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Immunomodulators in tick saliva and their benefits

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Summary. – Ticks are significant bloodsucking ectoparasites. Apart from causing blood loss and host skin damage, ticks are important vectors of tick-borne pathogens that cause disease in humans and animals as well as significant economic loss. For biological success, ticks evolved these substances with immunomodulatory activities capable of inhibiting host defence reactions (haemostasis, inflammation and immunity reactions), and which have a radical significance for their survival. The resulting feeding site represents a favourable environment and many pathogens began exploiting ticks to facilitate their transmission to the host. The structural-functional relationships of some salivary compounds have been outlined; however research on tick sialomas indicates that further extensive exploration is required on the subject. Also, tick saliva is a complex pharmacological component with great therapeutic potential for the treatment for some diseases.

Keywords: ticks; tick saliva; immunomodulation; haemostasis; inflammation; therapeutical tools

Contents:

1. Introduction
2. Hard ticks vs. haemostasis.
3. Hard ticks vs. inflammation and immunity
4. Tick saliva proteins as therapeutical tools in treatment of human disease
5. Conclusion

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Abbreviations: ADP = adenosinediphosphate, ATP = adenosinetriphosphate, BIP = B-cell inhibitory protein, CCL3 = MIP1α (Macrophage Inflammatory Protein alpha), CCL4 = MIP1β (Macrophage Inflammatory Protein beta), CCL5 = RANTES (Regulated upon Activation, Normal T cell Expressed and presumably Secreted), CCL11 = Eotaxin, CCR = CC chemokine receptor, CD = cluster of differentiation, CXCL1 = mouse GROα/MGSAα (MIP2α/KC), CXCL8/KC = human interleukin 8, C1-C9 = proteins of complement cascade, DC = dendritic cell, FGF = fibroblast growth factor, FII-FXII = coagulation factors, HBP = histamine binding protein, HGF = hepatocyte growth factor, IFN = interferon, Ig = immunoglobulin, IL = interleukin, NK = natural killer cells, NKA = natural killer activity, PDGF = platelet-derived growth factor, PG = prostaglandin (A, B, D, E, F, I), RGD = arginine, glycin, aspartic acid, Salp = tick salivary protein, SG = salivary gland, SGE = SG extract, SHBP = serotonin and histamine binding protein, TGF = transforming growth factor, Th = T helper lymphocyte, TNF = tumor necrosis factor

1. Introduction

Ticks are significant bloodsucking ectoparasites. Their lifestyle cause blood and therefore weight loss to their prey, damage their skin. Ticks are vectors of various pathogens and transmit a number of important diseases. Ixodid or hard ticks are characterized by their protracted blood-feeding. The penetration of tick mouthparts into the host skin causes dermal and epidermal damage.

Host defence against tick and tick feeding comprises of haemostasis, aimed to heal the bite injury and to prevent blood loss; innate immunity that consists of the inflammatory response and complement cascade which have an antimicrobial effect and lead to remodelling of damaged tissue; and of antigen specific acquired immunity due to the long duration of attachment to the host, resulting from the repeated exposure of the same animals to ticks. Defensive mechanisms and compartments are redundant. Signalling events during host defence elicited by the ectoparasites should lead to host wound healing and tick rejection responses.

However, ixodid ticks require large bloodmeals for their development and survival and ticks have their own defences. Unlike short term feeding Argasidae ticks, ixodid ticks are exposed to all defensive mechanisms during feeding, including acquired immunity.

Bioactive molecules in tick saliva sabotage wound healing responses at the level of haemostasis, inflammation and tissue repair and block signalling molecules of cellular communication during innate and adaptive immunity responses (mainly during secondary or subsequent infestations). Because of the redundancy of host defence, tick effectors target various host defensive pathways.

In addition, the immunosuppressive feeding site is therefore exploited by different tick-borne pathogens for their establishment and replication in hosts. Organisms that use ticks as vectors include viruses (Flaviviruses), bacteria (Ehrlichia, Borrelia, Coxiella, Rickettsia and Anaplasma) and protozoa (Babesia and Theileria). For example, Nuttall and Labuda (2003) illustrate the importance of immunosuppression by the tick vector for transmission of flaviviruses.

In contrast, the pharmacological properties of tick saliva proteins and their possible therapeutic use in the treatment of haemostatic disorders, tumours and autoimmunity diseases are being explored.

2. Hard ticks vs. haemostasis

During penetration of tick mouthparts into the skin, capillary and small blood vessels are lacerated, host cells are ruptured and haemorrhage occurs. Rupture of vessel walls immediately triggers three major mechanisms that support haemostasis, as a first line of host defence.

Firstly, blood loss is minimized by the contraction of muscle cells in vessel walls (vasoconstriction). Secondly, platelet aggregation begins. Activated thrombocytes adhere and finally attach to the exposed subendothelial collagen of damaged vessels, and release adhesion proteins (fibrinogen and thrombospodin), serotonin (to promote retention of procoagulants), and other platelet-activated factors in blood coagulation. The vasoconstrictors produced by platelets are responsible for early vasoconstriction. Coagulation leads to the production of fibrin clots. All these phenomena are redundant and counteract in a synergistic manner. To overcome these processes, ticks have developed potent components within their salivary secretions such as anticoagulants, anti-platelet and vasodilators (Francischetti, 2010; Chmelar *et al*., 2012). However, there is no existing species of tick whose full anti-haemostatic capacities have been fully explored and outlined.

Differences in the anti-haemostatic repertoires suggest independent evolution of anti-haemostatic mechanisms in hard and soft ticks (Mans *et al*., 2008). However, thrombin, factor X, and platelet aggregation and adhesion are shared obstacles that every tick species has to overcome. Platelets play a critical role in haemostasis. Also, these cells are crucial for vertebrate immunity, because of the release of many inflammatory mediators (chemokines and biogenic amines (Semple *et al*., 2011). These cells are activated by several independent agonists of platelet aggregation. The platelet activation and aggregation cascade can be targeted by ticks at several stages. First, ticks inhibit the activation of platelets by producing substances that remove agonists or compete with agonists for binding to their receptors. Tick saliva inhibits activation of platelets by adenosintriphosphate (ATP) and adenosindiphosphate (ADP) hydrolyzing to adenosine monophosphate (AMP) and monophosphate used by tick saliva apyrase (ATP diphosphohydrolase) (Ribeiro *et al*., 1985., Titus and Ribeiro, 1990, Liyou *et al*., 1999; Mans *et al*., 2000; Ribeiro and Fracischetti, 2003). Hard tick apyrase (from *Boophilus microplus*) belongs to the 5′-nucleotidase family (Liyou *et al*., 1999). Apyrase activity from soft tick is illustrated in table 1.

Antiplatelet activity is associated with group E tick prostaglandins and prostacyclins. At the host-parasite interface, ticks secrete saliva containing an extremely high concentration of prostaglandins (PGs) into the host and are thought to aid the parasite by modulating the inflammatory and immune response. To date, prostacyclin PGI_2 and prostaglandins PGA_2/PGB_2, PGD_2, PGE_2, and PGF2 have been identified in the saliva of some tick species (Ribeiro *et al*., 1988; Aljamali *et al*., 2002). Tick prostaglandins increase the concentration of intraplatelet cAMP, inhibit the secretion of ADP and as such inhibit aggregation and cause disaggregation of platelets that have aggregated and also have vasodilator and immunosuppressive activity (Dickinson *et al*., 1976; Inokuma *et al*., 1994; Bowman *et al*., 1996; Jones *et al*., 2009; Oliveira *et al*., 2011). PGI_2 is the most powerful known inhibitor of platelet aggregation and ADP secretion (Ribeiro *et al*., 1990). Via binding to its receptor to mediate two physiological processes in tick salivary glands, PGI_2 acts in salivary secretions and acts in receptor-mediated protein exocytosis (Stanley and Kim, 2011). Due to its ability to downregulate the function of T and B cells, several authors have suggested that PGE_2 is responsible for the impairment of T cell proliferation after mitogen stimulation.

The collagen-induced aggregation of platelets, and subsequent activated process, including granule release and increasing of cytosolic free calcium of platelets in response to collagen was very strongly inhibited by longicornin from the salivary glands of hard tick *Haemaphysalis longicornis.* Longicornin inhibited not only aggregation but also the activating process, possibly binding to a collagen receptor (Cheng *et al*., 1999).

