Tick-host-pathogen interactions in Lyme borreliosis

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Since its discovery approximately 30 years ago, Lyme borreliosis has become the most important vector-borne disease in the Western world. This thesis describes in molecular detail novel tick-host-pathogen interactions in Lyme borreliosis, contributing to the understanding of the pathogenesis of this emerging zoonotic disease. We have focused on the interaction of the *Ixodes* tick salivary gland protein, Salp15, with both *B. burgdorferi* as well as the mammalian immune system. The inhibition of host immune responses on the one hand, and the protection of *B. burgdorferi* on the other hand, by this pleiotropic tick salivary protein are exemplary of the complexity of tick-host-pathogen interactions that collectively determine the outcome of an infection with *B. burgdorferi*. In addition, we show a delicate role for the host immune response in the genesis of Lyme borreliosis symptoms. The mammalian immune system should not generate a weak immune response, since this may fail to eradicate the spirochete, however an excessive immune response will lead to (irreversible) tissue damage and clinical symptoms; this is not an enviable task with both the arthropod vector as well as the bacterium trying to tip the balance of this fragile equilibrium. Importantly, better understanding of the host immune response to *Borrelia* and ticks will bring us closer to the development of clear-cut diagnostic tests and therapeutic compounds that can specifically and favorably target the immune response against the bacterium.

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Colofon

Tick-host-pathogen interactions in Lyme borreliosis
Dissertation, Academic Medical Center, University of Amsterdam

Author: Joppe Willem Robert Hovius
Cover picture: The character on the cover of this thesis is the fictional character 'The Tick'. 'The Tick' is a surreal parody of superheroes and the protagonist of the homonymous cartoon. The Tick is TM & © 2009 Benjamin Edlund and is used with permission.
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The cover of this thesis has been designed in its current form because of several reasons. Firstly, ticks can be a nuisance to mankind and can transmit a variety of pathogens to humans, including *Borrelia burgdorferi*, the causative agent of Lyme borreliosis. The clouds underscore this ever emerging danger. On the other hand tick saliva contains many physiologically active molecules that could serve as a template for the development of therapeutical agents to be used in daily clinical practice, making them, like 'The Tick', little superheroes. The sun is to underscore this side of the tick. Furthermore, the author of this thesis spent some time at Yale University (New Haven, United States of America) and at the Academic Medical Center (AMC) (Amsterdam, The Netherlands). This is represented by the red building in the back, a distorted animation of the AMC, and the colors used in the picture, red, white and blue; colors used in both the Stars & Stripes and the Dutch national flag. Finally, it is not a coincidence that the thesis describes the presence and function of a specific tick protein present in both American and European ticks.

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Dit is Hovius' geschenk, waaruit de heelkracht blijkt, die nog natuur bezit, wanneer de kunst bezwijkt.

– Jacobus Hovius (1710-1786) –

Voor mijn ouders, Taco & Wendy en mijn lieve nichtjes

Preface

Historical aspects of Lyme borreliosis

Lyme borreliosis was first recognized as a distinct clinical entity in 1975 in Old Lyme, Connecticut, USA (Fig. 1A and B), in children attending the Yale-New Haven Hospital and initially thought to have juvenile rheumatoid arthritis [1], although certain clinical signs that were part of the disease were previously described in Europe [2,3]. In the early 1980s Burgdorfer and colleagues were the first to culture the causative spirochetal agent, *Borrelia burgdorferi* sensu stricto from ticks in an endemic area [4], and this spirochete was later cultured from patients with Lyme disease symptoms [5,6]. More than 30 years after the identification it has become clear that the causative agent of this emerging zoonosis belongs to a broader group of spirochetes referred to as the *Borrelia burgdorferi* sensu lato group, encompassing several pathogenic *Borrelia* species [7]. Their corkscrew-shaped appearance allows the bacterium to move through tick and host tissues (Fig. 1C).

Ecological and biological aspects of the life cycle of *Borrelia burgdorferi*

The obligate enzootic life cycle of the spirochetes involves ticks (Fig. 1D) and a variety of vertebrate hosts, including small rodents, large mammals and birds [8,9]. In general, uninfected tick larvae acquire the bacterium by feeding on infected animals. Ticks remain infected during their consecutive molting periods, enabling both nymphal and adult ticks to transmit spirochetes to other (larger) animals and humans. After their final bloodmeal adult female ticks, which have already mated, lay uninfected eggs; vertical transmission only rarely occurs. In the United States *Borrelia burgdorferi* sensu stricto [4], from here on referred to as *B. burgdorferi*, is the causative agent of Lyme borreliosis [5] and is mainly transmitted by the deer tick, *Ixodes scapularis*. By contrast, in Europe at least three major pathogenic *Borrelia* species, i.e. *B. burgdorferi*, *Borrelia garinii* and *Borrelia afzelii* are prevalent and able to cause Lyme borreliosis [10,11] and are primarily transmitted by the sheep tick, *Ixodes ricinus*. Both *Ixodes* species are capable of transmitting spirochetes while feeding. Importantly, during the course of the bloodmeal, ticks also introduce saliva into the host skin, containing a wide range of physiologically active components that interfere with host processes or interact with pathogens. The aim of this thesis was to elucidate tick-host-pathogen interactions that could directly or indirectly affect the ability of *Borrelia burgdorferi* sensu lato to survive in the host, since these could be important factors in the pathogenesis of Lyme borreliosis. Apart from the introduction (**Chapter 1**), we have divided the thesis in four sections: tick-host interactions (**Part I**), tick-pathogen interactions (**Part II**), host-pathogen interactions (**Part III**) and spin-off of research on tick-host-pathogen interactions (**Part IV**).

Part I: Tick-host interactions

Adult *Ixodes* ticks require five to seven days to feed to repletion [12]. In order to secure attachment of the tick and to ensure susceptibility of reservoir hosts for future tick infestations, tick saliva contains modulators of host immune responses. In the first section of the thesis we investigated

the immunosuppressive activity of a 15 kDa feeding induced *I. scapularis* salivary protein, Salp15 (**Chapter 2 and 3**).

Part II: Tick-pathogen interactions

Salp15 was originally identified in *I. scapularis* [13,14]. In chapter 4, we determined whether *I. ricinus*, the major vector for Lyme borreliosis in Europe, express *salp15* related genes (**Chapter 4**). Besides possessing immunosuppressive activity *I. scapularis* Salp15 has been shown to bind to *B. burgdorferi* Outer surface protein (Osp)C [15]. This interaction protects the spirochete from antibody-mediated complement dependent killing by the immune host. In this thesis we characterized the interaction of the three major pathogenic European *Borrelia* species with one of the Salp15-related proteins in *I. ricinus* saliva, Salp15 Iric-1 (**Chapter 5**)**.** In addition, we investigated the binding capacity of the *I. ricinus* Salp15 homologue to OspC from other, complement-sensitive, *Borrelia* isolates and assessed the effect of this interaction on antibody-independent complement-mediated killing *in vitro* (**Chapter 6**).

Part III: Host-pathogen interactions

The host immune response is crucially important in the pathogenesis of Lyme borreliosis [16]. Therefore, in a cohort of naturally *Borrelia* infected asymptomatic and symptomatic pet dogs we investigated the extent of the humoral immune response (**Chapter 7**). In humans, all three *Borrelia* species frequently cause an expanding migrating red skin lesion, classically accompanied by central clearing, and designated *erythema migrans* [17]. When untreated, the spirochete can disseminate and cause disease that affects various organs, however *B. burgdorferi*, *B. garinii* and *B. afzelii* are associated with different disease manifestations [11]. In this thesis we studied the effect of simultaneous infection with *B. burgdorferi* and *B. garinii* on the course of experimental murine Lyme borreliosis (**Chapter 8**). In *in vitro* studies *B. burgdorferi* has been shown to upregulate expression of the urokinase Plasminogen Activator Receptor (uPAR; CD87; Plaur) [18,19], a receptor expressed on a variety of immune cells and important in the instigation of both innate and adaptive immune responses. In this thesis we investigated the role uPAR in the course of experimental murine Lyme borreliosis *in vivo* (**Chapter 9**).

Part IV: Spin-off of research on tick-host-pathogen interactions

Tick saliva contains a wide range of physiologically active molecules that are crucial for attachment to the host or for the transmission of pathogens [15], and that interact with host processes, including coagulation and fibrinolysis, and immunity and inflammation [20,21]. In this chapter we speculate how immunologically targeting specific tick salivary proteins could prevent the transmission of tick-borne pathogens, such as *Borrelia burgdorferi*, from the tick to the host (**Chapter 10**). Importantly, we also discuss molecules in tick saliva that have been intensively studied in vitro

or in animal models for human diseases, and that, due to their specificity, are potential future anticoagulant or immunosuppressive agents (**Chapter 10**).

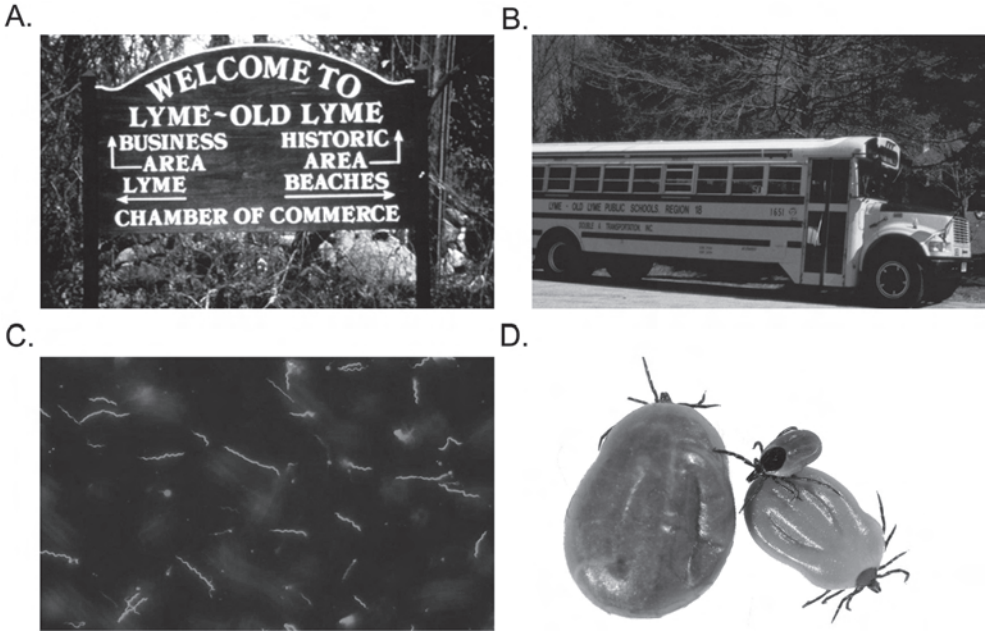

Figure 1. Historical, ecological and biological aspects of Lyme borreliosis.
(A) Lyme disease was named after the town Old Lyme, Connecticut, USA.
(B) The Old Lyme public schoolbus. Picture: K.E. Hovius.
(C) *Borrelia burgdorferi* visualized by microscopy.
(D) *Ixodes* ticks are the main vector for Lyme borreliosis. Picture: G.A. Oei.
For color figure see page 257.

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Tick-host-pathogen interactions in Lyme borreliosis

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Chapter 1

Abstract

Borrelia burgdorferi, the spirochetal agent of Lyme borreliosis, is predominantly transmitted by *Ixodes* ticks. Spirochetes have developed many strategies to adapt to the different environments that are present in the arthropod vector and the vertebrate host. This review focuses on *B. burgdorferi* genes that are preferentially expressed in the tick and the vertebrate host, and describes how selected gene products facilitate spirochete survival throughout the enzootic life cycle. Interestingly, *B. burgdorferi* also enhances expression of specific *Ixodes scapularis* genes, such as *TROSPA* and *salp15*. The importance of these genes and their products for *B. burgdorferi* survival within the tick, and during the transmission process, will also be reviewed. Moreover, we discuss how such vector molecules could be used to develop vector-antigen-based vaccines to prevent the transmission of *B. burgdorferi* and, potentially, other arthropod-borne microbes.

Ixodes–mouse–*Borrelia* interactions

Ticks are acarid ectoparasites that, while taking a bloodmeal, can transmit a variety of human and animal illnesses. Lyme borreliosis is a common tick-borne disease in parts of the USA, Europe and Asia. *Ixodes scapularis*, *Ixodes ricinus* and *Ixodes persulcatus* are the most important vectors for Lyme borreliosis in the USA, Europe and Asia, respectively. Lyme borreliosis is caused by spirochetes of the *Borrelia burgdorferi* sensu lato group [1]. In Europe and Asia, three major *Borrelia* genospecies (*B. burgdorferi* sensu stricto, *Borrelia garinii* and *Borrelia afzelii*) are the causative agents. By contrast, only *B. burgdorferi* sensu stricto strains are present in the USA. In humans, all three species frequently cause an expanding, migrating red skin lesion, classically accompanied by central clearing, and designated erythema migrans. When the infection is untreated, the spirochete can disseminate and cause disease that affects various organs, including the joints, central nervous system and skin. *B. burgdorferi* sensu stricto has been linked with arthritis, whereas *B. garinii* is often associated with neuroborreliosis and *B. afzelii* is the dominant cause of a late cutaneous disease manifestation – acrodermatitis chronica atrophicans [2-3].

The enzootic life cycle of *B. burgdorferi* sensu stricto, from here on referred to as *B. burgdorferi*, involves the tick and vertebrate host. Generally, uninfected *I. scapularis* larvae acquire the bacterium by feeding on infected small animals, such as the white-footed mouse, *Peromyscus leucopus*. Ticks remain infected during the molting period. Nymphs can then transmit spirochetes to other mammals, including mice and humans, while taking their next bloodmeal. Nymphs thereafter molt to become adults that can also transmit the spirochetes during feeding. After the final bloodmeal, adult female ticks (which have already mated) lay eggs. These eggs are not infected with *B. burgdorferi* because the spirochetes are usually not transovarially transmitted. *B. burgdorferi* has developed mechanisms to survive in both the arthropod vector and reservoir host, and differentially expresses certain genes depending on the environment. Some of these gene products have direct interactions with tick proteins, whereas others bind to, or interact with, reservoir host proteins. Interestingly, *B. burgdorferi* enhances expression of certain *I. scapularis* genes that are beneficial for either vector colonization or for spirochete transmission from the vector to the host. Both tick and *Borrelia* proteins that influence successful *Borrelia* infection represent potential candidates for vaccine and/or drug development, and are the focus of this review.

The *B. burgdorferi* genome

B. burgdorferi is an extracellular organism belonging to the order Spirochaetales. The genome is roughly 1.5×10^6 base pairs [4] comprising a linear chromosome and 21 linear and circular plasmids, together containing 1780 genes [5-6]. A large portion of the genome encodes lipoproteins [4], such as the well-studied outer surface proteins (Osp) A and C. Several *Borrelia* proteins have been identified that have interactions either with host or tick ligands and assist pathogen survival [7-14].

B. burgdorferi gene expression in the mammalian host

Upon entry into the host, the first obstacle *B. burgdorferi* has to overcome is the host innate immune system. A crucial line of defense in the innate immune response against invading microorganisms is the complement cascade, consisting of the classical, lectin and alternative pathways. The three pathways merge at the common intersection of complement factor C3. C3 is cleaved by C3 convertase to give C3a and C3b. Binding of C3b to the surface of microorganisms generally results in phagocytosis. A fraction of C3b can bind to C5, which is the first step towards formation of the membrane attack complex (MAC). Formation of the MAC on the microorganism's surface causes cell lysis. Many bacteria, such as *Streptococcus pneumoniae* [15] and *Neisseria meningitidis* [16], have evolved mechanisms to inhibit complement-mediated killing by binding to host plasma factor H or factor-H-like (FHL) protein resulting in factor I-mediated degradation of C3b. *B. burgdorferi* utilizes complement regulating-acquiring surface proteins (CRASP) [9] and Osp E/F related proteins (Erp) [8, 17-18] to bind factor H or FHL protein, and consequently inhibit complement-mediated borreliacidal activity. The ability to inhibit complement varies between the different *Borrelia* genospecies [19]. Also, Erp proteins have different relative affinities for factor H proteins from various potential animal hosts. Stevenson et al. suggest this enables the bacterium to inhibit complement-mediated killing in the diverse hosts it encounters during its enzootic life cycle [20].

Early in infection, *B. burgdorferi* expresses several genes that might be important for survival. For instance, decorin-binding protein A and B (DbpA and B) [7] and the fibronectin-binding protein BBK32 [21] bind to the host extracellular matrix components decorin and fibronectin, respectively, as has been shown by biochemical studies [7, 12]. Adhesion to extracellular host components might assist in survival of the few initial spirochetes that are transmitted through a tick bite [22]. Decorin-deficient mice infected with *B. burgdorferi* show diminished *Borrelia* numbers and less severe arthritis than infected wild-type controls [23]. It has also been shown that immunization of mice with recombinant DbpA induced protective immunity against *B. burgdorferi* [24]. The significant attenuation of Lyme borreliosis in mice infected with BBK32-deficient *B. burgdorferi* would suggest the importance of BBK32 [25], although other studies suggest that BBK32 is not essential for spirochete infectivity and pathogenicity [26]. By contrast, other *B. burgdorferi* genes are downregulated during the course of infection. Liang et al. showed that of the 116 lipoprotein encoding genes, which *B. burgdorferi* expresses early in infection, less than 40 were expressed several weeks after infection [27]. Host immune pressure is likely to be involved in this selection [28].

