UNIVERSITY OF SOUTH BOHEMIA

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THE ROLE OF TICK SALIVA AND TICK SALIVARY CYSTATINS IN THE TRANSMISSION OF *BORRELIA BURGDORFERI* AND THE CYSTATIN EFFECT ON EXPERIMENTAL ASTHMA IN MICE

Ph.D. Thesis

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Annotation

The thesis focuses on the investigation of the role of tick salivary components in the course of Lyme disease in mice. It includes studies on the saliva-facilitated transmission of *Borrelia burgdorferi* in vivo and the effect of tick cysteine protease inhibitors (cystatins) both on murine immune cells and the transmission of *B. burgdorferi* spirochetes in mice. The thesis also reveals practical applications of salivary cystatins for the development of anti-tick vaccine and the application of the pharmacological action of a tick salivary cystatin for the therapy of the disease symptoms in a mouse model of experimental asthma.

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Coauthors´ statement

The coauthors confirm the participation of Helena Horká on following publications and the manuscript and agree with their presentation in the PhD. thesis.

I) H. H. designed and performed the experiments, analyzed data and partially wrote the manuscript.

II) H. H. participated in the biological experiments.

III) H. H. performed *Borrelia*-related experiments and analyzed the data.

IV) H. H. performed and analyzed in vitro tests and participated on the manuscript.

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1 Introduction

1.1 *Borrelia burgdorferi* – a human pathogen

Borreliae are Gram-negative microaerophilic spirochetes of high medical importance, belonging to the eubacterial family Spirochetaceae. They are vigorously motile, corkscrew-shaped bacteria. Two groups of bacteria can be distinguished within the *Borrelia* genus: the etiologic agents of relapsing fever (*B. recurentis*, *B. duttoni*, *B. hermsii*) and those connected with the Lyme disease, forming the *Borrelia burgdorferi* sensu lato group. The latter group currently includes 17 species that differ in the antigenic repertoire, genotype, geographic occurrence, level of pathogenicity, natural vector and preferred reservoir host, as summarized in Table 1.

Species	Principal vector	Geographic location	Reference
B. burgdorferi sensu stricto	*Ixodes scapularis* *I. pacificus* *I. ricinus*	Northern/northeastern USA Western USA Europe	Johnson et al. 1984
B. garinii	*I. ricinus* *I. persulcatus*	Europe Asia	Baranton et al.1992
B. afzelii	*I. ricinus* *I. persulcatus*	Europe Asia	Canica et al. 1993
B. spielmanii	*I. ricinus*	Europe	Richter et al. 2004
Species with minimal or unknown pathogenicity			
B. andersoni	*I. dentatus*	Eastern USA	Marconi et al. 1995
B. bissetti	*I. spinipalpis* *I. pacificus*	Western USA	Postic et al. 1998
B. valaisiana	*I. ricinus*	Europe, Asia	Wang et al. 1997
B. lusitaniae	*I. ricinus*	Europe, North Africa	Le Fleche et al. 1997
B. japonica	*I. ovatus*	Japan	Kawabata et al. 1993
B. tanukii	*I. tanukii*	Japan	Fukunaga et al. 1996
B. turdi	*I. turdus*	Japan	Fukunaga et al. 1996
B. sinica	*I. persulcatus*	China	Masuzawa et al. 2001
B. californiensis	*I. spinipalpis* *I. pacificus*	USA (CA)	Postic et al. 2007
B. bavariensis	*I. ricinus*	Europe	Margos et al. 2009
B. carolinensis	*I. minor*	USA (SC)	Rudenko et al. 2009a
B. americana	*I. pacificus* *I. minor*	USA (CA, SC)	Rudenko et al. 2009b
B. kurtenbachii	*I. scapularis*	Western USA, Canada	Margos et al. 2010

Table 1. *Borrelia burgdorferi* sensu lato genospecies (adapted from Steere et al. 2004).

1.1.1 Morphology and genome of *Borrelia* pathogens

The spirochetal cell wall consists of a cytoplasmic membrane surrounded by a peptidoglycan layer, flagella and a loosely associated outer membrane. Helical

flagella enable the motility in low and high viscose environments inside the tick and the vertebrate host tissues (Sadziene et al. 1991). The location of the flagella underneath the outer membrane is advantageous for concealing the highly immunogenic flagellar proteins from the host immune system. For the same reason, *Borrelia* spirochetes can form non-motile spherical cysts under hostile conditions (Alban et al. 2000).

Borrelia burgdorferi (strain B31) genome consists of a small linear chromosome containing approx. 911 kbp and nine circular and twelve linear plasmids of 611 kbp total size. The genome encodes for very few proteins with recognizable biosynthetic activity (Fraser et al. 1997). Therefore, the in vitro cultivation of *B. burgdorferi* is rather difficult and requires a special culture medium called BSK-H which is supplemented with rabbit serum (Barbour 1984). In contrast, remarkably high content of *B. burgdorferi* genome encodes for lipoproteins, including the outer surface proteins (Osp) A to F. Plasmid loss during in vitro passages can induce attenuation or loss of virulence. For instance, bacteria deficient for the linear plasmid 28-1 have significantly impaired ability to infect mice, are promptly eradicated by host immune system and unable to set up a persistent infection (Labandeira-Rey and Skare 2001).

The outer membrane of *Borrelia* spirochetes has a unique structure compared to other bacteria in the sense that it contains a higher proportion of lipoproteins, less transmembrane proteins and glycolipid antigens distinct from bacterial lipopolysaccharide (LPS). This unusual arrangement of the bacterial surface probably contributes to the better survival of spirochetes in the vector, as well as in the vertebrate host (Haake 2000).

1.1.2 *Borrelia* life cycle

The coordinated production of outer surface proteins is crucial for the *Borrelia* life cycle that alternates between an arthropod tick vector and various vertebrate hosts. Spirochetes resided in the tick midgut express high levels of OspA which is required for pathogen adherence to the vector through the interaction with tick protein TROSPA (tick receptor for OspA) (Pal et al. 2004). In response to a fresh blood meal the pathogen dramatically changes its gene expression. OspC and other RpoS-dependent genes are upregulated in order to get the bacteria prepared for the infection of the host.

Indeed, *B. burgdorferi* maintains intensive OspC synthesis during early mammalian infection (Grimm et al. 2004, Liang et al. 2002).

1.1.3 Lyme disease

Lyme disease (LD) is the most spread vector-borne human disease in Europe and Northern America (Dennis and Hayes 2002). In humans, the infection has usually three phases: (1) localized skin infection termed erythema migrans, (2) early disseminated infection accompanied with flu-like symptoms and (3) if untreated, persistent (chronic) infection developing after several months or years.

As an extracellular pathogen, *B. burgdorferi* resides primarily in the extracellular matrix, connective tissues and between host cells. During the first few weeks the bacteria can invade tissues of the nervous system, heart, joints, muscles and skin, causing Lyme encephalopathy, carditis, arthritis, myositis or dermatitis. The particular clinical outcome of LD depends on the *Borrelia* species involved (van Dam et al. 1993). Similarly, laboratory mice inoculated with *B. burgdorferi* also develop arthritis and carditis over the course of three weeks and then undergo antibody-mediated remission and global reduction of spirochete loads in tissues; however, the infection is not eliminated (Hodzic et al. 2003).

In the late infection phases the bacteria in target organs are phagocyted by macrophages and presented to T lymphocytes. Long-term stimulation of T cells leads to excessive production of proinflammatory cytokines and tissue destruction. The cross-reactivity of antibodies against 41 kDa flagellar *Borrelia* protein with host myelinated nerve fibers, heart muscle cells and joint synovia has severe pathological consequences (Aberer et al. 1989).

1.1.4 *Borrelia* – vertebrate host interactions

Borrelia burgdorferi sensu lato infection induces strong innate as well as adaptive immune response. Despite this fact, the Lyme disease agents are capable of persistent infection in mice and humans, even after antibiotic treatment. Similar to other bacteria, these spirochetes have evolved evasion mechanisms to control host immunity, such as antigenic variability (gene conversion, mutations and genome

recombination events, antigen expression regulation) and physical barriers (cyst formation, colonizing immunologically hidden tissues).

Borrelia surface antigens activate host alternative complement pathway in the tick feeding site. Spirochetal sensitivity to complement highly depends on the species of the bacteria and host, respectively (Breitner-Ruddock et al. 1997). *B. burgdorferi* express at least five functionally related, but structurally heterologous complement regulator-acquiring surface proteins (CRASPs). The CRASPs belong among lipoproteins that bind to host C3b complement regulators, factor H, factor H-like protein 1 and factor H-related protein 1 for the protection against the complement cascade. The end-result is that the complement deposition is inhibited and the spirochetes protected from cell lysis (Kraiczy et al. 2001, Haupt et al. 2007).

B. burgdorferi infection also activates macrophages that engulf the spirochetes and kill them inside the phagolysosome (Montgomery et al. 2002). To understand how *B.burgdorferi* initiates innate immune cell activation, the first studies in the field focused on the borrelial lipoproteins expressed in relatively high abundance on their outer membrane. Lyme disease innate immunity activation was thus considered to occur predominantly through the interaction of the bacterial lipoproteins with CD14 and/or Toll-like receptors (TLR) 1/2 on the surface of macrophages and dendritic cells (Wooten et al. 1998, Hirschfeld et al. 1999). However, significant differences in immune activation by borrelial lysates and viable spirochetes have been described. The interaction of human peripheral blood mononuclear cells (PBMCs) and isolated monocytes with viable spirochetes elicited greater secretion of tumor necrosis factor-alpha (TNF-α), interleukin-1-beta (IL-1β), IL-6 and IL-10 compared to *Borrelia* lysates. On the other hand, viable *B. burgdorferi* can suppress early macrophage responses via enhanced IL-10 production (Lazarus et al. 2008). Moreover, TLR-2 deficiency had no significant effect on the uptake and degradation of spirochetes and only moderately suppressed cytokine response, suggesting that both TLR-2 dependent and independent mechanisms are involved in the innate immune responses against live *B. burgdorferi*. Interestingly, live bacteria induced the transcription of interferon-beta (IFN-β) which is known to be regulated by a TLR-2 independent pathway and is critical for Lyme arthritis development in mice (Salazar et al. 2009, Miller et al. 2008).