If platelets are activated, aggregation can still be inhibited by targeting the platelet fibrinogen receptor. Many ticks produce RGD molecules (arginin, glycin, aspartic acid) and non-RGD disintegrins which block the binding of fibrinogen to the integrin αIIbβ3, a fibrinogen receptor on the surface of activated platelets. The αIIbβ3 antagonists can displace fibrinogen from its receptor thereby allowing

Table 1. Imunomodulators in soft ticks – anti-haemostatic activities

Molecule	Tick species	Source	Target/function	References
Platelet aggregation inhibitors				
Apyrase	*Ornitodoros savignyi*	SG	Hydrolyses ATP and ADP to inactive AMP	Sutzer *et al.,* 2009
Moubatin	*O. moubata*	SG	TXA2 binding, inhibits collagen, ADP and thrombin-stimulated platelets aggregation, vasoconstrictor	Waxman and Connolly, 1993; Keller *et al*., 1993
TSGP3	*O. savignyi*	SG	TXA2 binding, vasonstrictor	Mans and Ribeiro, 2008
TAI	*O. moubata*	SG	Inhibitors of platelet adhesion to soluble collagen or to fibronectin, integrin α2β1 antagonist	Karczewski *et al*., 1995
Anticoagulants				
Disagregin	*O. moubata*	SG	Binds to αIIbβ3 fibrin receptor on platelets, Kunitz	Karczewski *et al*., 1994
Savignygrin	*O. savignyi*	SG	Binds to αIIbβ3 fibrin receptor on platelets, Kunitz with RGD motif	Mans *et al*., 2002
Savignygrin-like	*O. coriaceus*		Binds to αIIbβ3 fibrin receptor on platelets, Kunitz with RGD motif	Francischetti *et al*., 2008
Monogrin	*Argas monolakensis*		Binds to αIIbβ3 fibrin receptor on platelets, Kunitz with RGD motif	Mans *et al*., 2008
Monotonin	*A. monolakensis*	SG	Binds serotonin	Mans and Ribeiro, 2008
TSGP1	*O. savignyi*	SG	Binds serotonin	Mans *et al*., 2008
TAP	*O. moubata*	Whole tick	Kunitz type serine protease inhibitor, (binds to exosite and active site of FXa)	Vlasuk, 1993
FXa1	*O. savignyi*	SG	Kunitz type serine protease inhibitor, (binds to exosite and active site of FXa)	Gaspar *et al.,* 1996
Enolase	*O. moubata*	SG	Glycolitic enzyme acting as a plasminogen receptor, P-selectin antagonist ligand	Diaz-Martina *et al*., 2012
Thrombin inhibitors				
Ornitodorin	*O. moubata*	SG	Inhibits thrombin via its insertion into active and inside domain, Kunitz	Van de Locht *et al*., 1996
Savignin	*O. savygnyi*	SG	Inhibits thrombin via its insertion into active and inside domain, Kunitz	Nienaber *et al*., 1999
Monobin	*A. monolakensis*	SG	Inhibits thrombin via its insertion into active and inside domain, Kunitz	Mans *et al*., 2008
Anticoagulin/argasin	*A. persicus*	SG	Anticoagulant	Arocha-Pinango *et al*., 1999

SG = salivary gland.

disaggregation. One of disintegrins containing RGD motifs is variabilin from the salivary glands of the hard tick *Dermacentor variabilis* which blocks ADP induced platelet aggregation and prevents activation of platelets to immobilized fibrinogen (Wang *et al*., 1996). Variabilin is the first RGD motif-containing antagonist to be isolated from ticks. Ixodegrins from *Ixodes pacificus* and *I. scapularis* display sequence similarity to variabilin, with two additional cysteins in RGD position (Francischetti *et al*., 2005a). Activation of platelets via integrin αIIbβ3 fibrinogen receptor is ultimately accompanied by the production of unstable prostaglandin TXA_2 by platelets or prostacyclin PGI_2 by endothelial cells. These „local hormones" regulating a plethora of physiological processes in mammals and other vertebrates are charged by specific PGE_2 receptor in the salivary glands (SGs) of female *Amblyomma americanum*, a lone star tick (Qian *et al*., 1997). This PGE_2 receptor is linked with mobilization of Ca^{2+} via a phosphoinositide signalling pathway and is associated with the stimulation of protein secretion.

Platelet activation and ADP release is followed by thrombin production and activation of coagulation cascade. As a result of the cooperation of coagulation and platelet aggregation, thrombus is formed. Plug formation leads to two, intrinsic and extrinsic, pathways. Extrinsic pathway begins with injury and formation of complex among tissue factor TF and circulating coagulation factor FVIIa and ultimately leads to thrombin-catalysed conversion of soluble fibrinogen to insoluble fibrin mesh (Corral-Rodriguez *et al*., 2009). Extrinsic pathway is strictly regulated by host enzymes and inhibitors (Francischetti, 2010). Intrinsic pathway begins autoactivation of FXII by contact with polyanionic surfaces extrinsic origin. This pathway is contact phase and activates the kinin-kallikrein system (Chmelar *et al*., 2012). Both the intrinsic (collagen-activated) and extrinsic (tissue factor-

activated) pathways are activated by tick feeding. Because the catalytic domains of major coagulation factors belong to the family of trypsin like serine proteinases, tick anticoagulants are serine protease inhibitors, or serpins; most of them are inhibitors of factor X and thrombin or both (Maritz-Olivier *et al*., 2007). Tick anticoagulants include members of the Kunitz family of protease inhibitors, which may have single or multiple (two or five) Kunitz domain, which enables interaction with X-nase or prothrombinase complexes. Kunitz domain-containing proteins are highly represented in the saliva of both soft (in table 1) and hard ticks.

Inhibitors of extrinsic pathways named Ixolaris (with two Kunitz domains) protein and Penthalaris (with five Kunitz domains) were identified from *I. scapularis*, (Francischetti *et al*., 2002, 2004; Monteiro *et al*., 2005). Ixolaris binds to the FX heparin-binding domain and in this way inhibits prothrombinase assembly (Monteiro, 2005). Ixolaris- and Penthalaris-related sequences were detected in In *I. pacificus* (Francischetti *et al*., 2005b). In SG of *I. scapularis*, salivary protein families (Salp) with anticoagulant activity have also been reported, namely Salp 14 together with some 30 paralogues. Salp9Pac homolog of Salp14 exhibits no anticoagulant activity; and an inhibitor the intrinsic pathway of coagulation with homology to Salp 14 and Salp9Pac was identified and named Salp 9,8 (Narasimhan *et al*., 2002). In SG of feeding *D. andersoni* ticks, inhibitors of intrinsic and extrinsic pathways were detected (Gordon and Allen, 1991). Also, five putative serpin of *I. ricinus* serpin were detected (Prevot *et al*., 2006) and seventeen in *Am. americanum* (Mulenga *et al*., 2007). Serpin from *I. ricinus*, Iris, prolonged the duration of fibrinolysis, platelet adhesion and the contact phase-activated pathway of coagulation, Iris inhibited FXa and TF (Prevot *et al*., 2006). The anticoagulant repressing thrombin activity was detected in salivary glands of *Haem. longicornis* (Nakajima *et al*., 2006). Others inhibitors of thrombin are boophilin, microphilin, BmGTI, BmAP from saliva or midgut *B. microplus* (Macedo-Ribeiro *et al*., 2008; Ciprandi *et al*., 2006; Horn *et al*, 2000; Ricci *et al*., 2007). Boophilin contains conserved cystein residues, protease-recognition loops and Kunitz domain. Microphilin and BmGTI inhibit fibrin clot formation and thrombin-induced platelet aggregation. BmAP inhibits thrombin by binding to both active site and exosite (Ricci *et al*., 2007). Thrombin is the main target for thrombin competitive inhibitor called americanin from salivary glands of *Am. americanum* (Zhu *et al*., 1997), for calcaratin from salivary glands of *B. calcaratus,* hemalin and madanins isolated from midgut and/or saliva of *Haem. longicornis* (Iwanaga *et al*, 2003; Liao *et al*. 2009). However rhipilin from the salivary gland of *Rhipicephalus haemaphysaloides* containing Kunitz domain has no determined mode of action (Gao *et al*., 2011). Madanins contain acidic residues in the central region similar to that of hirudin, tsetse thrombin inhibitor anophelin and thrombostatin, which probably bind to exosite 1(Iwanaga *et al*., 2002). Chimadanin was identified from the same species. Experiments suggest binding in the thrombin active site (Nakajima *et al*., 2006). Two thrombin inhibitors with anticoagulant activity, named NTI-1 and NTI-2, were isolated from nymphal ticks *Hyalomma dromedarii,* probably with two Kunitz domains (Ibrahim *et al*., 2001). Recently, a new direct thrombin inhibitor, variegin, was characterized from the tropical bont tick *Am. variegatum* with structural similarity to, but much more potent than, hirulog, synthetic thrombin inhibitor based on the natural leech peptide hirudin (Koh *et al*., 2007). From the salivary glands of the ixodid tick *R. appendiculatus*, non enzyme anticoagulant which inhibits Fxa-induced clotting at a different site to the active site of FX or other components of the prothrombinase complex has been isolated (Limo *et al*., 1991). Thrombin is main target of enzyme ixodin, from extracts of the whole tick *I. ricinus*. In extracts of the same species of tick, another thrombin-inhibiting substance named ixin was observed (Arocha-Pinango *et al*., 1999). Following activation of factor XII by tissue-exposed collagen, bradykinin is produced (Ribeiro and Francischetti, 2003).Ticks evolved inhibitors of these contact phase proteins such as serine protease which participate in the intrinsic coagulation pathways. Hemaphysalin, isolated from salivary glands of *Haem. longicornis*, belongs to Kunitz type inhibitor and inhibits the reciprocal activation between FXIIa and kallikrein (Kato *et al*., 2005). Kunitz domain inhibitor of FIIa and FXIa and kallikrein was identified in the tick *I. ricinus* and named Ir-CPI (Decrem *et al*., 2009). Others such as RsTI Q2 and RsT Q7 were identified in the larvae of *R. sanguineus.* RsTIs also target plasmin, and neutrophil elastase in addition to plasma kallikrein as discussed above (Azzolini *et al*., 2003). Because of the need for dynamic equilibrium between fibrin formation by thrombin, its stabilization and the fibrin degrading system is responsible for dissolving fibrin and eventually blood clots also. Ticks produce protein with enzymatic activity that potentially inhibits platelet aggregation by lysis of fibrin. Metalloprotease activity targeting the aa chain of fibrinogen and fibrin has been found in *I. scapularis* saliva (Francischetti *et al*., 2003, Francischetti *et al*., 2010), *Haem. longicornis* (Harnnoy *et al*., 2007) Metalloproteases have exhibited gelatinase and fibrinogenlytic activity and probably have an effect on *Borrelia burgdorferi* transmission (Francischetti *et al*., 2003) In addition, ticks have developed proteases and protease inhibitors that affect fibrinolysis, because clots may be formed during feeding as well as the prevention of clotting. Tick carboxypeptidase inhibitor (TCI) from *R. bursa* accelerates fibrinolysis *in vitro* (Arolas *et al*., 2005). An *I. ricinus* serpin, Iris, has been shown to affect fibrinolysis by binding to leukocyte elastase, also inhibiting serine proteases and increased platelet adhesion (Prevot *et al*., 2006).

