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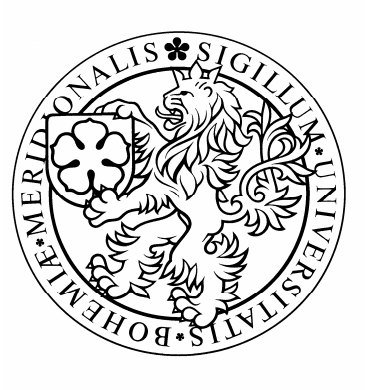

Ph.D. Thesis

Tick saliva-activated transmission of *Borrelia burgdorferi*

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Annotation:

The effect of the tick saliva on the mechanisms of innate immunity against *Borrelia* spirochetes was studied in vitro, with the following in vivo study of the saliva activated transmission of *Borrelia burgdorferi.* The in vivo study was focused on spirochetes loads in host tissues in early stages of *Borrelia* infection and transmission of spirochetes from the infected host to the uninfected ticks. The part of this study was aimed on the artificial infection of *Ixodes ricinus* ticks with *Borrelia* spirochetes, comparing immersion of larvae in the suspension of spirochetes with feeding on an infected host.

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I hereby declare that I did this work on my own, in collaboration with co-authors of the presented papers and using only the cited literature.

I declare that in accordance wit the Czech legal code § 47b law No. 111/1998 in its valid version, I consent to the publication of my PhD. thesis (in an edition made by removing marked parts archived by the Faculty of Science) in an electronic way in the public access to the STAG database run by the University of South Bohemia in České Budějovice on its web pages.

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Preface

This Ph.D. Thesis is based on following papers:

I) Kýčková K., Kopecký J. 2006: Effect of tick saliva on mechanisms of innate immune response against *Borrelia afzelii*. *J. Med. Entomol.* 43(6): 1208-1214.

II) Horká H., **Černá-Kýčková K.**, Skallová A., Kopecký J. 2009: Tick saliva affects both proliferation and distribution of *Borrelia burgdorferi* spirochetes in mouse organs and increases transmission of spirochetes to ticks. *Int. J. Med. Microbiol.* (in press).

III) Horká H., **Černá-Kýčková K.**, Fišerová L. Kopecký J. 2008: Efficiency of experimental infection of *Ixodes ricinus* ticks with *Borrelia burgdorferi* spirochetes. *Int. J. Med. Microbiol.* 298 S1: 177-179

IV) Fišerová L., **Černá K.**, Horká H., Kopecký J. 2008: Two ways of experimental infection of *Ixodes ricinus* ticks (Acari: Ixodidae) with spirochetes of *Borrelia burgdorferi* sensu lato complex. *Folia Parasitol. (Praha)*. 55(2): 150-154.

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1. Abstract

Causative agents of Lyme disease, bacteria from the *Borrelia burgdorferi* sensu lato complex, belong to the most widespread vector-borne human pathogens in Europe and North America. Seven genomic species from this group are thought to be etiological agents of Lyme disease in humans: *Borrelia burgdorferi* sensu stricto, *B. afzelii*, *B. garinii*, *B. spielmanii*, *B. valaisiana, B. bissetii, and probably B. lusitaniae*. They are known to be transmitted by hard ticks from the genus *Ixodes*, in the Czech Republic namely by *Ixodes ricinus*. Hard ticks take their blood meal for days, even weeks, depending on the stage of development, hence tick saliva has to contain high amount of pharmacologically active molecules with antihemostatic, vasoactive and immunomodulatory properties. Tick-transmitted pathogens, including *Borrelia* spirochetes, exploit immunomodulatory properties of tick saliva, which originally helps the tick to feed on the host successfully, to evade the host immune reactions.

In these studies, we targeted the early immune reaction against *Borrelia* infection, which could be directly influenced by the immunomodulatory molecules secreted in the tick saliva into the wound. We focused on the effect of tick saliva exerted on the mechanisms of innate immunity against *Borrelia* spirochetes, especially on killing of *Borrelia* by the activation of alternative pathway of complement cascade and phagocytosis of spirochetes by macrophages.

The in vitro experiments revealed significant inhibition of *Borrelia* killing by complement cascade in the presence of tick saliva, as well as reduction of the percentage of macrophages which phagocytosed *Borrelia*. Moreover, the tick saliva diminished the production of nitric oxide (NO) and tumor necrosis factor alpha (TNF-α), an important pro-inflammatory cytokine, by mouse peritoneal macrophages after stimulation with interferon gamma (IFN-γ) in combination with *Borrelia* spirochetes. Considering results of in vitro experiments we suggested an in vivo model to reveal if the tick saliva could influence the proliferation and distribution of the bacteria in the host tissues during the first stage of *Borrelia* infection and if the affection of the local immune reaction at the site of tick feeding could influence the transmission of spirochetes from the infected host to the uninfected ticks.

Comparing three different sources of the saliva molecules (SGE, saliva and feeding of uninfected nymphs), we detected that saliva significantly influences *Borrelia* loads in the skins, urinary bladders, and hearts of mice. Both SGE and saliva enhanced the percentage of ticks that became infected after feeding on infected mice, nevertheless absolute numbers of spirochetes per one tick was markedly higher in the group of mice co-inoculated with SGE than with saliva.

The last part of this study was aimed at artificial infection of *Ixodes ricinus* ticks with *Borrelia* spirochetes, because obtaining of a cohort of ticks with a known rate of infection is useful for studies on both pathogen transmission and *Borrelia*-vector interactions. The efficiency of introduction of *Borrelia* spirochetes into tick larvae by immersion in a suspension of spirochetes was tested on three of the most medically important European *Borrelia* genomic species, and results were compared with infection of nymphs by feeding on infected mice. Both methods yielded comparable results with the exception of *B. afzelii*, which produced better results using the immersion method compared with feeding on infected mice. The second experiment described the efficiency of nymph artificial infection with *B. burgdorferi* sensu stricto using the immersion method, reaching up to 90%, and monitored the development of infection in hosts that were used for the immersed ticks feeding. In both experiments, co-feeding of ticks on one host, owing to the effects of saliva, presumably contributed to the enhancement of percentage of infected ticks.

2. Introduction

Lyme borreliosis is a widespread zoonosis in the North America, Asia and Europe. It is maintained by tick-mediated transmission between mammalian hosts, usually small rodents. The competent vector of this disease in the Czech Republic is the common tick - *Ixodes ricinus* - in all its developmental stages (adult female, nymph and larva) (Burgdorfer 1984). Although humans are not natural hosts for the ticks, they often serve as alternative hosts for all bloodsucking tick stages.

The causative agent of Lyme disease was first isolated from the gut of *Ixodes scapularis*, and subsequently from the blood of patients who suffered from erythema migrans or meningoencephalitis, and was later described as *Borrelia burgdorferi* Johnston, 1984 (Burgdorfer et al. 1982, 1983, Johnson et al. 1984). Over the next years, many variants, so called genomic species, were discovered. Currently no less than 14 genomic species belong to the *B. burgdorferi* sensu lato complex, thereout 3 of them are demonstrably infectious for humans, causing the symptoms of Lyme borreliosis. However, next 4 genomic species were isolated from patients suffering from Lyme disease (Tab. I).

Genomic species	**Region**	**In *I. ricinus***	**LB**	**References**
B. burgdorferi sensu stricto	Am., Eu., As.	+	+	Johnson 1984
B. afzelii	Eu., As.	+	+	Canica et al. 1993
B. garinii	Eu., As.	+	+	Baranton et al. 1992
B. valaisiana	Eu., As.	+	+/-	Wang et al. 1997
B. lusitaniae	Eu., Af.	+	+/-	Le Fleche et al. 1997
B. bissettii	Eu., Am.	+	+/-	Postic et al. 1998
B. spielmanii	Eu.	+	+/-	Richter et al. 2004, 2006
B. andersonii	Am.			Marconi et al. 1995
B. californiensis	Am.			Postic et al. 2007
B. carolinensis	Am.			Rudenko et al. 2008
B. sinica	As.			Masuzawa et al. 2001
B. tanukii	As.			Fukunaga et al. 1996
B. turdi	As.			Fukunaga et al. 1996
B. japonica	As.			Kawabata et al. 1993

Tab. I: Genomic species of *Borrelia burgdorferi* sensu lato. Eu.-Europe, Am.-America, As.-Asia, Af.-Africa, LB-Lyme disease causing spirochetes.

2.1 Pathogen - *Borrelia burgdorferi*

2.1.1 Characterization

B. burgdorferi bacteria, as well as spirochetes causing recurrent fevers, were classified to the genus *Borrelia*, order Spirochaetales, family Spirochaetaceae and described as microaerophilic, gram-negative bacteria, despite Gram-staining (Barbour and Hayes 1986). The cork-screw shaped bacterial body of spirochetes is covered by two membrane structures; the inner membrane that encloses the cytoplasmic content, and the outer multilayer membrane (Johnson 1977). Bacterial flagella are anchored in the basal discs on both ends of the bacterium and concealed under the outer membrane whip spirochetal body in the periplasmic area (Hovind-Hougen 1984). The borrelial 1,5-Mb genome is very complex, consisting of a 0,91-Mb linear chromosome and a set of 12 linear and 9 circular plasmids (Fraser et al. 1997, Casjens et al. 2000). The evolutionary adaptation to both arthropod vectors and warm-blooded hosts involved the evolution of outer membrane proteins, largely lipoproteins, enabling bacteria to recognize and respond to assorted tissues. Proteins and lipoproteins of the outer membrane that are responsible for the antigenic specificity and adaptive antigenic variability of *Borreliae* are coded by the genes located on plasmids (Zhang et al. 1997, Zhang and Norris 1998).

2.1.2 Route of transmission

Ticks become infected via feeding on infected host, when *Borrelia* spirochetes, during tick's blood meal, enter the midgut. Transovarial transmission is not common (Nefedová et al. 2004, Zhioua et al. 1994). During the blood feeding in the tick's next life stage, *Borreliae* migrate from the tick gut to the salivary glands and are subsequently transmitted with the saliva to the host organism. This process is connected with the alterations in the surface lipoprotein expression pattern, which favors spirochetes to migrate through the vector's body and to invade the vertebrate host (Grimm et al. 2004, Pal et al. 2004).

OspA is a lipoprotein molecule of the *Borrelia* outer surface membrane responsible for the attachment to the tick gut cells, where it binds to the tick receptor for the OspA (TROSPA), which is important in the colonization of the tick midgut (Pal et al. 2000, Pal et al. 2004b). It has been proposed that expression of TROSPA is markedly higher in *Borrelia*-infected then in uninfected ticks, suggesting that *Borrelia* induces the expression of receptor molecule in the vector (Pal et al. 2004b).

The expression of OspA, but also OspB, encoded on adjacent paralogous genes present on linear plasmid lp54 (Wilske et al. 1992), underlies strict control of regulatory pathways insuring that OspA is up-regulated, as *Borreliae* enter the tick vector, and remains a major surface antigen during midgut colonization, and it is down-regulated in a mammalian host (Schwan et al. 1995). This regulation, as well as regulation of other surface proteins, was thought to be only temperature and pH dependent, thus OspA expression is triggered in ticks after beginning of blood-meal digestion (Schwan et al. 1995, Yang et al. 2000, Alverson et al. 2003). However, for the *Borrelia* organism, it is fundamental to display OspA molecules on its surface even before entering the tick midgut during the tick blood meal. Therefore, host neuroendocrine stress hormones, epinephrine and norepinephrine, which are secreted after tick attachment, are specifically bound by *B. burgdorferi* present in host tissues and result in increased expression of OspA (Scheckelhoff et al. 2007).

Borreliae are acquired from infected animal rapidly into the tick's gut lumen (Hodzic et al. 2002), where they remain attached and bound to the gut cells during blood digesting via OspA-TROSPA cooperation until tick molts to the next life stage and starts feeding on the next host (Pal et al. 2004). Nevertheless, the absolute amount of spirochetes in the gut decreases in molting ticks (Piesman et al. 1990). OspB also specifically binds to the gut cells, because OspB antibody prevents *Borrelia* spirochetes colonization of *I. scapularis* ticks (Fikrig et al. 2004) and additionally, OspB-deficient *B. burgdorferi* strain manifests lower adherence to the tick gut, indicating a synergic interaction of OspA, OspB and TROSPA (Neelakanta et al. 2007). Surprisingly, the Battisti group observed the ability of OspA mutant to persist in the vector after molting and they considered the role of this molecule only as antibody protection during vector blood meal uptake from immune hosts (Battisti et al. 2008). Moreover, another surface lipoprotein, OspD, has also been shown to have a function in the adherence of *Borrelia* to the tick gut cells (Li et al. 2007), which indicates that the functions of these molecules could complement and overlap.