Once a disseminated infection has been established, *B. burgdorferi* needs to evade adaptive host immune responses. One mechanism that might be important in immune evasion is recombination at the variable major protein-like sequence (*vls*) locus [29-30], which has also been described in other *Borrelia* genospecies [31]. The *vls* locus consists of an expression site for the lipoprotein VlsE and 15 unexpressed upstream silent cassettes [32]. Upon infection of the host, segments of

the silent *vls* cassettes randomly recombine into the *vlsE* gene, resulting in multiple variations of the VlsE protein during the course of infection [32-34]. In mice experimentally infected with *B. burgdorferi* [29-30, 34] and in human Lyme disease patients [35], strong antibody responses directed against conserved regions of the VlsE protein have been reported. In addition, in these experimentally infected mice, antibody responses against surface exposed [36] variable regions of VlsE protein variants were observed [34]. The ability of *B. burgdorferi* to survive in the presence of this robust anti-VlsE antibody response indicates that *vls* antigenic variation, resulting in changes of the variable regions and altered antigenicity [34] of the VlsE protein, protects the spirochete from destruction by anti-VlsE antibodies. Recombination seems to be promoted by host inflammatory responses [29]. In line with this, there is no detectable variation of VlsE in ticks [37].

We have discussed several *B. burgdorferi* genes that are differentially expressed in the mammalian host, but *B. burgdorferi* also differentially expresses selected genes in various tissues within the same host [38]. To study gene expression of *B. burgdorferi* in neuroborreliosis in primates, Narasimhan et al. modified a PCR-amplification strategy, differentiation expression using customized amplification libraries (DECAL), originally developed by Alland et al. to examine mycobacterial genes in vivo [38-39]. A large number of *B. burgdorferi* genes were found, both on the chromosome and plasmids, that were either up- or downregulated in the central nervous system (CNS) compared with other tissues. These genes are promising candidates for future research on the molecular interaction of the spirochete with the CNS.

B. burgdorferi gene expression in the tick and *B. burgdorferi*-induced tick gene expression

While infected *Ixodes* ticks engorge, the antigenic composition of *B. burgdorferi* changes drastically. *B. burgdorferi* genes upregulated in engorging *I. scapularis* nymphs include those encoding putative lipoproteins and periplasmic proteins [40]. The biological function of OspA, a *B. burgdorferi* protein produced while the spirochete resides in the *I. scapularis* gut [41-43], but downregulated during tick engorgement, has been established. A strain of *B. burgdorferi* deficient for OspA and OspB was able to infect mice and cause arthritis [44], but could not colonize the tick gut [45]. This strongly suggests that OspA and/or OspB have an important role in persistence of the spirochete within the vector. Indeed, Pal et al. identified a tick receptor for OspA, designated the tick receptor for OspA (TROSPA) [11] (Figure 1). *TROSPA* was highly expressed in the guts of tick larvae and nymphs, and, to a lesser extent, in the gut of adult ticks. In infected nymphs *TROSPA* was expressed more abundantly than in uninfected controls. It might be advantageous for the spirochete to induce the tick to produce high levels of TROSPA so that the spirochete can persist in the tick gut during the long interval between bloodmeals. In line with this, TROSPA mRNA levels in flat ticks were significantly higher than in engorged ticks. Recently, it was shown that an OspB-deficient *B. burgdorferi* strain had impaired ability to adhere to the tick gut [10]. Neelakanta et al. suggest a synergistic interaction of OspA, OspB and TROSPA [10].

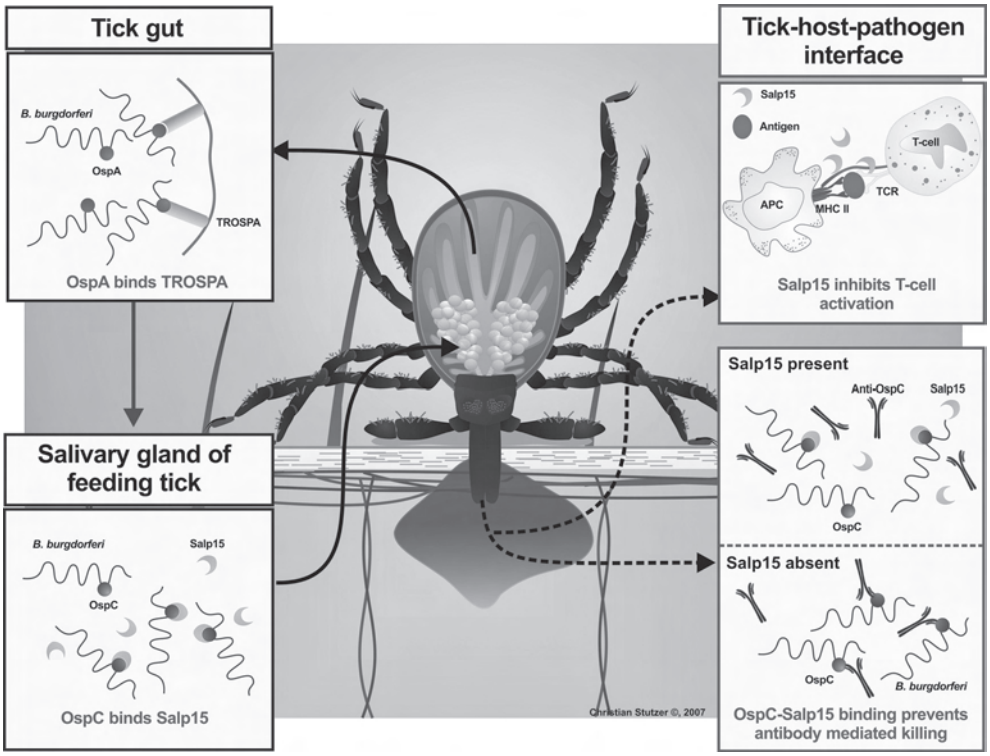

Figure 1. Schematic overview of tick–host–pathogen interactions important in the development of Lyme borreliosis. In the gut of *Ixodes scapularis* ticks, *Borrelia burgdorferi* upregulates *ospA* expression. OspA binds to the tick receptor for OspA (TROSPA) [11]. During tick feeding, *B. burgdorferi* downregulates *ospA* expression, starts producing OspC and migrates to the salivary gland. OspC binds to a tick salivary protein, Salp15. Salp15 has immunosuppressive properties, such as inhibiting $CD4^+$ T-cell activation [48], and immunoprotective effects, such as inhibiting antibody-mediated killing of *B. burgdorferi* by the host [13]. For other tick–host–pathogen factors that are of importance in Lyme borreliosis see the text. Unbroken arrows represent processes that take place within the tick, and broken arrows represent processes that take place at the tick–host–pathogen interface. Abbreviations: APC, antigen presenting cell; MHC, major histocompatability complex; OspA and OspC, outer surface protein A and C; TCR, T cell receptor. For color figure see page 258.

While ospA is downregulated within the gut of the engorged tick, the spirochete produces OspC during migration to the salivary glands and initial mammalian infection. Pal et al. have shown that *B. burgdorferi* recombinant OspC binds to *I. scapularis* salivary gland extracts [46]. Moreover, IgG F(ab)2 fragments from polyclonal OspC antisera reduce invasion of the salivary gland by *B. burgdorferi*. They also created an OspC-deficient spirochete that migrated poorly to the salivary glands and transmission of the OspC mutant to the murine host was dramatically impaired. This is in line with data from Grimm et al. that showed that OspC mutants were unable to infect severe combined immunodeficient mice (SCID); however, they showed (by immunofluorescense assays (IFA) and confocal microscopy) that OspC mutants were able to invade the tick salivary gland [47].

The spirochete enhances expression of certain tick genes, among which *salp15*, a gene encoding for a 15 kDa feeding-induced salivary gland protein. Salp15 inhibits activation of T lymphocytes by binding to the CD4 co-receptor on the surface of T lymphocytes [14, 48]. A recent study showed that Salp15 also interacts with *B. burgdorferi* by binding to OspC [13]. This binding protects the spirochete from antibody-mediated killing (Figure 1). Syringe infection of naive mice with *B. burgdorferi* and recombinant Salp15 resulted in significantly higher *Borrelia* numbers compared to infection with *Borrelia* alone. Moreover, mice with an acquired immune response to *B. burgdorferi* – mice infected with *B. burgdorferi* and thereafter treated with ceftriaxone – could not be re-infected with *B. burgdorferi* given as an intradermal challenge. However, these mice could be infected when they were (re-)infected with a cocktail of *B. burgdorferi* and recombinant Salp15. RNA interference-mediated repression of *salp15* in *I. scapularis* ticks drastically reduced the capacity of these ticks to transmit spirochetes to mice [13]. In nature, increased Salp15 levels could be beneficial for both vector and spirochete, because the tick might use Salp15's immunosuppressive capacities to avoid rejection and engorge more effectively, whereas the spirochete, by binding to Salp15, is capable of invading hosts that have previously encountered *B. burgdorferi*. Interestingly, Salp15 homologues were recently also identified in the European vector for Lyme borreliosis, *Ixodes ricinus* [49].

Implications for tick-antigen-based vaccine development to prevent Lyme borreliosis

A large number of human pathogens are transmitted by specific arthropod vectors. Understanding the interactions between vector and pathogen might help in developing strategies to combat arthropod-borne infections. The rationale for a tick-antigen-based vaccine is that repeated exposure of animals to tick bites results in an inability of ticks to successfully take a bloodmeal from these animals, as measured by impaired attachment, lower post engorgement weights, increased levels of tick mortality and lower fertility rates [48]. Acute basophil hypersensitivity, altered cytokine production profiles and circulating antibodies against tick antigens might all contribute to this phenomenon, also known as 'tick immunity' [50]. Interestingly, tick immune animals are less susceptible to transmission of several pathogens, including *B. burgdorferi* [51].

Several tick-antigen-based vaccines have been developed with mixed success and some of them also affect pathogen transmission [52]. It was shown that mice vaccinated with a recombinant *Rhipicephalus appendiculatus* cement protein, 64TRP, and challenged with tick-borne encephalitis virus (TBEV)-infected *I. ricinus*, were largely protected from lethal infection [53]. De la Fuente et al. identified an *I. scapularis* protein, sobulesin, that is involved in tick feeding and reproduction, and is highly conserved among a broad range of tick species [54]. They showed that ticks fed on mice vaccinated with recombinant sobulesin were unable to efficiently acquire *Anaplasma phagocytophilum* [52]. Recently, 500 new predicted salivary gland proteins were identified by comparing random clones from salivary gland cDNA expression libraries derived from fed, unfed,

B. burgdorferi-infected, uninfected, nymphal and adult *I. scapularis* ticks [55]. Understanding the function of these novel proteins in tick biology is crucial for development of new tick-antigen-based vaccines.

Because the interactions of TROSPA and Salp15 with *B. burgdorferi* have been established, in the next paragraphs, we will focus on TROSPA and Salp15 as potential candidates for tick-antigen-based vaccines to prevent Lyme borreliosis. A TROSPA vaccine could lead to decreased *B. burgdorferi* colonization and survival in the tick gut, and might diminish pathogen transmission to subsequent hosts. Previously, it had been shown that vaccinating wild mice with an OspA-based vaccine results in a reduction of the prevalence of *B. burgdorferi*-infected ticks, thus potentially lowering the risk of pathogen transmission to humans [56-58]. In theory, uninfected *I. scapularis* ticks feeding on *B. burgdorferi*-infected, yet also TROSPA-vaccinated, mice, will not acquire the spirochetes because TROSPA antibodies could interfere with *B. burgdorferi* binding to the tick gut. Therefore, vaccination of wild mice in endemic areas with a TROSPA-based vaccine, alone or in combination with an OspA vaccine, could lead to decreased *B. burgdorferi* infection rates of *I. scapularis* populations. Appropriate vaccine delivery systems would need to be developed. However, oral OspA-based preparations have been shown to protect laboratory mice against *B. burgdorferi* infection [59].

Interfering with OspC and Salp15 binding could, theoretically, result in diminished transmission of spirochetes from the vector to the host. Salp15 was originally identified by screening an *I. scapularis* salivary gland cDNA expression library with tick immune rabbit sera, suggesting that antibodies against Salp15 participate in tick rejection [60]. A vaccine against Salp15 could inhibit or diminish pathogen transmission from the tick to the host in two distinct ways. Salp15 antibodies could neutralize the immunosuppressive effects of Salp15 and thereby impair tick engorgement, making the tick–host–pathogen interface a more hostile environment for both tick and *B. burgdorferi*. In addition, Salp15 antibodies could bind to Salp15 that has previously bound to OspC on the surface of *B. burgdorferi* in the tick salivary gland, and thereby enhance clearance by host phagocytotic immune cells. The Salp15 antibodies would need to recognize a different Salp15 epitope than the epitope that is required for binding of Salp15 to OspC.

Summary

We have reviewed some of the interactions between *B. burgdorferi*, *I. scapularis* and the mammalian host. *B. burgdorferi* genes that are preferentially expressed in the tick and the mammalian host are crucial for spirochete survival throughout the enzootic life cycle. *B. burgdorferi* might also enhance the production of *I. scapularis* proteins that are crucial for *Borrelia* survival within the tick and transmission to the host. Future studies should focus on the identification of both vector and pathogen proteins that are important in pathogen transmission or vector colonization because these could be potential candidates for novel vaccination or therapeutic strategies for Lyme

borreliosis. The mechanism by which *I. scapularis* TROSPA and Salp15 interact with *B. burgdorferi* could serve as a model to understand the interaction of other vectors and pathogens, and lead to new prevention strategies.

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PART I

TICK-HOST INTERACTIONS

Salp15, an *Ixodes scapularis* salivary protein, inhibits CD4$^+$ T cell activation

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Chapter 2

Abstract

Tick saliva has pleiotropic properties that facilitate persistence of the arthropod vector upon the host. We now describe a feeding-inducible protein in *Ixodes scapularis* saliva, Salp15, that inhibits $CD4^+$ T cell activation. The mechanism involves the repression of calcium fluxes triggered by TCR ligation resulting in lower production of interleukin-2. Salp15 also inhibits the development of $CD4^+$ T cell-mediated immune responses in vivo, demonstrating the functional importance of this protein. Salp15 provides a molecular basis for understanding the immunosuppressive activity of *I. scapularis* saliva and vector-host interactions.

Introduction

Ixodes scapularis ticks are the vector for several medically important pathogens, including the causative agents of Lyme disease and human granulocytic ehrlichiosis (Burgdorfer et al. 1982 and Chen et al. 1994). Tick-host interactions have been the subject of extensive investigation (Allen 1989 and Wikel and Bergman 1997). *I. scapularis* requires a 5 to 7 day attachment period on the mammalian host in order to feed to repletion (Binnington and Kemp, 1980). The long period of tick engorgement allows the host to generate immune responses directed toward secreted components of the vector. In turn, *I. scapularis* attempts to modulate these responses in order to effectively attach to and feed upon its host (Binnington and Kemp 1980; Kopecky et al. 1999; Ramachandra and Wikel 1992; Urioste et al. 1994 and Wikel 1999).

Tick salivary proteins that enter the host during feeding have multiple effects, including the ability to inhibit the complement cascade (Ribeiro, 1987, 1995; Valenzuela et al., 2000), impair NK cell function (Kopecky and Kuthejlova, 1998), reduce circulating antibody titers (Wikel and Bergman, 1997), repress production of cytokines, such as interleukin-2 (IL-2) and IFN-γ (Ferreira and Silva 1998; Ramachandra and Wikel 1992 and Schoeler and colleagues 1999), and inhibit the proliferation of T lymphocytes (Ramachandra and Wikel 1992 and Urioste et al. 1994). Immunosuppression of the host by tick saliva may result in more efficient transmission of several tick-borne pathogens (Wikel 1999 and Zeidner et al. 1996). Recently, a protein-associated IL-2 binding activity (Gillespie et al., 2001) has been described in tick saliva.

Several animal species acquire resistance to ticks after repeated exposure to the vector, a phenomenon known as tick immunity (Brown and Askenase 1985; Trager 1939; Wang and Nuttall 1999 and Wikel 1996). Altered cytokine expression levels (Schorderet and Brossard, 1994), acute basophil hypersensitivity at the site of the tick bite (Askenase et al., 1982), and circulating antibodies to several tick salivary gland proteins are all thought to contribute to this phenomenon (Girardin and Brossard 1989 and Worms et al. 1988). Tick-immunity results in impairment of attachment of adult and nymphal ticks, lower postengorgement weights, and increased levels of mortality. Therefore, it is not surprising that tick saliva contains components that modulate the host immune response. Assessing the components in tick saliva that are responsible for immunosuppression may lead to a further understanding of vector-pathogen-host interactions. We here report the characterization of a 15 kDa tick salivary protein that inhibits the activation of $CD4^+$ T cells through the repression of calcium signals triggered by TCR engagement.