Antiplatelet properties, shared with monotonin from *Argas monolakensis* and TSGP1 from *Ornithodoros savigni* (Table 1), were detected in *D. reticulatus* saliva, and this protein has been labelled serotonin and histamine binding protein, SHBP (Sangamnatdej *et al*., 2002). Despite no existence of specific tick´s vasodilatators, tick histamine release factor that binds to basophiles and induces histamine release was detected in the saliva of *I. scapularis*, and may be considered as vasodilatators (Dai *et al*., 2010). Cathepsin G inhibitors, IRS-2, serpins were also identified in *I. ricinus* SG that inhibit cathepsin G and thrombin induced aggregation (Chmelar *et al*., 2011), as IRS-2 is a chymase inhibitor.

Tick saliva has anti-angiogenic properties. Angiogenesis is critical to the formation of granulation tissue, a hallmark of wound healing characterized by proliferation of endothelial cells, fibroblast accumulation and collagen synthesis. Francischetti *et al*. (2005a) detected inhibition of endothelial cell proliferation and angiogenesis by tick, and Fukumoto with colleagues identified a potent inhibitor of angiogenesis, tick troponin-like molecule (Fukumoto *et al*., 2006). Growth factors coordinating this process could be targets for salivary binder of cytokines and chemokines. We also identified the ability of several tick SGEs to bind growth factors (Hajnická *et al*., 2011).

3. Hard ticks vs. inflammation and immunity

The challenge to the immune system begins before contact with host blood. During penetration of tick mouthparts to the skin, resident epidermis and dermis leukocytes, mast cells, eosinophiles, dendritic cells, and macrophages, as well as keratinocytes are the first to make contact with mouthparts and tick saliva. These cells release mediators in addition to producing chemotactic factors to recruit inflammatory cells such as neutrophils to the attachment site. Additional components, prostaglandins, leukotrienes, chemokines and cytokines contribute to the recruitment of inflammatory cells to the site of injury (Andrade *et al*., 2005).When ticks feed on a naïve host, the cellular infiltrate is first dominated by neutrophils followed by mononuclear cells and later a small amount of basophils and eosinophils can by observed (Gill, 1986). Subsequent infestation may activate adaptive responses involving T cells and B cells by production of antibodies and sensitization of mast cells and basophils (den Hollander and Allen, 1985, Gill, 1986). Together with eosinophils, these are predominant cells at the attachment site.

Because of crosstalk among all components of haemostasis and innate immunity, many mediators of haemostasis are linked to pain production in inflammation (ATP, histamine, bradykinin, etc). ATP released by injured cells activates neutrophils. Neutrophil activation is accompanied by thrombin from blood-coagulation cascade, by platelet-activating factor, through the release of proteases modulating platelet function, such as cathepsin G, and or enzymes which act on the tissue matrix, as an elastase. Neutrophils constitute the first line of defence of the innate immune system, engulf and degrade microorganisms, and produce prostaglandins and platelet-activated factor and several chemokines, moderating early cell trafficking, and major pro-inflammatory cytokines (Scapini *et al*., 2000). Neutrophils are the most abundant cells in the acute inflammatory infiltrate induced by primary infestation, but not subsequent infestations of all hard tick species (Brown *et al*., 1983, 1984; Brown 1982; Gills and Walker, 1985). Tick saliva itself generates a neutrophil chemotactic factor by cleavage of C5 (Berenberg *et al*., 1972). Ribeiro and colleagues (1985) identified anti-inflammatory and immunosuppressive properties of *I. scapularis* tick saliva caused by inhibition of neutrophils, including their oxidative and phagocytic activity. Neutrophils infiltration and activation is orchestrated by chemokines as CCL3, CXCL8/KC. We detected that salivary gland extracts of many ixodid ticks are able to effectively bind and block in action a broad spectrum of pro-inflammatory cytokines and chemokines. All hard tick species tested were shown to posses anti CXCL8 activity mediated by one or more molecules (Hajnicka *et al*., 2001, 2005; Vancova *et al*., 2010a). In our earlier studies we confirmed an inhibition of CXCL8-coordinated neutrophil migration by tick *D. reticulatus* salivary gland extracts (SGE). This inhibition of neutrophils migration was made via inhibition of CXCL8-binding to the cell receptors (Kocakova *et al*., 2003). In salivary glands of *R. sanguineus*, Evasins, a family of chemokine-binding proteins (CHBP) were identified. Evasin-1 was able to block adhesion and CCL3-induced emigration of leukocytes. Neutrophil recruitment inhibition was most striking, however inhibition of $CD3^+$ lymphocytes was also observed. The inhibition of granulocyte recruitment by Evasin-1 was then further investigated in a murine model of a Th1-predominant delayed-type hypersensitivity and a Th2-predominant, late phase reaction. Evasin-3 has also subsequently been shown to have potent anti-inflammatory properties. It inhibited CXCL8-induced chemotaxis of human neutrophils *in vitro* and /or KC induced mouse neutrophil recruitment into the peritoneal cavity in a dose-dependent manner *in vivo.* Moreover, Evasin-3 treatment significantly reduced the inflammatory hypernociception associated with a mouse model of antigen-induced arthritis, and interestingly, the treatment resulted in decreased local production of tumour necrosis factor alpha (TNF-α) (Déruaz *et al*., 2008). Evasin-3 is likely to be the most potent inhibitor of neutrophil recruitment produced by the tick, whereas Evasin-1 may be produced to inhibit (later) monocyte recruitment in non-rodent hosts, as well as T lymphocytes (Déruaz *et al*., 2008). We proposed an existence of Evasin-3 functional or structural homologues (Evasin-3 like molecules) as we detected differences in CXCL8 and KC binding activities

of CXCL8-binding molecules purified from *D. reticulatus* saliva by affinity chromatography compared with Evasin-3 (Vancova *et al.*, 2010b). Our results suggest that Evasin-3 like activity is common amongst metastriate ixodid tick species because of anti-CXCL8 and anti-mouse CXCL1/KC activity in detected *Am. variegatum* SGE, *R. appendiculatus* SGE and *D. reticulatus* SGE, both males and females, during blood feeding. An absence of CXCL-8-binding molecules in tick saliva had no impact on TBEV and/or *Bor. afzelli* transmission and multiplication and had no significant statistic effect (I. Vancova, data not published). In *I. ricinus* saliva, lipocalin family LIRs was identified, only lipocalin LIR6 bound neutrophils chemoattractant LTB4 (Beaufays *et al.*, 2008). Table 2 summarises anti-inflammatory inhibitors of neutrophils from soft ticks.