Once being in the flat tick after molting, starting of blood meal triggers the downregulation of OspA in *Borreliae*, allowing them to leave the tick gut receptors and to migrate into the salivary glands. Simultaneously other lipoproteins, mainly OspC, but also the variable surface antigen VlsE, fibronectin-binding protein BBK32 and decorin-binding proteins (Dbps) A and B, are upregulated (Hodzic et al. 2002, Kočí et al. 2006). Whereas the levels of OspA on *Borrelia* spirochetes decrease in the midgut and salivary glands during tick feeding, the mRNA levels of OspC increase significantly in both organs (Piesman et al. 2003). This specific regulation is controlled by the complex mechanisms of alternative sigma factors - regulatory pathway RpoS/RpoN (Schwan et al. 1995, Alverson et al. 2003, Schwan at Piesman 2000, Hűbner et al. 2001). When infected ticks start their blood meal, RpoS is produced, which leads to *ospC* induction and *ospA* repression (Caimano et al. 2005, Srivastava and de Silva 2008), whereas the promoter recognition of these genes by RpoS is modulated by mammalian host signals (Caimano et al. 2007). Dissolved CO_2 levels contribute to the modulation of *B. burgdorferi* gene expression. Quantitative RT-PCR indicates that the levels of the *dbpA, ospC*, and *bbk32* transcripts are increased in the presence of CO_2 (Hyde et al. 2007). *Borrelia* spirochetes with all the expressed molecules on their surface enter host organism along with the tick saliva.

2.1.3 Development of infection

It was postulated previously that OspC plays an important role in the migration of spirochetes to the vector's salivary glands and is essential for colonization of host tissues (Pal 2004a, Grimm et al. 2004), although other authors admit only the impact of OspC expression on the higher infectivity of *Borrelia* organisms (Stewart et al. 2006, Fingerle et al. 2007). However Xu and colleagues have recently suggested that OspC as a dominant lipoprotein, present on the spirochetal membrane, only maintains of the surface membrane integrity, therefore protects the bacterium against host innate immune defenses. Furthermore, the function of OspC in the mammalian infection could be replaced in OspC mutants by the overexpression of another randomly chosen surface lipoprotein, as Erp, Dbps, or OspA (Xu et al. 2008a, b).

OspC is an inductor of host humoral response (Fung et al. 1994), hence *Borrelia* downregulates OspC expression and is able to evade the first line of host antibody response that starts soon after infection (Xu et al. 2006). This regulation is coupled with the

upregulation of other surface exposed proteins, including VlsE (Liang et al. 2002, Liang et al. 2004, Crother et al. 2004).

The VlsE locus is responsible for the antigenic variation of *B. burgdorferi* spirochetes during mammalian infection, enabling them to evade the host adaptive immunity (Zhang et al. 1997). The VlsE lipoprotein is encoded on the linear plasmid lp28-1, where recombination between the active vlsE locus and 15 nonexpressed silent cassette sequences leads to the generation of novel alleles at the expression site (Zhang et al. 1997), with the contribution of point mutations (Sung et al. 2001). Unlike in vertebrates, vlsE locus is stable in ticks (Indest et al. 2001, Nobisch and de Silva 2007). The expression levels of VlsE protein in *Borrelia* present in unfed tick are very low, but they increase significantly in salivary glands at the end of tick's feeding (Piesman et al. 2003). In the case of rabbit infection, VlsE is the most prominent antigen, starting with one week after tick detachment, while the expression of OspC antigen is silenced (Crother et al. 2004, Liang et al. 2004), which could allow *Borrelia* to resist immune attack.

In addition to proteins mentioned above, *Borrelia* spirochetes express a large number of surface lipoprotein adhesins that could be involved in host infection via binding fibronectin, integrins, plasminogen, and proteoglycans, thus enabling the easier attachment to the host extracellular matrix and penetration through the tissue barriers.

The Erp surface lipoprotein family attracts a lot of attention. Genes coding Erp proteins are located on 32-kb circular plasmids of *B. burgdorferi* (Stevenson et al. 1996). Expressed proteins are related to the antigenic OspE and OspF proteins, exercising a function of binding host-derived complement regulators, which may thereby help protect bacteria from complement mediated killing during host infection. Most studied members of Erp protein family, located on the linear plasmid lp54 and lp28-3, are complement regulator-acquiring surface proteins (CRASPs). The CRASPs interaction with host derived negative regulators of the alternative pathway of complement, factor H and factor H-like protein 1, brings resistance of *Borreliae* to complement mediated killing (Kraiczy et al. 2004a,b, Hartmann et al. 2006). Serum-resistant *B. burgdorferi* clones were demonstrated to express up to five distinct CRASPs (Hartmann et al. 2006). Moreover, some Erp proteins serve as bacterial adhesins, binding not only factor H, but also extracellular matrix protein laminin, contributing to the establishment of mammalian infection (Brissette et al. 2008). Erp protein expression underlies the regulation throughout the host-tick infectious cycle, for example while BbCRASP-2 is present on *Borrelia* surface in early stages in the host supporting the establishment of mammalian infection, BbCRASP-1 is produced only

during host-tick transmission stages. Other Erps are expressed during the whole process of host infection, including chronic stages (Von Lackum et al. 2005, Miller and Stevenson 2006, Bykovsky et al. 2007).

Early dissemination from the site of primary infection to the target organs and tissues of the host body is fundamental for *Borrelia* spirochetes, enabling them to evade the host immune system at the site of the tick bite, including the potency to induce secondary manifestations. *B. burgdorferi* binds host plasminogen to penetrate tissue barriers and to disseminate in the whole body, because plasminogen bound on the plasminogen-binding proteins could be converted to the active enzyme plasmin using host-derived plasminogen activator (urokinase-type). Plasmin is responsible for extracellular matrix degradation (Fuchs et al. 1994, Hu et al. 1995). Spirochetes highly expressing plasminogen-binding proteins exhibit enhanced penetration of endothelial monolayers (Coleman et al. 1995).

The first reported plasminogen-binding protein was OspA (Fuchs et al. 1994, Hu et al. 1995), however, the affinity for plasminogen is not as strong as that of OspC, the most important plasminogen-binding protein. The differences in OspC affinity for plasminogen in individual *Borrelia* genomic species are correlated with different invasiveness patterns in mice (Lagal et al. 2006). Surprisingly, ErpP, A and C are also able to bind plasminogen, and, moreover, to bind simultaneously factor H and plasminogen, which indicate separate binding sites for both ligands (Brissette et al. 2009)

The Lyme disease spirochete occurs primarily in the extracellular matrix and connective tissues during mammalian infection, where decorin, fibronectin and glycosaminoglycans are abundantly expressed, thus the ability of *Borrelia* spirochetes to bind decorin has been hypothesized to be important for the survival and dissemination to the bladder, heart, joints and skin (Barthold et al. 1991, Guo et al. 1998). *Borreliae* use DbpA and B to bind decorin (Guo et al. 1998), conducing to the strong dermatotropism of *Borrelia* spirochetes. All the same, the role of Dbps, as well as fibronectin-binding adhesin BBK32, in mammalian infection remains unclear. The results show that they are not required for the infection (Li et al. 2006, Shi et al. 2006), although at least Dbps play a role in overall virulence of *Borrelia* spirochetes (Shi et al. 2008) and inactivation of the gene bbk32 significantly attenuates the infectivity of *B. burgdorferi* (Seshu et al. 2006). DbpA-deficient *Borrelia* mutants, when needle inoculated, exhibit strong decrease in infectivity. On the contrary, the efficiency the transmission into the naïve mouse via tick bite is not affected (Blevins et al. 2008). A protective niche for spirochetes in the hosts seems to be in the tissues with the highest decorin expression, in particular in joints and

skin, while relatively low decorin expression in bladder and heart does not offer the sufficient protection (Liang et al. 2004). BBK32 protein that was shown to mediate the interaction between *Borrelia* spirochetes and host fibronectin, as well as glycosaminoglycans, may play a role in initial dissemination by interlocking of transient and dragging interactions. Moreover, BBK32 probably contributes to stationary adhesion of *Borreliae* to the vascular endothelium (Norman et al. 2008).

2.2 Effect of tick saliva on host immune reactions, in relation to *Borrelia* infection

Tick feeding stimulates host immune response pathways; triggers complement activation, skin inflammation, and induces adaptive immunity. Tick saliva contains a large number of molecules whose activities include the ability to inhibit hemostasis, vasoconstriction and development of inflammation with the subsequent adaptive immune responses. The immunomodulatory properties of tick saliva serve the tick to complete its blood-meal successfully; therewithal the locally suppressed immunity at the site of tick bite could be exploited by tick-borne pathogens. The fate of the Lyme disease agent, *B. burgdorferi*, is close-knit with its vector's saliva. Actually, *Borrelia* species infect the host more successfully when they are supported with the saliva of the natural tick vector (Zeidner et al. 2002). Saliva of the noncompetent tick vector *Amblyomma americanum* even itself contains borreliacidal activity; therefore *Amblyomma* is not able to transmit *Borrelia* spirochetes at all. Presence of competent vector (*I. scapularis*) saliva in the contrary enhances the percentage of surviving spirochetes in vitro (Ledin et al. 2005).

2.2.1 Innate immunity

Innate immune response has the main role in the defense against *Borrelia* spirochetes from the time point of *Borrelia* transmission by the tick vector into the host skin to the development of adaptive immunity in the host body. Even a small number of spirochetes induces vigorous local inflammatory response at the site of infection (Szczepanski and Benach 1991, Ma et al. 1994). Phagocytosis and degradation of spirochetes by phagocytosing cells as well as the degradation due to the complement cascade are essential parts of the innate immunity (Kochi end Johnson 1988, Rittig et al. 1992, Montgomery et al. 1993, van Dam et al. 1997).

Complement cascade (Fig. 2). Binding of *Borrelia*-specific antibodies on borrelial surface triggers the classical pathway of complement activation after being recognized by

C1q subunit of C1 compound (Kochi and Johnson 1988). The mannan-binding lectin pathway is homologous to the classical complement pathway. This pathway uses a lectin similar to C1q of the classical complement pathway, which binds to mannose residues and other sugars on bacterial surface. The alternative pathway of complement is activated independently of anti-*Borrelia* antibodies and serves as an early defense mechanism before the production of antibodies by the humoral immune response (Kochi and Johnson 1988).

The activation of the complement system triggers a proteolytic cascade leading to the C3 cleavage with the subsequent C3b deposition on bacterial surfaces, resulting in both opsonization and formation of membrane attack complexes (MAC) (Pickering et al. 1969, Gordon et al. 1988). Vertebrate organisms cover their own cell surfaces with serum complement regulatory proteins like factor H that inactivates C3b complement compound, protecting their own cells from complement mediated lysis.

Although the classical pathway participates in the killing of spirochetes, in particular after adaptive immune responses have evolved, it may affect spirochete numbers in some tissues even before the elicitation of a substantial humoral response, because IgM antibodies that bind *B. burgdorferi* may be naturally present in uninfected mammals (Belperron and Bockenstedt 2001).

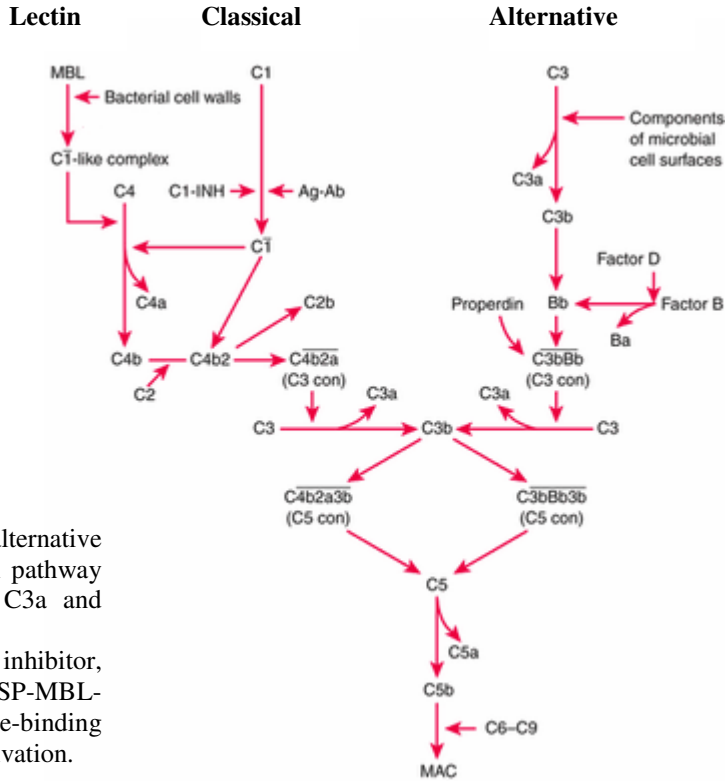

Fig. 1: The classical, lectin, and alternative pathways converge into a final common pathway when C3 convertase cleaves C3 into C3a and C3b.
Ab-antibody, Ag-antigen; C1-INH-C1 inhibitor, MAC-membrane attack complex, MASP-MBL-associated serine protease, MBL-mannose-binding lectin, P-properdin. Overbar indicates activation.