Results

Salp15 inhibits $CD4^+$ T cell proliferation

At least 14 antigens in *I. scapularis* saliva that are recognized by the mammalian host during tick exposure were identified by probing an *I. scapularis* cDNA expression library with sera from rabbits

that had been fed upon by ticks (Das et al., 2001). The sequence analysis of one of those antigens, Salp15, revealed weak similarities with two motifs of Inhibin A, a member of the TGF-β superfamily (FingerPRINTScan search tool at http://bioinf.man.ac.uk/cgi-bin/dbbrowser/fingerPRINTScan/muppet/FPScan.cgi) (Mason et al., 1985) (Figures 1A and 1B; E value: 1.9 × 103). Similar to tick saliva, TGF-β inhibits the production of cytokines, such as IL-2, and the proliferation of T cells (Wahl, 1994), suggesting that Salp15 may have immune-modulatory properties. Salp15 was expressed and purified in a *Drosophila* cell line-based system for use in functional assays (Figure 1C, lane 1). Tick-immune rabbit sera reacted with Salp15 in a Western blot assay (Figure 1B, lane 2), confirming that antibodies against tick saliva recognize the recombinant protein. Recombinant Salp15 migrated at 24–27 kDa in denaturing SDS-PAGE (Figure 1B), due to posttranslational modifications, consistent with other *I. scapularis* proteins (Das et al., 2000). The glycosylation of the protein was confirmed by staining with periodate-Schiff's reagent (data not shown). Rabbit

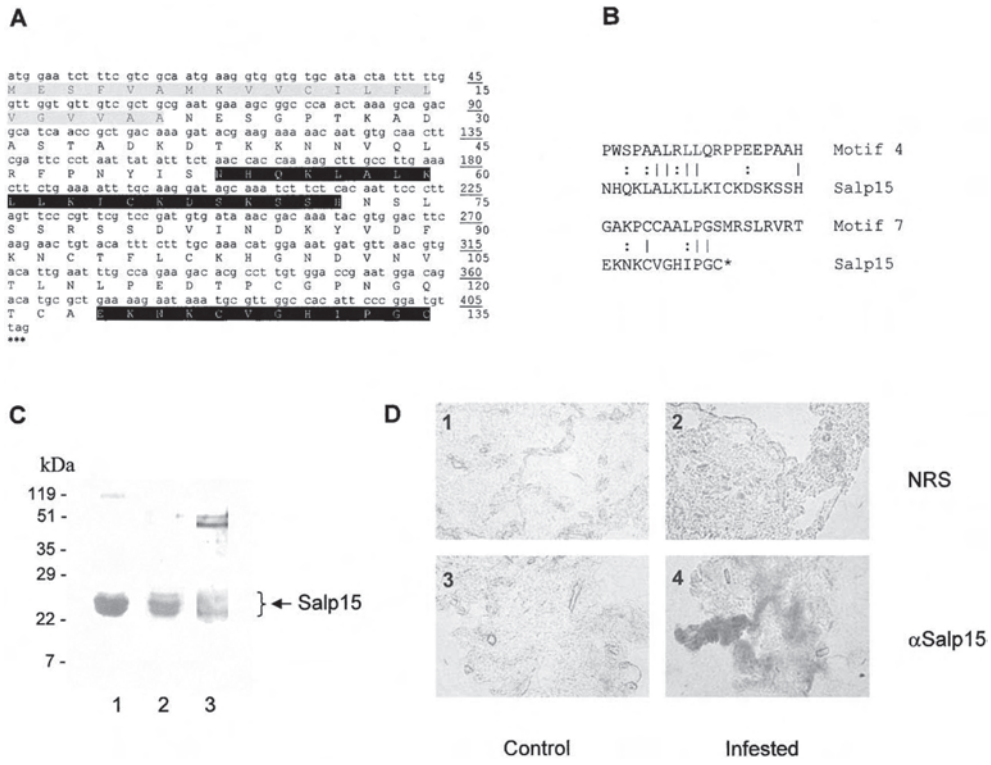

Figure 1. Characterization of Salp15. (A) Nucleotide and amino acid sequence of Salp15. The putative 21 amino acid signal sequence is highlighted in gray. The amino acid sequences highlighted in black represent regions that are similar to Inhibin A fingerprints. (B) Comparison of motifs 4 and 7 of the rat Inhibin A molecule and Salp15. Identical residues are linked with (|); similar residues are marked with (:). The * indicates the end of the sequence. (C) Recombinant Salp15 produced in *Drosophila* cells stained with Coomassie blue (1), Western blot analysis of the recombinant protein probed with tick-immune rabbit sera (2), and whole saliva probed with anti-Salp15 antibodies produced in rabbits (3). (D) Immunohistochemistry to detect Salp15 at the site of tick attachment. Skin biopsies of tick-infested mice were probed with Salp15 antisera (4) or normal rabbit sera (2) 2 days after tick detachment. Controls included the skin of naive mice (1 and 3).

antisera raised against the recombinant protein recognized Salp15 in whole tick saliva (Figure 1C, lane 3). Some crossreaction was observed with proteins in the range of 50–55 kDa. Sera from naive rabbits did not react with proteins in tick saliva (data not shown). Salp15 could be readily detected in skin biopsies of mice 2 days after tick detachment (Figure 1D), indicating that the protein is present in vivo at the site of natural inoculation.

We examined the effect of Salp15 on the proliferation of purified $CD4^+$ T cells in response to anti-CD3 and anti-CD28 monoclonal antibodies (mAb). Proliferative responses were impaired by Salp15 in a dose-dependent manner (Figure 2A). To rule out a cytotoxic effect of Salp15 on $CD4^+$ T cells, their viability was tested by trypan blue exclusion at different time points of activation. No differences were observed in cell viability between control and Salp15-treated $CD4^+$ T cells at 12, 24, and 42 hr of activation (Figure 2B). Salp15 activity was not due to induction of TGF-β during the activation process (data not shown). We then assessed the ability of Salp15 to bind $CD4^+$ T cells. Purified $CD4^+$ T cells were incubated with fluorescein-labeled Salp15 and analyzed by flow cytometry. Increasing concentrations of Salp15FITC bound to $CD4^+$ T cells (Figure 2C). We also tested the binding capacity of Salp15 in a microtiter assay. Increasing concentrations of Salp15 bound to the membrane preparations (Figure 2D). The binding capacity of Salp15 was abolished

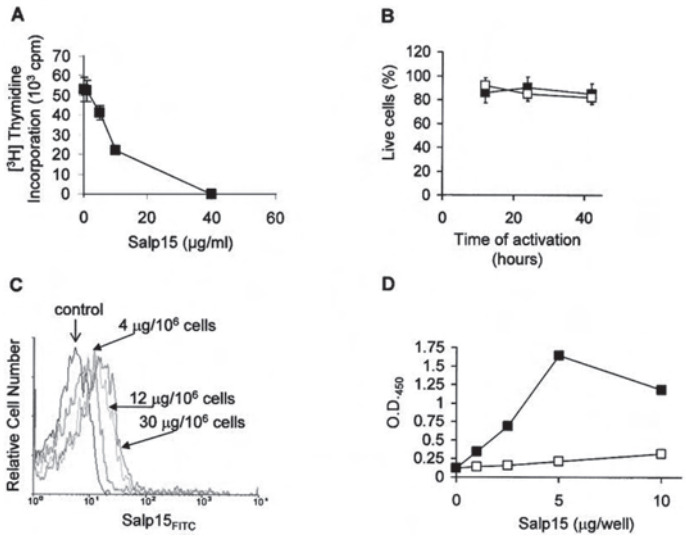

Figure 2. Salp15 inhibits $CD4^+$ T cell proliferation. (A) 10^6 naive $CD4^+$ T cells per ml were activated with anti-CD3/CD28 mAbs for 78 hr in the presence of different concentrations of Salp15. The cells were pulsed with 1 µCi [^{3}H] thymidine for the last 18 hr of the assay. (B) The viability of $CD4^+$ T cells (10^6 cells/ml) activated with anti-CD3/CD28 mAbs in the presence of Salp15 (50 µg/ml) (open squares) was analyzed by trypan blue exclusion at different times of activation. Closed squares represent control studies without Salp15. (C) Purified $CD4^+$ T cells were incubated with $Salp15_{FITC}$ (4 µg, left line; 12 µg, middle line; and 30 µg per 10^6 cells, right line). The cells were then analyzed by flow cytometry. (D) Membrane fractions were purified from $CD4^+$ T cells and used in a microtiter assay to determine binding of Salp15 (closed squares). Preincubation of the membrane fractions with trypsin resulted in elimination of Salp15 binding (open squares). The results are representative of at least three independent experiments.

when the membrane preparations were preincubated with trypsin (Figure 2D), suggesting that Salp15 is binding to a protein component on the surface of $CD4^+$ T cells.

Salp15 inhibits IL-2 production and CD25 expression in $CD4^+$ T cells

We then examined the effect of Salp15 on IL-2 production by $CD4^+$ T cells activated with anti-CD3 and anti-CD28 mAbs in the presence of Salp15. The level of IL-2 produced by $CD4^+$ T cells stimulated in the presence of Salp15 was lower (Figure 3A) than in the control untreated cells. The inhibitory effect of Salp15 on $CD4^+$ T cell IL-2 production was dose dependent (Figure 3A) and evident throughout the activation period (Figure 3B). The inhibition of IL-2 production was also observed at the mRNA level in purified $CD4^+$ T cells that had been activated for 16 hr

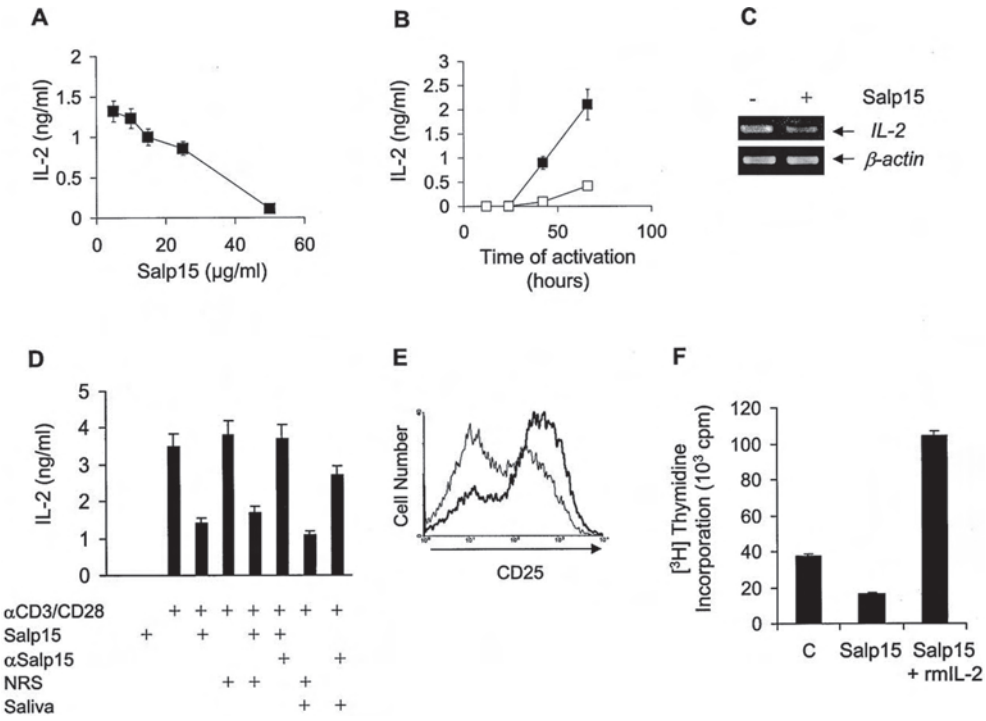

Figure 3. Salp15 inhibits IL-2 production and CD25 expression of anti-CD3/CD28 mAbs-activated $CD4^+$ T cells. (A and B) 10^6 purified naive $CD4^+$ T cells per ml were activated in vitro with plate-bound anti-CD3 mAb and soluble anti-CD28 mAb in the presence of different concentrations of Salp15 for 44 hr (A) or 50 μg per ml of Salp15 (open squares) during different time periods (B). At the specified time points, IL-2 levels in the culture supernatants were measured by capture ELISA. Black squares represent controls without Salp15. (C) IL-2 mRNA was detected by RT-PCR in 16 hr stimulated $CD4^+$ T cells in the presence of anti-CD3/CD28 mAbs ± 50 μg/ml of Salp15. β-actin was used as a control to ensure equal loads of RNA. (D) 10^6 purified $CD4^+$ T cells were incubated with anti-CD3 + anti-CD28 in the absence or presence of 50 μg/ml of Salp15 or tick saliva (1/100 dilution) ± rabbit Salp15 antisera (1/500 dilution). Normal rabbit sera (NRS) was used as a control. The supernatants were analyzed at 48 hr of activation for IL-2. (E) 10^6 purified $CD4^+$ T cells/ml were activated with anti-CD3/CD28 mAbs in the absence (thick line) or presence (thin line) of 50 μg/ml of Salp15. At 40 hr the cells were washed and stained for CD25 expression. (F) 10^6 $CD4^+$ T cells/ml were activated with anti-CD3/CD28 mAbs in the presence of 50 μg/ml of Salp15 ± exogenous recombinant murine IL-2 (10 ng/ml). At 60 hr of activation, the cells were pulsed with [^{3}H] thymidine for 18 hr.

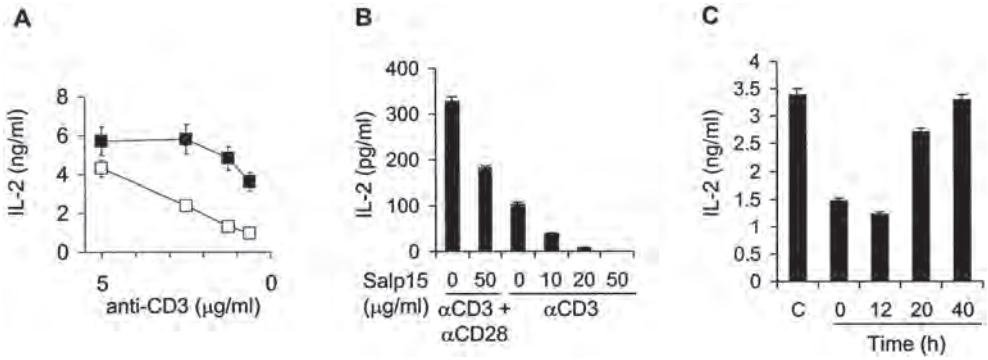

Figure 4. Salp15 inhibits early TCR-mediated signals. (A) 10^6 naive $CD4^+$ T cells per ml were activated with different concentrations of anti-CD3 mAb plus 1 μg/ml of anti-CD28 mAb for 44 hr in the presence of 20 μg of Salp15 per ml (open squares) or without Salp15 (black squares). (B) 10^6 $CD4^+$ T cells per ml were activated with plate-bound anti-CD3 (5 μg/ml) in the absence of costimulation and in the presence of different concentrations of Salp15. The supernatants were analyzed for IL-2 after 24 hr. As a control, purified $CD4^+$ T cells were incubated in the same conditions in the presence of anti-CD28 (1 μg/ml) ± 50 μg/ml of Salp15 (control). (C) Purified $CD4^+$ T cells (10^6 cells/ml) were activated with anti-CD3/CD28. At different time points (0, 12, 20, and 40 hr), 50 μg/ml of Salp15 was added to the cultures. The supernatants were recovered at 72 hr and analyzed for IL-2. The results shown are representative of two to four individual experiments.

(Figure 3C). The inhibition of IL-2 production was completely abolished by the addition of Salp15 antisera during the activation period (Figure 3D). Furthermore, saliva-mediated inhibition of IL-2 production by $CD4^+$ T cells was reversed by Salp15 antisera (Figure 3D), suggesting that the proteins recognized by the antibodies are the primary inhibitors of IL-2 production by $CD4^+$ T cells.

We then assessed whether the inhibitory effect of Salp15 on IL-2 production by $CD4^+$ T cells influenced CD25 expression. Salp15 caused a substantial reduction in the level of CD25 expression at 40 hr of activation (Figure 3E). The reduction was evident as soon as 12 hr and persisted throughout the activation period (data not shown). These data show that Salp15 specifically inhibits IL-2 production and CD25 expression by $CD4^+$ T cells activated by anti-CD3 and anti-CD28 mAbs. To further determine whether the lack of activation could be due to specific inhibition of IL-2 production, we assessed the effect of exogenously added recombinant IL-2 during activation. The proliferation of $CD4^+$ T cells activated in the presence of Salp15 increased markedly with the addition of 10 ng/ml of recombinant murine IL-2 (rmIL-2) (Figure 3F). Furthermore, the expression of CD25 in the presence of Salp15 also increased to similar levels in the presence of recombinant IL-2 (data not shown). Interestingly, a stronger inhibitory effect of Salp15 on IL-2 production was observed at lower concentrations of anti-CD3 mAb (Figure 4A) or when $CD4^+$ T cells were activated in the absence of costimulation provided by anti-CD28 (Figure 4B). These data suggested that Salp15 was interfering with T cell receptor (TCR) signals.

The initial production of IL-2 by $CD4^+$ T cells that are undergoing activation provides strong signals that permit the activation process to continue and the cells to enter the proliferative cycle. We therefore tested whether the addition of Salp15 would have the same effect on $CD4^+$ T cell activation when added at later time points, once IL-2 is already available to the cell. IL-2 production was decreased when the cells had been activated with anti-CD3 and anti-CD28 for 12 hr prior to the addition of Salp15 (Figure 4C). In contrast, the levels of IL-2 produced by $CD4^+$ T cells incubated with anti-CD3 and anti-CD28 for 20 hr before the addition of Salp15 were less affected (Figure 4C), and no effect was found on IL-2 production when Salp15 was added at 40 hr of activation (Figure 4C). These data indicated that the inhibition of $CD4^+$ T cell activation by Salp15 occurred in the initial period of activation. Overall, our results indicated that Salp15 inhibited $CD4^+$ T cell activation by inhibiting IL-2 production, and suggested that Salp15 interfered with TCR-signaling.

Salp15 inhibits Ca^{2+} mobilization after TCR ligation

Several regulatory elements have been identified within the proximal IL-2 promoter, including the activator protein (AP)-1, nuclear factor of activated T cells (NF-AT), and nuclear factor κB (NF-κB) elements (Jain et al., 1995). We assessed the effect of Salp15 on the binding activity of these transcription factors in $CD4^+$ T cells that had been activated with anti-CD3/CD28 mAbs. AP-1 DNA binding activity was not affected by Salp15 (Figure 5A). However, NF-AT and NF-κB DNA binding activities were substantially reduced in $CD4^+$ T cells activated in the presence of Salp15 (Figure 5A). As a control, we also tested the DNA binding capacity of the cyclic AMP response element binding protein (CREB), which was not affected by the presence of Salp15 (Figure 5A). In agreement with these results, NF-AT transcriptional activity was compromised when $CD4^+$ T cells from NF-AT-luciferase reporter transgenic mice were activated in the presence of Salp15 (Figure 5B). Salp15 did not induce AP-1 or NF-κB-DNA binding activity in unstimulated cells (data not shown), indicating that it exerted its effect through the inhibition of TCR-mediated transcriptional activation. Together, these results suggested that Salp15 was interfering with early signals induced by TCR crosslinking.