The saliva of ixodid ticks reduces polymorphonuclear leukocyte adhesion via downregulation of β2-integrins (CD18) and decreases the efficiency of PMN in the uptake and killing of spirochetes (Montgomery *et al.*, 2004).

Following injury, mast cells are activated by ATP released by platelets. These cells produce preformed mediators, such as vasoactive amines, proteoglycans, serine proteases, sulfatases and cytokines. Their activation triggers the degranulating process and they are able to synthesize mediators as growth factors, chemokines and lipid mediators for inflammatory cell recruitment. The increased number of mast cells/degranulated mast cells is observed in tick infested skin during secondary or tertiary infestation but not in primary infestation (den Hollander *et al.*, 1985; Steeves *et al.*, 1991; Brossard *et al.*, 1982; Gills and Walker, 1985, Gills, 1986). Histamine released by mast cells produces pruritus and triggers scratching by the host. The importance of histamine to tick feeding is emphasized by the possibly universal existence of the histamine-binding tick salivary lipocalin family. Antihistamine, which prevents inflammation, is also present. Male specific histamine – binding salivary protein [RaHBP (M)] and two female-specific histamine-binding salivary proteins [RaHBP (F)-1.2] were isolated from the saliva of *R. appendiculatus* (Paesen *et al*, 1999). A gene for both serotonin and histamine binding protein was detected in *D. reticulatus* tick salivary glands (SHBP) (Sangamnatdej *et al.*, 2002). Das *et al.* (2001) identified *I. scapularis* salivary proteins Salp 25B and Salp 25C which showed some similarities with *Rhipicephalus* tick histamine-binding proteins. Proteins IS-14 and IS-15, which bind histamine and serotonin (5-HS) have also been detected in *I. scapularis* saliva (Ribeiro *et al.*, 2006). The results of work by Mulenga and colleagues suggest evidence of a tick-derived multifaceted control mechanism for levels of histamine at feeding sites which have been identified in *D. variabilis* functional IgE dependent histamine release factor homolog which induced histamine secretion from rat basophilic leukemic cell line (Mulenga *et al.*, 2003). Like mast cells, basophiles possess high affinity immunoglobulin E (IgE) receptors. Basophil-derived histamine inhibits tick salivation and engorgement (Paine *et al.*, 1983). Basophils have long been documented as the predominant cell type that infiltrates to the tick-bite site in the skin (Allen, 1973) and recognized as important effectors in tick rejection (Askenase, 1977). Tick-infested animals have developed anti-tick antibodies IgE, which are able to bind to Fc receptors on basophils, and mast cells also (Nithiuthai and Allen, 1985). Basophils are a source of histamine, which inhibits tick salivation and engorgement (Paine *et al*, 1983). Cutaneous basophil hypersensitivity (CBH) is a form of delayed hypersensitivity mediated by Th1 lymphocytes (Mosmann and Coffman, 1989). CBH responses are associated with immune skin rejection of blood-sucking ticks (Brown and Askenase, 1983). Insulin like growth factor (IGF) is a selective chemotactic factor for basophils (Hartnell *et al.*, 2004), produced by activated platelets after aggregation; that facilitates tissue remodelling that leads to the acceleration of wound healing (Holly and Perks, 2006). Because of this, it is not surprising that tick SG of *Am. americanum* express an insulin-like growth factor-binding lipocalin, AamIGFBP, in multiple organs and prevent ticks from feeding to repletion. Its homologues were detected in *I. scapularis* S, *B. microplus*, *R. appendiculatus* N and *Am. variegatum* F, respectively (Mulenga *et al.*, 2007; Mulenga and Khumthong, 2010). Also a soluble receptor of histamine, tick histamine binding protein (HBP), is thought to suppress inflammation by preventing histamine to reach the target cell (Paesen *et al.*, 1999). However, histamine-binding is not likely to be its primary function (Paesen *et al.*, 2000; Sangamnatdej *et al.*, 2002)

Besides the histamine, bradykinin is an important mediator of itching and pain, which induces the release of TNFα by neutrophil and stimulates host grooming and the removal of the feeding ticks.

The effect of inflammatory mediators is blocked, perhaps by the same kininase enzyme or other carboxypeptidase (Valenzuela *et al.*, 2002).Tick salivary kininase, for example dipeptidyl carboxypeptidase from *I. scapularis* tick saliva (Ribeiro and Mather, 1998), hydrolyzed circulating kinins (e.g. bradykinin). Recently, a tick-derived protease inhibitor (TdPI) was detected and characterized from *R. appendiculatus*. This protein inhibits the activity of human β-tryptases and mast cell specific serine proteases important to inflammation and tissue remodelling (Paesen *et al.*, 2007).

The next cell population from body surface tissue that interacts with external environment is eosinophil population. They are the source of several cytokines, chemokines and lipid mediators. Furthermore, their granules are rich in cytotoxic granules containing eosinophil peroxidase, eosinophil cationic protein, eosinophil-derived neurotoxin and major basic protein, which is a mast cell-, and probably basophile-, degranulation factor. Eosinophils are a very im-

portant source of tissue repair and inflammation molecules, such as tenascin, transforming growth factors TGF-α and TGF-β1 (Paesen *et al.*, 1999). A high number of eosinophils are found after repeated infestation of guinea pigs with *Am. americanum*, *R. appendiculatus*, but were virtually absent in dermis of *I. holocyclus*-infested guinea pigs in primary and secondary feedings. Other animal species present similar eosinophil infiltration at the attachment site upon repeated hard tick infestation (Francischetti *et al.*, 2010). Because eotaxin is a very potent chemoattractant of eosinophils, recruitment of eosinophils could be blocked by very strong anti-eotaxin activity identified in the salivary gland extracts of many hard ticks (Hajnická *et al.*, 2005; Vancova *et al.*, 2010a). In the Th2 sensitization model, Evasin-1 inhibited eosinophil recruitment induced by antigen challenge into the lungs of mice immunized with *Schistosoma mansoni* eggs (Frauenschuh *et al.*, 2007). Also, Evasin-4 identified in *R. sanguineus*, specifically binds to CCL5 (RANTES) a CCL11 (Eotaxin). Because the over-expression of IL-5 significantly increases eosinophil numbers in vivo (Takatsu and Nakajima, 2008; Kouro and Takatsu, 2009) and concurrently, IL-5 has been recognized as the major maturation and differentiation factor for eosinophils in mice and humans, subsequent studies focused on infestation of IL5 deficient or anti-IL5 treated animals are needed.

Even though dendritic cells do not cause direct damage to ticks, they seem to be important in the generation of acquired immunity leading to resistance against the tick. *In vitro*, the treatment of dendritic cells by tick saliva induces T-cell proliferation obtained from the lymph node of bite sensitized tick-resistant guinea pigs (Nithiuthai *et al.*, 1985). A decrease in the number of these cells around sites of *D. andersoni* tick attachment suggests probable Langerhans cell migration to lymph nodes following contact with tick saliva components, in next step T-cells responses. More recently, tick saliva has been shown to affect several dendritic cell functions. PGE2 from *I. scapularis* saliva is a major inhibitor of dendritic cell maturation and function (Sa-Nunes *et al.*, 2007). Tick saliva inhibits the chemotactic function of CCL3 (MIP-1α) and selectively impairs chemotaxis of immature dendritic cells by down regulating cell-surface CCR5. Saliva inhibited DC migration in response to CCL3 (migration via receptors CCR1 or CCR5), to CCL4 (MIP-1β) (via CCR1) and to CCL5 (RANTES) (migration via CCR1, CCR3, CCR5). Also, Evasin-1 (from *R. sanguineus*) is able to bind to human CCL3 and mouse CCL3 (Dias *et al.*, 2009). Co-incubation of dendritic cells with tick saliva leads to attenuation of antigen-specific T-cells cytokine production stimulated by dendritic cells (Oliveira *et al.*, 2008). Tick saliva inhibits differentiation, maturation and function of murine bone-marrow-derived dendritic cells (Cavassani *et al.*, 2005). Saliva from *R. sanguineus* ticks inhibits the maturation of dendritic cells (DCs) stimulated with lipopolysacharide (LPS), a toll-like receptor (TLR) -4 ligand, leading to increased production of interleukin (IL)-10 and reduced synthesis of IL-12p70 and TNF-α (Oliveira *et al.*, 2010). Two salivary cystatins, cysteine protease inhibitors, from *I. scapularis* have been functionally characterized as inhibitors of cathepsins L and S, to inhibit inflammation, suppress dendritic cell maturation, and serve as vaccine targets (Kotsyfakis *et al.*, 2006; Kotsyfakis *et al.*, 2008; Sa-Nunes *et al.*, 2009)