Adapted from: http://www.merck.com/mmpe/sec13/ch163/ch163d.html

In the alternative pathway of complement activation, invading spirochetes are coated with C3b and then lysed by the formation of the membrane attack complex (MAC) or phagocytosed by macrophages and neutrophils (Gordon et al. 1988). Production of proinflammatory mediators anaphylatoxins, which is connected with the C3 cleavage process, attracts neutrophils and monocytes to the site of complement activation (Kochi and Johnson 1988, Morgan 1989). Complement sensitivity has a crucial importance for *Borrelia* ecology and pathogenicity (Kurtenbach et al. 1998, van Dam et al. 1997, van Dam 2002). *B. garinii* is sensitive to the killing by rodent complement, but relatively very resistant to the avian complement, in contrast, *B. afzelii* is effectively killed by avian, but not rodent complement, supporting the host specificity (Kurtenbach et al. 1998). Within the particular genomic species it was demonstrated that the acquisition of host-derived complement regulators, such as factor H and factor H-like protein 1, on the bacterial surface of some strains allows spirochetes to resist complement-mediated killing (Kraiczy et al. 2004). These strains are the most pathogenic for their hosts (van Dam et al. 1997).

However spirochetes of Lyme borreliosis manage their own complement regulators, they exploit certain molecules from their vector's saliva to evade the host complement lysis, which increases *Borrelia* infectious potential. The saliva contains very potent complement inhibitors that are necessary for tick feeding. Saliva from the members of the family Ixodidae is able to inhibit the alternative pathway of complement activation in varying degrees, whereas this effect is closely dependent on the animal source of complement (Lawrie et al. 1999). Saliva, or salivary gland extract (SGE), inhibits the alternative pathway of complement activation via preventing bacterial surfaces from being coverage by C3b and C5b complement components and, moreover, they prevent phagocytosis of organisms covered by these components (Ribeiro 1987). Saliva contains 49 kDa inhibitor of C3 hydrolysis, decreasing this way the production of C3a (an anaphylatoxin) that is important for the activation and degranulation of mast cells and basophils (Ribeiro 1987). *I. ricinus* saliva is able to markedly inhibit the formation of the anaphylatoxin C3a (Lawrie et al. 2005), which relates with the carboxypeptidase activity in tick saliva (Ribeiro and Spielman 1986). Carboxypeptidase B present in the saliva is responsible for the inhibition of bradykinin, a useful pain mediator and blood vessel permeabilizer. Indeed, by means of bradykinin inhibition, saliva prevents edema formation (Ribeiro and Mather 1998). An 18,5 kDa complement inhibitor Isac, with activity very similar to factor H, downregulates the function of C3bBb complex (C3 convertase), is present in *I. scapularis* saliva (Valenzuela et al. 2000). Silencing of *isac* significantly

reduced fed-tick weight, spirochete loads in these ticks were also significantly reduced (Soares et al. 2005). In fact, *I. ricinus* saliva contains two different Isac homologues – Irac I and II – that have broad and complementary inhibition activities against the complement of different host species (Daix et al. 2007, Schroeder et al. 2007). Other inhibitory proteins, including Salp9 and Salp20, have been recently identified in the Isac protein family (Das et al. 2001). The Salp20, an *I. scapularis* 48 kDa protein, inhibits the alternative complement pathway by dissociating the C3 convertase (Tyson el al. 2007). It acts directly against properdin, a stabilizer of C3 convertase, thereby accelerating the decay of C3b molecule (Tyson et al. 2008).

In the presence of anti-*Borrelia* antibodies, the classical pathway of complement activation could lead to the *Borrelia* spirochetes degradation. The Salp15 molecule, originally identified as CD4+ T-cell activation inhibitor (Anguita et al. 2002), is present in the saliva of *I. scapularis* as well as *I. ricinus* ticks (Hovius et al. 2007, Das et al. 2001). Both Salp15 homologues bind to the borrelial OspC and prevent preferentially *B. burgdorferi* sensu stricto antibody mediated killing (Ramamoorthi et al. 2005, Hovius et al. 2008a), Salp15 also protects spirochetes against the complement itself reducing the deposition of terminal C5bC9 complement complex on their surface (Schuijt et al. 2008). Furthermore, Salp15 production in salivary glands is selectively enhanced by the presence of *Borrelia* spirochetes in the tick, indicating the targeted exploitation of vector's molecules (Ramamoorthi et al. 2005). Tick saliva also contains further proteins with the competence to bind host IgG antibodies, what is important not only for the inhibition of the adaptive immune responses but also vicariously for the reduction in killing of bacteria through the classical pathway of complement activation (Wang and Nuttall 1994, 1995a, b).

Macrophages play a significant role in the early immune responses to the tick feeding as well as to the starting *Borrelia* infection. They belong to the first cells migrating to the site of infection, where they phagocytose the antigenic material, present it to lymphocytes and produce huge amounts of proinflammatory cytokines (Flavell et al. 1986). Macrophages, activated after *Borrelia* phagocytosis or alternatively after direct induction by the borrelial lipoproteins (Radolf et al. 1995), produce a variety of chemokines and cytokines (Sprenger et al. 1997).

Classical and coiling phagocytosis (Fig. 3) are two the most frequent mechanisms of internalizing of *Borrelia* spirochetes into the macrophages. The classical one is triggered

when antibody-opsonized bacteria are linked to the Fc receptor on phagocytic cell, and leads to lysosomal degradation. Unopsonized bacteria are internalized independently on Fc receptors by coiling phagocytosis and subsequently degraded in the cytosol (Rittig et al. 1992, Montgomery et al. 1994). Complement receptor CR3 (integrin $\alpha_m\beta_2$) can serve as a receptor for iC3b-coated spirochetes, whereas unopsonized bacteria are captured by the mannose receptor that recognizes sugar residues on bacterial surfaces (Cinco et al. 1997, Cinco et al. 2001). The activation of relevant signaling pathways is able to trigger intracellular killing mechanisms (Cinco et al. 1997). The inner adapter molecule MyD88, a key signaling molecule utilized by most of the Toll-like receptors (TLRs), is demonstrably important for the uptake of *B. burgdorferi* (Shin et al. 2008).

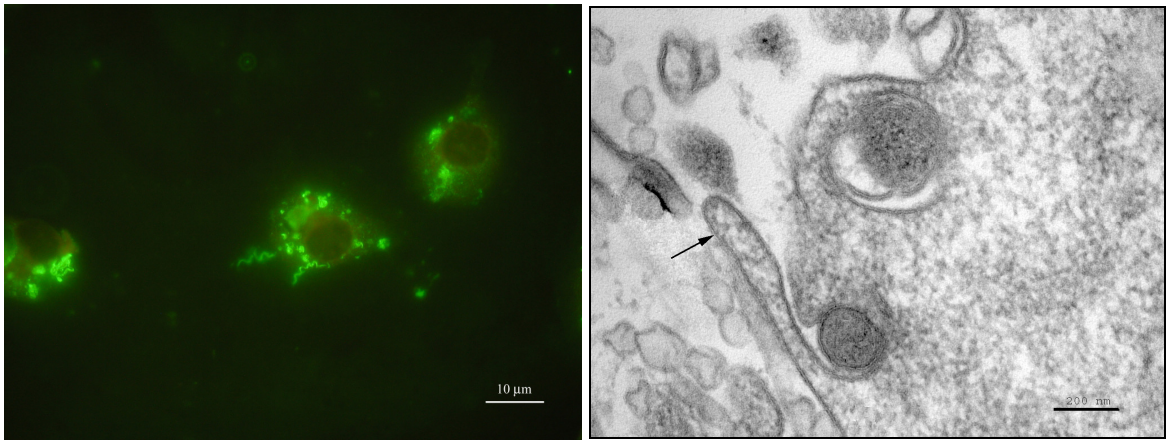

Fig. 2: Mouse peritoneal macrophages phagocytosing *B. afzelii*. Attached and engulfed (coiled) spirochetes are FITC-stained by indirect immunofluorescence, nuclei are visualized using Evans blue (A). Coiling phagocytosis of *B. afzelii* visualized by transmission electron microscopy, arrow indicates one coiling pseudopodium around the spirochete (B).

After stimulation with *B. burgdorferi*, macrophages and monocytes produce large amounts of proinflammatory cytokines, especially IL-1β (Habicht et al. 1986, Miller et al. 1992, Schulze et al. 1996), IL-6 (Radolf et al. 1995, Schulze et al. 1996) and TNF-α (Defosse and Johnson 1992, Radolf et al. 1995). The direct contact between *Borrelia* lipoproteins and TLR receptors present on phagocytes surface also leads to macrophage activation, inducing NF-κB nuclear transcription factors followed by the production of cytokines (IL-1β, IL-6, IL-8, IL-12), expression of adhesive molecules, co-stimulatory molecules (CD80 a CD86) and generation of nitric oxide (NO) and superoxide by macrophages and neutrophils (Ma et al. 1994, Morrison et al. 1997). Recent data suggest that the TLR1/TLR2 heterodimer is the main receptor molecule, probably cooperating in

the signalization with some other TLR molecules. Surprisingly, TLR5 (flagellin receptor) is downregulated after TLR1/TLR2 stimulation (Cabral et al. 2006, Shin et al. 2008).

Monocytes and macrophages are able to intracellulary kill and degrade bacteria. Phagocytosis of *Borrelia* spirochetes induces respiratory burst, connected with generation of reactive oxygen intermediates (Peterson et al. 1984, Rittig et al. 1992, Montgomery et al. 1993). These intermediates cooperate in the killing of bacteria with reactive nitrogen intermediates, especially NO, which are synthesized after stimulation with *Borrelia* lipoproteins due to the activation of the gene for inducible nitric oxide synthase (iNos) (Ma et al. 1994). Other oxygen-independent mechanisms, as acid pH in phagolysosome, lysozyme, defensins, and proteinases, are included in the elimination of Lyme disease spirochetes (Modolell et al. 1994).

Macrophages influenced by SGE produce lower amounts of the cytokines IL-1, IL-12 and TNF-α, whose function is to activate other leukocytes (Ramachandra and Wikel 1992, 1995, Schoeler et al. 1999, Kročová et al. 2003). Moreover, TNF-α in the cooperation with IFN-γ induces the expression of MHC II membrane molecules required for the antigen recognition by T-lymphocytes. Recently, anti-TNF-α activity was demonstrated in *I. ricinus* saliva. Anti-TNF-α factor is a protein with estimated molecular mass 23 kDa probably reacting through direct binding of the cytokine (Koník et al. 2006). A novel protein Iris (*Ixodes ricinus* serpin) which is upregulated in the salivary glands during feeding was identified in *I. ricinus* (Leboulle et al. 2002a). In addition to anti-hemostatic effects, Iris was proved to be a potent immunosuppressive protein with numerous modulatory functions on host immune system (Prevot et al. 2006). Considering innate immunity, Iris has no effect on the IL-10 expression level in macrophages, nevertheless it enhances the number of macrophages producing this cytokine. On the other hand, expression of the proinflammatory cytokines IL-6 and TNF-α is inhibited by macrophages owing to Iris, whereas IL-1 expression remains unaffected (Leboulle et al. 2002b).

Tick saliva or SGE decrease the killing and degradation of *Borreliae* in macrophages and suppress the production of two main defense molecules of phagocytic cells – NO and superoxide anion (Urioste et al. 1994, Ferreira and Silva 1998, Kuthejlová et al. 2001). Whereas the downregulation of NO production could be caused by the cytokine levels modulated by saliva, the effect on the production of superoxide is probably direct, due to its prompt effect.

Lyme borreliosis spirochetes induce phagocytosis, oxidative burst and calcium mobilization in **neutrophils**, these actions seem to be complement dependent (Suhonen et al. 2000). *I. scapularis* saliva inhibits the functions of neutrophils, decreases the phagocytosis of *B. burgdorferi*, neutrophil aggregation and also production of superoxide by these cells (Ribeiro et al. 1990). Actually, tick saliva reduces the adherence of neutrophils by the inhibition of β2-integrins expression, what leads to the failure of phagocytosis and killing of spirochetes in the phagocytes (Montgomery et al. 2004).

Saliva from different tick species was shown to have chemokine neutralizing activity. **Chemokines,** small chemoattractant cytokines produced by different immunocompetent cells, play an important role in recruiting specific leukocyte populations to the site of infection. Sialostatin L was shown to inhibit neutrophil recruitment to the inoculation site (Kotsyfakis et al. 2006). Anti-chemokine activity was revealed in the saliva of several tick species, whereas the inhibitory effect covered CXCL8/IL-8, CCL2/MCP-1, CCL3/MIP-1α, CCL5/RANTES, and CCL11/eotaxin activity (Hajnická et al. 2001, Kocáková et al. 2003, Hajnická et al. 2005). A family of chemokine binding proteins Evasins with the specificity to CCL3, CCL4, CCL18 (Evasin-1), CXCL8, CXCL1 (Evasin-3), CCL5 and CCL11 (Evasin-4), respectively, was identified in the saliva of *Rhipicephalus sanquineus* ticks (Frauenschuh et al. 2007, Déruaz et al. 2008). Moreover, tick saliva directly inhibits the chemotactic functions of MIP-1α, which affects the dendritic cell (DC) migration. Additionally, cell surface CC chemokine receptors on DCs are reduced under the influence of saliva, resulting in the impaired migration towards MIP-1α and MIP-1β, important macrophage-derived chemokines (Oliveira et al. 2008).