Further studies revealed an important role of the adaptor molecule myeloid differentiation antigen 88 (MyD88), which is required not only for TLR2- and TLR5-

mediated responses, but also for the production of the IL-1β and IL-18 proforms. MyD88-defficient cells show also a major defect in the uptake of the spirochetes by phagocytosis (Shin et al. 2008).

Neutrophil granulocytes are able to phagocyte spirochetes opsonized with antibodies and to destroy them by reactive oxygen and nitrogen intermediates (Montgomery et al. 2002). It has also been demonstrated that insufficient neutrophil recruitment and activation during the initial host inflammatory response may allow *B. burgdorferi* to effectively colonize the mammalian host (Xu et al. 2007).

The dissemination of *Borrelia* spirochetes within the host organism is also mediated by their ability to bind molecularly defined host receptors on the cell surface or extracellular matrix (ECM) of host cells. Host-derived plasmin plays a critical role in mammalian infection by *B. burgdorferi* and the Lyme disease spirochetes express several plasminogen-binding proteins. Indeed, OspA and CRASP-3, -4 and -5 were shown to bind host plasminogen (Fuchs et al. 1994, Brisette et al. 2009). Bound plasminogen converted to plasmin may facilitate the bacterium´s penetration from the tick feeding site into the bloodstream and its dissemination throughout the host (Coleman et al. 1995). Moreover, CRASP-1 binds to several new human ligands, including BMP-2 (bone morphogenetic protein 2), collagen I, III and IV, fibronectin, laminin and plasminogen (Hallström et al. 2010).

Interactions of *B. burgdorferi* with decorin and glycosaminoglycans, abundantly found in the extracellular matrix and connective tissues as well as on cell surfaces of mammals, are mediated by decorin-binding proteins A and B (DbpA/B). These proteins potentially play a role in various molecular events upon *Borrelia* infection. Neither DbpA nor DbpB is essential for mammalian infection, but both are critical for the overall virulence of *B. burgdorferi*, significantly changing bacterial colonization of the target host tissues such as joints, skin and heart (Shi et al. 2008a, b). Importantly, in contrast to needle-inoculation experiments, DbpA/B proteins are not required for the natural tick-to-mammal transmission process, implying the possibility that tick salivary proteins overlap with DbpA/B function in facilitating the transmission of tick-borne pathogens (Blevins et al. 2008, see also section 1.2.2).

OspC, a *Borrelia* protein highly expressed during early mammalian infection, seems to be another dissemination-facilitating factor. Spirochetes with truncated OspC

were protected against innate elimination, but they failed to disseminate to remote tissues in the host (Seemanapalli et al. 2010). To conclude, all the above-mentioned interactions are likely to be important in *Borrelia* pathogenicity, particularly in the context of adhesion, bacterial colonization, organ tropism and persistence of the infection.

In response to host immunity activation the spirochetes change or turn down the expression of their surface antigens and inhibit certain defensive mechanisms. OspC is a strong immunogen and its production ultimately induces a robust humoral response that imposes enormous stress on the pathogen. To evade adaptive immunity, *B. burgdorferi* downregulates OspC and the development of anti-OspC antibodies cannot be protective (Liang et al. 2002). Recently, a *Borrelia* protein binding to the complement inhibitor C4b of the classical complement pathway was described as a molecular mechanism that allows chronic infection even in the presence of specific antibodies (Pietikainen et al. 2010). Most notably, OspC downregulation occurs concurrently with the upregulation of VlsE (variable major protein-like sequence) encoded by the lp28-1 plasmid. This locus undergoes high frequency of recombination during the first 30 days of infection suggesting an antigen replacement in the surface of the spirochetes that results in high antigen variability upon host colonization (Liang et al. 2004).

1.2 Ticks as disease vectors

Ticks are bloodsucking mites and the second most common vector of human pathogens after the mosquitoes. Based on taxonomy, they are classified in the phylum Arthropoda, class Arachnida, subclass Acarina, order Parasitiformes and suborder Ixodida. They are obligate ectoparasites of terrestrial amphibians, reptiles, birds and mammals. Ticks are of high human and veterinary medical importance, since they are responsible for a significant percentage of all vector-borne diseases, with Lyme borreliosis being the most spread tick-borne human infection in the northern hemisphere (Dennis and Hayes 2002).

Suborder Ixodida can be further divided into two major families, the Argasidae (soft ticks) and the Ixodidae (hard ticks) (Black and Piesman 1994). These two families differ in the feeding strategies. More specifically, the soft ticks usually feed on their

hosts for less than an hour, but repeatedly in each of their developmental stages. The most important representative of the argasids is *Ornithodoros moubata*, a tick that is widely distributed in south and east Africa. It is a vector of *Borrelia duttoni*, the pathogen that causes relapsing fever in humans, and of African swine fever virus, the causative agent of a highly lethal hemorrhagic disease of domestic swine (Varma 1956, Plowright et al. 1970).

Concerning tick-borne diseases in humans, the ticks of the *Ixodes* genus are the most prominent vectors. *Ixodes ricinus* complex (*I. ricinus*, *I. scapularis*, *I. pacificus* and *I. persulcatus*) is responsible for the transmission of at least six disease agents (*Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum*, tick-borne encephalitis virus, Powassan virus, *Babesia microti* and *Babesia divergens*). Tularemia and Q fever are also transmitted by *Ixodes* ticks; however, the main vectors for these pathogens are *Dermacentor* and *Amblyomma* ticks, respectively (Gray 2002).

The life cycle of the castor bean tick *I. ricinus* and the deer tick *I. scapularis* consists of three developmental stages – a larval, a nymphal and an adult stage. During each developmental stage the ticks suck blood only once and for a prolonged period of time, varying from a few days to over one week. After the tick mouthparts penetrate the host epidermis, the disruption of blood capillaries creates a feeding pool of blood. The proteinaceous content of the ingested blood is concentrated in the tick midgut and the mostly aqueous leftover returns into the feeding pool as a component of tick saliva. The fully engorged tick digests the blood and molts to the next stage or lays eggs (in the case of adult females) in the soil detritus. The whole life cycle lasts two or three years in favorable conditions (Gray 2002).

I. scapularis can transmit Lyme disease spirochetes as late as after 36 – 48 hours of feeding, which is exactly the time that the spirochetes need to migrate from the midgut to the salivary glands (de Silva and Fikrig 1995). In contrast, it is hypothesized that *Borrelia* transmission via *I. ricinus* can take place earlier (Crippa et al. 2002).

1.2.1 The tick – host interface

The hard tick feeding period sometimes exceeds ten days and makes tick infestation a considerable intervention to the host physiology. A broad range

of inflammatory and immune reactions take place locally at the feeding site and also systemically within the host body. Active modulation of the host immune response by tick saliva is thus required for the tick to complete its blood meal. Upon attachment, the ticks inoculate their saliva containing a repertoire of bioactive molecules that help them to attach to the host, to overcome host hemostasis, to prevent host pain and itching and the concomitant host scratching. More importantly, tick saliva in addition facilitates the transmission of tick-borne pathogens (see section 1.2.2). Accordingly, tick saliva contains antihemostatic, antiinflammatory and immunomodulatory molecules (Ribeiro 1995, Ribeiro and Francischetti 2003).

Antihemostatic components of tick saliva

Immediately after the damage of the vascular endothelium by the penetrating tick mouthparts, a tick has to face a range of host physiological responses such as platelet aggregation, blood coagulation, angiogenesis and wound healing.

Platelets play a critical role in mammalian hemostasis, with adenosine diphosphate (ADP) and collagen to be the primary activators of platelet aggregation. Crude tick saliva or salivary gland extracts (SGE) were shown to inhibit platelet aggregation in vitro. Inhibitors targeting platelets have been found in saliva of both soft and hard ticks; among others the enzyme apyrase that hydrolyzes ADP, prostacyclin and disintegrins that prevent binding of fibrinogen to platelets (Ribeiro et al. 1988, 1991, Wang et al. 1996, Karczewski et al. 1994). Of note, the pharmacologic mediators released from the activated platelets contribute to local inflammatory processes and are hence relevant in tick – host interactions (Weyrich and Zimmerman 2004).

Blood coagulation is a very complex process regulated at several levels. The saliva from most tick species studied so far displayed inhibitory activity against factor Xa of the coagulation cascade and/or thrombin. Ixolaris, a salivary protein from *I. scapularis* was shown to inhibit the coagulation factor VIIa/tissue factor-induced activation of the factor X. The protein has two Kunitz domains; however, the interaction with factor Xa is mediated by heparin-binding exosite (Francischetti et al. 2002, Monteiro et al. 2005). Moreover, the administration of Ixolaris in rats caused a dose-dependent reduction in thrombus formation (Nazareth et al. 2006). Salp9 and Salp14 represent another novel family of tick immunogenic proteins that play important roles

in tick anticoagulation (Narasimhan et al. 2002). The multifunctional protein Iris, previously described as an immunomodulator secreted in the saliva, also interferes with hemostasis, namely with platelet adhesion, with the contact phase-activated pathway of coagulation, and increases fibrinolysis times (Prevot et al. 2006).

