominantly kallikrein, matriptase, trypsin and thrombin, with which it formed covalent complexes. Iripin-3 was upregulated by blood feeding in the salivary glands and was detected directly in tick saliva by immunodetection methods. Iripin-3 was the first tick serpin to inhibit extrinsic pathway of coagulation, however its most interesting activity was in immunomodulation. The serpin reduces the number of surviving T and B cells and the proliferation and differentiation of surviving T cells was strongly diverged from proinflammatory Th1, as assessed by flow cytometry, RT-qPCR and ELISA. On the other hand, regulatory and anti-inflammatory T cells (Tregs) were promoted. Moreover the production of IL-6 by macrophages was decreased after the treatment with the serpin. Overall, Iripin-3 showed a pluripotency in its activities with the ability to inhibit the development of proinflammatory Th1 cell subpopulation and to impair coagulation. The 3D structure of cleaved serpin was solved at the resolution of 1,95 Å.

This study brought strong immunological approach to study serpins' activities and it appeared to be fruitful. Iripin-3 is one of the first tick salivary proteins that modulates host immune response from pro- to anti-inflammatory.

Author's contribution:

Author designed and coordinated the study and participated on the writing and revising of the manuscript

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Iripin-3, a New Salivary Protein Isolated From *Ixodes ricinus* Ticks, Displays Immunomodulatory and Anti-Hemostatic Properties *In Vitro*

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Tick saliva is a rich source of pharmacologically and immunologically active molecules. These salivary components are indispensable for successful blood feeding on vertebrate hosts and are believed to facilitate the transmission of tick-borne pathogens. Here we present the functional and structural characterization of Iripin-3, a protein expressed in the salivary glands of the tick *Ixodes ricinus*, a European vector of tick-borne encephalitis and Lyme disease. Belonging to the serpin superfamily of protease inhibitors, Iripin-3 strongly inhibited the proteolytic activity of serine proteases kallikrein and matriptase. In an *in vitro* setup, Iripin-3 was capable of modulating the adaptive immune response as evidenced by reduced survival of mouse splenocytes, impaired proliferation of $CD4^+$ T lymphocytes, suppression of the T helper type 1 immune response, and induction of regulatory T cell differentiation. Apart from altering acquired immunity, Iripin-3 also inhibited the extrinsic blood coagulation pathway and reduced the production of pro-inflammatory cytokine interleukin-6 by lipopolysaccharide-stimulated bone marrow-derived macrophages. In addition to its functional characterization, we present the crystal structure of cleaved Iripin-3 at 1.95 Å resolution. Iripin-3 proved to be a pluripotent salivary serpin with immunomodulatory and anti-hemostatic properties that could facilitate tick feeding via the suppression of host anti-tick defenses. Physiological relevance of Iripin-3 activities observed *in vitro* needs to be supported by appropriate *in vivo* experiments.

Keywords: tick, serpin, X-ray crystallography, blood coagulation, inflammation, adaptive immunity, *Ixodes ricinus*, saliva

INTRODUCTION

The European tick *Ixodes ricinus* (Acari: Ixodidae) is an obligate blood-sucking ectoparasite that transmits several medically important pathogens such as Lyme disease spirochetes from the *Borrelia burgdorferi* sensu lato complex and tick-borne encephalitis virus (1). The insertion of the tick hypostome and two chelicerae into host skin disrupts the surrounding tissue and capillaries, to

which the host responds by activating a series of physiological defense processes including hemostasis and innate and adaptive immune responses (2–5). Cutaneous tissue injury and tick antigens are sensed by cells in the vicinity of the tick attachment site, such as keratinocytes, fibroblasts endothelial cells, mast cells, macrophages and dendritic cells (3). These cells release pro-inflammatory and chemotactic molecules that stimulate the recruitment of neutrophils and other immune cells to the area of tick feeding (3, 4, 6). Moreover, Langerhans cells and macrophages trap tick antigens and present them to T cells, which triggers T cell proliferation and ultimately results in the development of the acquired immune response (7). If unopposed, the host defense reaction rejects the tick via detrimental effects on tick viability and reproduction (8). Therefore, ticks surpass the host response by secreting hundreds of bioactive molecules via their saliva into the wound (9–11). Since these salivary molecules can target hemostasis and almost every branch of the immune response, they might be useful in the development of novel pharmaceuticals for the treatment of immune-mediated inflammatory diseases, hypercoagulable states, diseases associated with excessive complement activation, or even cancer (11–14). Moreover, tick salivary proteins represent potential targets for the development of anti-tick and/or transmission blocking vaccines (15).

Protease inhibitors form the largest functional group of tick salivary proteins (16). Based on their specificity, tick protease inhibitors can be divided into inhibitors of cysteine proteases (e.g., cystatins) and inhibitors of serine proteases (e.g., Kunitz domain-containing proteins and serpins) (17). Serpins (serine protease inhibitors) are mid-sized proteins consisting of about 330–500 amino acids (18, 19) with a conserved serpin domain and an exposed region near the carboxyl-terminal end referred to as the reactive center loop (RCL) (20). Cleavage of the scissile P1-P1′ bond in the RCL by a target serine protease results in the formation of a covalent serpin-protease complex and permanent inactivation of both the serpin and the protease (18, 20).

Serpins have been identified in many species of hard-bodied ticks of medical and veterinary importance such as *Amblyomma americanum* (21), *Haemaphysalis longicornis* (22), *I. ricinus* (23), *I. scapularis* (24), *Rhipicephalus appendiculatus* (25), and *Rhipicephalus microplus* (26, 27). Some of the functionally characterized tick serpins have been shown to suppress the enzymatic activity of blood clotting factors (mainly thrombin and factor Xa) and consequently inhibit the intrinsic and common coagulation pathways (28–31). Tick serpins that inhibit thrombin and cathepsin G can block platelet aggregation triggered by these two serine proteases (30–33). In addition to anti-hemostatic activities, many of the functionally characterized tick serpins interfere with the host innate immunity, since they inhibit the enzymatic activity of mast cell and neutrophil serine proteases, reduce vascular permeability and paw edema formation, suppress neutrophil migration *in vivo* and attenuate the production of pro-inflammatory cytokines by activated innate immune cells, such as macrophages and dendritic cells (32, 34–37). Last but not least, tick serpins can modify the host adaptive immune response via suppression of T lymphocyte proliferation and inhibition of Th1 and Th17 cell differentiation (35, 37–40). A number of RNA interference and vaccination experiments have demonstrated the important role of tick serpins in successful completion of a blood meal by prolonging the feeding period, reducing engorgement weight, or resulting in higher mortality rates or impaired oviposition (41–45).

To date, only two serpins from the tick *I. ricinus* have been assigned functions: Iris (*I. ricinus* immunosuppressor) (38) and IRS-2 (*I. ricinus* serpin-2) (32). Due to possible confusion arising from the previously used abbreviation IRS for *I. ricinus* serpins (32) (with insulin receptor substrates), we decided to name *I. ricinus* serpins Iripins (*Ixodes ricinus* serpins). Here we present the structural and functional characterization of Iripin-3 (*I. ricinus* serpin-3). Iripin-3 primarily inhibited two trypsin-like serine proteases, kallikrein and matriptase. When tested in various *in vitro* assays, Iripin-3 displayed several distinct functions: it inhibited the extrinsic blood coagulation pathway, attenuated interleukin-6 (IL-6) production by LPS-activated bone marrow-derived macrophages (BMDMs), impaired the survival and proliferation of $CD4^+$ T cells, and suppressed the Th1 immune response. The presence of Iripin-3 protein in tick saliva suggests that this serpin could play a role at the tick-host interface by suppressing various aspects of the host defense to *I. ricinus* feeding. Further *in vivo* studies, however, are necessary to confirm herein presented results. Finally, we determined the crystal structure of cleaved Iripin-3 at 1.95 Å resolution.

MATERIALS AND METHODS

Animals

C57BL/6N mice were purchased from Velaz, Ltd (Praha-Lysolaje, Czechia). C3H/HeN mice and OT-II transgenic mice were obtained from Charles River Laboratories (Wilmington, MA). Mice were maintained under standard, pathogen-free conditions in the animal house facility of the Department of Medical Biology, Faculty of Science, University of South Bohemia in České Budějovice, Czech Republic. Guinea pigs utilized for *I. ricinus* feeding and a rabbit used for the production of anti-Iripin-3 antibodies were bred and maintained at the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences (IP BC CAS), Czech Republic. All animal experiments were performed 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 Ministry of Education, Youth and Sports of the Czech Republic (protocol No. 19085/2015-3) and the responsible committee of the IP BC CAS. Pathogen-free *I. ricinus* ticks were obtained from the tick colony maintained at the IP BC CAS.

Bioinformatics Analyses

The molecular weight and isoelectric point of Iripin-3 were computed by ProtParam (46). The presence of a signal peptide was predicted using the SignalP 4.1 server (47). The ScanProsite tool (48) was utilized to identify the serpin signature motif

PS00284 as well as two other consensus amino acid motifs N-[AT]-[VIM]-[YLH]-F-[KRT]-[GS] and [DERQ]-[VL]-[NDS]-E-[EVDKQ]-G (26, 49). The reactive central loop together with the amino acid residue at the P1 site were determined based on the eight-residue pattern p17[E]-p16[E/K/R]-p15[G]-p14[T/S]-p13[X]-p12-9[AGS]-p8-1[X]-p1′-4′ [X] (26, 49). NetNGlyc 1.0 (Gupta et al., unpublished) and NetOGlyc 4.0 (50) servers were used to predict potential N-glycosylation and O-glycosylation sites, respectively. To compare Iripin-3 with other known serpins, the Iripin-3 protein sequence was tested against the GenBank database of non-redundant protein sequences using BLASTP (51). Alignment of IRS-2 and Iripin-3 amino acid sequences was conducted with ClustalW (52). Visualization of the alignment and addition of secondary structure elements were performed using ESPript 3.0 (53).

Crystal Structure Determination

The production of recombinant Iripin-3 in an *Escherichia coli* expression system is detailed in the **Supplementary Materials**. Crystallization experiments were conducted using the sitting-drop vapor diffusion technique, and the obtained crystals were used to collect X-ray diffraction data on the beamline BL14.1 at the BESSY II electron storage ring operated by the Helmholtz-Zentrum Berlin (54). The structure of Iripin-3 was solved by the molecular replacement method, in which the known structure of IRS-2 (Protein Data Bank (PDB) code 3NDA) (32) was used as a search model. The whole procedure of Iripin-3 structure determination, starting with crystallization and ending with structure refinement and validation, is described in detail in the **Supplementary Materials**. Complete data processing and refinement statistics are summarized in **Supplementary Table 1**. Atomic coordinates were deposited in the PDB under accession code 7AHP.

Phylogenetic Analysis

For the purpose of phylogenetic analysis, the amino acid sequences of 27 tick serpins and one human serpin were retrieved from GenBank. Accession numbers of these sequences are provided in **Supplementary Table 2**. Retrieved sequences were aligned and edited manually using BioEdit 7.2.5 (55). Evolutionary history was deduced from the protein sequences without a signal peptide by using the maximum likelihood method and Jones-Taylor-Thornton (JTT) matrix-based model (56). Initial trees for the heuristic search were obtained automatically by applying the neighbor-joining (57) and BIONJ (58) algorithms to a matrix of pairwise distances estimated using the JTT model, and then the topology with a superior log likelihood value was selected. The reliability of individual branches was determined by bootstrapping. Bootstrap values were calculated for 1000 replicates. Evolutionary analyses were conducted in MEGA X (59).

Iripin-3 Expression in Ticks

I. ricinus nymphs were fed on C3H/HeN mice for 1 day, 2 days, and until full engorgement (3–4 days). *I. ricinus* adult females were fed on guinea pigs for 1, 2, 3, 4, 6, and 8 days. Tick removal from host animals at given time points was followed by the dissection of nymphs and adult female salivary glands, midguts, and ovaries under RNase-free conditions. RNA was isolated from tick tissues using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH), and 1 μg of total RNA was reverse transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions. Five-fold diluted cDNA mixed with FastStart Universal SYBR Green Master (Roche Applied Science) and gene-specific primers were used for the analysis of *iripin-3* expression in the Rotor-Gene 6000 thermal cycler (Corbett Research, Saffron Walden, UK). Cycling conditions were 95°C for 10 min followed by 45 cycles of 95°C for 15 s, 60°C for 10 s and 72°C for 30 s. The relative quantification of *iripin-3* transcripts in tick tissues was performed using the ΔΔCt method (60). The *I. ricinus* gene encoding ribosomal protein S4 (*rps4*, GenBank accession number MN728897.1) was utilized as a reference gene for the calculation of relative expression ratios (61, 62). Nucleotide sequences of forward and reverse primers as well as amplicon lengths are provided in **Supplementary Table 3**.

Presence of Iripin-3 in Tick Saliva

Polyclonal antibodies against Iripin-3 were produced in a rabbit injected subcutaneously with 100 μg of purified Iripin-3 in 500 μl of complete Freund's adjuvant. The first immunization was followed by another two injections of Iripin-3 in 500 μl of incomplete Freund's adjuvant at 14-day intervals. On day 14 after the last injection, the rabbit was sacrificed, and its blood was collected. Prepared rabbit antiserum to Iripin-3 was subsequently utilized for the detection of Iripin-3 in tick saliva by indirect ELISA and western blotting. The saliva was collected from *I. ricinus* ticks feeding for 6–7 days on guinea pigs as described previously (63). ELISA and western blot analyses are detailed in the **Supplementary Materials**.

Inhibition of Serine Proteases

Preliminary screening of Iripin-3 inhibitory activity against a set of 17 serine proteases was performed as described previously (32), with the exception of factor VIIa (FVIIa). Human FVIIa (Haematologic Technologies, Inc., Essex Junction, VT) at 20 nM concentration was pre-incubated for 10 min at 30°C with 400 nM Iripin-3 before the addition of 250 μM fluorogenic substrate Boc-QAR-AMC. The assay buffer used consisted of 20 mM Tris, 150 mM NaCl, 0.01% Triton X-100, 5 mM $CaCl_2$, and 0.1% polyethylene glycol 6000, pH 8.0. After the determination of the substrate hydrolysis rate, the six most strongly inhibited proteases were chosen for more detailed analysis. The assessment of covalent complex formation between Iripin-3 and selected serine proteases and the determination of second-order rate constants of protease inhibition are detailed in the **Supplementary Materials**.

Blood Coagulation

The effect of Iripin-3 on blood coagulation was tested by prothrombin time (PT), activated partial thromboplastin time (aPTT), and thrombin time (TT) assays. All chemicals were purchased from Technoclone (Vienna, Austria). Citrated human plasma (Coagulation Control N) was mixed either with 6 μM

Iripin-3 or with five different Iripin-3 concentrations and then incubated for 10 min at room temperature. To perform the PT test, 100 µl of plasma with added Iripin-3 was incubated for 1 min at 37°C before the addition of 200 µl of Technoplastin HIS pre-warmed to 37°C. Plasma clotting time was measured on the Ceveron four coagulometer (Technoclone). In the aPTT test, the incubation of 100 µl of plasma mixed with Iripin-3 at 37°C for 1 min was followed by the addition of 100 µl of Dapttin TC. After incubating the mixture of plasma and Dapttin at 37°C for 2 min, 100 µl of 25 mM $CaCl_2$ was added to initiate the coagulation cascade. Plasma clotting time was determined as described above. To perform the TT test, 200 µl of plasma mixed with Iripin-3 was incubated at 37°C for 1 min. At the end of incubation, 200 µl of thrombin reagent was added, and plasma clotting time was measured as in the PT and aPTT assays.

Pro-Inflammatory Cytokine Production by BMDMs

Bone marrow cells were isolated from femurs and tibias of C57BL/6N mice. Both ends of the bones were cut with scissors, and bone marrow was flushed with complete medium. The complete medium was prepared by supplementation of RPMI 1640 medium containing glutamine (Biosera) with 10% heat-inactivated fetal bovine serum (FBS, Biosera), 50 µM 2-mercaptoethanol (Sigma Aldrich, St Louis, MO), 100 U/ml penicillin G (Biosera, Kansas City, MO) and 100 µg/ml streptomycin (Biosera). After erythrocyte lysis in RBC lysis buffer (eBioscience, San Diego, CA), bone marrow cells resuspended in complete medium were seeded into 10 cm Petri dishes and incubated in the presence of 10 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF, Sigma Aldrich) at 37°C and 5% CO_2 for 10 days. On days 4 and 7, non-adherent cells were removed and the medium was replaced with fresh complete medium containing 10 ng/ml GM-CSF. On day 10, adherent cells (macrophages) were collected, resuspended in RPMI 1640 medium supplemented only with 0.5% bovine serum albumin (BSA, Biosera), and seeded into 24-well culture plates (2×10^5 cells in 500 µl of culture medium per well). After 5 h incubation at 37°C and 5% CO_2, the medium was replaced with fresh RPMI 1640 medium containing 0.5% BSA, and BMDMs were pre-incubated for 40 min with 3 µM or 6 µM Iripin-3. Finally, 100 ng/ml of LPS (Sigma Aldrich; *E. coli* serotype O111:B4) was added, and macrophages were incubated in the presence of Iripin-3 and LPS for another 24 h. At the end of incubation, cells and cell-free supernatants were collected for RNA isolation and protein quantification, respectively. Relative expression of *Tnf*, *Il6*, and *Il1b* in macrophages was determined by RT-qPCR and concentrations of tumor necrosis factor (TNF), IL-6, and interleukin-1β (IL-1β) cytokines in collected supernatants were measured by DuoSet ELISA Development Kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions with only minor modifications. The RT-qPCR analysis is described in detail in the **Supplementary Materials**.

Splenocyte Isolation and Culture in the Presence of Iripin-3

Spleens harvested from OT-II mice were forced through a Corning 70 µm cell strainer to obtain a single cell suspension. Red blood cells (RBCs) were removed from the suspension by the addition of 1× RBC lysis buffer (eBioscience), and the erythrocyte-free spleen cells were resuspended in RPMI 1640 medium with stable glutamine (Biosera) supplemented with 10% heat-inactivated FBS (Biosera), 50 µM 2-mercaptoethanol (Sigma Aldrich), 100 U/ml penicillin G (Biosera), and 100 µg/ml streptomycin (Biosera). Splenocytes were then seeded into 24-well or 96-well culture plates and pre-incubated with 3 µM or 6 µM Iripin-3 for 2 h. Pre-incubation with Iripin-3 was followed by the addition of ovalbumin (OVA) peptide 323–339 (Sigma Aldrich) at a concentration of 100 ng/ml. Splenocytes were incubated in the presence of Iripin-3 and OVA peptide at 37°C and 5% CO_2 for either 20 h (assessment of cell survival) or 72 h (analysis of cell proliferation and transcription factor expression).

Survival of B and T Cells

Mouse splenocytes were seeded into 96-well culture plates (5×10^5 cells in 200 µl of complete medium per well), pre-incubated with Iripin-3, and stimulated with OVA peptide. After 20 h incubation at 37°C and 5% CO_2, cells were harvested for flow cytometry analysis. First, splenocytes were stained with fixable viability dye eFluor 780 (eBioscience). Subsequently, Fc receptors were blocked with anti-CD16/CD32 antibody (eBioscience, clone 93), and surface antigen staining was performed with following monoclonal antibodies purchased from eBioscience: anti-CD45-PerCP-Cyanine5.5 (clone 30-F11), anti-CD19-PE (clone eBio1D3(1D3)), and anti-CD3e-APC (clone 145-2C11). Finally, the active form of caspase 3 in splenocytes was labeled using the FITC Active Caspase-3 Apoptosis Kit (BD Biosciences). The percentage of live $CD19^+$ and $CD3e^+$ splenocytes as well as the level of active caspase 3 were analyzed on the BD FACSCanto II flow cytometer using BD FACSDiva software version 6.1.3 (BD Biosciences).

Proliferation of $CD4^+$ T Cells

Erythrocyte-free splenocytes were stained with red fluorescent dye eFluor 670 (eBioscience), which allows monitoring of individual cell divisions. The stained splenocytes were seeded into 96-well culture plates (5×10^5 cells in 200 µl of complete medium per well), pre-incubated with Iripin-3, and stimulated with OVA peptide. Cells were allowed to proliferate for 72 h and then were harvested for flow cytometry analysis. Collected cells were stained with FITC-labelled anti-CD4 monoclonal antibody (clone GK1.5, eBioscience) and propidium iodide (eBioscience), and the percentage of proliferating live $CD4^+$ splenocytes was measured on the BD FACSCanto II flow cytometer using BD FACSDiva software version 6.1.3 (BD Biosciences).

Transcription Factor Expression in $CD4^+$ T Cells (RT-qPCR)

Splenocytes were seeded into 24-well culture plates (4.5×10^6 cells in 500 µl of complete medium per well), pre-incubated with Iripin-3, and stimulated with OVA peptide. At the end of 72 h incubation, non-adherent cells were collected, stained with FITC-labeled anti-CD4 monoclonal antibody (clone GK1.5, eBioscience), and $CD4^+$ splenocytes were separated from the rest of the cell population using

the S3e Cell Sorter (Bio-Rad Laboratories, Hercules, CA). RNA was extracted from $CD4^+$ cells with the help of NucleoSpin RNA isolation kit (Macherey-Nagel, Düren, Germany), and 1 µg of total RNA was reverse transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science). RT-qPCR was performed in the CFX384 Touch thermal cycler (Bio-Rad) by utilizing five-fold diluted cDNA, SsoAdvanced Universal SYBR Green Supermix (Bio-Rad), and gene-specific primers. The PCR cycling conditions were 95°C for 3 min followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. The relative quantification of *Tbx21* (*Tbet*), *Gata3*, *Rorc*, and *Foxp3* transcripts in $CD4^+$ splenocytes was performed using Pfaffl's mathematical model (64). Based on the results of geNorm analysis (65), *Actb* and *Gapdh* were utilized as reference genes for the calculation of relative expression ratios. Nucleotide sequences of forward and reverse primers as well as amplicon lengths are given in **Supplementary Table 3**.

Transcription Factor Expression in $CD4^+$ T Cells (Flow Cytometry)

Splenocytes were seeded into 24-well culture plates (2×10^6 cells in 500 µl of complete medium per well), pre-incubated with Iripin-3, and stimulated with OVA peptide. After 68 h incubation at 37°C and 5% CO_2, 20 ng/ml of phorbol 12-myristate 13-acetate (PMA; Sigma Aldrich) together with 1 µM ionomycin (Sigma Aldrich) were added to re-stimulate the cells. Brefeldin A (eBioscience) at a concentration of 3 µg/ml was added 1 h later, and splenocytes were incubated in the presence of PMA, ionomycin, and brefeldin A for another 4 h. At the end of incubation, non-adherent cells were collected and stained with fixable viability dyes eFluor 520 and eFluor 780 (eBioscience). Subsequently, Fc receptors were blocked with anti-CD16/CD32 antibody (eBioscience, clone 93), and surface antigen staining was performed with anti-CD4-Alexa Fluor 700 (BD Biosciences, clone RM4-5) and anti-CD25-PerCP-Cyanine5.5 (eBioscience, clone PC61.5) monoclonal antibodies. Surface antigen staining was followed by intracellular staining of transcription factors and cytokine IFN-γ, for which the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) was used in conjunction with following monoclonal antibodies: anti-T-bet-APC (clone eBio4B10 (4B10)), anti-GATA-3-PE (clone TWAJ), anti-RORγt-PE-CF594 (clone Q31-378), anti-Foxp3-PE-Cyanine7 (clone FJK-16s), and anti-IFN-γ-PE (clone XMG1.2). All antibodies were purchased from eBioscience except for the anti-RORγt antibody, which was obtained from BD Biosciences. Analysis was performed on the BD FACSCanto II flow cytometer using BD FACSDiva software version 6.1.3 (BD Biosciences).

Statistical Analyses

Data are presented in all graphs as mean ± the standard error of the mean (SEM). Differences between the mean values of two groups were analyzed by the unpaired two-tailed *t*-test. Differences between the mean values of three or more groups were analyzed by one-way ANOVA or randomized block ANOVA, which involved two variables: a fixed effect factor (treatment) and a random effect factor/block (an experimental run) (66). In the case of a statistically significant result ($p < 0.05$), Dunnett's *post hoc* test was performed to compare the mean of a control group with the means of experimental groups. All statistical tests were conducted using the software package STATISTICA 12 (StatSoft, Inc.). Statistically significant differences between groups are marked with asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

RESULTS

Iripin-3 Belongs to the Serpin Superfamily

A full-length nucleotide sequence of Iripin-3 was obtained during a salivary gland transcriptome project (16) and was submitted to GenBank under accession number GADI01004776.1. This sequence, consisting of 1182 base pairs, encodes a 377-amino acid (AA) protein with predicted molecular weight of approximately 42 kDa and with theoretical isoelectric point (pI) 5.23. The SignalP 4.1 server found a 16-AA signal peptide at the N terminus of the protein sequence (**Figure 1A**), which indicates that Iripin-3 is a potentially secreted protein. Using ScanProsite, the serpin signature motif PS00284 was identified at AA positions 366-376 (**Figure 1A**). Moreover, two other serpin consensus AA motifs N-[AT]-[VIM]-[YLH]-F-[KRT]-[GS] and [DERQ]-[VL]-[NDS]-E-[EVDKQ]-G were recognized: NAMYFKG at AA positions 183-189 and EVNEEG at AA positions 338-343 (**Figure 1A**), suggesting that Iripin-3 belongs to the serpin superfamily. The hinge region of the Iripin-3 RCL has glycine at the P15 position, threonine at the P14 position, and residues with short side chains (alanine and valine) at positions P12-P9 (**Figure 1A**), which correspond to the RCLs of inhibitory serpins (68). The P1 site is occupied with the basic amino acid residue arginine (**Figure 1A**), suggesting Iripin-3 might target trypsin-like rather than chymotrypsin-like or elastase-like serine proteases (69). Using NetNGlyc 1.0 and NetOGlyc 4.0 servers, the Iripin-3 AA sequence was predicted to contain two potential N-glycosylation sites (N-X-[S/T]) and one putative O-glycosylation site (**Figure 1A**).

Iripin-3 Adopts a Typical Serpin Fold

Employing X-ray crystallography, we determined the 3D structure of Iripin-3 at 1.95 Å resolution. The crystal used exhibited symmetry of the $P6_222$ space group and contained one molecule in the asymmetric unit with a solvent content of 42.68%. The tertiary structure of Iripin-3 matched the 3D structures of other serpins, including the tick serpin IRS-2 (**Figure 1B**), with which it had the highest sequence similarity of all the serpin structures currently deposited in the PDB. More specifically, the Iripin-3 tertiary structure was composed of ten α-helices and three β-sheets, which were sequentially arranged in the order α1-β1-α2-α3-α4-α5-β2-α6-β3-α7-β4-β5-β6-β7-β8-α8-α9-β9-β10-α10-β11-β12-β13-β14-β15 (**Figures 1A**, **2**). The sheet A consisted of six β-strands (β2, β3, β4, β10, β11, β12), sheet B of five β-strands (β1, β7, β8, β14, β15), and sheet C of four β-strands (β5, β6, β9, β13) (**Figure 2**). Iripin-3 in the crystal adopted a conformation known as the relaxed (R) state, since its RCL was probably cleaved by some contaminating

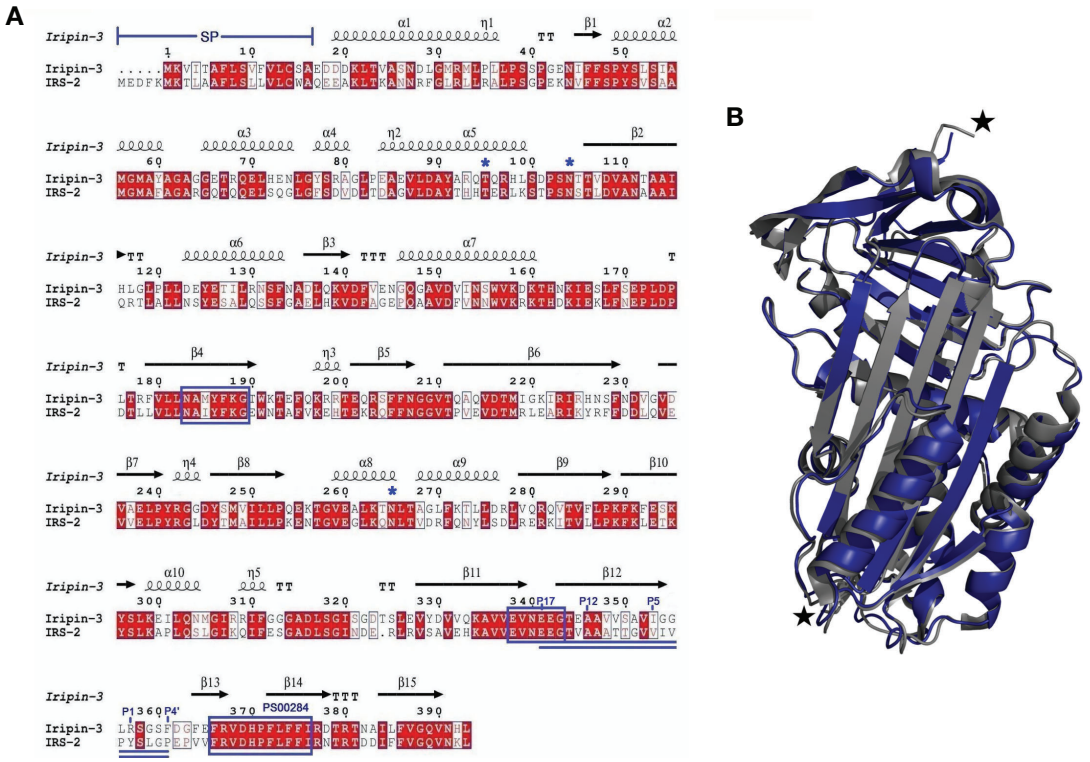

FIGURE 1 | A comparison of the primary, secondary and tertiary structures of Iripin-3 and IRS-2. **(A)** Structure-based sequence alignment of Iripin-3 and IRS-2. Secondary structure elements, which are shown above the aligned sequences, are depicted as spirals (α-helices, 3_{10}-helices) and arrows (β-sheets). Both Iripin-3 and IRS-2 possess a signal peptide (SP) at the N terminus of their sequences. Conserved AA motifs PS00284, N-[AT]-[VIM]-[YLH]-F-[KRT]-[GS], and [DERQ]-[VL]-[NDS]-E-[EVDKQ]-G are boxed in blue. The RCLs of both serpins are double underlined. Numbering of amino acid residues in the RCL is based on the standard nomenclature developed by Schechter and Berger (67). Putative N-glycosylation and O-glycosylation sites are marked with blue asterisks. **(B)** Superposition of the cleaved Iripin-3 structure (blue) on the structure of cleaved IRS-2 (gray). Cleavage sites are marked with black stars.

proteases before or during the crystallization experiment. A protein sample can contain traces of contaminating cysteine and serine proteases, as demonstrated previously (70). The cleavage of the RCL led to the insertion of the RCL hinge region into the β-sheet A as an additional β-strand S4 (**Figure 2**). The 3D structure of Iripin-3 contained 367 amino acid residues. The first 19 residues, which basically corresponded to the signal peptide of the protein, were missing. Moreover, the region $_{356}$LRSGSFD$_{362}$, in which the cleavage occurred, could not be modelled in the Iripin-3 structure due to its absence in the electron-density map. To compare the tertiary structure of Iripin-3 with that of IRS-2, the molecular structure of Iripin-3 was superposed with Cα atoms of IRS-2 with root-mean-square deviation of 0.8085 Å. The secondary structure elements were well conserved in both serpins, but there was a certain degree of divergence in disordered loop regions (**Figure 1B**).

Iripin-3 Is Most Closely Related to Serpins From *I. scapularis*

The BLASTP search of the GenBank non-redundant protein sequences identified three *I. scapularis* serpins (accession numbers XP_029826754.1, EEC19555.1, and AAV80788.1) whose sequences were highly similar to the Iripin-3 sequence (percentage identities 95.4%, 94.9%, and 93.6%, respectively). These homologs have not been functionally characterized. The phylogenetic relationship of Iripin-3 with 26 tick serpins, whose function was deciphered either by using recombinant protein or at least by gene knockdown via RNA interference in ticks, was determined by using the maximum likelihood method and JTT matrix-based model. The resulting phylogenetic tree, with human alpha-1-antitrypsin as an outgroup, showed two distinct groups of tick serpins (**Figure 3A**). The first group at the bottom of the tree included eight serpins without a signal peptide with presumably intracellular function (**Figure 3A**). Notably, these serpins usually contained one or more cysteines and methionines in their RCL (**Figure 3B**). The second, larger group at the top of the tree comprised 19 serpins with a signal peptide, including Iripin-3 (**Figure 3A**). Iripin-3 formed a small branch with one serpin from *I. scapularis* (IxscS-1E1) and one serpin from *I. ricinus* (IRS-2) (**Figure 3A**). In addition to the construction of the phylogenetic tree, we aligned the RCLs of the serpins used in the phylogenetic analysis (**Figure 3B**). Serpins that clustered together usually had similar RCLs, and the RCL of Iripin-3 resembled that of IxscS-1E1 (**Figure 3B**).

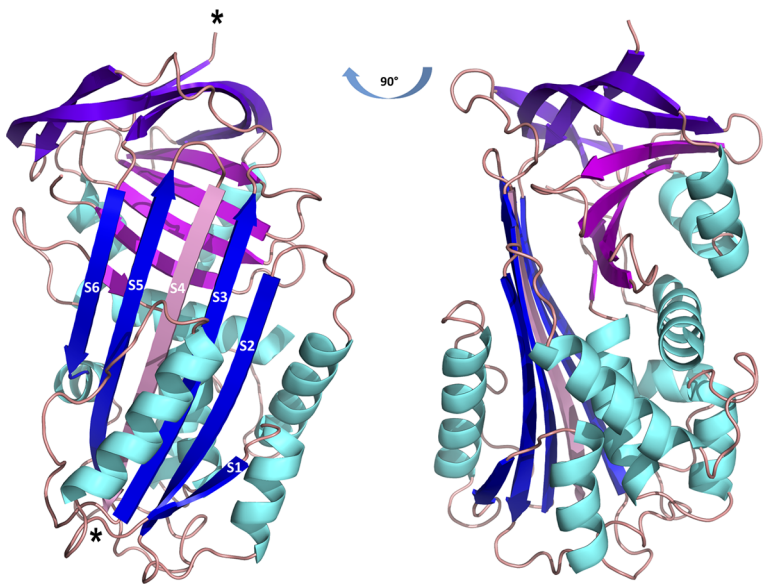

FIGURE 2 | Cartoon representation of the structure of cleaved Iripin-3. α-helices are colored cyan, β-sheet A is blue, β-sheet B is magenta, β-sheet C is purple, and loops are colored wheat. The insertion of the RCL hinge region between β-strands S3 and S5 (depicted in blue) resulted in the formation of an additional β-strand S4 (depicted in pink). Cleavage sites are marked with asterisks.

Iripin-3 Is Expressed in Feeding Ticks and Is Secreted Into Tick Saliva

In order to see how *iripin-3* expression changes during blood feeding, nymphal and adult ticks were allowed to feed on blood from host animals for various periods of time, and the amount of *iripin-3* transcript in tick tissues was subsequently determined by RT-qPCR. Overall, *iripin-3* expression was significantly induced in response to blood feeding in nymphs as well as in the salivary glands and ovaries of adult females (**Figure 4A**). In adults, the highest levels of *iripin-3* mRNA were detected in the salivary glands (**Figure 4A**). To prove the presence of Iripin-3 protein in tick saliva, we collected saliva from ticks that were feeding for 6 to 7 days on guinea pigs. By ELISAs, markedly higher optical density values were obtained after exposure of tick saliva to anti-Iripin-3 serum than to pre-immune serum (**Figure 4B**), suggesting that Iripin-3 is a salivary protein. This result was further confirmed by western blotting. Rabbit pre-immune serum did not recognize recombinant Iripin-3, and there was no band of appropriate size (around 42 kDa) in tick saliva (**Figure 4C**). Conversely, the use of anti-Iripin-3 serum led to the recognition of recombinant Iripin-3 and appearance of an approximately 45 kDa band in tick saliva, which might represent native Iripin-3 (**Figure 4D**). The difference in the sizes of native and recombinant Iripin-3 was probably caused by the fact that native Iripin-3 is glycosylated, whereas recombinant Iripin-3 was prepared in the *E. coli* expression system and therefore lacks glycosylation. The other bands with sizes greater or less than 45 kDa that appeared in the lanes with tick saliva after exposure of membranes to either pre-immune serum or anti-Iripin-3 serum are most likely a result of non-specific binding of antibodies to some components of tick saliva (**Figures 4C, D**).

Iripin-3 Primarily Inhibits Kallikrein and Matriptase

An initial screen for Iripin-3 inhibitory activity was carried out against 17 different serine proteases. Statistically significant reductions in enzymatic activity were observed for ten proteases, but only six of these, namely kallikrein, matriptase, trypsin, plasmin, thrombin, and FVIIa, had their proteolytic activity reduced by >20% (**Figure 5A**). Iripin-3 formed covalent complexes, typical for the serpin "suicide" mechanism of inhibition (71), with kallikrein, matriptase, thrombin, and trypsin, as shown by SDS-PAGE (**Figure 5B**). There was no visible complex between Iripin-3 and plasmin on the gel (**Figure 5B**). It is possible that the complex was hidden within an approximately 70 kDa protein band, which was also present in the lane with plasmin only (**Figure 5B**). Moreover, no SDS- and heat-stable complex was formed between Iripin-3 and FVIIa in the absence or presence of tissue factor under given conditions (**Supplementary Figure 1**), suggesting Iripin-3 probably does not reduce the proteolytic activity of FVIIa through the classic serpin inhibitory mechanism. Finally, the second-order rate constants k_2 for the interactions between Iripin-3 and kallikrein, matriptase, thrombin, and trypsin were measured by a discontinuous method under pseudo first-order conditions. Iripin-3 most potently inhibited kallikrein with $k_2 = 8.46 \pm 0.51 \times 10^4\ M^{-1}\ s^{-1}$ (**Figure 5C**). The k_2 for the interactions between Iripin-3 and matriptase and trypsin were determined as $5.93 \pm 0.39 \times 10^4\ M^{-1}\ s^{-1}$ and $4.65 \pm 0.32 \times 10^4\ M^{-1}\ s^{-1}$, respectively (**Figures 5D, F**). Thrombin was inhibited by Iripin-3 with the lowest potency ($k_2 = 1.37 \pm 0.21 \times 10^3\ M^{-1}\ s^{-1}$) (**Figure 5E**). Interface analysis between the active sites of matriptase, thrombin, kallikrein and trypsin and the P4-P4′ part of Iripin-3 RCL revealed possible polar interactions that could indicate the binding selectivity of

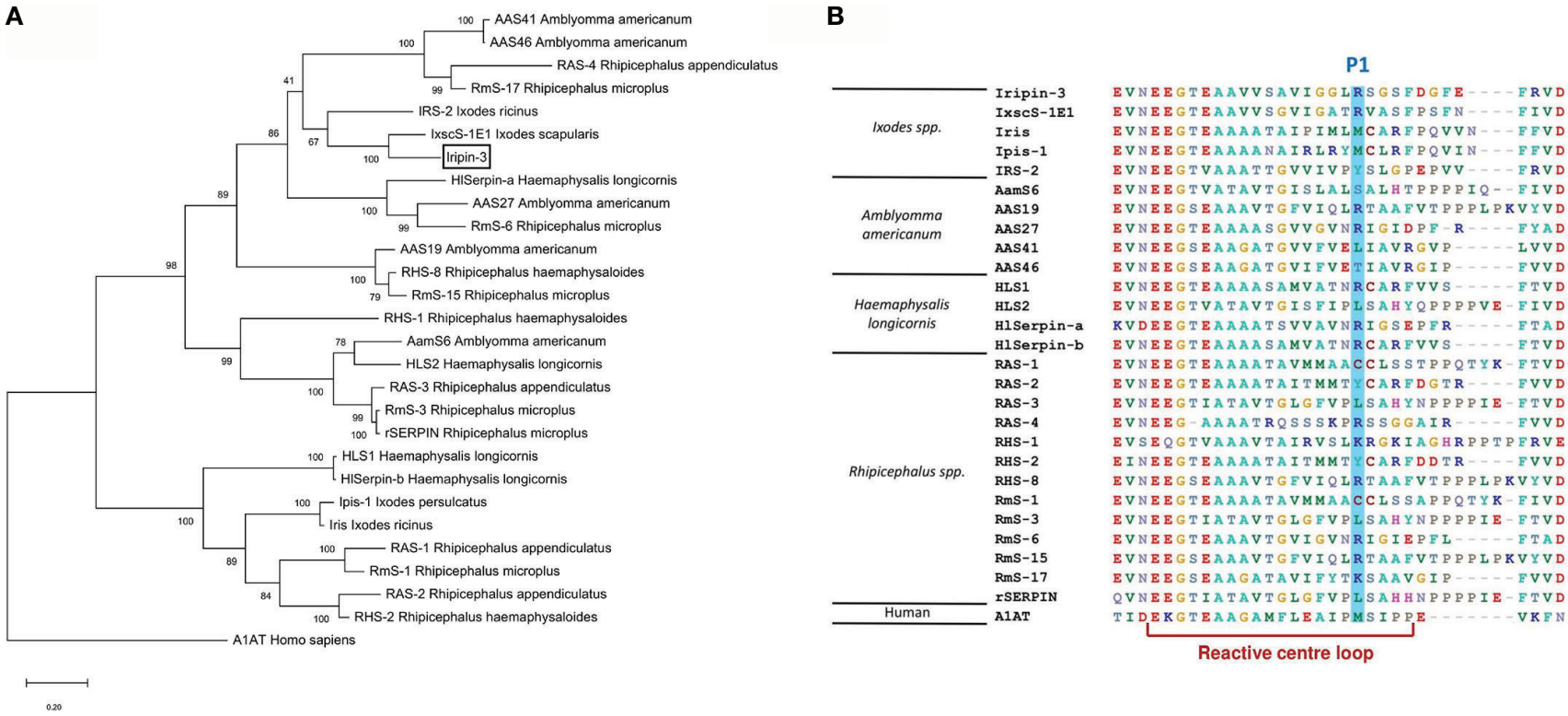

FIGURE 3 | Phylogenetic analysis of selected tick serpins. Protein sequences of previously characterized tick serpins were aligned and analyzed to determine phylogenetic relationships. **(A)** A phylogenetic tree was built using the maximum likelihood method and JTT matrix-based model. Alpha-1-antitrypsin (A1AT) was utilized as an outgroup to root the tree. The branch length represents the number of substitutions per site. The reliability of individual branches, assessed by bootstrapping, is expressed as a percentage of trees in which a given topology was present out of 1,000 replications. Iripin-3 is boxed. **(B)** Alignment of reactive center loop (RCL) regions of 27 tick serpins and one human serpin was performed using BioEdit. RCLs were determined based on the eight-residue pattern p17[E]-p16[E/K/R]-p15[G]-p14[T/S]-p13[X]-p12-9[AGS]-p8-1[X]-p1′-4′ [X] typical for inhibitory serpins (68). Amino acid residues at the predicted P1 site are highlighted in blue.

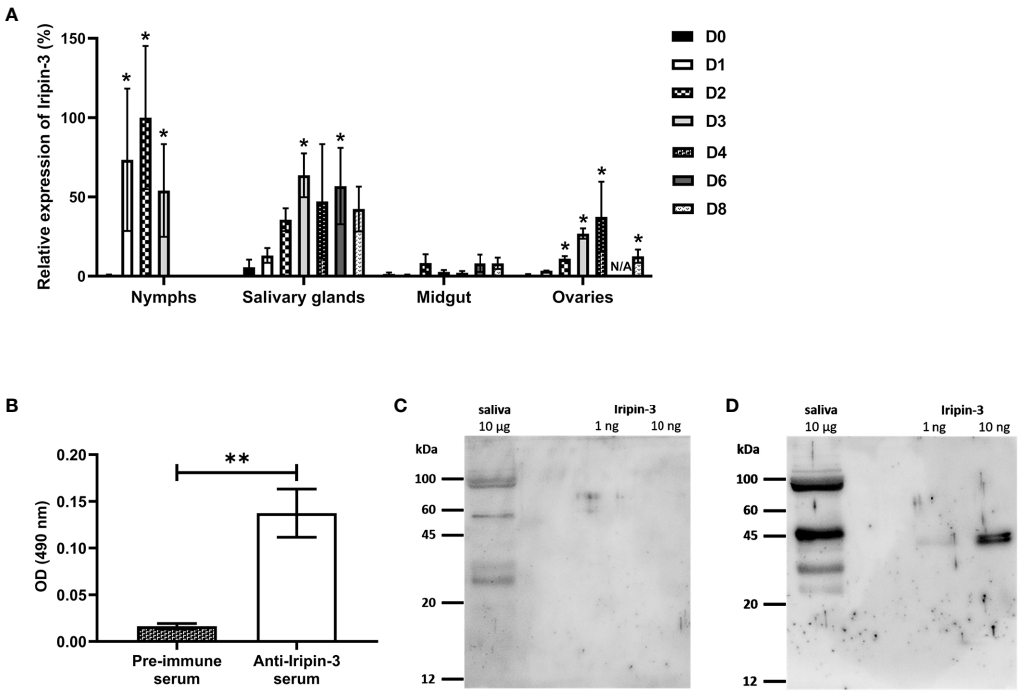

FIGURE 4 | *Iripin-3* transcription in *I. ricinus* ticks is increased in response to blood feeding, and Iripin-3 protein is present in the saliva of feeding ticks. **(A)** *Iripin-3* mRNA expression in nymphs and in the salivary glands, midguts and ovaries of adult females feeding for 1 (D1), 2 (D2), 3 (D3), 4 (D4), 6 (D6), and 8 (D8) days or not feeding at all (D0). In nymphs, the last column represents fully engorged ticks that completed their blood meal in 3 or 4 days. N/A – data not available. Relative expression values were calculated using the ΔΔCt (Livak) method (60), with *rps4* serving as a reference gene. A group with the highest *iripin-3* expression (nymphs feeding for 2 days) was utilized as a calibrator during calculations, and its expression value was set to 100%. Data are presented as mean of three biological replicates ± SEM. Statistically significant induction ($p < 0.05$) of *iripin-3* expression as compared to unfed ticks is marked with an asterisk. **(B)** ELISA results expressed as optical density (OD) values measured after exposure of tick saliva to either rabbit pre-immune serum or rabbit antiserum to Iripin-3. Data are presented as mean ± SEM of three values (**$p < 0.01$). **(C, D)** Tick saliva (10 µg) and Iripin-3 (1 ng or 10 ng) were resolved by SDS-PAGE and transferred to PVDF membranes. The membranes were incubated with rabbit pre-immune serum **(C)** or rabbit antiserum to Iripin-3 **(D)**.

