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Anti-inflammatory and immunomodulatory activities of *Ixodes ricinus* salivary proteins

Ph.D. Thesis

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Annotation:

Ticks are highly specialized obligate ectoparasites whose life cycle is fully dependent on obtaining a blood meal from a host. To prevent blood clotting and counteract the host's immune and wound healing responses, ticks secrete saliva, containing hundreds to thousands of proteins and non-proteins, into the feeding cavity. This thesis focuses on deciphering the function of three serpins (inhibitors of serine proteases) expressed in the salivary glands of the European tick *Ixodes ricinus*. Iripin-1, Iripin-3, and Iripin-8 were produced in the *Escherichia coli* expression system, and their effects on the immune response were evaluated using various *in vitro* assays and a mouse model of acute inflammation. Furthermore, the thesis involves one review article that summarizes the role of serpins in tick physiology and tick-host interaction.

Declaration:

I hereby declare that I am the author of this dissertation and that I have used only those sources and literature detailed in the list of references.

České Budějovice, 24. 8. 2022

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Mgr. Adéla Chlastáková

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List of papers and author's contribution

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

Paper I:

Chlastáková, A., Kotál, J., Beránková, Z., Kaščáková, B., Martins, L.A., Langhansová, H., Prudnikova, T., Ederová, M., Kutá Smatanová, I., Kotsyfakis, M., Chmelař, J., 2021. Iripin-3, a new salivary protein isolated from *Ixodes ricinus* ticks, displays immunomodulatory and anti-hemostatic properties *in vitro*. Frontiers in Immunology 12, 626200. DOI: 10.3389/fimmu.2021.626200.
[IF = 8.786]

AC designed and conducted some of the experiments, analyzed data, and performed statistical analyses. AC also wrote the manuscript, prepared the figures, and participated in the revision of the manuscript. Her contribution was 50 %.

Paper II:

Kotál, J., Polderdijk, S.G.I., Langhansová, H., Ederová, M., Martins, L.A., Beránková, Z., **Chlastáková, A.**, Hajdušek, O., Kotsyfakis, M., Huntington, J.A., Chmelař, J., 2021. *Ixodes ricinus* salivary serpin Iripin-8 inhibits the intrinsic pathway of coagulation and complement. International Journal of Molecular Sciences 22(17), 9480. DOI: 10.3390/ijms22179480.
[IF = 6.208]

AC participated in designing experiments and data analysis. AC also edited the manuscript. Her contribution was 15 %.

Paper III:

Abbas, M.N., **Chlastáková, A.**, Jmel, M.A., Iliaki-Giannakoudaki, E., Chmelař, J., Kotsyfakis, M., 2022. Serpins in tick physiology and tick-host interaction. Frontiers in Cellular and Infection Microbiology 12, 892770. DOI: 10.3389/fcimb.2022.892770.
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AC wrote some parts of the manuscript and edited the rest of the manuscript. Moreover, AC helped prepare the tables and edited the figures. AC also participated in the revision of the manuscript. Her contribution was 30 %.

Paper IV:

Chlastáková, A., Kaščáková, B., Kotál, J., Langhansová, H., Kotsyfakis, M., Kutá Smatanová, I., Tirloni, L., Chmelař, J., 2022. Iripin-1, a new anti-inflammatory tick serpin, inhibits leukocyte recruitment *in vivo* while altering the levels of chemokines and adhesion molecules. Manuscript.

AC designed and conducted most of the experiments, analyzed data, and performed statistical analyses. Moreover, AC wrote and edited the manuscript and prepared the figures. Her contribution was 75 %.

Co-author agreement:

Jindřich Chmelař, the supervisor of this thesis and the corresponding author of papers I–IV, fully acknowledges the contribution of Adéla Chlastáková as stated above.

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RNDr. Jindřich Chmelař, Ph.D.

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1 INTRODUCTION

1.1 *Ixodes ricinus*, its life cycle, and transmitted pathogens

I. ricinus, the castor bean tick, is a highly specialized obligate blood-feeding ectoparasite that belongs to the phylum Arthropoda, subphylum Chelicerata, class Arachnida, order Ixodida, and family Ixodidae [1]. *I. ricinus* is the most abundant tick species in Europe [2,3]. Due to rising global temperatures, *I. ricinus* geographical distribution is not only expanding northwards but the tick is also spreading to higher altitudes in several European mountain ranges [2]. Castor bean ticks prefer moister habitats, such as deciduous and mixed forests, pastures, moorlands, and urban parks, where they quest for their vertebrate hosts [3]. The life cycle of *I. ricinus* consists of three active developmental stages – larva, nymph, and adult (male or female) (Figure 1). Each tick stage (except for adult males) must feed on a different host in order to molt or lay eggs (Figure 2) [4]. The immature stages (larvae and nymphs) commonly feed for three to six days, while the blood feeding of adult females can take more than a week [1,4]. The host spectrum of the castor bean tick is very broad and includes small rodents, lizards, birds, and larger mammals, such as hedgehogs, hares, squirrels, wild boar, deer, livestock, and companion animals. The immature stages of *I. ricinus* can be found on hosts of all sizes, from lizards, birds, and small mammals to ungulates, whereas adults feed on larger hosts, such as cattle and deer [1,2]. The whole life cycle usually spans two to three years but can last even longer under unfavorable conditions [1].

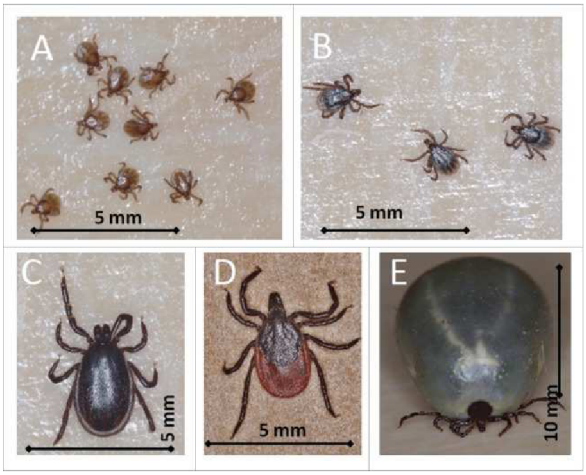

Figure 1. Developmental stages of the tick *I. ricinus*. (A) six-legged larvae, (B) eight-legged nymphs, (C) adult male, (D) unfed adult female, (E) fully engorged adult female. The figure was adapted from [5].

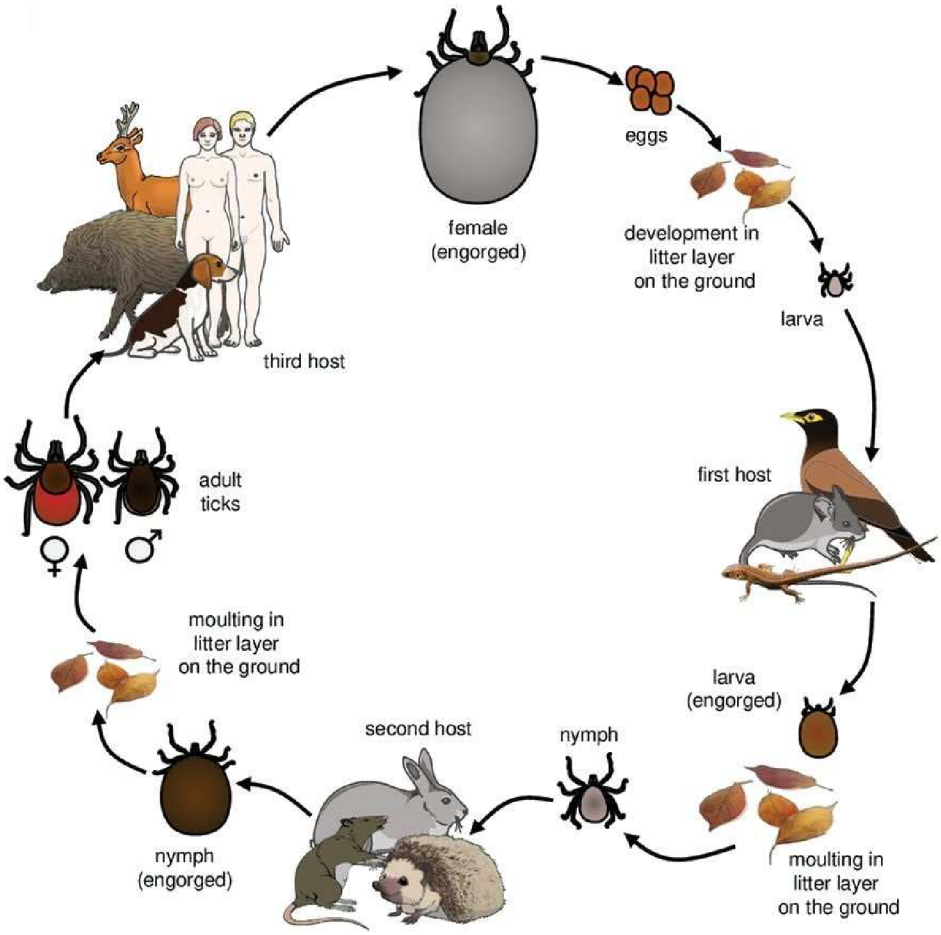

Figure 2. The life cycle of *I. ricinus*. The castor bean tick is a three-host tick, which means that each active developmental stage feeds on a different host. Larvae hatch from eggs and then wait in vegetation for the first host. After completion of a blood meal, larvae detach from the host and drop to the ground where they molt into nymphs that subsequently feed on the second host. The fully engorged nymphs again drop to the ground for a final molt to the adult male or female. The male and female mate already on vegetation or on the last host, with the male actively seeking the female. Following mating, the male dies, and the fully fed female drops from the last host and lays thousands of eggs into the layer of litter on the ground where a high relative humidity will ensure their survival [1,4]. The figure was adapted from [1].

I. ricinus ticks transmit a variety of viral, bacterial, and protozoan pathogens of medical and veterinary importance, of which tick-borne encephalitis virus and *Borrelia burgdorferi* sensu lato, the causative agent of Lyme borreliosis, have the greatest impact on human health. In addition to the two agents mentioned above, the castor bean tick is also a vector of *Anaplasma phagocytophilum* causing granulocytic anaplasmosis, *Francisella tularensis*, the causative agent of tularemia, *Rickettsia*

helvetica and *Rickettsia monacensis* causing spotted fever rickettsiosis, and *Babesia divergens*, *Babesia venatorum*, and *Babesia microti* responsible for babesiosis [6]. Tick-borne diseases have been reported to be on the rise in Europe, which might be caused by more factors, such as expansion of *I. ricinus* geographical distribution and suitable habitats, increase in tick and host numbers, prolonged periods of tick questing activity, and more frequent recreational activities of people in areas inhabited by infected ticks [6-8].

1.2 Reaction of a host to tick feeding

The penetration of skin by tick mouthparts triggers a host's defensive reaction that involves hemostasis and innate and adaptive immune responses [1,9]. Hemostasis and inflammation, accompanied by itch or pain, are the first two phages of a dynamic and complex process of wound healing [10]. The purpose of the host's defensive reaction is to limit blood loss, to cleanse the wound of contaminating microorganisms and damaged tissue, and to restore tissue integrity. The following sections are focused on a more detailed description of hemostasis, fibrinolysis, and the immune reaction.

1.2.1 Hemostasis

Hemostasis is a physiological process that stops bleeding at the site of an injury while maintaining normal blood flow elsewhere in the circulation [11]. Hemostasis is classified into primary hemostasis, which involves blood vessel contraction and platelet plug formation, and secondary hemostasis that refers to the deposition of insoluble fibrin generated by the activated coagulation cascade [12].

1.2.1.1 Primary hemostasis

The first response to the injury is vasoconstriction mediated by the contraction of vascular smooth muscle cells. Vascular spasm temporarily restricts or even stops blow flow through the wound, thus reducing the extent of bleeding [10]. Besides vasoconstriction, the platelet plug (white thrombus) starts to form to limit blood loss. The injury to the blood vessel wall results in the exposure of subendothelial matrix. Collagen and laminin of the subendothelial matrix together with bound von Willebrand factor

are recognized by platelet receptors, leading to the adhesion of platelets to damaged blood vessel wall and their activation [13]. Platelet activation is associated with the change in the conformation of integrins on platelet surfaces, enabling platelet aggregation and thus white thrombus formation [11,13]. Activated platelets release chemicals stored in their granules, such as adenosine diphosphate (ADP) and thromboxane A_2 (TXA_2). ADP and TXA_2 activate other platelets in the vicinity of the injury site in an autocrine manner, thus promoting further aggregation and propagation of the platelet plug [11,12]. TXA_2 is also a potent vasoconstrictor [14]. In addition to ADP and TXA_2, activated platelets release pro-inflammatory cytokines, chemokines, and factors enhancing vascular permeability, thus promoting the recruitment of leukocytes to the site of the injury [15].

1.2.1.2 Secondary hemostasis

The coagulation system consists of 12 originally identified clotting factors designated by Roman numerals (I, II, III, IV, V, VII, VIII, IX, X, XI, XII, XIII), of which factors I, II, III, and IV are commonly known as fibrinogen, prothrombin, tissue factor (TF), and calcium, respectively. Some coagulation factors are serine proteases that circulate in the inactive form and must be activated by proteolytic cleavage so that they could activate other factors in the cascade. The activation of each zymogen is indicated by the addition of letter "a" to the Roman numeral [16].

The cascade model of fibrin formation divides the coagulation system into two separate pathways (extrinsic and intrinsic) that, at the level of activated factor X (FXa), merge into a common pathway (Figure 3). The cascade model is particularly useful for understanding of how coagulation processes occur in plasma-based *in vitro* coagulation assays, but it does not adequately explain the *in vivo* hemostatic process [16,17]. Thus, a new cell-based model was created that incorporates the role of TF-bearing cells and platelets in blood coagulation. According to this model, vascular injury initially leads to the activation of the extrinsic pathway on the surfaces of TF-bearing cells. At this stage, only a small amount of thrombin is formed that is insufficient to produce an adequate amount of fibrin. However, the generated thrombin diffuses away from the TF-bearing cells and binds to platelets, leading to the activation of platelets as well as clotting factors V, VIII, and XI. Thus, the small amount of thrombin produced by the extrinsic

pathway activates the intrinsic pathway that augments thrombin generation and subsequent cleavage of fibrinogen to fibrin [11,16,17].

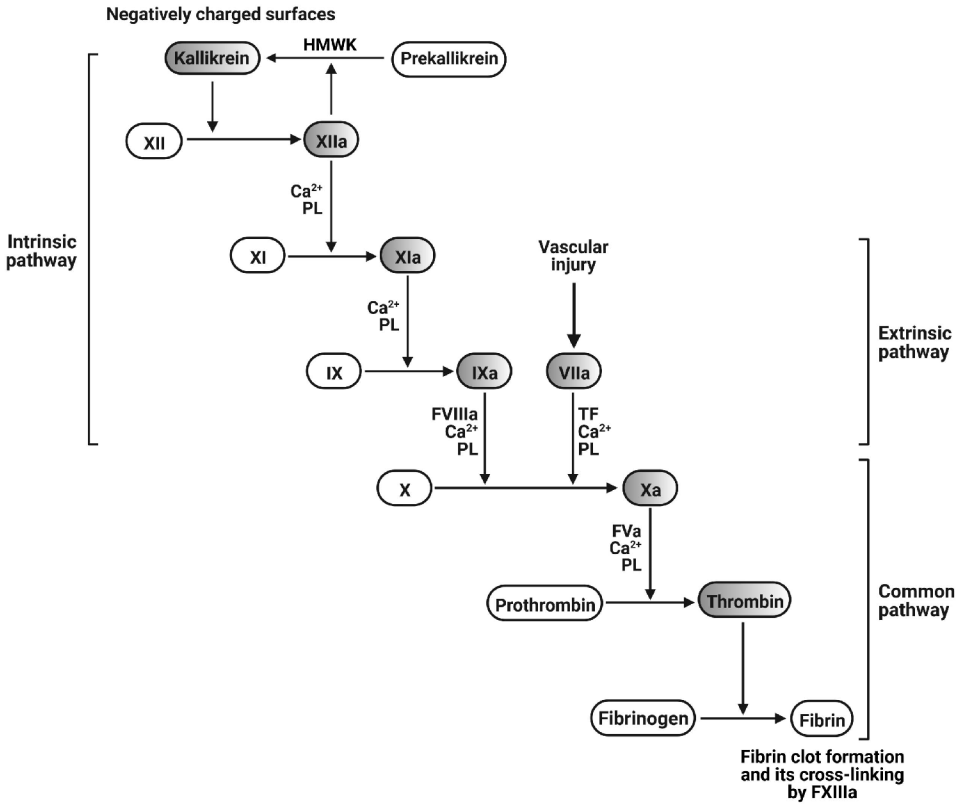

Figure 3. The cascade model of fibrin network generation. The extrinsic pathway is initiated by the formation of a complex between tissue factor (TF) exposed due to vascular injury and FVIIa. This complex (extrinsic tenase) activates FX. FXa can also be generated by the intrinsic pathway that is initiated by the activation of FXII on negatively charged surfaces. This initial step is followed by the consecutive activation of FXI, FIX, and FVIII, resulting in the formation of a complex between FIXa and FVIIIa (intrinsic tenase), which cleaves FX. The common pathway involves the conversion of prothrombin into thrombin by the prothrombinase complex (FXa + FVa) and the thrombin-mediated cleavage of fibrinogen to fibrin. Fibrin molecules polymerize into fibrin strands that are then cross-linked by thrombin-activated FXIII. Thus, in the end of the coagulation cascade, a stable insoluble fibrin network (red thrombus) is created that strengthens the platelet plug. Majority of the steps in the cascade occur on phospholipid membrane surfaces (PL) and require calcium (Ca^{2+}) [16,17]. Besides activating FXI, FXIIa converts the zymogen prekallikrein bound to high molecular weight kininogen (HMWK) to kallikrein. Kallikrein reciprocally activates additional molecules of FXII and liberates the pro-inflammatory mediator bradykinin from HMWK [18]. Coagulation factors that are active serine proteases are colored grey. The figure was created with BioRender.com.

Besides their crucial role in blood coagulation, thrombin, FXa, and FVIIa bound to TF activate protease-activated receptors (PARs) expressed on many cell types, including platelets, endothelial cells, and leukocytes [19]. The N-terminal proteolytic cleavage of PARs induces multiple downstream signaling pathways, leading to platelet activation, expression of cell adhesion molecules on endothelial cells, increased vascular permeability, promotion of leukocyte migration, and secretion of pro-inflammatory cytokines, chemokines, and growth factors [20]. Therefore, some clotting factors link blood coagulation with inflammation via PARs activation.

1.2.2 Fibrinolysis

Fibrinolysis is a highly regulated enzymatic process that prevents the unnecessary accumulation of intravascular fibrin and enables the removal of fibrin clots formed during hemostasis. Fibrin is lysed by plasmin, which is a serine protease generated from the zymogen plasminogen by the action of another two serine proteases, tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) [21]. While tPA co-localizes with plasminogen on the surface of the fibrin clot, uPA binds to its high-affinity receptor uPAR expressed on various cell types, including cancer cells. Thus, tPA is primarily responsible for thrombus dissolution, whereas uPA is mostly involved in the activation of cell-bound plasminogen [22,23]. As a broad-spectrum protease, plasmin, bound to the cell surface, can facilitate immune cell migration either directly through the lysis of some extracellular matrix (ECM) proteins and fibrinogen/fibrin or indirectly through the activation of ECM-degrading matrix metalloproteinases [24,25]. Moreover, plasmin can potentiate the inflammatory response via the activation of PARs and stimulation of PAR-independent release of pro-inflammatory mediators by many different cell types [20,26,27]. Proteolysis of fibrin by plasmin gives rise to soluble fibrin degradation products, some of which have immunomodulatory activities and are chemotactic for neutrophils and monocytes [28-30]. Thus, fibrinolysis and inflammation are interrelated processes.

1.2.3 Immune reaction

The host's immune reaction to the feeding tick consists of both innate and adaptive immune responses. The innate immune response is triggered immediately or within hours after recognition of microbial infection and/or

tissue damage, is non-specific, and does not lead to the generation of immunological memory. Unlike innate immunity, the adaptive immune response requires several days to develop, recognizes specific antigens, and produces memory cells that are responsible for an accelerated and more robust secondary immune response to the antigens re-encountered by the immune system [31]. The following text provides a more detailed description of innate and adaptive branches of immunity. Furthermore, one section concerns the complement system, which bridges innate and adaptive immune responses.

1.2.3.1 Innate immunity

Tissue damage, which is induced by the insertion of tick mouthparts into the host's skin, and the presence of microbial antigens are detected by pattern recognition receptors (PRRs) of resident cells, such as keratinocytes, fibroblasts, endothelial cells, mast cells, macrophages, and dendritic cells. The activation of resident cells via PRR engagement leads to the production of pro-inflammatory cytokines and chemokines [32,33]. Chemokines assist in the firm adhesion of leukocytes to endothelium and likewise create a concentration gradient that directs extravasating leukocytes to the inflammatory site [34]. Furthermore, pro-inflammatory cytokines tumor necrosis factor (TNF) and interleukin (IL)-1β stimulate endothelial cells to produce more cytokines/chemokines and to express cell adhesion molecules, such as E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) [35]. Thus, production of cytokines and chemokines by resident cells stimulate the migration of immune cells, like neutrophils and monocytes, to the tick attachment site. Neutrophil and monocyte recruitment can be further potentiated by mast cell degranulation, which leads to the release of chemokines and mediators inducing vasodilation and increased vascular permeability [36].

Extravasation of neutrophils and monocytes from blood into surrounding tissue is initiated by tethering and rolling of cells along the inflamed endothelium. This first step is followed by the firm adhesion of leukocytes to endothelial cells and transendothelial migration that most frequently occurs through endothelial junctions [37]. Once they cross the vascular basement membrane and reach the inflammatory site, neutrophils start to phagocytose microbes as well as dead cells in an attempt to clear the

wound. Neutrophils also eliminate extracellular microorganisms by releasing antibacterial proteins via degranulation. The released antibacterial proteins include defensins, lysozyme, and lactoferrin. Moreover, highly activated neutrophils can immobilize and kill extracellular microbes by extruding neutrophil extracellular traps that are composed of neutrophil DNA with attached histones, antibacterial proteins, and enzymes, such as neutrophil serine proteases cathepsin G, elastase, and proteinase 3 [38,39]. Besides clearing the wound, activated neutrophils secrete a wide variety of chemokines that are able to recruit neutrophils themselves, monocytes, dendritic cells, and T cell subsets. Thus, by producing chemokines, neutrophils can amplify both innate and adaptive immune responses [40].

Concomitantly with the influx of neutrophils, circulating monocytes enter the wound and differentiate into macrophages [41]. During the early inflammatory phase, macrophages produce numerous inflammation-promoting mediators, including some cytokines (e.g., TNF, IL-1, IL-6, IL-12), chemokines recruiting other leukocytes, enzymes, bioactive lipids, reactive oxygen and nitrogen species, and complement components [42,43]. Furthermore, macrophages phagocytose microbes as well as apoptotic and damaged cells and are able to present processed antigens to T cells, thus linking innate and adaptive immunity [44,45]. In response to the engulfment of apoptotic neutrophils and other external factors, macrophages eventually reduce the secretion of pro-inflammatory mediators and instead start to produce factors that promote the resolution of inflammation and tissue repair. These pro-wound healing factors include anti-inflammatory cytokines (e.g., IL-10 and TGF-β), resolvins, protectins, maresins, and growth factors [41,45].