Dendritic cells are the most potent antigen presenting cells (APCs) in priming naïve T-lymphocytes. Dermal DCs and blood-derived DCs phagocytose *B. burgdorferi* spirochetes, preferentially using coiling phagocytosis. DCs also process spirochetes and present the bacterial antigen to T-lymphocytes (Filgueira et al. 1996). Saliva from the tick *R. sanquineus* inhibits DC differentiation, reduces production of IL-12, whereas the generation of IL-10 is unaltered. Saliva-affected DCs fail to stimulate antigen-specific T cells (Cavassani et al. 2005). In the presence of tick saliva, DCs initiate Th2 differentiation of CD4+ T-cells, moreover, *I. ricinus* saliva impairs maturation of DCs as well as their migration from the site of infection and antigen presentation capacity (Skallová et al. 2008). These findings can be supported by the results of Mejri and Brossard (2007) showing that either in vitro co-cultures of DCs and saliva or ex vivo

cultures of DC-primed T-cells influenced by saliva exhibit higher IL-4/IFN-γ ratios compared with controls. Prostaglandin E2 plays a role in some, but certainly not in all, saliva-mediated alterations of DC functions. For example, IL-12 and TNF-α production after TLR-ligand stimulation is reduced in its presence, also is the expression of LPS-induced CD40. In the contrary, expression levels of maturation indicators CD80, CD86, or MHC class II remain unchanged (Sá-Nunes et al. 2007). Recently, *I. scapularis* protein Salp15 was revealed to inhibit adaptive immune responses via suppressing DC functions. It was shown to suppress *B. burgdorferi*-induced cytokine production in DCs, and subsequently T-lymphocyte activation. Salp15 interacts with DC receptor DC-SIGN, resulting in decrease of IL-6 and TNF-α mRNA stability and additionally, to the declension of nucleosome remodeling at the IL-12p35 promoter (Hovius et al. 2008b).

Although the mechanisms mentioned above have the major importance during the early phase of *Borrelia* infection, other saliva-derived immunomodulatory proteins can be helpful for the establishment of Lyme disease. For example, histamine-binding proteins that block the activity of histamine and serotonin released from the granules of basophils and mast cells were identified in the *I. ricinus* saliva (Paesen et al. 1999). Sequences of histamine-binding protein homologues were subsequently found in other tick species (Valenzuela et al. 2002, Mulenga et al. 2007). Another member of this lipocalin family that simultaneously binds histamine and serotonin was isolated from larval and nymphal salivary glands of *Dermacentor reticulatus*. Serotonin also functions as an important mediator of inflammation, whose activities are similar to those of histamine (Sangamnatdej et al. 2002). Although leukotriene-binding protein belongs to the same protein family, it binds highly specifically only leukotriene B4, resulting in the negative influence of neutrophil functions, especially on the chemotactic migration into the site of tick feeding and neutrophil activation (Beaufays et al. 2008). SGE from the partially fed females of *D. reticulatus* also decreases the activity of natural-killer (NK) cells (Kubeš et al. 1994), as well as does SGE from *I. ricinus* (Kopecký and Kuthejlová 1998). Inhibited NK cells produce lesser amounts of IFN-γ, the well-known Th1 cytokine, what contributes to the shift of the immune response toward Th2 (Hajnická et al. 2000).

2.2.2 Adaptive immunity

Adaptive immune response is generated relatively quickly, for example the detectable levels of IgM antibodies in mice arise on day 7 post infection (Barthold et al. 1991). Saliva of the tick vector modulates adaptive immune mechanisms, indeed. Besides the mentioned IgG binding proteins (Wang and Nuttall 1995a, b), tick saliva has an inhibitory effect on the production of cytokines by the LPS-stimulated B-lymphocytes and on the LPS and *Borrelia*-induced B-cell proliferation. The 18 kDa protein from *I. ricinus* was denoted to be responsible for these effects (Hannier et al. 2003, Hannier et al. 2004). *I. ricinus* SGE contains molecule(s) with direct inhibitory effect on B-cells, resulting in reduced expression of early activation marker CD69 and IL-10 production (Hannier et al. 2003). Actually, B-cell inhibitory protein, expressed from the cDNA library of the hard tick *Hyalomma asiaticum asiaticum*, was shown to affect LPS-induced B-cell proliferation (Yu et al. 2006).

T-lymphocyte functions are impaired under the influence of tick saliva in general (Wikel 1982, Kovář et al. 2001). T-lymphocytes from the hosts that underwent vaccination with SGE or repeated feeding of uninfected ticks are less reactive after the stimulation with T-mitogenes (Wikel 1982, Urioste et al. 1994, Ganapamo et al. 1996b, Rolníková et al. 2003), but surprisingly, the suppression correlates negatively with the number of infestations (Wikel 1982). Proteins present in tick saliva are responsible for the inhibition of T-lymphocyte proliferation in vitro (Urioste et al. 1994, Bergman et al. 1995, Ferreira and Silva 1999). Actually, a 65 kDa protein with this function was found in *I. ricinus* saliva (Ganapamo et al. 1997). In *Dermacentor andersoni* female salivary glands, a 36 kDa soluble protein that is produced during the tick feeding, mainly the sixth day of feeding, was identified, but the mechanism on its action has not been revealed yet (Bergman et al. 1998, Bergman et al. 2000, Alarcon-Chaidez et al. 2003). Both sialostatin L, and L2, respectively, have impact on the proliferation of cytotoxic T-lymphocytes and generation of inflammatory cytokines, which help the tick to feed properly (Kotsyfakis et al. 2006, Kotsyfakis et al. 2007, Kotsyfakis et al. 2008). Immunomodulatory protein Iris affects naive BALB/c spleen cell proliferation and human T-lymphocyte IFN-γ production after specific activation, whereas IL-5 and IL-10 levels remained unchanged or were inhibited (Leboulle et al. 2002b). Other members of Iris gene family were later found in other tick species (Mulenga et al. 2003).

The amount of cytokines produced by the Th1 T-lymphocyte subset, namely IL-2, IL-12, and IFN-γ, is significantly reduced in the presence of tick saliva (Ramachandra and Wikel 1992, 1995, Ferreira and Silva 1999). IL-2-binding protein, found in the saliva of *I. scapularis*, could be responsible for the reduction of free form of IL-2, as well as for the inhibition of proliferation, because IL-2 acts as the main inducer of T-cell proliferation (Gillespie et al. 2001). Moreover, Salp15, an immunomodulatory protein, was identified in the saliva of the same tick species (Das et al. 2001). The Salp15 binds specifically to the CD4 co-receptor on mammalian host cells, affects the tyrosine phosphorilation of several early signal components and represses hence the calcium fluxes triggered by TCR ligation, what results in lower IL-2 production and inhibition of CD4+ T-cell-mediated immune responses (Anguita et al. 2002, Garg et al. 2006, Juncadella et al. 2007). The production of Th2 cytokines is not reduced in the presence of tick saliva, in the contrary, levels of some Th2 cytokines as IL-10, IL-4, and TGF-β are elevated (Kopecký et al. 1999, Ganapamo et al. 1996a, Zeidner et al. 1997, Ferreira and Silva 1999). However, suppression of IFN-γ and IL-2 cytokines could be caused just by the elevated level of IL-10 (Ganapamo et al. 1996a, Kopecký et al. 1999).

Tick saliva effects mentioned above lead to the polarization of immune responses towards Th2 T-cell responses, which is effectual neither against the tick feeding, nor against *Borrelia* spirochetes (Jarefors et al. 2006). Immune response polarization was repeatedly demonstrated in hosts after feeding of various tick species (Ferreira and Silva 1999, Schoeler et al. 1999, Mejri et al. 2001) and in the co-cultures of immunocompetent cells with SGE (Kovář et al. 2002). Advantageously for the tick, some of the Th2 cytokines have substantial anti-inflammatory properties. As described previously, feeding of *I. scapularis* nymphs infected with *B. burgdorferi* spirochetes leads to the Th2 polarization in the C3H/HeJ mouse strain, known as *Borrelia*-susceptible, whereas in the *Borrelia*-resistant strain BALB/c this polarization was shown only transiently (Zeidner et al. 1997). Moreover, suppression of Th2 cytokines IL-4 and IL-5 before the feeding of *Borrelia* infected *I. scapularis* ticks notably reduced the spirochete burden in joints, bladder, heart, and skin of disease-susceptible host (Zeidner et al. 2008).

2.2.3 SAT

As mentioned above, saliva is essential for ixodid ticks, when they need to suck the host blood for relatively long time without being affected by the host immune system. Tick-induced host immunomodulation is increasingly recognized as a contributing factor in successful transmission and establishment of tick-borne pathogens, the transmission between co-feeding vectors could be also facilitated. Promotion of vector-borne pathogen transmission and activity, via the action of vector saliva on the host, has been termed saliva-activated transmission (SAT) (Nuttall and Jones 1991).

SAT was experimentally described as a facilitated transmission of pathogens in uninfected ticks feeding together (co-feeding) with the infected ones, whereas the high host viremia (bacteremia) was not required for the transmission (Jones et al. 1987, Jones et al. 1989, Labuda et al. 1993a, 1993c, Ogden et al. 1997). This is consider to be an indirect evidence of the SAT phenomenon. The increased number of ticks that became infected during feeding on nonviremic hosts inoculated with a mixture of pathogen and SGE (saliva), compared with ticks feeding on hosts inoculated with pathogen alone, is counted among direct evidences of SAT (Labuda et al. 1993b). Direct evidence is also specified as increased pathogen infectivity for the host in that case when the pathogen is syringe-inoculated in the combination with SGE or saliva, compared with the level of infectivity when the pathogen is injected alone (Macháčková et al. 2006).

The SAT phenomenon has been indirectly demonstrated for several tick-borne pathogens, for the first time for Thogoto virus (Jones et al. 1987, Jones et al.1989). Nonviremic transmission was subsequently observed for other tick-borne transmitted viruses, such as tick-borne encephalitic virus (Labuda et al. 1993a, Labuda et al. 1993b), Crimean-Congo hemorrhagic fever virus (Gordon et al. 1993), Bhanja virus, Palma virus (Labuda et al. 1997), and West Nile virus (Lawrie et al. 2004). Similar enhancement of tick infection rate in the presence of SGE was noticed for the Lyme disease pathogen, *B. afzelii* (Pechová et al. 2002), whereas it was not confirmed for other tick-borne bacterial pathogens, *Anaplasma marginale* and *Anaplasma phagocytophilum* (Hodzic et al. 2001, Kocan and de la Fuente 2003).

Enhancement of pathogen infectivity for the host induced by tick saliva was observed for some bacterial pathogens (Pechová et al. 2002, Zeidner et al. 2002, Kročová et al. 2003). Accelerated proliferation of *Francisella tularensis* was reported in the presence of *I. ricinus* SGE in mice, in the dermal site of infection, draining lymph nodes, and spleen (Kročová et al. 2003). Pechová with colleagues revealed the enhancement of *B. afzelii* loads in mouse blood and urinary bladder 1 day after subcutaneous injection of spirochetes mixed with SGE from *I. ricinus*, compared with spirochetes alone, while the tissue samples were cultivated 6 days and subsequently microscopically examined. Tick saliva had also the effect on the increase of incidence of spirochetes in blood and urinary bladders several days after infection (Pechová et al. 2002).

The first example of application of Real-Time PCR detection of *Borrelia* spirochetes in the SAT phenomenon studies was presented by Zeidner and his colleagues (Zeidner et al. 2002). They demonstrated increased numbers of *Borrelia* spirochetes in heart, bladder, joint and skin tissues eight weeks after infection using SGE from *I. scapularis* or *I. ricinus* nymphs, while the effect of SGE was both *Borrelia*- and tick-species dependent. It means that *I. scapularis* SGE promoted increase in *B. burgdorferi* N40-strain loads, whilst *I. ricinus* SGE enhanced amounts of *B. lusitanie* in mouse tissues. This indicates that *Borrelia* species are adapted on their vectors exploiting specific immunomodulatory properties of their saliva.

Immunomodulatory molecules present in tick saliva are probably responsible for the SAT effect; however, they may vary for different pathogens and vectors (Zeidner et al. 2002). It is not revealed so far, whether the SAT phenomenon for one pathogen is caused by one particular molecule, or if it is the result of combinations of several immunomodulatory molecules. In the case of Thogoto virus, SAT factor is not present in the salivary glands of flat ticks, while it is secreted into the saliva after the beginning of their blood meal (Jones et al. 1989, Jones et al. 1992). Actually, *Borrelia* spirochetes migrate along SGE gradient that attracts them, which could contribute to the transmission of *Borreliae* among co-feeding ticks (Shih et al. 2002).

Although there are several SAT factor candidates (Nuttall and Labuda 2004), only one molecule in the tick saliva has been proved to enhance pathogen activity in the host. The first identified SAT factor is well known Salp15 protein from the *I. scapularis* saliva, soon afterwards the SAT activity was also confirmed for its homologue from *I. ricinus* tick. Probably due to the protection of *Borrelia* spirochetes against complement-mediated killing and other immunomodulatory properties, Salp15 facilitate the infection of mice with *B. burgdorferi*. Moreover, the capability of spirochetes to infect mice is dramatically reduced after knocking down of Salp15 gene in *I. scapularis* by RNA *interference* (Ramamoorthi et al. 2005, Hovius et al. 2008a).