Iripin-3 for target proteases (**Supplementary Figure 2**). The strongest interaction with the catalytic triad was calculated for matriptase, followed by trypsin, kallikrein and thrombin (data not shown). According to this analysis, thrombin and kallikrein should be inhibited by Iripin-3 with similar potency. This, however, was not supported by enzyme-substrate kinetic analyses (**Figures 5C–F**), in which kallikrein displayed 60 times higher k_2 value than thrombin. Therefore, the specificity of Iripin-3 is probably dependent on more factors. As shown in **Supplementary Figure 3**, matriptase and trypsin have open and shallow active sites, easily accessible to various substrates, including Iripin-3 RCL. Thrombin and kallikrein, on the other hand, possess narrower and deeper cavities with the catalytic triad (**Supplementary Figure 3**). It is possible that some subtle differences in spatial arrangement hinder the access of Iripin-3 RCL to the thrombin's active site, while facilitating its access to the kallikrein's active site cleft.

Iripin-3 Prolongs Plasma Clotting Time in the Prothrombin Time Assay

Since tick serpins commonly inhibit the host coagulation system (72), we tested the effect of Iripin-3 on the extrinsic coagulation pathway, intrinsic coagulation pathway, and common coagulation pathway by using prothrombin time (PT), activated partial thromboplastin time (aPTT), and thrombin time (TT) tests, respectively (73). Iripin-3 at 6 µM final concentration did not significantly prolong plasma clotting time in the aPTT and TT assays (data not shown). However, there was a statistically significant delay in blood clot formation in the PT test when plasma was treated with 1.5, 3, and 6 µM Iripin-3 (**Figure 6**). The highest Iripin-3 concentration prolonged the prothrombin time by 8.8 s when compared to control plasma (**Figure 6**). These results therefore indicate that Iripin-3 slightly inhibits the extrinsic pathway while not affecting the intrinsic and common pathways of blood coagulation.

Iripin-3 Decreases Production of IL-6 by BMDMs

Serpins secreted in tick saliva can facilitate blood meal uptake not only by inhibiting coagulation but also by suppressing host inflammatory responses (37, 72, 74). Therefore, we next investigated whether Iripin-3 attenuates pro-inflammatory cytokine production by LPS-stimulated BMDMs. The production of TNF, IL-6, and IL-1β was assessed at the mRNA level by RT-qPCR as well as at the protein level by ELISA. Iripin-

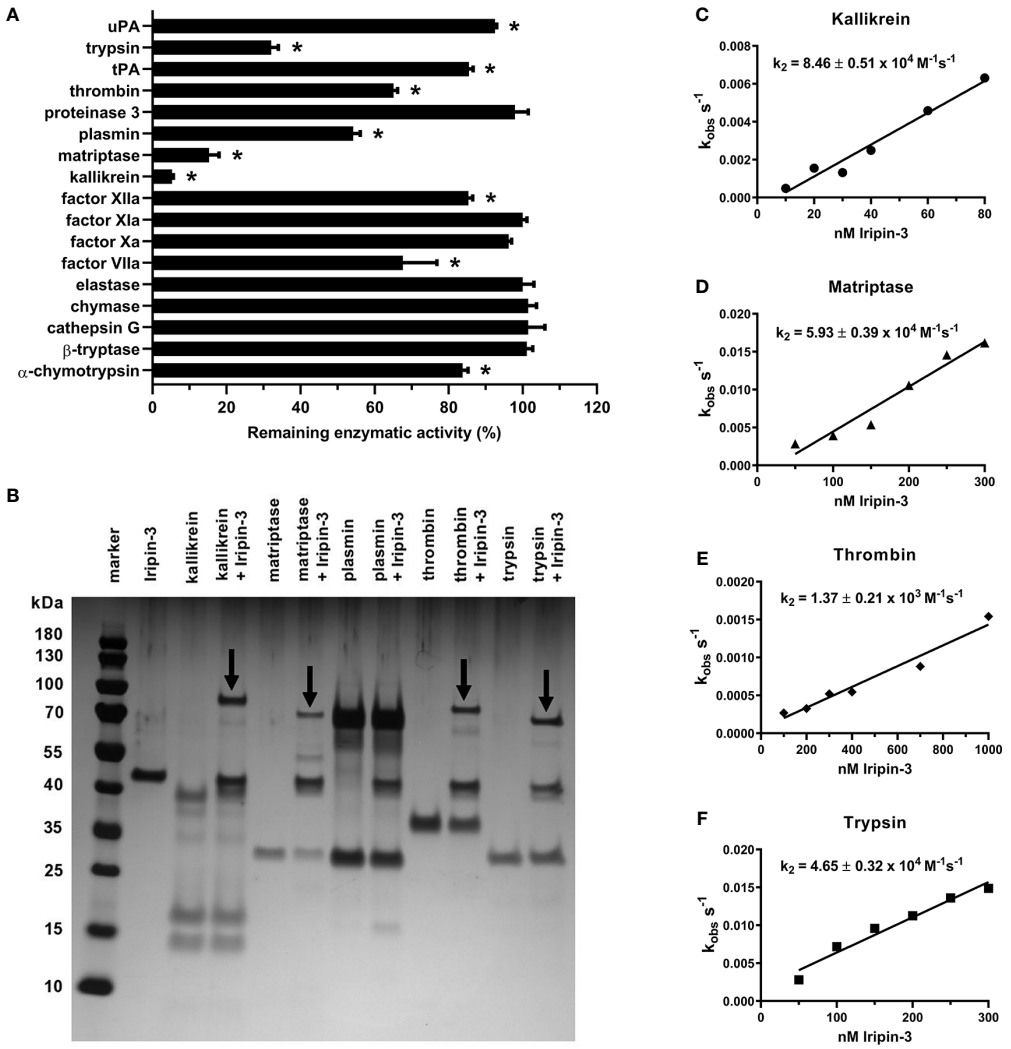

FIGURE 5 | Iripin-3 suppresses the enzymatic activities of kallikrein, matriptase, thrombin, and trypsin through the classic serpin inhibitory mechanism. **(A)** The residual enzymatic activities of 17 selected serine proteases in the presence of 400 nM Iripin-3. The experiment was performed in triplicate, and data are expressed as mean ± SEM. The enzymatic activities of individual proteases in the absence of Iripin-3 (control groups) were considered as 100%, and differences between control groups and Iripin-3-treated groups were analyzed by the unpaired two-tailed *t*-test. Enzymes labelled with an asterisk were inhibited with statistical significance ($p < 0.05$). **(B)** Formation of SDS- and heat-stable complexes between Iripin-3 and kallikrein, matriptase, plasmin, thrombin, and trypsin. Proteins were resolved on 4 to 12% NuPAGE Bis-Tris gels and visualized by silver staining. Covalent complexes between Iripin-3 and target proteases are marked with black arrows. **(C–F)** The apparent first-order rate constant k_{obs} was plotted against Iripin-3 concentration, and linear regression was performed to obtain the line of best fit. The slope of the line represents the second-order rate constant k_2 for the inhibition of kallikrein **(C)**, matriptase **(D)**, thrombin **(E)**, and trypsin **(F)** by Iripin-3. For each determination, the standard error of the slope is given.

3 caused a dose-dependent and statistically significant reduction in the transcription of all three genes (**Figures 7A–C**). However, decreases in the transcription of *Tnf* and *Il1b* did not result in corresponding changes in the concentrations of these two pro-inflammatory cytokines at the protein level (**Figures 7D, F**). Conversely, Iripin-3 was an efficient inhibitor of both IL-6 synthesis and secretion (**Figure 7E**).

Iripin-3 Impairs B and T Cell Viability *In Vitro*

In addition to inhibiting innate immune mechanisms, tick serpins can alter the host adaptive immune response (35, 37, 72). First, we tested whether Iripin-3 had an effect on B and T lymphocyte viability. Incubation of splenocytes derived from OT-II mice for 20 h in the presence of two different concentrations of Iripin-3 (3 μM and 6 μM) resulted in a pronounced dose-dependent reduction in the viability of both B cells ($CD45^+$ $CD19^+$ splenocytes) and T cells ($CD45^+$ $CD3e^+$ splenocytes), with B cell survival more negatively affected by the serpin presence than T cell survival (**Figures 8A–D**). B and T cell viability was impaired irrespective of whether the splenocytes were left unstimulated or were stimulated with OVA peptide (**Figures 8C, D**). Conversely, Iripin-3 did not reduce the viability of BMDMs or dendritic cells (**Supplementary Figures 4A, B**),

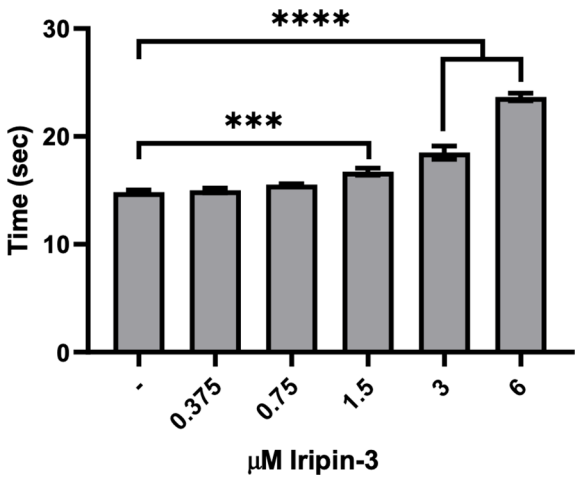

FIGURE 6 | Iripin-3 inhibits the extrinsic pathway of blood coagulation. Human plasma was treated with no Iripin-3 or with 0.375, 0.75, 1.5, 3, and 6 μM Iripin-3 and the time required for blood clot formation in the prothrombin time assay was subsequently determined on a coagulometer. Data are presented as mean ± SEM of three independent experiments (***p < 0.001, ****p < 0.0001).

and the viability of LPS-activated neutrophils was impaired only in the presence of the highest (6 μM) concentration of Iripin-3 (**Supplementary Figure 4C**). Therefore, Iripin-3 might selectively induce B and T cell death. To investigate the possibility that Iripin-3 triggers lymphocyte apoptosis, we measured active caspase-3 levels in both unstimulated and OVA peptide-stimulated splenocytes. Treatment of splenocytes with Iripin-3 did not lead to a statistically significant increase in the level of active caspase-3 (**Figures 8E, F**). Therefore, Iripin-3 probably does not induce B and T cell death through activation of a caspase-3-dependent pathway.

Iripin-3 Inhibits *In Vitro* CD4$^+$ T Cell Proliferation

Since Iripin-3 reduced T cell viability, we tested whether it also affected the survival and proliferation of CD4$^+$ helper T cells. OT-II splenocytes were pre-incubated with 3 μM or 6 μM Iripin-3 for 2 h before being stimulated with OVA peptide for 72 h. Propidium iodide staining in combination with the application of anti-CD4 antibody revealed a lower percentage of live CD4$^+$ cells in Iripin-3-treated groups than in the control group (**Figure 9A**), suggesting Iripin-3 has a negative effect on CD4$^+$ T cell viability. After the exclusion of dead cells, we assessed the

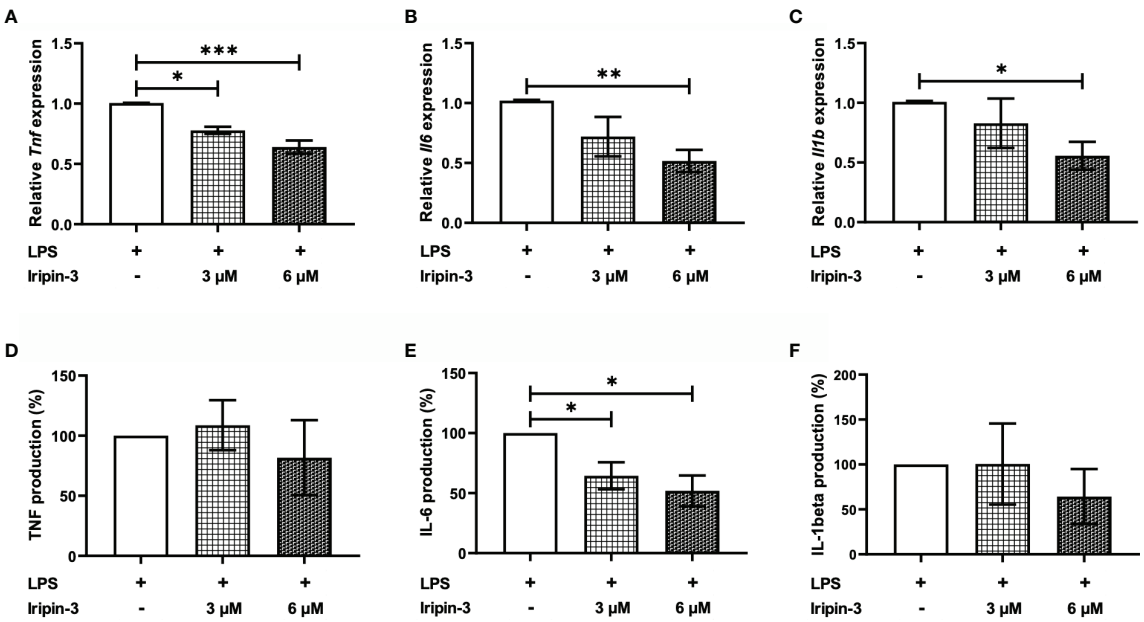

FIGURE 7 | Iripin-3 inhibits the expression of pro-inflammatory cytokines in LPS-stimulated BMDMs. Macrophages derived from bone marrow cells isolated from C57BL/6N mice were pre-incubated with 3 μM or 6 μM Iripin-3 for 40 min and were then stimulated with LPS (100 ng/ml) for 24 h. **(A–C)** At the end of 24 h incubation, cells were harvested for RNA extraction and the expression of *Tnf* **(A)**, *Il6* **(B)**, and *Il1b* **(C)** was determined by RT-qPCR. Relative expression values were calculated using the delta-delta Ct (Livak) method (60), with *Gapdh* serving as a reference gene. Cells incubated only in the presence of LPS were utilized as a calibrator during calculations. Data are presented as mean ± SEM of four independent experiments (*p < 0.05, **p < 0.01, ***p < 0.001). **(D–F)** Supernatants were collected, and TNF, IL-6, and IL-1β concentrations in these supernatants were measured by sandwich ELISA. TNF **(D)**, IL-6 **(E)**, and IL-1β **(F)** production by Iripin-3-treated BMDMs is expressed as the percentage of the cytokine production by control macrophages, since there were large differences in the concentrations of the same cytokine between three independent repeats of the experiment. Data are expressed as mean ± SEM, and statistically significant differences (p < 0.05) are marked with an asterisk.

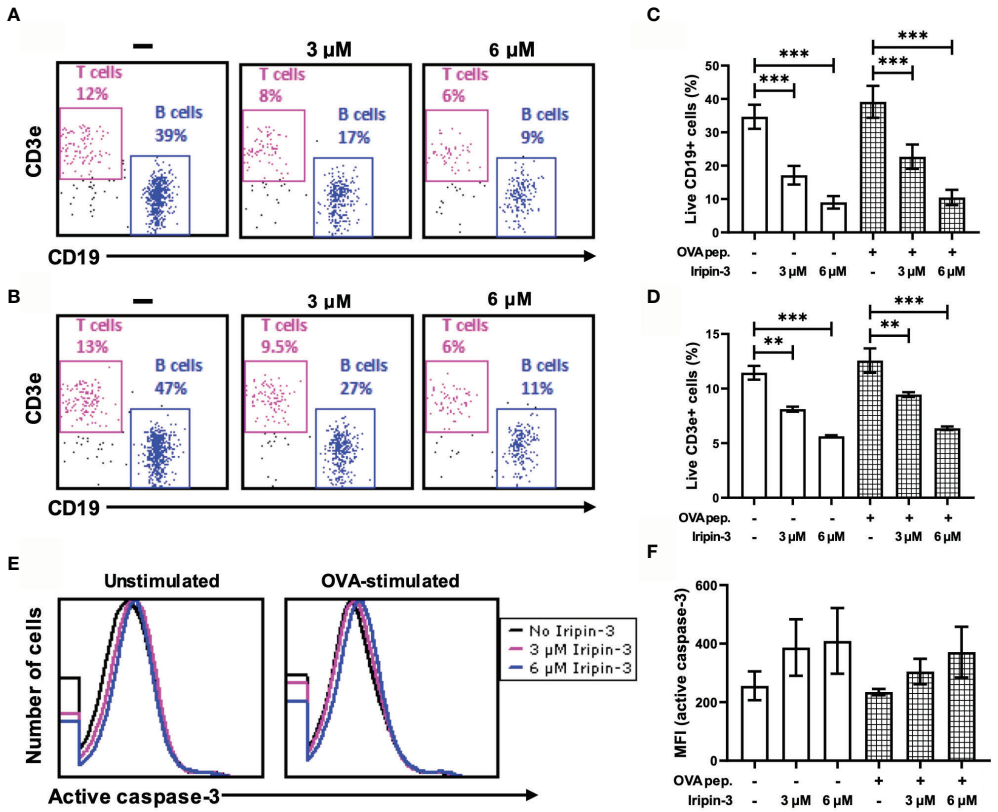

FIGURE 8 | Iripin-3 reduces B and T cell viability and does not significantly alter active caspase-3 levels. **(A, B)** Dot plots depicting the percentage of live $CD45^+CD19^+$ cells (B cells) and live $CD45^+CD3e^+$ cells (T cells) in unstimulated splenocytes **(A)** or OVA peptide-stimulated splenocytes **(B)**. Splenocytes were not treated with Iripin-3 (left) or were treated with 3 µM (middle) or 6 µM (right) Iripin-3. **(C, D, F)** The percentage of live B cells **(C)**, live T cells **(D)**, and median fluorescence intensity (MFI) corresponding to the level of active caspase-3 **(F)** after incubating the splenocytes for 20 h in the absence of Iripin-3 or in the presence of 3 µM and 6 µM Iripin-3. The cells were left either unstimulated or were stimulated with 100 ng/ml of OVA peptide. Data are presented as mean ± SEM of three independent experiments (**$p < 0.01$, ***$p < 0.001$). **(E)** Histograms showing the level of active caspase-3 in either unstimulated splenocytes (left) or splenocytes stimulated with OVA peptide (right). Splenocytes were incubated for 20 h without Iripin-3 or were treated with 3 µM or 6 µM Iripin-3.

proliferation of $CD4^+$ T cells. Unstimulated $CD4^+$ cells did not proliferate at all (**Figure 9C**), whereas addition of OVA peptide triggered proliferation in approximately 95% of cells (**Figures 9B, D**). Treatment with Iripin-3 caused a dose-dependent decrease in $CD4^+$ splenocyte proliferation (**Figure 9B**). While about 84% of cells proliferated in the presence of 3 µM Iripin-3 (**Figures 9B, E**), only 35% of cells were capable of proliferation after addition of 6 µM Iripin-3 (**Figures 9B, F**). Therefore, Iripin-3 impairs both the viability and proliferation of $CD4^+$ T cells.

Iripin-3 Inhibits a Th1 Immune Response and Promotes Differentiation of Regulatory T Cells (Tregs) *In Vitro*

To examine whether Iripin-3 alters the differentiation of naïve $CD4^+$ T cells into Th1, Th2, Th17, or Treg subpopulations, we evaluated the expression of transcription factors T-bet, GATA-3, RORγt, and Foxp3 in OVA peptide-stimulated $CD4^+$ splenocytes by RT-qPCR and flow cytometry. T-bet, GATA-3, RORγt, and Foxp3 are considered lineage-specifying transcription factors that govern Th1, Th2, Th17, and Treg differentiation, respectively (75–79). Iripin-3 markedly and dose-dependently inhibited the expression of T-bet in $CD4^+$ T cells at both the mRNA and protein levels (**Figures 10A–C**). Since T-bet controls *Ifng* transcription (76), we also tested the ability of Iripin-3 to inhibit the production of this hallmark Th1 cytokine. As with T-bet, Iripin-3 induced a pronounced and dose-dependent reduction in the percentage of $CD4^+$ T cells producing IFN-γ (**Figures 10D, E**). Despite the inhibition of the Th1 immune response, we did not observe significant changes in the differentiation of T cells into Th2 or Th17 subpopulations (**Figures 10F–K**). GATA-3 expression was slightly increased only in $CD4^+$ T cells treated with 3 µM Iripin-3 (**Figures 10G, H**). Similarly, both Iripin-3 concentrations induced only a small and non-significant increase in the percentage of $CD4^+$ T cells expressing RORγt (**Figures 10J, K**). Finally, Iripin-3 moderately stimulated the expression of Foxp3 at both the mRNA and protein levels (**Figures 10L–N**). Therefore, Iripin-3 might

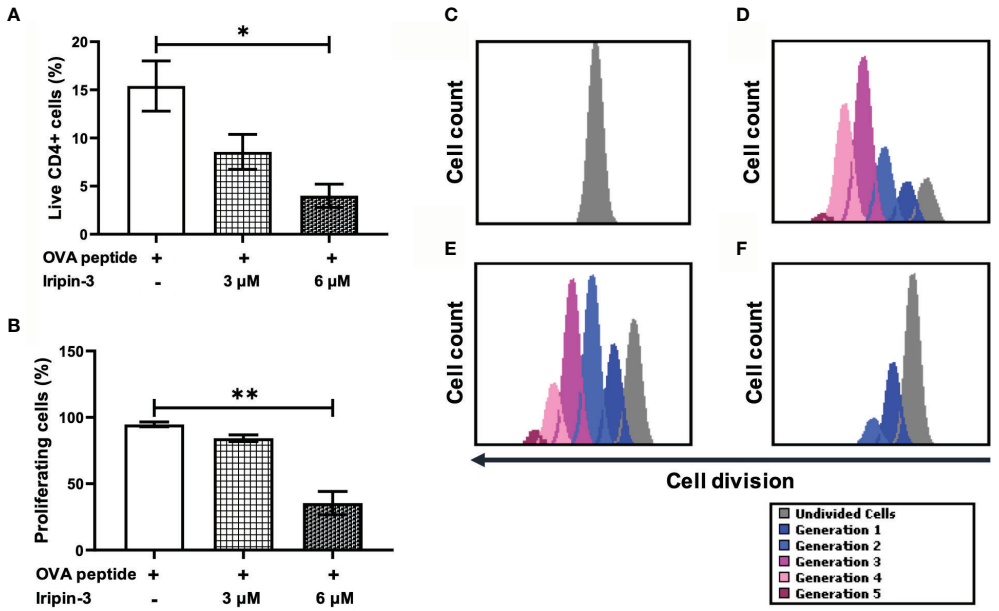

FIGURE 9 | Iripin-3 impairs the survival and proliferation of CD4$^+$ splenocytes. **(A, B)** The percentage of live CD4$^+$ cells **(A)** and the percentage of proliferating live CD4$^+$ cells **(B)** after exposure to 3 µM or 6 µM Iripin-3. Cells not treated with Iripin-3 were used as control. After 2 h pre-incubation with Iripin-3, cells were cultured in the presence of OVA peptide (100 ng/ml) for 72 h. Data are presented as mean ± SEM of three independent experiments (*$p < 0.05$, **$p < 0.01$). **(C–F)** Histograms showing the number of live CD4$^+$ cells that managed to divide once (blue), twice (light blue), 3 times (pink), 4 times (rose), 5 times (plum), or did not divide at all (gray) within the 72 h culture period. Cells were incubated in the absence of Iripin-3 and OVA peptide **(C)**, in the presence of OVA peptide only **(D),** or were treated with the combination of 3 µM Iripin-3 and OVA peptide **(E)** or 6 µM Iripin-3 and OVA peptide **(F)**.

induce the differentiation of Tregs in addition to inhibiting Th1 cell development.

Iripin-3 Is Not Essential for Feeding Success of *I. ricinus* Nymphs

Since *iripin-3* expression is induced in nymphs in response to blood feeding, we decided to assess the role of this serpin in the blood-feeding process by silencing *iripin-3* expression in nymphs via RNA interference. *Iripin-3* expression in *iripin-3* dsRNA-treated ticks was 34% when compared to *gfp* dsRNA-treated ticks (data not shown), suggesting that the knockdown of the target gene was successful. Despite diminished *iripin-3* expression, the time course of blood feeding and overall feeding success (i.e. the number of nymphs that reached full engorgement) did not significantly differ between control ticks and *iripin-3* dsRNA-treated ticks (**Supplementary Table 4**). The weight of fully engorged nymphs was not significantly affected by *iripin-3* silencing as well (**Supplementary Table 4**). Therefore, we can conclude that the deficiency of Iripin-3 alone is not sufficient to impair the blood meal acquisition and processing by nymphal *I. ricinus* ticks.

DISCUSSION

Tick saliva contains hundreds to thousands of proteins from diverse protein families (80). These salivary proteins are differentially expressed over the course of blood feeding and enable ticks to feed to repletion by maintaining blood fluidity and suppressing host defense responses (80). Serpins form one of four serine protease inhibitor families that have been discovered in ticks (72). Serpins are particularly intriguing to study, not only due to their unique trapping inhibitory mechanism but also because they regulate a variety of physiological processes in many organisms. The functional diversity of the serpin superfamily is exemplified by the widely studied human serpins, which have been shown to regulate blood pressure, transport hormones, and control blood coagulation, fibrinolysis, angiogenesis, programmed cell death, inflammation, or complement activation (81–84). We presume that ticks employ some of their serpins to modulate host defenses, as evidenced by several tick serpins with anti-platelet, anti-coagulant, anti-inflammatory, and/or immunomodulatory properties that have been shown to be secreted via saliva into the host (34–37, 72).

Here we determined the structure and partially deciphered the function of *Ixodes ricinus* serpin Iripin-3 by using several *in vitro* models. The size (377 amino acids), molecular weight (42 kDa), and 3D structure of Iripin-3, consisting of three β-sheets, ten α-helices, and a cleaved RCL, correspond to the structural parameters of typical serpins (18, 20, 71). *Iripin-3* expression was induced by blood feeding in both nymphs and adult females, suggesting Iripin-3 contributes to feeding success in both developmental stages. Of the three organs of adult ticks, the highest levels of *iripin-3* transcript were detected in the

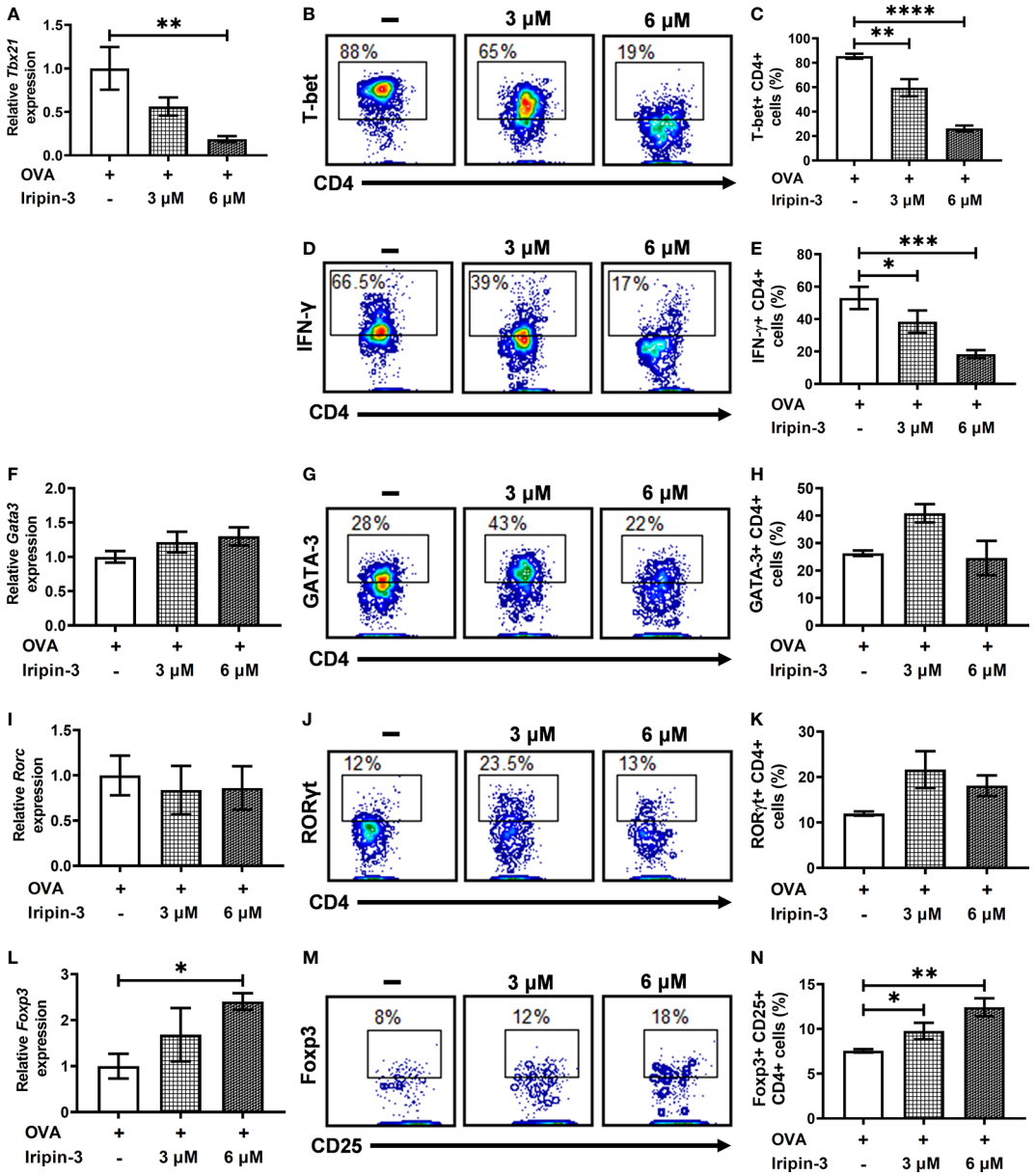

FIGURE 10 | Iripin-3 alters the expression of CD4$^+$ T cell transcription factors at both the mRNA and protein levels. **(A, F, I, L)** Expression of *Tbx21* **(A)**, *Gata3* **(F)**, *Rorc* **(I)**, and *Foxp3* **(L)** in CD4$^+$ cells stimulated with OVA peptide for 72 h. Cells were untreated with Iripin-3 or were treated with 3 µM or 6 µM Iripin-3. Cells incubated only in the presence of OVA peptide were utilized as a calibrator during calculations of relative expression values. Data are presented as mean ± SEM of four independent experiments (* $p < 0.05$, ** $p < 0.01$). **(B, D, G, J, M)** Representative contour plots showing the proportion of OVA peptide-stimulated CD4$^+$ splenocytes expressing T-bet **(B)**, IFN-γ **(D)**, GATA-3 **(G)**, RORγt **(J)** and the combination of CD25 and Foxp3 **(M)**. The cells were incubated in the absence of Iripin-3 (left) or in the presence of two different Iripin-3 concentrations: 3 µM (middle) and 6 µM (right). **(C, E, H, K, N)** The percentage of CD4$^+$ T cells producing the cytokine IFN-γ **(E)** and expressing transcription factors T-bet **(C)**, GATA-3 **(H)**, RORγt **(K)**, and Foxp3 together with CD25 **(N)**. Cells were cultured in the presence of Iripin-3 (3 µM or 6 µM) and OVA peptide for 72 h. Cells incubated without Iripin-3 were used as control. Data are presented as mean ± SEM of three or four independent experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

salivary glands. The presence of Iripin-3 protein in the saliva of partially engorged adults was confirmed by immunodetection. Thus, we can assume that Iripin-3 is secreted via saliva into the tick attachment site where it interferes with host anti-tick defenses. Statistically significant increase of *iripin-3* expression in response to blood feeding occurred not only in the salivary glands but also in the ovaries of adult ticks, which indicates that Iripin-3 might be somehow involved in the reproductive process.

The role of serpins in tick reproduction has been evidenced recently by *Rhipicephalus haemaphysaloides* serpin RHS-8, the knockdown of which impaired oocyte maturation due to the inability of oocytes to uptake adequate amount of vitellogenin (45).

The presence of the basic amino acid residue arginine at the P1 site of the Iripin-3 RCL indicates that Iripin-3 might inhibit trypsin-like rather than chymotrypsin-like or elastase-like serine proteases (69, 85). Indeed, out of 17 selected serine proteases, Iripin-3 most potently inhibited trypsin-like serine proteases kallikrein and matriptase and exhibited weaker inhibitory activity against trypsin, thrombin, plasmin, and factor VIIa. Kallikrein participates in the activation of the intrinsic blood coagulation pathway, promotes fibrinolysis, and is also responsible for the release of the potent inflammatory mediator bradykinin, which further induces vasodilation, increases vascular permeability, and evokes pain and itch (86, 87). Matriptase is a type II transmembrane serine protease that is primarily expressed in epithelial cells and is essential for the maintenance of skin barrier function (88). Moreover, matriptase seems to be involved in cutaneous wound healing (89, 90) and might contribute to the amplification and perpetuation of the inflammatory response through the activation of protease-activated receptor-2 (PAR-2) (91). Therefore, we speculate that Iripin-3-mediated inhibition of kallikrein and matriptase contributes to tick feeding success by suppressing the inflammatory response and consequent itch and pain and by impairing wound healing.

A phylogenetic analysis of 27 functionally characterized tick serpins revealed a close phylogenetic relationship between Iripin-3 and *I. scapularis* serpin IxscS-1E1. Both serpins possess arginine at the P1 site and inhibit trypsin and thrombin (30). However, while IxscS-1E1 prolonged plasma clotting time in aPTT and TT assays and had no effect on blood clot formation in the PT assay (30), Iripin-3 inhibited only the extrinsic coagulation pathway. This indicates that the Iripin-3-mediated inhibition of kallikrein and thrombin was not sufficient to significantly impair the intrinsic and common coagulation pathways. Other blood clotting factors (XIIa, XIa, Xa) involved in the intrinsic and common pathways were not markedly inhibited by Iripin-3. Several tick serpins are capable of inhibiting the common (and perhaps intrinsic) pathway of blood coagulation (28–31, 41, 92); however, none have shown any effect on the extrinsic coagulation pathway. The extrinsic coagulation pathway is initiated by damage to a blood vessel and subsequent formation of a FVIIa/tissue factor (TF) complex, which further activates factor X (93). In view of the fact that Iripin-3 exhibited weak inhibitory activity only in the PT test and not in the aPTT test or TT test, we hypothesized that it might target either FVIIa or TF, since these two proteins are the only unique components of the extrinsic pathway. FVIIa seemed to be a more likely target for Iripin-3 given that it is a serine protease (94), and some human serpins, such as antithrombin III or protein C inhibitor, have been shown to inhibit the proteolytic activity of FVIIa (95–97). In our hands, Iripin-3 did not form a covalent complex with FVIIa either in the absence or in the presence of TF. However, the proteolytic activity of FVIIa was reduced by approximately 30% in the presence of 400 nM Iripin-3 in the kinetic enzyme-substrate assay. Therefore, the prolongation of blood clot formation in the PT assay might be caused by the non-canonical inhibition of FVIIa by Iripin-3. Alternatively, a possible interaction between Iripin-3 and TF could also prevent FVIIa/TF complex formation, leading to a lower rate of FXa generation and inhibition of blood coagulation.

In addition to the inhibition of blood coagulation, Iripin-3 displayed anti-inflammatory activity *in vitro*, since it significantly and dose-dependently attenuated the production of pro-inflammatory cytokine IL-6 by LPS-stimulated bone marrow-derived macrophages. The decreased IL-6 production was probably caused by the inhibition of *Il6* transcription and not by reduced viability of macrophages, since the metabolic activity of macrophages remained unchanged in the presence of Iripin-3. Several tick serpins have been shown to inhibit IL-6 transcription and secretion (37–39, 74, 98), which can occur as a result of serpin-mediated inhibition of proteases such as cathepsin G and cathepsin B (37). However, the inhibition of pro-inflammatory cytokine production does not have to be dependent on serpin anti-protease activity because some serpins, like Iris and α-1-antitrypsin, can alter pro-inflammatory cytokine production by binding to immune cells via exosites (98, 99). An inflammatory environment with reduced IL-6 might favor differentiation of Tregs (100–102). Splenocytes, incubated in the presence of Iripin-3 for 72 h, increased the expression of Treg-specific transcription factor Foxp3 (77, 78), suggesting that Iripin-3 indeed induces the differentiation of naïve $CD4^+$ T cells into anti-inflammatory Tregs. Tregs would facilitate the suppression of the host immune response (103), which would be beneficial for feeding ticks. There is scarce evidence that tick saliva induces Treg differentiation (104, 105). The results of our *in vitro* assay indicate that salivary serpins could contribute to this particular activity of tick saliva.

Besides the reduction in IL-6 production and increase in Foxp3 expression, Iripin-3 caused a pronounced, dose-dependent decrease in B and T cell viability *in vitro*. This effect appears to be B and T cell-specific since macrophage and dendritic cell survival was not affected by Iripin-3 and the viability of LPS-stimulated neutrophils was slightly impaired only at the highest (6 μM) concentration of Iripin-3. Serpins usually protect cells from dying by reducing the proteolytic activity of enzymes (such as granzymes and caspases) involved in programmed cell death (106). However, certain serpins, e.g., kallikrein-binding protein, pigment epithelium-derived factor, or maspin, induce apoptosis of endothelial cells and some cancer cells through distinct mechanisms such as the activation of the Fas/FasL/caspase-8 signaling pathway or the permeabilization of the outer mitochondrial membrane followed by a loss of transmembrane potential (107–111). Active caspase-3 levels were only slightly and non-significantly increased in Iripin-3-treated splenocytes. Therefore, the induction of caspase-dependent apoptosis was not the main cause of impaired splenocyte viability. Various forms of caspase-independent cell death have been described such as autophagy, paraptosis, necroptosis, or necrosis (112, 113). Elucidation of the exact

mechanism behind the extensive splenocyte death in the presence of Iripin-3 is, however, beyond the scope of this paper.

I. ricinus saliva and salivary gland extracts inhibit T cell proliferation and suppress Th1 cell differentiation while simultaneously augmenting the Th2 immune response (114–117). Iripin-3 might contribute to this immunomodulatory effect of saliva, since in our *in vitro* assays it inhibited $CD4^+$ T lymphocyte proliferation and impaired the differentiation of naïve $CD4^+$ T cells into Th1 cells. Impaired Th1 cell generation was evidenced by decreased expression of the Th1 lineage-specifying transcription factor T-bet and a reduced percentage of $CD4^+$ T cells producing the hallmark Th1 cytokine IFN-γ. Several studies have reported inhibition of splenocyte and peripheral blood mononuclear cell proliferation in the presence of tick serpins (35, 37, 38, 40). Interestingly, the inhibition of mitosis observed in these studies was usually accompanied by decreased IFN-γ production (35, 38, 40), which might indicate, among other things, the suppression of Th1 cell differentiation. The causative mechanism of reduced cell proliferation and impaired Th1 cell differentiation in the presence of tick serpins remains unknown, but it could be associated with decreased production of certain cytokines such as IL-2, IL-12, and IFN-γ. In the case of Iripin-3, there might be a connection between the inhibition of cell proliferation and impaired viability of splenocytes, i.e., the mechanism behind B and T cell death could be also responsible for the suppression of $CD4^+$ T cell division. Iripin-3-mediated differentiation of naïve $CD4^+$ T cells into Tregs might also contribute to the reduction in $CD4^+$ T cell proliferation, since Tregs can inhibit cell multiplication by various mechanisms including the production of immunosuppressive cytokines TGF-β and IL-35, consumption of IL-2, and conversion of ATP to adenosine (103, 118).

It is worth mentioning that the Iripin-3 concentrations used in *in vitro* experiments (3 μM and 6 μM) are probably higher than the amount of Iripin-3 at the tick feeding site. This fact, however, does not make the anticoagulant, ant-inflammatory and immunomodulatory activities of Iripin-3 observed *in vitro* physiologically irrelevant. Tick saliva is a complex mixture of proteins from the same or different protein families, and some of these salivary proteins can share the same function (119). Therefore, even a low concentration of one tick protein may be sufficient to achieve a desired effect at the tick attachment site if this protein acts in concert with other tick proteins (119). For instance, the ability of *I. ricinus* saliva to inhibit $CD4^+$ T cell proliferation is probably a result of combined action of more proteins with anti-proliferative properties, such as the serpins Iripin-3 and Iris, the cystatin Iristatin and the Kunitz domain-containing protein IrSPI (38, 120, 121). That *I. ricinus* saliva may contain other proteins possessing Iripin-3-like activities was demonstrated by the RNA interference experiment. *Iripin-3* knockdown did not significantly affect the overall feeding success, time course of blood feeding and weight of fully engorged nymphs, which indicates that other similarly acting salivary proteins might compensate for the loss of *iripin-3* expression.

It is also important to note that native Iripin-3 is most likely glycosylated. However, recombinant Iripin-3 was prepared in an *E. coli* expression system, and therefore it lacks glycosylation. Glycosylation has been shown to reduce the propensity of serpins for polymerization (122) and increase the stability and half-life of circulating serpins by conferring resistance to proteolytic degradation (123, 124). The impact of glycosylation on the biological function of serpins is less clear. Recombinant Iripin-3 inhibited the proteolytic activity of some serine proteases, suggesting that its functions dependent on anti-protease activity (like anticoagulant properties) may not be affected by missing glycosylation. However, the absence of glycosylation might have an impact on anti-inflammatory and immunomodulatory activities of Iripin-3 mediated by its binding to cell surfaces and soluble immune mediators. For example, only glycosylated, but not non-glycosylated, α-1-antitrypsin was capable of binding IL-8, thus inhibiting IL-8-CXCR1 interaction (125).

CONCLUSION

To conclude, Iripin-3 is a pluripotent salivary protein secreted by *I. ricinus* ticks via saliva into the feeding site, where it might suppress various aspects of host anti-tick defenses. The attenuation of IL-6 production, suppression of $CD4^+$ T cell proliferation, and inhibition of Th1 immune responses have also been observed with other tick serpins and are consistent with the previously reported immunomodulatory effects of *I. ricinus* saliva and salivary gland extracts (114–117). On the other hand, our study is the first to describe the inhibition of the extrinsic pathway of blood coagulation, impaired B and T cell survival, and the induction of Treg differentiation by a tick serpin. The pluripotency and redundancy in Iripin-3 functions are consistent with the theory about the importance of these protein features for successful tick feeding (119). Although several distinct *in vitro* activities of Iripin-3 were observed in this study, their physiological relevance, mechanisms behind them and potential of Iripin-3 to be a candidate for drug or vaccine development remain to be determined. Therefore, further *in vivo* experiments and mechanistic studies are needed to validate and elucidate the Iripin-3 functions described in this work.

DATA AVAILABILITY STATEMENT

The data sets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

All animal experiments were performed 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 Ministry of Education, Youth and Sports of the Czech Republic (protocol no. 19085/2015-3) and the responsible committee of

the IP BC CAS. Pathogen-free *I. ricinus* ticks were obtained from the tick colony maintained at the IP BC CAS.

AUTHOR CONTRIBUTIONS

AC designed and performed experiments, analyzed data, and wrote the manuscript. JK, ZB, BK, LAM, HL, TP, ME, and IKS designed and performed experiments and analyzed data. MK edited the manuscript. JC directed the study, designed experiments, analyzed data, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.626200/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Article 5

Structural and biochemical characterization of the novel serpin Iripin-5 from Ixodes ricinus

Kascakova B, Kotal J, Martins LA, Berankova Z, Langhansova H, Calvo E, Crossley JA, Havlickova P, Dycka F, Prudnikova T, Kuty M, Kotsyfakis M, **Chmelar J**, Kuta Smatanova I

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In the collaboration with the group of Prof. Kutá-Smatanová, we published an article about iripin-5, the most abundantly expressed serpin in *I. ricinus*. The article is mostly structural analysis of the serpin and its complexes with target proteases, which are mostly neutrophil elastase and protease 3 and with lesser affinity also cathepsin G, trypsin, chymotrypsin and chymase. Docking studies *in silico* showed that amino acid glutamin at the position 310 could be important for the interaction between proteases and the inhibitor. Iripin-5 inhibited neutrophil migration and the production of nitric oxide by activated macrophages. Moreover it proved to be an inhibitor of complement dependent erythrocyte lysis. The structure of Iripin-3 was solved in its cleaved and most stable state, which proved arginine in its P1 site

Author's contribution:

Author designed and coordinated the immunological part of study and participated on the writing and revising of the manuscript

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Structural and biochemical characterization of the novel serpin Iripin-5 from *Ixodes ricinus*

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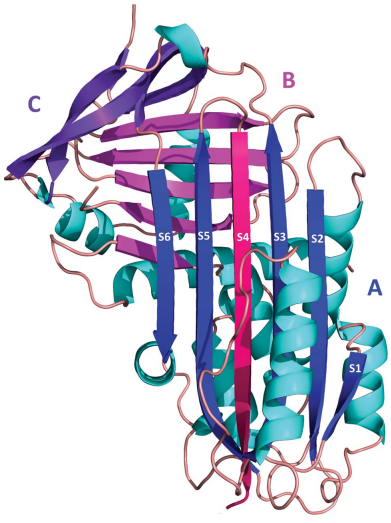
Iripin-5 is the main *Ixodes ricinus* salivary serpin, which acts as a modulator of host defence mechanisms by impairing neutrophil migration, suppressing nitric oxide production by macrophages and altering complement functions. Iripin-5 influences host immunity and shows high expression in the salivary glands. Here, the crystal structure of Iripin-5 in the most thermodynamically stable state of serpins is described. In the reactive-centre loop, the main substrate-recognition site of Iripin-5 is likely to be represented by Arg342, which implies the targeting of trypsin-like proteases. Furthermore, a computational structural analysis of selected Iripin-5–protease complexes together with interface analysis revealed the most probable residues of Iripin-5 involved in complex formation.

1. Introduction

The castor bean tick (*Ixodes ricinus*) has a wide geographical distribution throughout the Northern Hemisphere of Europe, Asia and Africa that points towards its resistance to various environmental conditions. This has helped this tick to become one of the major factors in the spread of zoonotic diseases, as it serves as a vector for multiple vector-borne pathogens (Tirloni *et al.*, 2014; Francischetti *et al.*, 2009). These include tick-borne diseases such as Lyme disease, Helvetica spotted fever, tick-borne meningoencephalitis, babesiosis and tick paralysis (Sprong *et al.*, 2018). *I. ricinus* represents a model organism used in the development of new sustainable tick-control approaches such as acaricides and repellents. The saliva of ticks helps them to stay attached to the host until the long-lasting blood-feeding process is finished. This is facilitated by many immunomodulatory, anti-inflammatory and antihemostatic proteins, peptides and nonpeptide molecules in the saliva (Francischetti *et al.*, 2009; Kotál *et al.*, 2015).