1.2.3.2 Complement

The complement system consists of a tightly regulated network of tens of proteins that are present either as soluble proteins in the blood or as membrane-associated proteins [46]. Complement has long been considered to be a component of the innate immune system; however, recent studies have shown that it is also involved in the modulation of the adaptive immune response [47]. Complement activation occurs via a classical, alternative, or lectin pathway and involves a sequential conversion of inactive zymogens into active enzymes by proteolytic cleavage. The

classical pathway is initiated by the interaction of the complement component C1q with antibodies bound to antigen. C1q can also bind to other proteins, like C-reactive protein. The alternative pathway is triggered by binding of C3b to pathogen surfaces, and the lectin pathway is activated when mannose-binding lectin (MBL) or ficolins attach to carbohydrate moieties on the surfaces of pathogens (e.g., bacteria, yeast, and parasites) and altered self-cells. All three pathways lead to the formation of multicomponent C3 and C5 convertases, which cleave C3 to C3a and C3b and C5 to C5a and C5b, respectively. Finally, the membrane attack complex (MAC) is assembled through the serial addition of complement components C6, C7, C8, and C9 to C5b. MAC insertion into the plasma membrane of target cells leads to pore formation and subsequent cell lysis (Figure 4) [46,48].

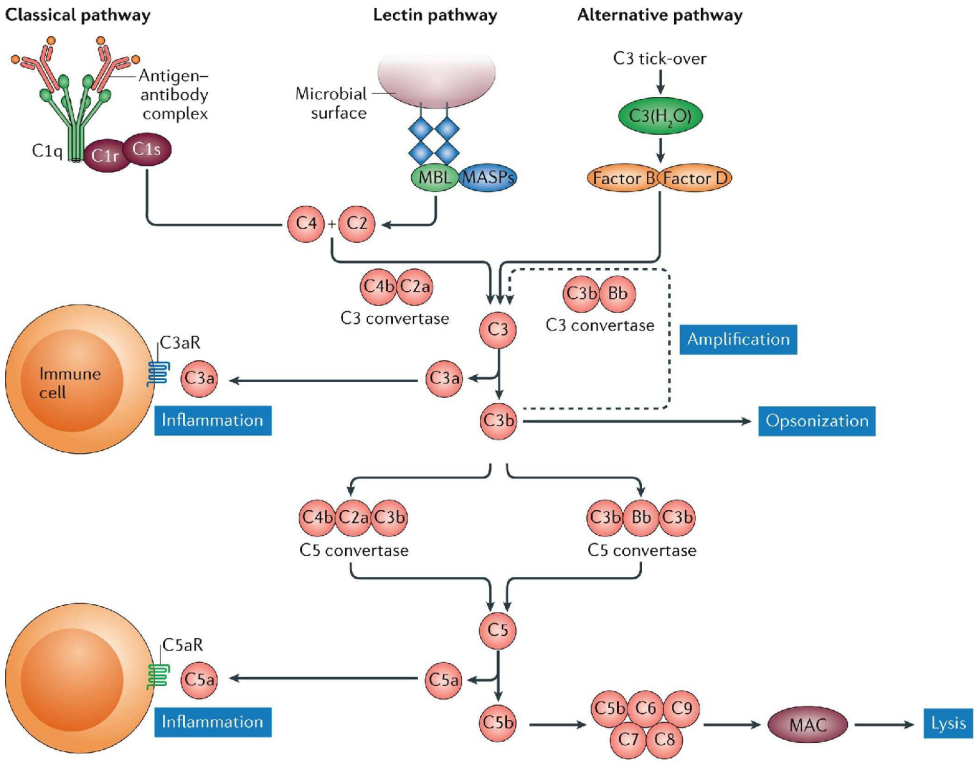

Figure 4. The overview of the complement system. The complement cascade can be initiated via the classical, lectin, or alternative pathway. All three pathways lead to the assembly of C3 convertases that cleave the complement component C3 into the anaphylatoxin C3a and the opsonin C3b. C5 convertases, formed from the C3 convertases by the addition of C3b, cleave C5 into the anaphylatoxin C5a and the component C5b. C5b subsequently associates with C6, C7, C8, and C9 to form the membrane attack complex (MAC). MBL, mannose-binding lectin. MASPs, MBL-associated serine proteases. The figure was adapted from [49].

Besides controlling microbial infection through MAC formation, some components of the complement system facilitate the clearance of pathogens, apoptotic and necrotic cells via promotion of phagocytosis, whereas other complement components are involved in the initiation of the inflammatory response. C3b, its degradation product iC3b, and C4b act as opsonins since they are able to bind simultaneously to target cells and complement receptors on the surface of phagocytes [48]. The anaphylatoxins C3a and C5a, on the other hand, induce degranulation of mast cells and basophils and can also trigger oxidative burst in neutrophils and macrophages. Additionally, C5a is a powerful chemoattractant for neutrophils, monocytes/macrophages, basophils, and mast cells [50]. Besides their role in inflammation, anaphylatoxins have been shown to regulate T cell survival, activation, and differentiation, thus modulating adaptive immunity [47].

1.2.3.3 Adaptive immunity

The adaptive immune system consists of bone marrow-derived B lymphocytes and thymus-derived T lymphocytes. T lymphocytes can be further divided into helper ($CD4^+$) T cells and cytotoxic ($CD8^+$) T cells [51]. Antigen-presenting cells (APCs) in the skin include dermal macrophages, epidermal Langerhans cells, and dermal dendritic cells. Other APCs can be recruited into the skin in response to an inflammatory stimulus [44]. Following tick attachment to the host, APCs engulf foreign proteins, cleave them into peptides, and then display the peptide antigens on their surface via major histocompatibility complex (MHC) class II molecules. Recognition of the antigen/MHC-II complex by naïve $CD4^+$ T cells and the presence of a co-stimulatory signal trigger $CD4^+$ T cell activation and proliferation. Based on the cytokines present in the surrounding microenvironment, antigen concentration, type of APCs, and co-stimulatory molecules, proliferating helper T cells differentiate into effector T cell subsets, including Th1, Th2, Th17 cells, and induced regulatory T cells (iTregs) [51].

The individual lineages of $CD4^+$ T cells produce a distinct set of cytokines and thus differentially regulate both innate and adaptive immune responses. Th1 cells through the secretion of interferon-γ (IFN-γ) and IL-2 stimulate macrophages to increase microbicidal activity and likewise promote the differentiation of cytotoxic T cells. Th2 cells, on the other hand, secrete IL-

4, IL-5, and IL-13, leading to the activation and recruitment of eosinophils, clonal expansion of B cells, and the production of immunoglobulin E by plasma cells. Effector cytokines produced by Th17 cells include IL-17A, IL-17F, IL-21, and IL-22. These cytokines support the migration of inflammatory cells, especially neutrophils, to the site of inflammation. Finally, iTregs secrete immunosuppressive cytokines TGF-β, IL-10, and IL-35, which allows them to maintain self-tolerance and inhibit immune responses [51,52].

In addition to $CD4^+$ T cells, another cell type responsible for adaptive immunity is $CD8^+$ T cell. The recognition of the antigen/MHC-I complex by naïve $CD8^+$ T lymphocytes triggers $CD8^+$ T cell proliferation and differentiation into cytotoxic T lymphocytes (CTLs), an event that is promoted by various cytokines, such as IL-2 produced by activated $CD4^+$ T cells. CTLs are effector cells of the immune system with the ability to induce death of altered self-cells, like pathogen-infected cells and cancer cells [53]. Target cells can be killed through the release of perforin and serine proteases, known as granzymes, from the granules of CTLs. Perforin forms pores in the membranes of target cells, which allows granzymes to enter the cells and induce cell death through various mechanisms, including the cleavage of caspase-3 and caspase-8. Furthermore, apoptosis of target cells can be triggered via the interaction between the Fas molecule (CD95) on the target cell and Fas ligand (CD178) on the CTL [53,54].

Unlike T cells, naïve B cells are able to recognize unprocessed antigens via B-cell receptors. After antigen encounter and help from activated $CD4^+$ T cells, which comes in the form of co-stimulatory ligands and cytokines, B lymphocytes start to proliferate and differentiate into plasma cells. Plasma cells subsequently produce antibodies, also known as immunoglobulins (Igs). There are five different classes of antibodies (IgM, IgG, IgD, IgA, and IgE) that differ in their effector functions. In general, antibodies are capable of opsonization, neutralization of targets, such as viruses, and activation of the classical pathway of the complement system. Igs produced by B cells are also responsible for antibody-dependent cell-mediated cytotoxicity. Besides the production of antibodies, B lymphocytes serve as APCs, secrete inflammatory cytokines (e.g., TNF, IFN-γ, IL-17), and a subgroup of them, known as Bregs, produce immunosuppressive cytokines IL-10 and IL-35. Therefore, B cells not only

are key players in humoral immunity but also modulate cell-mediated immune responses [55].

The adaptive immune response generates long-living memory B and T cells in addition to effector cells. Memory cells formed during primary exposure of a host to a tick bite might trigger a fast and robust secondary immune reaction during subsequent tick infestations, leading to the rejection of feeding ticks. Indeed, tick-resistant animals develop a response at the tick bite site that is characterized by the rapid recruitment of immune cells, predominantly composed of basophils and eosinophils. Especially degranulation of basophils and release of histamine seem to play a critical role in tick rejection [56,57]. Memory cells are involved in this acquired anti-tick immunity since antibodies against tick salivary proteins can bind to basophils, resulting in degranulation [56,58]. Moreover, the recruitment of basophils to the tick attachment site is promoted by IL-3 secreted by skin memory $CD4^+$ T cells [59].

1.3 Modulation of the host defensive response by tick salivary components

During blood meal acquisition, ticks secrete saliva into the wound. Tick saliva contains hundreds to thousands of non-proteins and proteins belonging to diverse protein families. For example, proteins synthesized in *I. ricinus* salivary glands include glycine-rich proteins, various enzymes, protease inhibitors, lipocalins, disintegrins, defensins, mucins, evasins, as well as members of the Salp15 and Da-p36 families [60,61]. Many of the proteins have been shown to have anti-hemostatic, anti-inflammatory, anti-complement, and immunomodulatory activities (see Table 1), suggesting ticks utilize salivary proteins to evade the host's defensive reaction, which would otherwise result in rejection and death of the tick. Interestingly, it seems that tick salivary proteins can either promote or suppress fibrinolysis (Table 1). Dissolution of fibrin clots would facilitate blood flow into the feeding cavity. Inhibition of plasmin and fibrinolysis, on the other hand, could attenuate the inflammatory response of the host.

Table 1. Effects of tick salivary proteins and peptides on hemostasis, fibrinolysis, and immune responses.

Hemostasis	
Inhibition of vasoconstriction by TXA_2 scavenging	[62]
Inhibition of platelet activation and aggregation	[63-66]
Dissolution of platelet aggregates	[67]
Inhibition of extrinsic, intrinsic, and common pathways of blood coagulation	[66,68-70]
Fibrinolysis	
Induction of fibrin clot lysis	[71]
Inhibition of fibrinolysis	[65]
Innate immunity	
Degradation of bradykinin	[72]
Binding of histamine, leukotriene B4, and chemokines, thus preventing their interaction with receptors	[62,73-75]
Decrease in the production of pro-inflammatory cytokines and chemokines	[76-80]
Increase in the production of immunosuppressive cytokines	[76,77]
Inhibition of neutrophil and monocyte/macrophage migration	[75,81-83]
Attenuation of the oxidative burst of activated neutrophils	[82]
Inhibition of nitric oxide production by macrophages	[83]
Complement	
Suppression of the alternative complement pathway by disrupting the activity of C3 convertase	[84,85]
Prevention of MAC formation through the inhibition of C5 activation	[62,86]
Inhibition of the lectin complement pathway by blocking of MBL and ficolin binding to their ligands	[87]

Table 1. Continued

Adaptive immunity	
Inhibition of dendritic cell differentiation from monocytes	[77]
Modulation of the expression of co-stimulatory and co-inhibitory molecules by dendritic cells/macrophages	[77,79,88,89]
Suppression of antigen processing and presentation by APCs	[79,88]
Inhibition of B cell, $CD4^+$ T cell and $CD8^+$ T cell activation and proliferation	[88,90-92]
Inhibition of $CD4^+$ T cell differentiation into Th1 or Th17 cells	[89,93]
Promotion of $CD4^+$ T cell differentiation into Th2 cells	[89]
Decrease in the lytic activity of cytotoxic T lymphocytes	[89]

Alteration of the host's immune response by tick salivary molecules can facilitate the transmission of tick-borne pathogens (TBP) from infected ticks to the uninfected vertebrate host and can also enhance the acquisition of TBP by uninfected ticks feeding on the infected host. This phenomenon, when TBP exploit saliva-induced modulation of host's defenses to promote their transmission and propagation, is called saliva-assisted transmission (SAT) [94]. SAT was observed for the first time in the experiments concerning the transmission of Thogoto virus. In one of the experiments, acquisition of infection by virus-free *Rhipicephalus appendiculatus* nymphs was enhanced when the nymphs fed on guinea pigs injected with a mixture of Thogoto virus and tick salivary gland extract [95]. Subsequent studies have demonstrated SAT also for tick-borne encephalitis virus [96] and *B. burgdorferi* sensu lato [97,98]. Tick salivary molecules can facilitate TBP transmission and dissemination not only through the modulation of the host's immune reaction but also through the direct interaction with pathogens. For example, a tick-derived SAT factor Salp15 protects *B. burgdorferi* sensu stricto from antibody- and complement-mediated killing by binding to the outer surface protein C of the spirochete [99,100].

Some tick salivary proteins belong to the group of protease inhibitors. Based on their inhibitory specificity, protease inhibitors can be divided into those targeting cysteine proteases (e.g., cystatins and thyropins) and those

targeting serine proteases (e.g., Kunitz domain-containing proteins, serpins, Kazal-type inhibitors, and trypsin inhibitor-like domain-containing inhibitors) [101,102]. Since the research articles, which are part of this dissertation, concern tick serpins, the following text will provide basic information about serine proteases and the members of the serpin superfamily.

1.4 Serine proteases

Proteases are a large group of enzymes that catalyze the hydrolysis of peptide bonds in proteins. Based on the mechanism of catalysis, proteases are classified into six distinct classes: aspartic, glutamic, cysteine, serine, threonine proteases and metalloproteases. Aspartic and glutamic proteases together with metalloproteases utilize an activated water molecule as a nucleophile to attack the peptide bond of the substrate. In cysteine, serine, and threonine proteases, on the other hand, the nucleophile is the amino acid residue (Cys, Ser, or Thr, respectively) located in the protease active site [103].

Serine proteases, which constitute over one third of all known proteolytic enzymes, are divided into many clans/superfamilies that are further classified into families. PA is a major clan of serine proteases of which the S1 family bearing the trypsin fold is widely studied. In general, serine proteases can be found in all three superkingdoms of life (Archaea, Bacteria, and Eukarya) as well as in many viral genomes. However, differences exist in the distribution of each clan. For example, proteases belonging to the PA clan are highly represented in eukaryotes but are rare constituents of prokaryotic genomes [104,105].

The active site of S1 proteases is generally made of the catalytic triad of Asp, His, and Ser amino acid residues, commonly referred to as the charge relay system. Based on the inhibitory specificity, S1 proteases can be divided into trypsin-like, chymotrypsin-like, and elastase-like proteases. Trypsin-like serine proteases (e.g., kallikrein, matriptase, plasmin, and some coagulation factors) prefer positively charged residues (Arg or Lys) at the P1 position of the substrate. In contrast, chymotrypsin-like proteases (e.g., cathepsin G and chymase) preferentially cleave peptide bonds at large hydrophobic residues (Phe, Tyr, or Trp). Finally, elastase-like serine

proteases, such as proteinase 3, tend to cleave bonds at smaller aliphatic amino acid residues (Ala, Gly, or Val) [104,105].

Serine proteases from the S1 family mediate many key biological processes, some of which involve cascades of sequential zymogen activation. More specifically, S1 proteases are involved in the breakdown of proteins in the digestive system, primary hemostasis, blood coagulation, fibrinolysis, extracellular matrix remodeling, regulation of vascular tone and permeability, inflammation, the complement system, apoptosis, and wound healing [104-106]. To assure that the correct substrates are processed at the right moment and in the appropriate environment, several mechanisms that control the action of serine proteases exist. These mechanisms include the regulation of gene expression, conversion of inactive zymogens into active enzymes, or blockade of the enzyme's proteolytic activity by endogenous inhibitors, such as serpins [103].

1.5 Serpins

Serpins (serine protease inhibitors) are a large family of proteins that are, like serine proteases, present in all three superkingdoms of life as well as in viruses [107,108]. Due to their ability to suppress the proteolytic activity of serine proteases, serpins can regulate all serine protease-dependent biological processes mentioned in the previous paragraph of this dissertation [109]. Although most of the serpins function as serine protease inhibitors, some members of the serpin superfamily can target papain-like cysteine proteases [110] and caspases [111,112] or can even lack inhibitory activity. Non-inhibitory serpins can serve as molecular chaperons [113] and are also involved in blood pressure regulation [114] and hormone transport [115].

Serpins typically consist of 350–400 amino acids and have an average molecular weight between 40–60 kDa. All members of the serpin superfamily share a highly conserved tertiary structure that is usually composed of three β-sheets (A, B, C) and eight to nine α-helices [109,116,117]. In the native metastable (so called stressed) state, a reactive center loop (RCL), which is a flexible stretch of approximately 20 amino acid residues, protrudes from the top of the serpin molecule into the surrounding environment (Figure 5A). The RCL serves as a substrate for target proteases, and its amino acid sequence (especially the amino acid

residue at the P1 position) determines the serpin inhibitory specificity [116,118-120].

Serpins inhibit proteases through a unique suicide substrate-like mechanism, which results in the inactivation of not only the protease but also the serpin [119]. Initially, the interaction of the protease with the amino acid residues flanking the scissile (P1–P1') bond of the RCL leads to the formation of a non-covalent Michaelis-Menten-like complex (Figure 5B). The subsequent cleavage of the scissile bond by the bound protease induces a dramatic change in the serpin conformation from the stressed state to a more stable relaxed state. This "stressed to relaxed" conformational transition involves the insertion of the cleaved RCL into the center of the β-sheet A and the translocation of the protease to the opposite end of the serpin molecule. The protease translocation is accompanied by the distortion of its active site. Thus, the inactive protease remains trapped in an irreversible covalent complex with the serpin (Figure 5C) [117,119]. However, if the RCL insertion is not rapid enough, the protease can dissociate from the Michaelis-Menten-like complex following RCL cleavage. Since the protease escapes just before the RCL inserts into the β-sheet A, it retains its activity and leaves the cleaved serpin inactive (Figure 5D) [117,120].

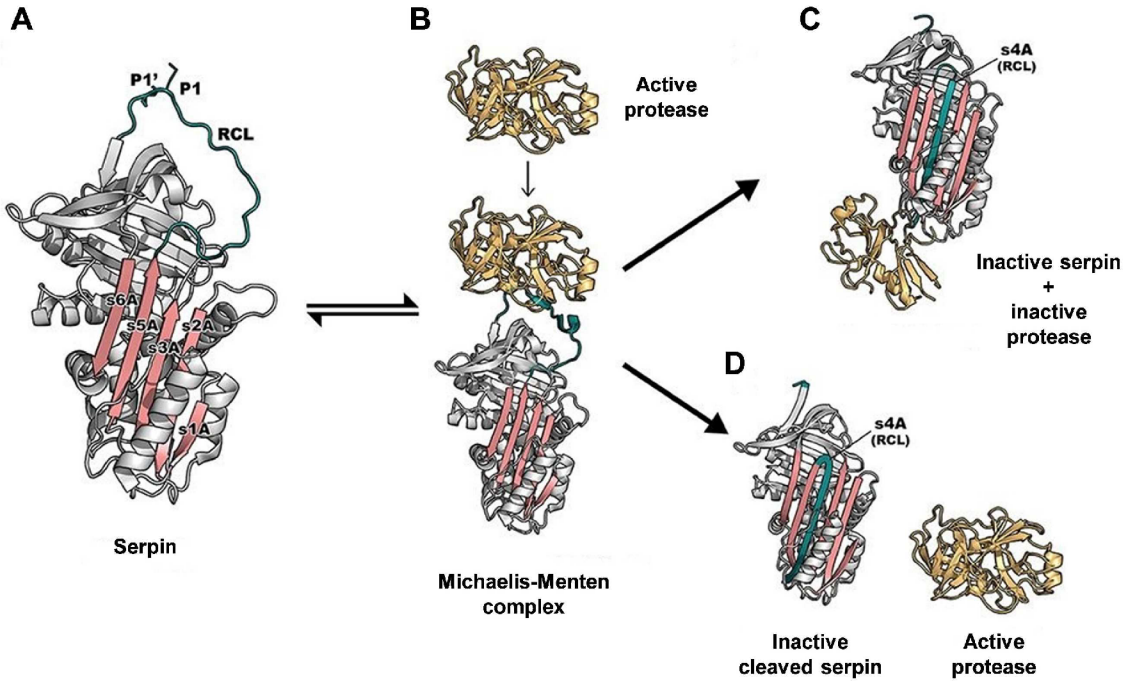

Figure 5. Interaction of a serpin with a protease. (A) The serpin in its native metastable conformation. The reactive center loop (RCL), which is located at the top of the serpin molecule and contains the protease cleavage site between P1 and P1' residues, is colored green. The five-stranded β-sheet A is pink. (B) A Michaelis-Menten-like complex between the protease (gold) and the serpin. (C) An irreversible covalent complex between the serpin and the protease. The N-terminal portion of cleaved RCL, inserted into β-sheet A as an additional strand, is colored green. (D) Serpins can sometimes act as substrates rather than inhibitors. In that case, the protease manages to dissociate from the serpin following RCL cleavage, thus retaining its activity and leaving the serpin inactive. The figure was adapted from [121].

1.6 Serpins in ticks

Due to a gradually increasing number of transcriptomic and proteomic studies, serpins have been detected in many species of ixodid (hard) ticks as well as argasid (soft) ticks [102,122,123]. To date, about 25 serpins of the hard ticks belonging to genera *Amblyomma*, *Haemaphysalis*, *Hyalomma*, *Ixodes*, and *Rhipicephalus* have been functionally characterized using *in vitro* assays, *in vivo* experimental models, and vaccination as well as RNA interference (RNAi) experiments [102,124,125]. Tick serpins have been reported to target trypsin-like, chymotrypsin-like, and elastase-like serine proteases, and some of them even inhibited papain-like cysteine proteases [102,125]. Biological processes, shown to be regulated by tick serpins, involve platelet

aggregation, blood coagulation, fibrinolysis, innate and adaptive immune responses, and complement activation [102,125,126].

Expression of serpins has been detected in all tick developmental stages (eggs, larvae, nymphs, male and female adults) and in some tick organs, especially salivary glands, midgut, and ovaries [102,125]. Serpins expressed in tick salivary glands are usually secreted via saliva into the tick feeding site where they counteract host's defenses against injury and infection [102,126,127]. Serpins expressed in tick midgut could maintain blood in a fluid state and might also participate in the regulation of blood meal digestion [127]. Finally, serpins present in ovaries appear to be involved in tick reproduction as evidenced recently by the functional characterization of *Rhipicephalus haemaphysaloides* serpin RHS8. This serpin has been reported to be expressed in the ovaries and fat bodies of partially engorged, fully engorged, and egg-laying ticks, and its knockdown by RNAi impaired the accumulation of vitellogenin in oocytes [128]. In addition to salivary glands, midgut, and ovaries, serpins are also present in tick hemolymph where they could regulate hemolymph clotting and tick innate immune responses, i.e., the Toll pathway and the activation of prophenoloxidase (melanization) [126,127]. The role of serpins in tick physiology and tick-host interaction is summarized in paper III of this dissertation.