Macrophages residing in the skin act as the antigen-presenting cells eliciting a potent proliferative response during secondary infestation. Macrophages recruit in increased number to the site of injury in response to inflammatory and immune stimulation, and produce cytokines and chemokines that attract inflammatory cells to the tick-bite site. Few studies have described the presence of macrophages/monocytes in feeding cavities and the area around the lesion of the first tick infestation of all development stages of *Am. americanum* (Brown and Knapp, 1980a,b). Tick macrophage migration inhibitor factor MIF has been identified only in the salivary glands of *Am. americanum*. This peptide inhibits the migration of macrophages and protects the tick from macrophage attack (Jaworski *et al.*, 2001). It remains to be shown whether or not tick MIF is secreted into the feeding lesion. Tick saliva also decreased the oxidative activity of mouse macrophages (Kuthejlova *et al.*, 2001). Activated macrophages released platelet-derived growth factor (PDGF) and TGF-β that attract fibroblast and smooth muscle cells to the wound site. TGF-β, master control signal of fibroblast function, is produced by activated platelets, macrophages and T-lymphocytes, has effects on extracellular matrix deposition, increases collagen, proteoglycans and fibronectin gene transcription, and stimulates the tissue metallo-protease inhibitor, and other cytokines (interleukins, fibroblast growth factor FGF, TNF-α). TGF-β binding activity of tick saliva was detected along with other growth factor binding activities (PDGF, hepatocyte growth factor HGF, FGF2) in *D. reticulatus, R. appendiculatus, I. ricinus, I. scapularis,* and *Am. variegatum*, also (Hajnická *et al.*, 2011). Kramer and colleagues (2011) identified the stimulating effect of *D. variabilis* tick saliva on basal-and PDGF-stimulated migration of macrophage derived cell line IC-21, saliva regulating cell signalling, and phagocytosis and gene expression skewed immune response toward a Th2 reaction, which is characterized by production of anti-inflammatory cytokines IL-4 and IL-10.

Recognition and defensive response activity of the host immune system during tick infestation depend on the production and release of several mediators, including those produced by activation of complement, and by cytokines. The complement system, like the clotting system, is proteolytic cascade. The complement system links the innate and adaptive responses of the host immune system and its most prominent role is to recognize and clear invading pathogens. Activation of the complement cascade can be done in four

different ways: the classical pathway (Rother *et al*., 1998), the lectin pathway (Ip *et al*., 2009; Matsushita, 2009), the alternative pathway (for amplification of complement activation initially triggered by the classical and/or lectin pathway) (Kishore and Reid, 2000), and the fourth pathway initiating by activation of C5 by thrombin (Huber-Lang *et al*., 2006). All of these ways result in opsonisation of the invading microorganism leading to enhanced phagocytosis, leukocyte chemotaxis and direct killing of pathogens by formation of the membrane attack complex (MAC) (Duncan *et al.*, 2008; Thiel *et al.*, 2007; Zipfel *et al.*, 2007a,b).

Complement inhibition is crucial for haematophagous parasites survival or facilitation of blood feeding and also contribute to their success as pathogen vectors (Schreoder *et al*., 2009). The complement-derived peptides C3a, C4a and C5a are evolved in the activation of histamine, cytokines and other pro-inflammatory substance production during basophile and mast cell degranulation as well as in other reaction triggers by complement peptides. Via these mechanisms, tick inhibitors of complement activation block inflammatory response or pain. C5a also acts as a neutrophil chemoattractant (Janeway *et al*., 2001). The anaphylatoxins C3a and C5a cause further release of vasoactive mediators (Andrade *et al*., 2005). Several tick salivary proteins from different tick species inhibit the host complement system.

The alternative pathway has been shown to be important in tick rejection reactions by guinea pigs, perhaps by the production of inflammatory anaphylatoxins (Wikel, 1979). In fact, the cleavage of host complement C5 protein by *D. variabilis* saliva substance(s) has been detected, generating chemotactic fragments for neutrophils (Berenberg *et al*., 1972). In addition to PGE2, other molecules with immunosuppressive properties, such as 49kDa molecules, inhibit the activation of an alternative complement pathway. The saliva of *I. scapularis* antagonizes anaphylatoxin and bradykinin likely in the presence of a carboxypeptidase and can inhibit C3a release and C3b deposition (Andrade *et al*., 2005). The first characterized molecule displaying these anaphylatoxin-inactivating activities was ISAC, *I. scapularis* anticomplement protein (Valenzuela *et al*., 2000). Additionally, other tick complement inhibitors Irac-1 and -2 (from *I. ricinus*), IxAC (from *I. scapularis*) and Salp20 (from *I. scapularis*), belong to the ISAC protein family and inhibit the alternative complement pathway by binding and displacing properdin thereby inhibiting the formation of C3 convertase (Couvreur *et al*., 2008; Tyson *et al*., 2007; Valenzuela *et al*., 2000). ISAC, a hard tick inhibitor of alternative complement pathways, has no effect on the classical pathway, and has no similarity with anti-complement proteins of the lipocalin family (Schroeder *et al*., 2009). Irac-1 and -2 have inhibition activity against the complement of different host species (Schroeder *et al*., 2007). These proteins are promising candidates for the development of an anti-tick vaccine (Gillet *et al*., 2009). IxACs have a completely new action mechanism as they bind and prevent the action of properdin and inhibit the AP in different hosts as a positive regulator of C3 convertase, but their expression varies depending on the individual (Couvreur *et al*., 2008).

Inhibition of complement by ISAC facilitates feeding and may predetermine vector competency to pathogen transmission, including *Bor. burgdorferi* (Schroeder *et al*., 2009). C5b-C9 complement binding on spirochetes surface is blocked by Salp15 from *I. scapularis*, and by its homologous Iric1 from *I. ricinus* and Iscap from *I. scapularis* (Schuijt *et al*., 2008). Soft tick lipocalins complement modulators (Table 2).

The haemolytic function of complement system mediated by C1q could be inhibited by calreticulin (CRT), the extracellular form of which can bind to a complement system (C1q) (Schroeder *et al* 2009). CRT has been identified in tick saliva of *Ixodidae* family ticks, *Am. americanum*, *D. variabilis* females and *B. microplus* (Jaworski *et al*., 1995; Fereira *et al*, 2002). Gao *et al*. demonstrated that immunisation of sheep against CRT expressed by the *Ixodidae* ticks *Haem. quinghaiensis* induced partially protective immunity against ticks, resulting in 54, 3% mortality in adult ticks (Gao *et al*., 2008). Tick calreticulin is not detectable in the saliva of unfed ticks, but begins to be secreted around the third day of feeding (Jaworski *et al.*, 1995).

A local inhibition of cytotoxic activity of natural killer (NK) cells at tick feeding sites may be a further addition to the repertoire of the immunomodulatory effects of ticks.

Table 2. Imunomodulators in soft ticks – anti-complement and anti-inflammatory activity

Molecule	Tick species	Function/activities	References
Anti-complement			
TSGP2, TSGP3	*Ornitodoros savignyi*	Lipocalin, bind and inhibit C5 convertase, inhibit both classical and alternative pathways of complement activation	Mans and Riberio, 2008
OmCI	*O. moubata*	Lipocalin, inhibits C5 activation by binding of C5 and preventing its activation by C5 convertase	Fredslund *et al*., 2008; Nunes *et al*., 2005; Hepburn *et al*., 2007
Anti-inflammatory activities			
TSGP3	*O. savignyi*	Binds leukotrien B4(neutrophil chemoattractant)	Mans *et al*., 2008
TSGP4	*O. savignyi*	Binds leukotrien C4, D4 and E4(neutrophil chemoattractants)	Mans and Ribeiro, 2008
AM-33	*Argas monolakensis*	Binds leukotrien C4, D4 and E4(neutrophil chemoattractants)	Mans and Ribeiro, 2008

NK cells are a subset of lymphocytes that kill various target cells without a need for additional activation and mediate spontaneous antibody independent non-major histocompatibility complex-restricted cytotoxicity (Trinchieri, 1989). Because of the expression of a low-affinity receptor for the Fc region of IgG, they can mediate antibody-dependent cellular cytotoxicity (ADCC) (Peritt *et al.*, 1998). The principal physiological role of NK cells is in defence against infection by viruses and some other intracellular microbes and tumours. In addition, they secrete several cytokines, particularly IFN-γ, TNF-α, interleukin 1 (IL-1), IL-3, and granulocyte-monocyte colony stimulating factor (GM-CSF) (See *et al.*, 1997).