Serpins (serine protease inhibitors) are the largest superfamily of protease inhibitors and are broadly distributed in nature (Silverman *et al.*, 2001; Spence *et al.*, 2021). The vast majority of serpins act as serine protease inhibitors, but during evolution some serpins switched to non-inhibitory functions such as molecular chaperones (for example heat-shock serpin 47; Nagata, 1996), tumour suppressors (for example maspin; Zou *et al.*, 1994), storage proteins (for example ovalbumin;



Mellet *et al.*, 1996; Law *et al.*, 2006) and hormone-binding globulins (for example thyroxine-binding globulin and cortisol-binding globulin; Pemberton *et al.*, 1988). The typical process of serpin inhibition is irreversible and leads to substrate suicide: inactivation of both the serpin and the target protease. At the beginning of the inhibitory pathway, serpins form a Michaelis complex with the protease (Huntington, 2011). Subsequently, translocation of the reactive-centre loop (RCL) with the bound protease takes place and leads to the formation of a covalent complex with the trapped protease and the addition of a new strand in β-sheet A (Silverman *et al.*, 2001). Inhibitory serpins vary in function according to their specificities, and their importance can be illustrated by serpinopathies, diseases caused by serpin dysfunction or deficiency (Belorgey *et al.*, 2007). Many well known diseases, for example emphysema, cirrhosis, angioedema, hypertension and familial dementia, are caused at least partially by serpin dysfunction (Law *et al.*, 2006; Huntington, 2011). This makes serpins interesting candidates for drug design and development, for which a high-resolution structure is necessary. All serpins possess a structurally similar core domain consisting of ~380 residues. This domain is made up of three β-sheets (A, B and C) and eight or more α-helices (hA–hI; Gettins, 2002). Another typical characteristic feature of serpins is the presence of an exposed, extended RCL that acts as a bait for the target protease during inhibition. The RCL consists of ~17 residues and is located between β-sheet A and β-sheet C (Dunstone & Whisstock, 2011). It was found that serpins show different structural conformations such as native (S, stressed state), cleaved (R, relaxed state), latent (a result of auto-inactivation due to a mutation or self-stabilization) and the δ-conformation (inappropriate partial insertion of the RCL due to a mutation) as well as the possible formation of complexes as a result of the inhibitory mechanism (Dunstone & Whisstock, 2011). The inhibitory mechanism can result in successful inhibition by covalent complex formation with the target protease or a cleaved conformation. During conformational change of both states, and the incorporation of the RCL into β-sheet A, energy release occurs and a rise in serpin stability is reported as a consequence of this transition. In the case where this process is not sufficiently fast, it results in unsuccessful inhibition of the protease and its release from the acyl-intermediate, followed by the formation of a cleaved conformation of the serpin (Gettins, 2002; Gettins & Olson, 2016; Yamasaki *et al.*, 2002).

Tick salivary serpins play important roles in tick physiology. They are necessary to modulate the immune-system responses of the host and to inhibit various defence mechanisms such as hemostasis, which can result in the facilitated transmission of the aforementioned tick-borne pathogens (Kotál *et al.*, 2015). High structural conservation of serpins across tick species has been observed (Porter *et al.*, 2015). The tick *I. ricinus* expresses over 30 serpins with different specificities, of which only Iris, IRS-2 and Iripin-3 have been characterized in detail functionally, while IRS-2 and Iripin-3 have also been structurally characterized (Prevot *et al.*, 2006; Chmelař *et al.*, 2011; Páleníková *et al.*, 2015; Chlastáková *et al.*, 2021).

Here, we present the structural, biochemical and functional characterization of the serpin from *I. ricinus* named Iripin-5 (*I. ricinus* serpin-5) that is highly expressed in the salivary glands of the tick; its expression is induced by feeding on blood and it displays anti-inflammatory and anticomplement features. Structural analysis revealed that Iripin-5 crystallized in a cleaved conformation and its structure was solved at 1.50 Å resolution. The structure was used for interface and computational analyses of its complexes with chosen proteases.

2. Materials and methods

2.1. Protein cloning, expression and purification

The full-length Iripin-5 sequence was cloned into pET-17b vector and transformed into *Escherichia coli* strain BL21-pLysS (Novagen, USA). 6 l LB medium (100 µg ml^{-1} ampicillin and 34 µg ml^{-1} chloramphenicol) was inoculated with an overnight culture of BL21-pLysS cells containing the Iripin-5 gene. Protein overexpression was induced by 1 m*M* isopropyl β-D-1-thiogalactopyranoside (IPTG) on reaching an OD_{600} of 0.6 and the cells were harvested 3 h after induction. Inclusion bodies were isolated by sonication in 20 m*M* Tris-buffered saline (TBS), 150 m*M* NaCl pH 8.0 with 1%(*v*/*v*) Triton X-100 buffer and washed three times with TBS to remove traces of Triton X-100. The inclusion bodies were dissolved in 20 m*M* TBS, 6 *M* guanidine–HCl pH 8 and undissolved impurities were removed by centrifugation (12 000*g*). Refolding was achieved by rapid dilution in a 160-fold excess of 50 m*M* Tris, 300 m*M* NaCl, 0.8 m*M* KCl, 250 m*M* L-arginine pH 8.5 with 0.25 g wet inclusion bodies per litre of refolding buffer. After filtration, Iripin-5 was purified by ion-exchange and size-exclusion chromatography (Supplementary Figs. S1 and S2). Pure protein was decontaminated from lipopolysaccharide (LPS) by Arvys Proteins (Trumbull, USA) using a detergent-based method. The LPS was removed from the sample because of its proven activation effect on cells, especially the stimulation of cells responsible for immune responses. This would interfere in subsequent experiments. The final concentration of protein was 1.14 mg ml^{-1} in 20 m*M* Tris, 150 m*M* NaCl pH 8.0 buffer and the protein was stored at −80°C.

2.2. Nitric oxide production by IC-21 macrophages

Macrophages of the IC-21 cell line were pre-incubated with various concentrations of Iripin-5 for 4 h. After stimulation with 100 ng ml^{-1} LPS and 5 ng ml^{-1} interferon-γ (IFNγ), the cells were incubated for 24 or 48 h. The nitric oxide (NO) concentration was assessed after incubation with a modified Griess reagent (Sigma–Aldrich, Germany).

2.3. Antiprotease selectivity

Assays were performed according to a previous publication (Chmelar *et al.*, 2011). The enzyme concentrations do not reflect their ratio in the plasma or skin of the tick host. The used concentrations were chosen based on the biochemical

Table 1
Antiprotease selectivity of Iripin-5.

Enzyme	Amount of enzyme used (n*M*)	Remaining enzymatic activity (%)
Thrombin	0.01	95.2 ± 3.2
Factor Xa	0.33	97.6 ± 4
Kallikrein	0.04	100.9 ± 2.5
Chymase	0.45	81.1 ± 3.3
Trypsin	0.1	55.9 ± 1.5
α-Chymotrypsin	0.05	68.6 ± 1.4
β-Tryptase	0.01	104.2 ± 1.4
Human neutrophil elastase	0.06	**13 ± 2.2**
Cathepsin G	8.8	80 ± 1.8
u-PA	0.5	101 ± 1.5
Plasmin	1.2	94.1 ± 2.2
Matriptase	0.03	100 ± 1.9
Factor XIa	0.06	98.8 ± 3
Factor XIIa	0.1	98.8 ± 1.2
t-PA	0.02	100.8 ± 3.7
Proteinase 3	1.7	**4.6 ± 0.8**

properties of particular proteases in order to detect substrate hydrolysis and do not reach saturation of reaction at the same time. Generally, the assay conditions were chosen as half of the V_{max} of each particular protease. Briefly, assays were performed at 30°C and tested in triplicate. The used protein concentration in the reaction was from 400 n*M* and the serpin was pre-incubated with the target enzyme (listed in Table 1) for 10 min before adding substrate (250 µ*M* final concentration). For each target enzyme, appropriate buffers at different final concentrations were used. The substrate-hydrolysis rate was determined using an Infinite 200 PRO 96-well plate fluorescence reader (Tecan, Switzerland; excitation at 365 nm, emission at 450 nm).

2.4. Complement assay

Fresh rabbit erythrocytes were collected in Alsever's solution from the rabbit marginal ear artery, washed three times in an excess of PBS buffer (1.8 m*M* KH_2PO_4, 137 m*M* NaCl, 10 m*M* $Na_2HPO_4{\cdot}7H_2O$) and finally diluted to a final 2%(*v*/*v*) suspension. Fresh human serum was obtained from three healthy individuals. The assay was performed in a 96-well round-bottomed microtiter plate (Nunc, Denmark). In each well, a concentration of 50% human serum in PBS premixed with different concentrations of Iripin-5 (156 n*M* to 5 µ*M*) was added to a volume of 100 µl. After 10 min of incubation at room temperature, 100 µl of erythrocyte suspension was added. Since the human serum lysed rabbit erythrocytes immediately after their addition to the reaction, we used only 50% concentration (*i.e.* a 25% final serum concentration after addition of the erythrocyte suspension); the final dilution had been empirically established as optimal. Reaction wells were observed individually under a Olympus SZX7 stereomicroscope with oblique illumination (Olympus KL 1500) using an aluminium pad. The time needed for erythrocyte lysis was measured using a chronometer. When full lysis was achieved, the reaction mixture turned from opaque to transparent. Negative controls did not contain either serpin or human serum. Additional controls were performed with heat-inactivated serum (56°C, 30 min) and the serpin Iripin-3 (156 n*M* to 10 µ*M*). The assay was evaluated in technical and biological triplicates.

2.5. Neutrophil-migration assay

Neutrophils were obtained from the bone marrow of C57BL/6J mice by magnetic separation using a Neutrophil Isolation Kit (Miltenyi Biotec, Germany). Isolated neutrophils were pre-incubated in RPMI1640 growth medium containing 0.5%(*m*/*v*) bovine serum albumin (BSA) in the presence or absence of Iripin-5 (3 µ*M*) for 1 h at 37°C and 5% CO_2. The cells were then seeded on the upper inserts of 3.0 µm pore Corning Transwell chambers (24-well format; Sigma–Aldrich, Germany). Chemoattractant solution (1 µ*M* *N*-formyl-L-methionyl-L-leucyl-phenylalanine-fMLP in RPMI1640 with 0.5% BSA) was placed in the lower compartments. After incubation for 1 h at 37°C and 5% CO_2, migration was determined by counting the cells in the lower chamber using a hemocytometer (Meopta, Czech Republic).

2.6. Iripin-5 expression profiles

I. ricinus nymphs were fed on C3H/HeN mice for one day, two days and until full engorgement (3–4 days); *I. ricinus* females were fed on guinea pigs for one, two, three, four, six and eight 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 (MRC). cDNA preparations were made from 1 µg total RNA from independent biological triplicates using a Transcriptor First Strand cDNA Synthesis kit (Roche, Czech Republic) according to the manufacturer's instructions. The cDNA was subsequently used for the analysis of Iripin-5 transcription by qPCR in a RotorGene 6000 cycler (Corbett Research, UK) using Fast Start Universal SYBR Green Master Mix (Roche, Czech Republic), forward primer 5′-CGA GAA CGC AAC CAC TAA GA-3′ and reverse primer 5′-GCT CAA CGT GAC CAA TGT AAT C-3′. Iripin-5 expression profiles were calculated using Livak's mathematical model (Livak & Schmittgen, 2001) and normalized to *I. ricinus* elongation factor 1α (ef1α; GU074829.1; forward primer 5′-CTG GGT GTG AAG CAG ATG AT-3′ and reverse primer 5′-GTA GGC AGA CAC TTC CTT CTG-3′). The amplicon lengths were ef1α, 105 bp; Iripin-5, 251 bp.

2.7. Protein crystallization, X-ray data collection and processing

Crystallization screening using commercial kits (JCSG++ from Jena Bioscience, SG1 and PGA Screen from Molecular Dimensions, and PEGRx and PEG/Ion from Hampton Research, USA) was carried out at room temperature (20°C) and at 4°C by the sitting-drop vapour-diffusion method using an OryxNano crystallization robot (Douglas Instruments). A suitable protein concentration for crystallization screening was determined using the Pre-Crystallization Test (Hampton Research, California, USA) as 1.14 mg ml^{-1}. Drops of protein solution composed of 20 m*M* Tris, 150 m*M* NaCl pH 8.0 buffer

(1 µl) mixed with reservoir solution (1 or 0.5 µl) were equilibrated against 50 µl reservoir solution and sealed in 96-well Swissci MRC 2-drop crystallization plates (Molecular Dimensions).

For data collection, crystals of Iripin-5 that grew for about one month were flash-cooled in liquid nitrogen with 20%(*v*/*v*) glycerol as an additional cryoprotectant. Measurements were carried out on beamline BL14.1 at the BESSY II electron-storage ring operated by Helmholtz-Zentrum Berlin (Mueller *et al.*, 2012). Collection of diffraction data was performed at 100 K with a 295.165 mm crystal-to-detector (PILATUS 6M) distance. Diffraction intensity data were processed using *XDS* (Kabsch, 2010) with the *XDSAPP* graphical user interface (Sparta *et al.*, 2016). Data-collection statistics are summarized in Table 2.

2.8. Structure determination and refinement

Crystallographic and structural analyses were performed using the *CCP*4 package (Winn *et al.*, 2011). The structure of Iripin-5 was solved by the molecular-replacement method using *MOLREP* (Vagin & Teplyakov, 2010) with the structure of the serpin IRS-2 (PDB entry 3nda; Chmelar *et al.*, 2011) as the search model. The structure was refined with *REFMAC*5 (Murshudov *et al.*, 2011) and further manually in *Coot* (Emsley *et al.*, 2010) from evaluation of the electron-density peaks. The improvement during refinement was monitored by structure validation throughout the refinement process. Water molecules were added to the model using the *REFMAC*5 interface. Accepted solvent molecules had tolerable hydrogen-bonding geometry contacts of 2.5–3.5 Å with protein atoms or with existing solvent. At this point, residues with two possible conformations were included and their alternative conformations were added for further refinement. In the last steps of refinement, glycerol was built into the appropriate $(2F_o - F_c)$ and $(F_o - F_c)$ electron-density maps using coordinates from the ligand data bank in *Coot* (Emsley *et al.*, 2010). The *MolProbity* server (Williams *et al.*, 2018) and wwPDB validation server (Berman *et al.*, 2003) were used for final qualitative validation of the model. All figures were prepared using *PyMOL* (DeLano, 2002). A summary of the data-collection and refinement statistics is given in Table 2.

2.9. Structural analysis and molecular dynamics of the modelled Michaelis complexes

The structures of the predominantly inhibited proteases proteinase 3 and human neutrophil elastase were fetched from the PDB as PDB entries 1fuj at 2.20 Å resolution (Fujinaga *et al.*, 1996) and 3q76 at 1.86 Å resolution (Hansen *et al.*, 2011), respectively. The cleaved Iripin-5 crystal structure was modelled to match the native conformation of serpins. The inserted RCL from β-sheet A was modelled above the Iripin-5 structure and the missing residues (Leu343, Ile344, Glu345, Val346 and Pro347) were modelled into the structure to complete the native structure. The crystal structures of the chosen proteases were modified by removing alternative conformations of the amino-acid side chains, ligands and ions from the structures as required for further docking calculations using the prepared native Iripin-5 model. The *HADDOCK*2.2 web server (van Zundert *et al.*, 2016) was used for Michaelis complex docking and the best results were used to run molecular-dynamics (MD) simulations. MD simulations were performed using the *GROMACS* simulation suite (Berendsen *et al.*, 1995) with the CHARMM27 all-atom force field and the SPC/E (extended simple point charge) model for water (Feller & MacKerell, 2000; Klauda *et al.*, 2005). Both Michaelis complex models were prepared for simulation by removing the solvent and were then solvated using the SPC/E water model (Berendsen *et al.*, 1987) in a rhombic dodecahedral box. The protein was centred in the box and the size of the box was such that the protein was at least 1 nm from all edges. Na^+ ions were added to the system at a concentration of

Table 2
X-ray data-collection and refinement statistics.

Values in parentheses are for the highest resolution shell.

Data collection	
X-ray diffraction source	BL14.1, BESSY II, Germany
Wavelength (Å)	0.9184
Detector	PILATUS 6M
Crystal-to-detector distance (mm)	295.165
Rotation range per image (°)	0.1
Total rotation range (°)	360
Exposure time per image (s)	0.1
Resolution range (Å)	48.09–1.50 (1.59–1.50)
Space group	$P12_11$
Molecules in asymmetric unit	2
a, *b*, *c* (Å)	76.24, 63.78, 81.99
α, β, γ (°)	90.0, 116.78, 90.0
Mosaicity (°)	0.199
Total No. of reflections	752984 (117495)
No. of unique reflections	112133 (17637)
Multiplicity	6.72
Average $I/\sigma(I)$	11.17 (1.41)
Completeness (%)	98.7 (96.5)
$CC_{1/2}$	99.8 (61.3)
R_{meas}† (%)	11.0 (124.1)
Overall *B* factor from Wilson plot (Å^2)	24.46
Refinement	
Resolution range (Å)	48.09–1.50
No. of reflections in working set	110024 (7612)
Final R‡/R_{free}§ (%)	0.153/0.185
Mean *B* value (Å)	17.725
No. of non-H atoms in the asymmetric unit	
Protein	6010
Water	1097
Magnesium	4
Chlorine	6
Total	7117
R.m.s. deviations	
Bonds (Å)	0.012
Angles (°)	1.672
Average *B* factor (Å^2)	17.517
Ramachandran plot	
Most favoured (%)	98.64
Allowed (%)	100.00
PDB code	7b2t

† $R_{meas} = \sum_{hkl}\{N(hkl)/[N(hkl)-1]\}^{1/2}\sum_i |I_i(hkl) - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i I_i(hkl)$, where the average intensity $\langle I(hkl)\rangle$ is taken over all symmetry-equivalent measurements and $I_i(hkl)$ is the measured intensity for the *i*th observation of reflection *hkl*. ‡ $R = \sum_{hkl}||F_{obs}| - |F_{calc}||/\sum_{hkl}|F_{obs}|$, where F_{obs} and F_{calc} are the observed and calculated structure factors, respectively. § R_{free} is equivalent to the *R* value but is calculated for 1.87% of the reflections that were chosen at random and omitted from the refinement process.

150 m*M* together with an appropriate amount of Cl^- ions to neutralize the system. The entire system was minimized using a steepest-descent minimization procedure. The energy-minimized structure was then further equilibrated in two phases for 100 ps each: first under an NVT ensemble (constant number of particles, volume and temperature) followed by an NPT ensemble (constant number of particles, pressure and temperature) to ensure that the system remained stable. Simulations were then performed for 100 ns each, during which time equilibrium of the system was achieved. MD simulations were performed fully in triplicate (*i.e.* from the minimization to production run) to ensure reproducibility. The results of the molecular simulations were analyzed using *VMD* (Humphrey *et al.*, 1996) with the use of the r.m.s.d. trajectory tool. An interface analysis of the resulting structures (the final frames of each simulation) was then performed using the *PDBePISA* web server (Krissinel & Henrick, 2007).

2.10. Structural analysis and protein docking of modelled covalent complex conformations

Structures were analyzed and compared with those of the other *I. ricinus* serpins using *PyMOL* version 2.0 (DeLano, 2002; Schrödinger). The *HADDOCK*2.2 web server (van Zundert *et al.*, 2016) was used for protein docking to generate covalent complexes. The possible target proteases selected as the best candidates from the antiprotease selectivity assays, namely proteinase 3, human neutrophil elastase, trypsin, α-chymotrypsin, cathepsin G and chymase, were used for analysis. The crystal structures of the human proteases were taken from the Protein Data Bank: PDB entries 1fuj at 2.20 Å resolution (Fujinaga *et al.*, 1996), 3q76 at 1.86 Å resolution (Hansen *et al.*, 2011), 1h4w at 1.70 Å resolution (Katona *et al.*, 2002), 4cha at 1.68 Å resolution (Tsukada & Blow, 1985), 1au8 at 1.90 Å resolution (F. J. Medrano, W. Bode, A. Banbula & J. Potempa, unpublished work) and 3n7o at 1.80 Å resolution (Kervinen *et al.*, 2010). Alternative conformations of the amino-acid side chains, ligands and ions were removed from the structures as required. Interface analysis was performed using the *PDBePISA* (Krissinel & Henrick, 2007) and *COCOMAPS* (Vangone *et al.*, 2011) web servers.

2.11. PDB deposition

The atomic coordinates of Iripin-5 have been deposited in the Protein Data Bank with accession code 7b2t.

2.12. Statistical analyses

All immunological experiments were performed as at least three 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 are marked as follows in the figures: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; n.s., not significant.

3. Results

3.1. Iripin-5 expression profiles

Expression of the Iripin-5 gene was upregulated during tick feeding in all tested tissues. The highest expression was found in semi-engorged nymphs (D2), and expression was also high in fully engorged nymphs (D3) as well as in female salivary glands during finishing of the blood meal (D6 and D8; Fig. 1). Furthermore, the Iripin-5 transcripts in engorged nymphs and female salivary glands were the most abundant among all tested tick serpins (data not shown). Thus, Iripin-5 is likely to be the most abundant serpin that is secreted from the salivary glands to the host.

3.2. Antiprotease selectivity and neutrophil migration

In the protease-selectivity assay, Iripin-5 needed to be in a high excess compared with the target enzyme in order to obtain even a low level of inhibition. The remaining protease inhibition after 10 min incubation with 200 n*M* Iripin-5 is given in Table 1. Iripin-5 showed the highest inhibitory specificity against two neutrophil proteases: human neutrophil elastase (87% inhibition) and proteinase 3 (95% inhibition). Statistically significant results are noted in bold. Based on

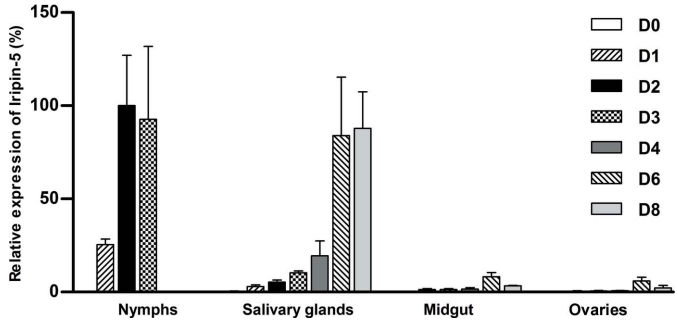


Figure 1
Iripin-5 expression is upregulated in *I. ricinus* nymphs and adults during feeding. The analysis was performed on tissues of flat, semi-engorged and fully engorged nymphs and female salivary glands, midguts and ovaries. RT-qPCR expression data are normalized against elongation factor 1α (ef1α) and the highest expression was set as 100%. The data represent mean + SEM from three biological replicates. D0–D8: days of feeding.

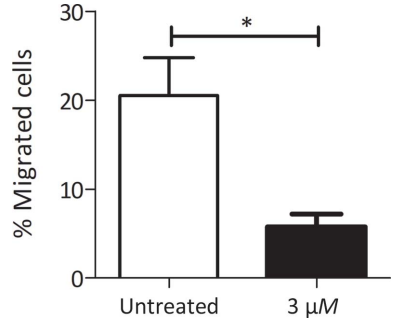


Figure 2
Iripin-5 inhibits neutrophil migration. Mouse bone-marrow neutrophils were pre-incubated with 3 µ*M* Iripin-5 and subjected to migration towards fMLP in a Transwell chamber. The average of three independent experiments (±SEM) is shown. *, $p \leq 0.05$

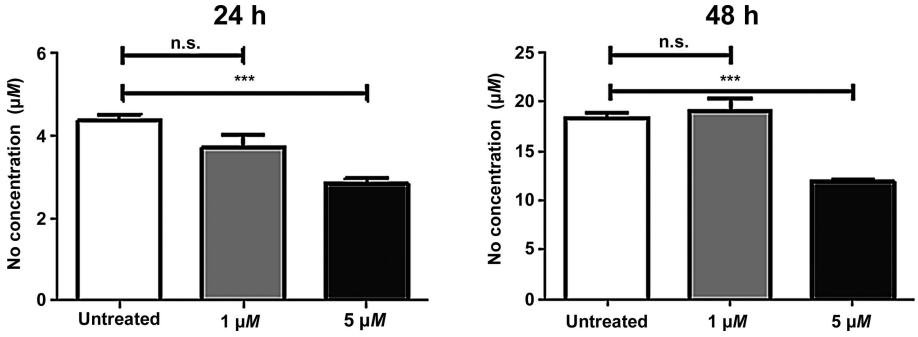


Figure 3
Iripin-5 decreased NO production by activated IC-21 macrophages. Iripin-5 inhibited NO production by IC-21 macrophages when used at high concentration. Macrophages were pre-incubated with 1 and 5 μ*M* Iripin-5, stimulated with LPS and IFN-γ, and the NO concentration was assessed after 24 or 48 h. The mean of three independent experiments (±SEM) is shown. ***, $p \leq 0.001$; n.s., not significant.

physiologically relevant proteases for tick–host interaction, it was found that only chymase and cathepsin G were inhibited significantly, and only very weakly. Another two inhibited proteases, trypsin and α-chymotrypsin, show importance during digestion.

Since Iripin-5 primarily inhibited neutrophil proteases, the effect on neutrophil functions was also studied. Static migration was tested using a Transwell chamber and purified mouse neutrophils isolated from bone marrow. Pre-incubation with 3 μ*M* Iripin-5 led to a greater than 70% decrease in neutrophil migration, thus showing a significant antineutrophil effect of Iripin-5 (Fig. 2).

3.3. NO production by IC-21 macrophages

The incubation of macrophages in the presence of Iripin-5 led to a decrease in NO production in a dose-dependent manner. At a concentration of 1 μ*M*, Iripin-5 inhibited NO production slightly, but not significantly, at 24 h, but not at 48 h. At a higher concentration of 5 μ*M*, Iripin-5 significantly decreased the amount of NO at both time points: by 35% and 36%, respectively (Fig. 3).

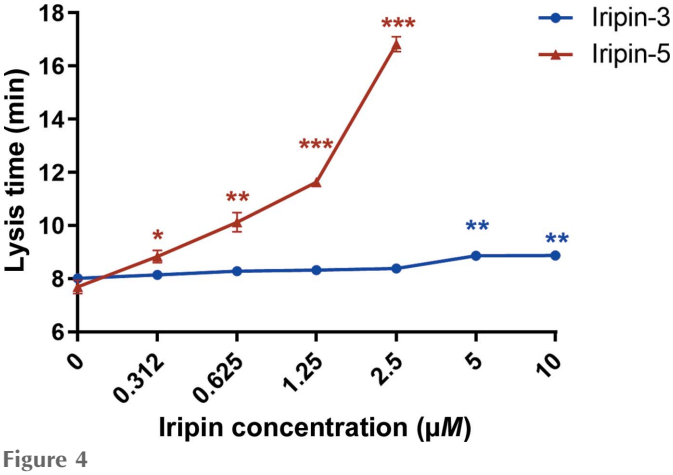


Figure 4
Inhibition of complement by Iripin-5 compared with another *I. ricinus* salivary serpin, Iripin-3. Human plasma was pre-incubated with an increasing concentration of Iripin-5 (156 n*M* to 5 μ*M*) or Iripin-3 (312 n*M* to 10 μ*M*). After the addition of rabbit erythrocytes, their lysis time by complement was measured. For each point in the graph, the mean of three independent experiments (±SEM) is shown. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

3.4. Complement assay

Since Iripin-5 affected two major immune-cell types involved in innate immune response, the interference of the tested serpin with another innate immune mechanism involved in antitick immunity of the complement was tested. Iripin-5 inhibited the lysis of erythrocytes by human complement. Human plasma was pre-incubated with different concentrations of Iripin-5 from 156 n*M* to 5 μ*M*. After the addition of rabbit erythrocytes, their lysis time by complement was measured. A statistically significant reduction in complement-driven lysis activity against erythrocytes when incubating human plasma with Iripin-5 at concentrations of 625 n*M* and higher was observed. No lysis of any erythrocytes was detected when using 5 μ*M* Iripin-5. The results were compared with those for another serpin, Iripin-3 (Chlastáková *et al.*, 2021), which had no effect on complement activity, demonstrating the specificity of our assay. The lysis of rabbit erythrocytes in the presence of 25% human serum was achieved within 7 min 57 s ± 0.12 s on average in the control group, which corresponds to the zero value in the graph (Fig. 4).

3.5. Crystal structure of Iripin-5

In order to obtain a deeper view into the mechanisms of Iripin-5 activity, a detailed structural analysis was performed. To generate a protein structure of Iripin-5, crystallization experiments were performed and the structure of the serpin was solved from the best-diffracting crystals to a resolution of 1.5 Å. Iripin-5 crystals with a monoclinic shape (Fig. 5) grew after one month at 4°C in a precipitant composed of 0.2 *M* magnesium chloride hexahydrate pH 8.5, 0.1 *M* Tris, 30%(*w*/*v*) PEG 4000 (condition No. 1-1 of SG1 from Molecular Dimensions). The crystal space group and unit-cell parameters are reported in Table 2.

The structure of Iripin-5 was solved by molecular replacement using the previously published structure of the serpin

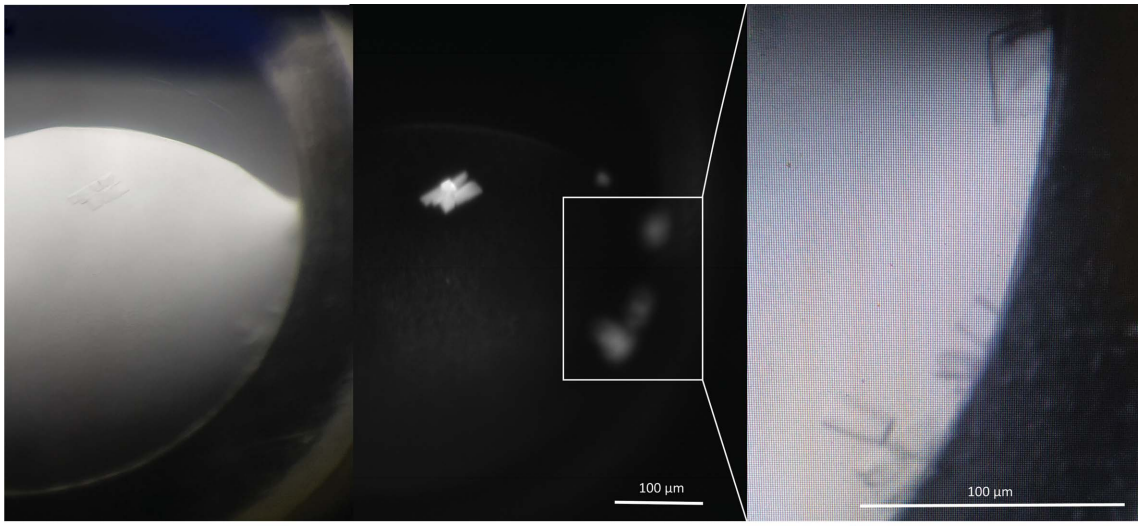


Figure 5
Crystals of Iripin-5 from *I. ricinus*. (*a*) Crystals of protein grown in 0.2 *M* magnesium chloride hexahydrate pH 8.5, 0.1 *M* Tris, 30%(*w*/*v*) PEG 4000. (*b*) The same crystallization droplet is shown under UV light. (*c*) Focus on the best-shaped crystals for diffraction measurements. (*a*) was taken using an Olympus SZX9 microscope and (*b*) and (*c*) were taken using a Minstrel Desktop Crystal Imaging System (Rigaku, Japan). The scale bar represents 100 µm.

IRS-2 (PDB entry 3nda) as the model structure, which has a sequence identity of 55.70% (Chmelar *et al.*, 2011). The crystal structure contains two molecules per asymmetric unit, with a solvent content of 39.97% and a Matthews coefficient of 2.05 Å^3 Da^{-1}. The Iripin-5 structure has a typical cleaved serpin secondary-structure fold in both molecules. The structure consists of a mixed $\alpha\beta$ secondary structure with an N-terminal helical region and a C-terminal β-sheet fold (Huntington, 2011; Fig. 6). The structure is composed of eight α-helices and three β-sheets sequentially arranged in the order α1–β1–α2–α3–β2–α4–β3–α5–β4–β5–β6–β7–β8–α6–α7–β9–β10–α8–β11–β12–β13–β14–β15. Sheet A consists of six β-strands (β2, β3, β4, β10, β11 and β12), sheet B of five β-strands (β1, β7, β8, β14 and β15) and sheet C of four β-strands (β5, β6, β9 and β13) (Fig. 6).

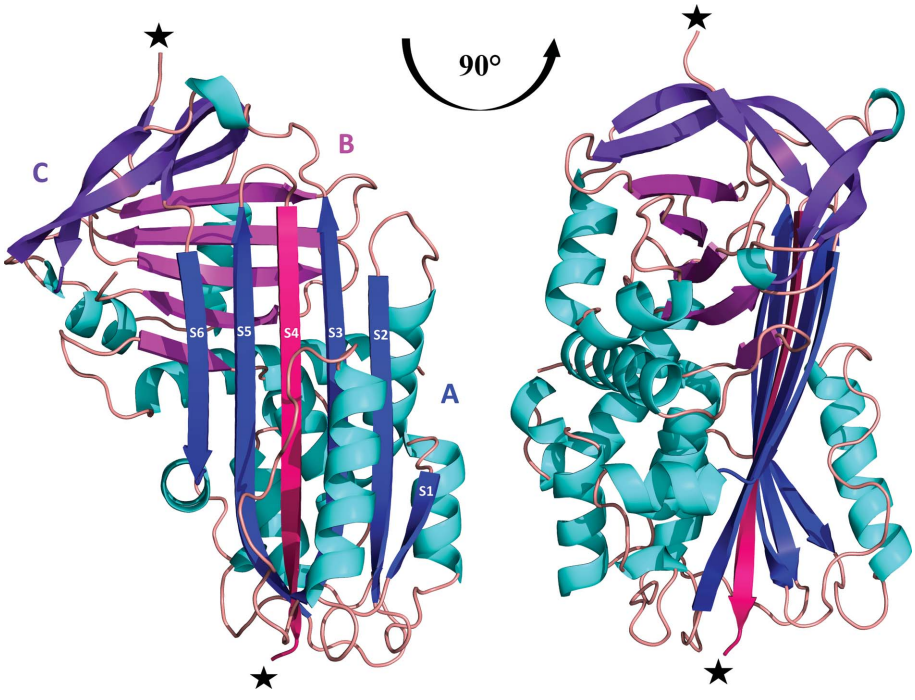


Figure 6
Cleaved protein form with colour-distinguished β-sheets: sheet A (blue), sheet B (magenta) and sheet C (violet). The insertion of the RCL between β-strands S3 and S5 (blue) is marked as β-strand S4 (dark pink). The location of protease cleavage is marked with black stars.

The final model of Iripin-5 contains 373 residues in chain *A* and chain *B* out of a total of 378, with five missing residues (Leu343–Thr347) in both chains. The missing residues in the crystal structure were detected as an absence of electron density due to the high flexibility of the cleaved regions (Fig. 6), and thus these regions were not modelled in the final structure. The cleavage is probably a consequence of the presence of protease, most probably during storage. The cleavage of the sample used for crystallization was confirmed by MALDI mass-spectrometric protein analysis (Supplementary Fig. S3), and led to the structural change and thus to the insertion of the cleavage site inside the β-sheet to form the extra β-strand (S4). The cleavage site is homologous to the RCL of other serpin inhibitors and the cleaved state is the most stable, so-called hyperstable or R form, of inhibitory serpins

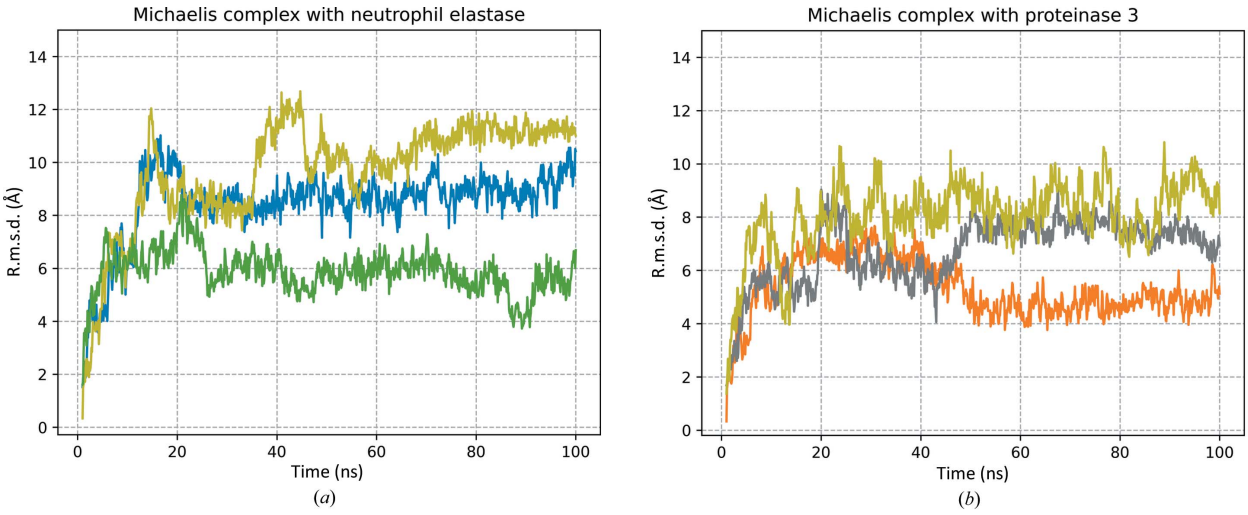


Figure 7
All-atom r.m.s.d. of MD simulations of Michaelis complex models. (*a*) R.m.s.d. for the Iripin-5–neutrophil elastase Michaelis complex and (*b*) r.m.s.d. for the Iripin-5–proteinase 3 Michaelis complex, each for 100 ns simulation. Triplicates are distinguished by different colours (corresponding to the visualization of Michaelis complex models in Fig. 8).

(Huntington, 2011; Fig. 6). Moreover, analysis of the protein interfaces by *PDBePISA* (Krissinel & Henrick, 2007) did not reveal any specific interactions resulting in the formation of stable quaternary structures. Most probably the structures do not form any complexes in solution (Schlee *et al.*, 2019).

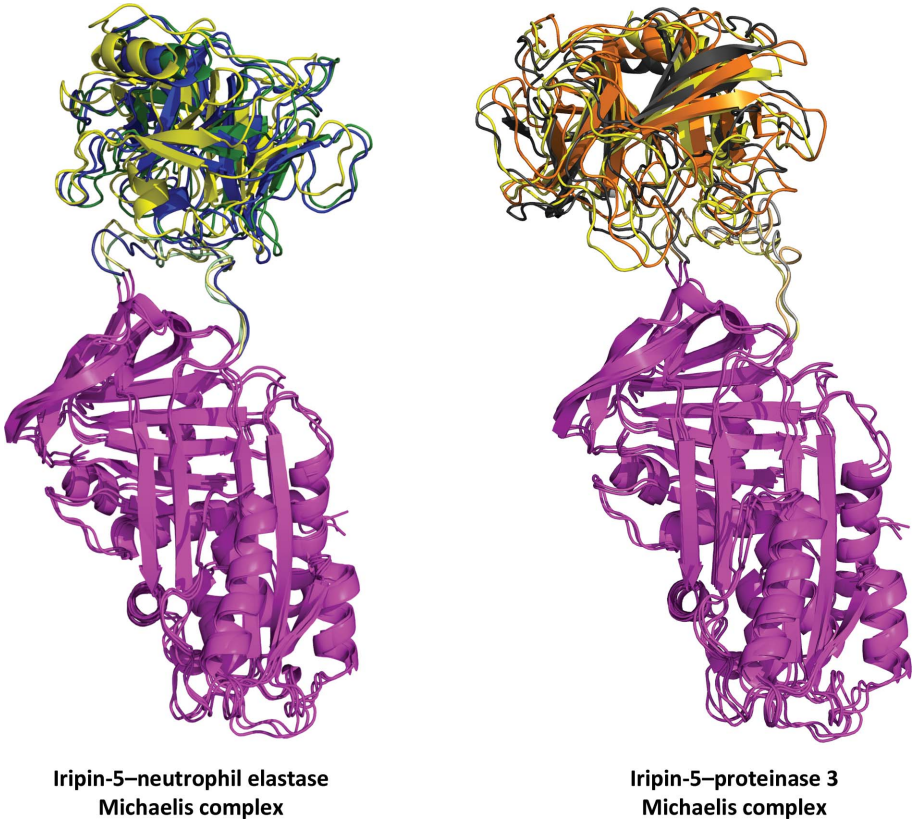


Figure 8
Results of MD simulation of the Michaelis complex. The structures are shown at the 100 ns point of simulation for each triplicate of the chosen target protease. The Iripin-5 (magenta) structures are aligned to show the RCL dynamics. Triplicates are distinguished by different colours for the target protease: neutrophil elastase, blue, green and yellow; proteinase 3, grey, orange and yellow. The Iripin-5 RCL is also distinguished in a corresponding colour to the interacting protease. A detailed view of the Michaelis complex interfaces is presented in Supplementary Fig. S6.

3.6. Structural analysis and molecular dynamics of the theoretical Michaelis complex

To identify the specific interactions that are potentially responsible for the mechanism of inhibition between the target proteases and Iripin-5, protein docking and subsequent MD simulations of the Michaelis complexes were performed. Three simulations for modelled Michaelis complexes with both neutrophil elastase and proteinase 3, each 100 ns long, were performed.

The stability of the complex was monitored by r.m.s.d. evaluation (Fig. 7). The results showed that triplicates of both simulated complexes reached equilibrium within the simulation time and the average r.m.s.d.s from the initial starting structure for the Michaelis complexes were 5.3 Å (Fig. 7*b*, orange), 6.7 Å (Fig. 7*b*, grey) and 8.0 Å (Fig. 7*b*, yellow) for the Iripin-5–proteinase 3 complex and 8.4 Å (Fig. 7*a*, blue), 9.6 Å (Fig. 7*a*, yellow) and 5.8 Å (Fig. 7*a*, green) for the Iripin-5–neutrophil elastase complex. The difference between the average r.m.s.d. of the Iripin-5–

Table 3
Area of the accessible surface interface between Iripin-5 and the tested proteases in the Michaelis complex conformation, the number of hydrogen bonds and the number of salt bridges formed after 100 ns of MD simulation (from *PDBePISA*; Krissinel & Henrick, 2007).

Protease	Surface interface (Å^2)	No. of hydrogen bonds	No. of salt bridges
Human neutrophil elastase	**684.9**	**5**	**4**
	662.9	4	5
	655.7	**2**	**2**
Proteinase 3	864.2	4	2
	804.2	3	2
	827.0	**2**	**2**

Table 4
Area of the accessible surface interface between Iripin-5 and the tested proteases, the number of hydrogen bonds and the number of salt bridges formed (from *PDBePISA*; Krissinel & Henrick, 2007).

Protease	Surface interface (Å^2)	No. of hydrogen bonds	No. of salt bridges
Proteinase 3	892.8	13	10
Human neutrophil elastase	733.6	8	4
Trypsin	919.5	12	10
α-Chymotrypsin	787.5	7	1
Cathepsin G	947.1	8	4
Chymase	849.8	5	6

proteinase 3 complex triplicates was 3.8 Å and that for the Iripin-5–neutrophil elastase complex was 2.7 Å.

Representations of the Michaelis complexes between Iripin-5 and neutrophil elastase and proteinase 3 are shown in Fig. 8. Structural alignment performed by *PyMOL* (DeLano, 2002) showed that the average r.m.s.d. between the Iripin-5–neutrophil elastase triplicates was 1.709 Å and that between the Iripin-5–proteinase 3 triplicates was 1.958 Å. These results show the flexibility of the Michaelis complex conformation and, more precisely, the flexibility of the Iripin-5 RCL (Fig. 8).

The interface analysis of the Michaelis complex triplicates is summarized in Table 3. The data in bold indicate the importance of the interface for complex formation (*PDBePISA*; Krissinel & Henrick, 2007). A more detailed summary of the interacting residues is presented in Supplementary Table S3.

3.7. Structural analysis of theoretical protein–protease covalent complex conformation

To test the hypothesis of the presence of polar contacts between Iripin-5 and six chosen proteases (proteinase 3, human neutrophil elastase, trypsin, α-chymotrypsin, cathepsin G and chymase), docking calculations of protein–protein interactions were performed using *HADDOCK* (van Zundert *et al.*, 2016). The results of the docking studies of interacting residues at the Iripin-5–protease complex interfaces, listed in Table 4 and shown in Fig. 9, show different characters for the interactions in complexes.

Only the α-chymotrypsin catalytic triad interacted with Arg342; thus, the potential Iripin-5 P1 site was a candidate for binding the protease. However, the proteinase 3 and chymase side-chain residues of the catalytic triad were not in contact with Arg342 of Iripin-5. The remaining proteases (cathepsin G, elastase and trypsin) interacted with Arg342 of Iripin-5 via side-chain residues other than the catalytic triad (Supplementary Fig. S5). Detailed information about atomic interface analysis is shown in Supplementary Table S2. These results were calculated using *PDBePISA* (Krissinel & Henrick, 2007).

4. Discussion

The *I. ricinus* sialome (transcriptome from the salivary glands) contains four major types of protease inhibitors, cystatins, TIL-domain inhibitors, Kunitz inhibitors and serpins, which are proven or presumed to be modulators of host-defence mechanisms (Chmelař *et al.*, 2017). Among them, the serpins stand out thanks to their omnipresence across all living organisms and their indispensability for many crucial biochemical pathways, such as coagulation or complement and other fundamental functions (Huntington, 2011; Law *et al.*, 2006). Considering the fact that tick serpins usually do not form multigenic families, as are typical for other salivary protease inhibitors such as Kunitz-domain and TIL-domain inhibitors, they seem to be suitable candidates for targeting in tick-control attempts. Moreover, the structural conservation and use of serpins by vertebrates makes them promising candidates for novel drug development combined with the use of protein engineering (Chmelař *et al.*, 2017). Tick serpins can be utilized as specific regulators of dysregulated processes, such as inflammation, immune-system regulation or hemostasis. Several tick serpins have been shown to interfere with vertebrate immunity (Chmelař *et al.*, 2017). To date, three of them have been functionally characterized in *I. ricinus*. It has been shown that the salivary serpin Iris modulates host innate and acquired immunity (Leboulle *et al.*, 2002). Likewise, IRS-2 and Iripin-3 modulated adaptive immune responses (Chmelar *et al.*, 2011; Chlastáková *et al.*, 2021). Moreover, crystal structures were determined for the last two, which are the only two tick serpins with resolved 3D structures to date.