Some members of the host coagulation cascade are considered to act in the interface between blood coagulation and inflammation; protease-activated receptors (PAR) on various mammalian cell types are activated by the serine proteases thrombin, factor VIIa or Xa. PAR activation in turn is associated with the production of proinflammatory cytokines, the expression of adhesion molecules and the activation of inflammatory cells, revealing that serine proteinases can be key modulators of several physiological procedures in the host. Thus novel compounds that can modulate PAR function may be potent candidates for drugs for the treatment of inflammatory or immune diseases (Coughlin 2005, Steinhoff et al. 2005).

The contact phase of the coagulation cascade initiates with the activation of the kallikrein-kinin system that leads to the production of active bradykinin. Bradykinin is a strong agonist of pain, also involved in vascular permeability and inflammation. Carboxypeptidase activity has been described in *I. scapularis* saliva that selectively inhibits plasma bradykinin (Ribeiro and Mather 1998). Recently, a contact phase inhibitor was identified in the *I. ricinus* saliva. It can inhibit the intrinsic coagulation pathway by binding to factor XII, prekallikrein and factor XI (Decrem et al. 2009). A similar kallikrein-kinin system inhibitor is present in *Haemaphysalis longicornis* saliva (Kato et al. 2005).

Concerning fibrinolysis, *I. scapularis* saliva has an abundant metalloprotease activity towards gelatinase, fibrinogen and fibrin clot formation. These metalloproteases may enhance the penetration of *B. burgdorferi* across extracellular matrix components and modulate its transmission (Francischetti et al. 2003).

Targeting wound healing and angiogenesis appears to be another strategy that ixodid ticks adopted for the suppression of inflammation and the successful completion of their blood meal. It has been shown that tick saliva inhibits the proliferation of microvascular endothelial cells and thus contains negative modulators of angiogenesis. Moreover, ixodid tick salivary gland products target host wound healing growth factors (Francischetti et al. 2005, Hajnická et al. 2011).

Immunomodulatory properties of tick saliva

Ticks have to deal with innate immunity during primary infestation and with both innate and adaptive immunity in subsequent infestations. Several animal species including guinea pigs, rabbits and bovines have been shown to develop resistance to tick feeding after a single or multiple tick infestation (Wikel 1996). The resistance is considered to be driven by an immune reaction from the host and it is characterized by early rejection of the ticks, followed by reduced numbers and weights of engorged ticks and impaired oviposition or metamorphosis to the next developmental stage. Acquired immune resistance of the host to ticks can also significantly contribute to the reduced transmission of pathogens from infected ticks (see below).

General strategy used by the ticks to evade host immune response is to sabotage the cellular communication necessary for immunity activation by neutralizing chemokines that normally recruit cells in the sites of exogenous antigens. Indeed, highly selective chemokine-binding proteins with anti-inflammatory features, termed evasins, were identified in *Rhipicephalus sanguineus* ticks (Déruaz et al. 2008).

Neutrophils (polymorphonuclear cells, PMN) are the most abundant cells in the acute inflammatory infiltrate induced upon primary tick infestation but not in subsequent infestations (Gill and Walker 1985). The saliva of ixodid ticks was previously found to inhibit neutrophil adhesion, granule release and to decrease the efficiency of PMN in uptake and killing of *Borrelia burgdorferi* (Ribeiro et al. 1990, Montgomery et al. 2004). Two *I. scapularis* salivary proteins ISL929 and ISL1373 have been identified to downregulate PMN ß2-integrins and inhibit the production of superoxide radicals by neutrophils in vitro. Mice immunized with these proteins had increased numbers of PMN at the site of tick attachment and a lower spirochete burden in the skin and joints 21 days after infection compared to control-immunized animals (Guo et al. 2009). More recently, a novel inhibitory serpin IRS-2 from the *I. ricinus* saliva inhibited neutrophil influx to the inflamed tissue in a mouse model of acute inflammation (Chmelař et al. 2011). Similar effect on the neutrophil migration has been addressed also to sialostatin L, an *I. scapularis* salivary cystatin (Kotsyfakis et al. 2006). Since neutrophils form the first line of immune defense against *Borrelia* spirochetes, the failure of sufficient neutrophil recruitment and activation during the initial

inflammatory response may allow spirochetes to effectively colonize the mammalian host (Xu et al. 2007).

Basophils have long been documented as the predominant cell type that infiltrates the tick-bite site in the skin and plays a key role in tick rejection (Askenase 1977). The migration of these cells upon tick feeding and the resulting basophil-rich responses are known as cutaneous basophil hypersensitivity (CBH). Mast cells, which are particularly concentrated beneath the epithelial surface of the skin and mucosal layers, have also been connected with the development of tick resistance. Mast cell-deficient mice failed to develop resistance and host anti-tick response was normalized after adoptive transfer of mast cells (Matsuda et al. 1985, 1987, Wada et al. 2010). The degranulation of basophils and mast cells and the local release of mediators including histamine and serotonin are considered to represent an important part of the protective immune response against ticks (Brown and Askenase 1983). Importantly, basophils but not mast cells are responsible for the antibody/IgFc-mediated acquisition of tick resistance, even though both types of cells are essential for the manifestation of resistance to *Haemaphysalis longicornis* feeding (Wada et al. 2010).

Histamine and other mediators released from mast cell granula cause pruritus and itching that can cause mechanical stress or even destruction of the tick. To prevent host scratching behavior, histamine-binding lipocalins are present in the saliva of several soft and hard tick species (Paesen et al. 1999, Mans et al. 2008). On the other hand, histamine-releasing factors modulate vascular permeability and increase blood flow to the feeding site, thus facilitating tick engorgement (Dai et al. 2010).

Similar to mast cells and basophils, eosinophilic granulocytes are also associated with resistance to ticks, since they were detected around the tick feeding site in guinea pigs after repeated infestation (McLaren et al. 1983).

The alternative pathway of complement is an important defense against pathogens and a key player in tick rejection reactions of the host. Several complement inhibitors have been described to be tick saliva constituents, among others Isac (*I. scapularis* anticomplement) and its *I. ricinus* homolog Salp20 (Valenzuela et al. 2000, Tyson et al. 2007), in addition to two paralogous proteins IRAC I and II that

protect *I. ricinus* from the complement-mediated immune response of a broad spectrum of vertebrate hosts (Schroeder et al. 2007).

Macrophages are important resident cells producing cytokines and chemokines that attract inflammatory cells to the tick-bite site. Activated macrophages are capable to engulf tick-borne *Borrelia* spirochetes by coiling phagocytosis and kill them by oxygen- and nitrogen-dependent mechanisms (Rittig et al. 1992, Modolell et al. 1994). Tick saliva has evolved mechanisms for regulating macrophage functions. Salivary gland extract (SGE) from *I. ricinus* impaired the production of superoxide and nitric oxide, two major defense molecules of macrophages (Kuthejlová et al. 2001). SGE reduced the proportion of active phagocytes and the number of engulfed bacteria. Saliva treatment of macrophages downregulated the production of proinflammatory cytokines (Kýčková and Kopecký 2006), maybe due to the presence of the serpin Iris that binds to monocytes/macrophages and inhibits their ability to secrete TNF-α. Notably, this activity is independent on the antiprotease activity of this protein, but it is driven by exosites (Prevot et al. 2009). *Rhipicephalus microplus* salivary gland molecules induced differential expression of the co-stimulatory molecule CD86 in murine macrophages which may serve to promote Th2 polarization of the immune response (Brake et al. 2010).

Dendritic cells (DCs) are the most efficient antigen-presenting cells and key drivers of T cell activation, thus connecting innate and adaptive immunity. Dendritic cells treated with ixodid saliva promote naïve $CD4^+$ T cells to Th2-type responses and the presence of saliva impairs their maturation, migration and overall function (Mejri and Brossard 2007, Skallová et al. 2008, Vesely et al. 2009). Non-protein molecules present in saliva, such as prostaglandin E2 (PGE_2) and adenosine (Ado) were reported as accounting for these effects (Sá-Nunes et al. 2007, Oliveira et al. 2011). Adenosine inhibits the production of the proinflammatory cytokines IL-12p40 and TNF-α and stimulates the production of the antiinflammatory cytokine IL-10 by murine DCs activated with Toll-like receptor (TLR) agonists. Additionally, PGE_2 suppresses the differentiation of DCs from blood cell precursors. Both Ado and PGE_2 exert their immunomodulatory effects on cytokine production by inducing a common cAMP-PKA signaling pathway. Furthermore, both Ado and PGE2 were able to inhibit expression of CD40 in mature DCs (Oliveira et al. 2011). Tick saliva also induces the regulatory DCs to secrete IL-10 and low levels of IL-12 and TNF-α when stimulated by TLR

ligands. Such regulatory DCs are associated with the expression of TLR-2 and the inhibition of ERK and p38 MAP kinases, which promotes the production of IL-10 and thus modulates the host immune response (Oliveira et al. 2010).

I. scapularis salivary protein Salp15 interacts with the DC-SIGN lectin on DCs, activating the Raf-1/MEK pathway that leads to impaired IL-6 and TNF-α mRNA stability and nucleosome remodeling at the IL-12p35 promoter (Hovius et al. 2008). Sialostatin L, an *I. scapularis* cystatin, inhibits DCs maturation by down-regulating the co-stimulatory molecules CD80 and CD86 and the production of IL-12p70, and to lesser extent also TNF-α, by DCs. Furthermore, it binds to cathepsin S inside the DCs and affects MHC class II invariant chain (Ii) processing. The resulting impaired DC maturation and Ii degradation correlates with the inhibition of antigen specific $CD4^+$ T cell proliferation by a cathepsin S-dependent mechanism (Sá-Nunes et al. 2009).

Argasid salivary proteins, such as the cystatin OmC2 from the soft tick *Ornithodoros moubata*, can also suppress the host adaptive immune response by reducing TNF-α and IL-12 production and the proliferation of antigen-specific $CD4^+$ T cells (Salát et al. 2010; **Chapter 3.2**).