1.6.1 Serpins of the tick *I. ricinus*

Approximately 35 serpins have been detected in *I. ricinus*. The serpins have distinct RCLs with many different amino acids at the P1 position, which suggests that individual serpins differ in their function. Many of the *I. ricinus* serpins seem to be secreted into the extracellular environment since they have a predicted signal peptide at the N terminus. A smaller portion of the serpins, however, lack a signal peptide, and thus have a presumable intracellular function. Differences also exist in the expression of individual serpins in ticks. While some of the serpins are expressed mainly in the salivary glands, other serpins are predominantly expressed either in the midgut or in the ovaries (unpublished data). To date, a function of six *I. ricinus* salivary serpins (Iris, Iripin-1, IRS-2, Iripin-3, Iripin-5, and Iripin-8) has been deciphered. This Ph.D. thesis is based on research papers concerning the structural and functional characterization of Iripin-1, Iripin-

3, and Iripin-8. The role of Iris, IRS-2, and Iripin-5 in the tick-host interaction is described below.

1.6.1.1 Iris

Iris (*I. ricinus* immunosuppressor) is the first functionally characterized serpin of the tick *I. ricinus*. It was shown to be expressed in feeding nymphs and in the salivary glands of feeding adult females. Moreover, Iris was detected in tick saliva using polyclonal serum raised against recombinant protein [129,130]. The presence of Iris in tick saliva is somewhat surprising since the serpin lacks a signal peptide and has cysteine and methionine in its RCL, which indicates an intracellular rather than extracellular function. Therefore, Iris either gets into saliva via a non-classical secretory mechanism, or the polyclonal serum used for Iris detection cross-reacted with another salivary serpin [102].

In the enzymatic assay, Iris primarily targeted elastase and also exhibited weaker inhibitory activity against thrombin, FXa, and tPA. Consistent with its inhibitory specificity, Iris suppressed the intrinsic coagulation pathway and fibrinolysis. Additionally, Iris increased platelet adhesion time [65]. Besides its effect on hemostasis and fibrinolysis, Iris also modulated innate and adaptive immune responses. The serpin inhibited the *in vitro* proliferation of concanavalin A-stimulated splenocytes and the production of IFN-γ, TNF, IL-6, and IL-8 by peripheral blood mononuclear cells (PBMCs) stimulated with a set of different activators [129,131]. Some *in vitro* findings were confirmed using the mouse model of lipopolysaccharide (LPS)-induced septic shock. In this *in vivo* model, Iris administration decreased the production of TNF, IL-6, and monocyte chemoattractant protein-1 (MCP-1) and concomitantly increased both survival rate and survival time of LPS-injected mice [131].

Interestingly, Iris devoid of anti-protease activity due to a mutation in the RCL lost its anti-coagulant and anti-fibrinolytic properties but still managed to interfere with platelet adhesion and TNF production by PBMCs [65,131]. Thus, Iris does not attenuate primary hemostasis and TNF secretion by inhibiting proteases. In fact, TNF production was shown to be reduced due to Iris binding to the monocyte population of PBMCs via exosites [131].

1.6.1.2 IRS-2

The second functionally characterized serpin of the castor bean tick is *I. ricinus* serpin-2 (IRS-2). The expression of the serpin was upregulated in the salivary glands and ovaries of adult females in response to blood feeding. Since the signal peptide was predicted at the N terminus of the IRS-2 sequence, it is likely that the serpin is secreted into tick saliva and participates in the modulation of the host defensive response at the tick attachment site. Moreover, its expression in ovaries suggests a role in tick reproduction [132].

In accordance with the presence of the aromatic amino acid tyrosine at the P1 position, IRS-2 predominantly inhibited α-chymotrypsin, chymase, and cathepsin G. In addition to the chymotrypsin-like serine proteases, IRS-2 also had a weaker inhibitory effect on thrombin and trypsin. Consistent with its anti-protease activity, IRS-2 attenuated cathepsin G- and thrombin-induced platelet aggregation. Besides targeting primary hemostasis, IRS-2 suppressed edema formation and neutrophil recruitment in the carrageenan-induced paw edema model. The anti-inflammatory activity of IRS-2 might be related to its ability to inhibit cathepsin G and chymase produced by activated neutrophils and mast cells [132].

Later it was shown that IRS-2 can also modulate the acquired immune response via inhibition of IL-6 production by activated dendritic cells. The decrease in IL-6 secretion resulted in attenuated phosphorylation of signal transducer and activator of transcription-3 (STAT-3) and impaired differentiation of naïve $CD4^+$ T cells into Th17 cells [93]. Thus, IRS-2, similarly to Iris, appears to be a multi-functional serpin that can facilitate tick feeding by suppressing the hemostatic and immune responses of the host.

1.6.1.3 Iripin-5

Just recently, a function of *I. ricinus* serpin-5 (Iripin-5) has been deciphered. This serpin is abundantly expressed in nymphs and in the salivary glands of adult females. More specifically, *iripin-5* expression gradually increases over the course of blood feeding and is the highest at the end of the feeding process. This suggests that Iripin-5 is involved in the modulation of the host's defenses at the tick attachment site [133].

Despite the predicted arginine at the P1 site, Iripin-5 had no significant effect on the proteolytic activity of blood clotting factors and plasmin, tPA, or uPA. Thus, Iripin-5 most likely does not modulate blood coagulation or fibrinolysis. In fact, Iripin-5 markedly inhibited neutrophil serine proteases elastase and proteinase 3, which indicates that ticks might use this serpin to interfere with neutrophil function. *In vitro* assays showed that Iripin-5 is able to diminish neutrophil migration, suppress nitric oxide production by activated macrophages, and inhibit the lysis of rabbit erythrocytes by human complement. Interestingly, Iripin-5 exerted significant anti-complement activity when used at low concentrations (312 nM and 625 nM). Therefore, the suppression of MAC formation might be the main role of Iripin-5 at the tick-host interface. However, it remains unknown what complement component(s) is targeted by this salivary serpin [133].

1.7 Reasons for studying tick serpins

Determination of the structure and function of each individual tick salivary serpin sheds more light on the mechanisms that ticks use to evade the host's defensive response. Additionally, tick serpins might be utilized as components of anti-tick vaccines and could also have therapeutic potential.

1.7.1 Anti-tick vaccines

The use of anti-tick vaccines is regarded as a promising, environmentally friendly, and sustainable strategy to control tick infestations and infection with tick-borne pathogens. The principle of transmission-blocking anti-tick vaccines is as follows. Immunization of a host with tick antigen(s) triggers the production of antigen-specific antibodies. When the tick starts to ingest a blood meal, the host's antibodies recognize the tick antigen used for immunization and affect/block its function, thus disrupting tick feeding and limiting the transmission of multiple TBPs [134-136]. The host can be immunized either with tick salivary antigens (which might act as SAT factors) or with concealed tick antigens that are not normally presented to the host, such as proteins found on the tick gut wall [134].

The role of some tick serpins in the process of blood feeding was evaluated using RNAi and vaccination experiments. Silencing of serpin genes in ticks by RNAi led to morphological deformities, decreased attachment and engorgement rates, reduced engorgement weight, longer feeding time,

impaired oviposition, and lower hatchability [128,137-139]. Moreover, immunization of animal hosts (rabbits, cattle) with recombinant proteins showed that tick serpins are immunogenic, and their neutralization by host antibodies can result in decreased engorgement weight, higher mortality, prolonged feeding time, and impaired oviposition [130,138,140-143]. Thus, serpins represent candidates for anti-tick vaccine development. To increase vaccine efficacy, serpins might be combined with other functionally non-redundant tick proteins, belonging to distinct protein families. In addition, the use of serpins conserved among different tick species could provide protection against infestations with multiple vectors [134,136].

1.7.2 Therapeutic application

Serine proteases are involved in the pathogenesis of many diseases. For example, excessive activity of neutrophil serine proteases elastase, cathepsin G, and proteinase 3 contributes to respiratory tissue damage in patients with cystic fibrosis and chronic obstructive pulmonary disease [144,145]. Furthermore, proteases chymase and tryptase, released from activated mast cells, have been linked to atherosclerotic plaque progression and destabilization as well as abdominal aortic aneurysm formation [146-148]. Finally, serine proteases, such as trypsin, uPA, plasmin, and matriptase, are implicated in the promotion of tumor growth, invasion, and metastasis [149,150]. Since tick serpins are capable of inhibiting many of the serine proteases mentioned above [102,125], they may find application in the treatment of diseases associated with protease/antiprotease imbalance.

To date, some tick serpins have been reported to exert anti-inflammatory activities in the mouse and rat models of acute inflammation. As mentioned previously, *I. ricinus* serpin Iris enhanced both survival rate and survival time in the mouse model of LPS-induced septic shock [131], and another *I. ricinus* serpin IRS-2 reduced edema formation and neutrophil influx in the carrageenan-induced paw edema model [132]. In addition to Iris and IRS-2, *Amblyomma americanum* serpins AAS27 and AAS41 and *Rhipicephalus microplus* serpins RmS-3, RmS-6, and RmS-17 reduced vascular permeability induced in the skin by compound 48/80 (an agonist of mast cell degranulation) or formalin (an activator of trypsin-like serine proteases) [139,151,152]. Furthermore, *Haemaphysalis longicornis* serpins

HlSerpin-a and HlSerpin-b relieved joint inflammation in the mouse model of collagen-induced arthritis. Surprisingly, a peptide containing the RCL of HlSerpin-a had similar anti-inflammatory activity as the full-length serpin [80]. All these results suggest that tick serpins (or their parts) could be employed in the treatment of inflammatory diseases. Moreover, tick serpins with anticoagulant and anti-complement properties might aid in the development of novel therapeutics for the treatment of hypercoagulable states and complement-related disorders.

2 SUMMARY OF RESULTS

The aim of this Ph.D. thesis was to perform the functional characterization of proteins expressed in the salivary glands of the tick *I. ricinus*. The emphasis was put on the determination of anti-inflammatory and immunomodulatory activities of tick salivary serpins in various *in vitro* and *in vivo* experiments. The thesis is based on four papers. Two already published research articles concern the structural and functional characterization of *I. ricinus* serpins Iripin-3 (paper I) and Iripin-8 (paper II). One review article (paper III) summarizes the current knowledge of the role of serpins in tick physiology and tick-host interaction. Finally, one manuscript (paper IV) reports on the structure and function of *I. ricinus* serpin Iripin-1.

2.1 Paper I

Paper I provides information about the structure and function of *I. ricinus* serpin-3 (Iripin-3). The expression of *iripin-3* was induced by blood feeding in nymphs as well as in the salivary glands and ovaries of adult females. Expression in tick salivary glands, prediction of a signal peptide at the N terminus of the serpin sequence, and detection of the Iripin-3 protein in tick saliva suggest that this serpin is transferred via saliva into the feeding cavity. Consistent with the presence of arginine at the P1 site, Iripin-3 suppressed the enzymatic activities of trypsin and trypsin-like serine proteases kallikrein, matriptase, and thrombin through the classic serpin inhibitory mechanism. Involvement of these four serine proteases in the inflammatory response [19,153-155] indicates that Iripin-3 might attenuate the inflammatory process triggered by tick attachment to the host.

In vitro assays showed that Iripin-3 is capable of suppressing/modulating various aspects of host anti-tick defenses, such as blood coagulation and innate/adaptive immune responses. As for blood clotting, Iripin-3, pre-incubated with plasma before addition of stimulants, had no significant effect on the intrinsic and common pathways of the coagulation cascade but delayed blood clot formation via the extrinsic coagulation pathway. This finding is interesting because to date, some tick serpins have been reported to inhibit intrinsic and common coagulating pathways but none of them significantly affected the extrinsic pathway [124,125]. Iripin-3 slightly reduced the proteolytic activity of FVIIa in the *in vitro* enzymatic

assay, but we did not observe the formation of a covalent complex between Iripin-3 and FVIIa under given experimental conditions. Thus, the mechanism behind the suppression of the extrinsic pathway by Iripin-3 remains unclear.

Besides inhibiting blood coagulation, Iripin-3 attenuated the production of the pro-inflammatory cytokine IL-6 by LPS-stimulated bone marrow-derived macrophages (BMDMs). This observation supports the role of Iripin-3 in the suppression of the host's inflammatory response.

Several *in vitro* experiments were focused on the ability of Iripin-3 to modulate the adaptive immune response. The serpin reduced the viability of unstimulated and ovalbumin (OVA) peptide-stimulated B lymphocytes and T lymphocytes, while not affecting the survival of BMDMs or dendritic cells. This fact implies that Iripin-3 selectively induces B and T cell death. Since the level of active caspase-3 was only slightly and insignificantly increased in Iripin-3-treated splenocytes, apoptosis associated with caspase-3 activation was not the main cause of impaired cell survival. In addition to reducing $CD4^+$ T cell viability, Iripin-3 dose-dependently inhibited the proliferation of live $CD4^+$ T cells. It cannot be excluded that the unknown mechanism behind B and T cell death might also be responsible for the suppression of $CD4^+$ T cell division. Finally, we assessed the effect of Iripin-3 on the differentiation of naïve $CD4^+$ T cells into Th1, Th2, Th17, and Treg subpopulations. Iripin-3 inhibited Th1 cell differentiation as evidenced by the decreased expression of the Th1-specific transcription factor T-bet in helper T cells and attenuated production of the cytokine IFN-γ [156]. Besides its inhibitory effect on Th1 cell formation, Iripin-3 enhanced the expression of the Treg-specific transcription factor Foxp3 in $CD4^+$ T cells, suggesting the induction of regulatory T cell differentiation [157,158]. In contrast to the differentiation of naïve $CD4^+$ T cells into Th1 or Treg cells, Iripin-3 did not significantly affect Th2 or Th17 differentiation.

Even though Iripin-3 modulates blood coagulation, inflammation, and the adaptive immune response, the RNAi experiment showed that the serpin is not essential for the feeding success of *I. ricinus* nymphs. This might be caused by the fact that tick saliva is a complex mixture of many proteins and non-proteins, some of which share the same function [159]. Salivary

molecules with Iripin-3-like activities might therefore compensate for the loss of *iripin-3* expression.

In addition to function, we determined the structure of Iripin-3 at 1.95 Å resolution by using the X-ray crystallography technique. Iripin-3 in the crystal was composed of ten α-helices and three β-sheets, and the cleavage of its RCL by some contaminating proteases before or during the crystallization experiment resulted in the RCL insertion into the β-sheet A as an extra β-strand.

2.2 Paper II

Paper II concerns the structural and functional characterization of *I. ricinus* serpin Iripin-8. RT-qPCR analysis showed that *iripin-8* is predominantly expressed in nymphs and in the salivary glands of adult females. Weaker expression of the serpin was also detected in midguts and ovaries of adult ticks. Furthermore, Western blot analysis revealed the presence of Iripin-8 in tick saliva, suggesting ticks secrete this serpin via saliva into the tick attachment site to suppress host's anti-tick defenses. In accordance with the basic amino acid arginine at the P1 position, Iripin-8 was shown to form covalent complexes with trypsin and nine trypsin-like serine proteases: activated protein C, FVIIa, FIXa, FXa, FXIa, FXIIa, kallikrein, plasmin, and thrombin. Of these proteases, Iripin-8 most efficiently inhibited plasmin and further exerted potent inhibitory activity against trypsin, kallikrein, FXIa, and thrombin.

Since Iripin-8 inhibited multiple blood clotting factors, we further tested its effect on blood coagulation *in vitro*. Although Iripin-8 reduced the proteolytic activity of FVIIa, it did not significantly affect the extrinsic coagulation pathway. In contrast to its lack of effect on the tissue factor pathway, Iripin-8 dose-dependently delayed fibrin clot formation via both the intrinsic and common coagulation pathways. A statistically significant inhibitory effect on the intrinsic coagulation pathway was already achieved with Iripin-8 used at 375 nM concentration, and fibrin clot formation in the common pathway was blocked completely with 800 nM Iripin-8. These results suggest that the inhibition of blood coagulation may be the main role of Iripin-8 at the tick-host interface. Given its expression in the *I. ricinus* midgut, Iripin-8 could also have a role in maintaining ingested blood in the tick midgut in an unclotted state.

Besides inhibiting blood coagulation, Iripin-8, similarly to another *I. ricinus* serpin Iripin-5 [133], suppressed the *in vitro* lysis of rabbit erythrocytes by human complement. A marked complement inhibition was achieved only with 10 μM Iripin-8, whereas Iripin-5 was already highly effective when used at 1.25 μM concentration. Therefore, Iripin-5 is a more potent inhibitor of MAC formation than Iripin-8. When tested in *in vitro* immunological experiments, Iripin-8 had no significant effect on OVA peptide-induced $CD4^+$ T cell proliferation and neutrophil migration towards the chemoattractant N-formyl-methionyl-leucyl-phenylalanine. Although we did not observe any inhibitory activity in the two *in vitro* assays, Iripin-8 may still be involved in the attenuation of the host's immune response at the tick feeding site due to its anti-complement activity and its ability to inhibit proteases involved in inflammation, such as trypsin, kallikrein, plasmin, thrombin, and FXa [19,20,153,155,160].

Downregulation of *iripin-8* expression in *I. ricinus* nymphs by RNAi resulted in prolonged feeding time and higher mortality of ticks, suggesting Iripin-8 contributes to successful blood feeding. However, the silencing of *iripin-8* had no significant effect on the transmission of *Borrelia afzelii* from infected nymphs to mice, which indicates that Iripin-8 probably does not act as a SAT factor. Interestingly, the RCL of Iripin-8 is fully conserved among the serpins of various species of hard-bodied ticks, including the functionally characterized serpins AAS19 from *A. americanum*, RHS8 from *R. haemaphysaloides*, and RmS-15 from *R. microplus*. Like Iripin-8, both AAS19 and RmS-15 inhibited the coagulation cascade [161,162]. Moreover, the knockdown of *aas19* expression by RNAi caused tick deformities and reduced engorgement weight [138], whereas *rhs8* silencing by RNAi prolonged feeding time, reduced engorgement weight, and impaired oviposition and hatching [128]. These facts indicate that serpins with Iripin-8-like RCL play an important role in the blood meal acquisition and tick reproduction, and therefore represent candidates for the development of a universal anti-tick vaccine that would be effective against more than one tick species.

Besides function, we also determined the structure of native (uncleaved) Iripin-8 at 1.89 Å resolution by employing X-ray crystallography. The crystal structure revealed that Iripin-8 has an unusually long and rigid RCL. The extension of the RCL may explain the ability of Iripin-8 to inhibit multiple proteases. Furthermore, several molecules of polyethylene glycol,

originating from the crystallization buffer, were bound to Iripin-8. This observation implies that Iripin-8 could bind small molecules either in ticks or at the tick-host interface.

2.3 Paper III

Paper III is a review summarizing the current knowledge about tick serpins. At the beginning of the paper, the tick-host-pathogen interaction is briefly described, and the basic information about the distribution, structure, function, and inhibitory mechanism of serpins is provided. The following section of the review deals with the expression of serpins in various tick developmental stages and tissues. Next, the role of serpins in the regulation of processes occurring within tick bodies, such as hemolymph clotting, innate immunity, vitellogenesis, and blood meal digestion, is discussed. The part of the paper concerning the role of serpins in tick physiology is followed by the section about the serpins' involvement in the tick-host interaction. This section mentions the host's anti-tick defenses (i.e., hemostasis, complement activation, innate and adaptive immune responses) and describes the ability of tick salivary serpins to counteract them. More specifically, the reader can learn here how individual tick serpins inhibit blood coagulation, platelet aggregation, and fibrinolysis, and how their anti-coagulant activity is affected by the interaction with glycosaminoglycans. The anti-complement, anti-inflammatory, and immunomodulatory activities of individual tick serpins are also summarized in this section. Finally, the potential application of tick serpins in the development of novel therapeutics and anti-tick vaccines is discussed.

The review article consists of two tables. Table 1 provides a brief description of each tick serpin mentioned in the text, including its expression in ticks, inhibited proteases, affected biological processes, and the results of vaccination experiments. Table 2 lists second-order rate constants of the interaction between tick serpins and their target serine proteases. Besides two tables, the paper contains five figures depicting the serpin's structure and inhibitory mechanism (Figure 1) and the regulation of biological processes by tick serpins (Figures 2–5).

2.4 Paper IV

Paper IV is a manuscript that describes the structure and function of *I. ricinus* serpin Iripin-1. The serpin was expressed most abundantly in the salivary glands of adult ticks where its expression reached a peak during the eighth day of blood feeding. Since the signal peptide was predicted at the N terminus of the Iripin-1 sequence, it can be concluded that ticks secrete this salivary serpin into the feeding cavity especially during the late, rapid feeding phase to facilitate blood meal acquisition.

Despite having arginine at the P1 site, Iripin-1, unlike Iripin-8, was not an efficient inhibitor of blood clotting factors, and accordingly did not significantly delay plasma clotting in *in vitro* coagulation assays. In contrast to its lack of effect on coagulation factors, Iripin-1 formed covalent complexes with trypsin and trypsin-like serine proteases kallikrein, matriptase, and plasmin. Of these four proteases, trypsin was inhibited most potently by Iripin-1. Since the proteases targeted by Iripin-1 possess pro-inflammatory activities [25,153-155,160], ticks might utilize Iripin-1 to attenuate the host's inflammatory response and concomitant immune cell migration. The role of Iripin-1 in alleviating inflammation is further supported by the fact that its RCL resembles the RCL of the functionally characterized serpins AAS27 from *A. americanum*, HlSerpin-a from *H. longicornis*, and RmS-6 from *R. microplus*. None of these three serpins has been shown to inhibit blood coagulation but all of them attenuated the inflammatory response *in vivo* [80,151,152].

Iripin-1 had no significant effect on the production of pro-inflammatory cytokines TNF, IL-1β, and IL-6 but increased the *in vivo* secretion of the immunosuppressive cytokine IL-10. Elevated concentration of IL-10 at the tick feeding site might contribute to the suppression of host's innate and adaptive immune responses [163]. Furthermore, Iripin-1 enhanced the secretion of chemokines for neutrophils (CXCL1, CXCL2), monocytes (CCL2), and eosinophils (CCL11) in the thioglycolate-induced peritonitis model. The chemokine MCP-1/CCL2 triggers histamine release from mast cells and basophils [164-166]. Since histamine induces vasodilation and enhances vascular permeability [167], its release at the tick attachment site could increase blood flow, thus facilitating tick engorgement during the late, rapid feeding phase [168].

Despite increased chemokine levels, Iripin-1 inhibited the recruitment of neutrophils and monocytes but not eosinophils to the inflamed mouse peritoneal cavities. To find a possible cause of impaired cell recruitment, we further tested the effect of Iripin-1 on the expression of molecules on the surface of either LPS-stimulated bone marrow-derived neutrophils or TNF-stimulated human umbilical vein endothelial cells (HUVECs). Iripin-1 decreased the expression of CXCR2, a receptor for chemokines CXCL1 and CXCL2 [169], on the surface of neutrophils. Moreover, Iripin-1 attenuated the expression of cell adhesion molecules ICAM-1, VCAM-1, and CD99 on the surface of HUVECs. These results suggest that Iripin-1 might inhibit immune cell recruitment to the site of inflammation by reducing the expression of chemokine receptors on immune cell surfaces and/or by decreasing the surface expression of certain cell adhesion molecules on endothelial cells. However, it is necessary to confirm the results of the *in vitro* experiments *in vivo*.