Iripin-5 belongs to the salivary serpins, the role of which is considered to be as modulators of host defence mechanisms. Iripin-5 seems to be one of the main salivary serpins since its mRNA expression is by far the highest compared with other *I. ricinus* serpins. This serpin is massively induced by the blood meal. Here, several effects supporting immunomodulatory and anti-inflammatory roles of Iripin-5 are reported. The observed inhibition of neutrophil migration suggests anti-inflammatory activity at the very beginning of the immune reaction. Macrophages play an important role in the interaction between ticks, the immune system of the host and transmitted pathogens. Activated macrophages secrete signalling molecules such as cytokines or NO to recruit immune cells to sites of inflammation or towards pathogens (Laroux *et al.*, 2001). The saliva of different tick species has been shown to suppress the ability of macrophages to produce NO (Kýčková & Kopecký, 2006). Since Iripin-5 inhibits this very feature of macrophages, Iripin-5 is likely to be at least partially responsible for this activity observed in *I. ricinus* saliva.

The inhibition of complement described here is interesting, but not surprising, as vertebrate serpins are natural regulators of the complement cascade (Bos *et al.*, 2002). There are other tick salivary protein families in which the members have been described as complement inhibitors (Daix *et al.*, 2007; Tyson *et al.*, 2008), but our case is the first observation of complement inhibition by a tick serpin. This finding confirms the hypotheses about the functional redundancy of tick salivary proteins (Chmelař *et al.*, 2016).

Structural analysis of Iripin-5 shows the typical serpin fold in the relaxed state that was observed in other known crystal structures of *I. ricinus* serpins (IRS-2 and Iripin-3; Chmelař *et al.*, 2017; Chlastáková *et al.*, 2021). The relaxed cleaved state of Iripin-5 was caused by the presence of contaminating proteases, probably during protein storage, and this cleavage has been observed previously (Kovářová *et al.*, 2010). The crystal structure of Iripin-5 was compared with those of IRS-2 (PDB entry 3nda; Chmelar *et al.*, 2011) and Iripin-3 (PDB

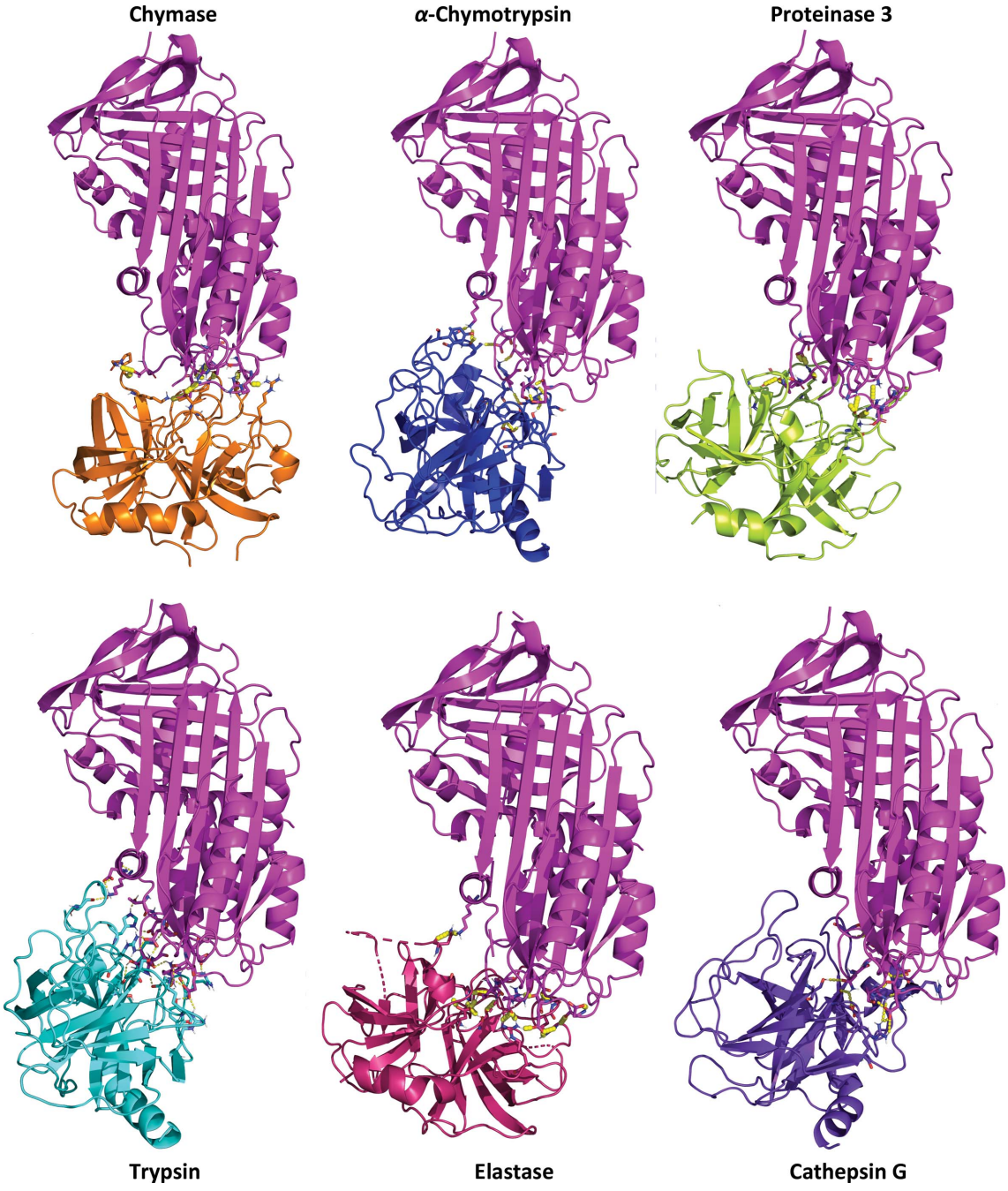


Figure 9
Cartoon representation of the docking results of Iripin-5 (magenta) with chosen proteases: cathepsin G (violet), trypsin (cyan), elastase (hot pink), α-chymotrypsin (blue), chymase (orange) and proteinase 3 (lemon). The residues interacting with the protease catalytic triad are shown in detail in Supplementary Fig. S5.

entry 7ahp; Chlastáková *et al.*, 2021) both by sequence alignment (Fig. 10*a*) and structural superimposition (Fig. 10*b*). The comparison of *I. ricinus* serpins with known structures reveals an almost identical fold (Fig. 10*b*) with some divergence in the

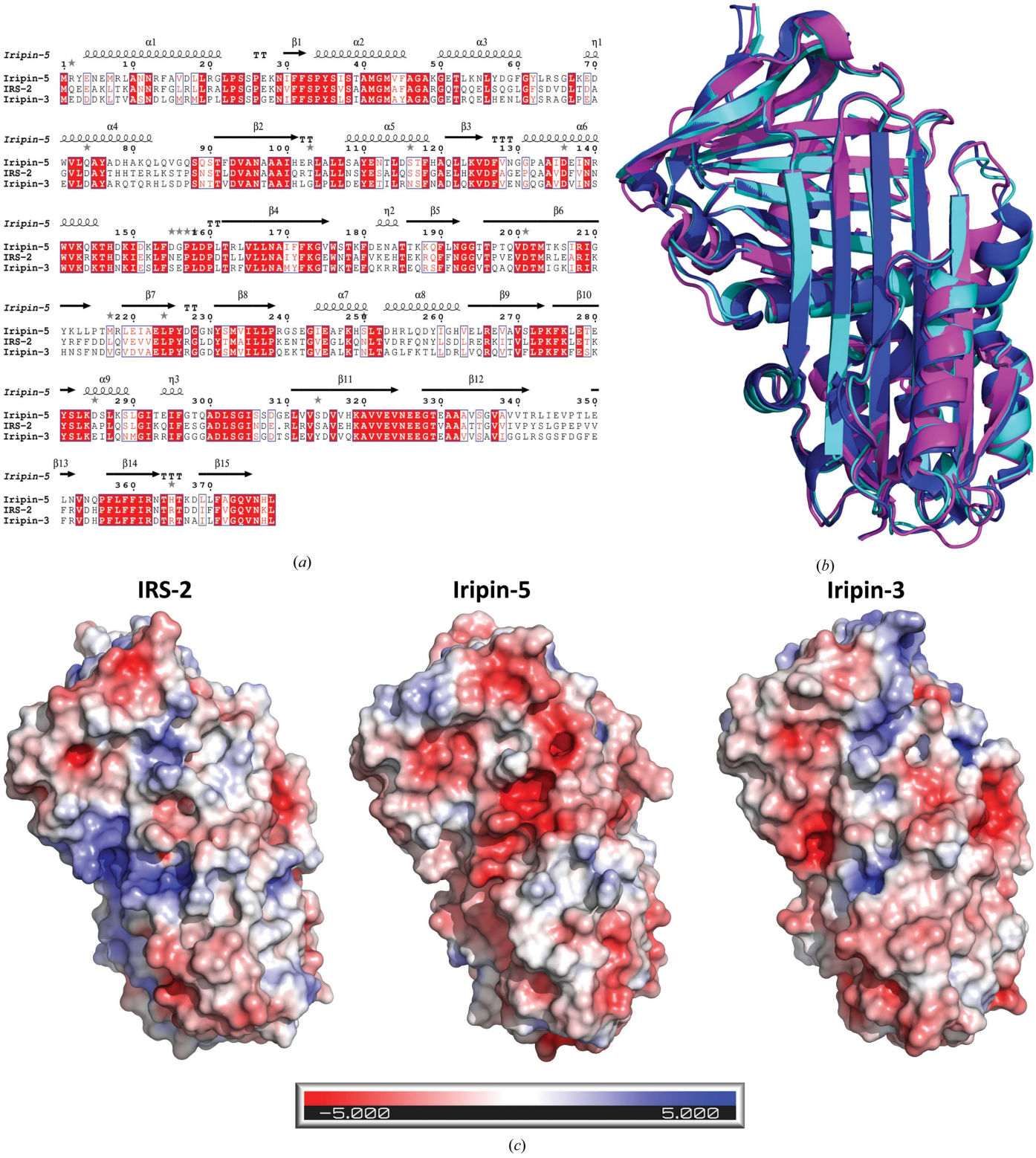


Figure 10
(*a*) Amino-acid sequence alignment between the serpins IRS-2, Iripin-3 and Iripin-5. Well conserved amino-acid motifs are indicated in red and the P1 site of the RCL is marked as a bold rectangle. This sequence alignment was obtained using *Clustal Omega* (Madeira *et al.*, 2019) and *ESPript* (http://espript.ibcp.fr; Robert & Gouet, 2014). (*b*) Superposition of *I. ricinus* crystal structures, namely Iripin-5 (magenta), IRS-2 (blue) and Iripin-3 (cyan). (*c*) Comparison of the electrostatic potentials of IRS-2 (PDB entry 3nda), Iripin-5 (PDB entry 7b2t) and Iripin-3 (PDB entry 7ahp). As shown in the figure, blue indicates positive potential and red indicates negative potential.

loop regions. The r.m.s.d. between molecules was calculated by *PyMOL* (DeLano, 2002). On alignment of Iripin-5 and Iripin-3 the r.m.s.d. was 0.616 Å, while the r.m.s.d. between Iripin-5 and IRS-2 was 0.804 Å across all atoms. In contrast, sequence alignments of Iripin-3 and IRS-2 with Iripin-5 showed only 53.89% and 55.70% sequence identity, respectively (Fig. 10*a*). Electrostatic surface potentials support complex formation and stability and consequently the inhibition of proteases. This can be achieved by charge–charge repulsion or attraction in accordance with their function as a protease substrate or inhibitor (Marijanovic *et al.*, 2019). A comparison of surface electrostatics among *I. ricinus* serpins reveals that Iripin-5 has a more negatively charged surface than the other two aforementioned serpins; Iripin-3 has only a slightly more negatively charged surface than Iripin-5, but shows much greater inhibition (Fig. 10*c*).

The amino acids of the RCL, specifically the P1 residue, determine the protease specificity (Marijanovic *et al.*, 2019). This was confirmed by structural analysis of the *I. ricinus* salivary serpins IRS-2, Iripin-3 and Iripin-5. Iris, with Met340 at the P1 site, is an inhibitor of leukocyte elastase and elastase-like serine proteases (Prevot *et al.*, 2007), although its inhibition is managed by several exosites in α-helices A and D (Prevot *et al.*, 2009). However, IRS-2 has Tyr341 at its P1 site, which signifies the inhibition of chymotrypsin-like proteases (Chmelař *et al.*, 2017), and Iripin-3 has Arg342 at the P1 site, indicating its trypsin-like protease inhibition (Chlastáková *et al.*, 2021). Nevertheless, diverse RCL residues can represent potential cleavage sites, but only a few residues (16–17 residues from the C-terminal β-sheet) manage to successfully inhibit the target protease (Gettins, 2002). For Iripin-5, the last visible residue of the inserted RCL is Arg342 (Supplementary Table S1 and Fig. S4), which is the potential P1 site, suggesting the targeting of trypsin-like proteases preferring Arg or Lys side chains at the P1 site rather than elastase-like (Ala, Gly and Val) or chymotrypsin-like (Tyr, Phe and Trp) proteases (Barrett *et al.*, 2004). However, Iripin-5 mainly inhibited neutrophil elastase and proteinase 3, which is the previously described behaviour of some serpins that inhibited serine proteases, despite that fact that these serpins have an inappropriate P1 recognition site and should have inhibited different proteases (Gettins, 2002). The presence of Arg at the P1 site is common for salivary serpins from prostriate ticks (Mulenga *et al.*, 2009) and led to the proposal of an interaction with blood-coagulation proteases.

Michaelis and covalent complex studies were performed to reveal the possible residues responsible for the inhibition of target proteases. The Michaelis complex is the initial step of protease inhibition; more specifically, it enables the cleavage of the scissile bond and the subsequent acylation step, and therefore represents the most informative structural conformation of serpins (Gettins, 2002). Apart from the primary recognition site of the serpin, some serpins also employ specific surface regions called exosites that can specify protease inhibition (Gettins & Olson, 2016). For this reason, MD simulations of Michaelis complexes were performed. No exosites were found to be directly involved in formation of the Michaelis complex (Fig. 8). In Iripin-5–neutrophil elastase the Michaelis complex was observed to involve engagement of Glu330 in the Iripin-5 RCL to form salt bridges with Arg36 of neutrophil elastase. Similarly, in Iripin-5–proteinase 3 the Michaelis complex was observed to involve the formation of salt bridges between Glu345 in the Iripin-5 RCL and Lys103 of proteinase 3 and between Val340 of the RCL and Glu101 after the MD simulation in triplicate. The two resulting structures of the Iripin-5–neutrophil elastase Michaelis complex and the single structure of the Iripin-5–proteinase 3 Michaelis complex were confirmed to involve interfaces that play important roles in complex formation (*PDBePISA*; Krissinel & Henrick, 2007). Previously, it was observed that not only the position of the specific residues in RCL but also the dynamics of the RCL play an important role in protease inhibition by serpins (Marijanovic *et al.*, 2019). It is probable that these two aspects are responsible for protease inhibition of the *I. ricinus* serpin Iripin-5.

Docking studies of covalent complexes revealed probable interactions between the chosen proteases and Iripin-5. The docking covalent complexes exhibit quite a large interface area, as observed previously for serpin–trypsin covalent complexes, with around 12 interacting interface residues. In the Iripin-5–trypsin complex more residues were involved in the formation of hydrogen bonds compared with other Iripin-5–protease complexes. These results are similar to the results of interface interaction comparison of antithrombin–trypsin and antithrombin–elastase complexes, in which the complex with trypsin made more hydrogen bonds (Rashid *et al.*, 2015). This could probably explain the important role of Glu310 in Iripin-5, which forms salt bridges in the complex with protease. Moreover, some residues of Iripin-5 were involved in hydrogen-bond formation more frequently, namely Gln299, Asp301, Glu51, Lys288, Glu294 and the abovementioned Glu310 and Arg342. We propose that these residues should play an important role in the formation of a covalent complex between Iripin-5 and protease.

5. Conclusions

The continuing structural studies of arthropod (ectoparasite) serpins provide an understanding of their specific functions and protease targets. Structural information on complexes with targets and cofactors would help to understand the exact mechanism of action of these functionally diverse serpins. Iripin-5 is the third described crystal structure of a tick serpin, and despite its cleaved form it provides important experimental proof of the specificity of Iripin-5 and its possible interactions with proteases. Iripin-5 appears to be an immunomodulatory and anti-inflammatory protein used by *I. ricinus* ticks to overcome host defensive mechanisms. The presence of Arg at the P1 site led to the proposal of an interaction with blood-coagulation proteases. MD simulations of the Michaelis complex revealed flexibility of the RCL to be one of the factors responsible for inhibition. A more detailed study of the dynamic behaviour of Iripin-5 during the inhibition mechanism may be beneficial for a better understanding of

inhibition. The residues with the most important roles in the formation of a covalent complex between Iripin-5 and proteases were proposed based on docking and MD simulations and it was found that Glu310 should play a crucial role in the interaction between Iripin-5 and proteases, with the exception of α-chymotrypsin.

6. Related literature

The following references are cited in the supporting information for this article: Cox & Mann (2008), Cox *et al.* (2011), Rappsilber *et al.* (2007) and Shevchenko *et al.* (2006).

Acknowledgements

The diffraction data were collected on BL14.1 at the BESSY II electron-storage ring operated by Helmholtz-Zentrum Berlin (HZB). We thank HZB for the allocation of synchrotron-radiation beam time. Author contributions were as follows. IKS, JC and MK designed the project. JK performed the cloning, expression and purification and determined the antiprotease selectivity and NO production. LAM performed the complement assay. ZB performed the neutrophil migration assay. HL determined Iripin-5 expression profiles. BK, PH and TP carried out crystallization experiments and performed X-ray diffraction analysis. BK analyzed the crystallographic data, solved the structure and drafted the manuscript. BK, MK and JAC performed the docking and analyzed the docking data.

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Article 6

Ixodes ricinus Salivary Serpin Iripin-8 Inhibits the Intrinsic Pathway of Coagulation and Complement

Kotál J, Polderdijk SGI, Langhansová H, Ederová M, Martins LA, Beránková Z, Chlastáková A, Hajdušek O, Kotsyfakis M, Huntington JA, **Chmelař J**.

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Iripin-8 may be the most interesting tick serpin. It displayed blood-meal-induced mRNA expression that peaked in nymphs and on day 8 of feeding in the salivary glands of adult females. Iripin-8 inhibited multiple proteases involved in blood coagulation and blocked the intrinsic and common pathways of the coagulation cascade *in vitro*. Moreover, Iripin-8 inhibited erythrocyte lysis by complement, and knock-down by RNA interference in tick nymphs delayed the feeding time. Interestingly, Iripin-8 did not show any immunomodulatory effects, therefore it seems to be mostly anti-hemostatic protein. We further resolved the crystal structure of Iripin-8 at 1.89 Å resolution. The structure revealed unusually long and rigid reactive center loop that is surprisingly conserved among several tick species. The P1 Arg residue is held in place, far from the serpin body by a conserved poly-Pro element on the P' side. The conservation of the RCL of Iripin-8 is intriguing and deserves more attention, as well as its unusual shape. Moreover, cavities that are able to bind small molecules, such as PEG from the crystallization buffer, were found in Iripin-8, including one deep cavity. This finding can have important functional relevance. Finally, Iripin-8 is the first tick serpin crystal structure in the native state.

Iripin-8 displays several unusual properties like long and rigid RCL, which is conserved among different tick species or the presence of cavities that are able to bind small molecules. The inhibitory specificity suggests a role either in host as an anti-coagulant or in tick as a regulator of hemolymph coagulation.

Author's contribution:

Author designed and coordinated the study and participated on the writing and revising of the manuscript

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Ixodes ricinus Salivary Serpin Iripin-8 Inhibits the Intrinsic Pathway of Coagulation and Complement

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Abstract: Tick saliva is a rich source of antihemostatic, anti-inflammatory, and immunomodulatory molecules that actively help the tick to finish its blood meal. Moreover, these molecules facilitate the transmission of tick-borne pathogens. Here we present the functional and structural characterization of Iripin-8, a salivary serpin from the tick *Ixodes ricinus*, a European vector of tick-borne encephalitis and Lyme disease. Iripin-8 displayed blood-meal-induced mRNA expression that peaked in nymphs and the salivary glands of adult females. Iripin-8 inhibited multiple proteases involved in blood coagulation and blocked the intrinsic and common pathways of the coagulation cascade in vitro. Moreover, Iripin-8 inhibited erythrocyte lysis by complement, and Iripin-8 knockdown by RNA interference in tick nymphs delayed the feeding time. Finally, we resolved the crystal structure of Iripin-8 at 1.89 Å resolution to reveal an unusually long and rigid reactive center loop that is conserved in several tick species. The P1 Arg residue is held in place distant from the serpin body by a conserved poly-Pro element on the P′ side. Several PEG molecules bind to Iripin-8, including one in a deep cavity, perhaps indicating the presence of a small-molecule binding site. This is the first crystal structure of a tick serpin in the native state, and Iripin-8 is a tick serpin with a conserved reactive center loop that possesses antihemostatic activity that may mediate interference with host innate immunity.

Keywords: blood coagulation; crystal structure; *Ixodes ricinus*; parasite; saliva; serpin; tick

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1. Introduction

Ticks are blood-feeding ectoparasites and vectors of human pathogens, including agents of Lyme disease and tick-borne encephalitis. *Ixodes ricinus* is a species of European tick in the Ixodidae (hard tick) family found also in northern Africa and the Middle East [1]. *I. ricinus* ticks feed only once in each of their three developmental stages (larva, nymph, imago), and their feeding course can last over a 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 into host skin causes mechanical injury that immediately triggers the hemostatic mechanisms of blood coagulation, vasoconstriction, and platelet aggregation to prevent blood loss [3]. Consequently, innate immunity is activated as

noted by inflammation with edema formation, inflammatory cell infiltration, and itching at tick feeding sites. Long-term feeding and/or repeated exposures of the host to ticks also activate adaptive immunity [4]. As an adaptation to host defenses, 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, we refer readers to several excellent reviews describing the impact 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 a fibrin clot. It can be initiated via the extrinsic or intrinsic pathway [9]. The extrinsic pathway starts with blood vessel injury and complex formation between activated factor VII (fVIIa) and tissue factor (TF). The 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 terminates the coagulation process through the activation of thrombin (fII) and the cleavage of fibrinogen to fibrin, the primary component of the clot [9,10].

Similar to blood coagulation, the complement cascade is based on serine proteases. Complement represents a fast and robust defense mechanism against bacterial pathogens, which are lysed or opsonized by complement to facilitate their killing by other immune mechanisms [11,12]. Complement can be activated via three pathways: the classical pathway, responding to antigen–antibody complexes; the lectin pathway, which needs a lectin to bind to specific carbohydrates on the pathogen surface; and the alternative pathway, which is triggered by direct binding of C3b protein to a microbial 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 the complement response and opsonize pathogens for phagocytosis. Together with other complement components, C3b forms C5 convertase, which cleaves C5 to C5a and C5b fragments. C5b initiates membrane attack complex (MAC) formation, leading to lysis of a target cell. Small C3a and C5a subunits promote inflammation by recruiting immune cells to the site of injury [11].

Both processes, coagulation and complement, are detrimental to feeding ticks, so their saliva contains many anticoagulant and anticomplement molecules, often belonging to the group of serine protease inhibitors (serpins) [13–16]. Serpins form the largest and most ubiquitous family of protease inhibitors in nature and can be found in viruses, prokaryotes, and eukaryotes [17,18]. Serpins are irreversible inhibitors with a unique inhibitory mechanism and highly conserved tertiary structure [19,20] classified in the I4 family of the MEROPS database [21]. Similar to other serine protease inhibitors, the serpin structure contains a reactive center loop (RCL) that serves as bait for the protease. The RCL amino acid sequence determines serpin's inhibitory specificity [22].

Arthropod serpins have mostly homeostatic and immunological functions. They regulate hemolymph coagulation or activation of the phenoloxidase system in insects [23]. Additionally, serpins from blood-feeding arthropods can modulate host immunity and host hemostasis [23]. Indeed, over 20 tick salivary serpins have been functionally characterized with described effects on coagulation or immunity [13]. However, according to numerous transcriptomic studies, the total number of tick serpins is significantly higher [13,24–27]. In *I. ricinus*, at least 36 serpins have been identified based on transcriptomic data, but only 3 of them have been characterized at the biochemical, immunomodulatory, anticoagulatory, or antitick vaccine levels [13,28–32].

Interestingly, one serpin has a fully conserved RCL across various tick species [24]. Homologs of this serpin have been described in *Amblyomma americanum* as AAS19 [33], *Rhipicephalus haemaphysaloides* as RHS8 [34], *Rhipicephalus microplus* as RmS-15 [35], and *I. ricinus* as IRS-8 [30], and it can also be found among transcripts of other tick species in which the serpins have not yet been functionally characterized.

2. Results

2.1. Iripin-8 Is Predominantly a Salivary Protein with Increased Expression during Tick Feeding

Analysis of Iripin-8 mRNA expression levels revealed its highest abundance in tick nymphs with a peak during the first day of feeding (Figure 1A). In salivary glands, increased Iripin-8 transcription positively correlated with the length of tick feeding on its host. A similar increasing trend was also observed in tick midguts; however, the total number of Iripin-8 transcripts was lower than in the salivary glands. Iripin-8 transcript levels were lowest in the ovaries of all the tested tissues/stages.

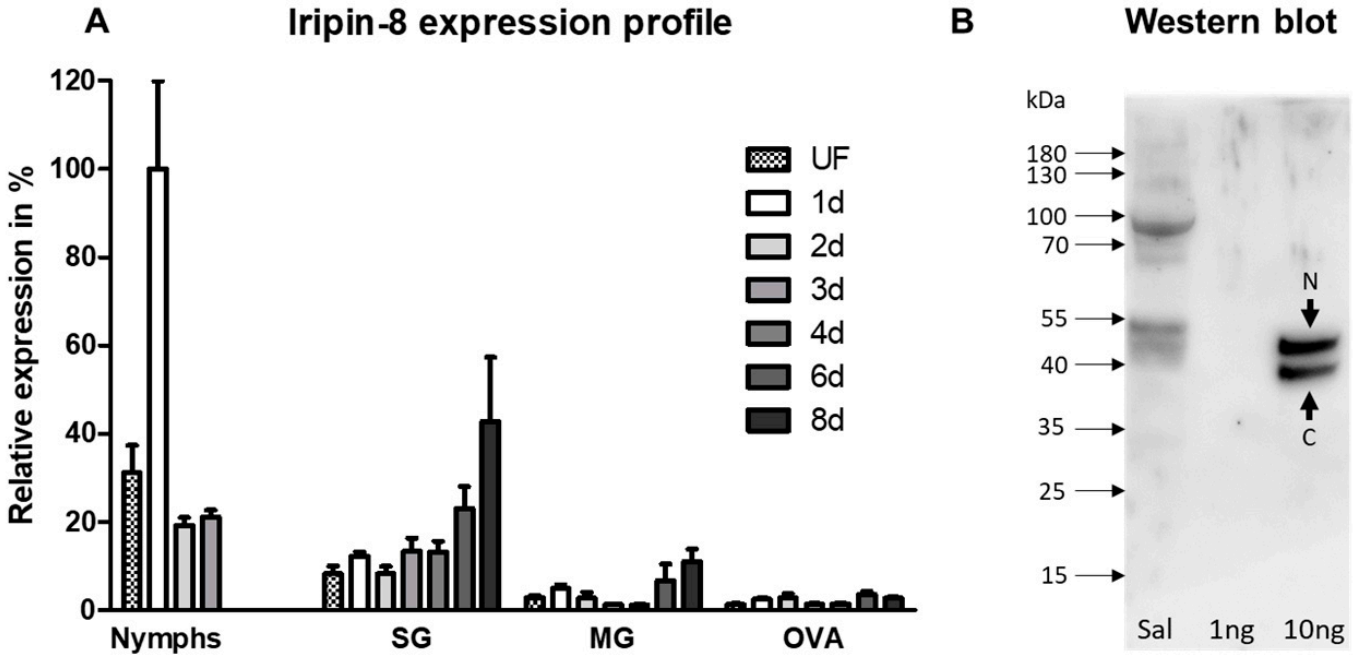

Figure 1. Iripin-8 expression in ticks and its presence in tick saliva. (**A**) Pools of *I. ricinus* salivary glands, midguts, and ovaries from female ticks and whole bodies from nymphs were dissected under RNase-free conditions. cDNA was subsequently prepared as a template for qRT-PCR. Iripin-8 expression was normalized to elongation factor 1α and compared between all values with the 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 = midguts; OVA = ovaries; UF = unfed ticks; 1 d, 2 d, 3 d, 4 d, 6 d, 8 d = ticks after 1, 2, 3, 4, 6, or 8 days of feeding. For nymphs, the last column represents fully fed nymphs. All feeding points for each development stage/tissue are compared with the unfed ticks of the respective group. (**B**) Iripin-8 can be detected in tick saliva by Western blotting. Saliva from ticks after 6 days of feeding and recombinant Iripin-8 protein were visualized by Western blotting using serum from naïve and Iripin-8-immunized rabbits. Sal = tick saliva; 1 ng, 10 ng = Iripin-8 recombinant protein at 1 ng and 10 ng load. N: native Iripin-8, C: cleaved Iripin-8.

Next, we performed Western blot analysis and confirmed the presence of Iripin-8 protein in tick saliva (Figure 1B). We detected two bands of the recombinant protein, representing the full-length native serpin (N) and a molecule cleaved in its RCL near the C-terminus, likely due to bacterial protease contamination (C). The proteolytic cleavage of RCL has previously been documented for serpins from various organisms, including ticks [36–38]. The ~5 kDa difference in molecular weight observed between native and

recombinant Iripin-8 was probably due to glycosylation, since two N-glycosylation sites are predicted to exist in this serpin. The signal at ~90 kDa in saliva was also detected when using serum from a naïve rabbit (data not shown) and is probably caused by nonspecific antibody binding.

Based on these results, we proceeded to test how Iripin-8 affects host defense mechanisms as a component of tick saliva. Despite the highest expression being observed in the salivary glands, activity in other tissues cannot be ruled out.

2.2. Sequence Analysis and Production of Recombinant Iripin-8

The full transcript encoding Iripin-8 was obtained using cDNA from tick salivary glands. Following sequencing, we found a few amino acid mutations (K10 → E10, L36 → F36, P290 → T290, and F318 → S318) compared with the sequence of Iripin-8 (IRS-8) published as a supplement in our previous work [30] (GenBank No. DQ915845.1; ABI94058.1), probably as a result of intertick variability. The RCL was identical to other homologous tick serpins [34], with arginine at the P1 position (Supplementary Figure S1A); however, the remainder of the sequence had undergone evolution, separating species-specific sequences in strongly supported groups (Supplementary Figure S1B). Iripin-8 has a predicted MW of 43 kDa and a pI of 5.85, with two predicted N-linked glycosylation sites.

Iripin-8 was expressed in 2 L 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 was formed from the native serpin and a fraction from a serpin cleaved at its RCL (Supplementary Figure S2). This mixed sample of native and cleaved serpin was used for all subsequent analyses because the molecules were inseparable by common chromatographic techniques. Proper folding of Iripin-8 was verified by CD spectroscopy (Supplementary Data) [39,40] and subsequently by activity assays against serine proteases, as presented below. Recombinant Iripin-8 protein solution was tested for the presence of LPS, which was detected at 0.038 endotoxin unit/mL, below the threshold for a pyrogenic effect [41,42].

2.3. Iripin-8 Inhibits Serine Proteases Involved in Coagulation

Based on sequence analysis of Iripin-8 and the presence of arginine in the RCL P1 position, we focused on analyzing its inhibitory specificity towards serine proteases related to blood coagulation. Considering the covalent nature of the serpin mechanism of inhibition, we analyzed by SDS-PAGE whether Iripin-8 forms covalent complexes with selected proteases. Figure 2 shows covalent inhibitory complex formation between Iripin-8 and 10 out of 11 tested proteases: thrombin, fVIIa, fIXa, fXa, fXIa, fXIIa, plasmin, APC, kallikrein, and trypsin. We did not detect complexes between Iripin-8 and chymotrypsin. All inhibited proteases could also partially cleave Iripin-8 as indicated by a C-terminal fragment and a stronger signal of cleaved serpin molecule. Chymotrypsin cleaved Iripin-8 in its RCL completely. Inhibition rates of Iripin-8 against these proteases were subsequently determined and are shown in Table 1. Among the tested proteases, plasmin was inhibited significantly faster than other proteases, with a second-order rate constant (k_2) of >200,000 M^{-1} s^{-1}. Trypsin, kallikrein, fXIa, and thrombin were inhibited with a k_2 in the tens of thousands range and the other proteases with lower k_2 values.

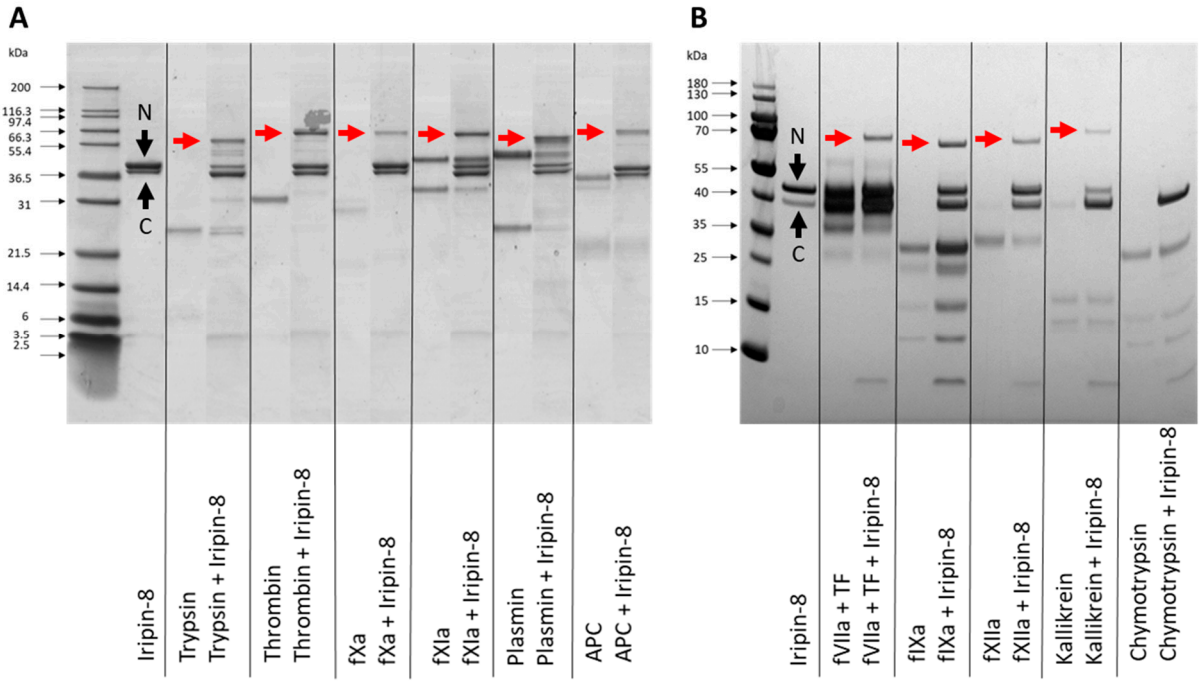

Figure 2. Formation of covalent complexes between Iripin-8 and serine proteases. Iripin-8 and selected serine proteases were incubated for 1 h and subsequently analyzed for complex formation by reducing SDS-PAGE. Protein separation differs between (**A**,**B**) due to the use of gels with different polyacrylamide contents. Gels show the profile of Iripin-8 serpin alone, various serine proteases alone, and proteases incubated with Iripin-8. Complex formation between fVIIa and Iripin-8 was tested in the presence of tissue factor (TF) at an equimolar concentration. Covalent complexes between Iripin-8 and protease are marked with a red arrow. N: native Iripin-8, C: cleaved Iripin-8.

Table 1. Inhibition rate of Iripin-8 against selected serine proteases.

Protease	k_2 (M^{-1} s^{-1})	±SE
Plasmin	225,064	14,183
Trypsin	29,447	3508
Kallikrein	16,682	1119
fXIa	16,328	948
Thrombin	13,794	1040
fXIIa	3324	409
fXa	2088	115
APC	523	35
fVIIa + TF	456	35
fIXa	N/A	N/A

2.4. Iripin-8 Inhibits the Intrinsic and Common Pathways of Blood Coagulation

Given the in vitro inhibition of coagulation proteases by Iripin-8, we tested its activity in three coagulation assays. The prothrombin time (PT) assay simulates the extrinsic pathway of coagulation, the activated partial thromboplastin time (aPTT) represents the intrinsic (contact) pathway, and thrombin time (TT) represents the final common stage of coagulation. Iripin-8 had no significant effect on PT, which increased from 15.3 to 16.7 s in the presence of 6 µM serpin (not shown). Iripin-8 extended aPTT in a dose-dependent manner, with a statistically significant increase already apparent at 375 nM. With 6 µM Iripin-8, the aPTT was delayed over five times from 31.8 ± 0.4 s to 167.9 ± 3.2 s (Figure 3A). Iripin-8 also inhibited TT in a dose-dependent manner and blocked fibrin clot formation

completely at concentrations of 800 nM and higher (Figure 3B). The other serpins presented for comparison in Figure 3C did not have any effect on blood coagulation except the inhibition of PT by Iripin-3, which we published elsewhere [32].

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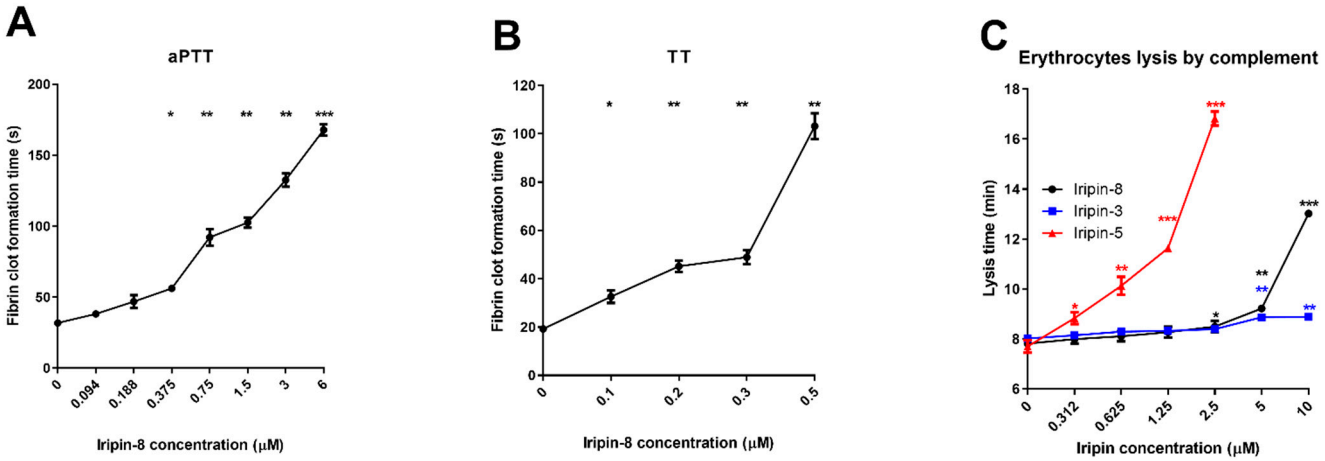

Figure 3. Inhibition of complement and coagulation pathways by Iripin-8. (**A**) Iripin-8 inhibits the intrinsic coagulation pathway. Human plasma was preincubated with increasing concentrations of Iripin-8 (94 nM–6 μM). Coagulation was triggered by the addition of Dapttin® reagent and $CaCl_2$, and clot formation time was measured. A sample without Iripin-8 was used as a control for statistical purposes. (**B**) Iripin-8 delays fibrin clot formation in a thrombin time assay in a dose-dependent manner. Coagulation of human plasma was initiated by thrombin reagent preincubated with various concentrations of Iripin-8, and thrombin time was measured. Samples without Iripin-8 were used as a control for statistical purposes. (**C**) Iripin-8 inhibits erythrocyte lysis by human complement. Human plasma was preincubated with increasing concentrations of Iripin-3, 5, and 8 (312 nM–10 μM). After the addition of rabbit erythrocytes, their lysis time by complement was measured. Values represent prolongation of time needed for erythrocyte lysis compared with the control group. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

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2.5. Anticomplement Activity of Iripin-8

The complement pathway readily lyses erythrocytes from various mammals, and those from rabbits were found to be the best complement activators [43]. We used human serum and rabbit erythrocytes to test the effect of tick protease inhibitors on the activity of human complement in vitro. Since the complement cascade is driven by serine proteases, we tested the potential effect of Iripin-8 as a complement regulator. There was a statistically significant reduction in complement activity against erythrocytes when human plasma was incubated with Iripin-8 at concentrations of 2.5 μM and higher (Figure 3C). We also compared Iripin-8 with other two tick salivary serpins: Iripin-3 [32] showed very weak anticomplement activity and was used as a control; compared with Iripin-5 [44], Iripin-8 had lower activity.

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2.6. Iripin-8 Knockdown Influences Tick Feeding but Not Borrelia Transmission

Since Iripin-8 is predominantly expressed in tick nymphs (Figure 1A), we decided to investigate its importance in tick feeding by RNA interference (RNAi) in the nymphal stage. Knockdown efficiency was 87% for transcript downregulation. Ticks with downregulated Iripin-8 expression showed a significantly lower feeding success rate and higher mortality, with only 51.0% (25/49) finishing feeding compared with 94.1% (48/51) in the control group. Moreover, in ticks that finished feeding, the feeding time was longer compared with control nymphs (Figure 4A). Despite this promising phenotype, we did not observe any effect of Iripin-8 RNAi on the weight of fully engorged nymphs (Figure 4B) or on *B. afzelii* transmission from infected nymphs to mice in any of the tested mouse tissues (Figure 4C).

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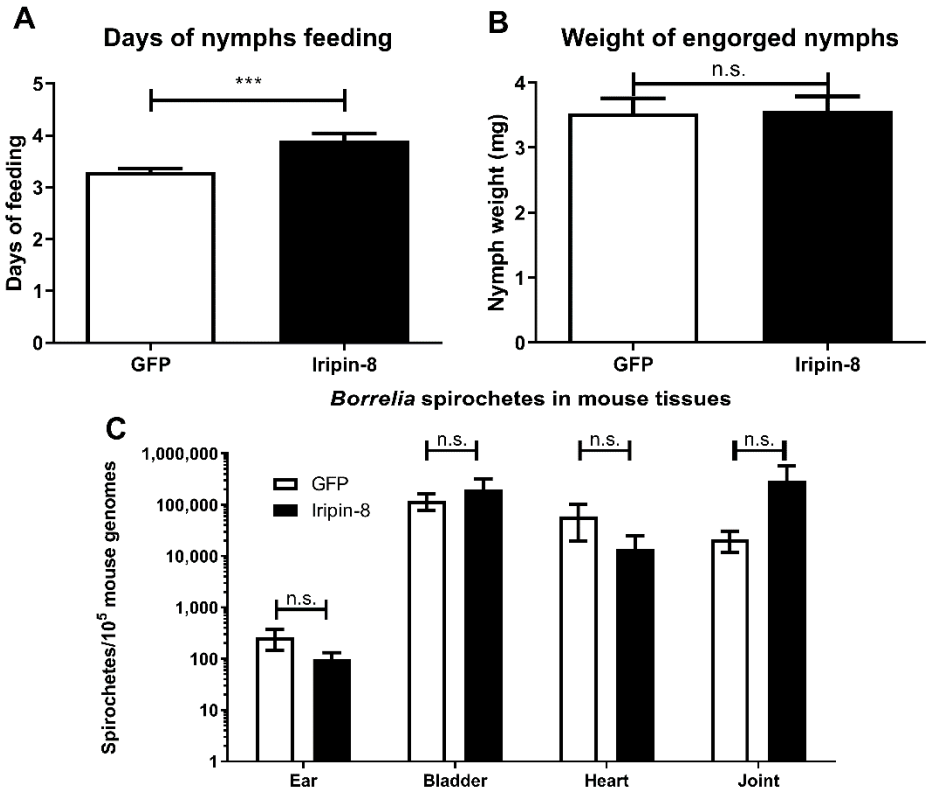

Figure 4. Effect of RNAi on tick fitness and *Borrelia* transmission. (**A**) RNAi of Iripin-8 prolonged the length of *I. ricinus* nymph feeding compared with the control group (GFP). (**B**) Weight of fully engorged nymphs with Iripin-8 knockdown was not different from the control group (GFP). (**C**) Presence of *B. afzelii* spirochetes in mouse tissues after infestation with infected *I. ricinus* nymphs. There were no significant differences between Iripin-8 knockdown and GFP control groups in any of the tested tissues. *** $p \leq 0.001$; n.s., not significant.

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2.7. Role of Iripin-8 in Modulating Host Immunity

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Next, we evaluated a possible role for Iripin-8 in the modulation of the host immune response to tick feeding via two assays (OVA antigen-specific CD4+ T cell proliferation model using splenocytes isolated from OT-II mice and neutrophil migration towards the chemoattractant (fMLP), in which we previously observed effects with other *I. ricinus* salivary protease inhibitors (the serpin Iripin-3 [32] and the cystatin Iristatin [45]). However, there was no inhibition by Iripin-8 in either assay (Supplementary Figure S4).

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2.8. Structural Features of Iripin-8

The crystallographic asymmetric unit contained a single molecule of native Iripin-8 (details in Supplementary Table S2). Electron density was of sufficient quality to model all residues from Ser5 to the C-terminus, including the entire RCL. Iripin-8 has the typical native serpin fold with a Cα RMSD (root-mean-square deviation) of 1.93 Å compared with the archetypal serpin α1-antitrypsin (α1AT, 342 of 352 residues, Figure 5A). The most remarkable feature of Iripin-8 is its long RCL (11 residues longer on the P′ side), which extends away from the body of the serpin, moving the P1 Arg364 17.8 Å further than the P1 residue of α1AT. This extended conformation is not the result of a crystal contact; rather it forms the basis of a crystal contact with the RCL of a symmetry-related molecule (Supplementary Figure S5). The P′ extension contains a stretch of proline residues that form a type II polyproline helix, conferring rigidity and extending the P1 residue away from the body of Iripin-8 (Figure 5B). We can infer from this that the extended RCL is a feature of native Iripin-8 in solution and that it has functional consequences in determining protease specificity and/or inhibitory promiscuity.