We hence assessed the effect of Salp15 on Ca^{2+} mobilization in response to TCR signals by flow cytometry using the fluorophore Indo-1. While anti-CD3 mAb caused a rapid increase of intracellular calcium levels, the presence of Salp15 delayed and reduced the Ca^{2+} flux induced by TCR ligation (Figure 5C). To determine whether the effect of Salp15 on Ca^{2+} mobilization was the cause of the inhibition in IL-2 production by the protein, we examined IL-2 production by $CD4^+$ T cells activated with PMA and ionomycin. The levels of IL-2 produced by $CD4^+$ T cells were equivalent in the absence or the presence of Salp15 (Figure 5D). Moreover, the addition of low doses of ionomycin (50 ng/ml) during activation of $CD4^+$ T cells with anti-CD3 and anti-CD28 prevented Salp15-mediated inhibition of IL-2 production (Figure 4E) without affecting the viability of these cells (data not shown). These data imply that the inhibitory effect of Salp15 on $CD4^+$ T cells is predominantly due to the inhibition of TCR-mediated calcium mobilization.

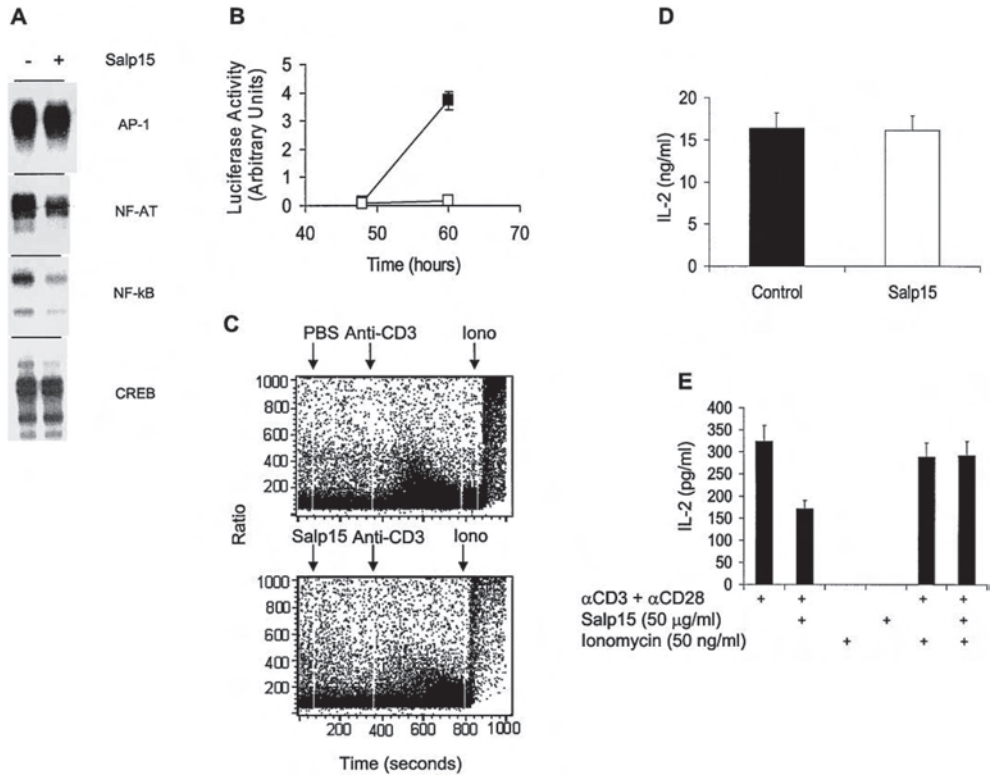

Figure 5. The immunosuppressive activity of Salp15 is mediated through the inhibition of calcium mobilization and IL-2 production. (A) Salp15 inhibits NF-κB and NF-AT DNA binding activity. Nuclear extracts were obtained from activated $CD4^+$ T cells (10^6 cells/ml) in the presence (+) or absence (–) of 50 μg/ml of Salp15. Electromobility shift analysis was then performed using specific ^{32}P end-labeled double-stranded oligonucleotides representing the consensus binding sites for AP-1, NF-AT, NF-κB, and CREB (control). (B) Purified $CD4^+$ T cells from NF-AT-luciferase reporter transgenic mice were activated with anti-CD3/CD8 in the presence of 50 μg/ml of Salp15 (open symbols) or without Salp15 (closed squares) and analyzed for luciferase activity at 48 and 60 hr of activation. (C) Salp15 inhibits calcium mobilization in response to TCR signals. $CD4^+$ T cells were loaded with Indo-1 for 45 min. The ratio of bound Indo-1 fluorescence (395 nm) to unbound Indo-1 fluorescence (525 nm) was determined for baseline during preincubation with Salp15 (100 μg/ml), after anti-CD3 plus anti-hamster IgG treatment (40 and 60 μg/ml, respectively), and after ionomycin induction (500 ng/ml). Phosphate-buffered saline (PBS) was used as a control. (D and E) Salp15 does not inhibit IL-2 production by $CD4^+$ T cells stimulated with PMA + ionomycin. 10^6 $CD4^+$ T cells/ml were activated in the presence of (D) PMA (5 ng/ml) + ionomycin (250 ng/ml) or (E) anti-CD3/CD28 mAbs + 50 ng/ml ionomycin in the absence or presence of 50 μg/ml of Salp15. The results are representative of two to four experiments.

Salp15 inhibits $CD4^+$ T cell activation in vivo

Activation of naive $CD4^+$ T cells requires a rise in IL-2 production, whereas the activation of effector $CD4^+$ T cells is less dependent on this cytokine (Yasui et al., 1998). To determine whether Salp15 inhibited the activation of effector $CD4^+$ T cells, we performed restimulation assays with $CD4^+$ T cells that had been differentiated in vitro for 4 days and from immunized mice. In vitro-generated effector $CD4^+$ T cells produced similar levels of IL-2, IFN-γ, and IL-4 in response to anti-CD3 in the absence or presence of Salp15 produced in the *Drosophila* expression system (Figure

6A). Similarly, Salp15 did not affect the proliferative capacity of restimulated effector cells (Figure 6B). For these experiments, we also cloned and expressed Salp15 as a fused partner to thioredoxin (TR) (Figure 6C) to specifically assess the effect of the immunomodulatory protein on CD4+ T cell activation and T cell-mediated antibody production in vivo. CD4+ T cells from TR-immunized animals responded similarly to TR, and TR fused to Salp15 (TR-Salp15) and produced high levels of IFN-γ (Figure 6D), suggesting that the stimulation of effector CD4+ T cells was not affected by the presence of Salp15. However, CD4+ T cells from TR-Salp15-immunized mice produced lower levels of IFN-γ in response to both TR and TR-Salp15 (Figure 6D), indicating that the presence of Salp15 inhibited the activation of antigen-specific CD4+ T cells in vivo. IL-4 production was below detectable levels in all cases (data not shown).

CD4+ T cells are also an important factor in the development of humoral antibody responses (Stavnezer, 1996). Therefore, we determined whether the inhibitory effect of Salp15 on CD4+ T cells influenced the generation of the murine antibody response. We found no difference in TR-specific IgM levels in the sera from TR- and TR-Salp15-immunized mice, indicating that T-independent B cell responses had not been affected by the presence of Salp15 (Figure 6E).

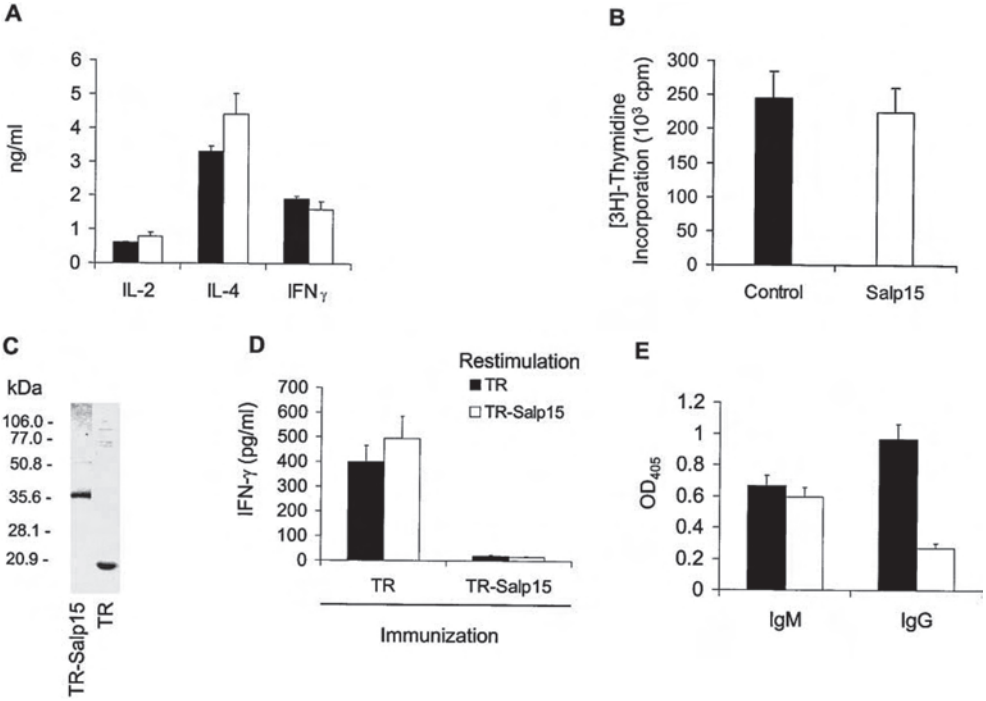

Figure 6. Salp15 inhibits CD4+ T cell activation in vivo. (A and B) In vitro-generated effector cells were activated for 24 hr with anti-CD3 in the absence (black bars) or presence (white bars) of Salp15 produced in *Drosophila* cells. The supernatants were collected after 24 hr and analyzed for IL-2, IL-4, and IFN-γ by capture ELISA (A) and proliferation (B). (C) Purified TR (1) and TR-Salp15 (2). (D) Balb/c mice were immunized with equimolar quantities of TR or TR-Salp15 (equivalent to 10 μg of TR-Salp15). Eleven days later, CD4+ T cells were purified and analyzed in recall responses to TR and TR-Salp15. IFN-γ levels were measured in 40 hr restimulation supernatants. (E) TR-specific IgM (1/160 dilution) and IgG (1/320 dilution) levels in the sera of the TR- (black bars) and TR-Salp15-immunized mice (white bars) were determined by ELISA using TR-bound plates. Experiments were repeated three times.

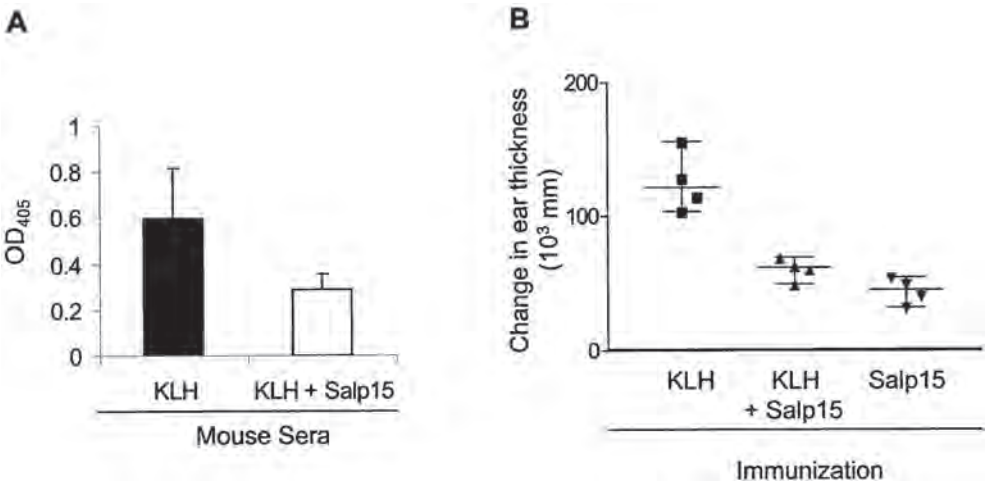

Figure 7. Salp15 inhibits DTH induced by KLH. (A) Groups of five mice were immunized with 1 μg of KLH in the absence (black bar) or presence (white bar) of 10 μg of *Drosophila*-produced Salp15 in CFA. The animals were boosted after 2 weeks, and 10 days later their sera were analyzed for KLH-specific IgG antibodies by ELISA. (B) Groups of four mice were immunized with KLH in the absence or presence of Salp15. Animals immunized with Salp15 alone served as controls. Four days later the mice were challenged with KLH in the ears. Ear swelling was recorded at 24 and 48 hr after challenge. Results indicate increase in ear thickness at 24 hr postchallenge compared to time 0 (prior to immunization).

Furthermore, Salp15 did not affect LPS-induced B cell proliferation (data not shown), agreeing with previous reports indicating that tick infestation does not affect, or slightly increases, B cell responses to LPS (Ganapamo et al., 1996). Similarly, Salp15 did not influence *Borrelia burgdorferi*-induced TNF-α production by $CD11c^+$ cells (data not shown). In contrast, TR-specific IgG antibody levels were significantly reduced in sera obtained from mice immunized in the presence of Salp15 (TR-Salp15), compared to control immunized mice (Figure 6E). To ensure that Salp15 had not influenced the antigenicity of the fusion protein, we immunized mice with keyhole limpet hemocyanin (KLH) in the absence or presence of 50 μg of Salp15 produced in *Drosophila* cells. The levels of KLH-specific IgG antibodies in the sera of the mice that had been injected with Salp15 were significantly lower than in control animals (Figure 7A, $p < 0.05$). These data are in agreement with reports describing absent IgG responses in Balb/c mice after repeated exposures to tick feeding (Christe et al., 1998). Furthermore, delayed-type hypersensitivity reactions elicited by KLH administration were also impaired in mice that had been immunized with the protein in the presence of Salp15 at 24 hr (Figure 7B) and 48 hr postchallenge (not shown). At both time points, the difference in ear swelling between KLH– and KLH+ Salp15-immunized mice were statistically significant (Student's t test, $P = 0.0017$ at 24 hr; $P = 0.0013$ at 48 hr). The impaired development of a strong IgG antibody and DTH responses in the presence of Salp15 reinforced the idea of an inhibitory effect of the protein on $CD4^+$ T cell activation.

Discussion

Tick saliva is abundantly secreted into the host during feeding (reviewed by Wang and Nuttall, 1999) and contains anticoagulant, vasodilatory, and immunosuppressive activities (Ribeiro et al. 1985; Ribeiro et al. 1995; Urioste et al. 1994; Valenzuela et al. 2000 and Wikel 1996). The interaction between arthropods and their hosts is a dynamic process and the result of coevolution (Wang and Nuttall, 1999). Upon attachment of the vector, the host elicits both specific and nonspecific immune responses to the vector. In turn, ticks have developed adaptive mechanisms to modulate the host immune response (Ferreira and Silva 1999 and Wikel 1999). The equilibrium between the host immune responses and the vector's immunosuppressive countermeasures partially determines the duration of attachment, the degree of engorgement, and the extent of pathogen transmission (Wikel and Bergman 1997 and Zeidner et al. 1996). In this study, we have characterized a 15 kDa *I. scapularis* saliva protein, Salp15, that inhibits $CD4^+$ T cell activation.

Our results show that Salp15 has an inhibitory effect on $CD4^+$ T cells in vitro and in vivo. IL-2 production, upregulation of the α-chain of the IL-2 receptor, and consequent proliferation are all readouts of $CD4^+$ T cell activation (Theze et al., 1996). Salp15 inhibited all three in a dose-dependent fashion without exerting a toxic effect on $CD4^+$ T cells. Moreover, the addition of exogenous IL-2 prevented the Salp15-mediated inhibition of $CD4^+$ T cell activation, indicating that the inhibitory effect of Salp15 is due to repression of IL-2 production and not due to toxic effects or interference with the response of $CD4^+$ T cells to IL-2.

Our results strongly suggest a quantitative effect of Salp15 that depends on the strength of the signal delivered through the TCR. Supporting this assertion, we have demonstrated that Salp15 activity is greatly increased when lower concentrations of anti-CD3 antibody are used to activate $CD4^+$ T cells in the presence of anti-CD28 or in the absence of costimulation, which provides an increase of the signal delivered through the TCR (Michel et al., 2001). Furthermore, differences in the activity between native tick saliva and the recombinant protein produced in vitro may be due to the degree of appropriate folding in the recombinant protein. These alterations may not, however, influence binding capacity to the cell surface. These data strongly suggest that, under physiological conditions, Salp15 is likely to induce high levels of inhibition, even though it represents 0.1% of the total protein content of tick saliva (1 mg/ml, which corresponds to a concentration of 1 μg/ml of Salp15, data not shown).

Crosslinking of TCR complexes and CD28 molecules on naive T cells induces multiple intracellular signaling pathways that lead to the activation of specific nuclear transcription factors, including AP-1, NF-AT, and NF-κB. *IL-2* is a key cytokine gene regulated by these transcription factors (Jain et al., 1995). Our data demonstrate that Salp15 inhibits IL-2 production upon TCR engagement by repressing calcium signals that result in decreased DNA binding activity of NF-AT and NF-κB. The effect of Salp15 on IL-2 production could be prevented by the activation of $CD4^+$ T cells with PMA

plus ionomycin or the addition of ionomycin during activation of $CD4^+$ T cells with anti-CD3/CD28. These data indicate that the inhibitory effect of Salp15 on IL-2 production of $CD4^+$ T cells is due to impaired TCR signaling, resulting in the inhibition of transcription of the *IL-2* gene.

Because of the potential immunosuppressive effect of the saliva protein in vivo, we tested whether the immune response against a foreign antigen was affected by the presence of Salp15. Our data suggest that the inhibitory effect of Salp15 on TCR-mediated $CD4^+$ T cell activation has an effect on the murine immune response in vivo. Interestingly, Schoeler and colleagues (1999) showed that T lymphocytes from Balb/c mice repeatedly infested with *I. scapularis* nymphs in vivo do not produce high levels of IFN-γ in response to ConA restimulation ex vivo. We suggest that the inhibitory effect of Salp15 on IL-2 production affects the activation of naive $CD4^+$ T cells in vivo and their differentiation to immune effector cells. On the other hand, $CD4^+$ T effector cells were not inhibited when incubated with Salp15, probably because these cells are less dependent on IL-2 and do not or hardly produce this cytokine in response to antigen stimulation (Mannie et al., 1996). Most interestingly, TR-specific IgG but not IgM antibody responses were diminished in mice immunized with Salp15. IgM antibodies are produced by B lymphocytes, whereas switching to IgG subclasses is dependent on $CD4^+$ T cell cooperation (Stavnezer, 1996). Indeed, Ganapamo and colleagues (1996) reported that tick infestation does not affect, or slightly increases, B cell responses to LPS. Consistent with these results, Salp15 did not exert any effect on LPS-induced B cell proliferation.