A decrease in the natural killer activity (NKA) of healthy human blood donors′ effector cells by SGE from *D. reticulatus* adult ticks was determined in our laboratory. This significant inhibitory effect was observed with SGE from female *D. reticulatus* partially fed (6 days), but not for SGE from unfed ticks (Kubeš *et al.*, 1994). In subsequent studies we have shown that anti-NK cell activity of *D. reticulatus* SGE correlates with blood-feeding but that such activity is not demonstrated by all ixodid tick species. Besides *D. reticulatus,* we demonstrated the inhibition effect of SGE from partially fed ticks *Haem. inermis* and *Am. variegatum* on cytotoxic activity of human NK cells. Despite the fact that SGE from *R. appendiculatus* ticks promotes transmission of several tick-borne viruses (Labuda *et al.*, 1996; Jones *et al.*, 1989), we determined no suppressive effect of SGE derived from these partially fed ticks. Similarly, SGE from *I. ricinus*, a vector of TBE virus, did not affect human NKA (Kubeš *et al.*, 2002).

Similarly, the inhibition effect on cytotoxic activity of mouse NK cells was detected in SGE from partially fed ticks *D. reticulatus* (both sexes), *Am. variegatum* (both sexes), *Haem. concina* (females). In contrast, no significant anti-NKA effect was detected neither in SGE from both sexes of unfed nor full engorgement *D. reticulatus* and *Am. variegatum*, respectively or in SGE derived from partially fed ticks *I. ricinus* (females), *R. appendiculatus* (both sexes), *R. pulchelus* (both sexes), *Haem. concina* (males) and *Haem. inermis* (females) (P. Kocakova-Bartikova, unpubl. observ.). However, Kopecky and Kuthejlova (1998) described the suppressive effect of SGE from *I. ricinus* females on mouse NKA. The suppression of NKA by SGE appears to have a direct effect on effector cells, because treatment of target cells did not affect NK activity. Furthermore, SGE affect the first step of NKA, i.e. the effector- target cell conjugate formation (Kubeš *et al.*, 2000, 2002). The reason ticks should seek to inhibit NKA during feeding is still unclear and requires identification of a molecular response to this effect and a better understanding of the function of NK cells. Tick-induced suppression of NKA may be directed at host cytokine response, as NK cells produce cytokines involved in the development of T helper (Th) 1-type host response. SGE of *I. ricinus* reduced polyinosinic-polycytidylic acid (polyIC)-induced production of IFN-α, IFN-β, and IFN-γ (Kopecky and Kuthejlova, 1998) and SGE from female *D. reticulatus* inhibited antiviral effects of IFN-α and IFN-β produced by mouse fibroblasts (Hajnicka *et al.*, 2000).

Subsequent tick infestations may activate adaptive immunity involving T cells and B cells by production of antibodies and sensitization of mast cells and basophiles, predominant cells at tick attachment sites.

The adaptive immune response is initiated by the recognition of foreign antigens by specific lymphocytes, which respond by proliferating and differentiating into effector cells (elimination of antigens) and into memory cells (enhancing responses on subsequent encounters with antigen). The two major subpopulations of lymphocytes are T and B cells. Both of these cell types are central to immunity to ticks (Wikel, 1996) and a target for tick-induced immune modulation (Gillespie *et al.*, 2000; Schoeler and Wikel, 2001).

T lymphocytes, based on their receptors subdivided on $CD4^+$ and $CD8^+$ T cells, are mediators of cellular immunity, recognizing the antigens of intracellular microbes and destroying these microbes or infected cells. T cell activity is altered by ticks. Inhibition of host T cell proliferation is induced by feeding as well as SGE of numerous tick species (Brossard and Wikel, 2004). Ticks impair *in vitro* different mitogen (concanavalin A – ConA, phytohemaglutinin – PHA, pokeweed – PWM, lipopolysaccharide – LPS) – driven proliferation of T lymphocytes from guinea pigs (Wikel, 1982), mice (Ramachandra and Wikel, 1992; Borsky *et al.*, 1994; Urioste *et al.*, 1994; Dusbabek *et al.*, 1995; Ganapamo *et al.*, 1996; Ferreira and Silva, 1998; Schoeler *et al.*, 2000a,b), rabbits (Schorderet and Brossard, 1993), dogs (Inokuma *et al.*, 1998; Ferreira and Silva, 1998; Ferreira *et al.*, 2003), cattle (Inokuma *et al.*, 1993; Ramachandra and Wikel, 1995; Turni *et al.*, 2002, 2004, 2007), sheep (Boppana *et al.*, 2004) and humans (Rolnikova *et al.*, 2003). Mbow and colleagues (1994) determined increasing ratios of $CD4^+$: $CD8^+$ T cells in host skin over the course of repeated infestations with *I. ricinus* nymphs. Similarly, this effect was shown in a study of *H. anatolicum anatolicum* and *Haem. bispinosa* ticks feeding on sheep (Boppana *et al.*, 2004).

T-lymphocyte function could be affected by tick prostaglandins which suppress IFN-γ and IL-2 production, inhibit bioactivity of IL-2 in cells by reduction of IL-2-receptor expression on IL-2-dependent cell surface – thus, PGE_2, and stimulate secretion of tick bioactive proteins (Urioste *et al.*, 1994; Singh and Grischick, 2003). Gillespie and colleagues (2001) demonstrated decreasing production IL-2 by spleen cells after treatment with tick saliva and these observations led to the identification of IL-2 binding protein in saliva from tick species *I. scapularis*. Besides the T cell modulation, this IL-2 binding protein has potentially far reaching

effects on the immune system due to the existence of IL-2 receptors on many cell types including B cells, macrophages and NK cells.

Another protein from SGE and saliva of *D. andersoni,* p36, suppresses T cell proliferation and probably alters host T-cell cytokine production (Bergman *et al*., 2000). Homologues genes related to the *D. andersoni*-derived *p36* gene have been isolated from *R. appendiculatus* (Ra-p36), *Am. variegatum* (Av-p36) (Nene *et al*., 2002) and *Haem. longicornis* (*Hl-p36*) respectively (Konnai *et al*., 2009). *In vitro* assays with HL-p36 protein showed significant reduction of IL-2 expression. *In vivo*, HL-p36 down-regulated immunomodulating factors H2-Ea (associated with MHC II molecule CD8) and Ifi (important in host defence) which resulted in the suppression of cell proliferation (Konnai *et al*., 2008).

In 2002, Leboulle and colleagues (2002a,b) identified a novel immunosuppressor, named Iris, in the salivary glands of *I. ricinus* during feeding. Besides inhibition of the production of pro-inflammatory cytokines, IL-6, and TNF-α (Leboulle *et al*., 2002b) and anti-haemostatic effects mentioned above (Prevot *et al*., 2006), Iris has been shown to suppress T cell proliferation and induces Th2 type immune response. Due to sialotranscriptome work on *I. scapularis* saliva (Valenzuela *et al*., 2002), a large number of protease inhibitors was detected in SG, including secreted 12.5 kDa Sialostatin L containing the conserved cystatin domain. It inhibits papain-like cysteine proteases, targeting mainly cathepsin L, C and S. This protein displays an anti-inflammatory effect and reduces the proliferation of cytotoxic T lymphocytes (CTL) (Kotsyfakis *et al*., 2006), as well as antigen specific $CD4^+$ T cells (Sa-Nunes *et al*., 2009). Cysteine proteases play several roles in biological events such as antigen presentation (Honey and Rudensky, 2003), immune system development (Lombardi *et al*., 2005), epidermal homeostasis (Reinheckel *et al*., 2005), neovascularization (Felbor *et al*., 2000), extracellular matrix degradation and neutrophil chemotaxis during inflammation (Reddya *et al*., 1995; Serveau-Avesque *et al*., 2005), proliferation and subsequent invasion of malignant cells (Joyce *et al*., 2004; Nomura and Katunuma, 2005). On the other hand, cystatins guard cells and tissues against damage that could be caused by cysteine proteases. Sialostatin L inhibition of cathepsin C should also prevent the activation of granule serine proteases in CTL, NK cells, mast cells and neutrophils. During inflammation, the balance between cystatins and cysteine proteasis changes in favour of proteolysis. In contrast, the presence of Sialostatin L at the site of inflammation changed the balance toward antiprotease activity and reduced both edema formation and granulocyte recruitment (Kotsyfakis *et al*., 2006).