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We also observed several molecules of PEG (polyethylene glycol) originating from the crystallization buffer bound to Iripin-8. One of the binding sites was a deep 109 Å^3 cavity in the core structure between helixes A, B, and C (Supplementary Figure S6). This observation suggests that Iripin-8 can bind small molecules, which may have functional implications. The coordinates and structure factors are deposited in the Protein Data Bank under accession code XXX (note: will be submitted before publication).

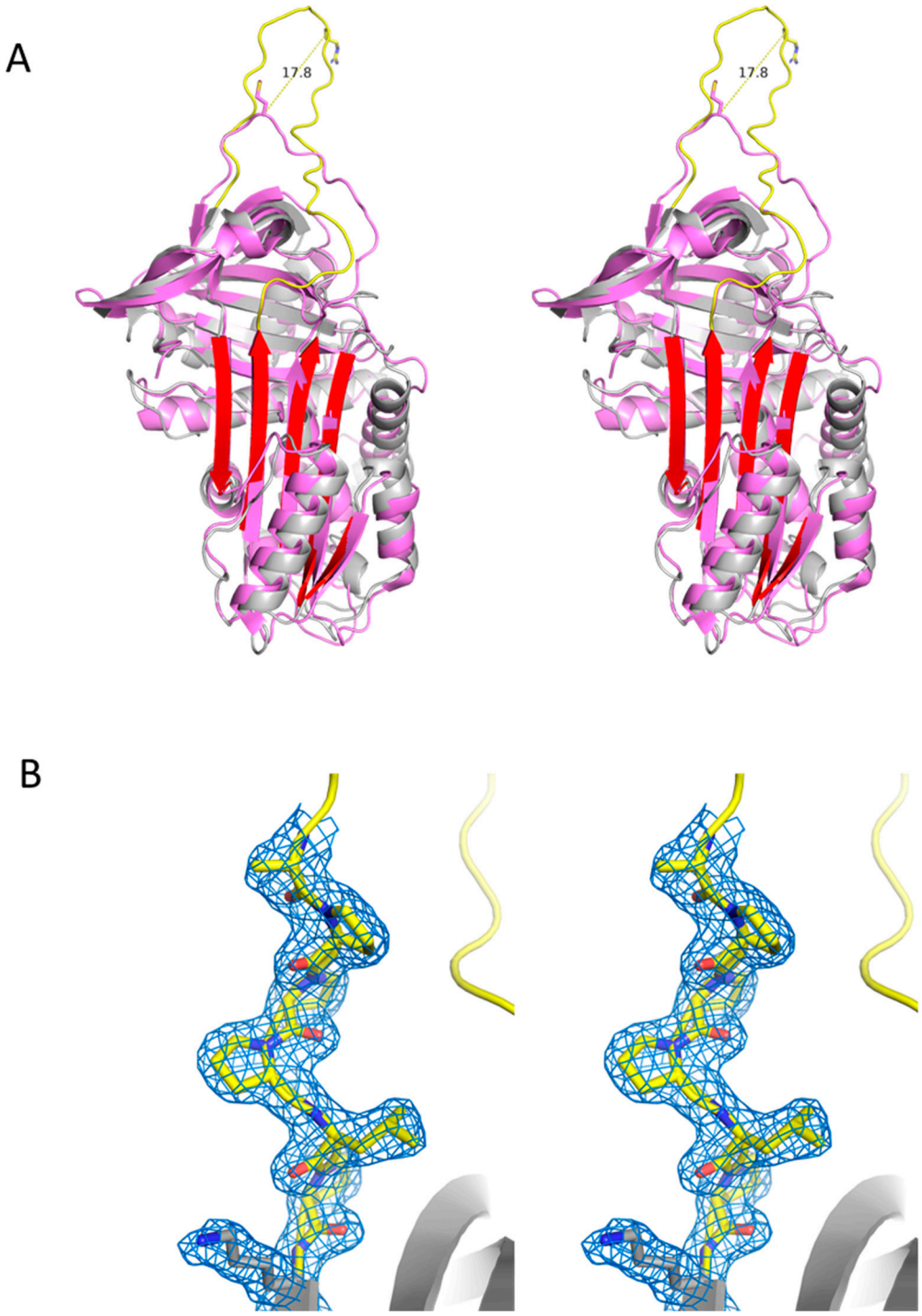

Figure 5. Crystal structure of native Iripin-8. (**A**) Stereo view of a ribbon diagram of Iripin-8 (gray with yellow RCL and red beta sheet A) superimposed with alpha-1-antitrypsin (PDB code 3ne4). The P1 side chains of both molecules are represented as sticks, and the distance between their Cα atoms is shown. (**B**) Stereo view of a close-up of the P′ region with surrounding electron density (contoured at 1 times the RMSD of the map), forming a rigid type II polyproline helix.

3. Discussion

Similar to other characterized tick salivary serpins [13], we found that Iripin-8 can modulate host complement and coagulation cascades to facilitate tick feeding [46].

Structurally, Iripin-8 has an unusually long, exposed, and rigid RCL, with an Arg in its P1 position. This potentially enables it to inhibit a range of proteases, as the RCL can interact independently from the body of the serpin molecule. We characterized Iripin-8 as an in vitro inhibitor of at least 10 serine proteases. The interference with the coagulation cascade through inhibition of kallikrein, thrombin, fVIIa, fIXa, fXa, fXIa, and fXIIa in vivo would be beneficial for tick feeding [3,47].

Iripin-8 also inhibited trypsin and kallikrein. Trypsin has a role in meal digestion and has also been linked to skin inflammation [48,49]. Potentially, trypsin inhibition in the host skin could be another mechanism by which the tick impairs the host immune response. Kallikrein has a role in the development of inflammation and pain. It is an activator of the nociceptive mediator bradykinin in the kinin–kallikrein system [50]. Through its inhibition, a deleterious inflammatory response could be altered to the tick's advantage.

Iripin-8 showed the greatest inhibition of plasmin, a protease involved in fibrin degradation and clot removal [51]. This was surprising, as clot removal should be beneficial for ticks. On the other hand, it is not fully understood whether fibrin clot formation occurs at a tick feeding site in the presence of tick anticoagulant molecules [52]. Apart from fibrinolysis, plasmin also modulates several immunological processes, interacting with leukocytes, endothelial cells, extracellular matrix components, and immune system factors [51,53,54]. Excessive plasmin generation can even lead to pathophysiological inflammatory processes [54]. Considering the proinflammatory role of plasmin, its inhibition by tick salivary serpin could be more relevant to the tick than unimpaired fibrinolysis. Although we did not see any effect of Iripin-8 in two immune assays, we cannot exclude the possibility that Iripin-8 exerts an immunomodulatory effect.

The anticomplement activity of tick saliva or its protein components has been known for decades and is described in numerous publications [14,15,55–57]. Although the active molecules originate from either unique tick protein families [58–62] or lipocalins [16], anticomplement activity has only recently been reported for a tick salivary serpin [44] as the only tick protease inhibitor with such activity. Since complement products might directly damage the tick hypostome or initiate a stronger immune response [11], we propose that the role of Iripin-8 is to attenuate these mechanisms. At the same time, an 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 Iripin-8 transcriptional downregulation on *Borrelia* transmission from ticks to the host. Although we saw some effect of RNA interference (RNAi) on tick fitness, it had no effect on the amount of *Borrelia* in host tissues. Such a result can be explained by a redundancy in tick salivary molecules, as ticks secrete a variety of effectors against the same host defense mechanism and knockdown of one molecule can be substituted by the activity of others [63].

The increased tick mortality after Iripin-8 knockdown might be due to a potential role for Iripin-8 within the tick body. As an anticoagulant, Iripin-8 can help to keep ingested blood in the tick midgut in an unclotted state for later intracellular digestion [64–66]. A similar principle has previously been suggested for other midgut serpins in various tick species [67]. Other potential functions of Iripin-8 include a role in hemolymph clotting [68,69] or in reproduction and egg development [70,71].

The broad inhibitory specificity combined with a conserved, long, and rigid RCL implies that Iripin-8′s role does not necessarily have to only be the modulation of host defense mechanisms. The function of a protruded RCL can be adapted to fit the active site of an unknown protease of tick origin, independently of the serpin body, thus regulating physiological processes in the tick itself, such as melanization and immune processes, which are also regulated by serpins in arthropods.

Interestingly, several PEG molecules from the crystallization buffer bind to Iripin-8, including one in a deep cavity, perhaps indicating the presence of a small-molecule binding

site. Considering that serpins can act as transport proteins independently of their inhibitory properties [72,73], the binding properties of Iripin-8 could have physiological relevance in ticks or their tick–host interactions.

By comparing Iripin-8 with other members of the tick serpin group with an identical RCL, we confirmed that the anticoagulant features have also been reported for AAS19 [33] and RmS-15 [35]. RNAi knockdown of Iripin-8 reduced feeding success, while RNAi of AAS19 decreased the blood intake and morphological deformation of ticks [74], and RNAi of RHS8 had an effect on body weight, feeding time, and vitellogenesis [34]. However, these findings are difficult to correlate due to the use of different tick species and life stages. Although Iripin-8 was detected in tick saliva and therefore most likely plays a role in the regulation of host defense mechanisms, further experiments to define Iripin-8 functions in tick tissues would be of interest. Similar to AAS19 [74], Iripin-8 might also regulate hemolymph clotting in the tick body, which is naturally regulated by serpins [67]. Iripin-8 could also contribute to maintaining ingested blood in the tick midgut in an unclotted state to preserve availability for intracellular digestion [64,66].

Although the concentration of Iripin-8 in tick saliva is not known, we can expect it to be lower than most of the concentrations used in our assays. Tick saliva, as a complex mixture, contains an abundance of bioactive molecules that are redundant in their activities and contribute to the inhibition of host defense mechanisms [63]. Therefore, despite the fact that the concentrations of Iripin-8 used in our experiments do not reflect a physiological situation, they can reflect the overall concentration of functionally redundant salivary proteins.

We conclude that the tick serpin Iripin-8 is secreted into the host as a component of *I. ricinus* saliva. Based on its inhibitory activity, mainly of proteases of the coagulation cascade [47], we suggest that its main role as a salivary protein is in the modulation of host blood coagulation and complement activity, with possible function in regulating the immune response. As such, Iripin-8 alters host defense mechanisms and most likely facilitates tick feeding on hosts.

Nevertheless, a more detailed comparative study of tick serpins with conserved RCLs might shed some light on the role of this particular subgroup in different tick species. The conservation of Iripin-8 among tick species suggests a potential for targeting this serpin as a tick control strategy.

4. Materials and Methods

4.1. Ticks and Laboratory Animals

All animal experiments were carried out in accordance with the Animal Protection Law of the Czech Republic No. 246/1992 Coll., ethics approval No. MSMT-19085/2015-3, and protocols approved by the responsible committee of the Institute of Parasitology, Biology Center of the Czech Academy of Sciences (IP BC CAS). Male and female adult *I. ricinus* ticks were collected by flagging in a forest near České Budějovice in the Czech Republic and kept in 95% humidity chambers under a 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 s.r.o. (Prague, Czech Republic). Mice were housed in individually ventilated cages under a 12 h light/dark cycle and used at 6–12 weeks. Laboratory rabbits were purchased from Velaz 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 mammals were fed a standard pellet diet and given water ad libitum.

4.2. 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 whole nymph bodies, were dissected under RNase-free conditions, and total RNA was isolated using TRI Reagent solution

(MRC, Cincinnati, OH, USA). 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, Basel, Switzerland) according to the manufacturer´s instructions. The cDNA was subsequently used for the analysis of *Iripin-8* expression by qPCR in a Rotor-Gene 6000 cycler (Qiagen, Hilden, Germany) using FastStart Universal SYBR® Green Master Mix (Roche). *Iripin-8* expression profiles were calculated using the Livak and Schmittgen mathematical model [75] and normalized to *I. ricinus* elongation factor 1α (ef1; GenBank No. GU074829.1) [76,77]. Primer sequences are shown in Supplementary Table S1.

4.3. RNA Silencing and Borrelia Transmission

Borrelia afzelii-infected *I. ricinus* nymphs were prepared as described previously [78,79]. A fragment of the *Iripin-8* gene was amplified from *I. ricinus* cDNA using primers containing restriction sites for ApaI and XbaI (Supplementary Table S1; Iripin-8 RNAi) and cloned into the pll10 vector with two T7 promoters in reverse orientations [80]. Double-stranded RNA (dsRNA) of *Iripin-8* and dsRNA of green fluorescent protein (*gfp*) used for control were synthesized using the MEGAscript T7 transcription kit (Ambion, Austin, TX, USA), as described previously [81]. The dsRNA (32 nl; 3 µg/µL) was injected into the hemocoel of sterile or infected nymphs using a Nanoject II instrument (Drummond Scientific, Broomall, PA). After 3 days of 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, mice were sacrificed, and the numbers of *Borrelia* spirochetes in the earlobe, urinary bladder, heart tissue, and ankle joint were estimated by qPCR [82] and normalized to the number of mouse genomes [83] (primer and probe sequences in Supplementary Table S1). The level of gene knockdown was checked by qPCR in an independent experiment.

4.4. Cloning, Expression, and Purification of Iripin-8

The full cDNA sequence of the gene encoding Iripin-8 was amplified with the primers presented in Supplementary Table S1 using cDNA prepared from the salivary glands of female *I. ricinus* ticks fed for 3 and 6 days on rabbits as a template. The Iripin-8 gene without a signal peptide was cloned into a linearized Champion™ pET SUMO expression vector (Life Technologies, Carlsbad, CA, USA) using NEBuilder® HiFi DNA Assembly Master Mix (New England Biolabs, Ipswich, MA, USA) and transformed into *Escherichia coli* strain Rosetta 2(DE3)pLysS (Novagen, Merck Life Science, Darmstadt, Germany) for expression. Bacterial cultures were fermented in autoinduction TB medium supplemented with 50 mg/L kanamycin at 25 °C for 24 h.

SUMO-tagged Iripin-8 was purified from clarified cell lysate using a HisTrap FF column (GE Healthcare, Chicago, IL, USA) and eluted with 200 mM 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 reapplied to the HisTrap column to separate tags from the native serpin. This step was followed by ion exchange chromatography using a HiTrap Q HP column (GE Healthcare) and by size exclusion chromatography using a HiLoad 16/60 Superdex 75 column (GE Healthcare) to ensure sufficient protein purity.

4.5. SDS-PAGE of Complex Formation

Iripin-8 and proteases were incubated at 1 µM final concentrations in a buffer corresponding to each protease (please see below) for 1 h at laboratory temperature. For the assay with fVIIa, we added 1 µM tissue factor (TF). Covalent complex formation was then analyzed in a reducing SDS-PAGE using 4–12% and 12% NuPAGE gels, followed by silver staining.

4.6. 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 at each time point by the addition of the chromogenic/fluorogenic substrate appropriate for the protease used. The slope of the linear part of absorbance/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, each of them consisting of 8 different time points 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 of the mean 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 and trypsin.

Substrates were: 400 µM S-2238 (Diapharma, Chester, OH, USA) for thrombin; 400 µM S-2222 for fXa (Diapharma); 400 µM S-2366 (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; and 250 µM Boc-G(OBzl)GR-AMC for fIXa.

Final concentrations and origin of human proteases were as follows: 2 nM thrombin (Haematologic Technologies, Essex Junction, VT, USA), 20 nM fVIIa (Haematologic Technologies), 20 nM TF (BioLegend), 200 nM fIXa (Haematologic Technologies), 5 nM fXa (Haematologic Technologies), 2 nM fXIa (Haematologic Technologies), 10 nM fXIIa (Molecular Innovations, Novi, MI), 8 nM plasma kallikrein (Sigma-Aldrich, St Louis, MO, USA), 1.25 nM plasmin (Haematologic Technologies), 15 nM APC (Haematologic Technologies), 20 pM trypsin (RnD); 10 nM chymotrypsin (Merck).

4.7. Anti-Iripin-8 Serum Production and Western Blotting

Serum with antibodies against Iripin-8 was produced by immunization of a rabbit with pure recombinant protein as described previously [84]. Tick saliva was collected from ticks fed for 6 days on guinea pigs by pilocarpine induction as described previously [85]. 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 membranes (Thermo Fisher Scientific). Subsequently, membranes were blocked in 5% skimmed milk in Tris-buffered saline (TBS) with 0.1% Tween 20 (TBS T) for 1 h at laboratory temperature. Membranes were then incubated with rabbit anti-Iripin-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 Technology; Danvers, MA, USA; 1:2000). Proteins were visualized using the enhanced chemiluminescent substrate WesternBright™ Quantum (Advansta, San Jose, CA, USA) and detected using a CCD imaging system (Uvitec, Cambridge, UK).

4.8. Coagulation Assays

All assays were performed at 37 °C using preheated reagents (Technoclone, Vienna, Austria). Normal human plasma (Coagulation Control N) was preincubated with Iripin-8 for 10 min prior to coagulation initiation. All assays were analyzed using the Ceveron four coagulometer (Technoclone).

For prothrombin time (PT) estimation, 100 µL plasma was preincubated with 6 µM Iripin-8, followed by the addition of 200 µL Technoplastin® HIS solution and estimation of fibrin clot formation time. For activated partial thromboplastin time (aPTT), 100 µL plasma was preincubated with various concentrations of Iripin-8 (94 nM–6 µM), followed by the addition of 100 µL of Dapttin® TC and incubation for 2 min. Coagulation was triggered by the addition of 100 µL 25 mM $CaCl_2$ solution. For thrombin time (TT), 200 µL of thrombin reagent was incubated with various concentrations of Iripin-8 for 10 min and subsequently added to 200 µL of plasma to initiate clot formation.

4.9. Crystal Structure Determination

Iripin-8 was concentrated to 6.5 mg/mL and dialyzed into 20 mM Tris pH 7.4, 20 mM NaCl. Crystals were obtained from the PGA screen [86] (Molecular Dimensions, Maumee, OH) in 0.1 M Tris pH 7.8, 5% PGA-LM, 30% *v/v* PEG 550 MME. Crystals were flash-frozen in liquid nitrogen straight from the well condition without additional cryoprotection. Data were collected at the Diamond Light Source (Didcot) on a beamline I04-1 and processed using the CCP4 suite [87] as follows: integration by Mosflm [88] and scaling and merging with Aimless [89]. The structure was solved by molecular replacement with Phaser [90]. The template for molecular replacement was generated from the structure of conserpin (PDB ID 5CDX [91]), which was truncated to remove flexible regions and mutated using Chainsaw [92] based on a sequence alignment to Iripin-8 using Expresso [93]. The structure was refined with Refmac [94]. Model quality was assessed by MolProbity [95,96], and figures were generated using PyMOL [97].

4.10. Complement Assay

Fresh rabbit erythrocytes were collected in Alsever's solution from the rabbit marginal ear artery, washed three times in excess PBS buffer, and finally diluted to a 2% suspension (*v/v*). Fresh human serum was obtained from three healthy individuals. The assay was performed in a 96-well round-bottomed microtiter plate (Nunc, Thermo Fisher Scientific). Each well contained 100 µL 50% human serum in PBS premixed with different concentrations of Iripin-8 (315 nM–10 µM). After 10 min incubation at laboratory temperature, 100 µL of erythrocyte suspension was added (i.e., 25% final serum concentration after the addition of erythrocyte suspension to a final 1%). Reaction wells were observed individually under a stereomicroscope using oblique illumination and an aluminum pad, and the time needed for erythrocyte lysis was measured. When full lysis was achieved, the reaction mixture turned from opaque to transparent. Negative controls did not contain either Iripin-8 or human serum. Additional controls were performed with heat-inactivated serum (56 °C, 30 min). The assay was evaluated in technical and biological triplicates.

4.11. Immunological Assays

Both the $CD4^+$ T cell proliferation assay and neutrophil migration assay were performed following the protocols described by Kotál et al. [45]. Briefly, for the $CD4^+$ T cell proliferation assay, splenocytes were isolated from OT-II mice, fluorescently labeled, pre-incubated with serpin for 2 h, and their proliferation stimulated by the addition of OVA peptide. After 72 h, cells were labelled with anti-CD4 antibody and analyzed by flow cytometry. For the migration assay, neutrophils were isolated from mouse bone marrow by immunomagnetic separation and preincubated with serpin for 1 h. Cells were then seeded in the inserts of 5 µm pore Corning® Transwell® chambers (Corning, Corning, NY, USA) and were allowed to migrate towards an fMLP (Sigma-Aldrich) gradient for 1 h. The migration rate was determined by cell counting using the Neubauer chamber.

4.12. Statistical Analysis

All experiments were performed as three biological replicates. 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. For RT-PCR, data for nymphs, salivary glands, midgut, and ovaries were analyzed separately using one-way ANOVA, followed by Dunnett's post hoc test. Statistically significant results are marked: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; n.s., not significant.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms22179480/s1: Supplementary Table S1: List of primers; Supplementary Table S2: Data processing, refinement, and model; phylogenetic analysis of Iripin-8 group between tick species; Supplementary Figure S1: Alignment and phylogenetic analysis of Iripin-8; Supplementary Figure S2: Analysis of Iripin-8 purity by SDS-PAGE; circular dichroism (CD) spectroscopy; Supplementary Fig-

ure S3: CD spectrogram of Iripin-8; Supplementary Figure S4: Effect of Iripin-8 on T cell proliferation and neutrophil migration; Supplementary Figure S5: A ribbon diagram of two Iripin-8 symmetry-related molecules; Supplementary Figure S6: Ribbon diagram of Iripin-8 with highlighted molecules of PEG; Supplementary methods: Evolutionary analysis by the maximum likelihood method.

Author Contributions: J.K. designed and performed experiments, performed the analyses, and wrote the manuscript; H.L., M.E., L.A.M., Z.B., A.C., and O.H. designed and performed experiments; S.G.I.P. and J.A.H. solved, refined, and analyzed the structure; J.C. designed experiments, performed analyses, and edited the manuscript; M.K. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Review 1

Modulation of host immunity by tick saliva

Kotál J, Langhansová H, Lieskovská J, Andersen JF, Francischetti IM, Chavakis T, Kopecký J, Pedra JH, Kotsyfakis M, **Chmelař J**.

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Since 1985, when the first works about tick saliva were published, many studies were carried out that attempted to decipher the role of tick saliva or salivary gland extracts (SGE) in tick-host interaction. These experiments more or less ended with rapid development of molecular techniques, including transcriptomics, which allowed researchers to use reverse genetic approach and search for individual molecules rather than for the effect of crude saliva or even SGE. Because it was obvious that the “era of saliva” was over, we decided to summarize up to date knowledge about saliva related modulation of host innate and adaptive immune system. The review was 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.

We overviewed in this review all 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. It may serve as a reference or a guide for the functional characterization of the numerous newly-discovered genes expressed in tick salivary glands.

Author’s contribution:

Author designed the structure of the review and participated on writing and revising of the manuscript

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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

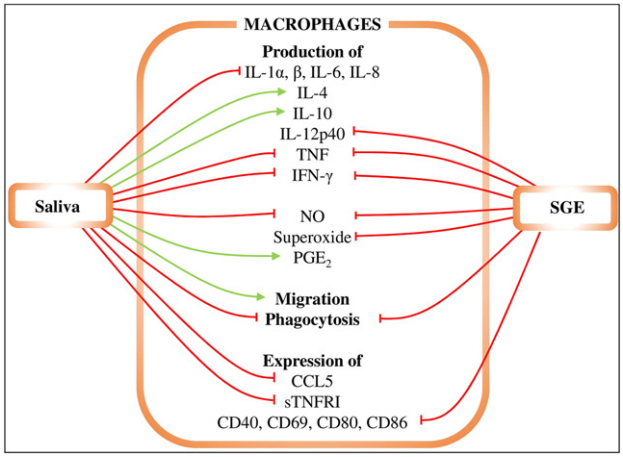

Fig. 1. The effects of saliva and SGE on macrophages. Red lines represent inhibition, green lines enhancement. Tick saliva inhibits production of IL-1α, IL-1β, IL-6, IL-8, TNF, IFN-γ, NO, superoxide, and CCL5, as well as expression of sTNFRI and phagocytosis. Tick saliva increases production of IL-4, 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.

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]

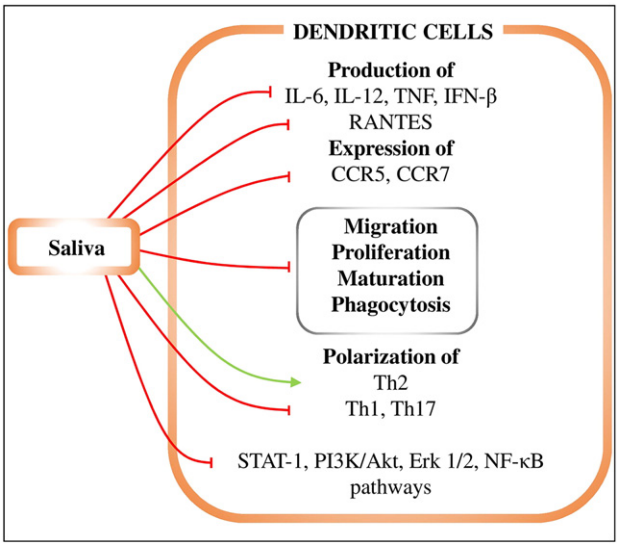

Fig. 2. The effects of saliva on dendritic cells. Red lines represent inhibition, green lines enhancement. Tick saliva inhibits production of IL-6, IL-12, TNF, IFN-β, and RANTES cytokines. It also inhibits expression of CCR5 and CCR7, DC migration, proliferation, maturation, and phagocytosis, and STAT-1, PI3K/Akt, Erk1/2, and NF-κB signaling pathways. The saliva induces Th2 polarization while suppressing Th1 and Th17 differentiation.

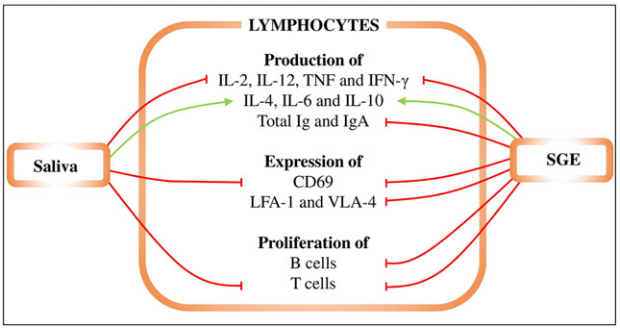

Fig. 4. The effects of saliva and SGE on B and T lymphocytes. Red lines represent inhibition, green lines enhancement. Tick saliva inhibits T cell proliferation, CD69 expression, and production of IL-2, IL-12, TNF, and IFN-γ by lymphocytes. In contrast, it increases production of IL-4, IL-6, and IL-10. 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.

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].

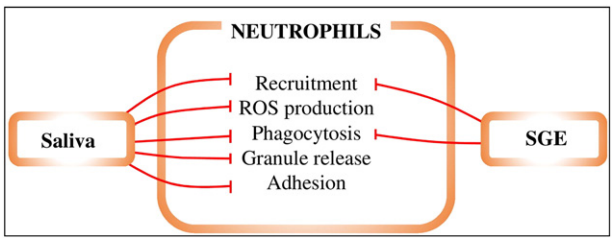

Fig. 3. The effects of saliva and SGE on neutrophils. Red lines represent inhibition, green lines enhancement. Tick saliva inhibits neutrophil recruitment, phagocytosis, adhesion, granule release, and production of ROS.

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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Review 2

All For One and One For All on the Tick-Host Battlefield

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The saliva of ixodid ticks contains a mixture of bioactive molecules that target a wide spectrum of host defense mechanisms to allow ticks to feed on the vertebrate host for several days. Tick salivary proteins cluster in multigene protein families, and individual family members display redundancy and pluripotency in their action to ameliorate or evade host immune responses. Members of different protein families can target the same cellular or molecular pathway of the host physiological response to tick feeding. At the same time, some proteins can display more activities. In this opinion paper, we presented and discussed our hypotheses that redundancy and pluripotency of tick salivary immunomodulators evolved in order to evade immune recognition by the host while retaining the immunomodulatory potential of their saliva. In the review, several examples of redundancy and pluripotency were described as well as some implication, such as the need of relatively high amount of pharmacoactive proteins, when they are tested in recombinant form. It is possible that *in vivo,* the redundancy leads to diminishing of efficacy of individual proteins, because usually they work in a concert.

This work was important, as it gathered and comprehensively presented several hypotheses that were circulating among researchers.

Author's contribution:

Author designed the structure of the review, wrote the text, created the figures and revised the manuscript.

Trends in Parasitology

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Opinion

All For One and One For All on the Tick–Host Battlefield

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The saliva of ixodid ticks contains a mixture of bioactive molecules that target a wide spectrum of host defense mechanisms to allow ticks to feed on the vertebrate host for several days. Tick salivary proteins cluster in multigenic protein families, and individual family members display redundancy and pluripotency in their action to ameliorate or evade host immune responses. It is now clear that members of different protein families can target the same cellular or molecular pathway of the host physiological response to tick feeding. We present and discuss our hypothesis that redundancy and pluripotency evolved in tick salivary immunomodulators to evade immune recognition by the host while retaining the immunomodulatory potential of their saliva.

Multigenic Protein Families in Ticks – Many Questions, Few Answers

Ticks are obligatory blood-feeding ecto-parasitic arthropods. There are two major tick families: Argasidae (soft ticks) and Ixodidae (hard ticks). Hard ticks remain attached to their hosts for up to 2 weeks, during which time they are exposed to host homeostatic and defense mechanisms [1]. To complete their blood meal, ticks need to evade host immune surveillance and/or suppress the host immune response. The evasion of host immune system is mediated mainly via tick salivary secretions that are injected into the tick-feeding cavity [2]: ixodid tick saliva is a highly complex natural mixture of low and high molecular weight bioactive molecules. The pharmacological effects of tick saliva and its individual components have been studied for almost half a century, and research in this field can be divided into the periods before and after transcriptomes, but this journey was outlined elsewhere [3] and its description exceeds the scope of this article. Transcriptomic studies have shown that secreted salivary proteins cluster in **multigenic protein families** (see Glossary) containing as many as 200 individual members differing by only a few amino acids [4,5]. Only a small number of family members have been functionally characterized at the protein level, recombinant or native. Therefore, the roles of individual family members remain to be elucidated. Moreover, a significant portion of the identified transcripts fall into the category of hypothetical proteins, without any relationship or similarity to known proteins from any living organism. Therefore, their (potentially important) role in the tick life cycle is currently unknown.

The existence of multigenic salivary protein families raises many questions. Which host homeostatic functions are affected by individual tick proteins? Do members of a given tick protein family target different, similar, or identical host physiological processes? Why are such a large number of functionally and structurally similar proteins produced, and what is the mechanism of their gene expression regulation? Although we can hypothesize that multigenic families exist to meet the need for large numbers of immunomodulatory proteins to block specific host defense mechanisms, is their effect additive and what are the immunological consequences? Indeed, expressing small quantities of many different antigens (at once or sequentially during tick feeding) can diminish immune recognition as a result of low-dose tolerance and/or antigenic competition

Trends

A major aim for tick research is the development of anti-tick or transmission-blocking vaccines.

Given the redundancy and pluripotency of tick salivary proteins, the biggest challenge in this area of research is unraveling the key immunomodulatory molecules to be targeted for anti-tick vaccine development.

Antigenic variation during tick feeding should be also considered as an important factor in the development of both anti-tick and transmission-blocking vaccines.

In addition to vaccine development, the discovery of powerful immunomodulatory proteins in tick salivary glands presents another important direction in tick research that might enrich the current repertoire of biotherapeutic molecules.

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effects, and impair immune cell activation [6]. As research has progressed in this field, it is now clear that tick salivary proteins display both **pluripotency** and **redundancy** in their actions. Recent publications show that the high level of redundancy, identified at the mRNA level, translates into the proteomic profile of the saliva [7,8]. In this short Opinion article we suggest that multigenic families of tick salivary secreted proteins evolved to reduce the **immunogenicity** of immunomodulatory tick effectors by lowering the amount of each individual antigen in tick salivary secretions.

Redundancy and Pluripotency in the Action of Tick Salivary Immunomodulators

To remain attached to their host and successfully complete their long (up to 2 weeks) feeding course, ticks block the actions of many host immune system components. From an evolutionary point of view, an efficient mechanism must exist to evolve high numbers of proteins with novel functions in the 'arms race' between ticks and their multiple and diverse hosts. There has been extensive research on how proteins with novel functions evolve after **gene duplication** events [9,10]. According to phylogenetic analyses, gene duplication is usually followed by rapid evolution driven by positive selection rather than by the accumulation of neutral mutations [10,11]. The concepts of redundancy and pluripotency in the action of tick salivary immunomodulators represent strategies that fulfill the need for many different low-immunogenicity effectors, while simultaneously reflecting evolutionary mechanisms and constraints such as the requirement for providing an evolutionary advantage [12]. Moreover, a single pluripotent tick salivary protein may exert multiple effects on the host either by targeting more than one mechanism or pathway in the same immune cell type or by targeting different functions in different cell types. For instance, particular tick inhibitors of vertebrate proteases usually target more than one protease involved in host immunity (Figure 1A). The evolutionary mechanisms by which protease inhibitors gain new specificities are well described in general [13], as well as for tick salivary protease inhibitors of the Kunitz family [14,15] where a positive evolutionary pressure on inhibitor functionality is known to exist. An additional element in host immunomodulation by tick saliva is redundancy (or overlap) in actions, in other words when a specific component of host immunity is targeted by more than one salivary protein (Figure 1A,B). Characterization of secreted tick salivary proteins has revealed at least two types of functional redundancy: (i) different tick proteins (from the same or different multigenic protein families) target an identical host cellular mechanism (Figure 1B), or (ii) different tick proteins affect different parts of the same cellular pathway to produce an identical final outcome and phenotype (Figure 1C). A combination of redundancy and pluripotency of secreted tick salivary proteins enables tick saliva to interfere with a wide range of host defense mechanisms (such as hemostasis, inflammation, and antigen-dependent acquired immunity) while evading the host response to compromised homeostasis due to the intrusion of tick mouthparts into the host skin. In the following sections we illustrate the aforementioned concepts with specific examples. Redundancy in targeting hemostasis, such as coagulation and platelet aggregation, has been already well described [16,17], and we therefore focus only on proteins with immunomodulatory features.

Pluripotency and Redundancy – Some Practical Examples

Thorough functional characterization of several individual recombinant tick salivary-secreted proteins has revealed their pluripotent immunomodulatory activities (Figure 1B). One example is the *Ixodes scapularis* salivary cystatin sialostatin L (SialoL), which is an inhibitor of human cathepsins with highest affinity for cathepsin L. Early SialoL studies in a mouse model of acute inflammation revealed its anti-inflammatory potential and inhibitory effect on cytotoxic T cells [18,19]. A follow-up study showed that SialoL also inhibited the proliferation of $CD4^+$ T helper cells, dendritic cell (DC) maturation, and the production of proinflammatory cytokines [19]. It was later shown that SialoL diminished interleukin-9 (IL-9) production in type 9 T helper (Th9) lymphocytes and mast cells [20,21]. Finally, SialoL impaired production of interferon-β (IFN-β) in DCs

Glossary

Antigenic variation: a mechanism developed independently by different parasitic species to evade or exhaust the host immune system. A parasite systematically switches the expression of antigens present at the host–parasite interface. The genetic mechanism underlying this phenomenon varies between different parasites. In ticks, the antigens present at the host–parasite interface are on the tick salivary proteins. Accordingly, different antigens are expressed either once or sequentially during tick feeding. The phenomena of silent antigens and antigenic variation in tick saliva are most likely interconnected.

Gene duplication: one of the dominant mechanisms in gene evolution. Gene duplication occurs due to recombination, and is followed either by positive or negative (purifying) selection. Positive selection results in a protein with a novel function, while negative selection eliminates the duplicated gene.

Immunogenicity: the ability of an antigen to induce humoral or cellular antigen-specific immune responses. Several factors influence immunogenicity: the type of molecule (proteins are usually the best antigens), molecular weight (larger proteins display higher immunogenicity), and concentration (low and high concentrations of antigens can induce immunotolerance or anergy, respectively).

Multigenic protein family: a group of phylogenetically closely related proteins that evolved from a single gene ancestor by the process of multiple gene duplications and subsequent evolution due to the pressure of positive selection. Individual members of one multigenic family can exert identical, similar, or completely different functions.

Pluripotency: in the context of this review, pluripotency means that a single salivary protein displays more than one function. For instance, a protease inhibitor can target more proteases with variable specificity and thus inhibit more physiological processes. Pluripotency can be misidentified if two different observed effects of a given tick protein actually result from the inhibition of the same upstream process.

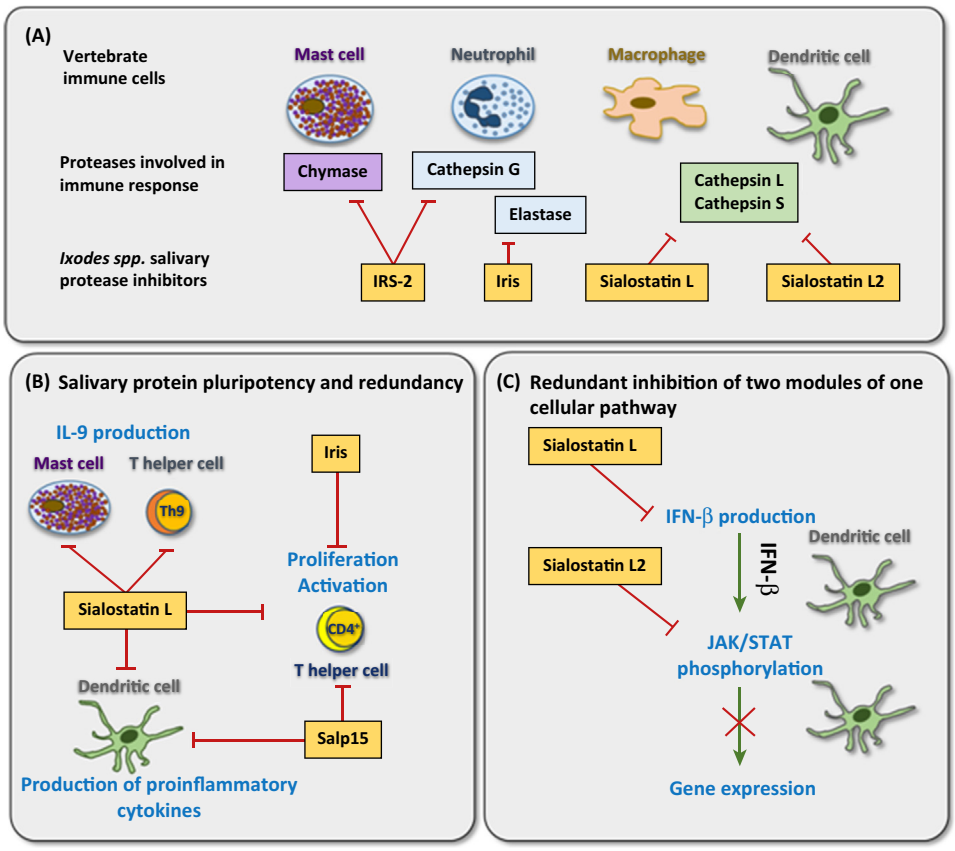


Figure 1. Redundancy and Pluripotency in the Action of Tick Salivary Proteins on Host Cellular Processes. Different members of different tick protein families target the same host immune function, or the same protein targets different modules of the host immune response. (A) By inhibiting different immune cell-derived proteases with specific inhibitors, *Ixodes* spp. ticks can block multiple defense mechanisms. The anti-inflammatory serpin IRS-2 (*Ixodes ricinus* serpin-2) inhibits mast cell chymase and neutrophil cathepsin G. Both proteases are known to be involved in promoting inflammation. The second of the three major neutrophil serine proteases involved in inflammatory responses, elastase, is inhibited by another serpin, Iris (*Ixodes ricinus* immunosuppressor). Sialostatin L and L2 are inhibitors of the intracellular cysteine proteases cathepsin L and cathepsin S that contribute to NLRP3 inflammasome activation and the production of the proinflammatory cytokines interleukin-1β (IL-1β) and IL-18 in macrophages and dendritic cells (DCs). (B) The cystatin from *Ixodes scapularis*, sialostatin L, was shown to inhibit the production of proinflammatory cytokines from DCs, the proliferation of T cells, and the production of IL-9 from type 9 T helper (Th9) cells and mast cells. Another member of an *I. scapularis* protein family, Salp (Salivary protein) 15, inhibited proliferation of T cells and the production of proinflammatory cytokines from DCs. Finally, the tick salivary serpin Iris also inhibited T cell proliferation. (C) Redundant inhibition of different modules of the interferon-β (IFN-β) signaling pathway by two members of the same multigenic family. Sialostatin L, unlike sialostatin L2, inhibits IFN-β production by DCs. Sialostatin L2, however, inhibits the phosphorylation of STAT-3 in the JAK/STAT signaling pathway and subsequent expression of several genes downstream from IFN-β-dependent DC activation.

Redundancy: several redundancy scenarios in tick salivary protein function can be anticipated and observed: two or more proteins from one or several different multigenic families (i) target an identical cellular mechanism, or (ii) target different modules of the same cellular or physiological pathway to exert the same final effect on host physiology.
Silent antigen: an antigen present on a tick salivary protein that can efficiently raise an antibody response when injected at high concentrations in recombinant form, but is unable to elicit such a response as a part of the physiological salivary mixture. This might be due to the lower concentration of the antigen in tick saliva. The concentration of a given effector in tick saliva is low due to the combination of functionally identical but antigenically different salivary proteins.

activated by *Borrelia burgdorferii* spirochetes [22]. When this latter study is evaluated in light of the data from another study [23], it appears that two members of the same multigenic family of *I. scapularis* cystatins (namely sialostatin L and sialostatin L2) target different modules of the same pathway. Specifically, the activity of sialostatin L2 (SialoL2) differed from that of SialoL: unlike SialoL, SialoL2 did not affect IFN-β production but instead attenuated STAT phosphorylation in spleen DCs, thus inhibiting JAK/STAT signaling downstream of IFN-β-dependent signaling. As a result, SialoL2 also inhibited IFN-β-dependent expression of the neutrophil-attracting chemokines MIP-1α and IP-10 in DCs [23] (Figure 1C). In summary, these two sialostatins demonstrate that: (i) a single protein can exert several functions, and (ii) members of

the same multigenic family, despite sequence similarity, can differ in their activities but have complementary effects (Figure 1B,C).

A second example of a pluripotent tick immunomodulator is the salivary protein (Salp) 15 (Figure 1B), a glycoprotein that belongs to another large protein family present in several *Ixodes* species [24]. Similarly to SialoL, Salp15 displays an effect on both $CD4^+$ T cells and DCs [25] and it carries out several activities, the most prominent being the direct binding of Salp15 to the CD4 receptor and subsequent inhibition of T cell activation, proliferation, and IL-2 production [26,27]. In DCs, Salp15 interacts with adhesion and receptor molecule DC-SIGN, leading to the inhibition of proinflammatory cytokine production (IL-6 and tumor necrosis factor, TNF) [28]. Inhibition of $CD4^+$ T cell proliferation has also been observed with another *Ixodes* species-specific protein, the serpin Iris (*Ixodes ricinus* immunosuppressor) [29]. Therefore, SialoL, Salp15, and Iris represent another type of redundancy in which specific host functions are targeted by phylogenetically distinct proteins (Figure 1B). The concept of pluripotency and redundancy of tick salivary proteins is supported by many other examples: two related lipocalins from *Ornithodoros savignyi*, tick salivary gland protein (TSGP) 2 and TSGP3, bind to and inhibit complement by inhibiting C5 convertase and also bind to the neutrophil chemoattractant leukotriene B4 [30]. Three lipocalins from *Rhipicephalus appendiculatus*, Ra-HBP-1, 2, and 3, display histamine-binding features [31], while two members of the Salp16-like family from *Ixodes persulcatus*, Salp16-Iper1 and 2, inhibit neutrophil migration and reactive oxygen species production [32], similarly to two disintegrin members from the ADAMTS (a disintegrin and metalloprotease with thrombospondin motifs) family (ISL 929 and ISL 1373) isolated from *I. scapularis* [33]. Because they are present in many ixodid species, Salp16-like and ADAMTS protein families represent another example of functional redundancy among different protein family members and among members of a single protein family. It is important to note that not every observed tick salivary protein effect on host immunity has a known mechanistic explanation. Therefore, some effects of a given recombinant tick protein may be indirect rather than direct, in other words they may be caused by modulating upstream components of the same molecular pathway or via pathway crosstalk. For instance, the *I. ricinus* salivary serpin IRS-2 (*Ixodes ricinus* serpin-2) was shown to inhibit T cell differentiation into Th17 cells [34]. This effect, however, resulted from upstream inhibition of DC-derived production of the proinflammatory cytokine IL-6 [34]. More detailed mechanistic studies are therefore necessary to determine the primary *in vivo* targets for each tick salivary protein in question.