In addition to function, we determined the structure of Iripin-1 in its native metastable conformation at 2.10 Å resolution by employing the X-ray crystallography technique. This method revealed that Iripin-1 consists of ten α-helices and three β-sheets, and its uncleaved RCL is 5 amino acid residues shorter than the RCL of Iripin-8.

3 PAPERS

3.1 Paper I

Iripin-3, a new salivary protein isolated from *Ixodes ricinus* ticks, displays immunomodulatory and anti-hemostatic properties *in vitro*

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ORIGINAL RESEARCH
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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 x 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

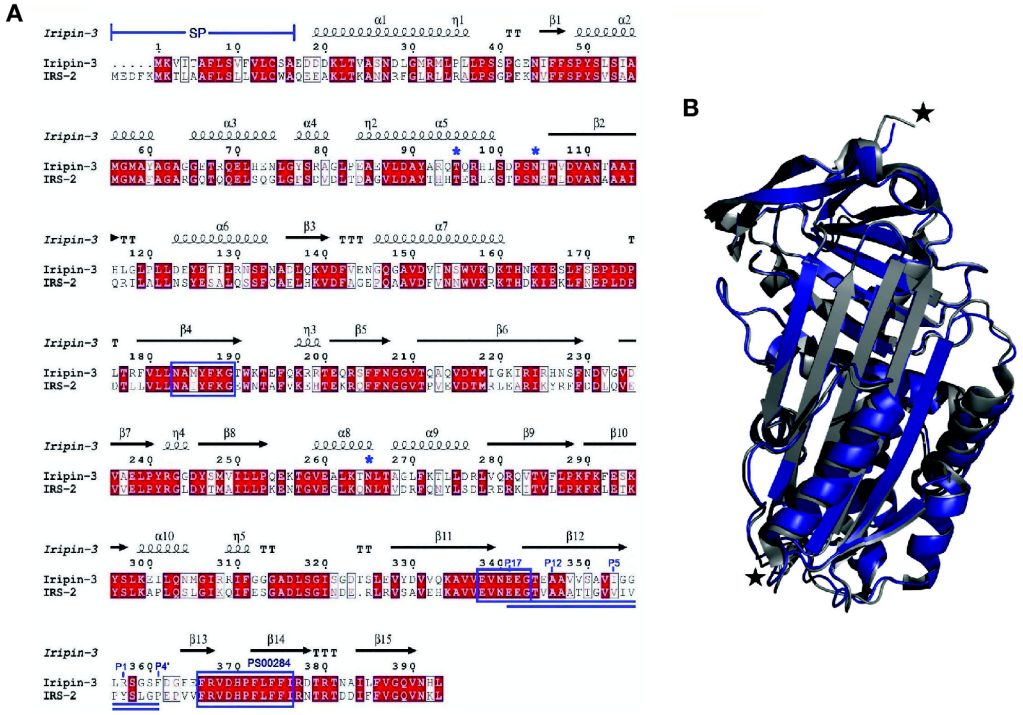

FIGURE 1 | A comparison of the primary, secondary and tertiary structures of Iripin-3 and IRS-2. **(A)** Structure-based sequence alignment of Iripin-3 and IRS-2. Secondary structure elements, which are shown above the aligned sequences, are depicted as spirals (α-helices, 3_{10}-helices) and arrows (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**).

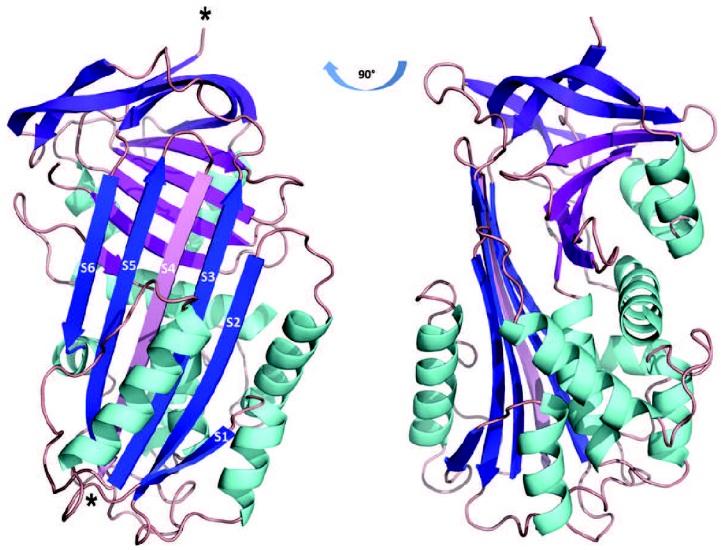

FIGURE 2 | Cartoon representation of the structure of cleaved Iripin-3. α-helices are colored cyan, β-sheet A is blue, β-sheet B is magenta, β-sheet C is purple, and loops are colored wheat. The insertion of the RCL hinge region between β-strands S3 and S5 (depicted in blue) resulted in the formation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

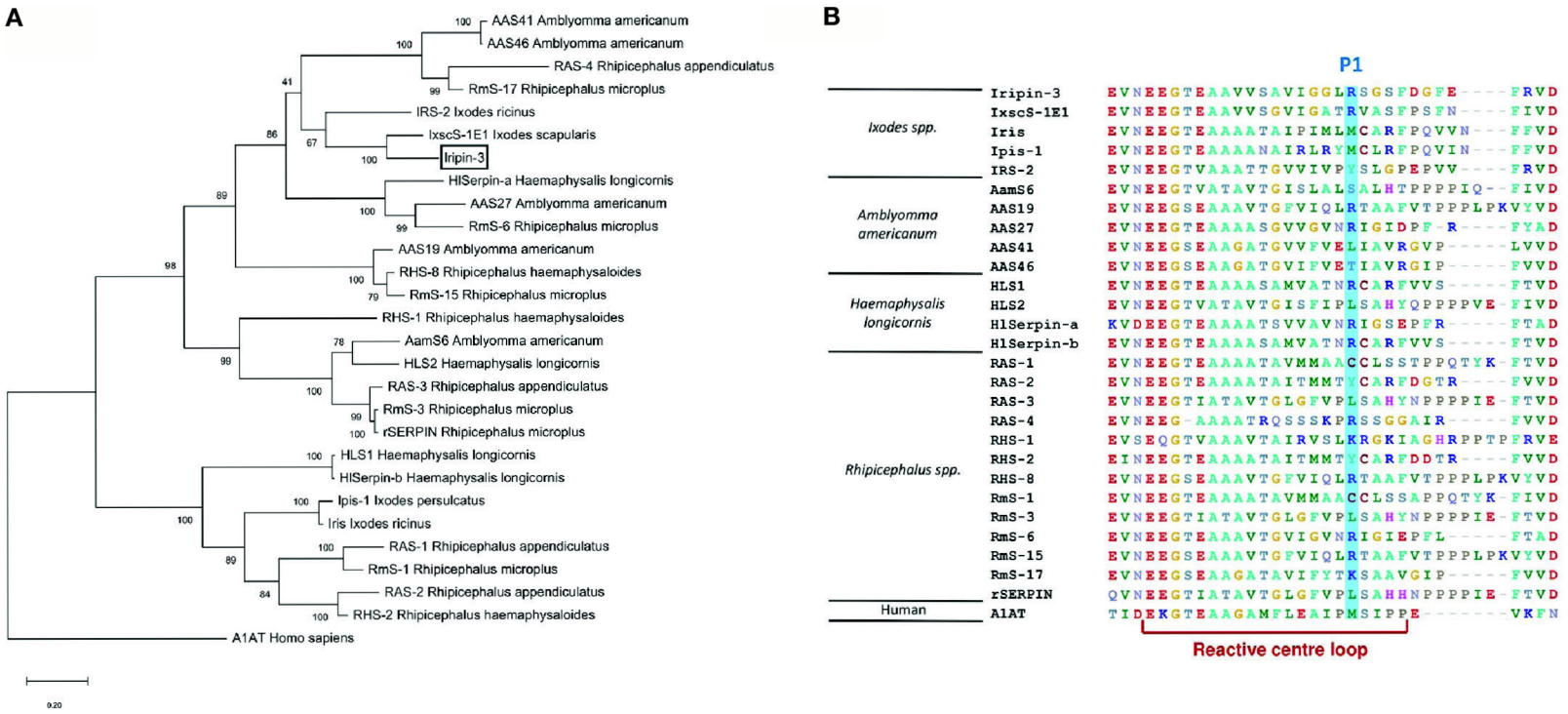

FIGURE 3 | Phylogenetic analysis of selected tick serpins. Protein sequences of previously characterized tick serpins were aligned and analyzed to determine phylogenetic relationships. **(A)** A phylogenetic tree was built using the maximum likelihood method and JTT matrix-based model. Alpha-1-antitrypsin (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.

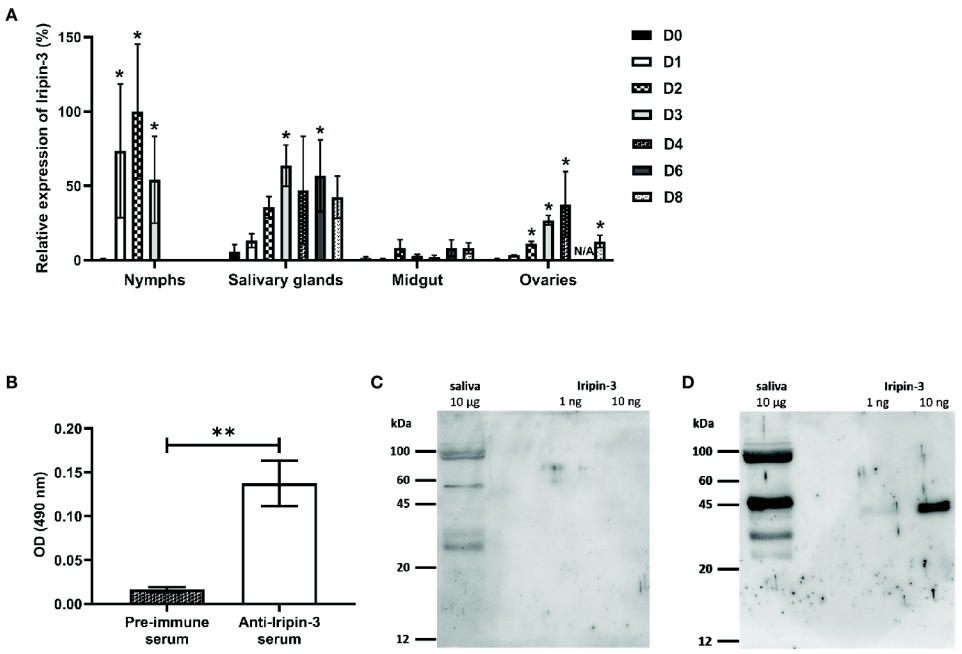

FIGURE 4 | *Iripin-3* transcription in *I. ricinus* ticks is increased in response to blood feeding, and Iripin-3 protein is present in the saliva of feeding ticks. **(A)** *Iripin-3* mRNA expression in nymphs and in the salivary glands, midguts and ovaries of adult females feeding for 1 (D1), 2 (D2), 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-

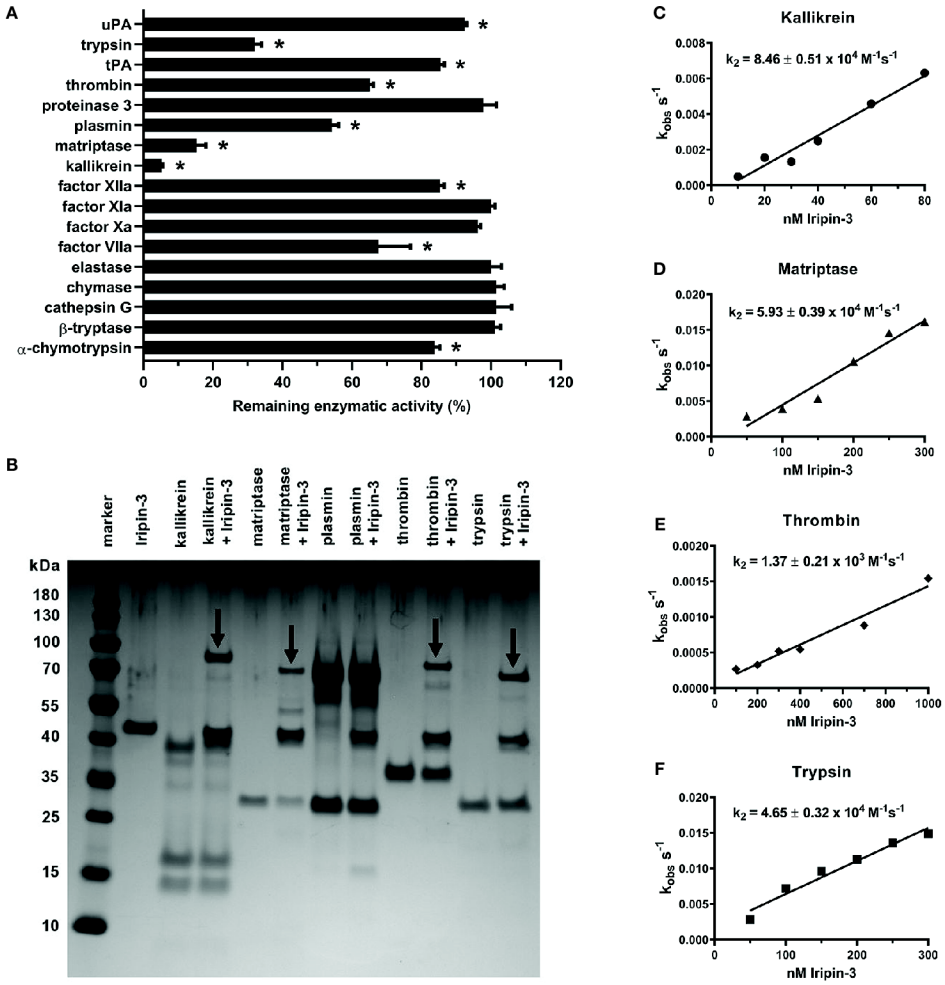

FIGURE 5 | Iripin-3 suppresses the enzymatic activities of kallikrein, matriptase, thrombin, and trypsin through the classic serpin inhibitory mechanism. **(A)** The residual enzymatic activities of 17 selected serine proteases in the presence of 400 nM Iripin-3. The experiment was performed in triplicate, 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**),

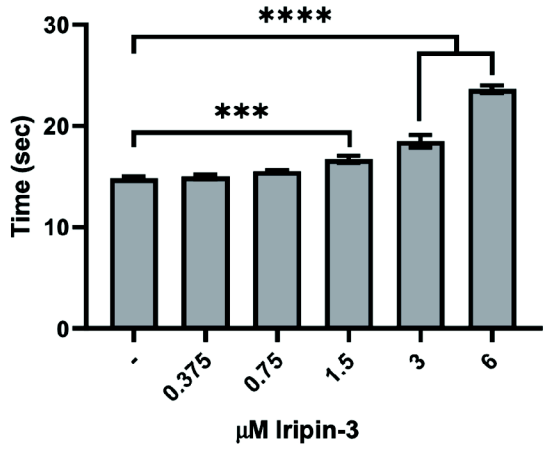

FIGURE 6 | Iripin-3 inhibits the extrinsic pathway of blood coagulation. Human plasma was treated with no Iripin-3 or with 0.375, 0.75, 1.5, 3, and 6 µM Iripin-3 and the time required for blood clot formation in the prothrombin time assay was subsequently determined on a coagulometer. Data are presented 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

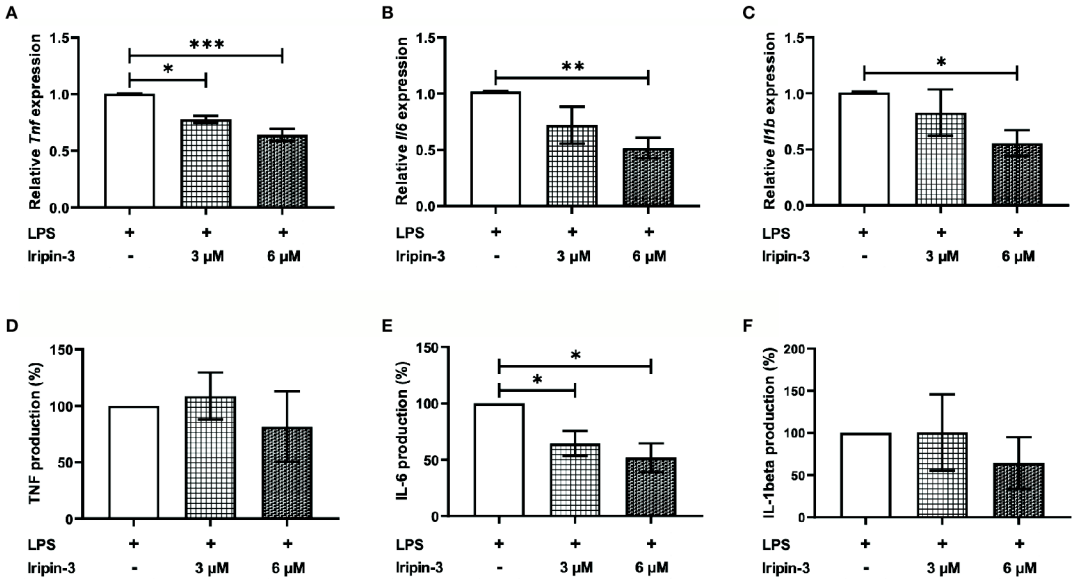

FIGURE 7 | Iripin-3 inhibits the expression of pro-inflammatory cytokines in LPS-stimulated BMDMs. Macrophages derived from bone marrow cells isolated from C57BL/6N mice were pre-incubated with 3 µM or 6 µM Iripin-3 for 40 min and were then stimulated with LPS (100 ng/ml) for 24 h. **(A–C)** At the end 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.

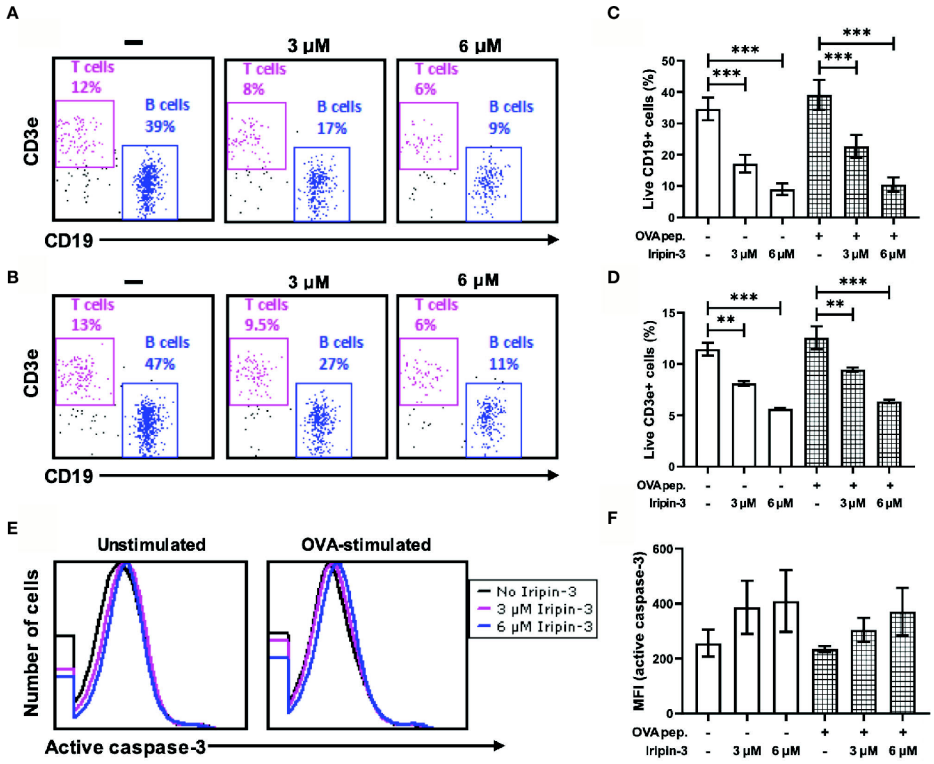

FIGURE 8 | Iripin-3 reduces B and T cell viability and does not significantly alter active caspase-3 levels. **(A, B)** Dot plots depicting the percentage of live $CD45^+CD19^+$ cells (B cells) and live $CD45^+CD3e^+$ cells (T cells) in unstimulated splenocytes **(A)** or OVA peptide-stimulated splenocytes **(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

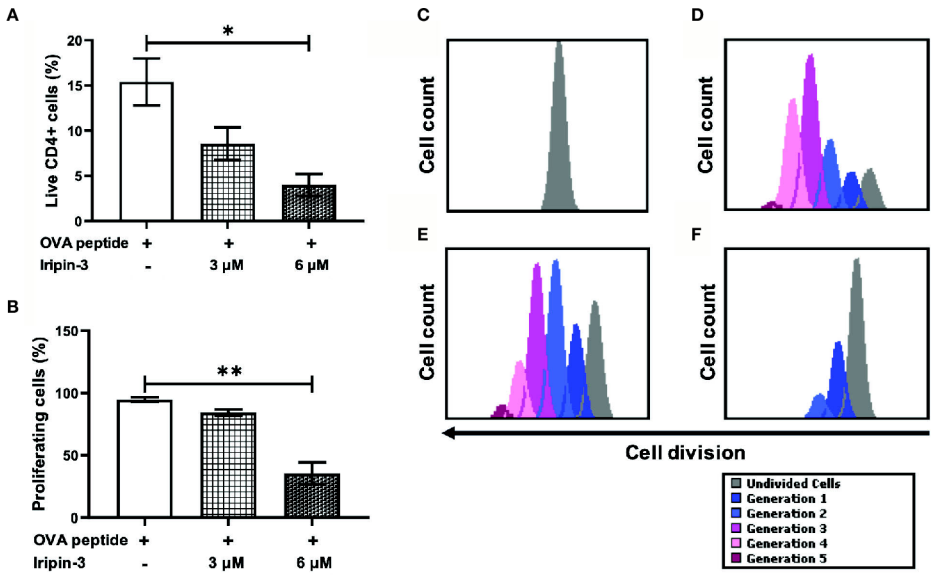

FIGURE 9 | Iripin-3 impairs the survival and proliferation of CD4$^+$ splenocytes. **(A, B)** The percentage of live CD4$^+$ cells **(A)** and the percentage of proliferating live CD4$^+$ cells **(B)** after exposure to 3 µM or 6 µM Iripin-3. Cells not treated with Iripin-3 were used as control. After 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

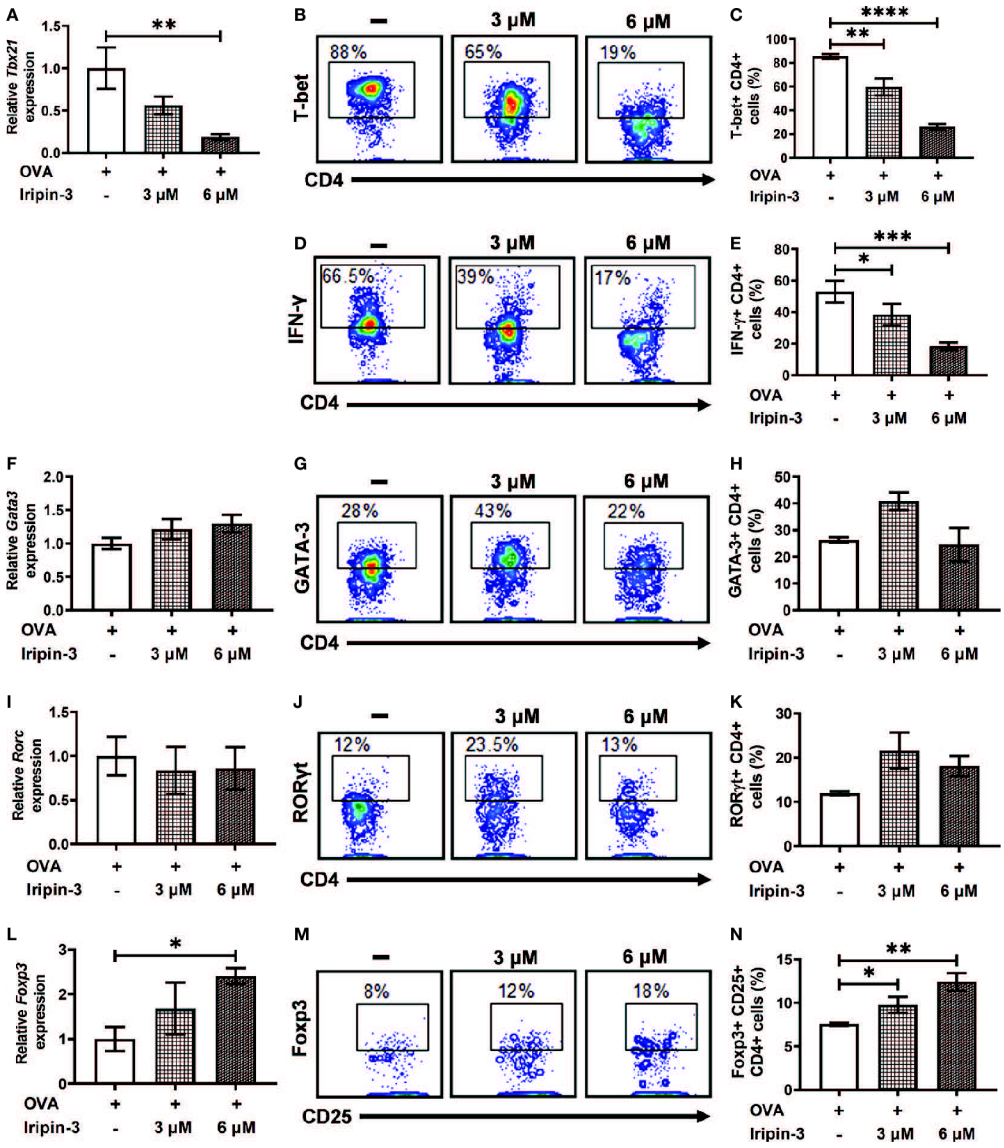

FIGURE 10 | Iripin-3 alters the expression of CD4$^+$ T cell transcription factors at both the mRNA and protein levels. **(A, F, I, L)** Expression of *Tbx21* **(A)**, *Gata3* **(F)**, *Rorc* **(I)**, and *Foxp3* **(L)** in CD4$^+$ cells stimulated with OVA peptide for 72 h. Cells were untreated with 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.