A recent study by team Karim *et al.* (2005) described how a cystatin is important for the feeding success of *Am. americanum* ticks, but unlike Sialostatin L, the target enzymes and the mechanisms of action are still unknown.

The modulation by tick infestation or tick saliva mentioned above have an indirect effect on T cells. On the other hand, the Salp15, a 15 kDa salivary protein from *I. scapularis,* with some sequence similarity to TGF (Das *et al*., 2001), is an example of a feeding-induced protein that directly inhibits the activation of T cells (Anguita *et al*., 2002). Salp15 specifically binds to the CD4 molecule on $CD4^+$ T (helper) cells, which results in the inhibition of T cell receptor–mediated signalling, leading to reduced interleukin 2 production and impaired T cell proliferation (Garg *et al*., 2006, Juncadella *et al*., 2007). Furthermore, Salp15 also inhibits inflammatory cytokine production by human monocyte-derived dendritic cells by interacting with the lectin receptor DC-SIGN (Hovius *et al*., 2008), indicating its potential role in modulating human adaptive responses.

Having established that ticks modulated lymphocyte proliferation, the next logical area to assess was cytokines. On the basis of cytokine patterns produced by $CD4^+$ Th cells, they are separated into two subsets Th1 (IL-2, IFN-γ and lymphotoxin) and Th2 (IL-4, IL-5, IL-10, and IL-13). Fuchsberger and colleagues (1995) showed that SGE from *R. appendiculatus* inhibited LPS-induced production of mRNA specific for different cytokines by normal human peripheral blood leucocytes. It has been observed in recent years that tick infestation on a natural or susceptible host leads to Th2 immune responses (Ferreira and Silva, 1999; Mejri *et al*., 2001). Splenocytes from BALB/c or C3H/HeN mice infested with pathogen-free *I. scapularis* nymphs secreted increased amounts of IL-4, and decreased levels of IFN-γ and IL-2 upon *in vitro* stimulation with ConA (Schoeler *et al*., 1999). This apparent Th2 polarization also occurred during infestation with *R. sanguineus* (Ferreira and Silva, 1999), *I. pacificus* (Schoeler *et al*., 2000b), *I. ricinus* (Ganapamo *et al*., 1995, 1996a, 1996b; Kopecky *et al*., 1999; Mejri *et al*., 2001), *I. scapularis* (Urioste *et al*., 1994; Zeidner *et al*., 1997) and *D. andersoni* (Macaluso and Wikel, 2001). Kovar *et al*. (2001, 2002) showed *in vitro* inhibition of T cell proliferation and modulation of the host immune response towards Th2 with SGE from *I. ricinus*. It has recently been demonstrated that splenic dendritic cells pulsed with saliva of *I. ricinus* initiated *in vitro* Th2 differentiation (Mejri and Brossard, 2007). Moreover, when ticks are infected with *Bor. burgdorferi*, the antispirochete immune response is also biased toward Th2 (Christe *et al*., 2000). In contrast to these previous *in vitro* studies, Muller-Doblies and colleagues (2007) examined the influence of tick feeding and tick SGE on the response of antigen-specific $CD4^+$ T cells by using TCR transgenic adoptive systems. Both infestation and SGE administration programmed a Th2 cytokine response, yet tick feeding did not diminish the strong Th1 response, but rather superimposed a Th2 response on top the Th1.

Antibody production is another of the host´s acquired responses to the salivary secretion introduced into the feed-

ing site. B lymphocytes, mediators of humoral immunity, are the only cells producing antibodies. Despite the absence of B cells in the skin of infested hosts, lymph nodes draining attachment sites are increased upon primary and secondary infestations (Boppana *et al.*, 2005), suggesting proliferation and differentiation of B lymphocytes. Suppression of B cell responses benefits the tick by inhibiting specific anti-tick antibody responses that could lead to rejection by the host.

Although *in vitro* analysis of the effect of tick saliva or SGE on the proliferation of B cells after LPS stimulation have been contradictory, *in vivo* studies have shown that ticks are able to modulate the specific antibody responses (Wikel, 1985, Fivaz, 1989; Inokuma *et al.*, 1997; Kashino *et al.*, 2005; Menten-Dedoyart *et al.*, 2008, 2011).

Recently, only two saliva proteins with inhibition effect on B cell proliferation have been identified and characterized. The B cell inhibitory protein (BIP) is one of the tick salivary proteins that suppress proliferation of murine B cells. First *I. ricinus* saliva induced a dramatic inhibition of host B cells by preventing IL-10 and TNF-α production, CD69 expression and proliferation after stimulation with LPS. This inhibitory activity acts directly on B cells, without inducing B-cell necrosis or apoptosis (Hannier *et al.*, 2003) and is due to a protein of approximately 18 kDa termed as the B-cell inhibitory protein (BIP) (Hannier *et al.*, 2004). BIP enriched fractions (after partial purification) dramatically inhibit the B-lymphocyte proliferation induced by the *Bor. burgdorferi* lipoproteins OspA and OspC, suggesting that BIP may be crucial to its transmission enhancement by preventing B-cell activation. BIP-enriched fractions did not suppress T-cell proliferation (Hannier *et al.*, 2004). Yu and colleagues (2006) identified and characterized an inhibitory protein of B cells in salivary glands of hard ticks *H. asiaticum asiaticum* and named it B-cell inhibitory factor (BIF). Molecular weight (MW) of mature BIF is different from MW of BIP from *I. ricinus*. The purified BIF significantly inhibited proliferation of LPS stimulated mouse B lymphocytes (Yu *et al.*, 2006).

Another way to alter host humoral immune responses presents immunoglobulin-binding proteins (IgGBPs) identified in haemolymph and SGE of ticks *R. appendiculatus* (males and females), as well as in SGE of *Am. variegatum*, *I. hexagonus* and *I. ricinus* ticks (Wang and Nuttall, 1999). During a tick′s feeding, a small fragment of host immunoglobulins pass through the midgut into the haemolymph of argasid and ixodid ticks (Aeckerman *et al.*, 1981; Brossard and Rais, 1984; Chinzei and Minoura 1987; Ben-Yakir *et al.*, 1996; Jasinskas *et al.*, 2000), still retain their biological activity (Fujisaki *et al.*, 1984) and can be localized in the internal organs (salivary glands and ovary) (Aeckerman *et al.*, 1981). During feeding, the concentration of host IgG in ixodid tick haemolymph increases (Ben-Yakir *et al.*, 1996). In studies of *R. appendiculatus*, Wang and Nuttall (1994) detected guinea-pig IgG not only in haemolymph of partially fed female ticks, but also in SGE and saliva and along with the existence of IgGBs in salivary glands or in the haemolymph, respectively, indicated that there is a mechanism in ticks for clearing host IgG via saliva back to the host. Inhibition or decrease of the host′s antibody response could help the tick complete its blood meal by preventing or reducing the neutralization of salivary factors. This effect may favour the transmission of pathogens during tick′s feeding.

4. Tick saliva proteins as therapeutical tools in the treatment of human disease

Immunity serves the important function of host defence against disturber like ticks, but, apart from ticks, immune responses are also capable of causing tissue injuries and diseases. Disorders may result from uncontrolled or excessive responses against either foreign antigens (hypersensitivity diseases) or self antigens (autoimmunity diseases). Ticks by their parasitical lifestyle and as vectors of numerous pathogens are associated with diseases in humans, wildlife and livestock. However, could they also be beneficial?

As described above, tick salivary glands produce a wide range of pharmacologically active molecules that not only facilitate blood feeding, but counteract haemostatic and both innate and adaptive immune responses. Some of the saliva proteins mentioned earlier in this work have promising potential for the treatment of a number of haemostatic disorders, tumours and autoimmune diseases.