When testing tick salivary recombinant proteins *in vitro* or *ex vivo*, especially candidate immunodulators, high concentrations of pure recombinant proteins are often needed to achieve a detectable effect. Such high protein concentrations are not usually observed in pure tick saliva, raising the question of whether these proteins really are responsible for the physiological immunomodulatory effects of tick saliva observed both *in vivo* and *in vitro*. The observed redundancy in tick salivary immunodulators could explain this need for high protein concentrations *in vitro*. Because each protein family contains many members with high sequence similarity, it is likely that many of these members share the same function. Therefore, although each protein is present only at low concentrations in the tick saliva, they may act in concert and display an additive (or even synergistic) functional effect that is quantitatively equivalent to a higher concentration of a single protein. Salivary proteins in recombinant form are usually biologically active at concentrations as high as 1–6 μM [22,28,34]. In the complex salivary mixture, this effective concentration could be achieved by combining numerous redundant proteins (Figure 2A). The functional characterization of five anti-complement multigenic family members from *I. ricinus*, IxAC-B1–B5, supports this hypothesis [35]: all five proteins inhibit the alternatively activated complement pathway by preventing C3 convertase complex formation despite their differing primary structures [35] and also their epitope structures (Figure 2A). By contrast, some other tick salivary activities are detected at much lower recombinant protein

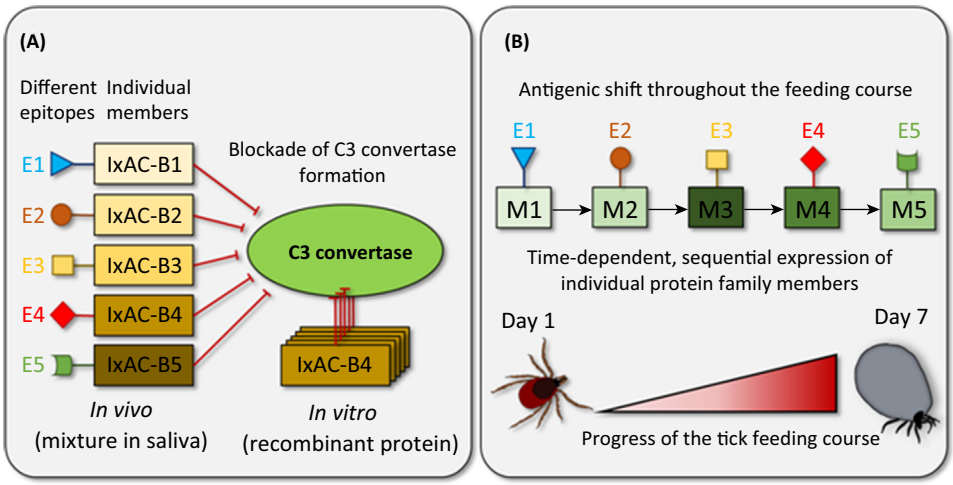


Figure 2. Redundancy in a Single Multigenic Family and Antigenic Variability. (A) Five members of the anti-complement protein multigenic family IxAC-B1–5 affect the same process: they bind to properdin and thus inhibit the stabilization of the C3 convertase complex which, in consequence, inhibits the activation of the alternative pathway of complement. Because there is a divergence in the primary structures of individual members of IxAC-B family, it is likely that there is epitope variability and, therefore, different antigenicity across family members, while all members exert the same function on the host. Indeed, the study of Couvreur and colleagues supports such a suggestion because the sera raised against IxAC-B1 did not recognize other members of the family [35]. The observed functional (but not antigenic) identity results in a reduction in the amount of protein of each family member that is needed to achieve inhibition of host complement, which in consequence leads to lower immunogenicity and helps to evade effective recognition by the host immune system (amount effect). By contrast, when a single recombinant family member (e.g., IxAC-B4, panel A) is tested in a functional bioassay, its concentration would need to be higher to achieve an observable effect, according to our hypothesis. E1–E5, epitopes 1–5. (B) The sequential expression of members from one multigenic family during feeding results in continuous antigenic shift, while the targeted host process remains blocked. Owing to this change in epitope exposure, an effective and timely antigen-specific response cannot be mounted against the tick salivary immunomodulators. Similarly to other parasites such as *Plasmodium* spp., *Trypanosoma* spp., and *Schistosoma* spp., the antigen switch takes place at the parasite–host interface, which – in the case of ticks – is between the blood and tick saliva. Both simultaneous and sequential expression of several antigenically different family members can result in a silent antigen phenomenon. M1–M5, members 1–5 of a hypothetical multigenic family.

concentrations. This might be explained by the hypothesis that a particular host function, although targeted by these proteins, is not blocked by numerous other tick salivary effectors (and thus assistance in their action is not provided by other salivary effectors) or that the mechanism in question is the primary target of a given salivary effector (the protein is a key modulator of the specific host homeostatic mechanism or pathway). For example, the *I. ricinus* serpin Iris significantly inhibited TNF production in human peripheral blood mononuclear cells at concentrations as low as 25 nM [36], but the contact phase of coagulation and fibrinolysis was affected at much higher concentrations (3–6 μM) [37]. The serpin superfamily, expressed in *Ixodes* spp. salivary glands, contains over 40 members [38]; the Iris reactive center loop with methionine at its main active site, the P1 site, however, is somewhat unique among them. We can therefore speculate that the inhibition of TNF production represents a major Iris function, whereas its anti-hemostatic activity plays only a minor role in concert with other tick inhibitors more specialized for hemostasis inhibition.

Antigenic Variation

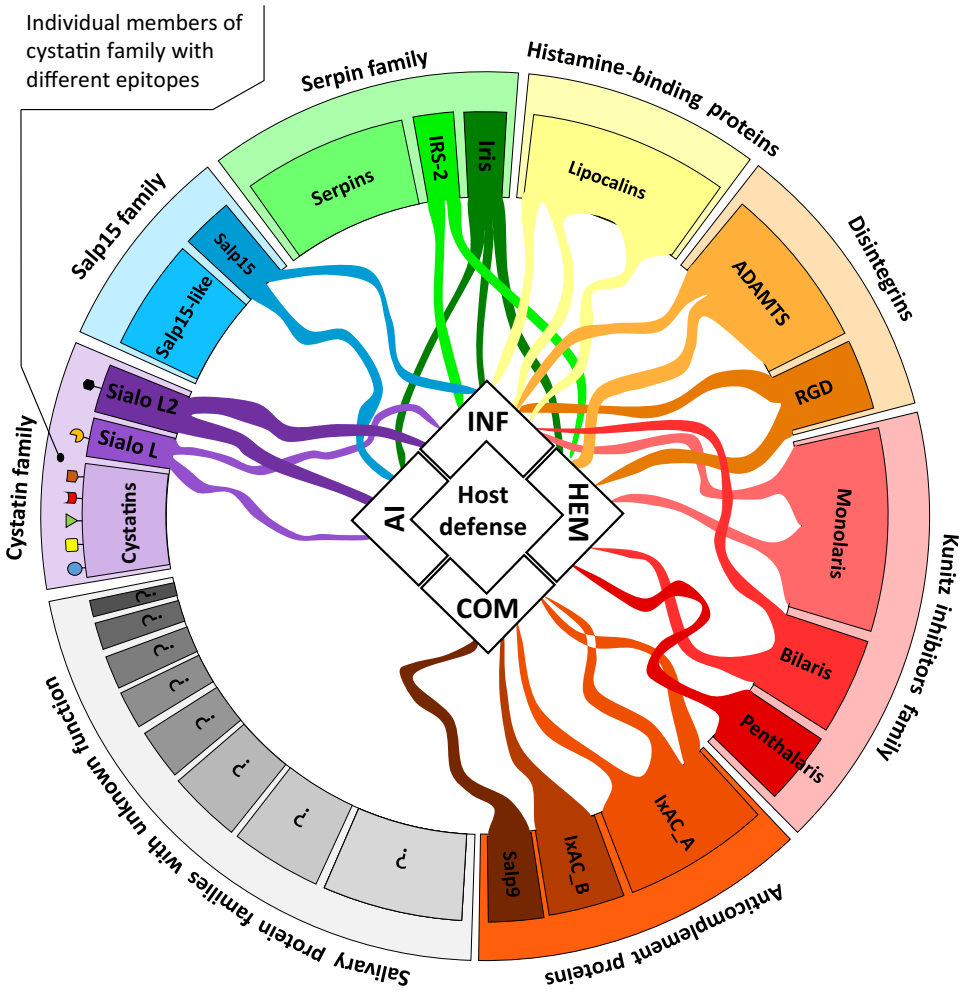
The genetic mechanism that drives multigenic family evolution in ticks is a combination of multiple gene duplications and subsequent mutations [15,39,40]. This mechanism, ubiquitous in eukaryotes [41], was also proposed as a hypothesis for multigenic evolution in ticks [42]. This was subsequently supported by a phylogenetic analysis [30] and confirmed experimentally by

the analysis of synonymous versus non-synonymous mutation rates in salivary proteins [43]. The latter analysis revealed significantly higher non-synonymous mutation rates in putative secreted proteins compared to other protein groups. During the evolution, newly emerging functional proteins/mutants either retain their original function or gain novel features/activities via a process of positive selection rather than by the accumulation of neutral mutations [10]. Although retaining their original function, divergent (in primary structure) proteins can change their epitope structure and immunogenicity by altering a few amino acids. A similar model was introduced in an attempt to experimentally decrease epitope immunogenicity of hirudin, a medically interesting anticoagulant of parasite origin [44,45]. Another participant in the race to evade and/or exhaust the host immune system is **antigenic variation**, the simultaneous or sequential change in multiple antigens upon host immune system exposure. The mechanism of immune system exhaustion by continuous antigen switching is known to occur in *Trypanosoma brucei*, the causative agent of sleeping sickness, which can sequentially express around 2000 different surface antigens [46]. Every newly expressed antigen stimulates a massive immune reaction that eliminates the majority of parasites, but some of the surviving individuals with altered surface antigen expression escape from the host antigen-specific reaction and start a new round of multiplication. This results in the typical wave-like course of the disease [47]. Similarly, malarial protozoa *Plasmodium spp.* also use antigenic diversity to evade immune recognition and elimination by the host immune system [48]. Among non-protozoan pathogens, antigenic variability is exploited by *Schistosoma* spp. [49] and it also plays an important role in pathogenesis of certain bacteria [50]. Interestingly, antigenic variability mechanisms have evolved several times independently, in other words the molecular and genetic mechanisms underpinning antigenic variability in parasites and pathogens are different. However, all these parasites have in common their contact with blood and the host immune system. All the aforementioned examples relate to endoparasites, and thus the variable antigens are expressed on their surface (which is in contact with host immunity). The equivalent in blood-feeding ectoparasites, such as ixodid ticks, should be the antigenic variability of the salivary proteins that are found at the actual interface between the host immune system and the tick. Indeed, as described in previous sections, similar mechanisms of antigenic variation do seem to exist in ectoparasites, as suggested by several transcriptomic analyses of tick salivary glands throughout feeding. Several multigenic protein family members are expressed sequentially during the progression of tick feeding; at each time-point during feeding a different multigenic family member is expressed in tick salivary glands and secreted into the host [43,51,52] (Figure 2B). The concept of antigenic variability, as a conserved mechanism to evade host immune recognition and responses, may therefore also be applied to blood-feeding ectoparasites, and may represent a ubiquitous mechanism exploited by parasites from very distant phylogenetic groups.

Recombinant Protein Versus Saliva – The Difference in Immunogenicity

In our study from 2008 we describe a discrepancy in the immunogenicity of a recombinant tick salivary protein that was artificially injected into the host at high concentrations versus when it was delivered naturally by multiple exposures of the host to tick saliva as a result of natural tick feeding [53]. Specifically, the tick salivary protein SialoL2 was highly immunogenic in recombinant form, and guinea pig vaccination with high amounts of the protein and subsequent exposure of the guinea pigs to ticks resulted in a strong anti-tick response and significantly higher tick rejection. However, natural exposure of guinea pigs to tick saliva by repeated tick feeding did not lead to SialoL2 recognition by their sera unless the animals were previously artificially vaccinated/sensitized with recombinant protein [53]. The observed discrepancy can have several causes. First, immunogenicity is correlated with antigen concentration, therefore exposure to low levels of an antigen can lead to immunotolerance. Similarly, time-dependent expression of different multigenic protein family members in tick salivary glands limits the time and intensity of the exposure of a given tick antigen to the host immune system, and thus reduces the likelihood of a host developing immunity to a specific protein (Figure 2A,B). Salivary

proteins are often of relatively low molecular weight, which is also an important factor that, in general, negatively correlates with antigen immunogenicity. Furthermore, the salivary proteins in native state could undergo post-translational modifications that diminish their immunogenicity. To summarize, the low molecular weight, post-translational modifications, time-limited exposure of a single antigen, and low antigen concentration due to the presence of several members of the same protein family in tick saliva (displaying the same function, but not antigenicity) impair



Trends in Parasitology

Figure 3. A Complex Network of Actions in the Vertebrate Host Is Mediated by Tick Salivary Proteins. Pluripotency and redundancy in the action of tick salivary effectors drive the formation of a complex network of molecular interactions between ticks and the hosts they parasitize. Host anti-tick defenses can be schematically divided into four branches – hemostasis (HEM), inflammation (INF), complement (COM), and antigen-specific acquired immune response (AI). Tick saliva contains multigenic protein families with known or unknown function that can together target all host defense mechanisms (the effect on host defense mechanisms is shown as a 'tentacle' in the figure). There is redundancy in their actions both in the frame of a single family and among the members of different families. The individual members of single family can exert the same effect on the host while expressing different antigenic epitopes (as shown in the example of the cystatin family), which leads to immune system evasion. Some pluripotent proteins (e.g., Iris, Sialo L, or Salp15 in the figure, and other so far uncharacterized proteins) could be considered as key factors because they can target more than one branch of immune reaction. We hypothesize that, by targeting these proteins with vaccines, the functional network of tick salivary immunomodulators could be disrupted, leading to stronger host resistance to ticks and to the pathogens that ticks transmit. Abbreviations: ADAMTS, a disintegrin and metalloprotease with thrombospondin motifs; Iris, *Ixodes ricinus* immunosuppressor; IRS, *Ixodes ricinus* serpin; RGD, proteins containing the integrin-binding motif (Arg-Gly-Asp); Salp, salivary protein; Sialo, sialostatin; IxAC, *Ixodes* anti-complement.

antibody production and T cell activation. This could explain why immunomodulatory proteins in tick saliva do not elicit humoral responses despite their immunogenic potential in the recombinant form.

Concluding Remarks

Several important questions persist in tick–host interaction research and the functional characterization of tick secreted salivary proteins, especially in testing them as candidate immunomodulators. Why are these proteins members of large multigenic families? What is the reason for sequential expression of individual family members during tick feeding? What is the purpose of redundancy and pluripotency in salivary protein function and what impact does it have on host humoral recognition and immune response? What are the implications of performing functional characterization of tick salivary proteins in recombinant form? In this article we have attempted to answer these questions by presenting the evidence-base for the herein proposed hypothesis. We hypothesize that the 'raison d'être' of multigenic families among tick salivary secreted proteins lies in their ability to provide sufficient amount of members with the same function, but different antigenicity. Combined with the pluripotency of some key salivary effectors, we can identify a very powerful network of salivary proteins that are functionally interconnected by the pluripotency and redundancy in their actions (Figure 3). This in turn leads to efficient suppression and modulation of important anti-tick defenses of the host, while the antigen-specific immune responses are diminished due to the presence of **silent antigens**; these silent antigens do not elicit a strong immune response because of their low amount in the tick saliva and low molecular weight. Our hypothesis brings possible explanation to (and is supported by) the fact that researchers need to use relatively high concentrations of pure recombinant tick candidate immunomodulators in bioassays (3–6 μM) to observe an effect [34,37]. Moreover, it prompts the suggestion that some pluripotent proteins (such as Iris, SialoL, Salp15, and other as yet uncharacterized proteins) could be considered as key factors because they can target more than one branch of the host immune response (Figure 3). We hypothesize that the functional network of tick salivary immunomodulators could be disrupted by targeting these proteins with vaccines, leading to stronger host resistance to ticks and to the pathogens that ticks transmit. To prove or disprove this hypothesis is one of the many possible future directions in anti-tick vaccine development (see Outstanding Questions).

Despite the fact that a larger body of experimental evidence from bioassays and/or *in silico* work on transcriptomic and proteomic datasets will be necessary to validate these hypotheses, there is already sufficient accumulated knowledge and data to at least direct the way of designing and developing anti-tick vaccines and to better understand the interface at which pathogen acquisition and/or transmission occur upon tick feeding.

Outstanding Questions

Are there some crucial tick salivary components that serve as cornerstones, without which tick feeding would be significantly disrupted?

What is the mechanism behind the regulation of sequential expression of individual protein family members? Is it dependent on tick feeding status?

How can we incorporate the antigenic shuffle during the progress of tick feeding into anti-tick vaccine development?

Are tick-borne pathogens adapted to the change in salivary protein expression and how?

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Review 3

Sialomes and Mialomes: A Systems-Biology View of Tick Tissues and Tick-Host Interactions

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High-throughput approach helped us to comprehensively characterize gene expression in tick salivary glands at both the transcriptome and the proteome level. Primary focus on salivary glands turned also to other organs, such as mid-gut, which is crucial from the blood digestion point of view. The start of employing transcriptomic analyses has been a milestone in the field of tick research, especially with the rapid development of next-generation sequencing (NGS). Furthermore, the application of quantitative proteomics to ticks with unknown genomes has provided deeper insights into the molecular mechanisms underlying tick hematophagy, pathogen transmission, and tick–host–pathogen interactions. In this review, we summarized current knowledge on the transcriptomics and proteomics of tick tissues from a systems-biology perspective. We overviewed different methodical pipelines for the identification of tick salivary proteins and discussed future challenges in the field. We covered all transcriptomic studies available to date and proposed different comparative studies, for which the transcriptomics and proteomics could be used.

Author's contribution:

Author designed the structure of the review, wrote the text, created the figures and revised the manuscript.

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Review

Sialomes and Mialomes: A Systems-Biology View of Tick Tissues and Tick–Host Interactions

Jindřich Chmelař,[1,2] Jan Kotál,[1,3] Shahid Karim,[4] Petr Kopacek,[3] Ivo M.B. Francischetti,[5] Joao H.F. Pedra,[6] and Michail Kotsyfakis[3,*]

Tick saliva facilitates tick feeding and infection of the host. Gene expression analysis of tick salivary glands and other tissues involved in host–pathogen interactions has revealed a wide range of bioactive tick proteins. Transcriptomic analysis has been a milestone in the field and has recently been enhanced by next-generation sequencing (NGS). Furthermore, the application of quantitative proteomics to ticks with unknown genomes has provided deeper insights into the molecular mechanisms underlying tick hematophagy, pathogen transmission, and tick–host–pathogen interactions. We review current knowledge on the transcriptomics and proteomics of tick tissues from a systems-biology perspective and discuss future challenges in the field.

Ticks, Hosts, and Pathogens

Ticks are obligatory ectoparasitic blood feeders that parasitize reptiles, birds, and mammals. Ticks are medically important because they transmit a plethora of pathogenic agents that cause human diseases, including anaplasmosis, ehrlichiosis, babesiosis, rickettsiosis, and others (www.cdc.gov/ticks/diseases/). Lyme borreliosis is a common tick-borne disease worldwide, while tick-borne encephalitis is a public health concern in Europe and Asia (http://ecdc.europa.eu/en/healthtopics/emerging_and_vector-borne_diseases/tick_borne_diseases/tick_borne_encephalitis/pages/index.aspx). Ticks are divided into two major groups: soft ticks (family Argasidae) and hard ticks (family Ixodidae), which differ in their life cycles and blood-feeding strategies [1,2] and, as a consequence, are exposed to different host homeostatic responses. Hemostasis and acute inflammation are common responses to both groups of ticks and form the basis of the host anti-tick response. Hard ticks, however, must also counteract chronic inflammatory responses and specific humoral and cellular immunity [3].

Bellum Omnium Contra Omnes

Dynamic, multi-directional interactions occur between ticks, hosts, and transmitted pathogens in both the tick and host environments, affecting all three members (Figure 1). These can be regarded as a continuous *bellum omnium contra omnes*, or war of all against all. When a tick ingests host blood, hemoglobin is digested and detoxified in the tick gut [4–6], and proteases of host or pathogenic origin are neutralized [7]. Tick midgut proteins and cells interact with ingested tick-borne pathogens, which migrate via the midgut and haemocoel [8–10] to invade the salivary

Trends

The development of high-throughput NGS methodologies has revolutionized research at the vector–pathogen–host interface.

The decrease in sequencing costs and in the quantity of required starting material, as well as higher transcriptome coverage, have improved drastically our understanding of gene expression regulation in tick tissues involved (or not) in pathogen transmission.

High-throughput quantitative proteomics are now feasible even for disease vectors with an unknown genome and have provided deeper insights into the molecular mechanisms underlying hematophagy, pathogen transmission, and tick–pathogen–host interactions.

Emerging high-throughput gene sequencing methodologies and other 'omics' methodologies, such as metabolomics, may soon be applied to this field.

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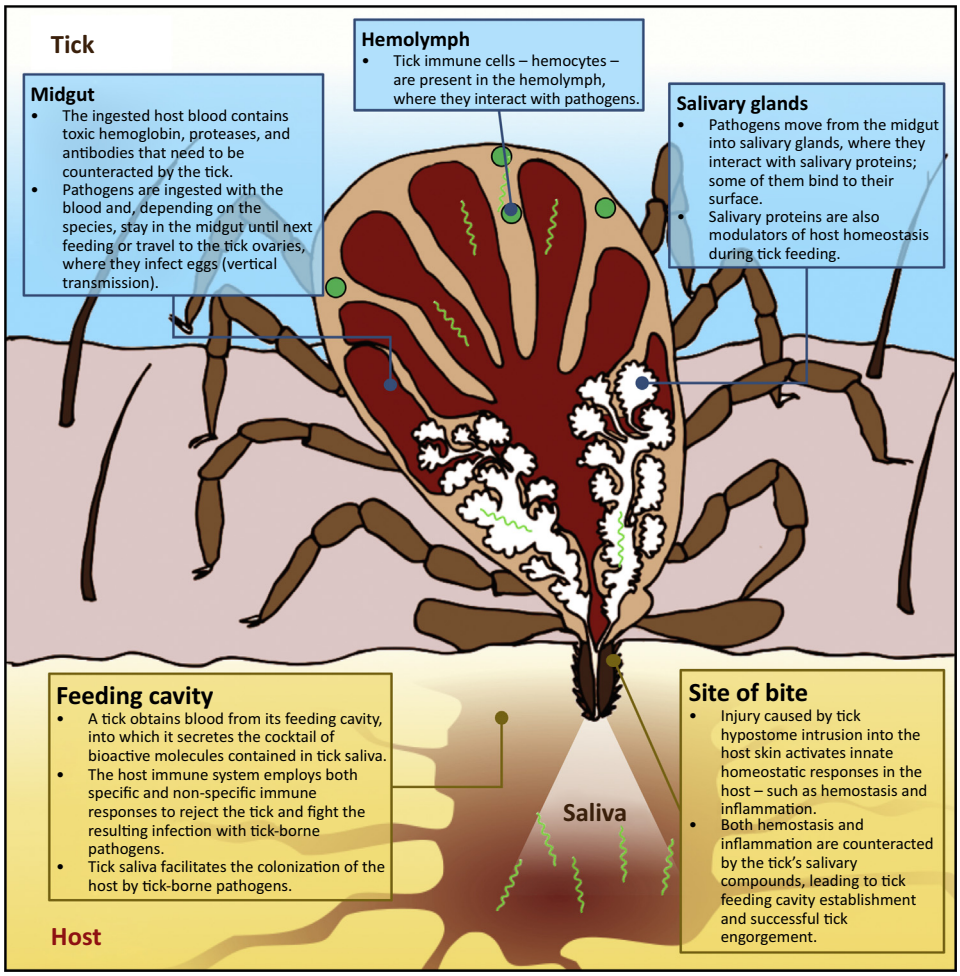

Figure 1. The Complex Interactions Between Ticks, Hosts, and Tick-Borne Pathogens. In ticks, the toxic properties of hemoglobin and the deleterious activities of leukocyte-derived proteins are attenuated by protease inhibition, enzymatic digestion of hemoglobin, and toxic iron scavenging [4,7,76]. Ingested pathogens interact with midgut proteins [11,12] and host blood-derived factors before migrating through the tick midgut and hemolymph to interact with the innate immune system of the tick [8,9]. Next, the pathogens migrate to the tick salivary glands, where they proliferate and acquire salivary proteins on their surface [13,14]. Pathogens are then injected into the host along with tick saliva, and tick salivary components begin to suppress the local host homeostatic response that is immediately raised against the tick bite-induced injury, the 'foreign' tick salivary antigens, and the tick-borne pathogens [3,3,16,19,77]. The overall outcome is facilitation of tick feeding and pathogen colonization of the tick, the host, or both.

glands, proliferate, and acquire salivary proteins on their surface. For example, the midgut proteins TROSPA (tick receptor for ospA) and Ixofin3D (*Ixodidae* fibronectin type III domain-containing tick gut protein) bind to *Borrelia* spirochetes and facilitate midgut colonization and subsequent pathogen transmission to the host [11,12]. Proteins of the Salp15 (salivary protein 15)-like multigene family are produced in the tick salivary glands and bind to *Borrelia* spirochetes to modulate host immunity, thus facilitating infection of the host [13,14]. Tick saliva secreted into the host suppresses local host immune responses, primarily to enable blood acquisition; however, the resulting host immunosuppression facilitates host infection [15–18]. Because tick salivary secretions are the main mediators of host immunosuppression or immunomodulation, salivary composition plays a crucial role in tick-borne pathogen transmission and represents a major topic of interest to researchers in the field [3].

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High-throughput approaches such as transcriptomics and proteomics have facilitated the systematic characterization of salivary composition and gene expression dynamics throughout tick feeding. Moreover, high-throughput technologies are useful for investigating the effects of other biological factors such as the sex or developmental stage of ticks or the presence/absence of pathogens in their tissues. We review and discuss the new high-throughput techniques used to study tick–host–pathogen interactions.

On the Path to Sialome Analysis

Tick saliva research has steadily progressed over the past three decades (Figure 2). The known immunomodulatory properties of tick saliva or salivary gland extracts (SGE) (recently reviewed by Kotál and colleagues [19]) has enabled the adoption of a 'function to protein' approach (Figure 2A), in which crude tick saliva fractions or SGEs that retain the biological activity of the starting material have been purified and isolated [20,21]. However, in the best-case scenario, this approach requires large amounts of starting material and only leads to the identification of individual salivary proteins [22,23].

Early reverse genetics approaches (Figure 2B) enabled the search for specific genes by nucleic acid hybridization-based screening of cDNA libraries produced from tick salivary glands [24–26]. Protein-coding cDNAs of interest were cloned, overexpressed using various systems, and their function characterized in bioassays [27,28].

These two low-throughput approaches were subsequently supplemented and supplanted by the rapid development of high-throughput approaches. These have led to the discovery of a hugely diverse set of salivary and midgut proteins acting at the interface of pathogen transmission in both the vector and host (Figure 2C). The terms '**sialome**' and '**mialome**' (see Glossary) were introduced to describe projects that identified hundreds of transcribed genes in tick salivary glands and the midgut, respectively, which were then extensively annotated and catalogued [29–36]. As the tick research community started to embrace transcriptomics many sialomes were published and hundreds of sequences were disclosed in GenBank, which was a real

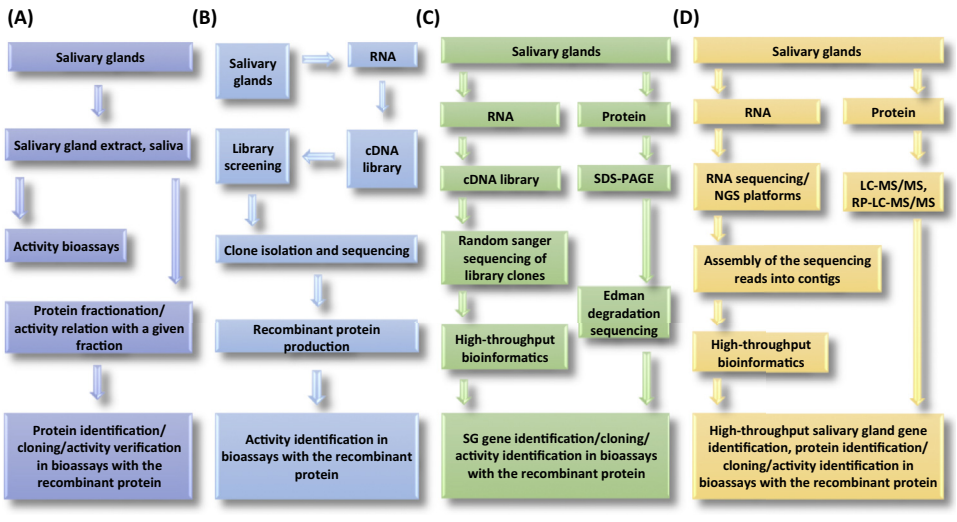

Figure 2. A Schematic of the Four Methodological Pipelines for Tick Salivary Protein Identification. (A) Activity to protein identification approach. (B) cDNA library screening for specific genes of interest. (C) Random cDNA library sequencing combined with proteomics. (D) Direct RNA sequencing with next-generation sequencing (NGS) combined with advanced reverse phase liquid chromatography-tandem mass spectrometry [(RP)-LC-MS/MS] proteomics. Main text for details.

Glossary

Contig: consensual DNA sequence assembled from several ESTs during bioinformatics analysis of transcriptomes. Contigs that encode proteins of the same family form clusters. The number of contigs per cluster is used in quantitative analyses, such as for describing gene expression dynamics or comparisons of gene expression at different tick developmental stages.

Edman degradation: Edman sequencing is still the most robust and fastest approach to sequencing the N terminus of peptides or proteins. Developed by Pehr Edman, in this method, the amino-terminal residue is labeled and cleaved from the peptide without disrupting the peptide bonds between other amino acid residues.

Expressed sequence tag (EST): a single sequence read, usually used in the context of classical Sanger sequencing.

Hemocytome: a hemocyte transcriptome.

Mialome: a midgut transcriptome sometimes coupled with its proteomic analysis. Derived from sialome and midgut.

Next-generation sequencing (NGS): general term for a group of sequencing platforms that enable massive sequencing of RNA or DNA. Platforms commonly used in this field are 454 pyrosequencing (Roche), Illumina dye sequencing (Illumina, Solexa), and SOLiD sequencing (Life Technologies). Their strength lies in the production of huge numbers of short sequence reads (70–150 nt for Illumina, 200–500 nt for 454) and powerful assembly software that allows the assembly of individual reads into contigs.

Proteomics informed by transcriptomics (PIT): protein identification based on a transcript sequence database.

(Reverse phase) liquid chromatography followed by tandem mass spectrometry (RP)-LC-MS/MS: a high-throughput proteomic analysis technique in which proteins are separated by liquid chromatography, followed by two rounds of MS for detection.

Sanger sequencing: the classical sequencing method used in most biological applications before NGS development. Sanger sequencing is based on the incorporation of

breakthrough: for the first time the complexity of gene regulation in tick salivary glands had been itemized. Similarly to earlier reverse genetics strategies, genes of interest were then expressed as recombinant proteins and extensively characterized at the biochemical and biological levels [37–42].

The next important development in studying tick–host–pathogen interactions was the introduction of **next-generation sequencing** (NGS) (Figure 2D). Compared to classical **Sanger sequencing**, NGS platforms such as Illumina or 454 provided unprecedented transcriptome coverage, making them pioneering tools for quantitative analysis of gene expression dynamics in different tick tissues (see below). Some transcriptomics projects were complemented with proteomics (Figures 2C,D). Early proteomic analysis of tick saliva employed **Edman degradation** protein sequencing; in most cases, individual SDS-PAGE gel bands were used for subsequent protein sequencing [29,43]. As transcriptomic coverage improved using NGS, more sensitive mass spectrometry (MS) methods such as **liquid chromatography followed by tandem mass spectrometry** (LC-MS/MS) or **reverse phase liquid chromatography followed by tandem mass spectrometry** (RP-LC-MS/MS) have simultaneously allowed more thorough and comprehensive analysis of protein expression dynamics [44–46]. This combination of transcriptomics and proteomics is referred to as a 'systems-biology approach' or systems-level analysis.

In the following sections we summarize the knowledge gained over 13 years of tick transcriptomics and proteomics research, and discuss the questions that can now be answered using new high-throughput analyses. Each advance in available technology over time was significant in its own right. As the theme of this review attests, the newest tools are always heralded as the greatest advances. Owing to space constraints, we focus on tick salivary glands, the midgut, and hemocytes.

Finding Missing Pieces of the Puzzle

Early transcriptomics projects on *Ixodes* spp. [29–31,33,35] provided insights into the qualitative aspects of tick salivary gene expression. Hundreds of transcripts were identified and the mechanisms and trends in salivary protein evolution were described. This provided a foundation for building a comprehensive overview of the molecular interface between ticks, hosts, and transmitted pathogens, which was further facilitated by the functional characterization of the discovered transcripts and proteins. The first tick sialome work by Valenzuela and colleagues revealed various mechanisms of host immune system evasion mediated by the salivary secretions of *Ixodes scapularis* and the existence of multigene protein families in secreted saliva [29]. These protein families were subsequently confirmed in other tick species [30,32,34–36,47]. The major groups of secreted proteins common in most tick species are described in Figure 3. Despite efforts to identify as many transcripts as possible using transcriptomics, new family members were still being identified using more specific approaches such as cDNA library screening with gene-specific probes or RT-PCR with degenerate primers [38,48]. It was clear, therefore, that coverage of genetic diversity using expensive Sanger sequencing alone was insufficient. Nevertheless, the annotated sequencing data supported the adoption of **proteomics informed by transcriptomics** (PIT), in other words, the identification of salivary proteins (mainly by Edman degradation) based on the discovered transcript sequences. The main qualitative improvement brought by NGS was the ability to detect large numbers of novel transcripts because of its capacity to detect even weakly expressed genes by extensive transcriptomic coverage. NGS has thus provided a more complete picture of tick gene expression and its regulation, and in doing so confirmed the presence of major protein families across tick species, the most represented (in terms of diversity and transcription rate) being Kunitz-domain proteins, lipocalins, metalloproteases, and basic tail proteins [49–51]. These families contain tens to hundreds of members of varying sequence similarity. The initial discovery of

fluorescently labeled dideoxynucleotides that stop polymerase activity and label DNA fragments, which are subsequently subjected to capillary electrophoresis and laser detection of fluorescence. The advantage of this method is the resulting long reads; one read can cover up to 1000 nt.

Sialome: a salivary gland transcriptome, sometimes coupled with its proteome analysis; usually used in the context of hematophagous arthropods. Sialome is a composite of the Greek word for saliva (*sialos*) and 'transcriptome'.

Unique transcript: a cDNA sequence originating from the reverse transcription of mRNA and coding for a single protein.

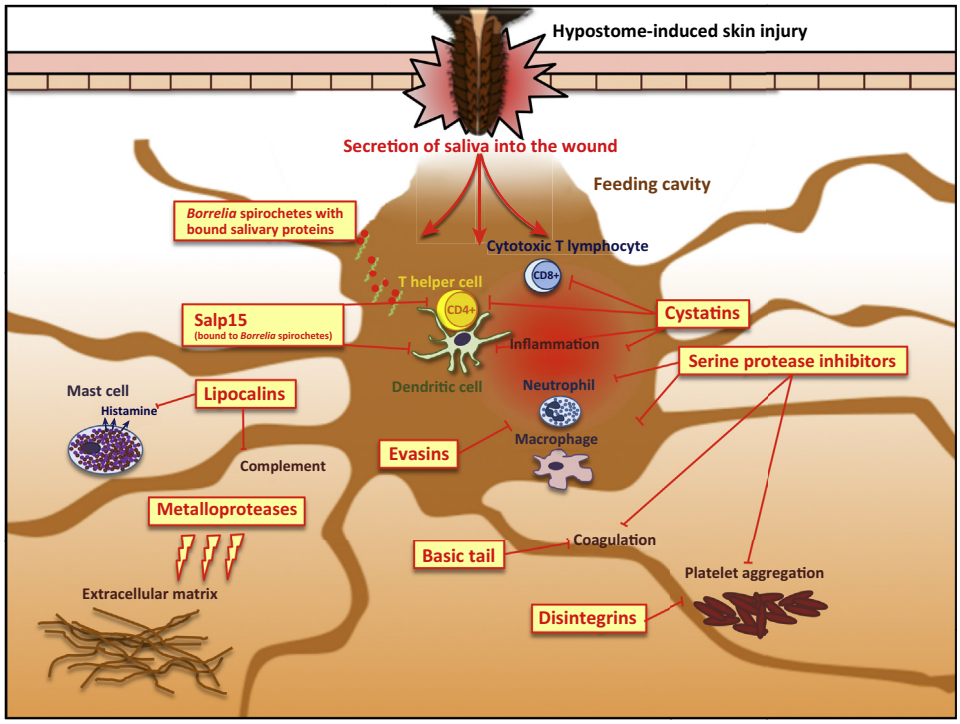

Figure 3. Major Multigene Families Identified by High-Throughput Transcriptomic Analysis of Tick Salivary Glands and their Effect on Host Homeostasis. These families include serine protease inhibitors [serpins: Kunitz or trypsin inhibitor-like (TIL)-domain proteins], cystatins, lipocalins (histamine-binding proteins), disintegrins, metalloproteases, and several novel protein families with little or no similarity to other proteins: basic tail, salivary protein 15 (Salp15), and evasins. Many other multigene families, although less abundant, are found in sialomes. Yellow-red rectangles represent the tick salivary contents.

multigene families in early transcriptomic studies provided the basis for efforts to decipher the function of each individual family member at the protein level [17,52,53]. The higher transcriptomic coverage and greater insights into the expression dynamics of individual transcripts provided by NGS significantly improved our ability to predict the function and importance of individual family members in tick survival and pathogen transmission.

Evolutionary Insights

High sequence similarity between members of multigene salivary protein families suggests that they originate from common ancestors that underwent multiple gene duplication events and subsequent mutations with divergent evolution [2,54,55]. Recent NGS-based *Ixodes ricinus* transcriptome analysis have provided convincing experimental support for this hypothesis [56]. The authors analyzed synonymous (Syn) and non-synonymous (NS) mutation rates for several multigene families. The highest NS mutation rate was detected in genes coding for unknown, secreted, and immunity-related proteins, suggesting an accelerated mutation rate in these gene groups. Phylogenetic analysis across tick species showed that certain branches (clades) of the phylogenetic tree were preferentially occupied by family members originating from a single tick species [49,50]; in other words, most gene duplications (and thus most proteins) in multigene families are tick lineage-specific, in accordance with previously published studies [55]. Thanks to the much higher transcriptomic coverage by NGS and new computational and bioinformatics approaches, phylogenetic analyses provide more information about multigene protein family evolution [57]. Furthermore, two NGS projects provided solid evidence that accelerated tick

evolution is related to hematophagy. In the first, Ribeiro and colleagues showed that non-hematophagous adult *Antricola delacruzi* females displayed the highest divergence from other hematophagous species with respect to secreted salivary proteins [58]. In the second, comparison of male and female *Rhipicephalus pulchellus* revealed a list of hematophagy-related genes because males do not feed on blood [46]. These examples show that large transcriptomic datasets can provide the foundation for studying the evolution of hematophagy in arthropods.

When More Is Better

The main advantage of NGS-based transcriptomics, however, lies in its ability to quantitatively describe transcriptome dynamics. Gene discovery from classical Sanger sequencing and NGS projects are compared in Table 1. The most extensive Sanger sequencing analysis revealed 13 643 and 12 319 **unique transcripts** in mixed libraries from *Rhipicephalus microplus* [47] and *Amblyomma americanum* [59], respectively; the usual number of unique sequences in similar projects is around 1000. NGS-based transcriptome projects, by contrast, can produce hundreds of thousands of assembled **contigs** [50] and over 50 000 unique transcripts [46,60]. On average, around 16 000 unique transcripts have been discovered in NGS projects on ticks, around 10-fold higher than Sanger sequencing in terms of novel transcript identification, and around 100–200-fold higher in terms of total contigs. The latter number is particularly important for the quantitative analysis of tick physiology: the unprecedented transcriptome coverage by NGS enables statistically reliable analysis of gene expression dynamics of secreted salivary proteins throughout the course of tick feeding, the comparison of tissue- and developmental stage-specific transcript accumulation, and metabolic pathway analysis [55,56]. Furthermore, different physiological conditions can be compared, for example changes in transcriptional regulation in the presence or absence of tick-borne pathogens and the influence of different host species on gene regulation in different tick tissues (see below and Figure 4).

Gene Expression Dynamics During Tick Feeding

One of the most important questions in tick–host–pathogen interactions is how tick salivary gland gene expression contributes to host homeostasis, pathogen transmission, and disease. In 2006, Ribeiro and colleagues observed that there were 20 genes at least twofold more abundant than expected in the cDNA library originating from the salivary glands of adult *I. scapularis* females 18–24 h after attachment [31]. These transcripts were collagen-like proteins, Kunitz domain-containing proteins, basic tail proteins, and several proteins of unknown function. Similarly, seven genes significantly differed from random in ticks 3–4 days after host feeding, but each transcript belonged to a different family. Interestingly, different members of the same protein family were expressed at different time-points of tick feeding [31]. Similarly, individual collagen-like protein family members were preferentially transcribed at specific tick feeding phases in the first *I. ricinus* sialome project [35]. This time-dependent preferential gene expression was recently confirmed in an NGS transcriptional analysis of metalloproteases, Kunitz domain-containing proteins, and lipocalins [56]. In the same study, greater than 10-fold tick feeding time dependent difference was observed for 1447 genes expressed in the salivary glands, of which 1135 encoded secreted proteins. The most represented protein families were Kunitz domain-containing proteins followed by Salp15/ixostatin family members, lipocalins, metalloproteases, and several novel protein family members of unknown function [56]. A very recent NGS study analyzed *Amblyomma americanum* salivary glands at four feeding time-points and confirmed time-dependent preferential gene expression in another medically important tick [61]. The genetic mechanisms underpinning sequential expression of individual members of a multigene family are unknown. However, transposable elements and genes of viral origin are consistently being detected in sialomes, suggesting that there are active changes in the tick genomic structure [49,56]. Together with the observed influence of histone modifications on

Table 1. Comparison Between Classical Sanger Sequencing and Two NGS Platforms (454 and Illumina)[a]

Year	Sequencing Method	Tissue	Species	Total Reads	Average Read Length	Good Quality EST	Unique Sequences	Refs
2002	Sanger	SG	*Ixodes scapularis*	735	–	735	410	[29]
2004	Sanger	SG	*Rhipicephalus appendiculatus*	28 416	670/780	19 046	7359	[66]
2004	Sanger	SG	*Rhipicephalus microplus*	–	–	324	188	[78]
2004	Sanger	HC	*Rhipicephalus microplus*	–	–	196	157	[78]
2005	Sanger	SG	*Ixodes pacificus*	–	487	1068	557	[30]
2006	Sanger	SG	*Ixodes scapularis*	8150	–	7476	3020	[31]
2007	Sanger	SG	*Dermacentor andersoni*	1440	600	1299	762	[32]
2007	Sanger	ML	*Rhipicephalus microplus*	42 512	–	–	13 643	[47]
2008	Sanger	SG	*Amblyomma cajannense*	1920	472	1754	1234	[79]
2008	Sanger	MG	*Dermacentor variabilis*	2304	–	1679	835	[33]
2008	Sanger	SG	*Ixodes ricinus*	2304	503	1881	1274	[35]
2008	Sanger	SG	*Ornithodoros coriaceus*	–	–	1089	726	[43]
2008	Sanger	SG	*Ornithodoros parkeri*	–	–	1 529	649	[34]
2009	Sanger	SG	*Amblyomma americanum*	–	–	3868	2002	[36]
2010	Sanger	SG	*Rhipicephalus sanguineus*	–	–	2034	1024	[80]
2010	454	AF	*Dermacentor variabilis*	233 335	203	–	38 683	[67]
2011	Sanger	SG	*Hyalomma marginatum rufipes*	–	–	2084	1167	[81]
2011	454	SG	*Amblyomma maculatum*	1 626 969	–	190 646	15 814	[49]
2011	454	AF	*Ixodes ricinus*	60 186	227	–	–	[74]
2012	Sanger	Larvae	*Rhipicephalus microplus*	–	–	–	775	[68]
2012	Sanger	SG	*Antricola delacruzi*	–	–	1147	923	[58]
2013	Sanger	ML	*Amblyomma americanum*	20 256	–	15 390	12 319	[59]
2013	Sanger	MG	*Rhipicephalus microplus*	5000	–	4054	1628	[82]
2013	Illumina	Nymphs	*Ixodes ricinus*	162 000 000	101	–	–	[72]
2013	454	SG	*Ixodes ricinus*	441 381	518	93 331	34 560[b]	[50]
2013	Illumina	SG	*Ixodes ricinus*	67 703 183	90	269 600	34 560[b]	[50]
2014	Illumina	SG	*Amblyomma americanum*	18 800 000	–	–	17 593	[64]

Table 1. (continued)

Year	Sequencing Method	Tissue	Species	Total Reads	Average Read Length	Good Quality EST	Unique Sequences	Refs
2014	Illumina	Larvae	*Dermacentor reticulatus*	21 677 414	207	18 946	3808	[45]
2014	Illumina	SG + MG	*Ixodes ricinus*	585 000 000	–	198 504	25 808	[44]
2014	454	SG	*Amblyomma cajennense*	67 677	–	–	4604	[51]
2014	454	SG	*Amblyomma parvum*	104 817	–	–	3796	[51]
2014	454	SG	*Amblyomma triste*	442 756	–	–	1124	[51]
2014	454	SG	*Ixodes ricinus*	778 598	379	–	24 539	[70]
2015	454	HC	*Ixodes ricinus*	926 596	498	–	15 716[b]	[65]
2015	Illumina	HC	*Ixodes ricinus*	49 328 982	148	–	15 716[b]	[65]
2015	Illumina	SG	*Amblyomma americanum*	344 909 378	101	–	5792	[61]
2015	Illumina	SG	*Haemaphysalis flava*	162 912 848	100	70 542	54 357	[60]
2015	Illumina	SG + MG	*Ixodes ricinus*	268 914 130	–	–	25 808	[56]
2015	Illumina	SG	*Ixodes scapularis*	28 000 000	101	–	11 105	[71]
2015	Illumina	MG	*Ixodes scapularis*	26 000 000	101	–	12 651	[71]
2015	Illumina	Nymphs	*Ixodes scapularis*	31 000 000	101	–	16 083	[71]
2015	Illumina	AF	*Ixodes scapularis*	3 700 000	–	–	9134	[63]
2015	Illumina	SG	*Rhipicephalus pulchellus*	241 229 128	–	–	50 460	[46]

[a]Abbreviations: AF, adult female, whole body; HC, hemocytes; MG, midgut; ML, mixed library from several tissues; SG, salivary gland; '–', not indicated in the study.
[b]Sum of transcripts identified by both 454 and Illumina sequencing.

gene expression in *Amblyomma maculatum* [62], we can hypothesize that sequential transcriptional regulation is epigenetically regulated [56]. The benefit of shifts in sequential gene expression between different members of the same protein family is still only speculative but may reflect the need to evade immune recognition by the host.