The results presented here argue against a relationship between Salp15 and the potential IL-2 binding protein activity described by Gillespie and colleagues (2001). First, the effects elicited by Salp15 on $CD4^+$ T cells occur very early during the activation process, before IL-2 is produced by T cells. Second, the temporal frame of Salp15 activity as an immunosuppressor is limited, since the addition of Salp15 at 40 hr of activation does not affect the completion of the activation process, indicating that Salp15 does not influence IL-2–IL-2 receptor interactions. Third, Salp15 acts by interfering with TCR-mediated signaling events. Overall, our data indicate that Salp15 acts early during the activation process by interfering with TCR-mediated signals that lead to IL-2 production. The activity is eliminated when the levels of IL-2 and the increase of surface CD25 allow for a continuation of the activation process that is less dependent on TCR stimulation (Bajenoff et al., 2002). Tick saliva contains multiple activities that can influence $CD4^+$ T and other cell type activation and effector functions. These include the inhibition of IFN-γ production by differentiating T cells in saliva of *I. ricinus* (Leboulle et al., 2002). Our data indicate that Salp15 has no effect on the commitment of these cells. Therefore, we can conclude that the overall activity of tick saliva on the immune system relies on the participation of several components, including Salp15.

Salp15 is the first *I. scapularis* protein associated with the immunosuppressive activity of tick saliva. Our data imply that the inhibitory effect of Salp15 on TCR-mediated activation of naive $CD4^+$ T

cells results in decreased numbers of responding immune effector cells. Immune modulation is probably most important for the vector at the site of the tick bite, since the responses that the arthropod encounters during feeding occur locally. The inhibition of acquired immune responses may also be ecologically significant, helping to ensure that the host remains susceptible to future tick infestations. Moreover, microorganisms that rely on vectors to complete their natural life cycle may take advantage of these immunomodulatory strategies to promote their survival in the mammalian host and their adaptation to their new environment. Therefore, delineating the mechanisms that ticks use to influence host immune responses may lead to an understanding of competency of the vector for specific hosts, the prevention of arthropod-borne diseases, and the characterization of new molecules that can be used as anti-inflammatory or immunosuppressive agents.

Experimental procedures

Purification of Salp15

salp15 was amplified from a pBluescript vector using specific primers: 5'-GAAAGCGGCCCAACT AAA-3' and 5'-CTAACATCCGGGAATGTG-3'. The PCR product was subcloned into the pMT/BiP/ V5-His A vector (Invitrogen, Carlsbad, CA) and transfected into *Drosophila* S2 cells (Invitrogen) in combination with the hygromycin selection vector pCOHYGRO for stable transfection. The stable transformants were selected using 300 μg/ml hygromycin-B for 3–4 weeks. The resistant cells were grown as large spinner cultures, switched to DES serum-free medium for 2 days, and induced with copper sulfate to a final concentration of 500 μM for 4 days. The cells were then centrifuged at 1000 × *g* for 5 min. The supernatant was used to purify the protein using the Talon metal affinity resin (Clontech, Palo Alto, CA). The protein was eluted using 100 mM imidazole, extensively dialyzed against PBS (pH 7.8), and concentrated by centrifugal filtration through a 10 kDa filter (Millipore Corp., Bedford, MA). The PCR product was also subcloned into the pBAD/Thio-TOPO vector (Stratagene, La Jolla, CA) and transformed into TOP 10 *Escherichia coli* cells (Invitrogen). The protein, fused to thioredoxin (TR), was then induced with 2% arabinose and purified using Ni^{2+} columns, following the manufacturer's specifications (Invitrogen). The poli-His tagged fusion protein was eluted with imidazole, dialyzed, and concentrated. TR control protein was expressed and purified in an identical fashion. The purity of the proteins was checked on SDS-PAGE.

Immunohistochemistry

Female Balb/c mice were challenged with 15 nymphal ticks on the back. Two days after tick detachment, skin punch biopsies of 4–5 mm were obtained at the site of tick attachment in the experimental animals. The biopsies were embedded in Optimun Cutting Temperature (O.C.T.) compound (Sakura Finetek, U.S.A., Inc., Torrance, CA) and quick frozen in a dry ice-ethanol bath. Skin biopsies from naive mice were also obtained and frozen in O.C.T compound to serve as negative controls. Frozen O.C.T.-embedded skin specimens were cut into 4 μm thick serial sections in a cryostat, collected on Superfrost Plus glass slides (Gallard-Schlesinger Industries,

Garden City, NY), and fixed with cold acetone for 20 min. Sections were allowed to air dry and were incubated with Tris-buffered saline (TBS) for 5 min. Endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 for 5 min at room temperature. Sections were rinsed with double distilled water (ddH_2O), followed by TBS for another 5 min, and then blocked with blocking buffer (5% normal goat serum and 1% triton-X 100 in TBS) at room temperature for 30 min. After the blocking step, sections were incubated overnight with Salp15 antisera (1:500 dilution). Normal rabbit sera were used as control. After three washes with TBS, the sections were incubated with peroxidase-conjugated anti-rabbit IgG (1:500 dilution) at 37°C for 30 min. Sections were washed with TBS followed by ddH_2O twice. The slides were developed with diaminobenzidine (Sigma Chemical Co., St. Louis, MO). The sections were rinsed in running water and mounted with Crystal/Mount (Biomed Corp. City State), followed by addition of a coverslip. The sections were then visualized under a light microscope.

Binding assays

Salp15 was equilibrated in carbonate buffer (pH 9.5) and labeled with fluorescein isothiocyanate (20 μg per mg of protein) for 2 hr at RT. Unbound fluorescein was then eliminated by size exclusion chromatography. The labeled protein was then buffer exchanged to a Tris-based buffer (Tris-HCl 50 mM and NaCl 150 mM [pH 7.0]). 10^7 per ml purified $CD4^+$ T cells were preincubated with 10 mg/ml of BSA for 10 min, incubated with Salp15-FITC for 30 min, washed, and analyzed by flow cytometry using a FACScalibur apparatus and the CellQuest software package (Becton Dickinson, Mountain View, CA). Negative controls were incubated in the same fashion with BSA-FITC (30 μg per 10^6 cells). $CD4^+$ T cell membrane fractions were obtained by passage of purified $CD4^+$ T cells through a nitrogen bomb at 1000 psi. The membrane fraction was then collected by centrifugation, and the protein content was measured using the Bradford method. Complete cell disruption was assessed visually under the microscope. Four hundred nanograms of protein-containing membrane fractions were coated in 96-well plates in bicarbonate buffer (pH 9.6) overnight at 4°C, blocked with PBS plus 10% FCS, and preincubated with PBS or trypsin (20 μg/ml) for 1 hr, followed by extensive washing. The wells were incubated for 1 hr with 30 μg/ml of soybean trypsin inhibitor and washed. Salp15 was then added at different concentrations in PBS + 10% FCS followed by incubation with rabbit Salp15 antisera and a biotinylated anti-rabbit IgG and streptavidin bound to HRP. Salp15 antisera was prepared by immunizing a rabbit with 100 μg of recombinant Salp15 produced in the *Drosophila* expression system in complete Freund's adjuvant and boosting the animal with 100 μg of Salp15 in incomplete Freund's adjuvant at 2 and 4 weeks. The assay was developed with TMB substrate and stopped with TMB 1 component stop solution. The wells were read at 450 nm.

Tick saliva collection and determination of Salp15 concentration

Adult ticks were allowed to feed on the backs of guinea pigs for 3 days and were removed mechanically. The ticks were then immobilized, and a finely drawn capillary tube was fitted over their mouthparts. Two to five microliters of 5% pilocarpine (Sigma Chemical Co.) in methanol was

applied topically to their dorsa, and saliva was collected over periods of 1–2 hr, pooled, and stored at −70°C until use. The concentration of total protein present in tick saliva was determined by the Bradford method (Bio-Rad). The concentration of Salp15 in saliva was estimated by Western blot analysis using rabbit antisera and known concentrations of recombinant Salp15 produced in the *Drosophila* expression system as standards.

CD4$^+$ T cell purification and activation

CD4$^+$ T cells from naive or immunized mice were purified by negative selection as described (Anguita et al., 2001). 10^6 CD4$^+$ T cells per ml were activated with plate-bound anti-CD3 (5 μg/ml, except where indicated) plus soluble anti-CD28 (1 μg/ml). At the specified time points, the supernatants were collected and analyzed for cytokines by ELISA, as previously described (Anguita et al., 1996). For surface expression analysis of IL2R, the cells were recovered and incubated with phycoerythrin (PE) and cy-chrome conjugated antibodies specific for CD25 and CD4, respectively, and analyzed by flow cytometry. Total RNA was extracted using the Ultraspec RNA isolation reagent (Biotecx Laboratory, Houston, TX) as recommended by the manufacturer. RT-PCR reactions were performed using the ProSTAR HF single-tube RT-PCR system (Stratagene), with primers specific for the murine IL-2 gene (Reiner et al., 1993). To ensure equal loads of RNA, β-actin primers were used (control). Lack of DNA contamination was assessed by running the same reactions in the absence of reverse transcriptase. Luciferase activity was analyzed as described (Rincón and Flavell, 1997). Effector CD4$^+$ T cells were generated in vitro as described (Rincón and Flavell, 1997). In brief, purified CD4$^+$ T cells were activated with plate-bound anti-CD3 + soluble anti-CD28 (5 and 1 μg/ml, respectively) for 4 days. The cells were extensively washed. 10^6 effector cells/ml were restimulated with 5 μg/ml of plate-bound anti-CD3 in the absence or presence of 50 μg/ml Salp15 for 24 hr, and the supernatants were analyzed for IL-2, IL-4, and IFN-γ by capture ELISA. The cells were also analyzed for proliferation by the addition of [^{3}H]-thymidine, as before.

EMSA

Nuclear extracts were obtained from 12 hr anti-CD3/CD28 mAbs activated and unstimulated CD4+ T cells in the presence (+) or absence (–) of Salp15 (Schreiber et al. 1989 and Tugores et al. 1992). Electromobility shift analysis was then performed using 2 μg of the extracts and 10^4 cpm of the specific ^{32}P end-labeled double-stranded oligonucleotides representing the consensus binding sites for AP-1, NF-AT, NF-κB, and CREB (control) (Schreiber et al., 1989). The oligonucleotides used are as follows (sense strand): IL2-NFAT, 5'-GCCCAAAGAGGAAAATTTGTTTCATACAG-3';AP-1,5' GTCGACGTGAGTCAGCGCG C-3' (Rincón and Flavell, 1997); NF-κB, 5'-GATCAGAGGGGACTTTCCGAG-3' (Millet et al., 2000); and CREB, 5'-AGAGATTGCCTGACGTCAGAGAGCTAG-3'.

Intracellular calcium levels

10^6 CD4$^+$ T cells were loaded with 7 μM Indo-1 for 45 min. Ten minutes before the assay, 2-aminoethoxydiphenyl borate (2-APB, 20 μM, a gift from M. Nelson) was added. The cells were

then transferred to a Standard Extracellular Solution (in mM: 140 NaCl, 4 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 1 KH_2PO_4, 10 glucose, and 10 HEPES, [pH 7.4]). The ratio of bound Indo-1 fluorescence (395 nm) to unbound Indo-1 fluorescence (525 nm) was then determined for baseline, during preincubation with Salp15 (100 μg/ml), after anti-CD3 plus anti-hamster IgG treatment (40 and 60 μg/ml, respectively), and after ionomycin induction (500 ng/ml).

Immunizations and DTH reactions

Groups of four Balb/c mice were immunized with equimolar quantities (0.5 μM, corresponding to 50 μg of TR-Salp15) of TR or TR-Salp15 in complete Freund's adjuvant (CFA). Eleven days later, CD4+ T cells were purified and analyzed in recall responses to TR and TR-Salp15. IFN-γ levels were measured by capture ELISA in 40 hr restimulation supernatants (Anguita et al., 1996). TR-specific IgM (1/160 dilution) and IgG (1/320 dilution) levels in the sera of the immunized mice were determined by ELISA (Anguita et al., 1996) using TR-bound plates. In another set of experiments, groups of five mice were immunized with 1 μg of KLH in the absence or presence of 10 μg of Salp15. The mice were boosted with KLH ± Salp15 after 10 days in incomplete Freund's adjuvant (IFA). Ten days later, KLH-specific IgG levels in the sera were measured by capture ELISA, as before. Delayed-type hypersensitivity reactions were performed as follows. Groups of four mice were immunized with 10 μg of KLH in the absence or presence of 50 μg of Salp15. Control animals were injected with 50 μg of Salp15 without KLH. Four days later, all mice were challenged in both ears with 1 μg of KLH. Ear swelling was recorded at 24 and 48 hr after challenge. The difference between ear thickness before immunization and after challenge was recorded and tabulated for each mouse.

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Salp15 binding to DC-SIGN inhibits cytokine expression by impairing both nucleosome remodeling and mRNA stabilization

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Chapter 3

Abstract

Ixodes ticks are major vectors for human pathogens, such as *Borrelia burgdorferi*, the causative agent of Lyme disease. Tick saliva contains immunosuppressive molecules that facilitate tick feeding and *B. burgdorferi* infection. We here demonstrate, to our knowledge for the first time, that the *Ixodes scapularis* salivary protein Salp15 inhibits adaptive immune responses by suppressing human dendritic cell (DC) functions. Salp15 inhibits both Toll-like receptor- and *B. burgdorferi*–induced production of pro-inflammatory cytokines by DCs and DC-induced T cell activation. Salp15 interacts with DC-SIGN on DCs, which results in activation of the serine/threonine kinase Raf-1. Strikingly, Raf-1 activation by Salp15 leads to mitogen-activated protein kinase kinase (MEK)-dependent decrease of IL-6 and TNF-α mRNA stability and impaired nucleosome remodeling at the *IL-12p35* promoter. These data demonstrate that Salp15 binding to DC-SIGN triggers a novel Raf-1/MEK-dependent signaling pathway acting at both cytokine transcriptional and post-transcriptional level to modulate Toll-like receptor–induced DC activation, which might be instrumental to tick feeding and *B. burgdorferi* infection, and an important factor in the pathogenesis of Lyme disease. Insight into the molecular mechanism of immunosuppression by tick salivary proteins might provide innovative strategies to combat Lyme disease and could lead to the development of novel anti-inflammatory or immunosuppressive agents.

Author Summary

Upon attachment of the tick, the host elicits both innate and adaptive immune responses directed against the vector. In turn, ticks have developed countermeasures to withstand and evade host immune responses. In the current paper we demonstrate how a tick salivary protein induces immunosuppression of human dendritic cells and discuss how this could facilitate infection with *B. burgdorferi*, the causative agent of Lyme disease. Insight into the molecular mechanism of immunosuppression by tick salivary proteins might provide innovative strategies to combat Lyme disease or other tick-borne illnesses and could lead to the development of novel anti-inflammatory or immunosuppressive drugs.

Introduction

Ixodes ticks are a major arthropod vector for human pathogens, such as *Borrelia burgdorferi*, the causative agent of Lyme disease [1]. *Ixodes* ticks require five to seven days to feed to repletion [2]. In order to secure attachment of the vector and to ensure susceptibility of reservoir hosts for future tick infestations, tick saliva contains modulators of host immune responses. Salp15, a 15-kDa salivary gland protein, is a major immunomodulatory protein in *I. scapularis* saliva [3]. Salp15 has been shown to bind to CD4, thereby inhibiting T cell receptor (TCR) ligation-induced signals, resulting in impaired interleukin (IL)-2 production and impaired $CD4^+$ T cell activation and proliferation [4–6]. While feeding on a host, ticks can introduce *B. burgdorferi* into the host's skin. Local immunosuppression of the host by tick molecules assists *B. burgdorferi* in establishing an infection. In addition, it has been shown that Salp15 binds to *B. burgdorferi* outer surface protein (Osp) C [7]. *B. burgdorferi* expresses OspC in the tick salivary glands and during the early stages of mammalian infection. Binding of Salp15 to OspC protects the spirochete from antibody-mediated killing by the immune host [7], and silencing of Salp15 by RNA interference in *I. scapularis* ticks resulted in a dramatically impaired ability to transmit *B. burgdorferi* to an immune host [7]. Thus, Salp15 is an important immunomodulatory protein in *I. scapularis* saliva that targets the T cell arm of adaptive immunity.

Dendritic cells (DCs) are essential in initiating adaptive immune responses in naive hosts [8]. After sensing invading pathogens in peripheral tissues, DCs capture them for processing and presentation to activate T cells in draining lymph nodes [8]. Previously we have shown that Salp15 is secreted by the feeding tick and is locally introduced in the host skin [4], where Salp15 also provides *B. burgdorferi* a survival advantage in a naive murine host, but only when coinjected, ruling out a systemic immunosuppressive effect of Salp15 [7]. However, local inhibition of immune responses by Salp15 could be responsible for the observed effect. Under normal circumstances there are very few T lymphocytes present at the site of the tick-bite, whereas DCs are abundantly present. Therefore, we hypothesized that DCs are a major target for immunomodulation by Salp15, since these cells are essential in initiating adaptive immune responses to exposed tick (salivary) antigens and *B. burgdorferi* in a naive host.