Many immunomodulatory molecules mentioned above may be potential SAT factors for *B. burgdorferi* or other tick-borne pathogens. Identification of factors responsible for the SAT effect is important step in the development of vaccines that enable to protect the host from the infection by most of pathogen species transmitted by one vector species (pathogen transmission-blocking vaccines). A vector based vaccine would not only protect against the pathogens known to be transmitted by the vector, but also against so far unidentified new ones (Wikel et al. 1997, Titus et al. 2006).

3. Objectives

The main aim of this thesis was to describe the influences of tick saliva on the establishment of *Borrelia* infection, including saliva activated transmission (SAT) of *B. burgdorferi* spirochetes.

Particular objectives:

- To describe the effect of tick saliva and salivary gland extract (SGE) on particular mechanisms of innate immunity operating against *Borrelia* infection, namely complement (alternative pathway of activation) and macrophages
- To evaluate the effect of tick saliva in vivo on the proliferation and distribution of *B. burgdorferi* s. s. in murine tissues during the early phase of infection
- To find out if the local effect of tick saliva in the skin influences the transmission of Lyme disease spirochetes from infected mice to uninfected ticks
- To determine the borrelial loads in these ticks
- To characterize the impact of anti-tick vaccination with salivary proteins on the saliva activated transmission of *Borrelia* spirochetes
- To obtain a cohort of nymphal ticks infected in known percentage with the specific strain of *Borrelia burgdorferi* sensu lato complex for further experiments considering searching for the SAT factors (using RNAi)

4. Summary of results and discussion

Exploring the effect of tick saliva and SGE on early mechanisms of innate immunity against *Borrelia* infection, which could be crucial for the establishment of Lyme disease, we focused on the alternative pathway of complement activation and macrophage functions.

The complement system has been shown to play a role in the tick rejection reactions (Wikel 1979) and also in the defence against tick-transmitted pathogens (Lawrenz et al. 2003). Not surprisingly, ticks have evolved complement inhibitors operating against the alternative pathway of complement activation (Ribeiro 1987, Lawrie et al. 1999, Valenzuela et al. 2000). For *I. ricinus*, anti-complement activity was demonstrated in SGE regardless of length of the feeding period (Lawrie et al. 1999), indicating that inhibition of complement activation is necessary from the beginning of tick feeding. Like other immunosuppressive activities of tick saliva, the anti-complement activity apparently could aid *Borrelia* spirochetes in their initial immune evasion and dissemination (Bubeck-Martinez 2005). We demonstrated the protection of *Borrelia* spirochetes against complement mediated killing owing to SGE in the presence of Mg-EGTA, the inhibitor of classical pathway of complement activation. Sera from different mammalian species were tested to select the suitable one with regard to sufficient killing potential in the 25% concentration. Eventually, calf serum was selected as complement source for further testing of the effect of SGE on the borreliacidal activity of complement. Calf serum is potent borreliacidal compound for most of *Borrelia* genomic species (Kurtenbach et al. 1998), in our study particular isolates of strains *B. burgdorferi* sensu stricto (B31, CB53) and *B. afzelii* (CB43) were lysed efficiently, surprisingly our isolate of *B. garinii* (BGH) was not susceptible to calf complement (Fig. 3). This discrepancy may be the consequence of the fact that some strains, respectively isolates, express molecules operating against host complement on their surface (Kraiczy et al. 2004a, b, Hartmann et al. 2006, von Lackum et al. 2005). Moreover, different isolates of *B. garinii* and even *B. afzelii* differ in the ability to bind complement regulator, factor H, which confers the resistance to complement-mediated killing (McDowell et al. 2003).

The influence of SGE on borreliacidal effect of complement was observed in all tested isolates (Fig. 3). SGE in the concentration 200 μg/ml (the dose of 10 μg of protein) effectively inhibited 12.5 μl of calf serum, which is comparable with that operate effectively against 10 or 12 μl of human or guinea pig serum, respectively, in a complement-mediated hemolysis assay (Lawrie et al. 1999).

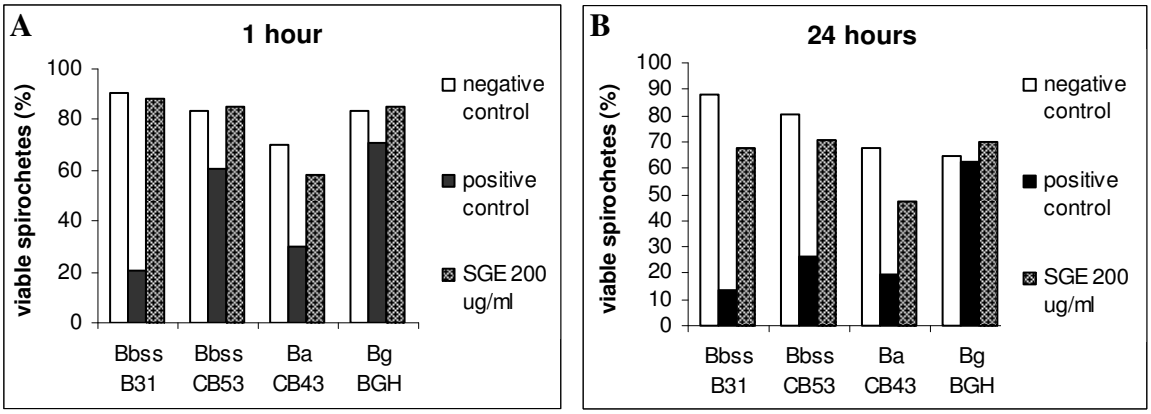

Fig. 3: Borreliacidal activity of calf serum complement on different *Borrelia* isolates, effect of SGE on this activity. *B. burgdorferi* sensu stricto (Bbss-B31 and CB53 isolates), *B. afzelii* (Ba-Cb43 isolate), and *B. garinii* (Bg-BGH isolate) were used for the experiment. *Borrelia* spirochetes were pretreated 10 minutes with SGE (200 μg/ml), fresh calf serum (25%) was added to the suspension. Classical pathway of complement activation was inhibited by EGTA. Heat activated serum served as a negative control; PBS instead of SGE was added to the positive control. Borreliacidal effects were estimated after 1 hour (A), or 24 hours (B) using dark-field microscopy.

We focused on the *B. afzelii* CB43 strain in our subsequent studies, considering that *B. afzelii* is the most prevalent genomic species transmitted among rodents and *I. ricinus* ticks in the Czech Republic (Bašta et al. 1999). Whereas higher concentrations of SGE (400, 200 μg/ml) protected the great majority of spirochetes against complement-mediated killing till the end of the experiment (48 hours), lower concentrations (50 and 20 μg/ml) significantly protected spirochetes just during the first 6 hours (Paper I. - Fig. 1).

The inhibition of complement-mediated *Borrelia* killing was not caused by the inhibitors of proteases (IP) present in SGEs, because IP added in appropriate concentration to the calf serum did not prevent lysis of spirochetes. Moreover, SGE had the same inhibitory effect when it was halved, and then one half was used supplemented with IP, the other without IP supplement (Fig. 4).

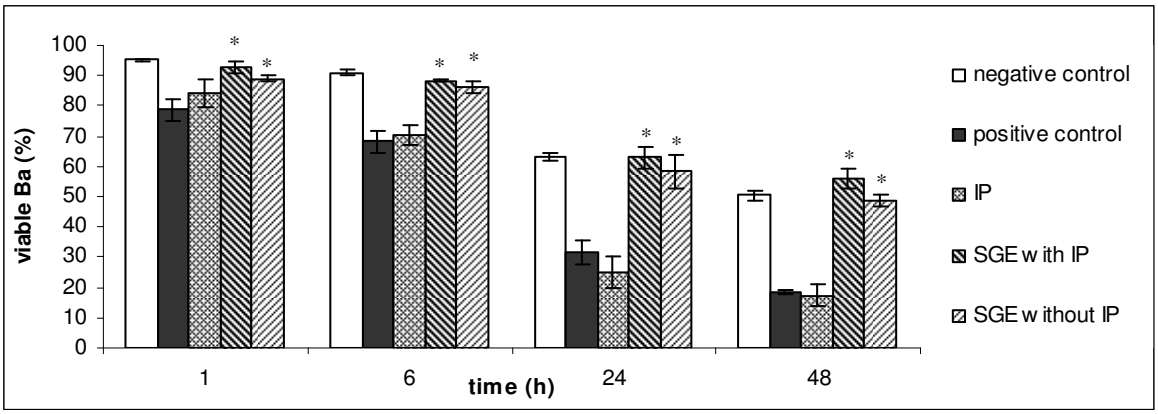

Fig. 4: Effect of IP on the borreliacidal activity of calf serum complement. *Borrelia afzelii* spirochetes (Ba-CB43 isolate) were pretreated with 200 µg/ml SGE with or without IP (diluted 500x), or with the corresponding concentration of IP in PBS. Fresh calf serum (25%) was added to the suspension. Classical pathway of complement activation was inhibited by EGTA. Heat activated serum served as a negative control; PBS instead of SGE was added to the positive control. Borreliacidal effects were estimated after 1, 6, 24, and 48 hours using dark-field microscopy. Each column represents an average of three cultures ± SD. The asterisk (*) indicates the difference between treated groups and control group was significant at $P<0.05$.

To my knowledge, this was the first report on the effect of tick saliva directly on the borreliacidal activity of serum complement. The observed effect could be caused by some of the molecules operating against alternative pathway of complement activation, namely by Salp15, Irac or other member of Isac family, eventually by other so far unidentified molecules, or by cooperation of these compounds (Schroeder et al. 2007, Tyson et al. 2007, 2008)

Phagocytosis was estimated by two ways, as the percentage of cells containing the spirochetes and the percentage of phagocytosed bacteria. Enumeration of phagocytosing cells showed that in both concentrations SGE significantly reduces their numbers at all three time-points of the experiment (Paper I - Fig. 2). The most remarkable effect of SGE was recorded at 60 minutes of incubation, when both SGE concentrations reduced the percentage of phagocytosing cells by more than one half.

Using ^{14}C-labelled spirochetes, we determined the effect of SGE on the proportion of phagocytosed bacteria (Paper I - Fig. 3). SGE significantly reduced the percentage of engulfed bacteria at 60 minutes as well as at 120 minutes of incubation with macrophages.

Ribeiro et al. (1990) reported the inhibitory effect of *I. scapularis* saliva on the phagocytosis of *B. burgdorferi* spirochetes by rat neutrophils. The reduced uptake of spirochetes by mouse peritoneal macrophages reported herein could reflect downregulation

of β2-integrins which mediate binding of unopsonized spirochetes to leukocytes. Such downregulation was caused by the action of *I. scapularis* saliva (Montgomery et al. 2004).

In addition to the effect of SGE on phagocytosis of *B. afzelii* spirochetes, we determined its impact on other macrophage functions, namely the production of nitric oxide (NO) and cytokines with proinflammatory activities, whereas the effect of SGE was compared with that of tick saliva. Both SGE and saliva markedly reduced production of TNF-α (Paper I – Fig. 4) by activated macrophages. Production of another pro-inflammatory cytokine IL-6 was not significantly influenced either by SGE or by saliva (Paper I - Fig. 5). Opposed results were obtained when the effect of SGE on the production of NO was compared with that of saliva (Paper I - Fig. 6). Whereas SGE increased production of NO by activated macrophages, saliva reduced elaboration of this killing molecule. We demonstrated that neither IP in SGE, nor pilocarpine (PC) used to the induction of salivation, have no effect on the production of any monitored compounds, except for the slight reduction of TNF-α production owing to PC (Fig. 5A-C). The PC contaminates tick saliva, hence we used the highest concentration of this substance determined by Ribeiro in *I. scapularis* saliva as a control (Ribeiro et al. 2004), even thought *I. ricinus* saliva contained lower amounts of PC in our experimental design. Reduced production of TNF-α could be caused by inhibition of the cytokine production by immunocompetent cells (Ramachandra and Wikel 1992, Gwakisa et al. 2001), however, a direct binding of TNF-α by tick salivary molecule has to be considered (Koník et al. 2006). The explanation of the discrepancy in NO production can consist in the presence of ballast proteins from salivary gland cells in SGE preparation, which can stimulate IFN-γ-activated macrophages to increased production of NO despite of the presence of NO down-regulating molecules. In the saliva, the effect of NO-inhibitory molecules predominates.