Supplementary Material

Materials and methods

Production of recombinant Iripin-3

A full-length Iripin-3 sequence was obtained during a salivary gland transcriptome project (1) and was submitted to GenBank under accession number GADI01004776.1. The Iripin-3 nucleotide sequence without a signal peptide and with an ATG codon inserted into its 5'-terminus was cloned into the pET-17b vector (Novagen, MilliporeSigma, Burlington, MA), and the resulting plasmid was transformed into BL21(DE3)pLysS chemically competent *E. coli* cells (Thermo Fisher Scientific, Waltham, MA). Cells were grown in LB medium containing ampicillin (100 µg/ml) and chloramphenicol (34 µg/ml), and when the OD600 of the culture reached approximately 0.7, isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.5 mM) was added to induce gene expression. Cells were harvested after 3.5 h incubation in the presence of IPTG and the cell pellet was resuspended in 20 mM Tris-HCl (pH 8). Cell disruption by sonication in inclusion bodies isolation buffer (20 mM Tris-HCl, 1% Triton X-100, pH 8) and repeated washing with 20 mM Tris-HCl (pH 8) resulted in a pellet that contained mainly inclusion bodies of insoluble recombinant Iripin-3. The inclusion bodies were dissolved by stirring in 6 M guanidine hydrochloride (pH 8) and 10 mM dithiotreitol for 1 h at room temperature. Following centrifugation, the supernatant, which contained denatured Iripin-3 released from the inclusion bodies, was diluted 150-fold in refolding buffer (20 mM Tris-HCl, 150 mM NaCl, pH 8), and the mixture was incubated overnight at 4°C. The precipitated protein was removed by filtration through filter paper and Steritop-GP (MilliporeSigma) and concentrated with a stirred chamber concentrator (MilliporeSigma). Properly refolded Iripin-3 was then dialyzed against 20 mM Tris-HCl (pH 8) and purified on a HiLoad 26/60 Superdex 200 pg gel filtration column (Cytiva) and a Mono Q column (Cytiva) with the 0-1 M gradient of NaCl. Endotoxin was removed by the company ARVYS Proteins, Inc. (Trumbull, CT) via a detergent-based method.

Crystallization

Crystallization experiments were performed in Swissci 96-well 2-drop MRC crystallization plates (Molecular Dimensions Ltd., Sheffield, UK) using the sitting-drop vapor diffusion technique and OryxNano crystallization robot (Douglas Instruments Ltd., Hungerford, UK). A suitable Iripin-3 concentration (1.88 mg/ml) was determined by the PCT Pre-Crystallization Test (Hampton Research, Aliso Viejo, CA). Iripin-3 crystals were grown with the precipitant composed of 0.2 M potassium thiocyanate, 0.1 M sodium cacodylate, and 8% w/v γ-polyglutamic acid, pH 6.5 at 21°C. The protein-to-precipitant solution ratios 2:1 (2 µl:1 µl) or 1:1 (1 µl:1 µl) were equilibrated against 50 µl of reservoir solution.

X-ray data collection and structure determination

Freshly grown crystals were flash frozen in a liquid nitrogen stream without additional cryoprotection, and X-ray diffraction data were collected at the BESSY II electron storage ring on the beamline BL14.1 operated by the Helmholtz-Zentrum Berlin (2). Data were processed using the XDS Program Package

(3) with the XDSAPP graphical user interface (4). The best diffracting crystal exhibited symmetry of the $P6_222$ space group and contained one molecule in the asymmetric unit. The structure of Iripin-3 was solved by the molecular replacement method using MOLREP (5). Of all the structures deposited in the PDB, IRS-2 (PDB code 3NDA) (6) displayed the highest sequence identity (56%) to Iripin-3 and was therefore used as a search model. The Iripin-3 structure was refined with the program REFMAC5 (7) from the CCP4 suite (8) and manually rebuilt in Coot (9). MolProbity (10) and wwPDB (11) were used for final qualitative validation of the model. Figures of the Iripin-3 structure were made using the PyMOL Molecular Graphic System (Schrödinger, LLC, New York, NY). Atomic coordinates were deposited in the PDB under accession code 7AHP. Data collection, processing, and refinement statistics are summarized in **Supplementary Table 1**.

Presence of Iripin-3 in tick saliva (ELISA)

Each well of a Corning 96-well microplate was coated overnight at 4°C with 50 µl of tick saliva diluted in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) to a final concentration of 10 µg/ml. The unoccupied binding sites of the plate were blocked by the addition of 300 µl/well of blocking buffer (5% precolostral calf serum in PBS) for 1 h at 37 °C. After washing the plate three times with wash buffer (0.05% Tween 20 in PBS), 50 µl of pre-immune serum or antiserum against Iripin-3, pre-diluted 1:1000 with 2% precolostral calf serum in PBS, was added and incubated for 1 h at 37°C. Another three washes with wash buffer were followed by the addition of 100 µl/well of peroxidase-conjugated goat antibody recognizing rabbit immunoglobulin G (Sigma Aldrich, St Louis, MO). The antibody was pre-diluted 1:1000 with 2% precolostral calf serum in PBS and incubated for 1 h at 37°C. At the end of incubation, the plate was again washed three times with wash buffer, and then 100 µl of a substrate solution (51.4 mM $Na_2HPO_4 \cdot 12\ H_2O$, 24.3 mM $C_6H_8O_7 \cdot H_2O$, 3.7 mM *o*-phenylenediamine, and 0.012% H_2O_2, pH 5) was applied to each well. The enzymatic reaction was stopped by the addition of 2 M H_2SO_4, and the optical density was measured at 490 nm on Synergy H1 microplate reader (BioTek Instruments, Inc., Winooski, VT).

Presence of Iripin-3 in tick saliva (Western blot)

Tick saliva (10 µg) and Iripin-3 (1 ng or 10 ng) were subjected to SDS-PAGE using a 10% gel, and the separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Thermo Fisher Scientific, Waltham, MA). Following blocking in Tris-buffered saline containing 5% fat-free milk and 0.1% Tween 20 for 1 h at room temperature, the blots were incubated overnight at 4°C with pre-immune serum or antiserum against Iripin-3. Both sera were pre-diluted 1:100 in Tris-buffered saline containing 5% milk and 0.1% Tween 20. After washing, the membranes were incubated with goat anti-rabbit antibody conjugated with horseradish peroxidase (Cell Signaling Technology, Danvers, MA) for 1 h at room temperature. The secondary antibody was pre-diluted 1:2000 in the same solution as the primary antibodies. The proteins were visualized using the enhanced chemiluminescent substrate WesternBright Quantum (Advansta, San Jose, CA), and the signal was detected using a charge-coupled device (CCD) imaging system (Uvitec Ltd., Cambridge, UK).

Inhibition of serine proteases

First, the formation of SDS- and heat-stable complexes between Iripin-3 and selected serine proteases (kallikrein, matriptase, plasmin, thrombin, trypsin, factor VIIa) was tested. All enzymes used were of human origin. Kallikrein and thrombin were purchased from Sigma Aldrich, matriptase and trypsin were purchased from R&D Systems (Minneapolis, MI), and plasmin and factor VIIa were obtained

from Haematologic Technologies, Inc. (Essex Junction, VT). Iripin-3 and proteases were diluted in assay buffer corresponding to each protease (described below), and then each of the six serine proteases was incubated with Iripin-3 at equimolar concentrations (1 μM) for 1 h at room temperature. Factor VIIa was incubated with Iripin-3 in the absence or presence of human tissue factor (1 μM, BioLegend, San Diego, CA). The 1 h incubation was followed by the addition of NuPAGE LDS Sample Buffer (Thermo Fisher Scientific) together with dithiotreitol and boiling of samples for 10 min. Finally, samples were analyzed by SDS-PAGE and protein bands were visualized by silver staining.

Second, second-order rate constants of protease inhibition were measured by a discontinuous method under pseudo first-order conditions using at least a 50-fold molar excess of Iripin-3 over serine proteases. Reactions were incubated at room temperature and were stopped at each time point by the addition of the fluorogenic substrate appropriate for the protease used. The slope of the linear part of fluorescence increase over time gave the residual protease activity at each time point. The apparent (observed) first-order rate constant k_{obs} was calculated from the slope of the plot of the natural log of residual protease activity against time. K_{obs} was measured for six different Iripin-3 concentrations and plotted against the serpin concentration. The slope of the line of best fit gave an estimate of the second-order rate constant k_2. The assay buffer was 20 mM Tris, 150 mM NaCl, 0.02% Triton X-100, pH 8.5 for kallikrein and plasmin; 50 mM Tris, 50 mM NaCl, 0.01% Tween 20, pH 9.0 for matriptase; 20 mM Tris, 150 mM NaCl, 0.01% Triton X-100, 5 mM $CaCl_2$, 0.1% polyethylene glycol 6000, pH 8.0 for thrombin and factor VIIa; and 50 mM Tris, 150 mM NaCl, 10 mM $CaCl_2$, 0.05% Brij 35, pH 7.5 for trypsin. The fluorogenic substrates used were Z-FR-AMC for kallikrein, Boc-QAR-AMC for matriptase, and Boc-VPR-AMC for trypsin and thrombin. All substrates were used at 250 μM final concentration. Kallikrein, matriptase, thrombin, and trypsin were used at 200 pM, 500 pM, 20 pM, and 2 pM final concentrations, respectively.

The HADDOCK2.2 web server (12) was used for docking of the peptide consisting of Iripin-3 RCL residues P4-P4' inside the active site of four proteases (trypsin, thrombin, kallikrein and matriptase). The Iripin-3 structure was modified, thus alternative conformations and ligands were removed. The visualization of the docking results together with electrostatic potential depiction and polar contact analysis were made in the PyMOL Molecular Graphics System (Schrödinger, LLC). Tertiary structures of proteases were retrieved from the Protein Data Bank. PDB accession codes are 5TJX for kallikrein, 1EAX for matriptase, 3U69 for thrombin and 1H4W for trypsin.

Pro-inflammatory cytokine expression in bone marrow-derived macrophages (RT-qPCR)

Total RNA was isolated from macrophages using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's instructions. Extracted RNA (500 ng) was treated with DNase I (Thermo Fisher Scientific) and reverse transcribed into cDNA using M-MLV Reverse Transcriptase (Thermo Fisher Scientific) as detailed in the manufacturer's protocol. The resulting cDNA mixed with Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific) and gene-specific primers were used for the analysis of *Tnf*, *Il6*, and *Il1b* expression in the QuantStudio 6 thermal cycler (Thermo Fisher Scientific)**.** Cycling conditions were 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Relative gene expression was calculated using the delta-delta Ct method, since the amplification efficiencies of target genes and a reference gene (*Gapdh*) were approximately equal (13). Nucleotide sequences of forward and reverse primers as well as amplicon lengths are provided in **Supplementary Table 3**.

Viability of macrophages, dendritic cells, and neutrophils

Dendritic cells and macrophages were obtained as described before (14). Briefly, dendritic cells were derived from bone marrow cells of a C57BL/6N mouse by incubating the cells for 8 days in the

presence of 20 ng/ml of GM-CSF (Sigma Aldrich). Macrophages were obtained from bone marrow cells by 7-day incubation in the presence of L929 cell-conditioned medium. Neutrophils were isolated from bone marrow cells by magnetic separation using a Neutrophil Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Dendritic cells, macrophages, and neutrophils were resuspended in RPMI 1640 medium with stable glutamine (Biosera, Kansas City, MO) 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) and then were treated with four different concentrations of Iripin-3 for 1 h at 37°C and 5% CO_2. Subsequently, macrophages and neutrophils were stimulated by the addition of LPS (100 ng/ml, Sigma Aldrich, *E. coli* serotype O111:B4). Dendritic cells were left unstimulated. After incubating the cells for 20 h at 37°C and 5% CO_2, alamarBlue HS Cell Viability Reagent (Thermo Fisher Scientific) was added. The fluorescence intensity was measured on Synergy H1 microplate reader (BioTek Instruments, Inc.; excitation 550 nm; emission 590 nm) 4 h (a 24h incubation period) and 28 h (a 48h incubation period) following alamarBlue addition.

***Iripin-3* knockdown in *I. ricinus* nymphs by the RNA interference technique**

A 550-bp fragment of the *iripin-3* gene was amplified from *I. ricinus* cDNA using primers 5′-ATTCTA GAGTCATTCTTTAACGGTGGCG-3′ and 5′-ATGGGCCCAAAAAGGATGGCGTTTGTGC-3′ that contained restriction sites XbaI and ApaI. The amplified fragment was cloned into the pLL10 vector with two T7 promoters in reverse orientations (15). Double-stranded RNA of *iripin-3* and green fluorescent protein (*gfp*) were synthesized using the MEGAscript T7 Transcription Kit (Thermo Fisher Scientific) as described previously (16). The dsRNA (32 nl; 3 μg/μl) was injected into the haemocoel of *I. ricinus* nymphs using Nanoinject II (Drummond Scientific Company, Broomall, PA). After a 3-day rest in a humid chamber at room temperature, ticks were fed on C3H/HeN mice (16-20 nymphs per mouse) until full engorgement. Three tick feeding parameters were evaluated - feeding duration, overall feeding success and engorgement weight. The level of gene knockdown was checked by RT-qPCR in an independent feeding experiment.

Supplementary Table 1. X-ray data collection, processing and refinement statistics.

Data collection	
X-ray source	BL14.1, BESSY II, Germany
Wavelength (Å)	0.9184
Detector	PILATUS 6M
Crystal-detector distance (mm)	222.687
Rotation range per image (°)	0.1
Total rotation range (°)	240
Exposure time per image (s)	0.25
Resolution range (Å)	48.32-1.95 (2.07-1.95)
Space group	$P6_222$
Unit-cell dimensions: a, b, c (Å)	132.94, 132.94, 88.89
Unit-cell dimensions: α, β, γ (°)	90.0, 90.0, 120.0
Mosaicity (°)	0.135
Total number of reflections	889221 (130341)
Number of unique reflections	34278 (5397)
Multiplicity	25.94 (24.15)
Average I/σ(I)	13.45 (2.45)
Completeness (%)	99.9 (99.4)
CC ½	99.8 (82.6)
R_{meas} (%)[a]	25.9 (157.3)
Overall B factor from Wilson plot (Å^2)	29.14
Refinement	
Resolution range (Å)	48.32-1.95 (2.07-1.95)
Number of reflections in working set	32559 (2340)
Final R value (%)[b] / Final R_{free} value (%)[c]	19.16 / 22.28
Mean B value (Å)	24.26
Number of atoms in the asymmetric unit	
Protein	2881
Ligand-Tris ion	3
Water	243
Total	3148
Root-mean-square deviations	
Bonds (Å)	0.015
Angles (°)	1.709
Average B factors (Å^2) Overall	24.264
Ramachandran plot	
Most favored (%)	98.90
Allowed (%)	100.00
PDB code	7AHP

The data in parentheses refer to the highest resolution shell.

[a] $R_{meas} = (|I_{hkl} -)I*|)/I_{hkl}$, where the average intensity)I∗ is taken over all symmetry equivalent measurements, and I_{hkl} is the measured intensity for any given reflection.

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

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

Supplementary Table 2. GenBank accession numbers of serpins used in the phylogenetic analysis.

Serpin	Species	GenBank accession number	Reference
A1AT	*Homo sapiens*	AAB59495.1	(17)
AamS6	*Amblyomma americanum*	ABS87358.1	(18)
AAS19		JAI08902.1	(19)
AAS27		JAI08961.1	
AAS41[a]		JAI08957.1	(19,20)
AAS46[a]		JAI08784.1	
HLS1[b]	*Haemaphysalis longicornis*	Not found	(21)
HLS2		BAD11156.1	(22)
HlSerpin-a		QFQ50847.1	(23)
HlSerpin-b		QFQ50848.1	
Ipis-1	*Ixodes persulcatus*	BAP59746.1	(24)
Iripin-3	*Ixodes ricinus*	JAA69032.1	
Iris		CAB55818.2	(25)
IRS-2		ABI94056.2	(6)
IxscS-1E1	*Ixodes scapularis*	AID54718.1	(26)
RAS-1	*Rhipicephalus appendiculatus*	AAK61375.1	(27)
RAS-2		AAK61376.1	
RAS-3		AAK61377.1	
RAS-4		AAK61378.1	
RHS-1	*Rhipicephalus haemaphysaloides*	AFX65224.1	(28)
RHS-2		AFX65225.1	
RHS8		QHU78941.1	(29)
RmS-1	*Rhipicephalus microplus*	AHC98652.1	(30)
RmS-3		AHC98654.1	
RmS-6		AHC98657.1	
RmS-15		AHC98666.1	
RmS-17		AHC98668.1	
rSERPIN[b]		Not found	(31)

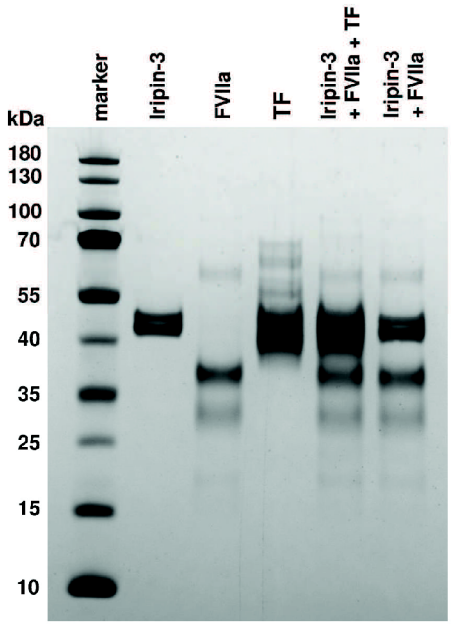
[a] Full-length protein sequences of AAS41 and AAS46 were obtained from the cited article (20), since GenBank contains only partial amino acid sequences of these two *A. americanum* serpins.

[b] In the case of two tick serpins (HLS1 and rSERPIN), no accession number was found, and therefore the amino acid sequences needed for phylogenetic analysis were derived directly from the cited articles.

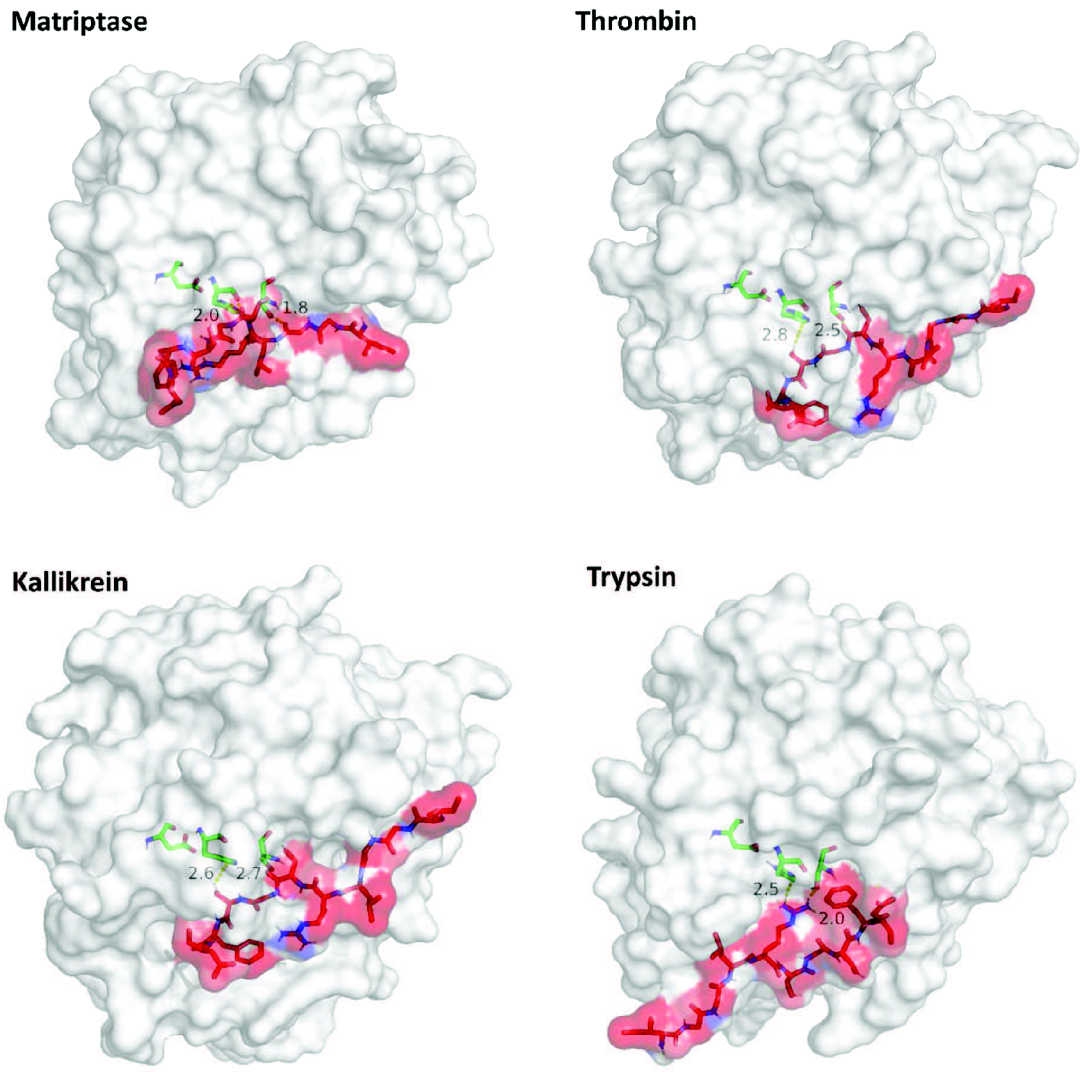
Supplementary Table 3. Sequences of primers used in the study.