Concerning natural killer (NK) cells, tick salivary proteins affect their ability to conjugate with their physiological cell targets and as a result they impair cell cytotoxicity (Kopecký and Kuthejlová 1998, Kubeš et al. 2002).

Immunomodulatory activities of tick saliva can be further demonstrated on the subsequent steps of immune activation such as the T and B cell mediated response. SGE treatment decreases γδ T lymphocyte load in infected skin (Severinová et al. 2005). Repeated infestations of pathogen-free ticks or saliva itself selectively promote an antiinflammatory Th2 cytokine profile; IL-4, IL-10 and transforming growth factor beta (TGF-β) secretion is upregulated, with a corresponding suppression of Th1 and Th17 responses (downregulation of IFN-γ, IL-2, IL-12 and IL-17) in ex vivo murine and human cell cultures (Ferreira and Silva 1999, Schoeler et al. 1999, Kovář et al. 2001, Skallová et al. 2008). The polarization is partially caused by the impairment of dendritic cell function; however, direct influence of tick salivary constituents on T cells also plays its role.

Salp15 binds directly to the CD4 receptor on the surface of T cells. It inhibits early T-cell activation via CD3/CD28 and MHC/CD4 by blocking T cell receptor

(TCR)-mediated Lck activation and thus improper downstream signaling, which results in reduced production of IL-2 (Anguita et al. 2002, Garg et al. 2006). A novel sphingomyelinase-like enzyme in *I. scapularis* saliva (IsSMase) directly programs host $CD4^+$ T cells to express IL-4, the hallmark Th2 cytokine, even if the cells were primed towards Th1 response by viral infection. Also TCR transgenic $CD4^+$ T cell proliferation was significantly increased by IsSMase (Alarcon-Chaidez et al. 2009). Humoral immunity can be also modulated by an 18 kDa B-cell inhibitory protein found in *I. ricinus* saliva (Hannier et al. 2004) or by immunoglobulin G binding proteins identified in the saliva of *R. appendiculatus* (Wang and Nuttall 1995).

1.2.2 Saliva-activated transmission of pathogens

Survival and successful proliferation of tick-borne pathogens is also dependent on their ability to exploit the pharmacological activities of tick salivary molecules. The facilitation of pathogen transmission and multiplication because of the presence of tick saliva in the transmission interface is termed saliva-activated transmission (SAT) (Nuttall and Jones 1991). The SAT phenomenon has been documented for several tick-borne pathogens, including Thogoto virus, tick-borne encephalitis virus, *Francisella tularensis* and several *Borrelia* species (Jones et al. 1987, Labuda et al. 1993, Kročová et al. 2003, Gern and Rais 1996, Sato and Nakao 1997, Zeidner et al. 2002, Pechová et al. 2002, Macháčková et al. 2006). Transmission of other vector-borne organisms, such as *Leishmania* spp. can be also facilitated by its *Phlebotomus* vector salivary secretion (Titus and Ribeiro 1988).

As already mentioned above, *Ixodes ricinus* salivary components inhibit killing of spirochetes by murine macrophages in vitro and negatively influence their phagocytosis (Kuthejlová et al. 2001, Kýčková and Kopecký 2006). Moreover, co-inoculation of *B. burgdorferi* in combination with SGE into naïve mice allows more efficient survival and higher infectivity of the spirochetes as demonstrated by increased loads of spirochetes in various tissues when compared to the injection of spirochetes without SGE (Zeidner et al. 2002, Macháčková et al. 2006). Salivary glands-derived borreliae show ten times higher infectivity than those derived from the gut, indicating an important role for arthropod salivary proteins in the transmission cycle of Lyme disease (Lima et al. 2005). Finally, we showed the changes in proliferation and distribution of the spirochetes in mice upon saliva treatment, focusing on target

tissues (skin, lymph nodes, heart and urinary bladder) at early time points after needle inoculation and comparing three different sources of salivary proteins (SGE, saliva and co-feeding of pathogen-free nymphs in the inoculation area). Accordingly, we also demonstrated that if spirochetes are co-inoculated with SGE/saliva, the transmission of Lyme disease agents from murine host to *I. ricinus* ticks is enhanced (Horká et al. 2009; **Chapter 3.1**).

In theory, any kind of immunomodulatory molecules present in saliva that facilitate tick feeding is a candidate SAT factor. For example, it is likely that polarization towards Th2 immune response, extensively described in section 1.2.1, facilitates the transmission of pathogens that would otherwise be neutralized by Th1 immunity. Accordingly, the suppression of IL-4 and IL-5 prior to the feeding of *B. burgdorferi*-infected ticks significantly decreased spirochete load in target organs of a Lyme disease-susceptible host (Zeidner et al. 2008).

The first molecularly characterized SAT molecule is Salp15 from *I. scapularis*. Salp15 expression is selectively induced in *Borrelia*-infected salivary glands. Salp15 binds to the spirochetal outer surface protein C (OspC) and thus protects bacteria from antibody-mediated killing and makes their proliferation in target organs easier (Ramamoorthi et al. 2005). Moreover, the presence of Salp15 increases the levels of spirochetes in host skin, joints and bladder. When *salp15* was silenced in ticks by RNA interference, the amounts of spirochetes transmitted to mice were significantly reduced (Ramamoorthi et al. 2005). The Salp15 homolog from *I. ricinus* protects a serum-sensitive isolate of *B. burgdorferi* against complement-mediated killing, since the deposition of membrane attack complex on the spirochetal surface is inhibited by Salp15 (Schuijt et al. 2008). Additionally, Salp15 in vitro inhibits keratinocyte inflammation induced by *Borrelia* via the downregulation of alarmins, essential mediators that mobilize and activate antigen presenting cells (Marchal et al. 2011).

A cysteine protease inhibitor derived from *I. scapularis* salivary glands, named as sialostatin L2, dramatically affects tick feeding success, most likely by influencing the activity of cathepsins involved in host inflammation, tissue remodeling and angiogenesis (Kotsyfakis et al. 2007). Co-administration of *Borrelia* spirochetes with sialostatin L2 exacerbates skin infections in a murine model, as determined by spirochetal load in infected mouse skin 4 days after inoculation. Sialostatin L2 does not influence spirochetal growth in vitro and unlike Salp15, the stimulatory effect seems

not to be due to direct interaction of the protein with the spirochetes (Kotsyfakis et al. 2010; **Chapter 3.3**).

1.2.3 Lyme disease and anti-tick vaccines

Previous efforts to develop a protective and safe vaccine against Lyme disease were based on the classical approach, where immunogenic proteins from *Borrelia* surface (OspA, OspC, DbpA) were used as the vaccination antigens (de Silva et al. 1996, Gilmore et al. 1996, Hanson et al. 1998). Although these vaccines were protective in certain conditions, their general usage is not feasible due to the high heterogeneity of the targeted antigens among different *Borrelia* strains, the insufficient efficacy and the side effects of the vaccines (Wilske et al. 1993, Hagman et al. 2000, Rosé et al. 2001, Alexopoulou et al. 2002).

Due to immune evasion mechanisms, the spirochetal proteins expressed on *Borrelia* surface inside the host are considered as not good vaccine candidates. The efforts are thus turned to the development of a universal anti-tick vaccine. In contrast to acaricides, anti-tick vaccines based on recombinant proteins are environmentally safe, may not be risky for human health and the development of resistance is less likely (Willadsen 2004). However, the identification of suitable antigenic targets seems to be the primary limiting factor of the whole process.

Generally there are two distinct types of vaccination antigens. The so-called 'exposed tick antigens' are salivary secreted proteins or cement components. They come into direct contact with host immune mechanisms; hence immunity against an exposed antigen is enhanced by repeated tick infestations (Opdebeeck 1994). Immunization with the *I. scapularis* serpin Iris, that is secreted as a component of saliva and interacts with both host immunity and hemostasis, established a significant protective immunity against tick infestation of rabbits, resulting in 30% tick mortality, diminution of weight gain and prolongation of blood feeding time (Prevot et al. 2007).

Notably, sialostatin L2, a cystatin with anti-inflammatory properties, has been termed silent antigen. Although the protein is found in the tick – host interface, it is not recognized by humoral immunity after repeated tick infestations, possibly due to its function or the amount of its secretion. However, sialostatin L2 used as a vaccination antigen of guinea pigs impaired tick feeding ability (Kotsyfakis et al. 2008).

In contrast to exposed antigens, the so-called ‘concealed antigens’ are derived from tick tissues, most often from tick gut wall, ovaries or fat body. If a concealed antigen is tightly connected with a physiological function, its reaction with antibodies produced due to the host vaccination for this antigen leads to increased tick poisoning and the surviving ticks have impaired ability to molt and reproduce. However, concealed antigens do not stimulate host immunity during tick feeding and host immunization has to be frequently repeated (Willadsen et al. 1993). Currently, the only anti-tick vaccine commercially available is derived from Bm86, a midgut membrane-bound protein of *Boophilus* (*Rhipicephalus*) *microplus* (Willadsen et al. 1995).

A promising candidate for the development of an efficient anti-tick vaccine is ferritin 2 that is expressed in the gut tissue and is required for iron transport to the ovaries and salivary glands. Immunization of rabbits and cattle with the ferritin 2 protein dramatically impaired the ability of ticks to feed and reproduce, most likely due to a resulting imbalance in iron metabolism (Hajdušek et al. 2010).

Another one of the tested principles for the development of transmission-blocking vaccines is the immunization of the host with an arthropod vector molecule that the microbe exploits during the infection of the mammalian host. Passive as well as active immunization with Salp15 significantly protected mice from *B. burgdorferi* infection due to an increased clearance of Salp15-coated bacteria by phagocytes (Dai et al. 2009).