Here we have investigated the interaction of the major immunomodulatory protein in *Ixodes scapularis* saliva, Salp15, with human DCs. Salp15 inhibits the production of the pro-inflammatory cytokines IL-12p70, IL-6, and TNF-α of DCs stimulated with the Toll-like receptor (TLR)2 and -4 ligands, LTA and LPS, respectively. Salp15 interacts with the C-type lectin DC-SIGN, which results in activation of the kinases Raf-1 and mitogen-activated protein kinase kinase (MEK). This leads to the inhibition of pro-inflammatory cytokine production and suppresses the T cell–stimulatory role of DCs. Strikingly, the Salp15/DC-SIGN-induced signaling pathway regulates the inhibition of pro-inflammatory cytokines at different levels: decreased nucleosome remodeling at the *IL-12p35* promoter impairs IL-12p70 production, whereas the inhibition of IL-6 and TNF-α is caused by an

increased decay of their respective mRNAs. A similar suppression of pro-inflammatory cytokines is observed when DCs are activated with viable *B. burgdorferi* in the presence of Salp15, suggesting that the spirochete uses Salp15 to induce immune suppression. Thus, local interaction of Salp15 and DCs will lead to immunosuppression, which potentially allows the tick to feed for a longer period of time, and *B. burgdorferi* to escape from human immune responses, and might therefore be an important factor in the pathogenesis of Lyme disease.

Results

Salp15 inhibits pro-inflammatory cytokine production by dendritic cells upon stimulation with LPS

To investigate the effect of Salp15 on human DC function, we incubated immature DCs with different concentrations of recombinant Salp15 for 18 h and analyzed DC maturation and cytokine production. Salp15 alone did not induce upregulation of the maturation markers CD80, CD83, or CD86 (Figure 1A). In addition, DCs incubated with Salp15 did not secrete detectable levels of IL-10, IL-6, IL-8, IL-12p70, or TNF-α (Figure 1B). Thus, Salp15 by itself does not affect activation of DCs.

Next, we investigated the effect of Salp15 on DC maturation and activation induced by the TLR4 ligand LPS. DCs incubated with LPS in combination with Salp15 showed an upregulation of the maturation markers CD80, CD83, and CD86 similar to DCs incubated with LPS alone (Figure 1A). LPS induced the secretion of the cytokines IL-6, IL-8, IL-10, IL-12p70, and TNF-α by DCs. Strikingly, production of the pro-inflammatory cytokines IL-6, IL-12p70, and TNF-α was inhibited by Salp15 in a dose-dependent manner (Figure 1B). This effect was observed at both protein and mRNA level (Figure 1B and 1C). IL-8 levels were increased at both the protein and mRNA level, whereas IL-10 production was only affected at the mRNA level (Figure 1B and 1C). The inhibition of pro-inflammatory cytokine production was not due to cytotoxicity of Salp15 as determined by 7-AAD staining (unpublished data). DCs incubated with the TLR2 ligand LTA showed a similar downregulation of pro-inflammatory cytokines in the presence of Salp15 (Figure S1). Thus, Salp15 suppresses TLR-induced cytokine responses by DCs.

Whole tick saliva obtained from *Ixodes ricinus* did not induce maturation by itself or cytokine production, but there was a decrease in pro-inflammatory cytokine production when saliva was added to DCs in combination with LPS similar to Salp15 (Figure 1A and 1D). These data indicate that Salp15 in *Ixodes* saliva is responsible, although not necessarily exclusively, for reducing pro-inflammatory cytokine production by DCs. The increase in IL-8 caused by recombinant Salp15 was not observed as clearly in tick saliva, indicating that other tick molecules could hamper IL-8 production by human DCs. Similar to Salp15, DCs stimulated with LPS in the presence of tick saliva did not have an increased production of the cytokine IL-10 compared to DCs stimulated with LPS alone (Figure 1D).

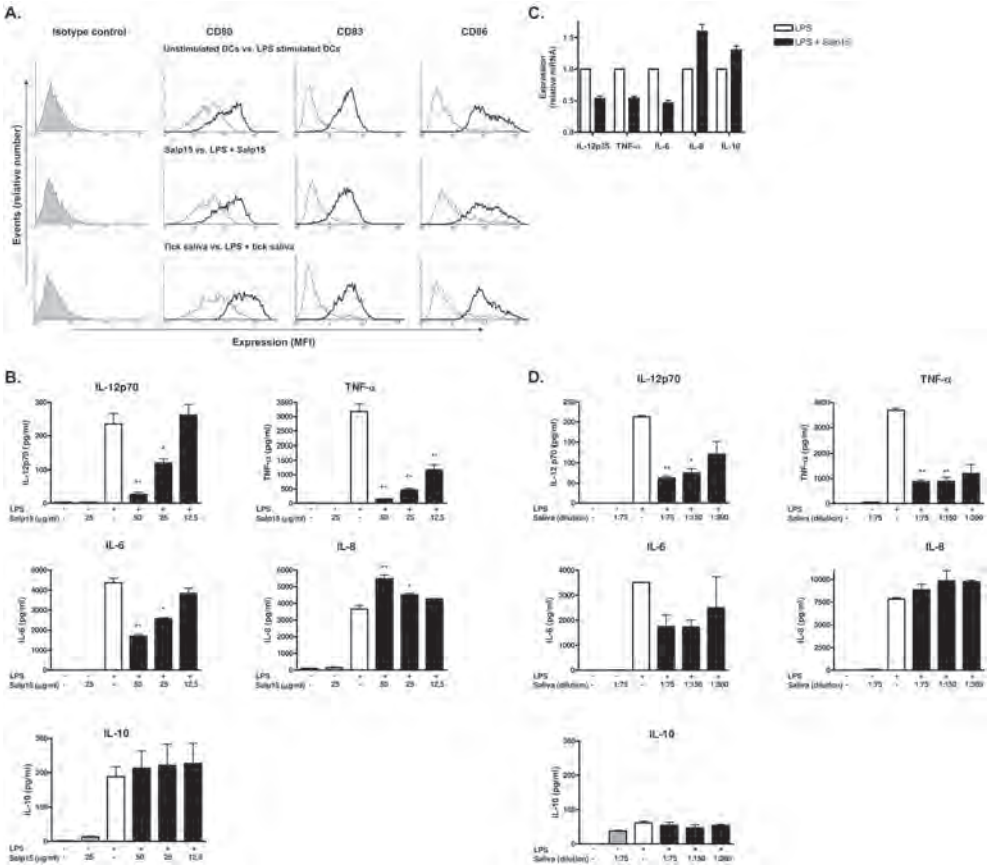

Figure 1. Salp15 and *Ixodes* tick saliva do not affect DC maturation, but dose-dependently inhibit IL-12, IL-6, and TNF-α production.

(A) Salp15 does not interfere with DC maturation. Immature DCs were stimulated with Salp15 or tick saliva alone in the presence or absence of 10 ng/ml LPS. 18 h post-stimulation DCs were analysed by flow cytometry (FACS) for surface expression of maturation markers CD80, CD83, and CD86. The thin lines represent DCs incubated without LPS and the bold lines those of DCs incubated with LPS. The isotype control is depicted in the left three graphs.

(B and C) Salp15 inhibits pro-inflammatory cytokine production by DCs. Immature DCs were stimulated with control medium (white bars), Salp15 (50, 25, or 12.5 μg/ml; black bars) in the presence of 10 ng/ml LPS. As a control, immature DCs were incubated with control buffer or Salp15 (25 μg/ml) in the absence of LPS. Supernatants were analyzed after 18 h for cytokine production (B), and for isolation of mRNA cells were lysed after 6 h of incubation and cytokine gene expression was measured by quantitative real time PCR. Relative mRNA expression of LPS-stimulated cells was set at 1 (C).

(D) Tick saliva inhibits pro-inflammatory cytokines by DCs. Immature DCs were treated with tick saliva (dilutions of 1:75, 1:150, and 1:300; black bars) in the presence of 10 ng/ml LPS as described in (B).

For (B) and (D) bars represent the average of triplicates from one experiment ± SE. The results are representative of at least three independent experiments. For (C) bars represent the mean of four to six independent experiments ± SE. Differences in cytokine production by DCs stimulated with LPS and DCs stimulated with LPS in the presence of Salp15 or tick saliva were analyzed by a two-sided one way ANOVA, implementing a one way analysis of variance and a Dunnett multiple comparison test. A P-value < 0.05 was considered statistically significant. (*) $0.01 < P < 0.05$; two asterisks (**) $0.001 < P < 0.01$.

Salp15 interacts with dendritic cells through the C-type lectin DC-SIGN

To further investigate the interaction of Salp15 and DCs, we investigated the binding of Salp15 to DCs. Salp15 interacts with DCs, and this interaction could be blocked to background levels by preincubating DCs with the calcium and magnesium-chelator EDTA, and the mannose-specific C-type lectin inhibitor mannan (Figure 2A). This strongly indicates that C-type lectins are involved in binding of Salp15 to DCs. Analysis of carbohydrate structures on Salp15 demonstrated that recombinant *Drosophila*-expressed Salp15 contains mannose and galactose structures, since the mannose- and galactose-specific plant lectins Con A and Peanut agglutin (PNA) strongly bound to Salp15 (Figure 2B). Similar to *Ixodes* ticks, *Drosophila* belongs to the phylum of Arthropoda, suggesting that Salp15 glycosylation is similar to native Salp15 in tick saliva. Salp15 binding to DCs could be blocked with mannan, N-acetyl-D-glucosamine (GlcNac), Lewis X (LeX), Lewis Y (LeY), and Fucose (Figure 2C). These carbohydrates all have high affinity for the C-type lectin receptor DC-SIGN, suggesting that DC-SIGN mediates binding of Salp15 to DCs. To investigate the cellular binding of Salp15 to DC-SIGN we used a Raji cell line transfected with DC-SIGN and demonstrated that Salp15 binds to DC-SIGN-transfected cells, but not to mock-transfected cells (Figure 2D). The interaction between Raji cells expressing DC-SIGN and Salp15 could be blocked with mannan, similar to the interaction of Salp15 to DCs. HIV-1 gp120 binding to DC-SIGN seems stronger than Salp15, although this could reflect differences in coating of the fluorescent beads. DC-SIGN binding to Salp15 was further assessed in a DC-SIGN-Fc binding ELISA. DC-SIGN-Fc interacted with plate-bound Salp15 and binding could be blocked with EDTA and mannan, suggesting that the binding is specific for DC-SIGN (Figure 2E). Furthermore, deglycosylation of Salp15 by N-glycosidase F (PGNaseF) abrogated the binding to DC-SIGN-Fc, demonstrating that the carbohydrate structures on Salp15 are essential to the interaction with DC-SIGN (Figure 2E). Due to interaction of murine anti-DC-SIGN blocking antibodies with goat-anti-mouse IgG antibodies used to generate the fluorescent Salp15-beads, we were unable to investigate whether these anti-DC-SIGN antibodies were able to block Salp15 binding to DC-SIGN in our bindings assays. Instead, we used Salp15-coupled Prot-A-Sepharose beads to immunoprecipitate the receptor for Salp15 from a cell-surface biotinylated DC lysate. The immunoprecipitate was analyzed by SDS-PAGE, transferred to a nitrocellulose membrane and the biotinylated cell-surface proteins were visualized by streptavidin-PO. The Salp15 immunoprecipitate showed only one distinct band with an apparent molecular weight of 45–50 kDa (Figure 2F). The size of the immunoprecipitate corresponded to the size of DC-SIGN, which was immunoprecipitated with beads coupled to anti-DC-SIGN antibodies. To confirm that the Salp15-coupled beads had selectively pulled down DC-SIGN, we also immunoprecipitated the receptor for Salp15 from non-biotinylated DC lysate. The immunoprecipitate was analyzed by SDS-PAGE, transferred to a nitrocellulose membrane, and visualized with antibodies against DC-SIGN showing a band at 45–50 kDa (Figure 2F) confirming that Salp15 binds to DC-SIGN on DCs.

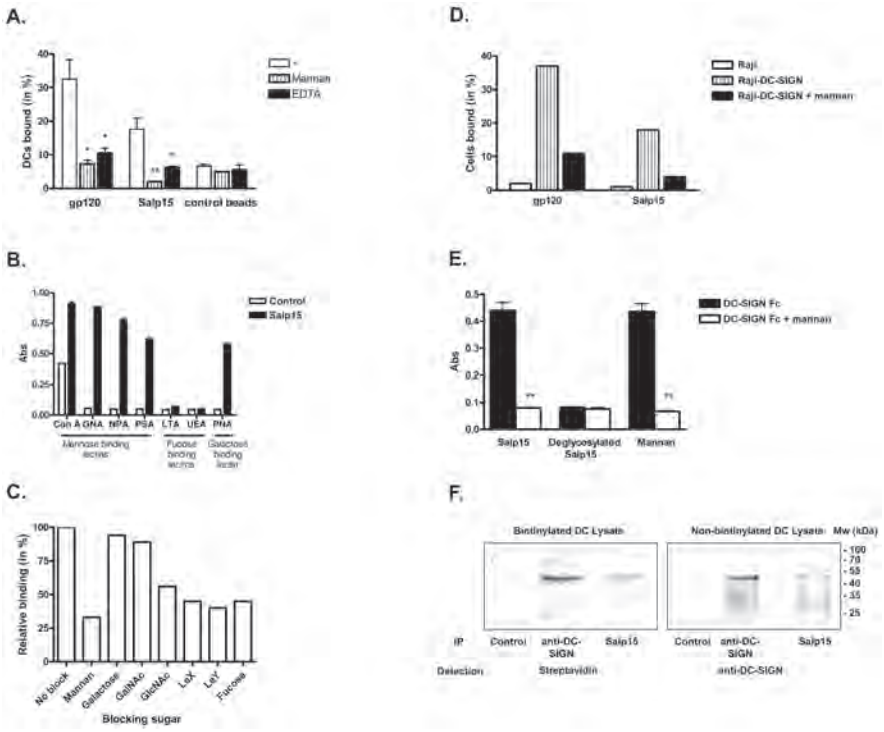

Figure 2. Salp15 interacts with DCs through the C-type lectin DC-SIGN.
(A) Salp15 binds to DC-SIGN on DCs. A fluorescent bead adhesion assay was performed using fluorescent beads coated with HIV-1 gp120 (positive control) and Salp15. As negative control beads we used antibody (anti-V5) coated beads. Immature DCs were incubated with the ligands and binding was measured by FACS analysis. Specificity was determined by preincubation of DCs with EDTA or mannan (significance determined by a two-sided one way ANOVA, implementing a one way analysis of variance and a Dunnett multiple comparison test).
(B) Salp15 contains mannose and galactose-specific glycosylations. The glycosylation pattern of Salp15 was analysed by a plant lectin ELISA. For abbreviations see Materials and Methods.
(C) Salp15 binding to DCs is inhibited by carbohydrates that interact with DC-SIGN. Specificity of binding of DCs to Salp15 was further assessed by pretreating DCs with mannan, free carbohydrate structures (Galactose, N-acetyl-galactosamine [GalNac], N-acetyl-D-glucosamine [GlcNac], or Fucose), or biotinylated Lewis-X/Y (LeX/Y) before performing the fluorescent bead adhesion assay as described in (A). Salp15 binding was set at 100%.
(D) Salp15 interacts specifically with DC-SIGN. The fluorescent bead adhesion assay was performed using the Raji-cell line transfected with DC-SIGN. Mannan and EDTA were used to determine specificity for C-type lectins.
(E) Glycosylation of Salp15 is important in the interaction with DC-SIGN. A DC-SIGN-Fc binding ELISA was performed on Salp15, deglycosylated Salp15 (by incubation with PGNaseF), and mannan (positive control). For Salp15 and mannan DC-SIGN-Fc binding could be blocked by preincubation with mannan (significance determined by a two-sided unpaired Student *t*-test).
(F) DC-SIGN is the major receptor on immature DCs for Salp15. Both a cell-surface biotinylated and non-biotinylated DC lysate were incubated with Salp15-coupled protein A-Sepharose beads. The biotinylated immunoprecipitate was analysed on SDS-PAGE, transferred to a nitrocellulose membrane, and detected by streptavidin-PO. The non-biotinylated immunoprecipitate was analyzed by SDE-PAGE, transferred to a nitrocellulose membrane, and detected by anti-DC-SIGN antibodies. As controls, anti-DC-SIGN protein A-Sepharose beads and anti-V5-protein A-Sepharose beads were used.
Adhesion experiments and ELISAs are representative of at least three independent experiments. Error bars, where depicted, represent the SE of triplicates within one experiment. A P-value < 0.05 was considered statistically significant. (*) $0.01 < P < 0.05$; two asterisks (**) $0.001 < P < 0.01$. The immunoprecipitations are representative of two experiments.

Salp15-induced activation of Raf-1 inhibits pro-inflammatory cytokine production

Next, we investigated the intracellular signaling pathways that Salp15 induces to inhibit the expression of pro-inflammatory cytokines by DCs. Recently, we have demonstrated that binding of mycobacterial ManLAM to DC-SIGN leads to activation of the serine/threonine kinase Raf-1 [9]. We investigated whether binding of Salp15 to DCs also activates Raf-1 by assessing phosphorylation of the Serine (S)338 and Tyrosine (Y)340/341 phosphorylation sites of Raf-1. Indeed, Salp15 induced phosphorylation of both sites (Figure 3A), demonstrating that Salp15 activates Raf-1 similar to ManLAM. Phosphorylation was partially blocked by blocking antibodies against DC-SIGN (Figure 3A).