Gene	Species	Sequence	Amplicon length (bp)
Iripin-3 expression in ticks			
rps4	*Ixodes ricinus*	Forward: 5′-GGTGAAGAAGATTGTCAAGCAGAG-3′ Reverse: 5′-TGAAGCCAGCAGGGTAGTG-3′	80
iripin-3		Forward: 5′-CACAGCGGCAATTCATTTAGG-3′ Reverse: 5′-CGGTACGTCTCTTCTGAAACTC-3′	269
Pro-inflammatory cytokine expression in macrophages			
Gapdh	*Mus musculus*	Forward: 5'-TGTGTCCGTCGTGGATCTGA-3' Reverse: 5'-TTGCTGTTGAAGTCGCAGGAG-3'	150
Il1b		Forward: 5'-TGACCTGGGCTGTCCTGATG-3' Reverse: 5'-GGTGCTCATGTCCTCATCCTG-3'	160
Il6		Forward: 5'-CTGCAAGAGACTTCCATCCAG-3' Reverse: 5'-AGTGGTATAGACAGGTCTGTTGG-3'	131
Tnf		Forward: 5'-CCCCAAAGGGATGAGAAGTTC-3' Reverse: 5'-GGCTTGTCACTCGAATTTTGAGA-3'	101
Transcription factor expression in CD4$^+$ T cells			
Actb	*Mus musculus*	Forward: 5'-CTCTGGCTCCTAGCACCATGAAGA-3' Reverse: 5'-GTAAAACGCAGCTCAGTAACAGTCCG-3'	200
Foxp3		Forward: 5'-CAGCTCTGCTGGCGAAAGTG-3' Reverse: 5'-TCGTCTGAAGGCAGAGTCAGGA-3'	190
Gapdh		Forward: 5'-TGTGTCCGTCGTGGATCTGA-3' Reverse: 5'-TTGCTGTTGAAGTCGCAGGAG-3'	150
Gata3		Forward: 5'-CTCGGCCATTCGTACATGGAA-3' Reverse: 5'-GGATACCTCTGCACCGTAGC-3'	134
Rorc		Forward: 5'-ACGGCCCTGGTTCTCATCA-3' Reverse: 5'-CCAAATTGTATTGCAGATGTTCCAC-3'	79
Tbx21		Forward: 5'-TCAACCAGCACCAGACAGAGA-3' Reverse: 5'-TCCACCAAGACCACATCCAC-3'	130

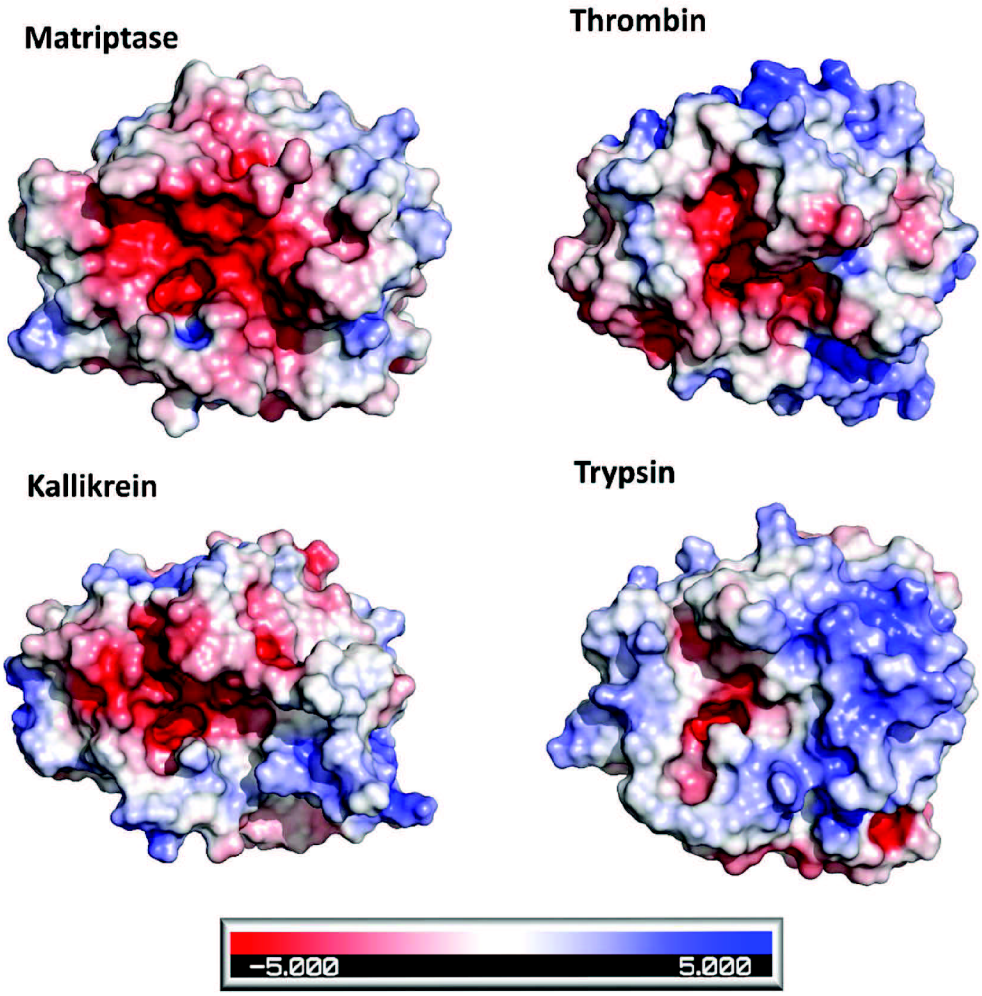
Results



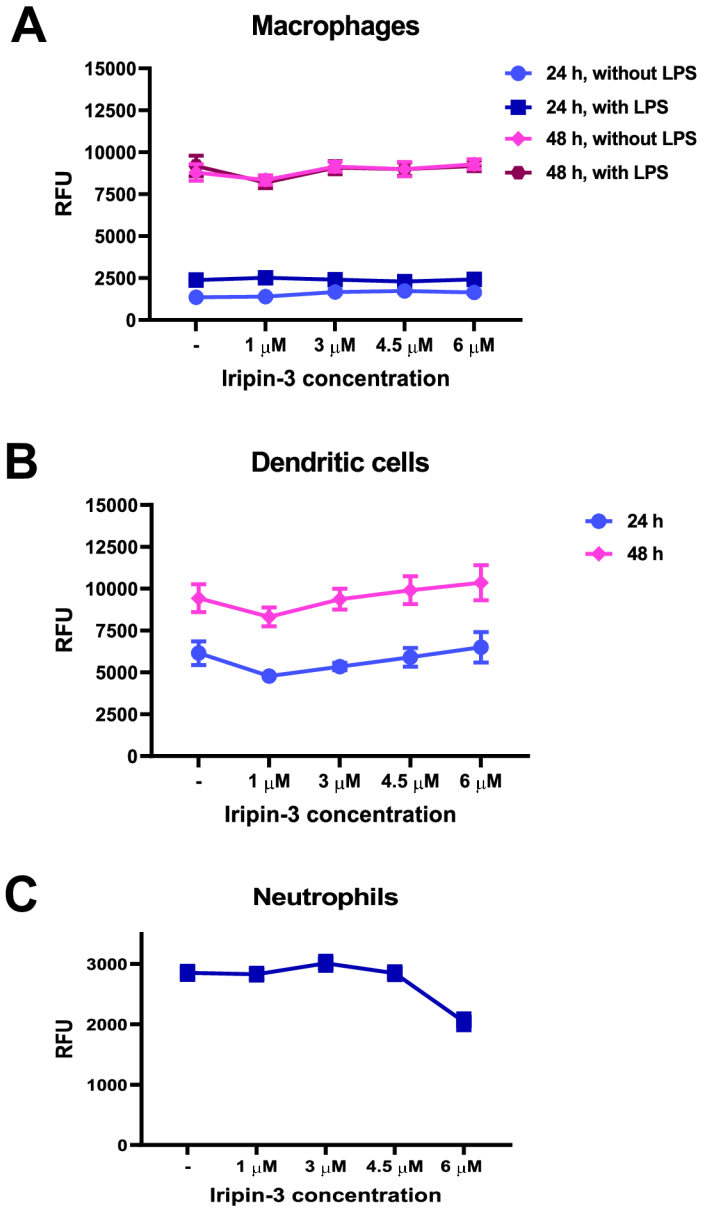
Supplementary Figure 1. Iripin-3 does not form a covalent complex with activated factor VII (FVIIa). A high molecular weight complex formation between FVIIa and Iripin-3 in the absence or presence of tissue factor (TF) was analyzed using SDS-PAGE. Proteins were resolved on 4 to 12% NuPAGE Bis-Tris gels and were visualized by silver staining.



Supplementary Figure 2. Interface analysis of the proteases, for which k_2 was determined, and the P4-P4' part of Iripin-3 RCL. The surfaces of the four proteases are colored white, and the P4-P4' region of Iripin-3 RCL is represented by a cartoon (red). Predicted interactions shorter than 2.8 Å are shown with dotted lines. The catalytic triad of the proteases is depicted in green.



Supplementary Figure 3. Electrostatic potential on the surfaces of matriptase, thrombin, kallikrein and trypsin. Active sites of these serine proteases are negatively charged (red color), which enables binding of suitable substrates and/or inhibitors.



Supplementary Figure 4. Iripin-3 does not negatively affect macrophage, dendritic cell, and neutrophil viability. Cell viability was evaluated by their ability to reduce virtually non-fluorescent resazurin, the active ingredient of alamarBlue, to highly fluorescent resorufin. **(A, B)** The viability of unstimulated or LPS-stimulated macrophages **(A)** and unstimulated dendritic cells **(B)** after exposure to four different concentrations of Iripin-3 for either 24 h or 48 h. Macrophages and dendritic cells unexposed to Iripin-3 were used as control. **(C)** The viability of LPS-stimulated neutrophils untreated with Iripin-3 or treated with four different concentrations of Iripin-3 for 24 h. All data in **(A, B, C)** are presented as mean ± SEM. The experiment was performed only once. RFU, relative fluorescence unit.

Supplementary Table 4. The overall feeding success, time course of blood feeding and weight of fully engorged *I. ricinus* nymphs were not significantly affected by *iripin-3* silencing via RNA interference.

Silenced gene	Feeding success[a]	FF < 48 h	FF 48-72 h[a]	FF 72-96 h[a]	FF > 96 h[a]	Weight (mg)[b]
gfp	92.1 % (70/76)	0	30.0 % (n=21)	61.4 % (n=43)	8.6 % (n=6)	3.51 ± 1.03
iripin-3	92.2 % (71/77)	0	33.8 % (n=24)	57.7 % (n=41)	8.5 % (n=6)	3.79 ± 0.92

FF, fully fed nymphs. Parentheses contain the number of nymphs that reached full engorgement within given time periods.

[a] Data were analyzed by the two-tailed Fisher's exact test.

[b] Data, expressed as mean ± standard deviation, were analyzed by the unpaired two-tailed *t*-test.

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3.2 Paper II

Ixodes ricinus salivary serpin Iripin-8 inhibits the intrinsic pathway of coagulation and complement

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Article

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.

Here we present the functional characterization of Iripin-8, the serpin from *I. ricinus* previously referred to as IRS-8 [30,34], whose RCL is conserved among several tick species. We demonstrate its inhibitory activity against serine proteases involved in coagulation and direct the inhibition of the intrinsic coagulation pathway in vitro. Moreover, we report for the first time the inhibition of complement by a tick serpin. Finally, we provide the structure of Iripin-8 in its native, uncleaved form, revealing an unusual RCL conserved among several tick serpins.

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.

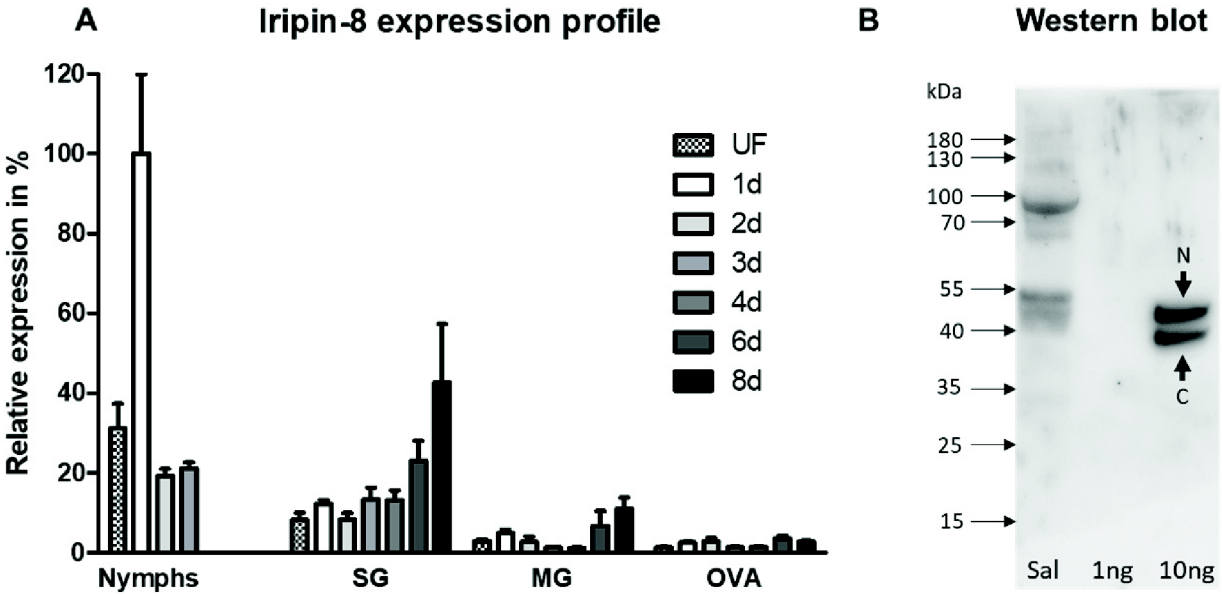

Figure 1. Iripin-8 expression in ticks and its presence in tick saliva. (**A**) Pools of *I. ricinus* salivary glands, midguts, and ovaries from female ticks and whole bodies from nymphs were dissected under RNase-free conditions. cDNA was subsequently prepared as a template for qRT-PCR. Iripin-8 expression 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.

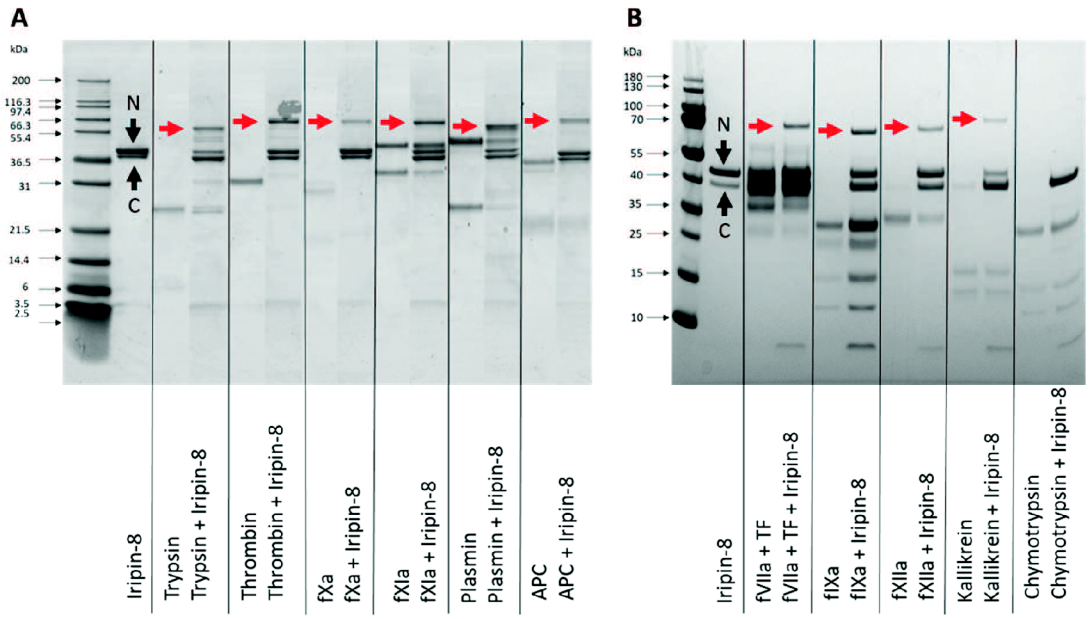

Figure 2. Formation of covalent complexes between Iripin-8 and serine proteases. Iripin-8 and selected serine proteases were incubated for 1 h and subsequently analyzed for complex formation by reducing SDS-PAGE. Protein separation differs between (**A**,**B**) due to the use of gels with different polyacrylamide 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].

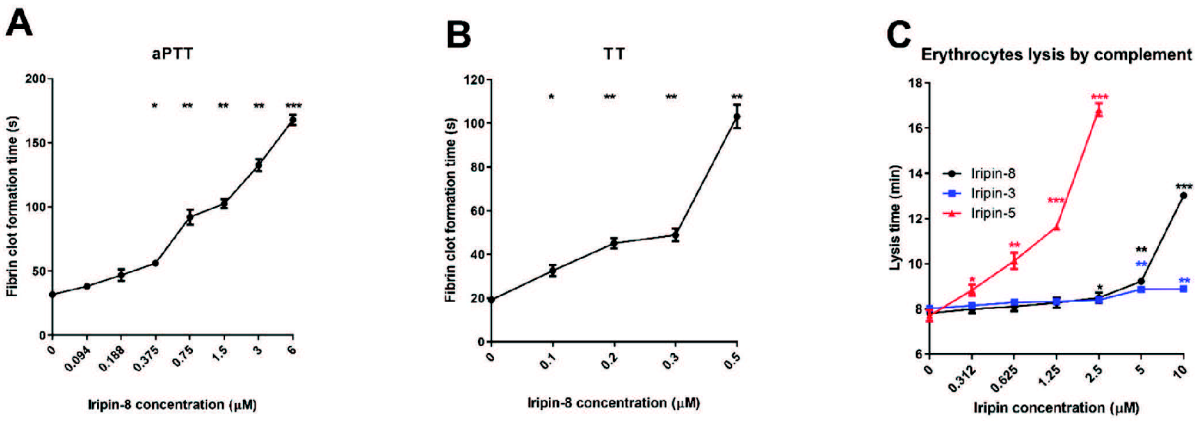

Figure 3. Inhibition of complement and coagulation pathways by Iripin-8. (**A**) Iripin-8 inhibits the intrinsic coagulation pathway. Human plasma was preincubated with increasing concentrations of Iripin-8 (94 nM–6 μM). Coagulation was triggered by the addition of Dapttin® reagent and $CaCl_2$, and 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. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

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.

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).

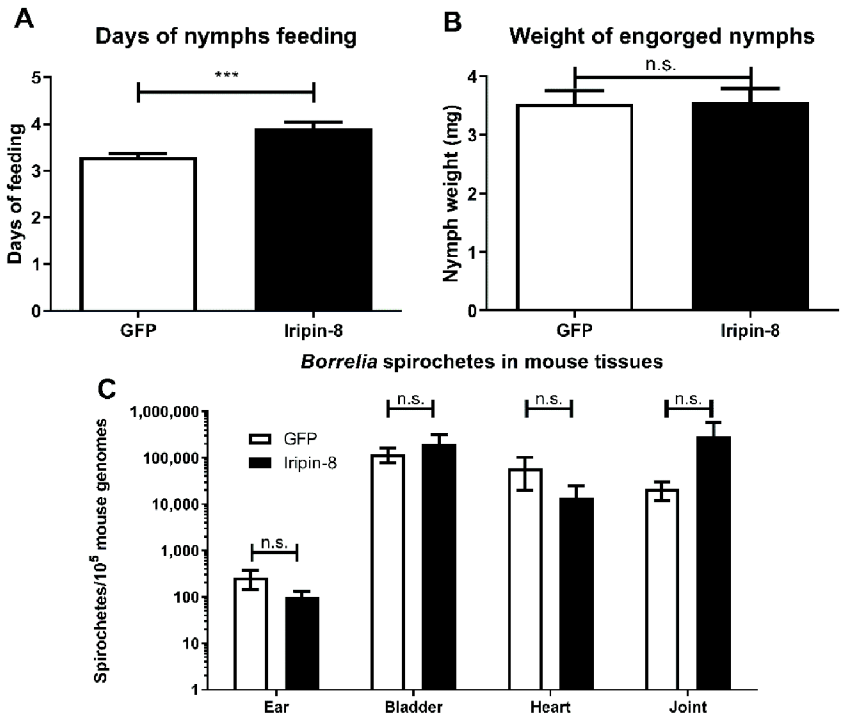

Figure 4. Effect of RNAi on tick fitness and *Borrelia* transmission. (**A**) RNAi of Iripin-8 prolonged the length of *I. ricinus* nymph feeding compared with the control group (GFP). (**B**) Weight of fully engorged nymphs with Iripin-8 knockdown was not different from the control group (GFP). (**C**) 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.

2.7. Role of Iripin-8 in Modulating Host Immunity

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).

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 alpha-1-antitrypsin (A1AT, 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 A1AT. 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.

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).