The development of vaccines against the so-called ‘dual antigens’ is a relatively new strategy followed in the field, targeting both exposed antigens in tick saliva and concealed antigenic epitopes in the tick midgut. This dual action stimulates specific protective immune response which is boosted by natural infestations, and at the same time, the host antibodies ingested with the blood meal react with antigenic epitopes on tick gut cells, followed by midgut rupture (Trimnell et al. 2002). A cement protein 64P derived from *R. appendiculatus* has been proposed as a candidate transmission-blocking vaccine antigen with dual function (Havlíková et al. 2009).

Another proposed step in anti-tick vaccine development could be the combination of different vaccine antigens composed of both *Borrelia* and tick antigens that act synergistically (Schuijt et al. 2011). Using antigen orthologs

for the control of multiple arthropod vectors has been also proposed for the development of an effective vaccine (Canales et al. 2009).

1.3 Cysteine proteases and their inhibitors (cystatins)

Cysteine proteases are wide spread in all living organisms including vertebrates and arthropods. Acidic cysteine proteases (cathepsins) have traditionally been considered as mediators of the proteolysis inside the lysosomes. Lysosomal cysteine proteases thus imply their role in antigen presentation and intracellular protein turnover. However, gene knockouts revealed their specific and important roles for the normal function of an organism.

Cathepsin L is involved in the development of vertebrate immune system, namely, in the expression of extracellular matrix in lymphoid organs and in the regulation of thymic and peripheral T cell numbers (Lombardi et al. 2005). Cathepsin L further plays a role in neovascularization, regulation of apoptosis and epidermal homeostasis by controlling keratinocyte proliferation (Felbor et al. 2000, Wille et al. 2004, Reinheckel et al. 2005). Cathepsin cysteine proteases play also a role in invasive growth and angiogenesis during tumor metastasis (Joyce et al. 2004). Importantly, active cathepsins B, H, K, L and S were found in human inflammatory bronchoalveolar lavage fluids (Serveau-Avesque et al. 2006).

Cystatins are natural tight binding reversible inhibitors of papain-like cysteine proteases. They are further divided into three separate families. The Family 1 members, also known as stefins, are cytosolic proteins, the have a single approximately 11 kDa domain, no disulfide bonds or carbohydrates. Family 2 cystatins are secreted proteins found in most biological fluids; they have a single domain approx. 13 kDa, two disulfide bridges at the C-terminal end and no glycosylation. The prototype family 2 cystatin, the chicken egg white cystatin, was the first cystatin described in 1968 (Fossum and Whitaker 1968). Family 3 cystatins called kininogens are multi-domain proteins with high molecular mass (60-120 kDa) found mostly in plasma.

A cystatin molecule is made up by a wedge-shaped structure composed of an amino-terminal segment and two hairpin loops. The reactive site is formed by a highly conserved sequence between residues 53 and 57 (Glu-x-Val-x-Gly) in a β-hairpin loop of the protein (Bode et al. 1988).

1.3.1 Immunomodulatory properties of cystatins

Human cystatin C, the most explored type 2 cystatin, has been shown to antagonize transforming growth factor beta (TGF-β) signaling in normal and cancer cells by interaction with the TGF-β receptor (Sokol and Schiemann 2004). Cystatin C also binds to the C4 complement factor and has been suggested as a regulator of the phagocytic function of neutrophils (Ghiso et al. 1990, Leung-Tack et al. 1990).

Secreted cystatins have been found in various invertebrate organisms including parasitic worms. These endoparasites release a variety of molecules that were optimized during the adaptation of the worms in their vertebrate hosts. They enable the worms to penetrate the host defense barriers, to bypass the immune response and survive in their host until they are ready to reproduce. Nematode cystatins inhibit proteases involved in antigen processing and presentation, leading to impaired T cell response. For example, the cystatin Bm-CPI-2, produced by the filarial nematode *Brugia malayi* and nippocystatin from *Nippostrongylus brasiliensis* inhibit cathepsins B, L, S and AEP inside the endosomes and lysosomes of antigen presenting B cells, helping these parasites to evade host immunity (Manoury et al. 2001, Dainichi et al. 2001). Nematode cystatins also modulate the host cytokine response; this is mainly demonstrated by the upregulation of the Th2 cytokine IL-10 by macrophages, which further contributes to antiinflammatory environment (Hartmann et al. 1997). Interestingly, cystatins from both parasitic and free-living nematodes differ substantially in their immunomodulatory properties. More specifically, two filarial cystatins from *Onchocerca volvulus* and *Acanthocheilonema viteae* suppressed the proliferation of human peripheral blood mononuclearcells (PBMC) and murine splenocytes and markedly increased the IL-10 production, while the homologous proteins from the free-living nematode *Caenorhabditis elegans* increased the production of IL-12 and IFN-γ (Schönemeyer et al. 2001, Schierack et al. 2003).

1.3.2 Cystatins in ticks

Although ticks and other ectoparasites have a completely different life strategy from the nematodes, cystatins are at least of similar importance for their life cycle. In the case of the lone star tick *Amblyomma americanum* disruption of the expression

of a salivary cystatin by RNA interference reduces the ability of ticks to feed successfully (Karim et al. 2005). *Haemaphysalis longicornis* cystatin Hlcyst-2 has been associated with tick innate immunity (Zhou et al. 2006). Another *H. longicornis* cystatin, named as HlSC-1, is tightly connected with feeding process (Yamaji et al. 2009).

Two secreted cystatins OmC1 and OmC2 have been described in the gut of the soft tick *Ornithodoros moubata* (Grunclová et al. 2006). OmC2 is present also in tick saliva, where it can contribute to the immunomodulation of the host. OmC2 has been shown to decrease TNF-α and IL-12 levels produced by LPS-activated dendritic cells in vitro and to reduce DC-mediated proliferation of $CD4^+$ T cells. Moreover, vaccination with this cystatin reduced argasid feeding success and fitness (Salát et al. 2010; **Chapter 3.2**).

Two cystatins found in the saliva of the black-legged tick *Ixodes scapularis* were extensively characterized so far. They were called sialostatin L and L2 due to their target specificity for cathepsin L (Kotsyfakis et al. 2006, 2007). Although they are most similar to the family 2 cystatins, their specificity profiles differ from their vertebrate homologues. Within the vertebrate members of the papain family, cathepsins L, V, C and X were inhibited by sialostatin L, whereas only a slight inhibition of human cathepsin B was observed. In agreement with an inhibitory activity against papain-like proteases, sialostatin L inhibits the proliferation of a cytotoxic T cell-like cell line. Additionally, sialostatin L applied in vivo reduces the paw edema formation driven by the administration of carrageenan in the mouse footpad and also inhibits neutrophil recruitment in the inflamed footpads (Kotsyfakis et al. 2006). The broad spectrum of sialostatin L effects on dendritic cells is described in the section 1.2.1. Notably, sialostatin L has also a strong therapeutic effect in a mouse model of experimental asthma (Horká et al. 2011, submitted manuscript; **Chapter 3.4**).

The second *I. scapularis* cystatin sialostatin L2 shares 75 % identity to sialostatin L at the protein level. It is equally potent inhibitor of cathepsins L and V, whereas the activities of cathepsins S and C were affected by the presence of sialostatin L2 to a lesser extent when compared to sialostatin L. Interestingly, the expression of sialostatin L2 in tick salivary glands is strongly upregulated after 4 days of tick feeding, suggesting its role for tick feeding success. Indeed, silencing of sialostatin L2 by RNA interference leads to tick feeding inhibition, reduced tick size and oviposition,

which can be attributed to an enhanced immune reaction from the vertebrate host (Kotsyfakis et al. 2007). Co-injection of this protein with *Borrelia* upon needle inoculation of mice assisted the pathogen establishment in the skin of the mice (Kotsyfakis et al. 2010; **Chapter 3.3**).

1.4 Tick-derived molecules in the treatment of human diseases

Several tick-derived molecules with immunomodulatory properties have been successfully tested against human disorders, mainly in animal models of human diseases. A histamine-binding protein from *Rhipicephalus appendiculatus* was tested as a treatment of allergic conjunctivitis in mice (Chapin et al. 2002). A complement C5 inhibitor from the soft tick *O. moubata* was successful in preventing experimental autoimmune myasthenia gravis (Soltys et al. 2009) and sialostatin L from the hard tick *I. scapularis* significantly prevented disease symptoms in a mouse model of multiple sclerosis, i.e. experimental autoimmune encephalomyelitis (Sá-Nunes et al. 2009). Two tick-derived proteins were successfully used in the prevention of experimental allergic asthma. The first one was a histamine-binding protein from *R. appendiculatus* (Couillin et al. 2004); the second one was Salp15, the immunomodulatory protein from *I. scapularis* (Paveglio et al. 2007). Both proteins reduced airway hyperresponsiveness, pulmonary eosinophilia and mucus hypersecretion.

1.5 Allergic asthma

Asthma is a chronic disease of the airways in which various exogenous and endogenous stimuli, most likely inhaled allergens, induce a chronic inflammation of the conducting airways. This leads to airway hyperresponsiveness (AHR), accompanied by enhanced irritability of the airways and increased mucus secretion. The disease is heterogeneous as far as it concerns immunopathology, clinical phenotypes, response to therapies and natural history.

Airway inflammation in asthma is a multicellular process involving mainly eosinophils, neutrophils, $CD4^+$ T lymphocytes and mast cells, with eosinophilic infiltration being the most striking feature (Kay 2005). The inflammatory response in the large airways appears to be predominantly in the submucosa, whereas in the case

of small airways, inflammation outside the smooth muscles dominates (Haley et al. 1998).