Tissue- and Life Stage-Specific Transcriptional Regulation

Quantitative transcriptomic analysis has also been performed in *I. ricinus* according to tissue (salivary glands vs midgut) and developmental stage (nymphs vs adults). Multiple pairwise comparisons between nymph and adult female midguts and salivary glands at different tick feeding time-points revealed over 8300 genes with at least 10-fold differences in gene expression in salivary glands and midguts [56]. A systems-biology analysis of *I. ricinus* salivary glands and midguts revealed some discrepancies between the transcriptomic and proteomic analyses [44]: of a total of 1510 genes were expressed at both transcriptomic and proteomic levels in the specific tick tissues, 373 proteins were more abundant in the salivary glands than in the midgut, but only 110 of these displayed corresponding transcript accumulation in the same tissue. Conversely, 217 proteins were significantly upregulated in the tick midgut versus salivary glands, but only 93 had a similar transcriptional pattern. The authors explained this discrepancy by the delay between activation of the transcriptional and translational machinery or by tissue-specific pre-synthesis (or secretion) of some tick proteins. More detailed analysis revealed that the majority of over-represented salivary gland proteins were secreted proteins or connected to the

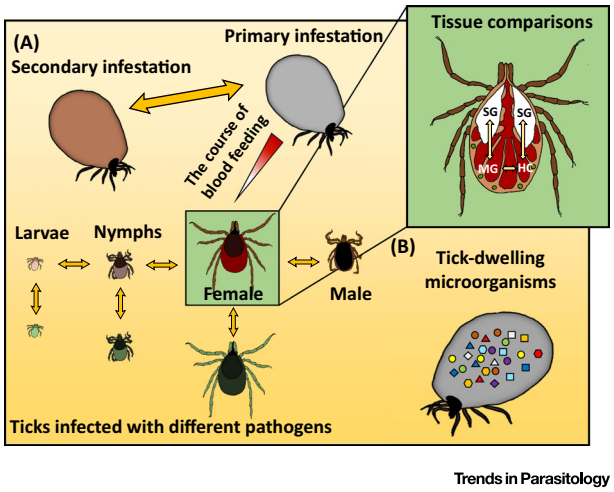

Figure 4. Schematic of Some Comparisons that Can Be Performed by Next-Generation Sequencing. (A) Comparisons were made between various tick developmental stages, [51,56] and as a function of tick blood feeding progress [50,56,60,61,64]. Differences in gene expression regulation were analyzed in the presence (infected ticks shown with green color in the figure) or absence of pathogens in ticks [70,71,82]. Transcriptional regulation was also compared between male and female adult ticks [63], and between adult female ticks upon primary (feeding on naive host) or secondary infestation of the same host (the host was exposed to ticks for the second time) [46]. Comparisons of transcription regulation in tick salivary glands (SG), midgut (MG), and hemocytes (HC) (see inset) were performed at different tick developmental stages either infected or non-infected with various tick-borne pathogens [44,56,65]. Other tick tissues analyzed by transcriptomics include synganglia and male and female reproductive organs; owing to space constraints we do not discuss these projects further, but details can be found in [78,83–87]. (B) The analysis of various tick-dwelling microorganisms (tick microbiome analysis and exploring host species-dependent pathogen diversity in ticks) revealed a high number of bacteria and protozoa in ticks [72,73,75].

protein modification machinery, while over-represented midgut proteins were mainly metabolic [44]. While transcriptomics provides only putative (although often precise) information on salivary composition, proteomics reveals the actual composition, making these two approaches complementary.

The experimental design of transcriptomic projects largely depends on the question being asked. Therefore, we can expect future NGS transcriptomic or systems level analyses to focus on specific questions concerning individual genes, protein families, or physiological phenomena related to the tick life cycle. For instance, Lewis and colleagues constructed and sequenced a cDNA library of tick immunogens by phage-display library screening of *I. scapularis* females fed for 24 h with serum collected from rabbits sensitized by repeat exposure to ticks. They found 182 contigs that were considered immunogenic and usable for potential vaccine development [63]. A similar approach was used to identify 895 potential immunogens in the salivary glands of *Ammblyoma americanum* [64]. The influence of stress on unfed *Dermacentor reticulatus* gene expression was studied using a systems-biology approach and revealed hundreds of stress-activated genes that could be potentially targeted to develop novel tick-control methods [45].

In addition to the midgut and salivary glands, hemocytes also appear to be an important interface between the tick and pathogens. Characterization of the *I. ricinus* hemocyte transcriptome (**hemocytome**) revealed the existence of important immune-related proteins in these ancient phagocytic ancestors of mammalian leukocytes [65]. The identified transcripts encoded proteins similar to defensins, pattern recognition receptors, proteases, protease inhibitors, and others. However, in contrast to 8300 genes with more than 10-fold difference in expression in salivary

glands or the midgut, only 327 were expressed at least fivefold more in hemocytes compared to other tissues [65].

Ticks Versus Pathogens

The question of how pathogens affect tick gene expression has been addressed in several studies. In 2004, Nene and colleagues compared the sialomes of *Rhipicephalus appendiculatus* ticks with or without *Theileria parva* infection but did not find any significant differences in gene expression [66]. Ribeiro and colleagues identified ten differentially expressed contigs in *I. scapularis* nymph salivary glands with or without *Borrelia burgdorferi* infection [31], and these belonged to the 5.3 kDa family, the basic tail protein family, or were histamine-binding proteins in the lipocalin superfamily. Notably, some lipocalin genes were overexpressed in infected ticks and others in uninfected ticks. Jaworski and colleagues used 454 pyrosequencing to characterize the immune response of *Dermacentor variabilis* after injection with different bacterial species [67]. In whole-body sample analyses, the authors identified over 30 immune-responsive genes, including genes encoding serpins, calreticulin, superoxide dismutase, galectin, and defensins. Interestingly, transcriptional upregulation in response to bacterial infection was only confirmed in seven genes by RT-PCR [67]. Similarly, 26 differentially expressed genes were identified by cDNA library subtraction and classical Sanger sequencing after infection of *Rhipicephalus microplus* with *Babesia bovis* [68]. The upregulated genes were mostly related to metabolism and tick immunity, indicating that *Babesia bovis* is a physiological burden in infected ticks. In contrast to the low number of genes identified in these studies, infection of *R. microplus* with *Anaplasma marginale* affected the expression of 888 midgut genes and 146 salivary gland genes, mostly of unknown function, as reveled by microarray analysis [69]. In another study, infection of *I. ricinus* with *Bartonella henselae* resulted in transcriptional upregulation of 829 genes and downregulation of 517 genes in salivary glands [70]. Similar to the observations of Ribeiro *et al.* [31], these genes belonged to the same multigene families, with particular members being upregulated and others downregulated on infection. In the same study, Liu and colleagues revealed that IrSPI (*I. ricinus* serine protease inhibitor 1), a Kunitz protease inhibitor, was most upregulated after infection with *B. henselae*. IrSPI was shown to facilitate tick feeding and the proliferation of the pathogen in the tick salivary glands [70]. A very thorough NGS-based analysis focused on apoptotic pathway changes in *I. scapularis* infected with *Anaplasma phagocytophilum* [71]. The authors revealed pathogen-driven inhibition of apoptosis that facilitated establishment of the pathogen via upregulation of Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling in the tick. This is an excellent example of how huge NGS datasets can be used to answer specific questions. Such analyses of specific metabolic and signaling pathways can be useful not only for disclosing differences in gene expression between two physiological states (e.g., non-infected vs infected ticks) but also for detailed characterization of the molecular pathways that mediate tick physiology.

An NGS approach was also successfully used to analyze the spectrum of pathogens present in *I. ricinus* [72]. In total, 12 bacterial pathogens were identified, including known pathogens such as *Borrelia afzeli, B. garini, B. burgdorferi* s.s., *Anaplasma phagocytophilum*, but also new and unexpected species such as *Borrelia miyamotoi* or *Bartonella henselae*, *Bartonella graham*, and *Rickettsia felis*. The same group used the same dataset to identify protozoan parasites [73]. Similarly, both known and unknown species infecting *I. ricinus* were revealed by data analysis. Carpi and colleagues combined *I. ricinus* 454 pyrosequencing data and cDNA libraries to identify over 100 bacterial genera, both pathogenic and symbiotic [74]. These projects highlight the potential of NGS for tick-borne pathogen identification. NGS has also been used several times in tick research to analyze symbiotic bacteria and the midgut microbiome, as recently reviewed by Narasimhan and Fikrig [75].

Concluding Remarks

Tick transcriptomics has undergone rapid and impressive progress owing to technical developments in NGS and proteomics; this, in turn, has allowed not only the identification of tick transcripts but also the detailed analysis of transcriptional and translational dynamics. It is now feasible to describe the detailed proteome dynamics of tick salivary glands and midguts for over 1500 proteins in ticks with unknown genomes [44]. Therefore, the latest technical developments have resulted in at least an order of magnitude increase in the number of identified transcripts and proteins compared to early sialome and mialome projects. There is no doubt that there is room for further research in this field, regardless of whether the focus is on the evolution of the tripartite interaction between ticks, hosts, and tick-borne pathogens, the gene expression dynamics of hematophagy-related genes, or even the physiological or ecological aspects of tick biology (see Outstanding Questions). Furthermore, there remains an open question about the real-time changes occurring in tick tissues in response to gene and protein expression changes in the host skin and in lymph nodes draining the bite site. More importantly, there are still many topics in disease vector genomics that remain barely investigated, such as the role of epigenetics, non-coding genomic regions, and non-coding RNAs in the tripartite interaction. Finally, we anticipate the development of research projects that employ single-cell/single-molecule sequencing methodologies and do not require nucleic acid amplification. Furthermore, other '-omics' approaches such as metabolomics may shed additional light on the physiology of this tripartite interaction. The increasing impact of vector-borne diseases in human and veterinary public health mandates the use of cutting-edge technologies to rapidly develop novel control methods and tools.

Outstanding Questions

Will the new, emerging nucleotide sequence analysis methods improve assembly of transcriptome reads into contigs for organisms with an unknown genome by providing longer sequencing reads?

What changes in knowledge can we expect from transcriptomic and proteomic analyses at the individual tick level, which are now becoming feasible given the decreased amount of starting material needed in such analyses?

Decreasing costs will boost the adoption of systems-biology approaches and genome sequencing projects in this field. How much of an impact will this have on our understanding of the tick life cycle and hematophagy in molecular and evolutionary terms?

What real-time changes occur in tick tissues in response to gene and protein expression changes in the host skin and the lymph notes draining the bite site?

How soon will we be able to deploy other '-omics' approaches, such as metabolomics, in this field?

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Resources

[i] 454: http://454.com/downloads/GSFLXApplicationFlyer_FINALv2.pdf

[ii] Illumina: www.illumina.com/technology/next-generation-sequencing.html

[iii] Ion Torrent: www.thermofisher.com/gr/en/home/brands/ion-torrent.html

[iv] PacBio: www.pacificbiosciences.com/

[v] Nanopore sequencing: https://nanoporetech.com/applications/dna-nanopore-sequencing

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Review 4

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

Chmelař J, Kotál J, Langhansová H, Kotsyfakis M.

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Thanks to long term collaboration with Dr. Michail Kotsyfakis, our attention has been focus time from time also to cystatins and not only on serpins. Therefore in 2017 we decided to sum up the knowledge about the role of these two families of protease inhibitors in the tick-host-pathogen interaction. Since both serpins and cystatins have their endogenous relatives in vertebrate host, often with crucial and indispensable function, we focused on the possibility of the use of tick inhibitors in the development of novel immunomodulators with the use in medicine and pharmacy.

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Author designed the structure of the review, participated from large part on text writing, created the figures and revised the manuscript.

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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

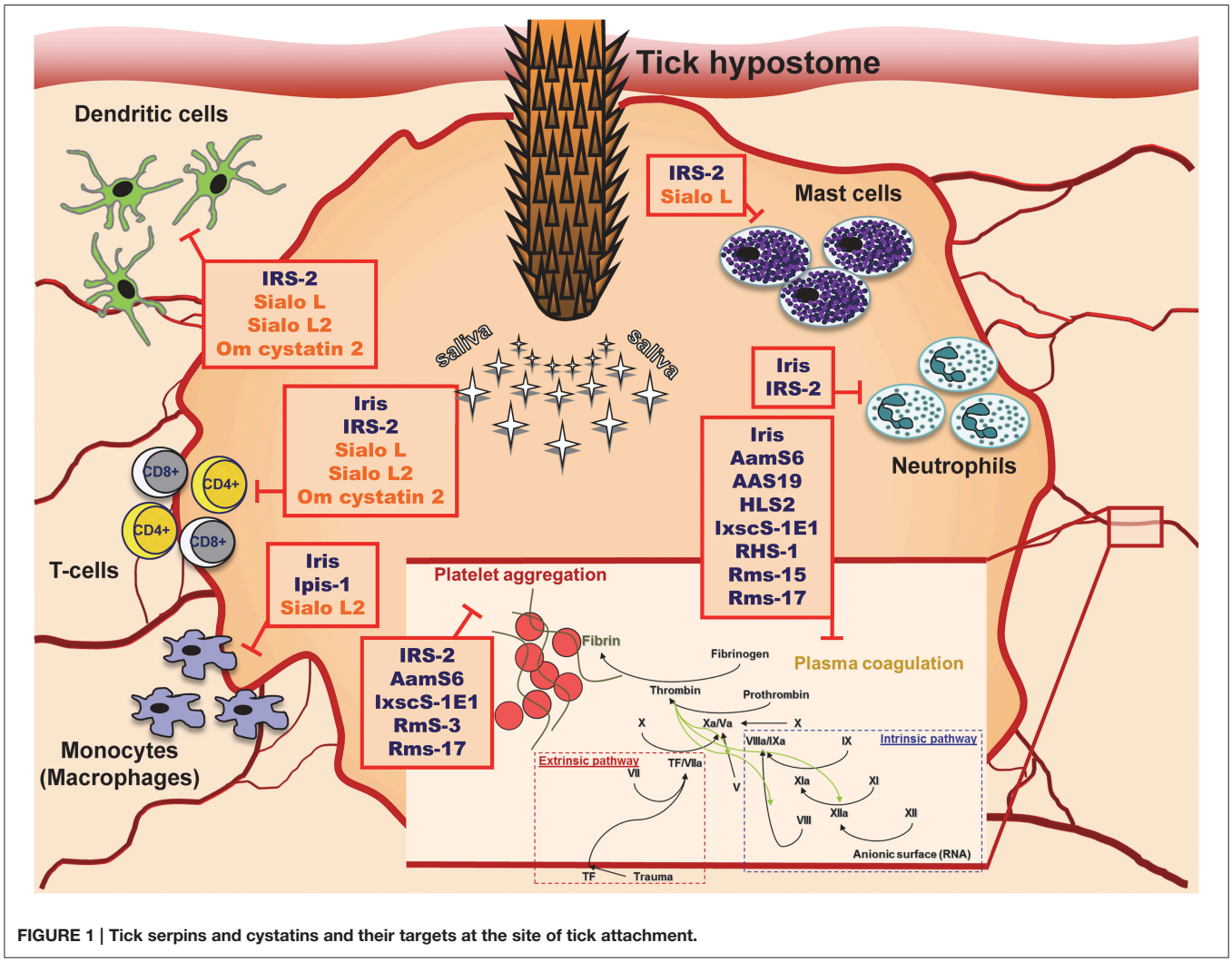

FIGURE 1 | Tick serpins and cystatins and their targets at the site of tick attachment.

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,

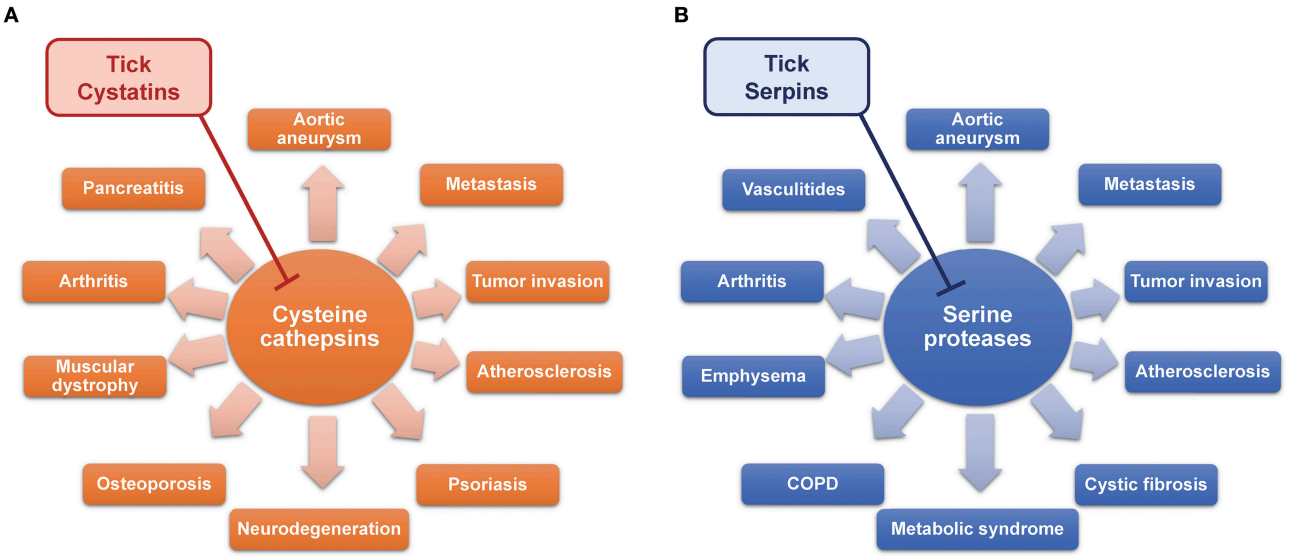

FIGURE 2 | The therapeutic potential of tick cystatins (A) and serpins **(B)** by inhibiting cysteine and serine proteases in various diseases.

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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Review 5

Serpins in Tick Physiology and Tick-Host Interaction

Abbas MN, Chlastáková A, Jmel MA, Giannakoudaki EI, Chmelař J and Kotsyfakis M.

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Last submitted review focused solely on the serpins, this time in tick physiology and tick-host interaction. As a difference from the previous review, instead of 17 serpins, we covered 30 serpins and the review was more thorough. The role of serpins in tick development, blood digestion and tick immunity as well as their role at the tick-host interface as anti-hemostatics, anti-inflammatory molecules and immunomodulators was discussed in this comprehensive review. In order to make it more complex, the mechanism of serpin inhibition of the proteases was also described.

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REVIEW
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Serpins in Tick Physiology and Tick-Host Interaction

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Tick saliva has been extensively studied in the context of tick-host interactions because it is involved in host homeostasis modulation and microbial pathogen transmission to the host. Accumulated knowledge about the tick saliva composition at the molecular level has revealed that serine protease inhibitors play a key role in the tick-host interaction. Serpins are one highly expressed group of protease inhibitors in tick salivary glands, their expression can be induced during tick blood-feeding, and they have many biological functions at the tick-host interface. Indeed, tick serpins have an important role in inhibiting host hemostatic processes and in the modulation of the innate and adaptive immune responses of their vertebrate hosts. Tick serpins have also been studied as potential candidates for therapeutic use and vaccine development. In this review, we critically summarize the current state of knowledge about the biological role of tick serpins in shaping tick-host interactions with emphasis on the mechanisms by which they modulate host immunity. Their potential use in drug and vaccine development is also discussed.

Keywords: tick saliva, serpins, immunomodulation, therapeutic effects, anti-tick vaccine, tick host interaction

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1 INTRODUCTION

1.1 Tick-Host-Pathogen Triad

Ticks (order Ixodida) are ectoparasitic arthropods with a wide global distribution which serve as vectors of a broad spectrum of transmitted pathogens, including bacteria, viruses, and parasites. Ticks are medically considered the second most important vector of disease (Dantas-Torres et al., 2012). Ticks comprise two main families, soft ticks (Argasidae) and hard ticks (Ixodidae), with different lifestyles and life cycles, but both are obligate blood-feeders, entirely dependent on parasitic life. Their feeding strategies differ markedly; while hard ticks feed for several days until complete engorgement and repletion, soft ticks can complete their blood meal in less than one hour. Both groups of ticks alternately inject saliva and suck blood during this feeding process. Digestion takes place in the lumen of the midgut, where lysis of blood cells occurs, and subsequent digestion of proteins, including hemoglobin and other blood components, occurs intracellularly in the epithelial cells of the midgut. The process is driven by a cascade of intracellular endopeptidases and

exopeptidases, in particular Cathepsins B, C, D, L and legumain, and leads to protein digestion down to single amino acids (Sojka et al., 2013).

Ticks penetrate the vertebrate skin with their saw-like hypostome, which serves to inject saliva and to draw blood but also opens the host skin to the external environment, leading to exposure to secondary infection. The resulting injury, transmitted pathogens, and superimposed infection trigger a host immune response. To avoid it, the tick releases its pharmacologically potent salivary constituents (Ribeiro and Mans, 2020) into the skin wound and alters all kinds of host immune responses. This action facilitates both tick feeding and pathogen transmission. The passage of transmitted pathogens within the tick tissues is usually described as pathogens entering the midgut from an infected host *via* the blood meal, then crossing the digestive epithelium and infiltrating the hemocoel, from where the pathogens can enter the salivary glands and infect the host while contained in tick saliva during the next feeding cycle (Šimo et al., 2017).

1.2 An Overview of Serpins

Serpins form the richest group of serine (but they have been reported also as cysteine) protease inhibitors, consisting of 350-500 amino acid residues and ranging in molecular weight from 40 to 60 kDa. Recent and the most extensive phylogenetic study on serpins analyzed more than 18 000 unique protein sequences, extracted from public protein databases. Around 10 000 sequences differed by more than 25% in their amino acid sequence, showing enormous abundance of serpins among the organisms (Spence et al., 2021). Serpins are found mostly in eukaryotes, but they can also be detected in archaea, bacteria, and viruses, although in much smaller numbers than in eukaryotes, and many of them have also been functionally characterized. (Silverman et al., 2001; Gettins, 2002; Irving et al., 2002; Silverman et al., 2010; Spence et al., 2021). The number of serpin genes may vary in different animal species, and their distribution patterns in eukaryotes indicate that they appeared early in eukaryotic evolution (Logsdon et al., 1998). Inhibitory serpins usually play an important role in the regulation of physiological pathways controlled by serine proteases in vertebrates and invertebrates, including blood and hemolymph clotting, fibrinolysis, inflammation, complement activation, or regulation of the enzyme phenoloxidase in the Toll pathway in arthropods (Silverman et al., 2001; Rau et al., 2007; Gulley et al., 2013). Moreover, serpins are implicated in diverse biological processes in invertebrates, including immunoregulation, dorsal-ventral formation, development, and the regulation of apoptosis (Levashina Elena et al., 1999; Ligoxygakis et al., 2003; Pak et al., 2004; Kausar et al., 2017; Kausar et al., 2018). In plants, serpins are involved in the defense against insect pests and are studied for their application potential in agriculture (Alvarez-Alfageme et al., 2011; Clemente et al., 2019). In addition to their inhibitory role, serpins have been shown to modulate biological processes such as blood pressure or hormone transport in humans (Gettins, 2002; Zhou et al., 2006a; Whisstock et al., 2010). Interestingly, the hormone release mechanism is also dependent on the dynamics of serpin conformational changes (Zhou et al., 2008). Serine protease inhibitors are phylogenetically grouped by species rather than by their biological role in animals. Thus, rather than coevolution with serine proteases, the evolution of serine protease inhibitors appears to be driven by speciation in order to fulfill the species-specific biological roles (Krem and Di Cera, 2003). Despite relatively low sequence homology, all serpins have almost identical three-dimensional structure. This feature was explored in a recent phylogenetic study that suggested that convergent evolution has occurred several times in different taxa for serpins to acquire similar structure and function. The same study showed a high degree of conservation among intracellular serpins from both prokaryotes and eukaryotes, presumably with some key homeostatic function, whereas secreted serpins formed more species-specific branches (Spence et al., 2021). Thanks to protein crystallography, we have gained substantial insights into the molecular mechanism of serpin mode of action, which is termed suicidal because serpins form covalent complexes with the target protease(s) and are ultimately eliminated by a protein degradation mechanism (Whisstock et al., 2010; Huntington, 2011; Mahon and McKenna, 2018). As shown in **Figure 1**, serpins are composed of conserved β-sheets and α-helices and several coils that form a typical tertiary structure. Proper amino acid composition of specific region, called hinge region, allows the serpin to undergo necessary conformational changes that are crucial for their activity as protease inhibitors. A flexible, Reactive Center Loop (RCL) with P1 site functions as a bait for the target serine protease. It is exposed at the top of the serpin molecule and forms an intermediate Michaelis-Menten complex, which can further lead to the formation of covalent complex with the target protease. The final conformation of the serpin in the complex results from the insertion of the RCL into the β-sheet A to form one additional β-strand (Silverman et al., 2001; Gettins, 2002; Huntington, 2011). In case the inhibitory complex is not produced, cleaved serpin becomes inactive and active protease is released.

Despite the acronym serpin (Serine Protease Inhibitor) suggesting that serpins inhibit only serine proteases, it was experimentally shown that they could act as 'cross-class' inhibitors of proteases (Bao et al., 2018). For example, CrmA, a viral serine protease inhibitor, can inhibit caspase-1 protein (Komiyama et al., 1994) and SERPINB3 can inhibit cathepsins S, K, and L, which are papain-like cysteine proteases (Schick et al., 1998). In addition, miropin, a human pathogenic bacterial serpin, has been reported to inhibit a variety of both serine proteases, such as pancreatic and neutrophil elastases, cathepsin G, trypsin, plasmin or subtilisin and the cysteine proteases cathepsin L and papain (Ksiazek et al., 2015; Goulas et al., 2017; Sochaj-Gregorczyk et al., 2020). Such a wide inhibitory range could represent an adaptation strategy to the highly proteolytic environment of the subgingival plaque, which is constantly exposed to a number of host proteases in the inflammatory exudate. Under such environmental conditions, miropin is thought to play a key role as a virulence factor by protecting bacterial pathogens from the damaging activity of neutrophil serine proteases (Ksiazek et al., 2015). Miropin or

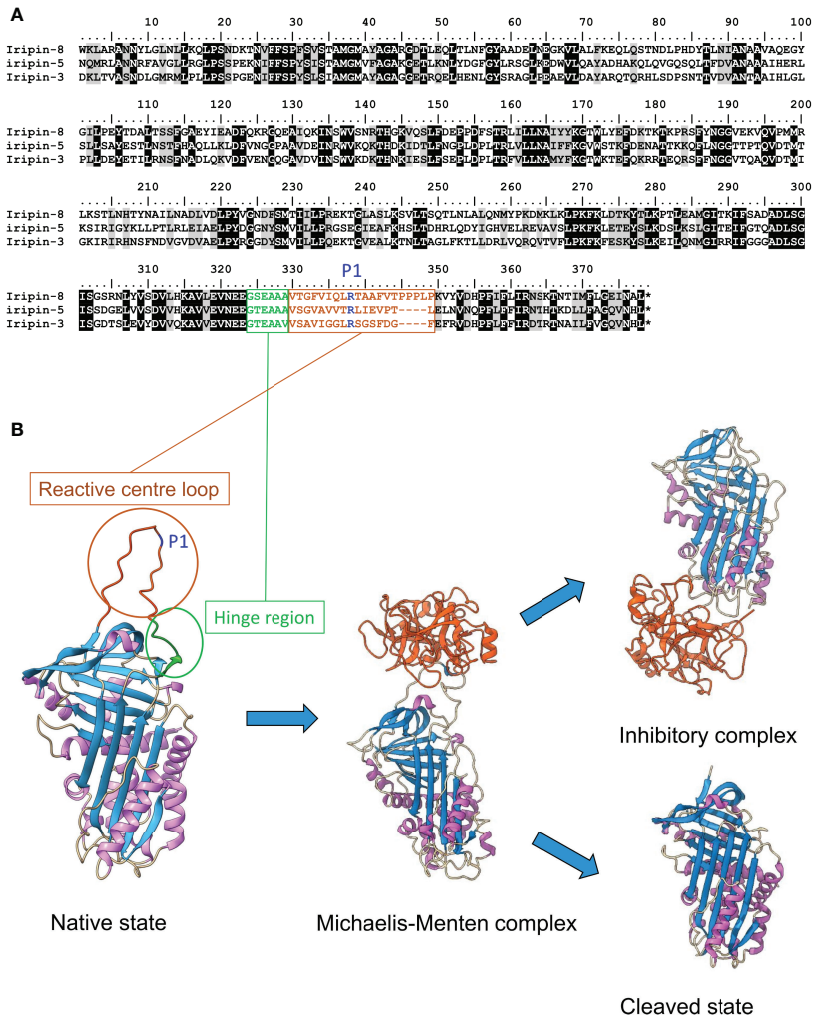

FIGURE 1 | Structure of serpins and their mechanism of inhibition. **(A)** Sequence alignment of three characterized serpins from *I. ricinus*. RCL is highlighted in brown, P1 site in blue and hinge region in green. **(B)** Tertiary structures of four most common serpin conformation states. Native state is presented with highlighted RCL, P1 site and hinge region highlighted with the same colors as in the alignment. It forms non-covalent Michaelis-Menten complex with target protease, which can further end up as a covalent inhibitory complex or as cleaved inactive state. Used structures were downloaded from RCSB Protein Data Bank and prepared in ChimeraX (Pettersen et al., 2021). Asterisk in the alignment represents the stop codon.

CrmA are examples of the use of serpins by pathogens to invade and survive in the host. However, serpins are also used by blood-feeding arthropod ectoparasites to evade the host immune response and facilitate blood uptake.

2 SERINE PROTEASE INHIBITORS IN TICKS

Since the discovery of the serpin superfamily of serine protease inhibitors (Hunt and Dayhoff, 1980), many biological roles of serpins from different organisms have been discovered. Among other animals, many tick serpins have been identified using classical molecular methods, cDNA library screening or transcriptomic approaches (Ribeiro et al., 2012; Yu et al., 2013; Chmelař et al., 2016). In this review, we discuss tick serpins and their role in tick physiology and tick-host interactions in detail. We will focus on their anti-hemostatic, anti-inflammatory, anti-complement, and immunomodulatory functions in the host, and how these activities are important for pathogen transmission. Observed effects on the host are summarized in **Table 1** and inhibitory specificities, expressed by measured Ki values, are summarized in **Table 2**.

2.1 Expression of Serpin Genes in Ticks

In ticks, serpins are usually expressed in different developmental stages and tissues but with some degree of stage and/or tissue specificity. For example, the serpin gene RHS8 has been shown to be expressed in all developmental stages, with mRNA levels being higher in *Rhipicephalus haemaphysaloides* larvae and

TABLE 1 | Tick serpins examined in the current review article.

Serpin name	GenBank accession number	Tick species	Expression profile	Inhibited proteases	Biological processes affected by a serpin	Vaccination experiments	References
AamS6	ABS87358.1	*A. americanum*	Adult females, SG, saliva, MG, OVA	Trypsin, chymotrypsin, elastase, chymase, plasmin, papain	Platelet plug formation Coagulation cascade	–	Mulenga et al., 2007 Chalaire et al., 2011 Mulenga et al., 2013
AAS19	JAI08902.1	*A. americanum*	Adult females, SG, saliva, MG, OVA, SYN, MT	Trypsin, plasmin, fXa, fXIa, fXIIa, fIXa, thrombin, chymotrypsin, tryptase, papain	Platelet plug formation Coagulation cascade	Immunization of rabbits Reduced engorgement weight and impaired oviposion in adult female ticks	Porter et al., 2015 Kim et al., 2015 Kim et al., 2016 Radulović and Mulenga, 2017
AAS27	JAI08961.1	*A. americanum*	Eggs, larvae, nymphs, adults, SG, saliva, MG, CAR, OVA, SYN, MT	Trypsin, plasmin, chymotrypsin	Inflammation	–	Porter et al., 2015 Tirloni et al., 2019 Bakshi et al., 2019
AAS41	JAI08957.1	*A. americanum*	Eggs, larvae, nymphs, adults, SG, MG, CAR	Chymase, mast cell protease-1, chymotrypsin, papain	Inflammation	–	Porter et al., 2015 Bakshi et al., 2019 Kim et al., 2020
HLS-1	–	*H. longicornis*	Adult ticks, MG	–	Coagulation cascade	Immunization of rabbits Increased mortality rate in nymphs and adults	Sugino et al., 2003
HLS2	BAD11156.1	*H. longicornis*	Nymphs, adults, hemolymph	Thrombin	Coagulation cascade	Immunization of rabbits Prolonged feeding time and higher mortality rate in nymphs and adults, impaired oviposition	Imamura et al., 2005
HlSerpin-a	QFQ50847.1	*H. longicornis*	–	Cathepsin G, cathepsin B, fXa, papain	Inflammation Adaptive immunity	–	Wang et al., 2020
HlSerpin-b	QFQ50848.1	*H. longicornis*	–	Cathepsin G, fXa, papain	Inflammation Adaptive immunity	–	Wang et al., 2020
Ipis-1	BAP59746.1	*I. persulcatus*	Adult females, SG	–	Adaptive immunity	–	Toyomane et al., 2016
Iripin-3	JAA69032.1	*I. ricinus*	Nymphs, adult females, SG, saliva, OVA	Kallikrein, matriptase, thrombin, trypsin	Coagulation cascade Inflammation Adaptive immunity	–	Chlastáková et al., 2021
Iripin-5	JAA71155.1	*I. ricinus*	Nymphs, adult females, SG	Trypsin, elastase, proteinase-3	Inflammation Complement system	–	Kascakova et al., 2021
Iripin-8	ABI94058.1	*I. ricinus*	Nymphs, adult females, SG, saliva, MG	Thrombin, fVIIa, fIXa, fXa, fXIa, fXIIa, plasmin, activated protein C, kallikrein, trypsin	Coagulation cascade Complement system	–	Kotál et al., 2021
Iris	CAB55818.2	*I. ricinus*	Nymphs, adult females, SG, saliva	Elastase, tissue plasminogen activator, fXa, thrombin, trypsin	Platelet plug formation Coagulation cascade Fibrinolysis Inflammation	Immunization of rabbits Higher mortality and lower weight gain in nymphs, prolonged feeding period and higher mortality rate in adult females	Leboulle et al., 2002a Prevot et al., 2006 Prevot et al., 2007

(Continued)

TABLE 1 | Continued

Serpin name	GenBank accession number	Tick species	Expression profile	Inhibited proteases	Biological processes affected by a serpin	Vaccination experiments	References
					Adaptive immunity		Prevot et al., 2009
IRS-2	ABI94056.2	*I. ricinus*	Adult females, SG, MG, OVA	Chymotrypsin, cathepsin G, chymase, thrombin, trypsin, and other proteases	Platelet plug formation Inflammation Adaptive immunity	–	Chmelar et al., 2011 Páleníková et al., 2015 Pongprayoon et al., 2020 Fu et al., 2021
IxscS-1E1	AID54718.1	*I. scapularis*	SG, saliva, MG	Thrombin, trypsin, cathepsin G, fXa	Platelet plug formation Coagulation cascade	–	Mulenga et al., 2009 Ibelli et al., 2014
RAS-1	AAK61375.1	*R. appendiculutus*	Larvae, nymphs, adults, SG, MG	–	–	Immunization of cattle with a combination of RAS-1 and RAS-2	Mulenga et al., 2003
RAS-2	AAK61376.1	*R. appendiculutus*	Larvae, nymphs, adults, SG, MG	–	–	Decreased engorgement rate in nymphs, higher mortality in nymphs and adult females	Imamura et al., 2006
RAS-3	AAK61377.1	*R. appendiculutus*	Male and female adults, SG, MG	–	–	Immunization of cattle with a combination of RAS-3, RAS-4, and RIM36	Mulenga et al., 2003
RAS-4	AAK61378.1	*R. appendiculutus*	Male and female adults, SG, MG	–	–	Higher mortality in female ticks	Imamura et al., 2008
RHS-1	AFX65224.1	*R. haemaphysaloides*	SG, saliva	Chymotrypsin, thrombin	Coagulation cascade	–	Yu et al., 2013
RHS-2	AFX65225.1	*R. haemaphysaloides*	MG	Chymotrypsin	Adaptive immunity	–	Yu et al., 2013 Xu et al., 2019
RHS8	QHU78941.1	*R. haemaphysaloides*	Eggs, larvae, nymphs, adults, SG, OVA, fat bodies	–	Tick reproduction (vitellogenesis)	–	Xu et al., 2020
RmS-3	AHC98654.1	*R. microplus*	Nymphs, adult females, SG, saliva, MG, OVA	Chymotrypsin, cathepsin G, elastase, chymase, mast cell protease-1	Platelet plug formation Inflammation Adaptive immunity	–	Rodriguez-Valle et al., 2012 Rodriguez-Valle et al., 2015 Tirloni et al., 2014 Tirloni et al., 2014 Tirloni et al., 2016 Coutinho et al., 2020 Pongprayoon et al., 2021
RmS-6	AHC98657.1	*R. microplus*	Adult females, SG, saliva, MG, OVA	Trypsin, chymotrypsin, plasmin, fXa, fXIa	Inflammation	–	Tirloni et al., 2014 Tirloni et al., 2014 Rodriguez-Valle et al., 2015 Tirloni et al., 2016 Coutinho et al., 2020

(Continued)

TABLE 1 | Continued

Serpin name	GenBank accession number	Tick species	Expression profile	Inhibited proteases	Biological processes affected by a serpin	Vaccination experiments	References
RmS-15	AHC98666.1	*R. microplus*	Eggs, nymphs, adult females, SG, saliva, MG, OVA	Thrombin	Coagulation cascade	–	Tirloni et al., 2014 Tirloni et al., 2014 Rodriguez-Valle et al., 2015 Xu et al., 2016
RmS-17	AHC98668.1	*R. microplus*	Adult females, SG, saliva, MG, OVA	Trypsin, chymotrypsin, cathepsin G, plasmin, fXIa	Platelet plug formation Coagulation cascade Inflammation Adaptive immunity	–	Tirloni et al., 2014 Tirloni et al., 2014 Rodriguez-Valle et al., 2015 Tirloni et al., 2016 Coutinho et al., 2020

SG, salivary glands; MG, midgut; OVA, ovaries; SYN, synganglion; MT, Malpighian tubules; CAR, carcass; RIM36, Rhipicephalus immunodominant molecule 36 (a putative cement protein of R. appendiculatus ticks).

nymphs (Yu et al., 2013; Xu et al., 2020). Similarly, serpins have been found to be transcribed in a number of tick tissues, suggesting a role either in tick physiology or in tick-host interactions. Such an interaction can occur either in the host or in the tick midgut. As an example, a study by Tirloni and co-workers analyzed the expression profiles of 18 serpins from *Rhipicephalus microplus* and found that 16 of them are transcribed in all tissues, but with quantitative differences for different serpins (Tirloni et al., 2014). Similarly, serpins from the Lone Star tick *Amblyomma americanum*, named Lospins, were also expressed in multiple tissues but with a tissue preference for individual serpins (Mulenga et al., 2007; Porter et al., 2015).

TABLE 2 | Second-order rate constants of the interaction between tick serpins and serine proteases.

Serpin name	Tick species	Protease	Second-order rate constant (M^{-1} s^{-1})	References
AAS27	*A. americanum*	trypsin	$6.46 \pm 1.24 \times 10^4$	Tirloni et al., 2019
AAS41	*A. americanum*	chymase	$5.6 \pm 0.37 \times 10^3$	Kim et al., 2020
		α-chymotrypsin	$1.6 \pm 0.41 \times 10^4$	
Iripin-3	*I. ricinus*	kallikrein	$8.46 \pm 0.51 \times 10^4$	Chlastáková et al., 2021
		matriptase	$5.93 \pm 0.39 \times 10^4$	
		trypsin	$4.65 \pm 0.32 \times 10^4$	
		thrombin	$1.37 \pm 0.21 \times 10^3$	
Iripin-8	*I. ricinus*	plasmin	$2.25 \pm 0.14 \times 10^5$	Kotál et al., 2021
		trypsin	$2.94 \pm 0.35 \times 10^4$	
		kallikrein	$1.67 \pm 0.11 \times 10^4$	
		fXIa	$1.63 \pm 0.09 \times 10^4$	
		thrombin	$1.38 \pm 0.1 \times 10^4$	
		fXIIa	$3.32 \pm 0.41 \times 10^3$	
		fXa	$2.09 \pm 0.12 \times 10^3$	
		activated protein C	$5.23 \pm 0.35 \times 10^2$	
		fVIIa + tissue factor	$4.56 \pm 0.35 \times 10^2$	
Iris	*I. ricinus*	leukocyte elastase	$4.7 \pm 0.64 \times 10^6$	Prevot et al., 2006
		pancreatic elastase	$2.2 \pm 0.15 \times 10^5$	
		tissue plasminogen activator	$2.9 \pm 0.15 \times 10^5$	
		fXa	$1.7 \pm 0.36 \times 10^5$	
		thrombin	$2.5 \pm 0.42 \times 10^4$	
		trypsin	$1.5 \pm 0.42 \times 10^4$	
RmS-15	*R. microplus*	thrombin	$9.3 \pm 0.5 \times 10^4$	Xu et al., 2016

All tick serpins with available data are presented.

The varying levels of expression across tissues suggest that serpins may have a broader biological role, i.e. serpins may be involved in development (present in the ovary) and in the regulation of blood digestion (present in the midgut). In addition, their expression and presence in salivary glands and/or saliva suggest that they play a role in tick feeding, possibly influencing host resistance mechanisms and facilitating pathogen transmission (Jmel et al., 2021). Therefore, in order to determine the role of individual serpins, we must not only investigate their capabilities in experimental models *in vitro* and/or *in vivo*, but we must also consider developmental stage and tissue specific expression, taking into consideration also the time during tick feeding that gene expression present peak(s). It is difficult to determine the concentration of tick salivary proteins in a host as the tick feeding site is a very complex and dynamic environment where the concentrations of both host and tick proteins constantly change (Mans, 2019). Therefore, we can only estimate roughly that the concentration of serpins can vary from nanomolar to micromolar range.

3 SERPINS MODULATE TICK BIOLOGICAL PROCESSES RELATED TO DISEASE VECTOR PHYSIOLOGY

As discussed in the previous section, the pattern of serpin expression in different tick developmental stages and tissues may suggest a biological significance in tick physiology (**Figure 2** and **Table 1**). The first area in which serpins have a definite role is in the biology and physiology of ticks.

3.1 Serpins in Tick Hemolymph

In ticks and arthropods in general, hemolymph clotting is a key defense mechanism that reduces hemolymph loss and blocks entry into the wound, thereby preventing entry of microbial pathogens and tick infection/death. To date, several tick serpins have been identified as being involved in hemolymph clotting. The RAS-3 and RAS-4 serpins of the tick *Rhipicephalus appendiculatus* have been found to share some degree of similarity with the horseshoe crab hemolymph clotting factors LICI-1 and LICI-2, suggesting that they also have hemolymph clotting potential (Mulenga et al., 2003). Serpin HLS2, which is comparable to serpins from *R. appendiculatus*, was found to be produced only in the hemolymph, indicating that it likely controls processes in the hemolymph of this tick species (Mulenga et al., 2001; Imamura et al., 2005). Apart from exceptions, such as HLS2, it is not known, whether hemolymph serpins are produced by hemocytes or secreted there by other organs. Anyway, serpins are definitely produced in arthropod hemocytes, as they can be found by BLAST in hemocytomes not only from ticks (Kotsyfakis et al., 2015), but also from *Drosophila melanogaster* (BioProject database at NCBI, no. PRJEB33170).

In addition to coagulation, innate immunity processes are also present in the tick hemolymph. These processes contribute to the protection of ticks from pathogens and thus, are important factors in determining vector competence (Hajdušek et al., 2013). Several inhibitors of serine proteases have been reported to control the innate immune response in tick hemolymph, either by direct antimicrobial activity (Fogaça et al., 2006) or by a more complex role in arthropod immune response (Kopacek et al., 2012; Blisnick et al., 2017). Although serpins have not been

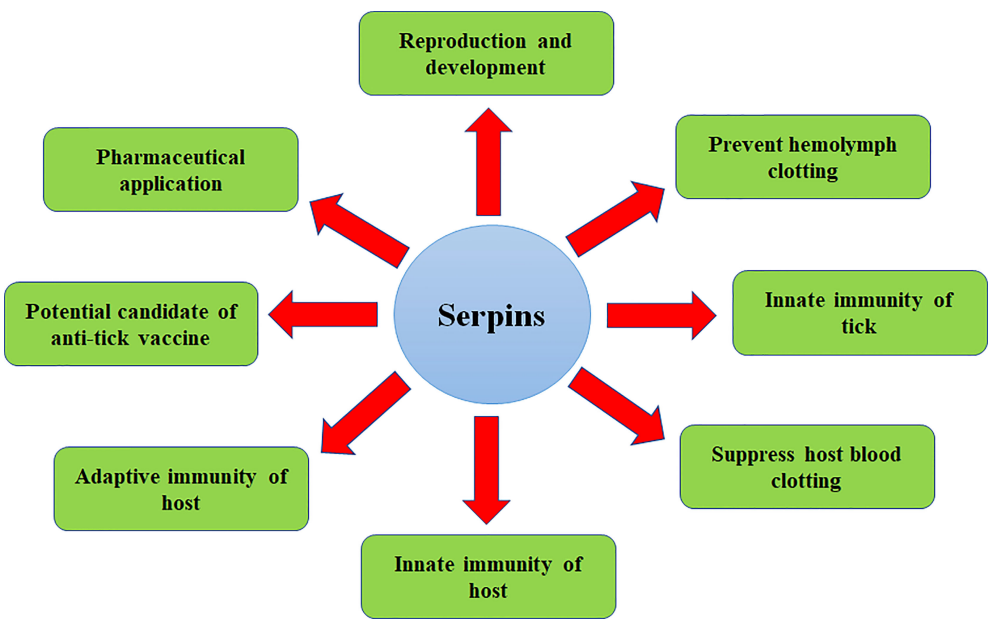

FIGURE 2 | An overview of various biological processes that are regulated by serpins.

experimentally proven to be involved in the tick immune response, their role in arthropod defense system was shown in *Anopheles stephensi*, in which serpin AsSRPN6 expression was induced by common microbiota bacterium *Enterobacter cloacae* and this correlated with inhibited development of *Plasmodium berghei* (Eappen et al., 2013). Thus, serpins can affect the composition of arthropod microbiota, which has direct implication in the defense against pathogens. Moreover, serpins are directly involved in the regulation of intracellular immune pathways, such as Toll pathway or myeloperoxidase production (Meekins et al., 2017). However, the main role of serpins in tick hemolymph appears to be in the regulation of proteolytic cascades, such as clot formation, rather than in the immune response *per se*.