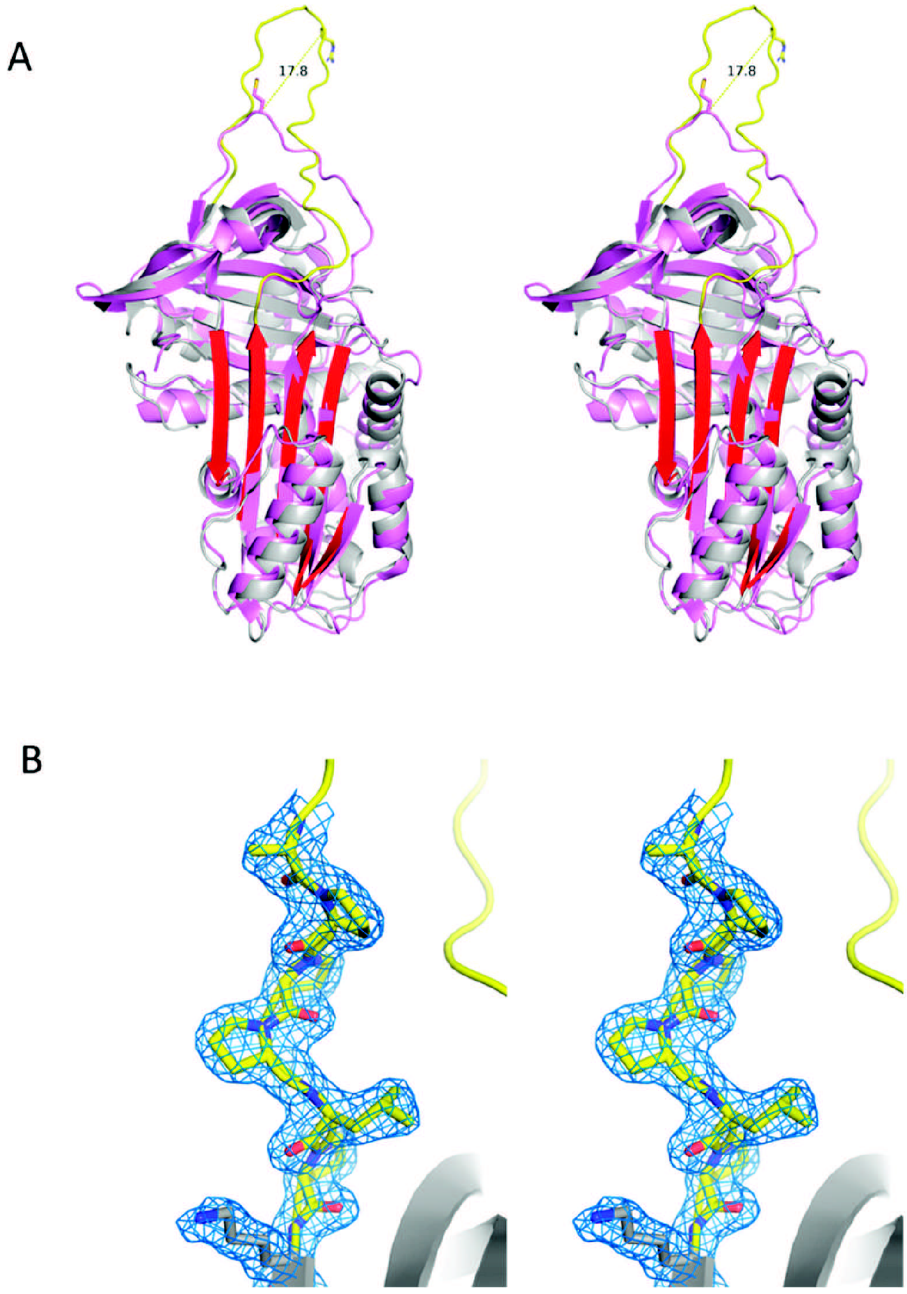

Figure 5. Crystal structure of native Iripin-8. (**A**) Stereo view of a ribbon diagram of Iripin-8 (gray with yellow RCL and red beta sheet A) superimposed with alpha-1-antitrypsin (PDB code 3ne4). The P1 side chains of both molecules are represented as sticks, and the distance between their Cα atoms 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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Supplementary Materials

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

Supplementary Table S1. List of primers.

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

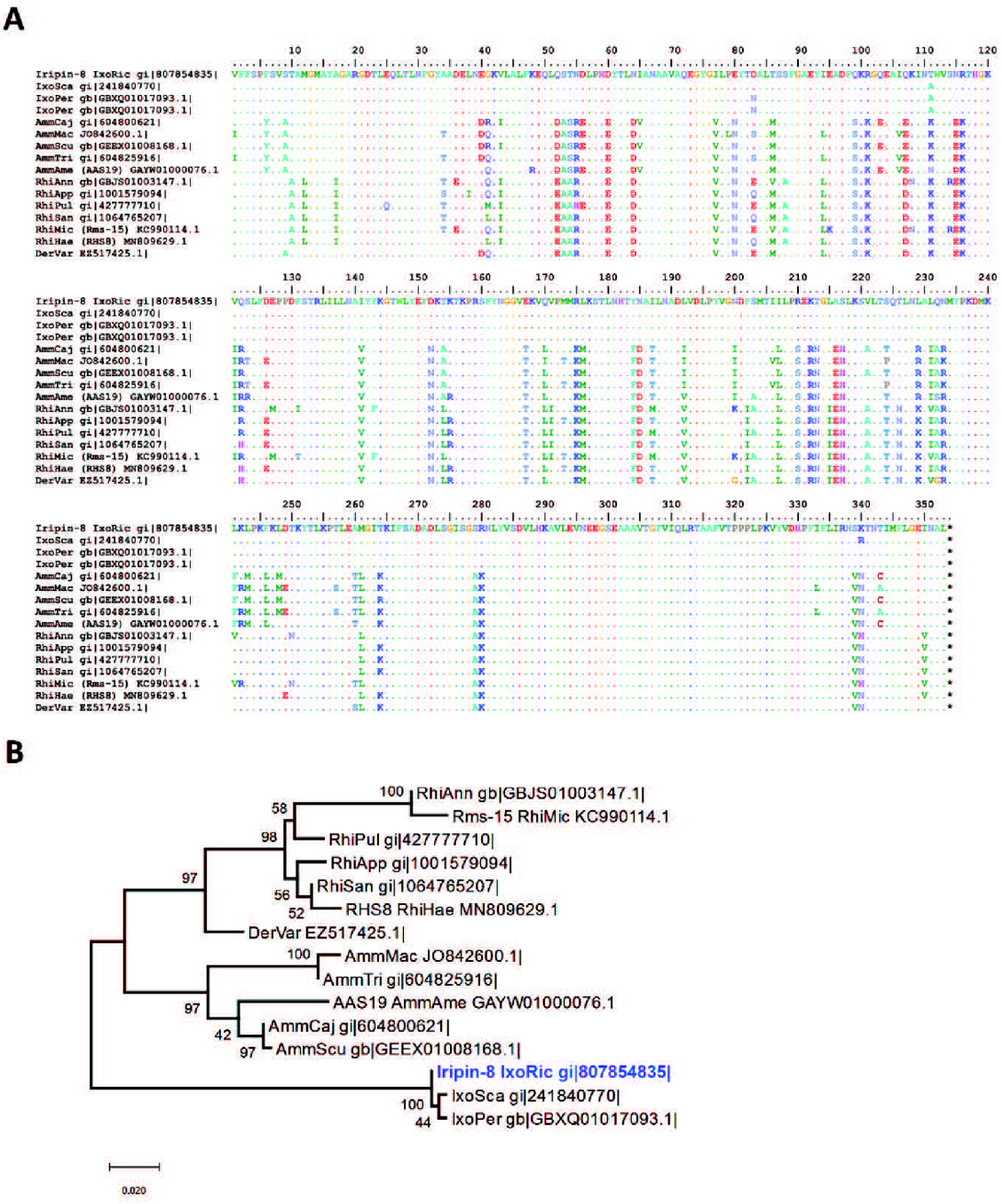
Supplementary Table S2. Data processing, refinement, and model.

Crystal		
Space Group	F222	
Cell dimensions (Å)	a = 96.95 b = 130.67 c = 151.09	
(°)	α = β = γ = 90	
Solvent content (%)	56.1	
Data Processing Statistics		
Wavelength (Å)	0.91587 (Diamond, beam line I04-1)	
Resolution (Å)	75.54-1.89	1.93-1.89
Total reflections	327544	22074
Unique reflections	38420	2465
Multiplicity	8.5	9.0
<I/σ(I)>	8.9	1.6
Completeness (%)	100.0	100.0
R_{merge}	0.124	1.441
CC(1/2)	0.998	0.594
Model		
Number of atoms modeled: Protein PEG Water	 2882 65 78	
B-factors (Å²)		
Average	41.0	
Protein	40.4	
Ions/ligand	67.8	
Water	42.1	
Refinement statistics	69.31-1.89Å	1.94-1.89Å
Reflections in working/free set	36491 / 1926	2677/ 136

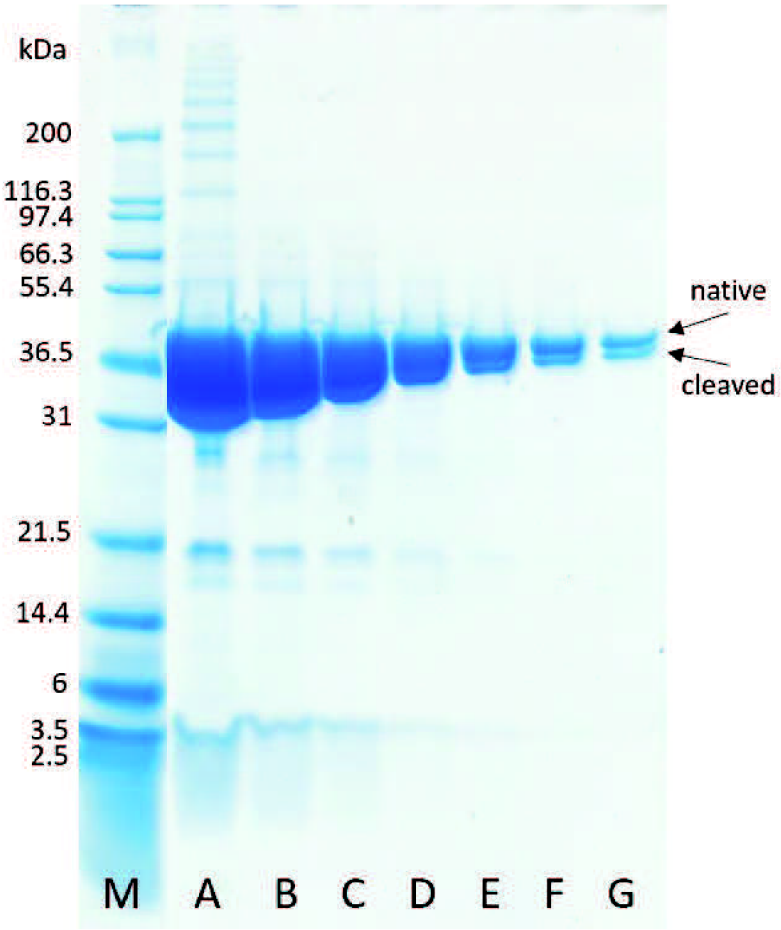
R-factor/R-free (%)	20.40 / 24.34	44.0 / 49.1
r.m.s. deviation of bonds(Å)/angles (°) from ideality	0.010 / 1.568	
Ramachandran Favoured	97.89%	
Molprobity Score	1.38 (98th percentile)	

Phylogenetic analysis of Iripin-8 group among tick species

In order to show the conserved nature of Iripin-8-like serpins in ticks, an alignment was produced by using the ClustalW algorithm in BioEdit version 7.2.5 [1] (Supplementary Figure S1A) and an unrooted phylogenetic tree was built using MEGA X software [2] (Supplementary Figure S1B). Interestingly, the RCL and adjacent regions are 100% conserved, unlike the rest of the sequence that seems to undergo evolutionary changes, as evidenced by strongly supported species-specific branches of the phylogenetic tree (Supplementary Figure S1B).



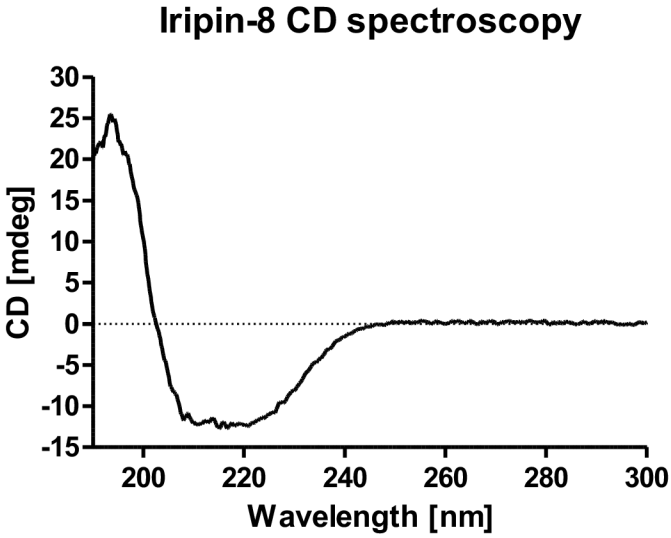
Supplementary Figure S1. Alignment and phylogenetic analysis of Iripin-8. (**A**) Alignment of members of the Iripin-8 group of tick serpins. The conserved area, including hinge region and RCL, is between residues 290-330. (**B**) Phylogenetic reconstruction of serpins from the Iripin-8 group. See Supplementary Methods for details of the analysis.



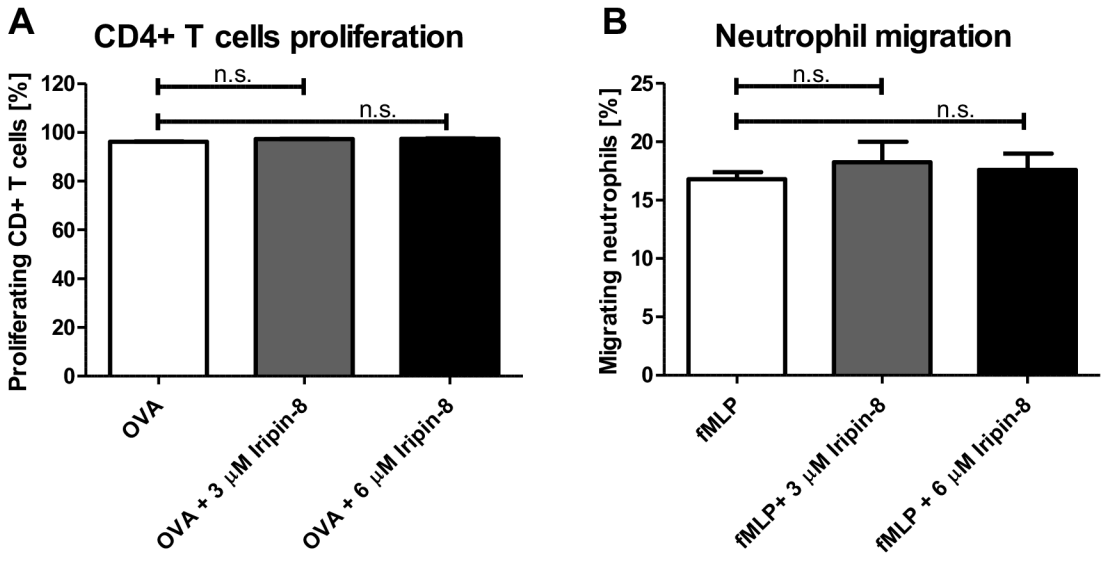
Supplementary Figure S2. Analysis of Iripin-8 purity by SDS-PAGE. Iripin-8 was analyzed by a reducing SDS-PAGE gel. M: Molecular weight marker, A-G: Iripin-8 with load of 50, 25, 12.5, 6.2, 3.1, 1.55, 0.8 mg per well. Arrows show Iripin-8 in its native and cleaved states.

Circular dichroism (CD) spectroscopy

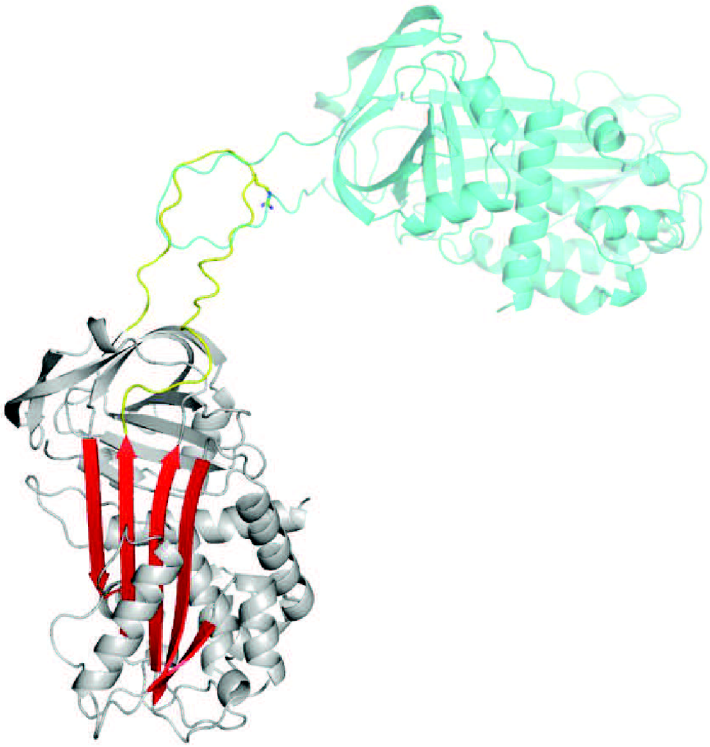
In order to verify a correct fold of Iripin-8 recombinant protein, we performed CD spectroscopy analysis. Prior to analysis, buffer of Iripin-8 solution was exchanged for 20 mM NaH_2PO_4, 150mM NaF, pH 7.4. CD spectra were obtained using a JASCO J-810 spectropolarimeter at 22°C at wavelengths ranging from 190 to 300 nm using a 0.1 mm path-length cuvette. As shown in Supplementary Figure 2, Iripin-8 displays properties typical for the serpin secondary structure [3, 4].



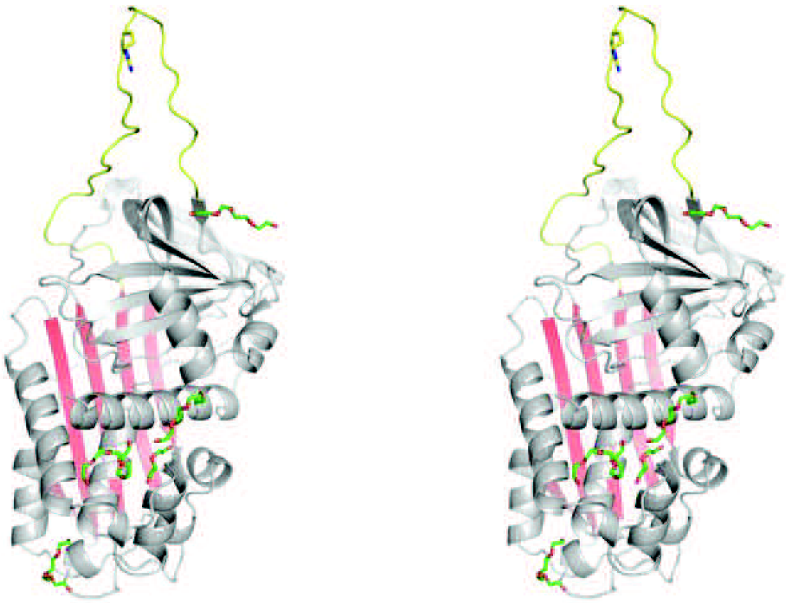
Supplementary Figure S3. CD spectrogram of Iripin-8. Iripin-8 shows a similar CD spectrum to other serpins due to their highly conserved structure.



Supplementary Figure S4. Effect of Iripin-8 on T cell proliferation and neutrophil migration. (**A**) Splenocytes from OT-II mice were pre-incubated with Iripin-8 and stimulated by OVA peptide. Percentages of proliferating $CD4^+$ T cells were evaluated after 72 hours by flow cytometry. (**B**) Mouse primary bone marrow neutrophils were pre-incubated with Iripin-8 and subjected to migration towards fMLP in a Boyden chamber. Figure shows the percentage of neutrophils migrating from an insert with a 3 μm membrane to a compartment with fMLP. Both experiments were performed as three biological replicates.



Supplementary Figure S5. A ribbon diagram of two Iripin-8 symmetry-related molecules in a crystal contact via their extended rigid RCL. RCL of one Iripin-8 molecule is depicted in yellow, while the second Iripin-8 molecule is all blue.



Supplementary Figure S6. Stereo view of a ribbon diagram of Iripin-8 with highlighted (green) molecules of PEG bound to the structure including a deep cavity in the core structure between helices A, B, and C.

Supplementary Methods

Evolutionary analysis by the maximum likelihood method

The evolutionary history was inferred using the maximum likelihood method and Tamura-Nei model [5]. The tree with the highest log likelihood (-1062.81) is shown. The percentage of trees out of 1000 replications by bootstrap method in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model and then selecting the topology with superior log-likelihood value. There were 355 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [2].