Tick saliva presents novel and more easily used anticoagulant agents (Maritz-Olivier *et al.,* 2007). For example, recombinant forms of TAP protein (rTAP) from soft tick *O. moubata*, have been tested on a variety of animal models for both venous and arterial thrombosis (Table 3). In a mouse carotid artery thrombosis model, it was more effective than enoxaparin without prolonged bleeding time in comparison to conventional anticoagulants (Stoll *et al.*, 2007). Ixolaris, a promising agent for anti-tumour therapy of human glioblastoma, blocks primary tumour growth and angiogenesis in the glioblastoma model (Carneiro-Lobo *et al.*, 2009). In a rat model, administration of recombinant Ixolaris resulted in effective antithrombotic activity, without haemorrhage and bleeding (Nazareth *et al.*, 2006). Because of cytotoxic activity in different tumour cells (among them pancreatic and human melanoma) occurring without causing changes in normal human fibroblasts, amblyomin-X from *Am. cajannense* could be promising candidate for anticancer therapy (Chudzinski-Tavassi *et al.*, 2011; Simons *et al.*, 2011).

Direct thrombin inhibitors are the next group of possible therapeutical tools. Variegin, a member of this group, is structurally and functionally similar to, but more potent

Table 3. Possible therapeutic use of blood-coagulation cascade inhibitor

Molecule	Tick species	Target/function	Disease models	References
TAP	*Ornitodoros moubata*	Inhibitor of FXa	Primate model thrombosis Rabbit model of venous thrombosis Canine model of acute coronary artery thrombosis Mouse model of carotid artery thrombosis Rat model of venous thrombosis	Schaffer *et al.*, 1991 Vlasuk *et al.,* 1991 Sitko *et al.*, 1992 Stoll *et al.,* 2007 Nazareth *et al.*, 2006
Ixolaris	*Ixodes scapularis*	Inhibitor of TF pathway	Glioblastoma model	Carneiro-Lobo *et al.*, 2009
Variegin	*Amblyomma variegatum*	Direct trombin inhibitor	Human *in vitro* studies	Koh *et al.*, 2007

than hirulog, a drug used for the treatment of patients with acute coronary syndromes (Lincoff *et al.*, 2004).

Other anticoagulant agents such as Salp14, Penthalaris, Savignin, Madanin and others, possibly for medical application have recently undergone both animal and human *in vitro* studies.

Events of innate and adaptive immune responses are involved in the pathogenesis of many autoimmune diseases, including rheumatoid arthritis, systematic lupus erythematosus (SLE), multiple sclerosis etc. Table 4 describes some tick saliva proteins that influence either innate or adaptive arms of immunity and have large potential as novel drugs in therapy.

Evasin-1, which is highly selective for CCL3 and CCL4, reduced symptoms in a mouse model of skin inflammation resembling psoriasis in humans. Evasins were subsequently tested in disease models in which neutrophils play an important role. In the CCL3-dependent bleomycin-induced lung injury, Evasin-1 reduced leukocyte influx, fibrosis, and lethality (Smith *et al.*, 1994; Ishida *et al.*, 2007). A blockade of CXCR2 prevented leukocyte influx and joint damage in several models of experimental arthritis in rats and mice (Barsante *et al.*, 2008; Cunha *et al.*, 2008). Evasin-3, which binds to both, human and mouse, CXCR2 ligands (CXCL8 and KC), prevented neutrophil influx into the joint and local production of TNF-α in a model of antigen-induced arthritis. Both Evasins were able to completely inhibit the neutrophil recruitment induced by their respective ligand in the knee joint. In an ischemia model, Evasin-3 was more efficacious, showing significant reduction in lethality compared with Evasin-1 (Deruaz *et al.*, 2008). The Evasin-1 was used in idiopathic pulmonary fibrosis therapy (Russo *et al.*, 2011).

The possible therapeutic usage of tick saliva constituent of *D. variabilis* with growth factor binding activity is indicated in a study by Poole *et al.* (2009). In this study, inhibition of basal and epidermal growth factor (EGF) stimulated migration and invasion of osteosarcoma cells by suppression of Akt signalling pathway in cells was identified (Poole *et al.*, 2009).

Complement is also considered a privilege target for new therapeutic agents. A promising tool in disease therapy could be OMCI which inhibited complement haemolytic activity and the development of pathological features in

Table 4. Possible therapeutic use of immunosuppressors of innate and adaptive immune responses

Molecule	Tick species	Effect	Disease models	References
Evasins (Evasin-1,3)	*Rhipicephalus sanguineus*	Inhibitor of CCL3, CCL4, CXCL8/KC chemokine	Mouse model of psoriasis Mouse model of antigen-induced lung injury Mouse model of antigen-induced arthritis Mouse model of ischemia and reperfusion	Deruaz *et al.,* 2008
Unknown	Various	Inhibitor of EGF	Human *in vitro*	Poole *et al.*, 2009
OMCI	*Ornitodoros moubata*	Inhibitor of C5	Roden model of myasthenia gravis	Hepburn *et al.*, 2007
IxAC	*Ixodes ricinus*	Inhibitor of C3 through properdin	Human *in vitro*	Couvreur *et al.*, 2008
Salp15	*I. scapularis*	Inhibitor of $CD4^+$ T cells	Mouse model of allergic airway disease	Paveglio *et al.*, 2007
Iris	*I. ricinus*	Inhibitor of T cells and several serine proteases	Animal and human in vitro studies Animal and human in vitro studies and in vivo animal studies	Prevot *et al.*, 2006 Prevot *et al.*, 2007
Sialostatin L	*I. scapularis*	Cytotoxic T cells	Animal in vitro studies Mouse model of multiple sclerosis	Kotsyfakis *et al.*, 2006 Sa-Nunes *et al.*, 2009
BIP	*I. ricinus*	Inhibitor of B cells	Animal *in vitro* studies	Hannier *et al.*, 2004

a rodent model for autoimmune myasthenia gravis (Hepburn *et al.*, 2007). The next candidate could be IxAC due to its positive clinical effect in conditions where inappropriate complement activation involves the binding of properdin (positive complement regulator) and C3b, leads to reduction of proinflammatory molecules, and reduces deposition of C3b and reduction cell lysis due to deposition of the MAC (Couvreur *et al.*, 2008).

T cell inhibitors, Salp 15, Iris, and Sialostatin L are promising therapeutic candidates. A possible use of Salp 15 protein in human disease, such as atopic asthma or T-cell driven autoimmune disease has been proposed in the work of Paveglio and colleagues (2007). They showed that Salp15 prevented development of atopic asthma in mouse models of allergic airway diseases.

Sialostatin L, a novel inhibitor of cysteine proteases, mainly cathepsin L and C, with a very stringent and unique specificity may be a strong tool and of great medical importance, as cysteine proteases have been associated with a number of pathological events such as cancer, rheumatoid arthritis, osteoarthritis, Alzheimers disease, multiple sclerosis and muscular dystrophy (Barrett *et al.*, 1998; Otto and Schirmeister, 1997). In a mouse model of multiple sclerosis, *in vivo* administration of Sialostatin L during the immunization phase of experimental autoimmune encephalomyelitis significantly prevented disease symptoms, which were associated with impaired IFN-γ and IL-17 production and specific T cell proliferation (Sa-Nunes *et al.*, 2009).

Potent B-cell immunosuppressants are rare and have to be effective in clinical studies of lymphoproliferative disorders and autoimmune diseases such as rheumatoid arthritis, multiple sclerosis and SLE (Cambridge *et al.*, 2003; Bugatti *et al.*, 2007; Klawiter and Cross, 2007; Anolik *et al.*, 2003). Up to now, only two B cell inhibitory proteins in tick saliva have been described which need further characterization to serve as a template for novel drugs, specifically targeting B cells. BIP could be an interesting new agent to treat autoimmunity thanks to its potent immunosuppressive effect on B cells and different mechanism of action compared with anti-CD20 immunoglobulin used today (Hannier *et al.*, 2004).

5. Conclusion

Tick salivary glands represent a rich source of various bioactive compounds that interfere with host haemostasis and immunity to facilitate blood feeding. The composition of saliva is complex and changes during feeding. This review has summarized the discoveries in tick saliva component with immunomodulatory effects that have been published to date and is an evidence of the impressive resourcefulness that ticks display in modulating host defences. Knowledge from tick saliva research can shed light on the complexity of vector-host interactions and help us to understand and combat tick-borne pathogens and ticks themselves by designing new and more effective vaccines. On the other hand, some tick saliva proteins have potential as new pharmaceuticals for treatment of a number autoimmune and chronic diseases and tumours. Undoubtedly, future research will reveal even more potential molecules to our benefit.

Advances in tick saliva component knowledge will have widespread benefits including in the biomedical, veterinary and pharmacological fields.

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