3.2 Serpins as Regulators of Tick Reproduction

Another process related to tick physiology in which serpins play a role is oviposition. Serpins appear to be involved in tick reproduction alongside other key proteins such as vitellogenin or lipophorin (Tufail and Takeda, 2009). To date, many serpins have been identified as highly expressed in tick eggs and larvae (Andreotti et al., 2001; Sasaki et al., 2004). For example, the serpin RmS-3 is transcribed in the ovaries of *R. microplus* (Rodriguez-Valle et al., 2012; Rodriguez-Valle et al., 2015). *In vitro* feeding assays revealed that female ticks fed with anti-RmS-3 sheep serum had reduced egg weight and larval hatching rates, suggesting that RmS-3 is likely to be involved in tick reproduction and egg development (Rodriguez-Valle et al., 2012). The *R. microplus* serpins RmS-6, RmS-19, and RmS-20 might also play a role in tick embryogenesis or vitellogenesis (Rodriguez-Valle et al., 2015). The *R. haemaphysaloides* serpin RHS8 appears to stabilize vitellogenin by inhibiting serine protease activity since the knockdown of this serpin caused a significant reduction of vitellogenin protein levels, impaired oocyte maturation, and reduced fecundity (Xu et al., 2020). Similar evidence of serpin involvement in tick reproduction has been observed in *H. longicornis* (Zhou et al., 2006b) and *A. americanum* (Kim et al., 2016) when analyzing the effects of serpins on tick reproduction and development by vaccination experiments against tick serpins or RNA interference targeting serpin genes in these ticks.

3.3 Serpins as Regulators of Blood Fluidity and Digestion in Tick Midgut

Tick serpins might also be involved in the regulation of blood fluidity and digestion in tick midgut. This claim is supported by the fact that many serpins, some of which are known to possess anti-coagulant activity, have been found to be expressed in the midgut of feeding ticks (see **Table 1**). However, these functions have not yet been experimentally demonstrated. By employing a transcriptomic approach, Tirloni and his co-workers identified a total of 22 serpins in *R. microplus* (Tirloni et al., 2014; Rodriguez-Valle et al., 2015) with some of them (e.g. RmS-1, RmS-19, RmS-20, and RmS-21) being expressed in both the salivary glands and midgut, suggesting that certain *R. microplus* serpins might maintain blood in a fluid state at both the feeding site and in tick midgut and could regulate the process of blood meal digestion. Likewise, many serpins have been found to be expressed in both the salivary glands and midgut of feeding *A. americanum* ticks (Mulenga et al., 2007; Porter et al., 2015), and the same also applies to some *I. scapularis* serpins (Bakshi et al., 2018). HLS-1, the serpin of the tick *H. longicornis*, was revealed to be expressed only in the midgut of partially-fed ticks and had anti-coagulant activity in the aPTT (Activated Partial Thromboplastin Time) assay, which indicates that this particular serpin might be involved in maintaining blood fluidity in the midgut (Sugino et al., 2003).

4 THE IMPORTANCE OF TICK SALIVARY SERPINS IN TICK-HOST INTERACTION

Saliva is a complex mixture of various peptidic and non-peptidic components that are crucial for successful tick attachment. There are many reviews on the effects of tick saliva (Kotál et al., 2015; Šimo et al., 2017) and its individual components (Kazimírová and Štibrániová, 2013), including serine protease inhibitors (Blisnick et al., 2017; Chmelař et al., 2017). Serpins target hemostasis and the innate and adaptive branches of the host immune system. In the following sections, we will focus on the role of serpins in tick attachment success and how they modulate host immunity.

4.1 Tick Serpins Inhibit Host Hemostasis

4.1.1 Host Hemostatic Response Against Tick Feeding

The first battle that a feeding tick must win is the battle against host hemostasis, a complex of host defense mechanisms that respond immediately to prevent blood loss from the physical injury caused by the tick mouthparts (once intruded into the host skin). Host hemostasis consists of vasoconstriction, plasma coagulation, and platelet aggregation. A number of cellular and biochemical processes take place in response to injury (LaPelusa and Dave, 2022). More specifically, after the resulting injury of the vascular epithelium, extrinsic clotting signaling is activated as epithelial cells begin to produce Tissue Factor (TF) to induce the clotting process. Tissue Factor interacts with pre-existing factor VIIa to form the TF-VIIa complex, which causes the cleavage of factor X. Factor XII activates a second intrinsic pathway in which high molecular weight kininogen and prekallikrein (PK) stimulate the cleavage of factors XI, IX, and the formation of the factor IXa-VIIIa complex, and the cascade ends with cleavage of factor X. Based on the above, it is clear that the activation cleavage of factor X to Xa is the target site of both coagulation pathways. The final product of both pathways is factor Xa, which binds to its cofactor Va and induces the prothrombinase complex. Finally, the factor Xa-Va complex converts factor II (prothrombin) to factor IIa (thrombin), which converts fibrinogen to fibrin and induces blood clotting (Jagadeeswaran et al., 2005; Kim et al., 2009).

Another process in hemostasis is platelet aggregation, which is an essential part of vertebrate defense against injury (Chmelar et al., 2011). Platelets are activated by contact with the extracellular matrix, which contains large amounts of adhesive macromolecules such as collagens and fibronectin (Jackson and Schoenwaelder, 2003; Furie and Furie, 2005; Watson et al., 2005). A number of surface protein interactions lead to the binding of the platelet GPVI receptor to collagen (Jandrot-Perrus et al., 2000). This causes integrins (e.g., α2β1) to switch to a high-affinity state, allowing them to mediate tight platelet adhesion to collagen while promoting the release of TXA2 and ADP, which are pro-inflammatory mediators (Jackson and Schoenwaelder, 2003; Furie and Furie, 2005; Watson et al., 2005). Vasoconstriction is the third hemostatic process mediated by smooth muscle cells and it is controlled by the vascular endothelium. Endothelial cells release molecules such as endothelin that control contractile properties of the blood vessels. Damaged blood vessels constrict to limit the amount of blood loss and the extent of bleeding. The presence of collagen exposed at the site of the damaged blood vessel promotes platelet adhesion. Salivary gland extract has been shown to impair vasoconstriction (Charkoudian, 2010; Pekáriková et al., 2015).

4.1.2 Tick Serpins Target Host Blood Coagulation Factors

Ticks have developed a variety of molecules that they inject into the host *via* saliva to stop blood clotting (Chmelar et al., 2012). Since coagulation is a cascade of serine protease-dependent activations, inhibitors of serine proteases, including serpins, are the major regulatory factors involved in this process. In this section, we will discuss the molecular mechanisms that serpins use to inhibit blood clotting and to facilitate blood feeding (**Figure 3** and **Table 1**).

4.1.2.1 Tick Serpins Interact With Host Thrombin

In vertebrates, thrombin is the main coagulation enzyme that catalyzes the conversion of fibrinogen to fibrin. Tick serpins are key regulators of this enzyme, as they control the balance between active and inactive thrombin. In ticks (but also in other hematophagous species), many thrombin inhibitors have evolved from different protein families, including serpins.

Of several serpins described and isolated from *R. microplus* (Rodriguez-Valle et al., 2015), only RmS-15 was found to substantially inhibit thrombin activity, as demonstrated by detailed enzymatic analysis (Xu et al., 2016). In addition, plasma clotting increased in the absence of serpin RmS-15, and higher titers of IgG antibodies to RmS-15 were detected in bovine serum after prolonged exposure to *R. microplus* challenge, suggesting its presence in tick saliva and its high immunogenicity (Rodriguez-Valle et al., 2015; Xu et al., 2016). The serpin RHS-1, which was identified from the closely related species *R. haemaphysaloides*, displayed strong expression in the salivary glands of fed ticks and inhibited chymotrypsin and thrombin activity *in vitro* (Yu et al., 2013). Consistent with its capacity to inhibit thrombin, RHS-1 prolonged plasma clotting time in the aPTT assay (Yu et al., 2013). These data suggest that RHS-1 may be involved in the inhibition of blood coagulation. Similarly, IxscS-1E1 is produced in both the salivary glands and midgut of *I. scapularis*, and its expression is increased after the first 24 h of tick feeding (Mulenga et al., 2009; Ibelli et al., 2014). This serpin formed stable complexes with thrombin and trypsin,

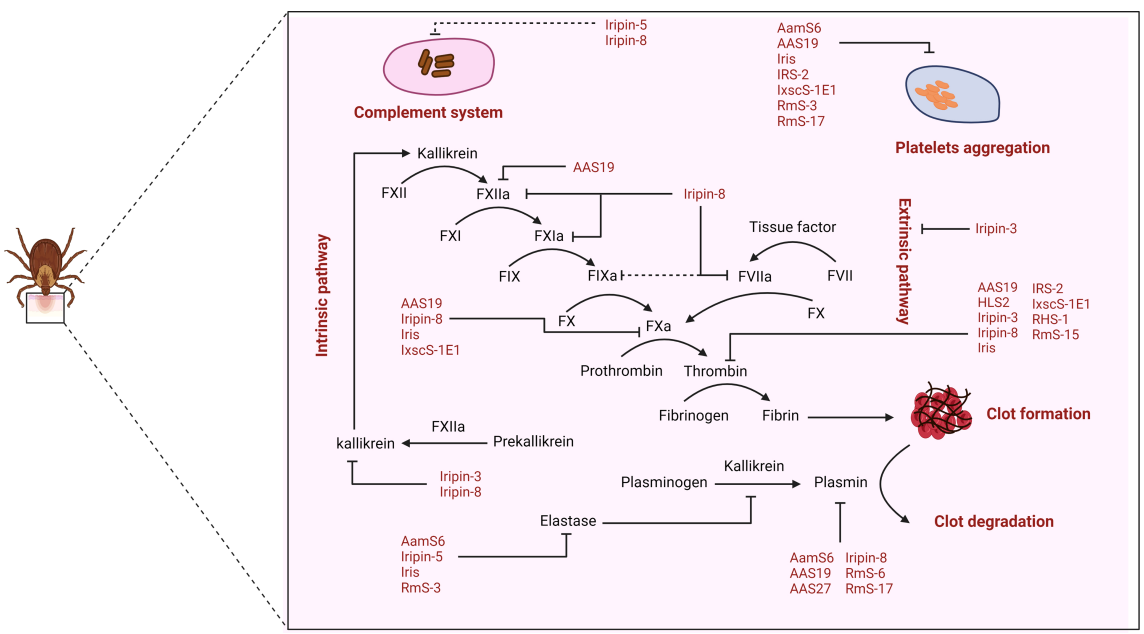

FIGURE 3 | The role of tick salivary serpins in the regulation of host hemostasis and complement.

inhibited platelet aggregation, and prolonged plasma clotting time, as demonstrated by *in vitro* experiments (Ibelli et al., 2014). The serpin Iripin-8 from *I. ricinus* also inhibited thrombin and other proteases of the coagulation cascade and it has been shown to be a potent inhibitor of the intrinsic and common pathways of the coagulation cascade, as evidenced by aPTT and TT (Thrombin Time) assays (Kotál et al., 2021). Other, rather weak inhibitors of thrombin from the same tick species are Iris (Prevot et al., 2006), IRS-2 (Chmelar et al., 2011), and Iripin-3 (Chlastáková et al., 2021). However, additional data on these serpins suggest a role other than anticoagulation.

4.1.2.2 Tick Serpins Regulate Host Blood Coagulation via Inhibition of FX(A) and Other Blood Clotting Factors

Activated factor X (FXa) is a central enzyme of coagulation that stands at the intersection of both coagulation activation pathways and is responsible for the activation of thrombin (Borensztajn et al., 2008). To date, several FX(a) inhibitors, including serpins, have been described in various tick species. In 2002, the first serpin named Iris (*Ixodes ricinus* immunosuppressor) was isolated from the tick *I. ricinus* (Leboulle et al., 2002b). Besides other immunomodulatory effects and the aforementioned inhibition of thrombin, Iris inhibited factor FXa in a dose-dependent manner and with higher specificity than thrombin (Prevot et al., 2006). Serpin Iripin-8 also inhibits factor FXa and other proteases of the coagulation cascade, including factors fVIIa, fIXa, fXIa, fXIIa, APC (activated protein C), kallikrein, and thrombin, demonstrating that it is an inhibitor of coagulation by targeting many different host enzymes at the same time (Kotál et al., 2021). Iripin-3 has also been shown to block coagulation, but only the extrinsic pathway. Thus, Iripin-3 was the first tick serpin to inhibit this type of coagulation activation (Chlastáková et al., 2021). The serpin AAS19, which was originally identified by RNA sequencing of *A. americanum* and is expressed in the salivary glands and midgut during tick feeding (Porter et al., 2015), was found to be able to inhibit a wide range of proteases of the coagulation cascade, such as FXa and FXIa. Reduced activity of the same serpin was also reported against FXIIa, FIXa and thrombin (Kim et al., 2015).

4.1.2.3 Inhibition of Fibrinolysis by Tick Serpins

Fibrinolysis is a highly regulated enzymatic process that prevents the unnecessary accumulation of intravascular fibrin and enables the removal of thrombi (Chapin and Hajjar, 2015). The cleavage of insoluble fibrin polymers into soluble fibrin degradation products is mediated by plasmin that is generated from the zymogen plasminogen by either tissue-type plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA) (Schaller and Gerber, 2011; Chapin and Hajjar, 2015). Plasmin, tPA, and uPA are serine proteases whose enzymatic activity is commonly regulated by serpins, such as plasminogen activator inhibitor-1, plasminogen activator inhibitor-2, and α2-antiplasmin (Schaller and Gerber, 2011; Chapin and Hajjar, 2015). Some tick serpins, e.g. *A. americanum* serpins AAS19 and AAS27 (Kim et al., 2015; Tirloni et al., 2019). *I. ricinus* serpin Iripin-8 (Kotál et al., 2021), and *R. microplus* serpin RmS-17 (Tirloni et al., 2016), reduced the proteolytic activity of plasmin *in vitro*; however, their effect on fibrinolysis has not been tested. The only tick serpin that has been shown to inhibit fibrinolysis thus far is Iris derived from the tick *I. ricinus* (Prevot et al., 2006). The anti-fibrinolytic effect of Iris is probably mediated though its ability to inhibit tPA since Iris devoid of any anti-protease activity due to a mutated RCL did not significantly affect fibrinolysis time (Prevot et al., 2006). Even though tick serpins can reduce the enzymatic activity of plasmin and tPA, the inhibition of fibrin clot dissolution makes no sense in the context of blood feeding since it is in tick's best interest to maintain host blood in a fluid state both at the feeding site and in tick midgut. However, beyond fibrinolysis, plasmin is also involved in the inflammatory response (Syrovets et al., 2012), as described later in this review in the section 4.2.2., dedicated to the effects of tick serpins on host inflammation. Unlike the aforementioned inhibition of fibrin clot dissolution, attenuation of inflammation by targeting plasmin might be beneficial for feeding ticks.

4.1.2.4 Tick Serpins and Their Interaction With Glycosaminoglycans

The inhibitory activity of some serpins involved in the regulation of blood coagulation and fibrinolysis can be altered by their interaction with glycosaminoglycans (GAGs), such as heparin or heparan sulfate (Gettins, 2002; Huntington, 2003; Rau et al., 2007). GAGs can influence the anti-proteolytic activity of serpins in two ways. First, they can simultaneously bind both the serpin and the protease, bringing them together in an appropriate orientation for the productive interaction of the serpin's RCL with the protease active site (Gettins, 2002). Second, GAGs binding to the serpin can lead to the alteration of the serpin conformation to one in which the serpin is more reactive toward the target protease (Gettins, 2002). The *A. americanum* serpin AAS19 has four predicted GAG-binding sites on its surface, suggesting it could be responsive to GAGs (Kim et al., 2015). Indeed, binding of heparan sulfate/heparin to AAS19 caused pronounced changes in the inhibitory profile of the serpin in that AAS19 inhibitory activity was significantly increased against thrombin and FIXa and was considerably reduced against FXa and FXIIa. Overall, AAS19 interaction with GAGs enhanced the capacity of this serpin to suppress the coagulation cascade (Radulović and Mulenga, 2017). It is likely that this observation is just an example of how glycosaminoglycans are involved in the regulation of tick serpins activity and more examples would be found if we focused in that direction.

4.1.3 Platelet Aggregation and Tick Serpins

Platelet aggregation is necessary for the formation of hemostatic plugs. It is a complex and dynamic multistep adhesion process involving various receptors and adhesion molecules, especially integrins (Jackson, 2007; Li et al., 2012). Importantly, platelet aggregation can be triggered by certain serine proteases, such as cathepsin G and thrombin. Cathepsin G, which is released by activated neutrophils, can induce platelet aggregation through the activation of protease-activated receptor-4 (PAR-4) (Sambrano et al., 2000), and blood clotting factor thrombin

can trigger platelet aggregation by activating PAR-1 and PAR-4 (Lisman et al., 2005). Tick serpins that were shown to reduce the enzymatic activity of cathepsin G and/or thrombin, such as *A. americanum* serpin AAS19 (Kim et al., 2015), *I. ricinus* serpin IRS-2 (Chmelar et al., 2011), *I. scapularis* serpin IxscS-1E1 (Ibelli et al., 2014), or *R. microplus* serpins RmS-3 and RmS-17 (Tirloni et al., 2016) inhibited *in vitro* platelet aggregation triggered by these two serine proteases (see **Table 1**). This suggests that tick serpins can suppress primary hemostasis through their capacity to inhibit serine proteases involved in the activation of platelet aggregation. However, the inhibitory effect of some tick serpins on platelet plug formation might be independent of their anti-proteolytic activity. For example, the RCL mutants of the serpin Iris from the tick *I. ricinus* lost their anticoagulant activity but still managed to inhibit platelet adhesion (Prevot et al., 2006). As discussed in this particular study, serpins may interact *via* exosites with other proteins such as von Willebrand factor and integrins to block platelet adhesion on endothelial cells (Prevot et al., 2006; Berber et al., 2014). Overall, tick serpins appear to have an important role in inhibiting platelet adhesion, thus blocking the specific host response to tick feeding, but other salivary protein families are also known to mediate the same effect.

4.2 Tick Serpins Regulate Host Innate Immunity

Injury caused by a tick hypostome, together with concomitant and/or transmitted infections, induces a host immune response, which begins with the activation of pattern recognition receptors (PRRs) by pathogen- or danger-associated molecular patterns (PAMPs or DAMPs). Activated resident cells begin to produce cytokines and chemokines that recruit from the bloodstream to the site of injury/infection various innate immune cells, such as neutrophils and monocytes. Complement activation further amplifies the local inflammatory response. The feeding period, which extends to several days in Ixodidae, provides sufficient time for the development of adaptive immunity, which includes both humoral and cellular branches. To prevent rejection by the host, ticks use a mixture of pharmacologically active molecules at the site of injury to manipulate all types of host immune responses. Many excellent and thorough review articles have been published describing both the immune response against tick attachment and the effects of tick saliva or of individual salivary compounds on the host immune system (Hovius et al., 2008; Francischetti et al., 2009; Kazimírová and Štibrániová, 2013; Kotál et al., 2015; Chmelař et al., 2016; Chmelař et al., 2017; Kazimírová et al., 2017; Šimo et al., 2017; Chmelař et al., 2019; Wen et al., 2019; Aounallah et al., 2020; Martins et al., 2020; Fogaça et al., 2021; Jmel et al., 2021; Narasimhan et al., 2021; Wikel, 2021; Wang and Cull, 2022). In the following section, we discuss how tick salivary serpins contribute to the evasion of immunity-mediated host defense mechanisms – both innate (**Figure 4**) and adaptive (**Figure 5**).

4.2.1 Tick Serpins and Host Complement

The vertebrate complement system enhances the ability of phagocytic cells to remove microbial pathogens and damaged cells by opsonization, by promoting inflammation and by directly attacking cell membrane components of pathogens (Kimura et al., 2009; Cagliani et al., 2016). Tick saliva and its protein components possess anti-complement activity, which has been reported in several publications (Schroeder et al.,

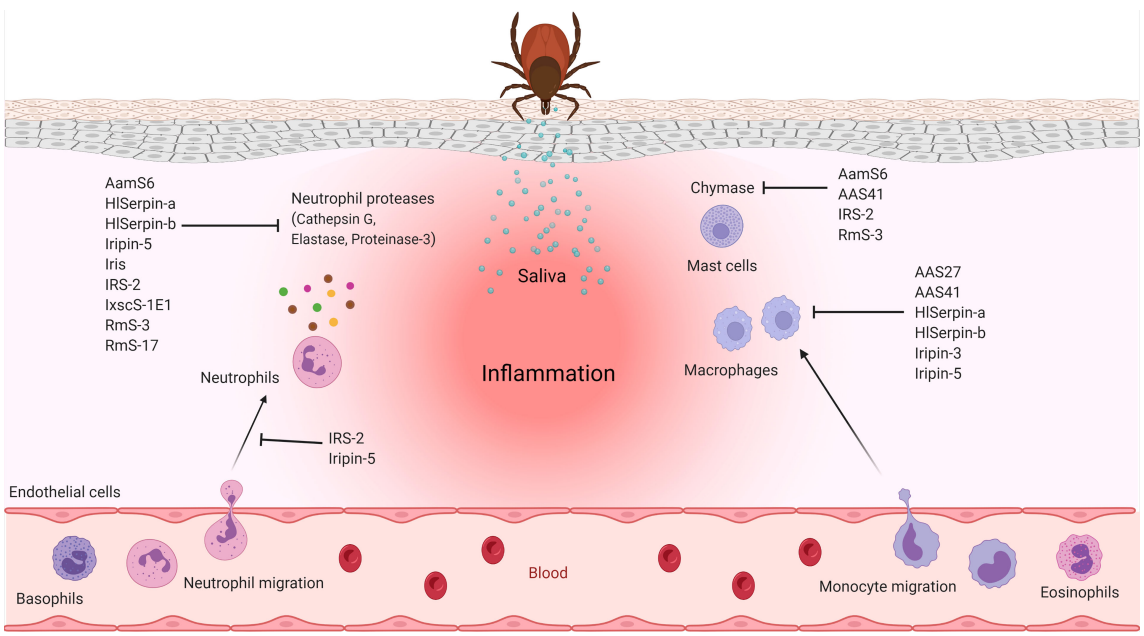

FIGURE 4 | Anti-inflammatory activities of tick salivary serpins.

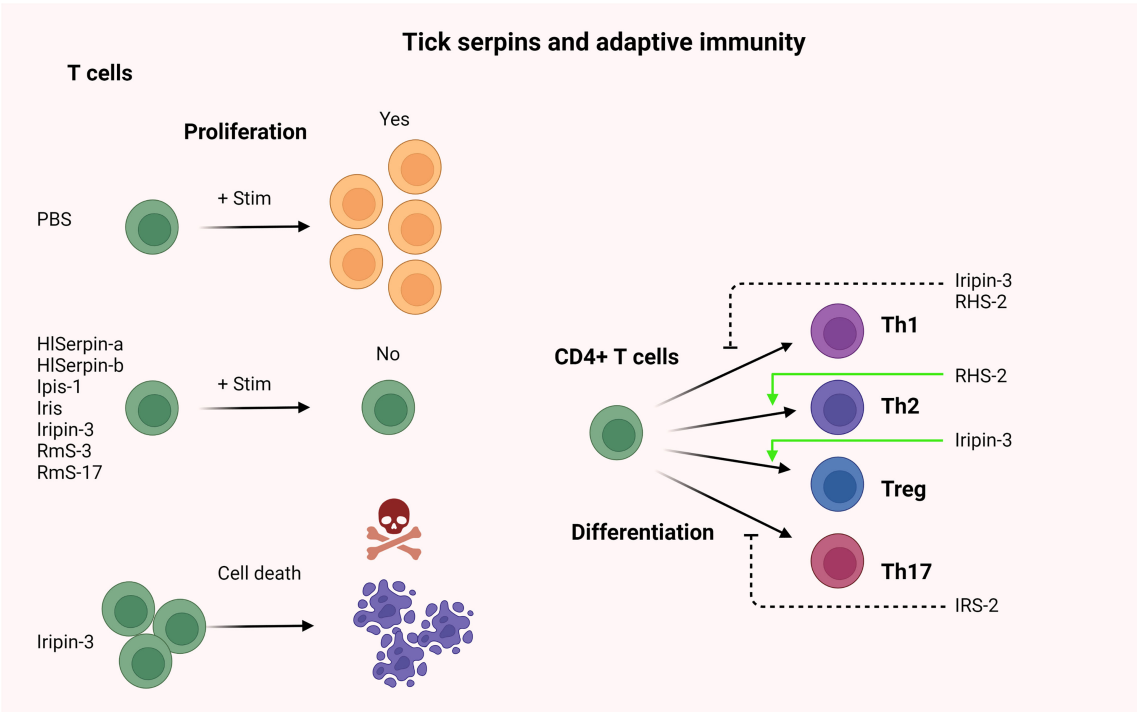

FIGURE 5 | The role of tick serpins in the modulation of vertebrates host adaptive immune system.

2009; Miller et al., 2011; Cong et al., 2013; Wikel, 2013). So far, a number of anti-complement proteins have been discovered in the saliva of several tick species. A well-characterized complement inhibitor that binds the C5 component and thereby inhibits its activation by C5 convertase has been isolated from the soft tick *Ornithodoros moubata* (Fredslund et al., 2008). It inhibited complement-mediated hemolytic activity as well as the development of pathological features in a rodent model of myasthenia gravis (Hepburn et al., 2007). Other tick complement inhibitors, such as Isac, Irac-1, and -2, and Salp20, belong to the ISAC/IRAC family of proteins and inhibit the alternative complement pathway by binding and displacing properdin, thereby inhibiting C3 convertase production (Valenzuela et al., 2000; Schroeder et al., 2007; Tyson et al., 2007).

Recently (and for the first time in ticks), anti-complement activities have been described for two *I. ricinus* serpins, namely Iripin-5 and Iripin-8 (Kascakova et al., 2021; Kotál et al., 2021), and their anti-complement activity was comparable to that of vertebrates serpins (Bos et al., 2002; Kascakova et al., 2021; Kotál et al., 2021). Iripin-5 has a dose-dependent inhibitory activity against complement system, as evidenced by a decrease in erythrocyte lysis when incubated with increasing concentrations of Iripin-5 (Kascakova et al., 2021). Iripin-8 serpin exhibited a similar effect, but approximately 10-fold weaker anti-complement activity when compared to Iripin-5 (Kotál et al., 2021). In summary, these findings suggest that tick serpins may also be involved in complement inhibition at the tick attachment site. However, further studies would be required to unravel the molecular mechanism by which these serpins regulate the complement cascade.

4.2.2 Tick Serpins and Host Inflammation

The role of serpins in the regulation of inflammation is well known because the most abundant serpin in human serum is alpha-1-antitrypsin, which is a major protective factor against the damaging effects of neutrophil elastase (Mangan et al., 2008; Yaron et al., 2021). Other human serpins, such as antichymotrypsin, also have an anti-inflammatory function. Not surprisingly, many serpins from tick saliva exhibit anti-inflammatory effects in both *in vitro* and *in vivo* experiments. These activities are thought to result from their inhibitory specificity towards important pro-inflammatory proteases such as neutrophil elastase, cathepsin G, plasmin or chymase.

Plasmin is a key protease in hemostasis, particularly in fibrinolysis, but it is also involved in the development of the inflammatory response by playing a major role in producing proinflammatory cytokines, in regulating monocyte and dendritic cell chemotaxis, and in attracting neutrophils to the site of inflammation (Syrovets et al., 2012). Several tick serpins inhibited plasmin, but the association between this inhibition and the observed anti-inflammatory phenotype has not been directly demonstrated. Antiplasmin specificity has been observed in serpins from *A. americanum* - AamS6, AAS19, AAS27 (Chalaire et al., 2011; Syrovets et al., 2012; Mulenga et al., 2013; Kim et al., 2015; Bakshi et al., 2019). In a recent study,

the serpin AAS27 was found to have a peak of expression at 24 h after tick attachment and formed SDS-stable irreversible complexes with trypsin and plasmin and blocked both formalin- and compound 48/80-induced inflammation in rats. Thus, AAS27 appears to be an anti-inflammatory protein, but the causal link to plasmin inhibition is not yet demonstrated (Tirloni et al., 2019). The most potent plasmin inhibitor among tick serpins so far is *I. ricinus* serpin Iripin-8 (Kotál et al., 2021), which however showed no immunomodulatory or anti-inflammatory effect in several assays.

Neutrophil elastase is one of the four neutrophil serine proteases with a key role in killing bacteria and in activating inflammatory mediators. Its inhibition should be beneficial to ticks. *I. ricinus* serpin Iris inhibited several elastase-like proteases, including leukocyte and pancreatic elastase, and also exhibited anti-inflammatory effects, but these were explained by exosite activity (Leboulle et al., 2002a; Prevot et al., 2006; Prevot et al., 2009). Another elastase inhibitor from *I. ricinus* is Iripin-5, which affects neutrophil migration, decreases nitric oxide production by macrophages, and modifies complement function, thus exhibiting potent anti-inflammatory activity (Kascakova et al., 2021). Anti-elastase activity was described for the other two tick serpins, namely AamS6 and RmS-3 (Chalaire et al., 2011; Syrovets et al., 2012; Mulenga et al., 2013; Pongprayoon et al., 2021).

Under normal physiological functions, mast cells are known to regulate vasodilation, vascular homeostasis, innate and adaptive immune responses, and angiogenesis (Krystel-Whittemore et al., 2016). Large granules in the cytoplasm of mast cells store inflammatory mediators, including histamine, heparin, a variety of cytokines, chondroitin sulfate, and neutral proteases, like chymase and tryptase (Moon et al., 2014). Cathepsin G and chymase, which are produced after mast cell activation, regulate the acute inflammatory response, particularly during the cross-talk of IL-2 between neutrophils and platelets (Zarbock et al., 2007). These proteases are often targeted by tick serpins, indicating their importance in host defense against tick feeding. Mast cell chymase affects inflammation at multiple levels, including cleavage of proinflammatory cytokines/chemokines and activation of protease-activated receptor 2, degradation of endothelial cell contacts, activation of extracellular matrix-degrading enzymes, and recruitment of eosinophils/neutrophils (Pejler et al., 2010). Serpin RmS-3 from *R. microplus* tick saliva inhibited rMCP-1, the major chymase produced by rat connective tissue-type mast cells in the peritoneum (Coutinho et al., 2020). It has also been shown that serpin RmS-3 reduces vascular permeability stimulated by compound 48/80, which can cause degranulation of plantar-type mast cells, thermal hyperalgesia, tissue edema, and neutrophil infiltration (Chatterjea et al., 2012). Thus, RmS-3 may be a key component in modulating the early steps of inflammatory reactions by blocking the chymase which is generated during mast cell activation (Coutinho et al., 2020). Chymase also appears to be crucial for the degradation of tick anticoagulants, so its inhibition should help the tick to maintain blood fluidity (Fu et al., 2021). A recent study showed that the serpin IRS-2 of *I. ricinus* can inactivate almost all connective tissue chymases from a range of animals, including human, hamster, rat, dog, and opossum, as well as mucosal mast cell proteases, rat blood vessel chymases, and also neutrophil proteases. However, this serpin fails to inactivate mast cell tryptases and the basophil-specific protease mMCP-8 (Fu et al., 2021). The first study of the tick serpin IRS-2 disclosed the protein as having a preferential specificity for chymase and cathepsin G and as having a significant anti-inflammatory effect *in vivo* by reducing swelling and neutrophil migration into inflamed tissues, while a later study showed that IRS-2 reduced spirochete *Borrelia burgdorferi*-induced IL-6 production in splenic dendritic cells (Chmelar et al., 2011; Páleníková et al., 2015). Moreover, IRS-2 impaired the development of proinflammatory Th17 cells by reducing STAT-3 phosphorylation (Páleníková et al., 2015). Overall, by inhibiting mast cell chymase, IRS-2 can affect host inflammatory response against tick feeding.

4.2.3 Tick Serpins and Host Cytokines

Cytokines play a central role in the communication between host immune cells, in their differentiation and maturation, and in the overall control of the immune response. Tick serpins altered the production of various cytokines in many experiments, modulating the immune response, mostly from a pro-inflammatory to an anti-inflammatory direction.

Haemaphysalis longicornis serpins HlSerpin-a and HlSerpin-b can suppress the expression of pro-inflammatory cytokines such as TNF-α, interleukin (IL)-6, and IL-1β from lipopolysaccharide (LPS)-induced mouse bone marrow-derived macrophages or mouse bone marrow-derived dendritic cells (BMDCs) (Wang et al., 2020). Furthermore, this study demonstrated that cathepsins B and G are required for sufficient LPS stimulated activation of mouse macrophages (Wang et al., 2020). This suggests that tick serpins may use their protease inhibitory activities to suppress the activation of host immune cells.

In addition, two serpins from *A. americanum* (AAS27 and AAS41) were shown to regulate evasion of host immune response by altering host cytokine secretion (Bakshi et al., 2019). Based on the results of this study, it seems that *A. americanum* saliva proteins can be divided into two groups, those with LPS-like activity causing the expression of pro-inflammatory (PI) markers by macrophages and those that suppress the expression of pro-inflammatory markers in activated macrophages. The PI group included the insulin-like growth factor binding-related proteins (AamIGFBP-P6S, AamIGFBP-P1, and AamIGFBP-P6L). These PI recombinant proteins could stimulate PBMC (peripheral blood mononuclear cell) derived macrophages and mouse RAW 267.4 macrophage lineage *in vitro*. Following this activation, PI co-stimulatory markers, such as CD40, CD80, and CD86, and cytokines (e.g. TNF-α, IL-1, and IL-6) were produced by these macrophages. In contrast, *A. americanum* tick salivary anti-inflammatory (AI) serpins, including AAS27 and AAS41, did not affect cytokine expression or PI markers production by macrophages. However, AI serpins could enhance the expression of AI cytokines (TGFβ and IL-10) in macrophages pre-activated by LPS or PI recombinant proteins. In addition, the injection of PI-tick

salivary proteins (individually or as a cocktail) into mice induced paw edema *in vivo*, resulting in increased levels of CD40, CD80, CD86, IL-1, TNF-α, IL-6, and chemokines (CCL2, CXCL1, CCL3, CCL5, and CCL11). In comparison, the AI serpins AAS27 and AAS41 (cocktail and individually) suppressed the activation of host immune cells. Overall, PI proteins activated host immune cells towards the production of pro-inflammatory cytokines, whereas AI serpins inhibited such production, implying that ticks may use a combination of PI and AI proteins to evade host immune defenses (Bakshi et al., 2019).

4.3 Tick Serpins Regulate Host Adaptive Immunity

Vertebrates are the only group with "Darwinian" type of adaptive immunity (Muller et al., 2018). This type of immunity is based on a large number of pre-formed clones with a wide range of specificities, which is able to further increase its accuracy in response to antigens. In anti-tick immunity, the adaptive branch plays a role, especially later during the feeding course in the case of primary exposure to ticks, but also earlier in the case of repeated tick infestation on the same host. During this process, a plethora of cytokines is released, each of which is responsible for steering towards distinct types of immune responses. Pro-inflammatory response mediated by Th1 cells have a crucial role in the defense against pathogen infection and is deleterious also for tick feeding (Raphael et al., 2015; Hirahara and Nakayama, 2016; Duan et al., 2019; Ng et al., 2021).

Several tick serpins were shown to modulate adaptive immunity (**Figure 5**), affecting mostly CD4+ T cell proliferation, survival, and differentiation to T cell subpopulations, but also the production of many cytokines. Iripin-3 from *I. ricinus* disrupted the survival and proliferation of CD4+ T cells; moreover, it suppressed the differentiation of T helper type into pro-inflammatory Th1 cells and promoted the differentiation into T regulatory cells (Chlastáková et al., 2021). Finally, the same study showed that Iripin-3 reduced the generation of the pro-inflammatory cytokine interleukin-6 by bone marrow-derived macrophages activated with LPS. Thus, Iripin-3 appears to be another pluripotent salivary serpin with immunomodulatory and anti-hemostatic properties that can facilitate tick feeding by suppressing host anti-tick immune reaction (Chlastáková et al., 2021). Some of these observations are similar to those with Iris, which also suppressed CD4+ T cell proliferation and the production of pro-inflammatory cytokines IFN-γ, IL-6, TNF-α, and IL-8 (Leboulle et al., 2002a). Dendritic cells play crucial role in the adaptive immunity as they can affect, which direction the immune response will proceed. Ticks can alter the biology of dendritic cells as described previously (Sa-Nunes and Oliveira, 2021). *R. haemaphysaloides* derived serpin RHS-2 blocked the differentiation of bone marrow-derived cells into dendritic cells while promoting the differentiation of these cells into macrophages. RHS-2 also inhibited dendritic cell maturation and the expression of CD80, CD86, and MHC-II. Moreover, this serpin suppressed the differentiation of Th1 cells, as evidenced by decreased production of the cytokines IL-2, IFN-γ, and TNF-α (Xu et al., 2019). The serpin Ipis-1 has been shown to be expressed in the salivary glands of unfed and feeding *Ixodes persulcatus* ticks and was reported to be associated with immunomodulatory effects on the acquired immune responses (Toyomane et al., 2016). More specifically, Ipis-1 inhibited the proliferation of bovine peripheral blood mononuclear cells (PBMCs) and IFN-γ production (Toyomane et al., 2016). However, the precise molecular mechanism behind the aforementioned Ipis-1 inhibitory activities is not known (Toyomane et al., 2016).

The immune cells that have been activated acquire additional biological roles such as cytokine production, proliferation, and chemotaxis (Moro-García et al., 2018; Zhang et al., 2020). A recent study analyzed the ability of *R. microplus* serpins RmS-3, RmS-6, and RmS-17 to reduce the metabolic activity of splenocytes and the production of the cytokine IFN-γ (Coutinho et al., 2020). This study showed that in the presence of 1 μM RmS-3, concanavalin A (ConA)-stimulated spleen cells displayed a partial decrease in their metabolic activity, whereas RmS-6 had no impact on the metabolic activity of these cells (Coutinho et al., 2020). RmS-17 serpin also lowered the metabolic activity of ConA-stimulated spleen cells in a dose-dependent manner, with a substantial effect at 300 nM and 1 μM concentrations (Coutinho et al., 2020). IFN-γ production in ConA-stimulated splenocytes treated with *R. microplus* serpins followed similar patterns. RmS-3 used at 1 μM concentration partially inhibited IFN-γ production, RmS-6 did not modify it, and RmS-17 strongly inhibited IFN-γ production at both 300 nM and 1 μM concentrations (Coutinho et al., 2020). The authors of the same study also investigated the effects of these three serpins on the proliferation of T lymphocytes. They showed that naïve T lymphocytes did not proliferate when incubated with medium or in the presence of RmS-3, RmS-6, and RmS-17 serpins alone. Under suboptimal activation conditions, T lymphocytes exhibited weak proliferation, which was partially inhibited in the presence of RmS-3, not affected by RmS-6, and completely inhibited by RmS-17 (Coutinho et al., 2020). However, under optimal activation conditions, RmS-3 and RmS-6 had no significant effect on the robust proliferation of T lymphocytes, and RmS-17 managed to inhibit T cell proliferation only partially (Coutinho et al., 2020). Overall, it seems that some tick serpins can suppress T cell proliferation and IFN-γ production to produce optimal conditions for tick feeding on vertebrate hosts. However, more research is needed to better understand this phenomenon and its molecular mechanism.

5 TICK SERPINS ARE PROMISING MOLECULES FOR THERAPEUTICS DEVELOPMENT

The presence of swollen joints indicates that there is an increase in the amount of fluid in the tissues around the joints. People who suffer from different types of arthritis, infections, and injuries may have swollen joints. A recent study has shown that tick serpins can also be used as a substance to treat these ailments. However, full-length serpins, which contain about 400

amino acids, have a number of disadvantages for use in drug development (Wang et al., 2020). The reactive center loop of serpins is the main inhibitory region that directly binds to serine proteases (Huntington et al., 2000; Whisstock et al., 2010), but without a conserved tertiary structure, the inhibitory potential of RCL should be lost. In a rather surprising study, Wang and co-workers synthesized a peptide corresponding to the RCL of HlSerpin-a from *H. longicornis* (Wang et al., 2020). The authors suggested that the minimal active region (i.e. RCL) of this tick serpin has similar inhibitory activity and immunosuppressive properties as the whole serpin. In a mouse arthritis model, the RCL peptide derived from HlSerpin-a substantially impaired cytokine production from immune cells and alleviated joint swelling and tissue inflammation. This preliminary observation surprisingly suggests that the RCL of a functional tick serpin could be used as a drug, because of its non-immunogenic nature due to small size and easy synthesis (Wang et al., 2020).

6 TICK SERPINS AS EPITOPES FOR ANTI-TICK VACCINE DEVELOPMENT

Ticks are effective vectors of a variety of viral and bacterial diseases in vertebrates. Therefore, ticks are studied extensively all over the world in order to develop management strategies to control them or to immunize vertebrate hosts against ticks. Some pesticides (e.g., acaricides) are routinely used to control tick populations (Nwanade et al., 2020). However, pesticides drastic impacts on non-target species, the evolution of resistant tick populations, and the resulting environment hazard are the major concerns against the use of pesticides (Nwanade et al., 2020). Researchers around the globe are attempting to develop environmentally friendly and sustainable strategies to control ticks. For example, the development of a vector-specific vaccine may immunize (and protect) the vertebrate hosts but also may have a detrimental influence on tick population growth in the areas where (immunized) host activity is localized. Many laboratories work on the potential development of vaccines that would use tick-derived epitopes. These vaccines should be effective in tick control while simultaneously reducing the transmission of viral or bacterial pathogens (**Table 1**).

Many molecules have been tested as targets for the development of such vaccines. Serpins that are found in a wide range of animals, including ticks, appear to be promising targets. Imamura and colleagues injected a mixture of two recombinant serpins (RAS-1 and -2) from *R. appendiculatus* into cattle for the first time. Nymphs and adult ticks that fed on the cattle immunized by these two serpins had higher mortality rates, and the egg-laying capacity of the female ticks was also reduced when compared to the control group. However, the feeding time of the ticks was approximately identical on both the vaccinated and unvaccinated hosts (Imamura et al., 2006). Another salivary serpin, Iris from *I. ricinus*, was also examined as a potential anti-tick vaccine target. Prévot et al. administered recombinant Iris protein into mice and rabbits, but only rabbits developed anti-tick immunity as evidenced by higher mortality and lower weight gain in nymphs and by a prolonged feeding period and a higher mortality rate in adult females (Prevot et al., 2007). Most of the functionally characterized tick serpins, such as RmS-3, AAS41, and others, have been suggested as prospective vaccine candidates (Kim et al., 2020; Pongprayoon et al., 2021). However, the majority of these serpins have not been evaluated in vaccination experiments (see **Table 1**). Therefore, further investigations are required to advance the vaccine development process.

Even though it has been established that the administration of some serpins can improve the immunity of the host against ticks, the way to get considerably higher levels of protection is to produce vaccines based on multiple members of the serpin family. Individual differences in the expression of different members of the serpin family may make it possible to target a larger number of ticks. Another possibility is to prepare anti-tick cocktail vaccine by combining members of different protein families. For example, Imamura et al. immunized cattle with a combination of *R. appendiculatus* serpins RAS-3 and RAS-4 and a putative cement protein RIM36 (Imamura et al., 2008). The administration of this coctail vaccine led to an increased mortality of female ticks feeding on immunized cattle (Imamura et al., 2008). Moreover, immunization of a host with serpins conserved in many different tick species (such as *I. ricinus* serpin Iripin-8, *A. americanum* serpin AAS19, *R. microplus* serpin RmS-15, and *R. haemaphysaloides* serpin RHS8) might be a more efficient strategy than relying on the serpins present only in a small number of closely related tick species since the conservation of these serpins suggests they might play an important role in tick biology. It was suggested previously that tick salivary proteins undergo some kind of antigenic variation in order to escape from the recognition by host adaptive immune system and that there is a redundancy in salivary proteins functions (Chmelar et al., 2016). Therefore, in order to prepare an effective vaccine, conserved epitopes or the cocktail with multiple antigens should be used. An interesting opportunity came up from the lesson we learned about mRNA vaccines during the Covid19 pandemic. Recently a research group employed a mixture of mRNAs coding for tick salivary proteins as an anti-tick vaccine, and they observed very promising effects against the transmission of *B. burgdorferi* (Matias et al., 2021; Sajid et al., 2021). It will be interesting to observe the development of new types of anti-tick vaccines in this direction.

7 FUTURE PERSPECTIVES

The interactions between arthropod parasites such as ticks and their hosts have always been of interest. Ticks developed strategies to evade host defensive response in order to successfully complete a blood meal. Ticks serve as a reservoir of pathogens that are transmitted to the host during blood feeding. In recent years, advances in molecular techniques have made it possible to investigate the factors which mediate this

interaction, providing a much-needed impetus to unlock previously unattainable insights into this phenomenon. A better molecular understanding of this phenomenon will help in the development of methods to identify a subset of antigens that could be used as potential vaccine targets. Many of the serpins identified are involved in various biological processes in ticks. Serpins also play a role in the maintenance of blood fluidity by inhibiting thrombin, FXa, and other factors. They are also involved in controlling the innate and adaptive immune responses of the host. Several serpins have been shown to be effective candidates for enhancing host anti-tick immunity.

Serpins display multiple functions in various *in vitro* and *in vivo* experiments. Their functional characterization usually requires recombinant proteins. Fortunately, functional recombinant serpins are usually relatively easy to produce in large quantities in bacterial expression system. This system, however, does not take into account possible post-translational changes. The mechanisms behind the observed effects are usually not known for tick serpins and this is the direction we should focus on in future studies. Their inhibitory mechanism can be altered by point mutation of P1 site, thus the indispensability of inhibitory function of serpins can be tested. According to published data, serpin RCL alone can display interesting activity (Wang et al., 2020) and application potential. Since the function of serpins is mostly dependent on structural changes, structural analyses could be employed in mechanistic studies as well. Finally, serpins represent great material for protein engineering to gain novel functions, as shown both for inhibitory and non-inhibitory serpins (Chan et al., 2014; Polderdijk et al., 2017).

Serpins definitely have application potential in drug development. Inflammation is a symptom of a variety of diseases, and currently available therapies are limited. Researchers are looking for natural compounds with potent anti-inflammatory activities and novel chemical structures. Ticks and other blood feeding arthropods can be considered as a rich source of proteins with unique biological activities against vertebrate homeostasis. Tick serpins appear to be useful for treatment of inflammatory diseases (Wang et al., 2020). Although the data are rather preliminary to support drug development based on tick serpins, further research can help to identify other medically relevant serpins and to translate the laboratory studies into preclinical and clinical trials. Finally, there is some evidence to suggest serpins as potential candidates for vaccine development against ticks at least as a part of the vaccine cocktail.

AUTHOR CONTRIBUTIONS

MA, JC and MK designed the structure of the article. All authors performed the literature search and wrote parts of the manuscript/assembled the data. MA, AC and JC extracted the data and prepared the tables. MA, JC and AC created and edited the figures. MK made critical revisions and proofread the manuscript. All authors read and approved the final manuscript.

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