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3.3 Paper III

Serpins in tick physiology and tick-host interaction

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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

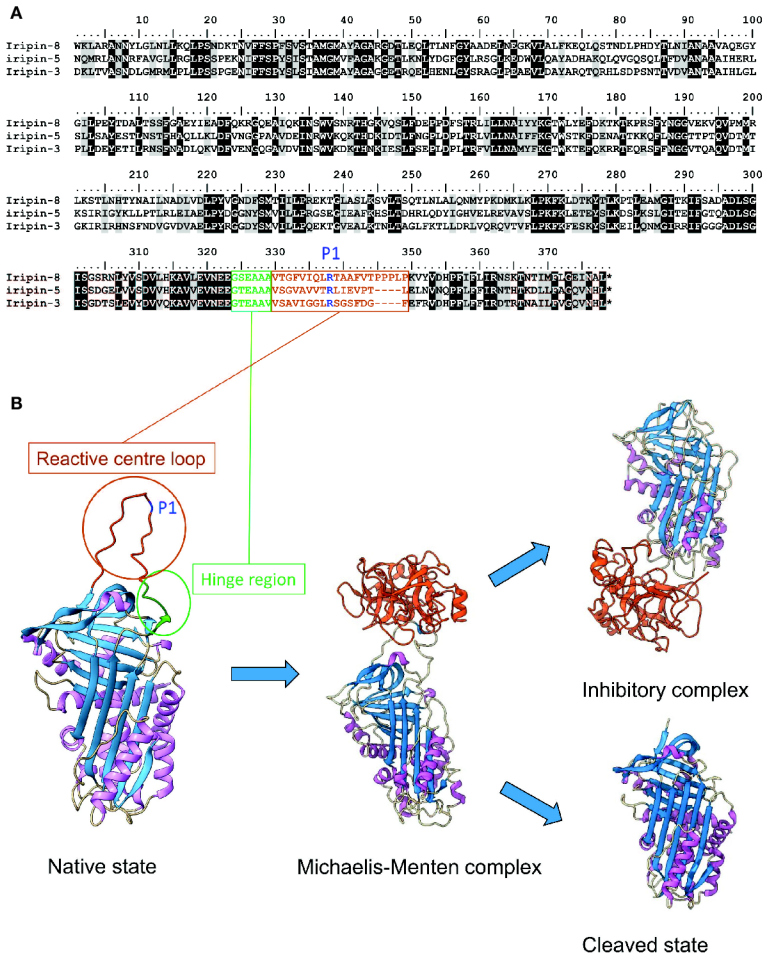

FIGURE 1 | Structure of serpins and their mechanism of inhibition. **(A)** Sequence alignment of three characterized serpins from *I. ricinus*. RCL is highlighted in brown, P1 site in blue and hinge region in green. **(B)** Tertiary structures of four most common serpin conformation states. Native state 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

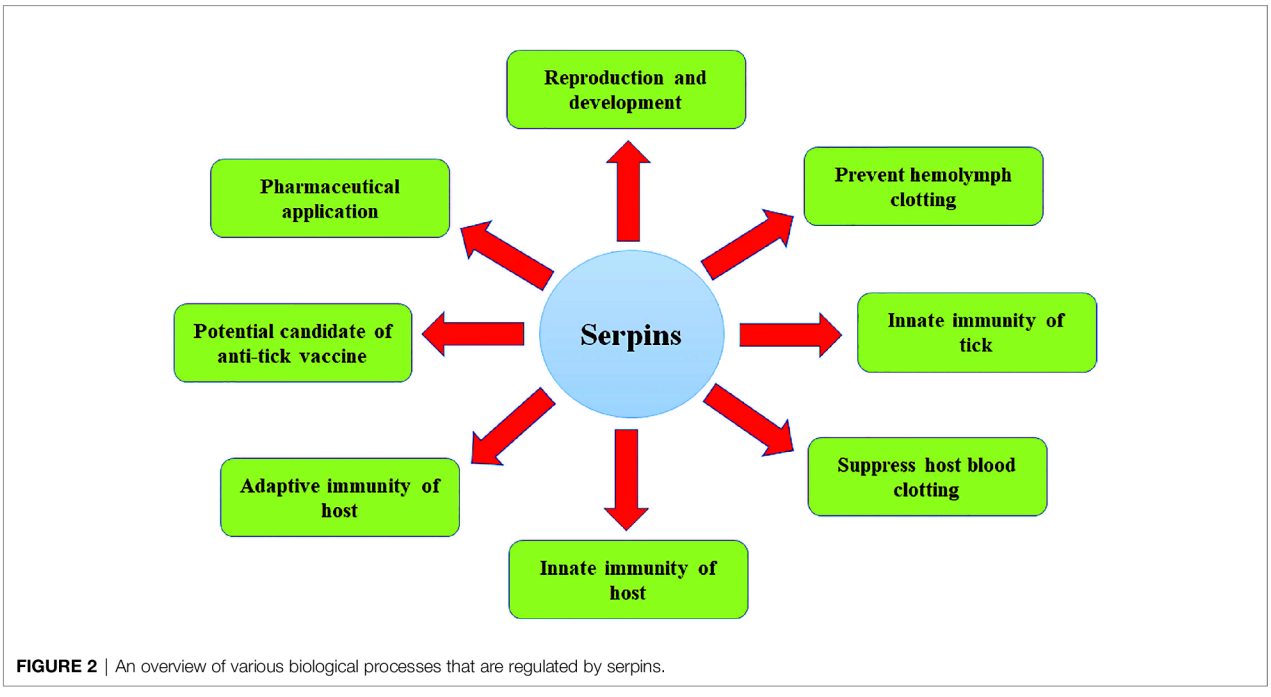

FIGURE 2 | An overview of various biological processes that are regulated by serpins.

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,

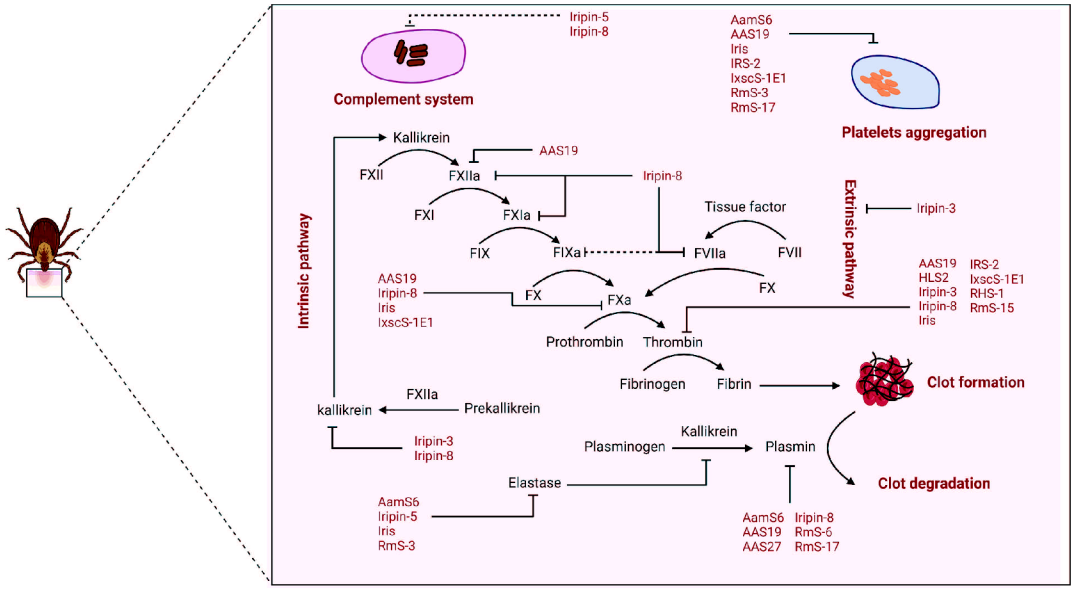

FIGURE 3 | The role of tick salivary serpins in the regulation of host hemostasis and complement.

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.,

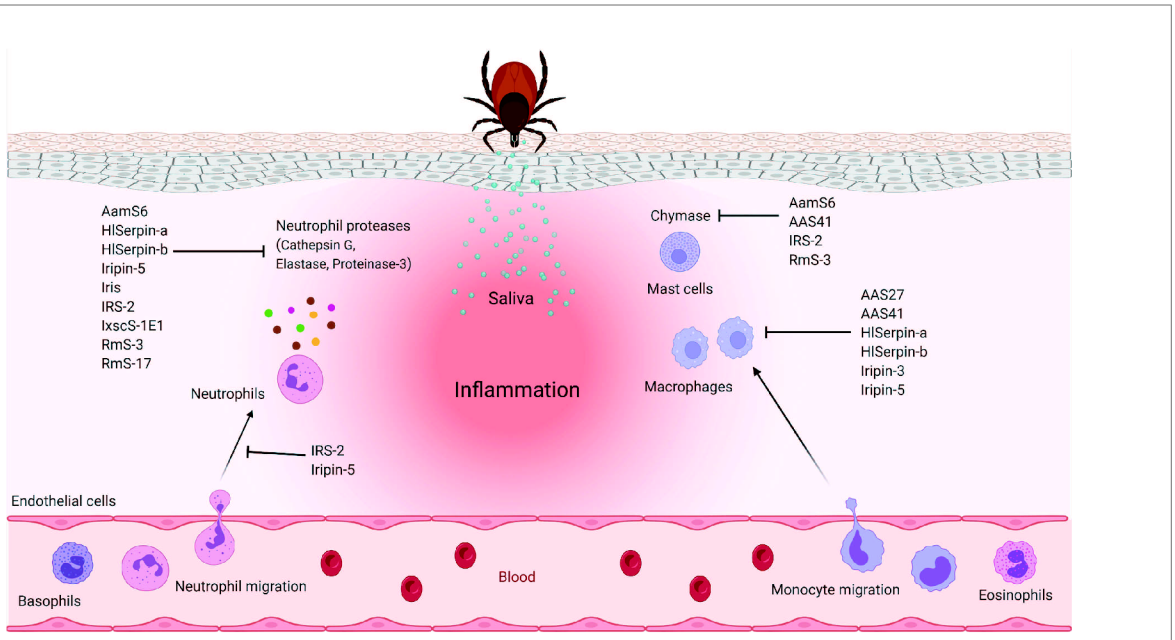

FIGURE 4 | Anti-inflammatory activities of tick salivary serpins.

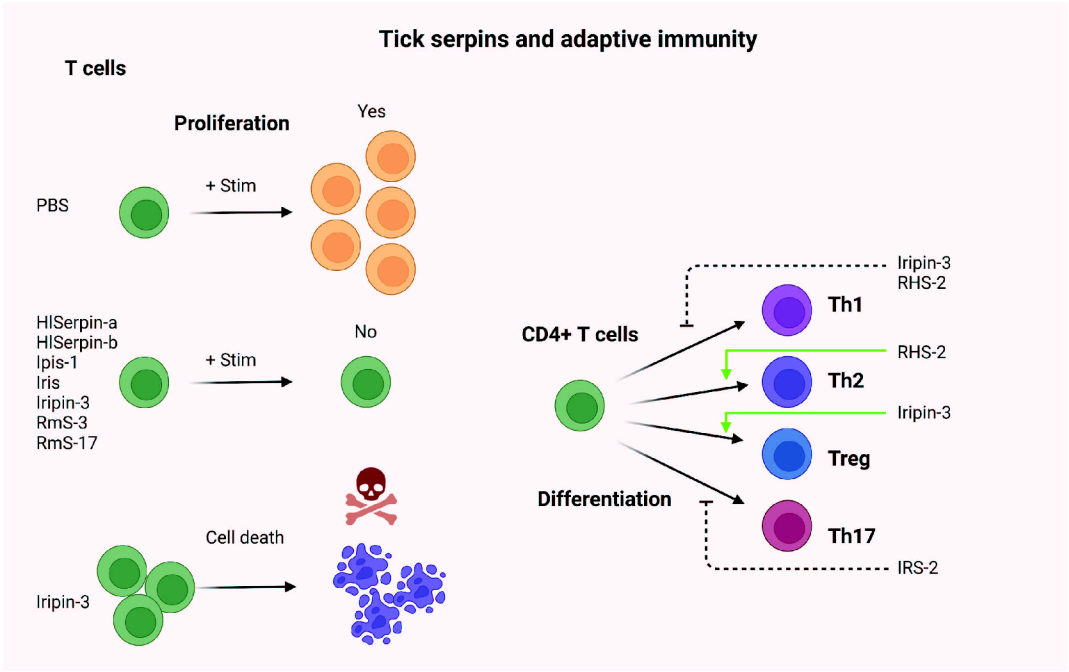

FIGURE 5 | The role of tick serpins in the modulation of vertebrates host adaptive immune system.

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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3.4 Paper IV

Iripin-1, a new anti-inflammatory tick serpin, inhibits leukocyte recruitment *in vivo* while altering the levels of chemokines and adhesion molecules

Iripin-1, a new anti-inflammatory tick serpin, inhibits leukocyte recruitment *in vivo* while altering the levels of chemokines and adhesion molecules

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Abstract

Serpins are widely distributed and functionally diverse inhibitors of serine proteases. Ticks secrete serpins with anti-inflammatory and immunomodulatory activities via their saliva into the feeding cavity to

modulate host's immune reaction initiated by the insertion of tick's mouthparts into skin. The suppression of the host's immune response not only allows ticks to feed on a host for several days but also creates favorable conditions for the transmission of tick-borne pathogens. Herein we present the functional and structural characterization of Iripin-1 (*Ixodes ricinus* serpin-1), whose expression was detected in the salivary glands of the tick *Ixodes ricinus*, a European vector of tick-borne encephalitis and Lyme disease. Of 16 selected serine proteases, Iripin-1 inhibited primarily trypsin and further exhibited weaker inhibitory activity against kallikrein, matriptase, and plasmin. In the mouse model of acute peritonitis, Iripin-1 enhanced the production of the anti-inflammatory cytokine IL-10 and chemokines for neutrophils and monocytes, including MCP-1/CCL2, a potent histamine-releasing factor. Despite increased chemokine levels, the migration of neutrophils and monocytes to inflamed peritoneal cavities was significantly attenuated following Iripin-1 administration. Based on the results of *in vitro* experiments, immune cell recruitment might be inhibited due to Iripin-1-mediated reduction of the expression of chemokine receptors in neutrophils and adhesion molecules in endothelial cells. Decreased activity of serine proteases in the presence of Iripin-1 could further impede cell migration to the site of inflammation. Finally, we determined the tertiary structure of native Iripin-1 at 2.10 Å resolution by employing the X-ray crystallography technique. In conclusion, Iripin-1 seems to facilitate *I. ricinus* feeding by attenuating the host's inflammatory response and by increasing blood flow at the tick attachment site through MCP-1-triggered histamine release from mast cells and basophils.

The manuscript is being prepared for the submission to the journal Frontiers in Immunology.

4 CONCLUSION AND FUTURE PERSPECTIVES

Papers, which are part of this dissertation, report on the function and structure of three novel *I. ricinus* salivary serpins, thus expanding our knowledge about the role of serpins at the tick-host interface. The three serpins exerted distinct effects on the host's anti-tick defenses. Iripin-8 inhibited intrinsic and common pathways of blood coagulation, while Iripin-1 attenuated inflammation and concomitant immune cell migration. Unlike Iripin-1 and Iripin-8, Iripin-3 suppressed the extrinsic coagulation pathway and modulated the adaptive immune response. These findings indicate that individual tick salivary serpins can interfere with different biological processes occurring at the tick feeding site.

To date, about 25 tick serpins have been functionally characterized [124,125], and some of them have been shown to possess similar inhibitory activities as Iripin-1, Iripin-3, and Iripin-8. More specifically, other tick serpins besides Iripin-1, -3, and -8 have been reported to inhibit pro-inflammatory cytokine production [80,129,170], T cell proliferation and Th1 cell differentiation [89,129,152,171], neutrophil migration [132,133], and activation of the intrinsic and common pathways of blood coagulation [65,66,124,137,140,141,161]. However, some Iripin-1, -3, and -8 activities represent novel observations in the field. For example, Iripin-3 is the first tick serpin that have been shown to inhibit the extrinsic coagulation pathway, decrease the survival of B cells and T cells, and promote the differentiation of Tregs. Furthermore, Iripin-8 together with Iripin-5 [133] are the first tick serpins that have been reported to possess anti-complement properties. The ability of Iripin-1 to enhance chemokine production is also a novel finding in the field of tick salivary serpins. Moreover, no other tick serpin except Iripin-1 has been shown to inhibit monocyte migration or decrease the expression of cell adhesion molecules on the surface of endothelial cells. Therefore, the functional characterization of Iripins clearly enriched our understanding of the impact of tick salivary serpins on the host's defensive response and likewise confirmed some previous observations made with other tick serpins.

Although the three papers concerning Iripin-1, Iripin-3, and Iripin-8 considerably expanded our knowledge of the function of *I. ricinus* serpins, there are still many questions that need to be answered. First, some

inhibitory effects of Iripins were observed only in *in vitro* experiments. This is especially true for the anti-inflammatory and immunomodulatory properties of Iripin-3 or the ability of Iripin-1 to decrease the surface expression of chemokine receptors and cell adhesion molecules. However, if we want to know whether the serpins can exert similar activities also in a more complex environment, such as the tick feeding site, it is necessary to confirm the results of *in vitro* assays in *in vivo* experiments. Testing of individual serpins in the animal models of human disease might also reveal their therapeutic potential.

Some tick serpins can utilize glycosaminoglycans (GAGs), such as heparin and heparan sulfate, to enhance inhibitory functions. For example, binding of GAGs to *A. americanum* serpin AAS19 increased the inhibitory activity of the serpin against thrombin and FIXa and reduced its capacity to inhibit FXa and FXIIa. Overall, GAGs binding to AAS19 dramatically enhanced the anti-coagulant effect of the serpin in the recalcification time assay [172]. The covalent complex between the *Hyalomma dromedarii* serpin Dromaserpin and thrombin also bound heparin, and the presence of this GAG accelerated thrombin inhibition by Dromaserpin approximately 25-fold [124]. Using *in vitro* coagulation assays, we showed that Iripin-3 inhibits the extrinsic coagulation pathway, and Iripin-8 suppresses the intrinsic and common pathways of blood coagulation. However, we have not tried to test the ability of these serpins to bind GAGs, nor have we assessed the effect of GAGs on the Iripin-3 and Iripin-8 anti-coagulant activities. Thus, the interaction between Iripin-3/Iripin-8 and GAGs and its impact on blood coagulation might be the subject of future investigation.

Iripin-5 and Iripin-8 are the first tick serpins that have been shown to possess anti-complement properties. However, the mechanism behind impaired MAC formation in the presence of both serpins remains unknown. MAC assembly can be initiated via three distinct pathways: classical, alternative, or lectin. Since all three pathways are dependent on the serine protease activity [173], serpins could suppress the complement cascade by targeting any of the three activation pathways. This is evidenced by the human serpin C1 esterase inhibitor that inhibits both the classical pathway by inactivating C1r and C1s [174] and the lectin pathway by reducing the proteolytic activity of MASP-1 and MASP-2 [175]. Besides regulating the classical and lectin pathways of complement activation, C1 esterase inhibitor can also suppress the alternative pathway by binding to C3b [176].

Thus, future experiments might focus on the ability of Iripin-5 and Iripin-8 to interfere with the classical, alternative, and lectin pathways of complement activation. The effect of these serpins on the proteolytic activity of serine proteases that are part of the complement cascade, including C1r, C1s, MASP-1, MASP-2, and factor D, might also be evaluated.

Although it is very likely that tick serpins inhibit blood coagulation by inactivating blood clotting factors, the mechanisms behind the serpins' anti-inflammatory and immunomodulatory activities are generally not known. For example, it is not clear how Iripin-3 diminishes IL-6 production, impairs lymphocyte viability, and inhibits $CD4^+$ T cell proliferation. The effects of tick serpins on innate and adaptive immune responses might be dependent or independent on their anti-protease activity. This point can be illustrated by the serpins HlSerpin-a from *H. longicornis* and Iris from *I. ricinus*. HlSerpin-a was reported to reduce the expression of TNF, IL-1β, and IL-6 by inhibiting the enzymatic activities of cathepsin B and cathepsin G in LPS-stimulated RAW264.7 macrophages [80]. Unlike HlSerpin-a, Iris decreased TNF production by LPS-activated PBMCs through binding to the monocyte population of PBMCs via exosites [131]. Therefore, the first step towards determining the mechanism of action of tick serpins could be the production of recombinant proteins devoid of anti-protease activity or with altered inhibitory specificity due to mutation in the RCL. The serpins' activities mediated by protease inhibition should be lost or altered following RCL mutation, whereas the functions independent of anti-protease activity should not be affected by a single-point mutation in the RCL. Comparison of the activities of mutated and unmutated serpins could help us decide what to do next. The following experiments might be focused on the capacity of serpins to inactivate proteases involved in the affected processes. Alternatively, the experiments assessing the binding of serpins to selected immune cell populations might be performed.

The evaluation of the importance of tick serpins for successful blood feeding by using RNAi or vaccination experiments facilitates the discovery of suitable candidates for anti-tick vaccine development. To date, we have only assessed the impact of serpin gene silencing on the feeding parameters of *I. ricinus* nymphs. However, in the future, we could also knock down serpin expression in *I. ricinus* adult females. RNAi experiments with adult

ticks would allow us to evaluate the role of individual serpins not only in blood meal acquisition but also in tick reproduction. This would be particularly useful in the case of IRS-2, which is highly expressed in ovaries of adult ticks [132], and Iripin-8, which has the same RCL as RHS8, a serpin involved in the process of vitellogenesis [128]. In addition to RNAi experiments, vaccination experiments with *I. ricinus* serpins might be conducted in the future.

5 LIST OF ABBREVIATIONS

AAS	*Amblyomma americanum* serpin
ADP	adenosine diphosphate
APC	antigen-presenting cell
BMDM	bone marrow-derived macrophage
Breg	regulatory B cell
C1–C9	complement components 1–9
CCL	CC chemokine ligand
CD	cluster of differentiation
CTL	cytotoxic T lymphocyte
CXCL	CXC chemokine ligand
CXCR2	CXC chemokine receptor 2
Da-p36	36-kDa protein from *Dermacentor andersoni*
DNA	deoxyribonucleic acid
ECM	extracellular matrix
Foxp3	forkhead box p3
FV–FXIII	blood clotting factors V–XIII
FVa–FXIIIa	activated blood clotting factors V–XIII
GAG	glycosaminoglycan
HlSerpin-a, -b	*Haemaphysalis longicornis* serpin-a, -b
HMWK	high molecular weight kininogen
HUVEC	human umbilical vein endothelial cell
iC3b	inactive complement component 3b
ICAM-1	intercellular adhesion molecule-1
IFN-γ	interferon-γ
Ig	immunoglobulin
IL	interleukin
Iripin	*Ixodes ricinus* serpin
Iris	*Ixodes ricinus* immunosuppressor
IRS-2	*Ixodes ricinus* serpin-2
iTreg	induced regulatory T cell
LPS	lipopolysaccharide

MAC	membrane attack complex
MASP	MBL-associated serine protease
MBL	mannose-binding lectin
MCP-1	monocyte chemoattractant protein-1
MHC	major histocompatibility complex
OVA	ovalbumin
PAR	protease-activated receptor
PBMC	peripheral blood mononuclear cell
PL	phospholipid
PRR	pattern recognition receptor
RCL	reactive center loop
RHS8	*Rhipicephalus haemaphysaloides* serpin 8
RmS	*Rhipicephalus microplus* serpin
RNA	ribonucleic acid
RNAi	RNA interference
RT-qPCR	reverse transcription-quantitative polymerase chain reaction
Salp15	15-kDa salivary protein
SAT	saliva-assisted transmission
STAT-3	signal transducer and activator of transcription-3
T-bet	T-box expressed in T cells
TBP	tick-borne pathogen
TF	tissue factor
TGF-β	transforming growth factor-β
Th cell	helper T cell
TNF	tumor necrosis factor
tPA	tissue-type plasminogen activator
Treg	regulatory T cell
TXA_2	thromboxane A_2
uPA	urokinase-type plasminogen activator
uPAR	receptor for urokinase-type plasminogen activator
VCAM-1	vascular cell adhesion molecule-1

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7 CURRICULUM VITAE

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Education

2017 – now

Institution: Faculty of Science, University of South Bohemia in České Budějovice, Czech Republic

PhD study program: Infection Biology

PhD thesis title: Anti-inflammatory and immunomodulatory activities of *Ixodes ricinus* salivary proteins

2014 – 2017

Institution: Faculty of Science, University of South Bohemia in České Budějovice, Czech Republic

Master's study program: Clinical Biology

Master's thesis title: Establishment and optimization of *in vivo* models of inflammation and their use for functional analysis of tick salivary protease inhibitors

2010 – 2014

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Bachelor's study program: Biomedical Laboratory Techniques

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Employment history

2017 – now Research assistant, Faculty of Science, University of South Bohemia in České Budějovice, Czech Republic

2022 Research assistant, Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, České Budějovice, Czech Republic

Research internships

2021 (3 months) Pre-doctoral visiting fellow, Rocky Mountain Laboratories, National institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana, USA

International conferences

06/2018 Gordon Research Conference on Proteolytic Enzymes and Their Inhibitors, Lucca (Barga), Italy, poster presentation

03/2019 13th International Symposium on Ticks and Tick-borne Diseases, Weimar, Germany, poster presentation

Research projects

2019 – 2020 Project title: Anti-inflammatory and immunomodulatory activities of *Ixodes ricinus* salivary serpins (105/2019/P)

Funding: Grant Agency of the University of South Bohemia

Role: principal investigator

Publications

Kotál, J., Stergiou, N., Buša, M., **Chlastáková, A.**, Beránková, Z., Řezáčová, P., Langhansová, H., Schwarz, A., Calvo, E., Kopecký, J.,

Mareš, M., Schmitt, E., Chmelař, J., Kotsyfakis, M., 2019. The structure and function of Iristatin, a novel immunosuppressive tick salivary cystatin. Cellular and Molecular Life Sciences 76(10), 2003-2013. DOI: 10.1007/s00018-019-03034-3. **[IF = 9.207]**

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