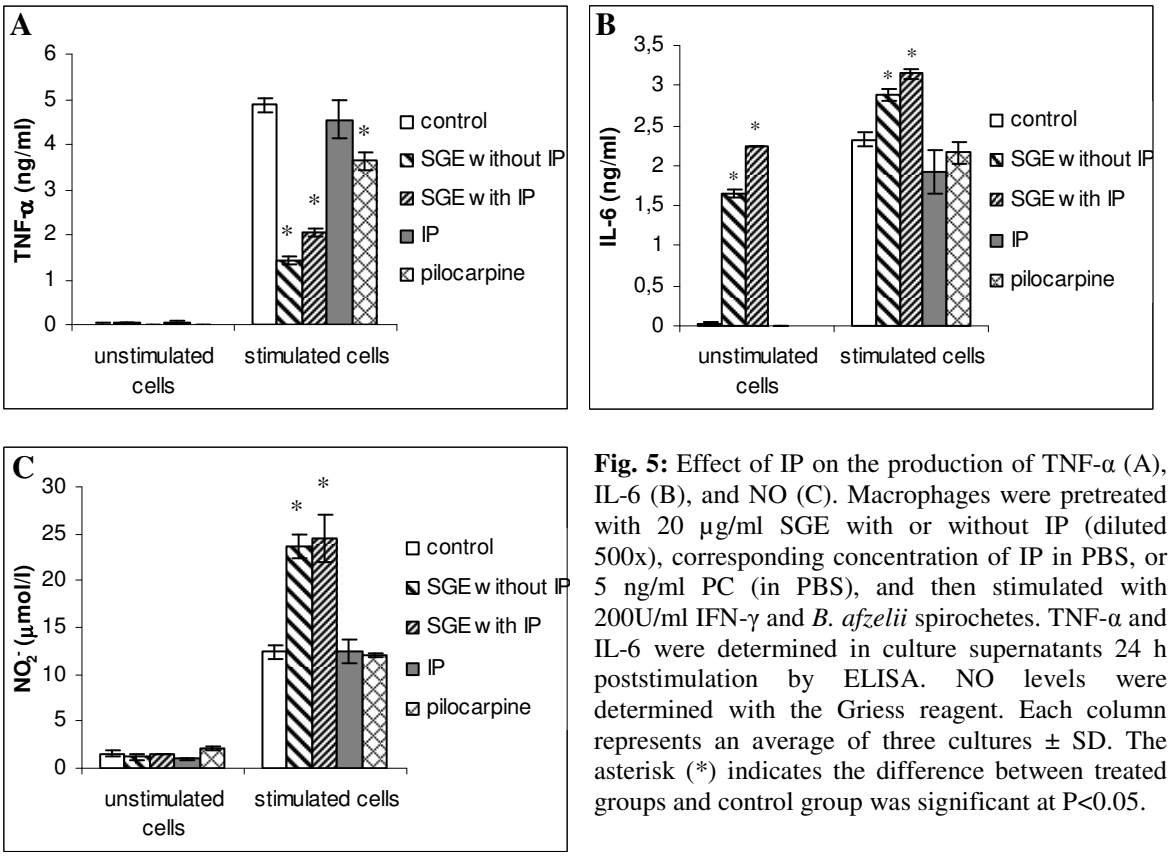

Fig. 5: Effect of IP on the production of TNF-α (A), IL-6 (B), and NO (C). Macrophages were pretreated with 20 μg/ml SGE with or without IP (diluted 500x), corresponding concentration of IP in PBS, or 5 ng/ml PC (in PBS), and then stimulated with 200U/ml IFN-γ and *B. afzelii* spirochetes. TNF-α and IL-6 were determined in culture supernatants 24 h poststimulation by ELISA. NO levels were determined with the Griess reagent. Each column represents an average of three cultures ± SD. The asterisk (*) indicates the difference between treated groups and control group was significant at $P<0.05$.

The following aim of our study was to find out if these in vitro observed remarkable affections of innate immune mechanisms induced by the tick saliva could influence in vivo the proliferation and distribution of *Borrelia spirochetes* in mouse tissues in the early phase of infection. We focused also on the effect of tick saliva on the transmission of Lyme disease spirochetes from infected mice to the uninfected ticks, including the evaluation of *Borrelia* loads in these ticks.

Increased levels of bacteremia and earlier appearance of *B. afzelii* spirochetes in the urinary bladder, as the effect of SGE, were reported based on the counting of spirochetes after a 6 day cultivation of organ specimens in BSK-H medium (Pechová et al. 2002). Cultivation of tissue samples with subsequent microscopically examination of spirochete presence and concentration is relatively poorly sensitive, that is the reason why Real-Time PCR (qPCR) is frequently used not only for the detection, but also for quantification of spirochetes (Pahl et al. 1999, Morrison et al. 1999). Although tick saliva-activated transmission of *Borrelia* spirochetes into the naive host has been already demonstrated using qPCR, rather late time points after infection were chosen for the detection of spirochetes. Therefore, the influence of tick saliva on mechanisms of adaptive immunity could be responsible for the observed effects (Zeidner et al. 2002).

We used the taq-man® probe-based method published by Zeidner (Zeidner et al. 2001), and focused on the early intervals during and just after tick sucking to evaluate, whether the direct effect of tick saliva on innate immunity mechanisms plays a role in saliva-activated transmission of *Borrelia* spirochetes. We continued with the work of our laboratory members, who observed accelerated proliferation of *B. burgdorferi* in the blood 1 day post infection (p.i.) due to the effect of SGE from *I. ricinus* (Macháčková et al. 2006), and focused on the dissemination of spirochetes to target organs, including the dynamics of this process.

Intradermal infection with *Borrelia* spirochetes resembles the natural infection via tick bite, whereas the site of infection can influence the development of *Borrelia* infection (de Souza et al. 1993). As little as 1 – 10 intrademally inoculated spirochetes are sufficient for mouse infection (Barthold 1991). We have chosen skin at the site of infection, draining lymph nodes, bladder, and heart for the examination of spirochete presence. Skin is the tissue of *Borrelia* initial entry, bladder is known to be a consistent source of spirochetes, and heart is the site of inflammation in mouse model of Lyme borreliosis (Liang et al. 2004). Microscopically, spirochetes were first found in the heart as late as 7 days p.i. within the walls of great vessels (Barthold et al. 1991).

Intradermal inoculation of spirochetes together with SGE, tick saliva or *I. ricinus* nymphs feeding in the vicinity of the inoculation site influenced both the proliferation and distribution of spirochetes in murine organs. While on day 1 post infection (p.i.) no significant difference between experimental groups was observed, mice inoculated with SGE showed a significantly increased spirochete number in the skin on days 4 and 6 p.i. (Paper II - Fig. 1A). Moreover, an insignificant increase of spirochete loads in the urinary bladder 6 days p.i. was observed (Paper II - Fig. 1B). On the other hand, there was a strong decrease of bacteria load in the heart of SGE-treated mice 4 and 6 days p.i. in comparison with control animals (Paper II - Fig. 1C). Inoculation of pure tick saliva increased the spirochete load in the urinary bladder on day 4 p.i. (Paper II - Fig. 1B), whereas spirochete burden in the heart 6 days p.i. was significantly decreased (Paper II - Fig. 1C). The feeding of *I. ricinus* nymphs positively influenced spirochete numbers in the bladder 6 days p.i. (Paper II - Fig. 1B), whereas the number of spirochetes found in the heart on day 6 p.i. was significantly lower than that in the controls. As for inguinal lymph nodes, a strong trend to increase spirochete loads due to the effect of saliva and nymph feeding 4 days p.i. was demonstrated (Paper II - Fig. 1D).

All three ways of influencing *Borrelia* infectivity decreased spirochete loads in the heart 6 days p.i., compared with the control group. One possible explanation could be the different expression of decorin-binding proteins on *Borrelia* surface, namely DbpA, that influence the colonization of host tissues. The lack of DbpA prevented the spirochetes from colonizing the heart tissue (Shi et al. 2008). It is tempting to speculate that factors present in tick saliva could downregulate expression of DbpA, eventually block its accessibility or reduce in some other way the occurrence of this protein on the spirochete surface.

However, only SGE significantly increased spirochete load in the skin within the inoculation site in our experiments, which was surprising, because the immunomodulatory effect of SGE in vitro mostly resembles that of saliva. The possible explanation can be the presence of IP in SGE, which could cause the retention of spirochetes in the host skin on day 1 p.i., due to blocking the activation of *Borrelia*-attached plasminogen (Coleman et al. 1995), thereby preventing the penetration of the bacteria into the blood stream. Indeed, besides the added IP cocktail, SGE contains many additional proteins originating from salivary gland cells, which are not present in saliva, causing probably the differences in the effect on NO production as well.

It is probable that *Borreliae* exploit the effect of early phase saliva proteins, because immunization with these proteins impairs *Borrelia* transmission (Narasimhan et al. 2007). The only SAT factor identified so far, Salp15, when inoculated together with *B. burgdorferi* spirochetes into the murine host, enhanced the spirochete load in the joints, bladder and skin 25 days post infection in both naïve and even *Borrelia*-immune mice (Ramamoorthi et al. 2005).

Ticks fed in the nymphal stage on mice infected with the spirochetes mixed with SGE, saliva or PBS, respectively, were in the adult stages tested on the presence of borrelial DNA. A marked increase in the percentage of infected ticks was reported in the group fed on mice co-inoculated with SGE or saliva (Paper II - Table 1). The effect of saliva was higher than that of SGE. The obtained results confirmed and extended our previous results considering SGE effects (Pechová et al., 2002) in terms of the determination of spirochete loads in infected ticks. The effect of SGE was not restricted to the percentage of infected ticks; the presence of SGE in the infectious inoculum also increased markedly the average number of spirochetes per one tick compared with the control group (Paper II - Fig. 2). The effect of saliva on spirochete numbers was only marginal. These differences between the results obtained using two different sources

of saliva may reflect much higher numbers of spirochetes present in the skin of mice co-inoculated with SGE (Paper II - Fig. 1A). The comparable percentage of infected ticks in both saliva-treated groups indicate that factors other than spirochete load in the skin influences the host infectivity for ticks or, in other words, that relatively low levels of spirochetes are sufficient for tick infection.

To contribute to the knowledge about in vivo effects of tick saliva on host immune mechanisms, we have started the experiments with tick saliva-based vaccination. We tried to determine if the vaccination with tick saliva can reverse the results obtained in the SAT experiment; that means, if the vaccination with the saliva suppresses the effects of salivary proteins on the proliferation and dissemination of Lyme disease spirochetes in mouse tissues. We have monitored *Borrelia* loads in tissues as well as production of typical Th1/Th2 cytokines by ex-vivo cell cultures derived from the draining lymph nodes of mice that had been vaccinated with either salivary proteins (10 µg) or BSA (10 µg) 3 times in 14 day intervals and then intradermally inoculated with 10^3 *Borrelia* spirochetes. Untreated mice served as a source of control lymph nodes.

Loads of *Borrelia* spirochetes were monitored in skin, heart, and urinary bladder of each *Borrelia*-inoculated mouse using Real-Time PCR. Spirochete burden was enhanced in skin of mice after feeding of *I. ricinus* nymphs, thus SAT effect developed, regardless of saliva vaccination (Fig. 6A). Regarding SAT effect in heart tissue, *Borrelia* load in heart of tick-exposed mice was markedly lower. This SAT effect was partially suppressed due to saliva vaccination (Fig. 6B). Spirochetes were detected in urinary bladder of non mice.

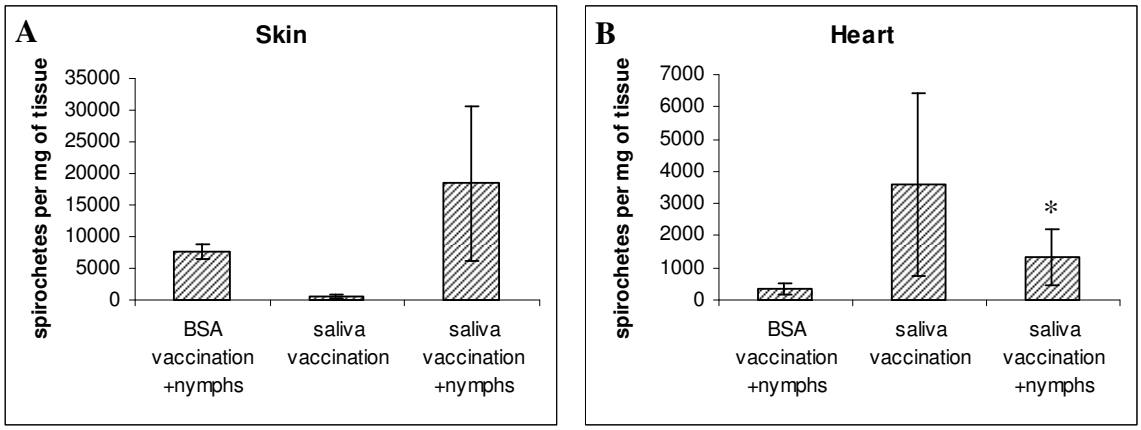

Fig. 6: Spirochete load in the skin (A) and heart (B) from C3H/HeN mice. Mice were vaccinated with either salivary proteins (10 µg) or BSA (10 µg) 3 times in 14 day intervals and then intradermally inoculated with 10^3 *B. burgdorferi* sensu stricto spirochetes. Twelve uninfected *I. ricinus* nymphs were allowed to feed on selected mice in the vicinity of inoculation site. Tissue specimens were collected on days 4 p. i. Each column represents an average number of spirochetes per mg of target tissue from 4 mice as measured by quantitative real-time PCR ± SD. The asterisk (*) indicates the difference between BSA and saliva vaccinated groups after feeding of *I. ricinus* nymphs was significant at $P<0.05$.