Allergic asthma has been initially characterized by T-helper type 2 inflammatory responses and the secretion of the Th2 cytokines IL-4, IL-5 and IL-13, together with IgE production, infiltration of eosinophils and mast cells and ultimately chronic inflammation (Fig. 1). Nowadays it seems valid that chronic allergic disease may not be solely a Th2-driven process. Indeed, human and animal studies demonstrate that IL-9 produced mainly by a newly designated Th9 cell subpopulation is playing an important role in asthma pathology. During the severe asthma and exacerbation following lung infections, Th1 cells are recruited to secrete TNF-α and IFN-γ (Truyen et al. 2006).

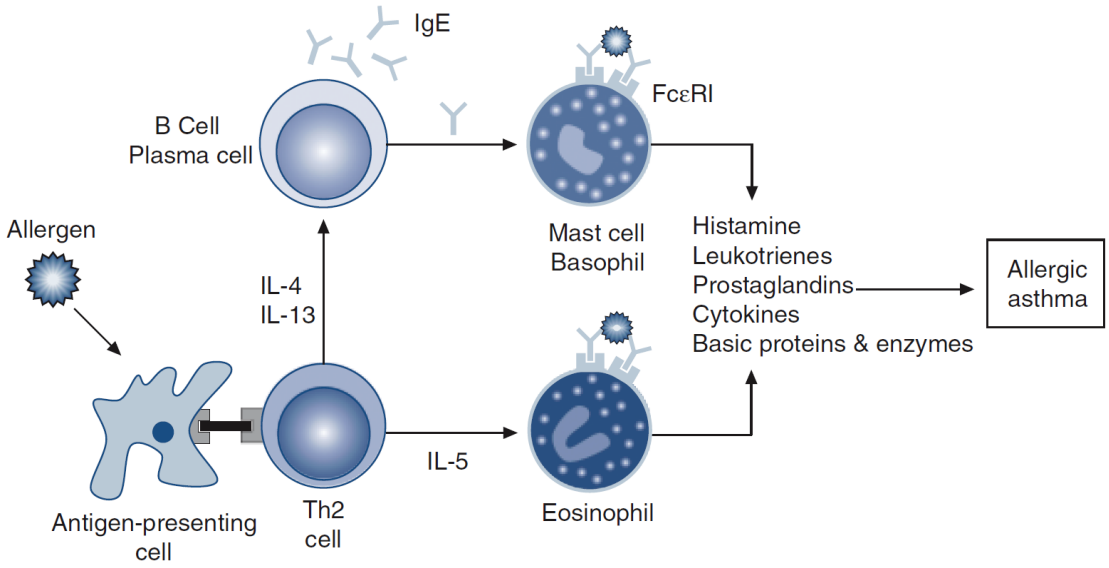

Figure 1. Schematic representation of the inflammatory cascade in Th2 allergic asthma (Holgate 2008).

1.5.1 Treatment of asthma

In asthma, airway inflammation is usually adequately minimized with standard-of-care treatments, such as high-dose inhaled corticosteroids, leukotriene modifiers, long-acting bronchodilators or IgE-binding antibodies. However, it may not be sufficient to provide asthma control in all patients, since asthma is a heterogeneous disorder and the development of other antiinflammatory approaches is required (Wenzel 2006).

Anti-cytokine therapy

In the case of an eosinophilic asthma phenotype, a therapeutic scheme that blocks the action of IL-5 could be indicated. However, such a therapy can only reduce the 'end product' of Th2-driven inflammation. Thus, it makes questionable any benefit to mild asthma cases, unless the administered corticosteroids are not able to control eosinophilia (Antoniu 2009).

Dual anti-IL-4/anti-IL-13 therapy targeting the shared receptor complex IL-4Rα/ IL-13Rα seems to be the most adequate approach to get sustained reduction in airway inflammation, assuming that the preclinical data of this scheme will be further confirmed (Tomkinson et al. 2001).

A neutrophilic phenotype is most prevalent in tobacco smokers and it is often refractory to corticosteroids (Chaudhuri et al. 2003). It is connected with more aggressive disease symptoms and increased tissue destruction and airway remodeling, most likely due to increased TNF-α activity (Holgate and Polosa 2006). However, anti-TNF-α therapeutic trials had to be ceased due to the increased risks of serious infections including tuberculosis and the development of malignancy (Lin et al. 2008).

Besides anti-cytokine therapy, the fusion protein abatacept was used as an immunomodulatory agent in an animal model of allergen induced airway inflammation. This cytotoxic T lymphocyte antigen 4 (CTLA4)-immunoglobulin fusion protein prevents CD80/CD86 co-stimulation of T cells (Wan et al. 2003).

Protease inhibitors

It is generally accepted that an excess of secreted proteases resulting from the accumulation of leukocytes in the airways and lungs contributes to the abnormal function of airways. Thus, targeting these proteases with their inhibitors seems to open a relatively new field in the treatment of allergic asthma. For example, a dual low-molecular weight inhibitor of cathepsin G and chymase was successfully tested in antigen-induced allergic asthma of sheep (Maryanoff et al. 2010).

The cystatin AV17 from the filarial nematodes *A. viteae* has been shown to reduce allergic and inflammatory asthma responses in mice. The cystatin suppressed

the Th2-related inflammation and the ensuing asthmatic disease in a murine model of chicken egg albumin (OVA)-induced allergic airway responsiveness, in terms of eosinophil recruitment, IgE levels, IL-4 production and suppressed AHR. Macrophages producing IL-10 were found responsible for these effects (Schnöller et al. 2008).

1.5.2 Role of IL-9 in allergic asthma

The pleiotropic cytokine interleukin 9, originally termed as p40, T cell growth factor III (TCGF III) or mast cell growth enhancing activity (MEA), was described independently by three different research groups (Uyttenhove et al. 1988, Schmitt et al. 1989, Hültner et al. 1989). Naïve $CD4^+$ T cells and Th2 cells were initially shown to be the sources of this cytokine. Subsequently, IL-9 was found to be produced also by mast cells and eosinophils (Hültner et al. 2000, Soussi-Gounni et al. 2000). Secretion of IL-9 in murine $CD4^+$ T cells depends on IL-2, and it is synergistically enhanced by the combination of TGF-β and IL-4 while it is inhibited by IFN-γ (Schmitt et al. 1994). In mast cells, the expression of IL-9 is driven by the p38 MAP kinase and the transcription factor GATA-1, whereas the transcriptional regulation by $CD4^+$ T cells highly relies on the transcription factor IRF-4 (interferon regulatory factor 4) (Stassen et al. 2007, Staudt et al. 2010).

On the basis of these findings a new IL-9 producing T-helper subpopulation, designated Th9, was defined very recently. It can develop from naïve $CD4^+$ T cells under the influence of IL-4 and TGF-β (Dardalhon et al. 2008). In parallel, it was demonstrated that Th9 cells can be developed by "reprogramming" Th2 cells in the presence of TGF-β (Veldhoen et al. 2008). Interestingly, IL-9 in combination with TGF-β promotes the development of Th17 T cells that have also the ability to produce IL-9 (Elyaman et al. 2009).

Functionally, IL-9 is involved in the host protective immunity to the parasitic worms *Trichuris muris* and *Trichinella spiralis*, by contributing to the intestinal muscle function (Khan et al. 2003). More importantly, IL-9 plays a detrimental role in asthma pathogenesis (Temann et al. 1998). In humans, IL-9 gene resides within the same cytokine gene cluster together with IL-3, IL-4, IL-5, IL-13, CD14 and GM-CSF genes on the chromosome 5 (van Leeuwen et al. 1989). Interleukin 9 was proposed as

a candidate gene for allergic hypersensitivity since linkage analysis showed significant association with elevated total IgE levels (Doull et al. 1996).

IL-9, originally described as a mast cell growth factor, primes mast cells to respond to an allergen via an increased expression of the high-affinity IgE receptor and the production of inflammatory mediators including IL-6 and several proteases (Louahed et al. 1995). IL-9 also enhances the IL-4 mediated production of IgE in human and murine B cells, promotes eosinophil maturation in synergy with IL-5 and stimulates mucin transcription in respiratory epithelial cells (Dugas et al. 1993, Louahed et al. 2001, Longphre et al. 1999).

Although initial studies evaluated IL-4, IL-5 and IL-13 levels in bronchoalveolar lavages (BAL) from asthmatic patients, IL-9 levels have also been increased after allergen challenge in asthmatics, compared to normal controls (Erpenbeck et al. 2003a). Additionally, bronchial biopsies from asthmatic patients have shown an elevated number of IL-9 mRNA-positive cells in the airways. IL-9 mRNA can also be localized to lymphocytes in the BALs (Shimbara et al. 2000, Erpenbeck et al. 2003b). These studies have revealed that IL-9 expression is increased during asthmatic inflammation; however, the precise elucidation of its role in asthma pathogenesis relies on mouse models.

IL-9-overexpressing transgenic mice were found to have increased AHR, lung eosinophilia and total IgE levels in their sera after allergen challenges (McLane et al. 1998). Also lung-restricted overexpression of IL-9 led to symptoms of asthmatic airways even in the absence of an allergen. These mice also had typical features of airway tissue remodeling associated with chronic asthma, in terms of mucus production and subepithelial fibrosis (Temann et al. 1998). Using a conventional acute asthma model that employs mice sensitization to OVA, Cheng et al. showed a significant decrease in AHR and BAL eosinophilia after administration of an IL-9 blocking antibody (Cheng et al. 2002). Furthermore, a humanized anti-IL-9 antibody was even tested in phase I clinical trials in healthy adult volunteers (White et al. 2009). Finally, in a Th9-dependent asthma model, neutralization of IL-9 substantially ameliorated asthma symptoms (Staudt et al. 2010).

We have demonstrated that *I. scapularis* salivary cystatin sialostatin L strongly inhibits the production of IL-9 by Th9 cells through an IL-2 independent mechanism,

and even in the case that the IL-9 production is promoted by IL-1. Most interestingly, intravenous application of this tick protein in a murine model of asthma almost completely abrogated AHR and eosinophilia, suggesting that sialostatin L can prevent this airway disease in an animal model, most likely by inhibiting the IL-9 production by Th9 cells (Horká et al. 2011, submitted manuscript; **Chapter 3.4**).