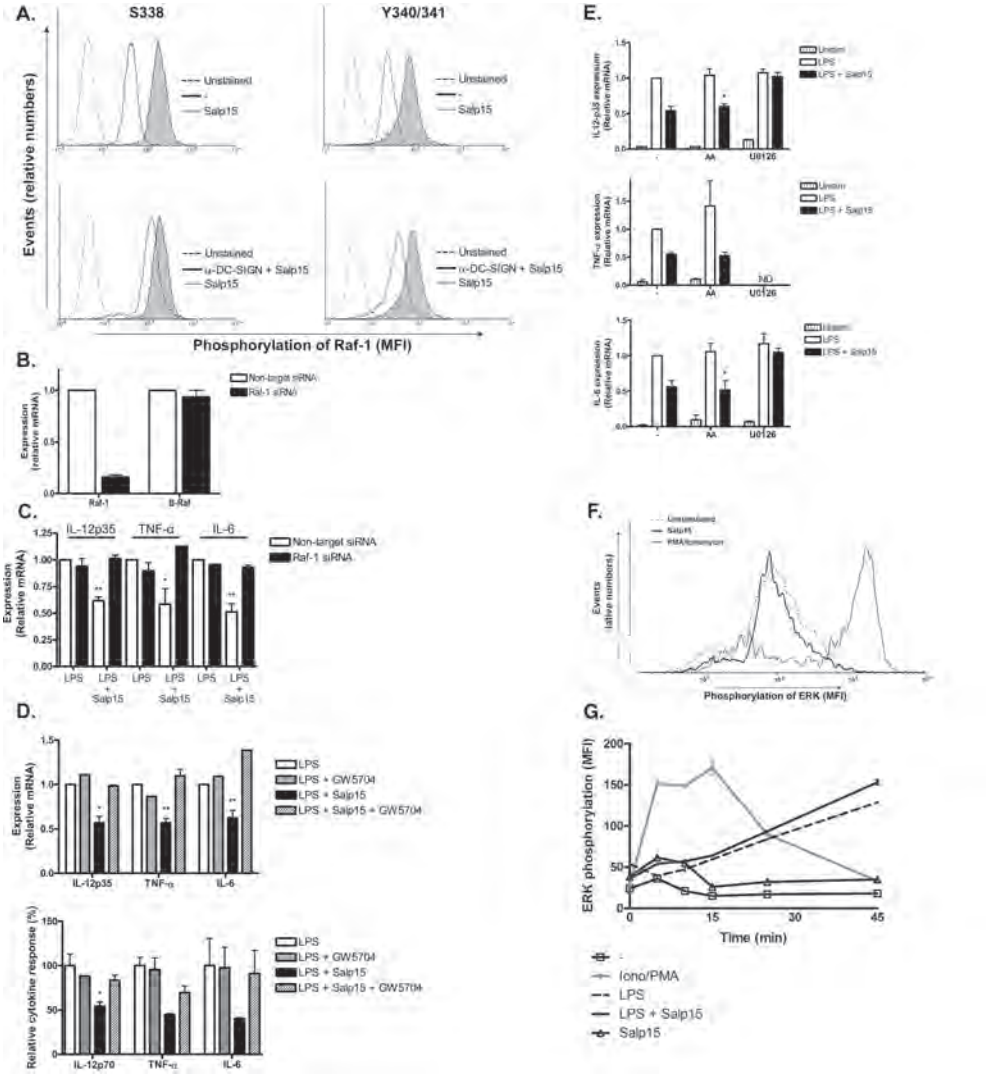

Figure 3. DC cytokine inhibition by Salp15 is Raf-1-dependent.
(A) Salp15 induces phosphorylation of Raf-1. DCs were pretreated with medium or with blocking anti-DC-SIGN antibodies (AZN-D2) before addition of 25 μg/ml Salp15. Intracellular phosphorylation of Raf-1 was measured by flow cytometry using rabbit anti-phospho-c-Raf (Ser338) mAb and rabbit anti-c-Raf (Tyr341) pAb.
(B) Raf-1 was specifically silenced by siRNA. DCs were transfected with 100 nM siRNA (Raf-1 or non-targeting siRNA as a control). Raf-1 and B-Raf mRNA was quantified by real-time PCR using gene-specific primers. Relative mRNA expression was corrected for GAPDH expression.
(C) RNAi-mediated silencing of Raf-1 abrogates Salp15-mediated cytokine inhibition. siRNA-treated cells were stimulated for 6 h with LPS in the presence or absence of 25 μg/ml Salp15. Cytokine gene expression was measured by real time PCR and corrected for GAPDH expression. Relative mRNA expression of LPS-stimulated non-targeting siRNA-treated cells was set at 1.
(D) Raf-1 inhibition by GW5704 abrogates Salp15-mediated cytokine inhibition. DCs were incubated with 1 μM GW5704, or DMSO as a negative control, for 2 h before stimulation with LPS for 6 h (mRNA) or 18 h (protein) in the presence or absence of 25 μg/ml Salp15. Cytokine gene expression was measured by real-time PCR and corrected for GAPDH expression. Relative mRNA expression of LPS-stimulated cells was set at 1. For protein levels, protein levels of LPS-stimulated cells were set at 100%, which is representative of 133 ± 17 pg/ml IL-12p70; 9,188 ± 850 pg/ml TNF-α, and 1,563 ± 800 pg/ml IL-6. GW5704 alone did not induce cytokine production (unpublished data).
(E) MEK1/2-inhibitor U0126 blocks Salp15-mediated cytokine inhibition. DCs were preincubated with anacardic acid (AA), an inhibitor of the histone acetyltransferases p300 and CBP, U0126, an inhibitor of MEK1 and MEK2, or DMSO as a negative control. Cells were stimulated as described in (C) and cytokine gene expression was measured by real-time PCR and corrected for GAPDH expression. Relative mRNA expression of LPS-stimulated cells was set at 1.
(F and G) Salp15 does not induce phosphorylation of ERK. DCs were stimulated for 0 to 45 min with Salp15 (25 μg / ml), LPS, or PMA/Ionomycin as a positive control. Phosphorylation of ERK was determined by flow cytometry using a rabbit anti-phospho-p44/42 MAPK (Thr202/Tyr402) mAb.
Bars represent the mean of at least three independent experiments ± SE. For (C) mRNA expression levels in non-target siRNA-treated DCs activated with LPS + Salp15 were compared to Raf-1 siRNA-treated DCs activated with LPS + Salp15. In (D) and (E) levels in DCs treated with LPS + Salp15 were compared to levels in DCs treated with LPS + Salp15 in the presence of the indicated inhibitor. A two-sided unpaired Student *t*-test was applied and a *P*-value < 0.05 was considered statistically significant. (*) $0.01 < P < 0.05$; two asterisks (**) $0.001 < P < 0.01$.

Next, we investigated whether Raf-1 silencing through RNA interference could prevent the Salp15-induced inhibition of DC cytokine production. Immature DCs were transfected with siRNA specifically targeting Raf-1, while expression of the closely related kinase B-Raf remained unaltered as determined by quantitative real-time PCR (Figure 3B). Raf-1 silencing at the protein level was complete since Raf-1 protein was not detected in silenced DCs as determined by immunofluorescence analysis (unpublished data). Silencing of Raf-1 in DCs completely abrogated the Salp15-induced inhibition of cytokines, since stimulation of Raf-1 silenced DCs with LPS in the presence of Salp15 restored IL-12p35, IL-6, TNF-α, IL-8, and IL-10 levels to those observed in DCs stimulated with LPS alone (Figures 3C and S2A). To confirm the Raf-1 silencing data, we also used the Raf inhibitor GW5074 and observed a similar restoration of pro-inflammatory cytokine levels in DCs treated with the Raf inhibitor in the presence of LPS and Salp15 (Figures 3D and S2B). IL-8 enhancement by Salp15 was abrogated by GW5704 mostly at the mRNA level, whereas IL-10 was not increased by Salp15 (Figure S2B). These data demonstrate that Raf-1 is essential for the inhibition of pro-inflammatory cytokine production by Salp15.

Next, we investigated the downstream effectors of Raf-1. Raf-1 activation by ligation of ManLAM to DC-SIGN leads to phosphorylation of the NF-kB subunit p65 at residue S276, which

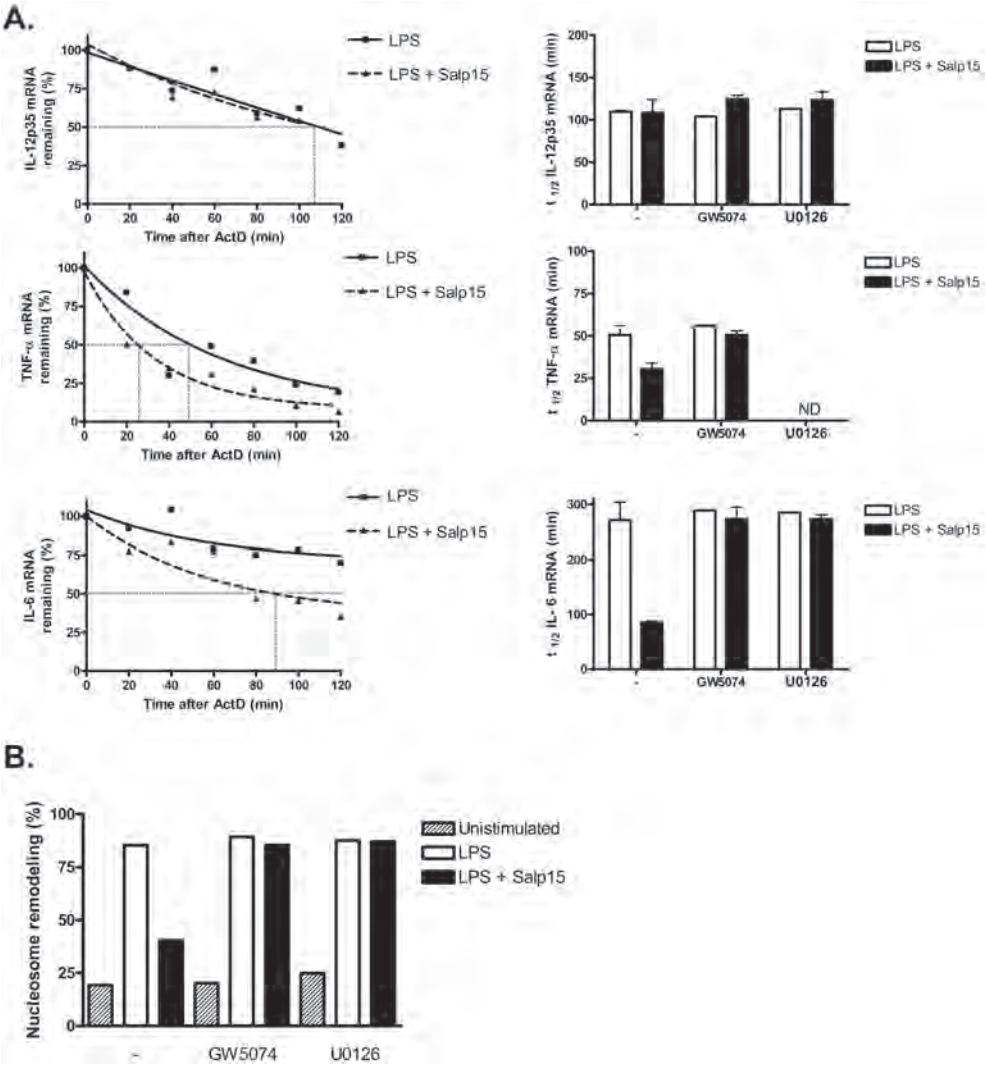

Figure 4. Salp15 destabilizes IL-6 and TNF-α mRNA and impairs nucleosome remodeling at the *IL-12p35* promoter.

(A) Salp15 destabilizes IL-6 and TNF-α mRNA. DCs were stimulated with Salp15 and LPS for 6 h. Actinomycin D (ActD) was added to block new mRNA synthesis. Cells were harvested for 2 h to determine mRNA half-life. IL-12p35 mRNA stability remained unaltered in the presence of Salp15, whereas IL-6 and TNF-α mRNA half-life was strongly reduced. This was completely restored using Raf (GW5074) or MEK (U0126) inhibitors. Bars represent the mean of two independent experiments ± SE. ND: Not determined.

(B) IL-12 inhibition by Salp15 is a result of decreased nucleosome remodeling at the *IL-12p35* promoter. Cells were stimulated as described before and harvested. Using enzymatic digestion, nucleosome remodeling was measured by quantitative real-time PCR. Results are expressed as a percentage of the remodeling observed in EcoRI-digested samples. The decreased remodeling induced by Salp15 could be completely restored using Raf (GW5074) or MEK (U0126) inhibitors. Results represent results from one donor.

subsequently results in acetylation of p65 by the histone acetyltransferases (HATs) p300 and CREB binding protein (CBP) [9]. Acetylation of p65 leads to an increased IL-10 cytokine response by DCs, which is inhibited by the CBP/p300-inhibitor anacardic acid [9]. Strikingly, anacardic acid (AA) did not prevent Salp15-induced modulation of IL-12p35, IL-6, TNF-α, IL-8, and IL-10 transcription (Figures 3E and S2C), whereas it did abrogate ManLAM-induced DC cytokine production (unpublished data, [9]), indicating that Salp15 induces downstream signaling cascades different from ManLAM-induced DC-SIGN signaling. Raf kinases are well known for their ability to activate MEK 1 and 2, that subsequently activate extracellular signal-regulated kinase (ERK) 1 and 2 [10]. Therefore, we investigated whether the MEK1/2 inhibitor U0126 could block Salp15-induced inhibition of DC cytokine production. Inhibition of MEK completely blocked the Salp15-induced modulation of cytokine production (Figures 3E and S2C). Strikingly, despite a role for MEK kinases in Salp15-induced signaling, stimulation of immature DCs with Salp15 did not lead to ERK activation as assessed by the phosphorylation of ERK (Figure 3F and 3G), which is a prerequisite for ERK activation [11]. Activation was followed over time but no phosphorylation of ERK in the presence of Salp15 could be detected (Figure 3G). In addition, ERK activation by LPS was not altered in the presence of Salp15, demonstrating that Salp15 does not sequester MEK away from ERK and thereby inhibiting LPS-induced cytokine production. Thus, Salp15 binding to DC-SIGN modulates DC function through a Raf-1- and MEK-dependent, but ERK-independent pathway that is distinct from the recently identified ManLAM/DC-SIGN-induced signaling pathway that results in Raf-1-dependent but MEK-independent phosphorylation and acetylation of p65 [9].

Salp15 downregulates pro-inflammatory cytokines by increasing IL-6 and TNF-α mRNA decay and impairing nucleosome remodeling at the *IL-12p35* promoter

Since we demonstrated that phosphorylation and acetylation of p65 was not responsible for the observed Salp15-induced modulation of cytokine responses, we set out to identify the mechanisms responsible for the Salp15-induced Raf-1/MEK-dependent decrease in TLR-induced pro-inflammatory cytokines. Gene expression is regulated by complex mechanisms at many stages, including chromatin accessibility, transcription activation, mRNA nuclear export, mRNA decay, and translation. Many cytokine genes are subject to regulation at the level of mRNA decay through the presence of mRNA-stability elements, particularly AU-rich elements, within their 3'untranslated regions, as it provides the means to use transcripts with optimal efficiency and to respond rapidly to cellular signals [12,13]. Therefore we determined the effect of Salp15 on mRNA stability. DCs were stimulated with LPS and Salp15 in the presence or absence of Raf and MEK inhibitors. After 6 h, actinomycin D was added to block mRNA synthesis, and cells were harvested every 20 min for 2 h and mRNA decay was determined. Both TNF-α and IL-6 mRNA showed a strongly decreased half-life in the presence of Salp15 and LPS compared to LPS alone (Figure 4A). IL-6 mRNA half-life was completely restored by the Raf and MEK inhibitors, GW5074 and U0126, respectively. Inhibition of Raf-1 by GW5074 also completely abrogated the effects of Salp15 on TNF-α mRNA half-life. We could not assess whether the MEK inhibitor abrogated the effect of Salp15 on TNF-α mRNA half-life, since inhibition of MEK has been shown to affect nucleocytoplasmic transport of

TNF-α mRNA [14]. Strikingly, IL-12p35 mRNA stability remained unchanged in LPS-activated DCs in the presence of Salp15 compared to DCs stimulated with LPS alone, indicating that the decrease in IL-12 is not regulated at the level of mRNA stability. In addition, there was no change in IL-8 and IL-10 mRNA stability in the presence of Salp15 (Figure S3).

Previously, it has been described that regulation at the level of chromatin accessibility is an important factor in the regulation of IL-12p35 transcription. IL-12p35 gene activation during DC maturation involves selective and rapid remodeling of nucleosome 2 in the *IL-12p35* promoter region [15]. To investigate the effect of Salp15 on nucleosome remodeling at the *IL-12p35* promoter we used a Chromatin accessibility by real time PCR (ChART) assay [16]. In the presence of LPS, rapid nucleosome remodeling at the *IL-12p35* promoter occurs in DCs, allowing for efficient transcription initiation (Figure 4B). Strikingly, Salp15 severely impaired this nucleosome remodeling, whereas both Raf and MEK inhibitors restored the level of remodeling to that observed with LPS alone (Figure 4B). Thus, Salp15 impairs nucleosome remodeling at the *IL-12p35* promoter through a Raf-1/MEK-dependent signaling pathway, which results in decreased IL-12p35 mRNA expression in DCs.

Therefore, Salp15-DC-SIGN signaling regulates cytokine production at both transcriptional and post-transcriptional levels; the decreased IL-12p70 production in the presence of Salp15 and LPS is dependent on nucleosome remodeling at the *IL-12p35* promoter, while the downregulation of IL-6 and TNF-α is a result of increased mRNA decay.

Salp15 blocks T cell activation by mature dendritic cells

T lymphocyte activation by mature DCs is essential to initiate an effective adaptive immune response against invading pathogens. Therefore we investigated whether Salp15 interfered with the T lymphocyte activation capacity of DCs by performing a mixed leukocyte reaction (MLR). DCs were incubated with Salp15 and LPS for 18 h. The DCs were washed extensively to remove remaining Salp15 before allogenic lymphocytes were added, because it has been shown that Salp15 can directly inhibit T cell activation [4,5]. DCs pretreated with Salp15 alone did not suppress lymphocyte proliferation. However, LPS-matured DCs pre-incubated with Salp15 or tick saliva were less capable of inducing lymphocyte proliferation compared to DCs matured with LPS alone (Figure 5A and 5B). These data demonstrate that Salp15 in tick saliva does not only alter cytokine responses of DCs, but also inhibits T cell proliferation induced by DCs.