Preliminary results showed that BSA vaccinated mice manifest after *Borreliae* inoculation in combination with feeding of *I. ricinus* nymphs greatly upregulated levels of IL-10 produced by ex vivo cultured cells. Saliva vaccinated mice produced higher amounts of IL-10 after *Borrelia* and nymph challenge only when ex vivo cultured cells were stimulated with ConA (Fig. 7A). Elevated levels of IL-10 were also observed in C3H mice post infestation with *I. scapularis* nymphs on day 10-12, then decreased to the base line (Zeidner et al. 1997). Only slightly elevated levels of IL-10 in both saliva vaccinated groups of mice 3 weeks after last saliva vaccination indicate that saliva vaccination is able to decrease the saliva-induced polarization of immune reactions. This effect of saliva vaccination was not evident in case of IFN-γ, since the production differed in dependence on particular stimulus (Fig. 7B). Ex vivo cultured cells produced detectable levels of an important Th1 cytokine IL-2 only after stimulation with ConA, whereas notably lower levels of this cytokine were generated in all three treated groups, compared to the untreated control (Fig. 7C). It is likely that the production of IL-2 is affected by tick saliva and that the decreased levels persist more than two weeks after saliva exposure. The Th1 cytokines IL-2 and IFN-γ were either suppressed or did not differed significantly from controls after the repeated infestations with *I. scapularis* nymphs (Schoeler et al. 1999). However, BSA vaccinated group of mice without subsequent nymph feeding is required to asses the impact of *Borrelia* spirochetes injection itself on IL-2 production.

Strong polarization towards Th2 cytokine profile was observed only in the group of BSA vaccinated mice after *Borrelia* and *I. ricinus* nymphs exposure, where higher amounts of IL-10 and concurrently lower levels of IL-2 were detected. Partial polarization of immune reaction was observed after saliva vaccination, because only IL-2 production was downregulated, whereas IL-10 levels were low and in both vaccinated groups of mice. Feeding of *I. ricinus* nymphs on saliva vaccinated mice did not have, in contrast to feeding on BSA vaccinated mice, an additional polarization effect. Saliva vaccination did not affect feeding of nymphs; neither feeding periods nor engorged tick weighs differed in saliva and BSA vaccinated groups.

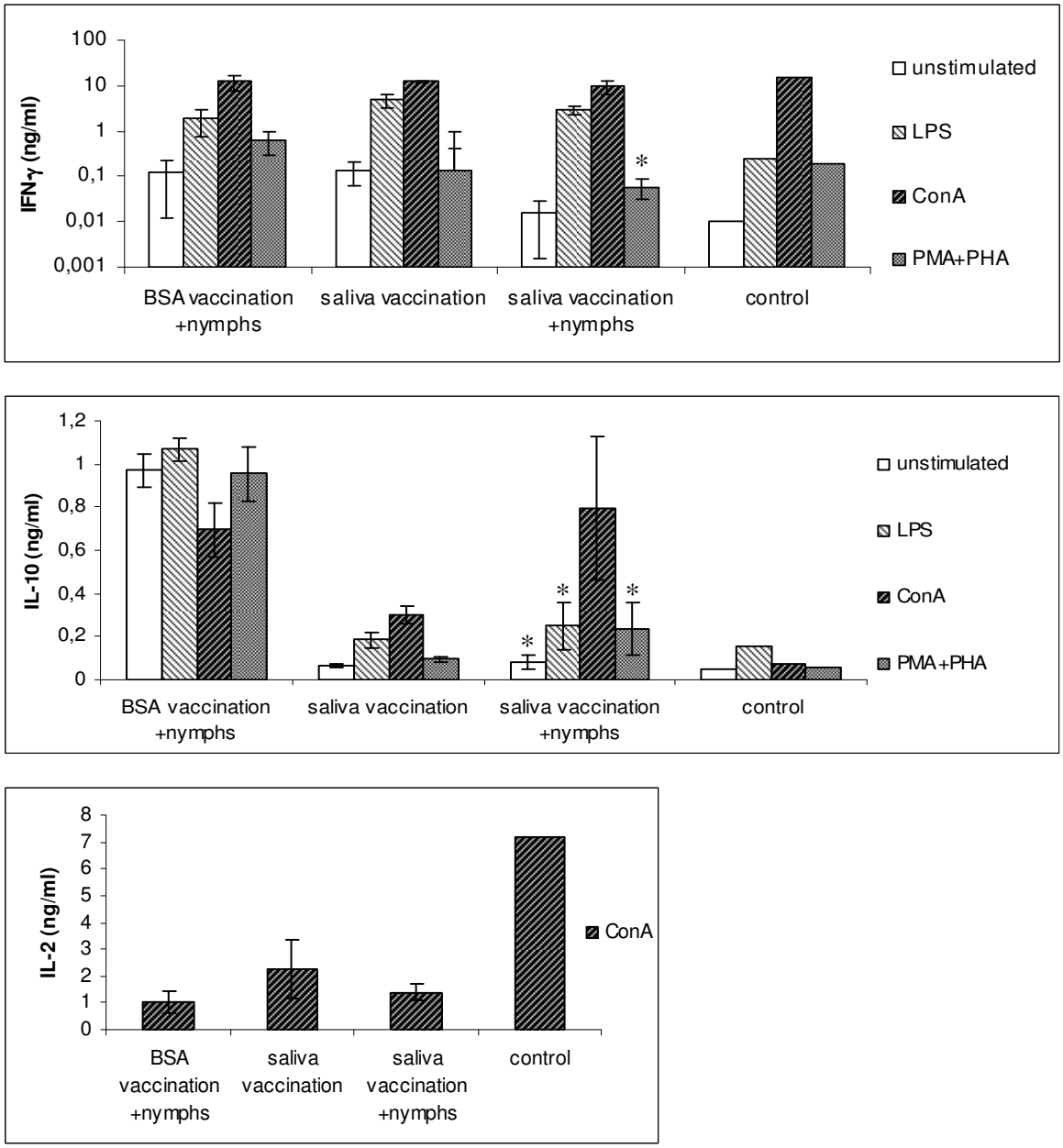

Fig. 7: The effect of saliva vaccination on cytokine production by ex vivo cultured lymph node cells. Mice were vaccinated with either salivary proteins (10 µg) or BSA (10 µg) 3 times in 14 day intervals and then intradermally inoculated with 10^3 *B. burgdorferi* sensu stricto spirochetes. Twelve uninfected *I. ricinus* nymphs were allowed to feed on selected mice in the vicinity of inoculation site. Axillary and brachial lymph nodes were collected on days 4 p. i., ex vivo cultures were stimulated with LPS, ConA, and PMA+PHA. Culture supernatants were collected on day 2 post stimulation and amounts of IFN-γ (A), IL-10 (B), and IL-2 (C) were measured by ELISA. Each column represents an average from 4 mice ± SD, except for the control group. The asterisk (*) indicates the difference between BSA and saliva vaccinated groups after feeding of *I. ricinus* nymphs was significant at $P<0.05$.

The third part of our study was aimed at searching for the best method for getting cohorts of *I. ricinus* nymphs infected in known rate with the defined *Borrelia* strain. These cohorts of nymphs infected with known *Borrelia* strain will be useful for the studies on saliva-activated transmission of *Borrelia* spirochetes, namely searching for the SAT molecule using gene knocking down by the RNA interference method. Furthermore, they

could be utilized for the study of different gene expression in infected and uninfected ticks, as well as for the pathogen-transmission studies and *Borrelia*-vector interactions, indeed.

In the first trail, only the method of *I. ricinus* larvae infection by the immersion in the suspension of *B. burgdorferi* sensu stricto was examined. We focused on the larvae viability, rate of infection in subsequent nymphal and adult stages and the capability to transmit *Borreliae* to naïve mice. All immersed larvae fed on naïve mice until full engorgement and 10 individuals from each group were PCR examined for *Borrelia* presence after molting. The metamorphosis to the nymphal stage was not affected by that way of infection. Nine of 10 tested individuals (90%) in *Borrelia*-immersed group and none in the control PBS-immersed group were PCR positive, however, mice fed on by *Borrelia*-immersed larvae did not produce specific anti-*B. burgdorferi* antibodies (Paper III – Tab. 1). It can be caused by the short time of *Borrelia* adaptation in the tick gut, because the larvae were placed on the mice immediately after immersion. The remaining nymphs infected in the larval stage were allowed to feed on naive mice until full engorgement and molt to adults. All of 10 individual adult ticks examined were PCR-positive. All the three mice, on which the infected nymphs had fed, were infected after tick feeding; spirochetal DNA was detected in at least one tested organ. Surprisingly, specific antibodies were not detected in the sera of these mice. Antibodies could often not be observed if the ticks were immersed in high-passage spirochetes (Policastro and Schwan 2003). However, the sixth passage of *B. burgdorferi* used in this experiment is commonly used in our laboratory to induce antibody response by syringe inoculation of mice. Moreover, spirochetes in larvae and nymphs were active, since they were able to infect naive mice. All control ticks and control mice were free of spirochetal DNA.

This method was described for *B. burgdorferi* sensu stricto and *I. scapularis* larvae (Policastro and Schwan 2003). It is alternative of the artificial infection of ticks by capillary feeding. Using capillary feeding, 30-70% on nymphal *I. scapularis* become infected and were capable to transmit infection to naïve mice although not as efficiently as naturally infected nymphs (Broadwater et al. 2002). The important advantage of immersion method is that we could treat already larvae and acquire nymphs with known rate of infection. These nymphs may be utilized for experiments using rodents as host animals for ticks, because rodents are not natural hosts for adult stages. Immersion is more reliable and less difficult to perform, if compared with capillary feeding, but it may last a rather long time until the ticks reach the next life stage and feed again. Due to the co-feeding of infected and uninfected ticks on the same host, which can result in the transmission of

B. burgdorferi to uninfected ticks (Gern and Rais 1996, Piesman and Happ 2001), the rate of infection in ticks can raise in every next stages of tick development. This is important for any experiment, in which ticks with a high rate of infection with defined *Borrelia* genomic species are necessary.

The second study was more complex and compared the previously reported procedure of immersion of tick larvae into the suspension of spirochetes to obtain infected nymphs with the infection of nymphs by feeding on infected mice. The rate of nymphal infection, the ability to transmit the infection to naïve mice and the reproducibility of the method were monitored.

Probably the best method for obtaining adult infected ticks is to feed nymphs on an infected rodent (Piesman 1993). It is less difficult to perform than capillary feeding, however the efficiency is dependent on the *Borrelia* genomic species. We have infected 15 mice in 3 groups, each group with different *B. burgdorferi* sensu lato genomic species known to cause Lyme disease in the Czech Republic – *B. burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii*. Almost all mice were tested for the presence of anti-*Borrelia* antibodies with positive results one month post infection. Nymphs of *I. ricinus* were allowed to feed on each mouse till repletion, and then the molted adults were PCR tested for the presence of borrelial DNA. Regardless of the *Borrelia* genomic species, 41% of nymphs became infected (Paper VI – Tab. 1). The highest infection rate was achieved with *B. burgdorferi* sensu stricto, the lowest with *B. afzelii*, which is in the contrast with the results of *Borrelia*-host complement sensitivity (Kurtenbach et al. 1998). Hence, it seems likely that the infectivity of particular isolates, dependent on the number of in vitro passages, can play a role in particular *Borrelia* genomic species transmission (Peavey and Lane 1996).

Immersion method is commonly used for delivery of various pathogens into the tick gut (Mitzel et al. 2007, Baldridge et al. 2007). To obtain *Borrelia*-infected nymphs, 3 groups of *I. ricinus* larvae were immersed into the suspensions of the same *Borrelia* genomic species and isolates as in the previous experiment. Nymphs molted from immersed larvae were *Borrelia*-positive in about 50%, the highest infection rate was observed for *B. burgdorferi* sensu stricto (65%), the lowest for *B. afzelii* (40%) (Paper VI - Tab. 2), which is not in agreement with the results of previous studies dealing with *Borrelia* complement sensitivity (Kurtenbach et al. 1998).

Generally, more consistent results of infection rates were obtained using the immersion method, where the percentage of infected ticks varied among tested genomic species between 40-65%. It was in contrast with the feeding on infected mice, where the infection rate varied from 16 to 64%. The advantage of immersion method was that we could obtain as many as 80 infected nymphs from 100 engorged larvae that completed their feeding on one animal, which means that all these ticks had the same conditions for becoming infected. This fact enables to test 10 individuals from each cohort before experiment and gain the information on the infection rate. Moreover, tick larvae feed very close together in one feeding place, therefore the probability of *Borrelia* transmission between among ticks raises. This situation fulfills the conditions for co-feeding, which could markedly enhance the percentage of infected ticks, whereas the bacteremia is not present in the host (Nuttall and Jones 1991). Infection of ticks by feeding on infected mice depends on the efficiency and phase of mouse infection. Additionally, maximum number of 15 nymphs can take the blood meal on one mouse, therefore no more than 15 infected adults are available for further experiments. Co-feeding can not assert in this case, because none of the nymphs have *Borrelia* spirochetes present in hemolymph or salivary glands before feeding.

Considering results presented in this thesis, as well as in the literature, we can conclude that tick saliva significantly affects mechanisms of host innate immunity operating against *Borrelia* infection. Tick saliva influences the onset of borreliosis and also the transmission of Lyme disease agent from infected host into feeding ticks this way. Vaccination of mice with the tick saliva does not seem to suppress *Borrelia* loads in host organism, but it partially modified the distribution of spirochetes and polarization of immune response after tick feeding.