2 Goals of the project

Ticks are bloodsucking mites of high importance in human and veterinary medicine, as well as in agriculture. Feeding of high tick numbers on the same animal and the subsequent blood loss causes significant economic problem in cattle breeding. Tick-borne pathogens including viruses, bacteria and protozoan parasites then represent a 'gratis service' of a tick feeding which is often even much more serious than the economic impact. There is a lack of efficient anti-tick vaccines and any novel information about the tripartite interaction of ticks with hosts and tick-borne pathogens can be essential for the development of novel methods to prevent the harmful consequences of tick feeding.

This Ph.D. thesis is a part of a big project that aims to understand and characterize the events within the triangle of interactions between the ticks, their vertebrate hosts and the tick-borne pathogens. In the first paper (**Chapter 3.1**) we focused on the proliferation and distribution of *Borrelia burgdorferi* in a mouse host in the presence of tick saliva and also in the effects that saliva has in the transmission cycle from the mice to ticks.

Further on, we aimed to characterize individual salivary molecules that could be crucial in the tick – host – pathogen interface. We focused on the cysteine protease inhibitors (cystatins) derived from the tick saliva, namely OmC2 from the soft tick *Ornithodoros moubata* (**Chapter 3.2**) and sialostatins L and L2 (**Chapter 3.3**) from the hard tick *Ixodes scapularis*. Apart from the crystal structures of these proteins, we intended to describe their biochemical, physiological and immunomodulatory features and their roles in *Borrelia* transmission.

Since new drugs for the treatment of human diseases are in demand, another goal of the project was to exploit the immunomodulatory properties of tick salivary molecules for the treatment of various immunity-related diseases. Towards this direction, as described in **Chapter 3.4**, sialostatin L was tested for the therapy of an asthmatic airway disease in mice.

3 Experimental section

3.1 Tick saliva affects both proliferation and distribution of *Borrelia burgdorferi* spirochetes in mouse organs and increases transmission of spirochetes to ticks

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International Journal of Medical Microbiology (2009) 299: 373-380.

Contribution of H.H.: 60 %

Abstract

Ixodes ricinus tick saliva-activated transmission of *Borrelia burgdorferi* sensu stricto spirochetes was studied on the C3H/HeN mouse model. The influence of the feeding of uninfected nymphs on the proliferation and distribution of intradermally inoculated spirochetes was compared with the effect of co-inoculated saliva or salivary gland extract (SGE), respectively. Spirochete loads in murine tissues were evaluated using real-time q-PCR. SGE induced significantly increased spirochete numbers in the skin on the days 4 and 6 post-infection (p.i.). On the other hand, decreased bacterial load in the heart of SGE-treated mice was demonstrated in comparison with control animals. The inoculation of tick saliva increased spirochete load in the urinary bladder on day 6 p.i., while the number of spirochetes in the heart declined on day 6 p.i. The feeding of *I. ricinus* nymphs raised the spirochete load in the bladder on the days 4 and 6 p.i. On day 6, the number of spirochetes found in the heart was significantly lower than in controls. The prevalence of spirochetes in ticks infected by feeding on mice was more than 10 times higher when the mice were infected with the mixture of spirochetes and saliva or SGE, in comparison with spirochetes alone. The presence of SGE in the infectious inoculum increased the spirochete burden per tick from 0 to almost 28,000. Taken together, these results show a very early effect of tick saliva on the proliferation and distribution of *Borrelia*

spirochetes in the host, probably due to the effect of saliva on the host innate immunity mechanisms.

Na myším modelu byl studován přenos spirochet *Borrelia burgdorferi* sensu stricto aktivovaný slinami klíštěte *Ixodes ricinus*, kdy vliv aplikace extraktu ze slinných žláz (SGE) na proliferaci a distribuci borelií v C3H/HeN myších byl srovnán s aplikací slin, resp. sáním neinfekčních nymf. Množství spirochet v myších tkáních bylo stanoveno pomocí kvantitativní PCR v reálném čase. Aplikace SGE významně zvýšila množství spirochet v kůži 4 a 6 dní po infekci (p.i.). Naopak v srdci došlo ke snížení počtu spirocet pod vlivem SGE. Inokulace slin zvýšila množství borelií v močovém měchýři 6 dní p.i., zatímco v srdci tomu bylo naopak. Sání neinfekčních nymf *I. ricinus* zvýšilo množství spirochet v močovém měchýři čtvrtý a šestý den po infekci. V srdci bylo šestý den opět signifikantně méně borelií než v kontrolní skupině. Prevalence borelií v klíšťatech infikovaných sáním na myších byla více než desetkrát vyšší, pokud myši byly nakaženy směsí borelií a slin nebo SGE, v porovnání se samotnými boreliemi. Přítomnost SGE v infekčním inokulu zvýšila obsah bakterií v jednom klíštěti z nuly na téměř 28 000. Celkově vzato, tyto výsledky ukazují velmi časný efekt klíštěcích slin na proliferaci a distribuci *B. burgdroferi* v hostiteli, a to zřejmě díky vlivu slin na mechanismy přirozené imunity hostitele.

3.2 Crystal structure and functional characterization of an immunomodulatory salivary cystatin from the soft tick *Ornithodoros moubata*

Jiří Salát, Guido C. Paesen, Pavlína Řezáčová, Michalis Kotsyfakis, Zuzana Kovářová, Miloslav Šanda, Juraj Majtán, Lenka Grunclová, **Helena Horká**, John F. Andersen, Jiří Brynda, Martin Horn, Miles A. Nunn, Petr Kopáček, Jan Kopecký and Michael Mareš

Biochemical Journal (2010) 429: 103-112.

Contribution of H.H.: 5 %

Abstract

The saliva of blood-feeding parasites is a rich source of peptidase inhibitors that help to overcome the host's defence during host–parasite interactions. Using proteomic analysis, the cystatin OmC2 was demonstrated in the saliva of the soft tick *Ornithodoros moubata*, an important disease vector transmitting African swine fever virus and the spirochaete *Borrelia duttoni*. A structural, biochemical and biological characterization of this peptidase inhibitor was undertaken in the present study. Recombinant OmC2 was screened against a panel of physiologically relevant peptidases and was found to be an effective broad-specificity inhibitor of cysteine cathepsins, including endopeptidases (cathepsins L and S) and exopeptidases (cathepsins B, C and H). The crystal structure of OmC2 was determined at a resolution of 2.45 Å (1 Å=0.1 nm) and was used to describe the structure–inhibitory activity relationship. The biological impact of OmC2 was demonstrated both in vitro and in vivo. OmC2 affected the function of antigen-presenting mouse dendritic cells by reducing the production of the pro-inflammatory cytokines tumour necrosis factor α and interleukin-12, and proliferation of antigen-specific $CD4^+$ T-cells. This suggests that OmC2 may suppress the host's adaptive immune response. Immunization of mice with OmC2 significantly suppressed the survival of *O. moubata* in infestation experiments. We conclude that OmC2 is a promising target for the development

of a novel anti-tick vaccine to control *O. moubata* populations and combat the spread of associated diseases.

Sliny krevsajících parazitů jsou bohatým zdrojem inhibitorů peptidáz, které pomáhají překonat obranu hostitele během interakcí parazita s hostitelem. Pomocí proteomické analýzy byl cystatin OmC2 detekován ve slinách měkkého klíštěte *Ornithtodoros moubata*, důležitého přenašeče viru Africké prasečí horečky a spirochet *Borrelia duttoni*. Tato studie obsahuje strukturní, biochemickou a biologickou charakteristiku tohoto peptidázového inhibitoru. Rekombinantní OmC2 byl testován proti panelu fyziologicky relevantních peptidáz a byl označen jako účinný širokospektrý inhibitor cysteinových proteáz kathepsinů, včetně endopeptidáz (katepsinu L a S) a exopeptidáz (katepsinů B, C a H). Krystalová struktura OmC2 byla stanovena v rozlišení 2,45 Å (1 Å = 0,1 nm) a byla použita pro popsání vztahu struktury a inhibiční aktivity. Následně byly in vitro i in vivo popsány biologické funkce OmC2. OmC2 ovlivňuje funkci myších dendritických buněk prezentujících antigen pomocí snížení produkce prozánětlivých cytokinů TNF-α a IL-12 a proliferaci antigen-specifických $CD4^+$ T lymfocytů. OmC2 tedy může potlačovat adaptivní imunitní odpověď hostitele. Imunizace myší s OmC2 významně snížilo přežití *O. moubata* po experimentální infestaci. OmC2 tedy představuje slibný cíl pro vývoj nové vakcíny proti populacím *O. moubata* a boji v rozšíření jimi přenášených chorob.

3.3 The crystal structures of two salivary cystatins from the tick *Ixodes scapularis* and the effect of these inhibitors on the establishment of *Borrelia burgdorferi* infection in a murine model

Michalis Kotsyfakis, **Helena Horká**, Jiří Salát and John F. Andersen

Molecular Microbiology (2010) 77: 456-470.

Contribution of H.H.: 35 %

Abstract

We have previously demonstrated that two salivary cysteine protease inhibitors from the *Borrelia burgdorferi* (Lyme disease) vector *Ixodes scapularis* – namely sialostatins L and L2 – play an important role in tick biology, as demonstrated by the fact that silencing of both sialostatins in tandem results in severe feeding defects. Here we show that sialostatin L2 – but not sialostatin L – facilitates the growth of *B. burgdorferi* in murine skin. To examine the structural basis underlying these differential effects of the two sialostatins, we have determined the crystal structures of both sialostatin L and L2. This is the first structural analysis of cystatins from an invertebrate source. Sialostatin L2 crystallizes as a monomer with an 'unusual' conformation of the N-terminus, while sialostatin L crystallizes as a domain-swapped dimer with an N-terminal conformation similar to other cystatins. Deletion of the 'unusual' N-terminal five residues of sialostatin L2 results in marked changes in its selectivity, suggesting that this region is a particularly important determinant of the biochemical activity of sialostatin L2. Collectively, our results reveal the structure of two tick salivary components that facilitate vector blood feeding and that one of them also supports pathogen transmission to the vertebrate host.

Sialostatiny L a L2, inhibitory cysteinových proteáz z klíštěte *Ixodes scapularis*, hrají důležitou úlohu v biologii tohoto klíštěte. Inaktivace obou genů současně silně

interferuje s úspěšným sáním. V této práci ukazujeme, že sialostatin L2, ale nikoliv sialostatin L, usnadňuje růst bakterií *B. burgdroferi* v myší kůži. Kvůli nalezení strukturních souvislostí podmiňujících rozdílné efekty těchto dvou proteinů jsme určili krystalovou strukturu obou sialostatinů. Jedná se o první strukturální analýzu cystatinů z bezobratlých. Sialostatin L2 krystalizuje jako monomer s „neobvyklou" konformací amino-konce, zatímco sialostatin L krystaluje jako dimer s N-terminální konformací podobnou ostatním cystatinům. Delece „neobvyklých" pěti aminokyselinových zbytků na N-konci sialostatinu L2 vede k výrazným změnám v selektivitě proteinu, což je směrodatné pro biochemickou aktivitu sialostatinu L2. Naše výsledky tedy ukazují strukturu dvou složek klíštěcích slin, které usnadňují sání krve a jeden z nich také podporuje přenos patogena z vektora do obratlovčího hostitele.

3.4 The tick salivary protein sialostatin L inhibits the Th9-derived production of the asthma-promoting cytokine interleukin-9 and is effective in the prevention of experimental asthma

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Contribution of H.H.: 25 %

Abstract

Ticks developed a multitude of different immune evasion strategies in order to obtain a blood meal. Sialostatin L is an immunosuppressive cysteine protease inhibitor present in the saliva of the hard tick *Ixodes scapularis*. Herein we demonstrate that sialostatin L strongly inhibits the production of IL-9 by Th9 cells. Since we could show recently that Th9-derived IL-9 is essentially involved in the induction of asthma symptoms, sialostatin L was used for the treatment of experimental asthma. Application of sialostatin L in a model of experimental asthma almost completely abrogated AHR and eosinophilia. Our data suggest that sialostatin L can prevent experimental asthma, most likely by inhibiting the IL-9 production of Th9 cells. Thus, alternative to IL-9 neutralization sialostatin L provides the basis for the development of innovative therapeutic strategies to treat asthma.

Klíšťata si vyvinula velké množství různých strategií, jak uniknout imunitnímu systému hostitele a získat dostatek potravy. Sialostatin L je inhibitor cysteinových proteáz s imunosupresivními účinky, přítomný ve slinách tvrdého klíštěte *Ixodes scapularis*. V této práci ukazujeme, že sialostatin L výrazně inhibuje produkci interleukinu 9 pocházejícího z Th9 buněk. Protože nedávno bylo dokázáno, že IL-9

produkovaný Th9 lymfocyty se zcela zásadním způsobem podílí na vyvolání astmatických symptomů, byl sialostatin L testován v terapii experimentálního astmatu. Aplikace sialostatinu L v myším astmatickém modelu téměř kompletně zabránila vzniku AHR a eosinofilie. Naše data naznačují, že sialostatin L může předejít astmatu, a to pravděpodobně díky inhibici IL-9 pocházejícího z Th9 buněk. Sialostatin L tedy představuje alternativní způsob neutralizace IL-9 a základ pro vývoj inovačních léčebných strategií pro léčbu astmatu.

4 Summary

We provided a detailed description of the effect of *Ixodes ricinus* saliva on the proliferation of *Borrelia burgdorferi* sensu stricto in various murine tissues, comparing three different sources of salivary effectors (salivary gland extracts (SGE), saliva and co-feeding of *Borrelia*-free nymphs in the sites of pathogen transmission). Intradermal inoculation of spirochetes together with SGE increased the spirochete load in the skin on days 4 and 6 post infection. Upon co-administration of tick saliva, the numbers of spirochetes in the urinary bladder were significantly higher 6 days post infection. Similarly, the co-feeding of nymphal ticks in the area of *Borrelia* inoculation had a positive effect on spirochete load in the bladder on day 6 post infection. In contrast, bacterial loads in the heart were substantially lower upon treatment with any of the three sources of salivary components, compared to untreated controls. A strong trend of increased spirochete loads 4 days post infection was also demonstrated in inguinal lymph nodes when the spirochetes were co-injected with tick saliva or administered in the presence of feeding nymphs.

The transmission of spirochetes upon the presence of tick saliva was affected also in the opposite direction, i.e. the transmission of the spirochetes to the feeding ticks. More specifically, co-inoculation of mice with spirochetes and SGE or saliva led to higher *Borrelia* transmission rate from the mice to the feeding ticks, as demonstrated by ten times higher percentage of infected ticks. Additionally, the presence of SGE in the infectious inoculum increased the mean spirochete load per tick from zero to almost 28000.

We next demonstrated that the cystatin OmC2 that is secreted in the saliva of the soft tick *Ornithodoros moubata* is a potent inhibitor of several cysteine proteases including cathepsins L, S, C, H and B. OmC2 was shown to affect the function of host immune cells. More specifically, OmC2 decreased the production of TNF-α and IL-12 by LPS-activated murine dendritic cells, whereas IL-6 levels remained unaffected. OmC2 also reduced the dendritic cell-mediated proliferation of antigen-specific $CD4^+$ T lymphocytes. Vaccination of mice with OmC2 led to impaired feeding of *O. moubata* nymphs, since only 60 % of the ticks that were attached on OmC2-vaccinated animals were able to fully engorge and survive compared to OVA-vaccinated controls.

The ability of the nymphs to survive and molt into the next developmental stage was negatively correlated with the anti-OmC2 antibody titer in the blood of the host mice.

Moreover, we determined the crystal structures of sialostatins L and L2, two cysteine protease inhibitors from *I. scapularis* saliva. In addition, we showed that co-administration of *B. burgdorferi* with sialostatin L2 (but not with sialostatin L) into mouse dermis led to a six-fold increase in the spirochete burden in the skin on day 4 post infection, in comparison to saline injection. Sialostatin L2 is thus a protein that facilitates the establishment of the infection in mice. However, sialostatin L2 presence does not stimulate or inhibit the proliferation of cultured spirochetes in vitro. In contrast to the mechanism described for the first and only (characterized to date) tick salivary effector that facilitates *Borrelia* transmission, the tick salivary protein Salp15, the stimulatory effect of sialostatin L2 is not caused by the direct interaction of the protein with the spirochete.

Finally, we described a novel mechanism of host immunomodulation by an arthropod salivary protein, the cystatin sialostatin L. This tick cystatin strongly inhibited IL-9 production by the recently described Th9 cells. Interleukin-9 was suppressed even in the presence of IL-9-promoting cytokine IL-1 and the inhibition was driven through an IL-2 independent mechanism. We also showed that sialostatin L could inhibit the Th9-derived IL-9 production in vivo. Since IL-9 is essentially involved in the development of airway inflammation, an intravenous application of sialostatin L almost completely abrogated the symptoms of experimental asthma-like airway disease, such as airway hyperresponsiveness and eosinophilia, in a Th9-dependent murine model of allergic asthma.

5 Proposed future lines of research

In our running projects we are trying to characterize additional tick proteins which are important for tick feeding and pathogen transmission. We would like to focus on additional cystatins also from European Lyme disease vector *I. ricinus*. Moreover, we would like to get information how the contact with a defined cystatin alters the kinome of Th9 cells and mast cells. Eventually, the obtained results could be used to develop innovative therapeutic strategies for the treatment of asthma and other allergic diseases by exploiting IL-9 modulating molecules from the tick saliva.

We also deal with several tick serine protease inhibitors (serpins). These proteins seem to be very important in the establishment of *Borrelia* infection in the host and their further studying could bring a new insight into the role of serine proteases in the successful pathogen transmission within the tick – host interface.

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7 List of abbreviations

Ado	adenosine
ADP	adenosine diphosphate
AHR	airway hyperresponsiveness
AS CR	Academy of Sciences of the Czech Republic
BAL	bronchoalveolar lavage
BMP	bone morphogenetic protein
BSK-H	Barbour-Stoenner-Kelly-H
cAMP	cyclic adenosine monophosphate
CBH	cutaneous basophil hypersensitivity
CD	cluster of differentiation
CRASP	complement regulator acquiring surface protein
CTLA	cytotoxic T lymphocyte antigen
Dbp	decorin-binding protein
DC	dendritic cells
ECM	extracellular matrix
ERK	extracellular regulated kinase
GM-CSF	granulocyte-monocyte colony stimulating factor
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IRF	interferon regulatory factor
kbp	kilobase pairs
LD	Lyme disease
lp	linear plasmid
LPS	lipopolysaccharide

MAP	mitogen activated protein
MEA	mast cell growth enhancing activity
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
MyD88	myeloid differentiation antigen 88
Osp	outer surface protein
OVA	chicken ovalbumin
PAR	protease-associated receptor
PBMC	peripheral blood mononuclear cells
PG	prostaglandin
PKA	protein kinase A
PMN	polymorphonuclear cell
RpoS	RNA polymerase sigma factor
SAT	saliva-activated transmission
SGE	salivary gland extract
TCGF	T cell growth factor
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper
TLR	Toll-like receptor
TNF	tumor necrosis factor
TROSPA	tick receptor for OspA