Identification of SAT molecules using advanced methods of immunology and molecular biology, isolation of candidate SAT factors, and testing their effects on *Borrelia* transmission, is the next step to better understand the complicated relationships between pathogen, vector and their host. The challenge for the future experiments will be to asses how the SAT factors could be used to control tick-borne diseases.

5. Conclusions

- SGE from *I. ricinus* inhibited borreliacidal activity of calf serum complement (alternative pathway of activation), decreased phagocytosis of *Borrelia* spirochetes by mouse macrophages, reduced production of proinflammatory cytokines, and induced NO production by these cells
- Saliva from *I. ricinus* decreased levels of proinflammatory cytokines and NO generated by mouse macrophages
- SGE, tick saliva or *I. ricinus* nymphs feeding in the vicinity of the inoculation site influenced both the proliferation and distribution of *B. burgdorferi* in murine tissues, namely they enhanced *Borrelia* load in skin and urinary bladder, on the other hand they decreases spirochete burden in heart during the first week post infection
- Tick saliva and SGE, when intradermally inoculated together with *Borrelia* spirochetes, caused higher infection rates in feeding ticks, in comparison with intradermal inoculation of pure spirochetes
- *Borrelia* loads in these ticks were significantly higher in SGE treated group
- Vaccination with salivary proteins did not seem to have an impact on high *Borrelia* loads in skin and it only slightly enhanced low spirochete burden in heart, however, the polarization of the immune reaction towards Th2 due to tick feeding was partly inhibited
- Cohorts of nymphal ticks infected in known percentage (40-90%) with the specific strain of *Borrelia burgdorferi* sensu lato complex were obtained by immersion method, they will be in used in further experiments

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7. List of abbreviations

BSA	-	bovine serum albumin
BSK-H	-	Barbour-Stoenner-Kelly medium
CO2	-	carbon dioxide
ConA	-	concanavalin A
CRASPs	-	complement regulator-acquiring surface proteins
Dbps(A, B)	-	decorin binding proteins (A, B)
DC	-	dendritic cell
DNA	-	deoxyribonucleic acid
EGTA	-	ethylene glycol tetraacetic acid
ELISA	-	enzyme-linked immunosorbent assay
IFN	-	interferon
Ig	-	immunoglobulin
IL	-	interleukin
IP	-	protease inhibitor
kDa	-	kilodalton
LB	-	Lyme borreliosis
LPS	-	lipopolysaccharide
MAC	-	membrane attack complex
Mb	-	mega base
MBL	-	mannan-binding lectin
MCP-1	-	monocyte chemotactic protein 1
MHC	-	major histocompatibility class
MIP-1	-	macrophage inflammatory protein 1
NF	-	nuclear factor
NK	-	natural killer
NO	-	nitric oxide
OspA-F	-	outer surface proteins A-F
p. i.	-	post infection
PBS	-	phosphate buffer saline
PC	-	pilocarpine
PCR	-	polymerase chain reaction
PHA	-	phytohemagglutinin
PMA	-	phorbol myristate acetate

qRT-PCR	-	quantitative polymerase chain reaction
RNA	-	ribonucleic acid
RNAi	-	ribonucleic acid interference
SAT	-	saliva activated transmission
SD	-	standard deviation
SGE	-	salivary gland extract
TLR	-	Toll-like receptor
TNF	-	tumor necrosis factor
TROSPA	-	tick receptor for the OspA

8. Appendices

Paper I) Kýčková K., Kopecký J. 2006: Effect of tick saliva on mechanisms of innate immune response against *Borrelia afzelii*. *J. Med. Entomol.* 43(6): 1208-1214.

Paper II) Horká H., **Černá-Kýčková K.**, Skallová A., Kopecký J. 2009: Tick saliva affects both proliferation and distribution of *Borrelia burgdorferi* spirochetes in mouse organs and increases transmission of spirochetes to ticks. *Int. J. Med. Microbiol.* (in press).

Paper III) Horká H., **Černá-Kýčková K.**, Fišerová L. Kopecký J. 2008: Efficiency of experimental infection of *Ixodes ricinus* ticks with *Borrelia burgdorferi* spirochetes. *Int. J. Med. Microbiol.* 298 S1: 177-179

Paper IV) Fišerová L., **Černá K.**, Horká H., Kopecký J. 2008: Two ways of experimental infection of *Ixodes ricinus* ticks (Acari: Ixodidae) with spirochetes of *Borrelia burgdorferi* sensu lato complex. *Folia Parasitol. (Praha)*. 55(2): 150-154.

Kýčková K., Kopecký J. 2006: Effect of tick saliva on mechanisms of innate immune response against *Borrelia afzelii*. *J. Med. Entomol.* 43(6): 1208-1214.

Abstract:

The effect of salivary gland extract (SGE) and saliva from the tick *Ixodes ricinus* on the interaction of *Borrelia afzelii* spirochetes with mouse macrophages and on the borreliacidal effect of calf serum was studied. SGE reduced both the number of phagocytosing cells and phagocytosed bacteria. An inhibitory effect of SGE on the killing of spirochetes by the alternative pathway of complement was also observed. Both SGE and saliva down-regulated production of pro-inflammatory cytokine TNF-α by macrophages stimulated with IFN-γ and live *B. afzelii* spirochetes. Production of another macrophage cytokine IL-6 remained unchanged. SGE and saliva exerted a different impact on the production of nitric oxide by stimulated macrophages. Whereas SGE up-regulated NO production, saliva decreased it. The significance of immunosuppressive effects of tick saliva for the transmission of *Borrelia* spirochetes is discussed.

Abstrakt:

V této práci byl studován vliv extraktu ze slinných žláz (SGE) a slin z klíštěte *Ixodes ricinus* na interakci mezi *Borrelia afzelii* a myšími makrofágy a na boreliacidní účinek telecího séra. SGE snižovalo jak množství fagocytujících buněk, tak i fagocytovaných bakterií. Zároveň byl pozorován inhibiční efekt SGE na zabíjení spirochét alternativní cestou aktivace komplementu. Zatímco sliny i SGE snižovaly produkci prozánětlivého cytokinu TNF-α makrofágy aktivovanými IFN-γ a živými spirochétami *B. afzelii*, tvorba dalšího makrofágového cytokinu IL-6 ovlivněna nebyla. Sliny a SGE měly ale rozdílný vliv na produkci oxidu dusnatého (NO) aktivovanými makrofágy, tedy SGE produkci NO zvyšoval, sliny ji snižovaly. Je zde diskutován význam imunosupresivních účinků slin klíšťat na přenos spirochét *B. afzelii*.

Horká H., Černá-Kýčková K., Skallová A., Kopecký J. 2009 (in press): Tick saliva affects both proliferation and distribution of *Borrelia burgdorferi* spirochetes in mouse organs and increases transmission of spirochetes to ticks. *Int. J. Med. Microbiol.* (2008), doi:10.1016/ j.ijmm.2008.10.009.

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 day 4 and 6 post infection. 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 6 dpi, while the number of spirochetes in the heart on day 6 p.i. declined. The feeding of *I. ricinus* nymphs raised spirochete load in the bladder 4 dpi and 6 dpi. 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 ten 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 28000. Taken together, these results show a very early effect of tick saliva on he proliferation and distribution of *Borrelia* spirochetes in the host, probably due to the effect of saliva on the host innate immunity mechanisms.

Abstrakt:

Na modelu myši kmene C3H/HeN byl sledován přenos *Borrelia burgdorferi* sensu stricto spirochét aktivovaný slinami klíštěte *Ixodes ricinus*. Byl sledován a porovnáván vliv sání neinfikovaných nymf klíšťat a inokulace samotných klíštěcích slin nebo extraktu ze slinných žláz (SGE) na proliferaci a distribuci spirochét po intradermální aplikaci. Množství spirochét v tkáních myší bylo stanovováno metodou Real-Time PCR. SGE průkazně zvyšoval množství spirochét v kůži 4 a 6 dní po infekci, a naopak ho snižoval v srdci, jak bylo zjištěno srovnáním s kontrolními zvířaty. Inokulace klíštěcích slin měla

vliv na zvýšení množství borelií v močovém měchýři 6 dní po infekci, zatímco počet spirochét v srdci byl stejný den naopak snížen. Sáním nymf *I. ricinus* došlo k nárůstu počtu spirochét v močovém měchýři 4 a 6 dní po infekci. Šestý den po infekci došlo také u této skupiny myší ke snížení množství borelií v srdci ve srovnání s kontrolní skupinou. Prevalence spirochét v klíšťatech nakažených sáním na myších byla více než desetkrát vyšší v případě, že byly myši infikovány směsí borelií a slin, případně SGE, než když byly infikovány pouze samotnými spirochétami. Byl-li v inokulu přítomný SGE, došlo ke zvýšení průměrného počtu spirochét v jednom klíštěti z 0 na téměř 28000. Tyto výsledky prokazují velmi časný efekt slin klíštěte na množení a distribuci spirochét *B. burgdorferi* v hostiteli, tento efekt je způsoben pravděpodobně především potlačením mechanizmů přirozené imunity hostitele.

Horká H., Černá-Kýčková K., Fišerová L. Kopecký J., 2008. Efficiency of experimental infection of *Ixodes ricinus* ticks with *Borrelia burgdorferi* spirochetes. Int. J. Med. Microbiol. 298 S1:177-179.

Abstract:

Feeding of an ixodid tick on a *Borrelia*-infected vertebrate is the natural route of tick infection. To obtain a cohort of nymphs with a high prevalence of infection, the immersion of *Ixodes ricinus* larvae in the suspension of *B. burgdorferi* sensu stricto spirochetes was used. Immersed larvae fed on C3H mice until full engorgement, molted to nymphs and 9 of 10 nymphs were shown to be infected by PCR with specific primers. Specific antibodies in the sera of the mice, on which either immersion-infected larvae or infected nymphs had engorged, were not detected. However, nymphs molted from immersed larvae were able to infect naïve mice, and all the resulting 10 adults were infected.

Abstrakt:

Přirozenou cestou infekce ixodního klíštěte boreliemi je sání na infikovaném hostiteli. Abychom získali kohortu nymf s vysokou prevalencí infekce, použili jsme rychlejší metodu ponořování larev *Ixodes ricinus* do suspenze spirochét *B. burgdorferi* sensu stricto. Larvy po ponoření sály na myši kmene C3H až do odpadnutí a po přeměně do stádia nymf byly vyšetřeny metodou PCR, přičemž 9 z 10 vyšetřených nymf bylo pozitivní na přítomnost boreliové DNA. V séru myši, na které sály larvy po ponoření, nebyly překvapivě detekovány specifické protilátky proti boreliím, nicméně ve stádiu nymf byly tyto klíšťata schopna během sání infikovat myši. Všech 10 dospělců metamorfovaných z těchto nymf bylo PCR pozitivních na přítomnost borelií.

Fišerová L., Černá K., Horká H., Kopecký J. 2008: Two ways of experimental infection of *Ixodes ricinus* ticks (Acari: Ixodidae) with spirochetes of *Borrelia burgdorferi* sensu lato complex. *Folia Parasitol. (Praha)* 55(2):150-154.

Abstract:

A previously reported procedure for the introduction of *Borrelia* spirochetes into tick larvae by immersion in a suspension of spirochetes was tested on *Ixodes ricinus* ticks and three of the most medically important European *Borrelia* genomic species, *B. burgdorferi* sensu stricto, *B. garinii* and *B. afzelii*. The procedure was compared with "classical" infection of nymphs by feeding on infected mice. Both methods yielded comparable results (infection rate 44-65%) with the exception of *B. afzelii*, which produced better results using the immersion method (44%) compared with feeding on infected mice (16%). Nymphs infected by the immersion method at the larval stage were able to transmit the infection to naïve mice as shown by serology and PCR detection of spirochetal DNA in organs. The immersion method is faster than feeding on infected mice and provides more reproducible conditions for infection. It can be exploited for studies on both pathogen transmission and *Borrelia*-vector interactions.

Abstrakt:

Testovali jsme možnost nakažení larev klíšťat boreliemi ponořením do suspenze spirochét, metodu již dříve publikovanou, pro klíště *Ixodes ricinus* a tři nejvýznamnější evropské poddruhy borelií – *B. burgdorferi* s. s., *B. garinii* a *B. afzelii*. Tuto metodu jsme porovnávali s metodou infekce nymf klíšťat sáním na infikovaných myších. Oběmi metodami bylo dosaženo srovnatelných výsledků (44-65% míra infekce), s výjimkou *B. afzelii*, pro kterou bylo dosaženo lepších výsledků metodou ponoření klíšťat do suspenze (44%) ve srovnání se sáním na infikovaných myších (16%). Nymfy klíšťat infikovaných v larválním stádiu ponořením do suspenze borelií byly schopné přenést infekci do myší, což bylo prokázáno jak sérologií, tak i detekcí bakteriální DNA v orgánech myší pomocí PCR. Metoda ponořování je na rozdíl od sání na infekčních zvířatech rychlejší, umožňuje reprodukovatelnější podmínky infekce a může být proto využita pro studie jak přenosu patogenu, tak i interakce mezi boreliemi a vektorem.