Salp15 suppresses DC-mediated cytokine responses induced by *Borrelia burgdorferi*

A recent study demonstrated that *B. burgdorferi* alone does not suppress DC functions [17]. Since *B. burgdorferi* has been shown to interact with Salp15 to evade host antibody-mediated killing [7], we investigated whether *B. burgdorferi* could also benefit from the inhibition of DC cytokine production by Salp15. Therefore, we determined DC cytokine production by stimulating DCs with viable *B. burgdorferi* alone or in the presence of Salp15. *B. burgdorferi* alone induced DC maturation

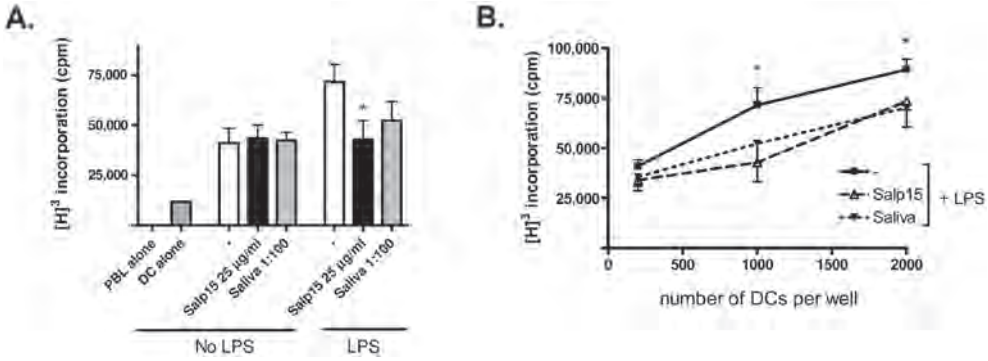

Figure 5. Salp15 inhibits DC-induced T cell proliferation.
(A and B) Salp15 impairs DCs in the induction of T cell proliferation as shown by a mixed lymphocyte reaction (MLR). After 18 h of stimulation with LPS in the presence of Salp15 (25 µg/ml), tick saliva (1:100), or controls, DCs were extensively washed and cultured with allogeneic PBLs (1×10^5) at different ratios (1:50 to 1:500) for 4 d. Lymphocyte proliferation was assessed by overnight incorporation of radioactive thymidine. Data are representative of results obtained from four donors. Bars represent the mean of triplicates within one experiment ± SE. Differences between the different conditions were analyzed by a two-sided one way ANOVA, implementing a one way analysis of variance with Dunnett multiple comparison test. A P value < 0.05 was considered statistically significant and indicated with one asterisk (*).

and pro-inflammatory cytokine secretion, as has been shown before [17]. Preincubation with Salp15 did not affect the upregulation of DC maturation markers by *B. burgdorferi* (Figure 6A). However, Salp15 inhibited pro-inflammatory cytokine production by *B. burgdorferi*-activated DCs, similar to DCs activated by LPS in the presence of Salp15 (Figure 6B). *B. burgdorferi*, in contrast to LPS, did induce IL-1β production by human DCs (Figure 6B). Similar to the other pro-inflammatory cytokines IL-1β was inhibited by Salp15. In addition, compared to *B. burgdorferi* alone, *B. burgdorferi* preincubated with Salp15 induced enhanced production of the immunomodulatory cytokine IL-10 by DCs; this was similar to what was observed for LTA (Figure S1), underscoring the fact that the set of cytokines produced by human DCs is dependent on the TLR-ligand. Thus, these data suggest that the interaction of Salp15 with *B. burgdorferi* assists *B. burgdorferi* in altering and potentially evading host adaptive immune responses during early human infection.

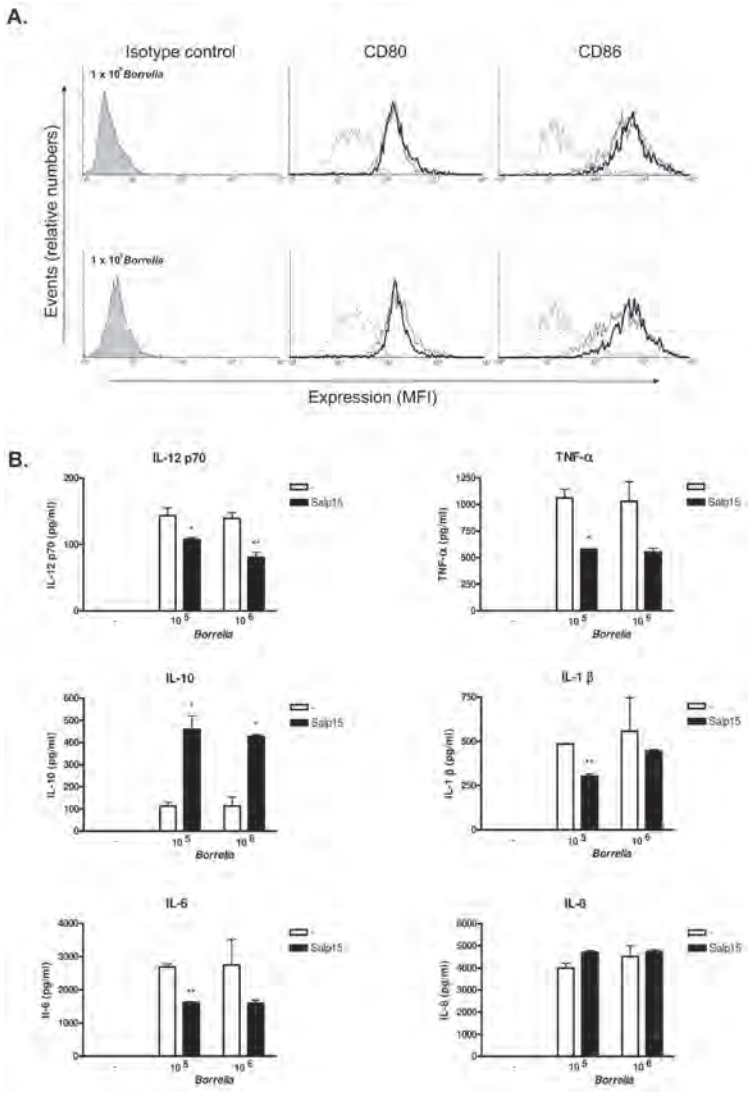

Figure 6. Salp15 does not affect DC maturation, but dose-dependently inhibits IL-12p70, IL-6, and TNF-α production by DCs stimulated with viable *Borrelia burgdorferi*.

(A) Salp15 does not interfere with viable *B. burgdorferi*-induced maturation of DCs. Immature DCs were either not stimulated (dotted line), or stimulated with 1×10^5 or 1×10^6 viable *B. burgdorferi* in the absence (solid line) or presence (bold line) of Salp15. The grey-filled graphs represent isotype controls. *B. burgdorferi* was preincubated with Salp15 for 30 min before addition of DCs. 18 h post-stimulation DCs were analysed by FACS for surface expression of CD80 and CD86.

(B) Salp15 inhibits viable *B. burgdorferi*-induced pro-inflammatory cytokine production by DCs. The experiment was performed as in (A). However, for this assay supernatant was analyzed for cytokine production by CBA.

The experiments were performed with DCs from four independent donors. Bars represent the mean of triplicates within one experiment ± SE. Differences in cytokine production between DCs stimulated with *B. burgdorferi* and DCs stimulated with *B. burgdorferi* and Salp15 were analyzed by a two-sided one way ANOVA, implementing a one way analysis of variance with Dunnett multiple comparison test. A P-value < 0.05 was considered statistically significant, indicated as one asterisk (*) $0.01 < P < 0.05$ or two asterisks (**) $0.001 < P < 0.01$.

Discussion

The main vectors for *Borrelia burgdorferi*, the causative agent of Lyme borreliosis, are ticks belonging to the *Ixodes* genus. In Europe, *B. burgdorferi* is transmitted by *I. ricinus*, whereas in the United States *B. burgdorferi* is predominantly transmitted by *I. scapularis*. Upon attachment of the tick, the host elicits both innate and adaptive immune responses directed against the vector. In turn, ticks have developed countermeasures to withstand and evade host immune responses. Therefore, tick saliva contains anticoagulant, vasodilatory, and immunosuppressive molecules and is abundantly secreted into the host's skin during tick feeding. The balance between host immune responses and the vector's immunosuppressive countermeasures determines the duration of tick attachment, the degree of engorgement of the tick, but also the extent of *B. burgdorferi* transmission [18–20].

Salp15 is a protein in *I. scapularis* saliva that is induced during feeding and it is abundantly secreted into the host. *I. ricinus* contains a Salp15 homologue, Salp15 Iric-1 (GenBank accession number: ABU93613) with 82% similarity to Salp15 from *I. scapularis* [21]. Previously, we have shown that native Salp15 can be readily detected in host skin at the site of the tick-bite [4]. Salp15 has been shown to inhibit $CD4^+$ T cell activation [4], and to interfere with T cell receptor signaling [6] through binding to CD4 [5], which results in the inhibition of IL-2 production upon recognition of cognate antigens. However, low numbers of T cells are present in the dermis of the skin, the site where Salp15 is secreted during tick feeding. In contrast, DCs, as sentinels and initiators of adaptive immune responses, are abundantly localized in the skin and might therefore be targeted by the tick to suppress the initiation of adaptive immune responses. Indeed, we demonstrate that *I. scapularis* Salp15 interacts with human DCs and that Salp15 impairs TLR-induced pro-inflammatory cytokine production by DCs and suppresses DC-induced T lymphocyte activation. DCs recognize antigens by TLRs and C-type lectins. DC-SIGN is a C-type lectin abundantly expressed by DCs. It is becoming clear that DC-SIGN is involved in pathogen recognition, but that several pathogens target this receptor to modulate DC functions [22]. We here demonstrate that Salp15 targets the C-type lectin DC-SIGN on DCs to activate a Raf-1/MEK-dependent signaling cascade that results in downregulation of pro-inflammatory cytokines induced not only by TLR2 and TLR4 ligands, but also by viable *B. burgdorferi*. This downregulation is a result of decreased IL-6 and TNF-α mRNA stability and impaired nucleosome remodeling at the *IL-12p35* promoter. Moreover, our data demonstrate that Salp15 and tick saliva inhibit T cell activation. Therefore, *B. burgdorferi* could use Salp15 in saliva to suppress DC-mediated cytokine production, which might assist the spirochete in establishing an infection of the host.

I. scapularis Salp15, similar to tick saliva, inhibited the production of the pro-inflammatory cytokines IL-12p70, IL-6, and TNF-α by LPS-activated DCs. These data indicate that Salp15 present in tick saliva induced the observed cytokine suppression. Indeed, anti-Salp15 antibodies prevented the suppression of IL-12 by tick saliva (unpublished data). This is also supported by Salp15 concentrations used in this study, since approximately 0.1% of tick saliva consists of Salp15

which equals a concentration of 1 μg/ml [4] and the concentration of Salp15 increases over time during tick feeding, reaching high concentrations locally. Our observations are in line with data from Cassavani et al. showing that saliva from the *Rhipicephalus sanguineus* tick inhibits IL-12 production by murine bone marrow–derived DCs [23]. Also, very recently, *I. scapularis* saliva was shown to dose-dependently inhibit IL-12 and TNF-α production by murine bone marrow–derived DCs [24]. Although the authors contribute this effect to Prostaglandin E2 (PGE2), they state that other DC modulators might be present in *I. scapularis* saliva. Importantly, both studies were performed with murine DCs, whereas our experiments are performed with human DCs. The fact that the addition of rabbit polyclonal anti-Salp15-antibodies abrogates the capacity of *Ixodes* saliva to inhibit IL-12, but not IL-6 and TNF-α (unpublished data), might indicate that saliva contains other molecules, such as PGE2, that are able to block IL-6 and TNF-α. However, silencing of Raf-1, and inhibitors of Raf and MEK restored Salp15-induced suppression of IL-12p35, IL-6, and TNF-α, suggesting that the anti-Salp15 antibodies are less effective than small molecule inhibitors or RNAi treatment. Possibly due to genetic variability [25], DCs derived from approximately half the donors failed to produce IL-10 upon stimulation with LPS. Moreover, the IL-10 production might be dependent on the TLR ligand used to activate DCs, since Salp15 did enhance IL-10 production by LTA- and *B. burgdorferi*-treated DCs (Figures 6 and S1). Neutralization of IL-10 using antibodies did not restore IL-12 levels, demonstrating that inhibition of pro-inflammatory cytokines is not due to IL-10 production (unpublished data).

B. burgdorferi by itself does not modulate DC function, since human DCs have been shown to react adequately to *B. burgdorferi* [17]. However, similar to the observed effects of Salp15 on LPS-stimulated DCs, preincubation of *B. burgdorferi* with Salp15 resulted in inhibited pro-inflammatory cytokine production (Figure 6). Immunosuppression of the host by Salp15 could be advantageous to the arthropod vector and the spirochete, since it could impair adaptive immune responses to both tick as well as *B. burgdorferi* antigens and could therefore be an important factor in the pathogenesis of Lyme disease. Interestingly, Ramamoorthi et al. have shown that Salp15 mRNA levels were 13-fold higher in *B. burgdorferi*-infected engorged ticks than in uninfected controls [7]. This symbiosis might be the result of millions of years of co-evolution of the spirochete and the arthropod vector.

Pro-inflammatory cytokines, such as IL-12, are essential to T cell activation as well as differentiation [26], and indeed Salp15, as well as tick saliva, inhibits DC-mediated T lymphocyte activation (Figure 5). This inhibition was not due to a direct effect of Salp15 on lymphocyte proliferation, since, besides extensive washing of the DCs before addition of lymphocytes, simultaneous addition of lymphocytes and Salp15 to DCs did not result in impaired lymphocyte proliferation (unpublished data).

The inhibition of DC cytokine production by Salp15 we describe here and the previously described direct inhibition of T cell activation through binding to CD4 [4,5] could be complementary to

each other. Salp15 could inhibit T cell activation by directly binding to CD4 on T cells entering the tick-host-pathogen interface. In addition, inhibition of cytokine production by DCs by Salp15 could inhibit these DCs from adequately activating T cells in regional lymph nodes or at the site of the feeding lesion. Both approaches will lead to decreased numbers of effector T cells and impaired adaptive immune responses. In contrast to T cells, DCs express only low levels of CD4 [27] suggesting that CD4 is not a major receptor for Salp15 on human DCs. Indeed, only DC-SIGN was immunoprecipitated by Salp15 from immature DC lysates (Figure 2), supporting our findings that DC-SIGN plays an important role in the observed immunosuppression. However, we can not exclude a low affinity/avidity interaction between Salp15 and another receptor on DCs, possibly CD4.

Several pathogens and parasites have been demonstrated to target DC-SIGN on DCs to modulate DC-mediated immune responses [22,28] leading to evasion of host immune responses and prolonged pathogen survival. Blocking antibodies against DC-SIGN were not effective in restoring cytokine responses inhibited by Salp15 (unpublished data) suggesting that even a decreased binding of Salp15 to DCs is sufficient to inhibit cytokine responses. Recently, we have demonstrated that binding of various pathogens such as *Mycobacterium tuberculosis* to DC-SIGN leads to activation of the serine/threonine kinase Raf-1 [9]. To further identify the mechanism by which Salp15 inhibits DC cytokine production, we also assessed the ability of Salp15 to activate Raf-1. Similar to the mycobacterial component ManLAM, Salp15 activates Raf-1 by inducing phosphorylation of Raf-1 at the residues S338 and Y340/341, which is partially blocked by antibodies against DC-SIGN (Figure 3), supporting an important role for DC-SIGN in Raf-1 activation. Furthermore, silencing of Raf-1 in human primary DCs by RNA interference completely restored *IL-12p35*, *IL-6*, and *TNF-α* transcription (Figure 3) and protein expression (unpublished data) after activation by LPS in the presence of Salp15. In addition, cytokine levels could be completely restored by blocking Raf-1 activation during stimulation of DCs with Salp15 and LPS using a Raf inhibitor (Figure 3). Thus, DC-SIGN-induced Raf-1 activation is essential to the observed immunosuppression by Salp15. Recently, we have demonstrated that Raf-1 activation by the mycobacterial component ManLAM leads to acetylation of the NF-κB subunit p65, but only after TLR-induced activation of NF-κB. Acetylation of p65 both prolonged and increased *IL-10* transcription to enhance anti-inflammatory cytokine responses [9]. Strikingly, our data demonstrate that acetylation of the NF-κB subunit p65 is not involved in the suppression of pro-inflammatory cytokines by Salp15, since an inhibitor of the HATs p300/CBP, responsible for p65 acetylation, did not abrogate pro-inflammatory cytokine suppression by Salp15 (Figure 3). Moreover, we did not see any effect on IL-8 and IL-10 cytokine levels (Figure S2C). These data indicate that Raf-1 activation by Salp15 leads to activation of different downstream effectors than ManLAM [9]. Indeed, Salp15-induced inhibition of cytokine production is completely blocked by the MEK1/2 inhibitor U0126, demonstrating that MEK kinases are essential downstream effectors of Raf-1 after DC-SIGN/Salp15 ligation, in sharp contrast to DC-SIGN/ManLAM ligation [9]. Raf kinases are known to activate MEK1 and MEK2 through phosphorylation of two serine residues [29]. MEK kinases are well-known to

signal through ERK kinases and there is evidence that suggests that antibody ligation of DC-SIGN leads to ERK activation [30]. However, DC-SIGN ligation by its natural ligands such as ManLAM and Salp15 does not result in ERK activation (Figure 3 and [9]), demonstrating that ERK is not involved in pathogen-induced DC-SIGN signaling. The mechanism of MEK-dependent and ERK-independent signaling induced by Salp15 binding to DC-SIGN might be dependent on the subcellular localization of the kinases [31–33]. Salp15 might interact with low avidity to other receptors on DCs such as CD4, which could result in an altered signaling cascade. A recent study has demonstrated that Salp15 binding to CD4 modulates actin polymerization [6]. This observation implies that coligation of DC-SIGN and CD4 by Salp15 might affect the subcellular localization of Raf-1 or its downstream effectors. This would result in the activation of different kinases compared to DC-SIGN ligands that do not affect actin polymerization, such as ManLAM. Thus, although Salp15 binding to DC-SIGN does induce Raf-1 similar to ManLAM, the downstream effectors are different. Indeed, Salp15 affects pro-inflammatory cytokine production by human DCs both at the transcriptional and the post-transcriptional level. We demonstrate that Salp15 increases IL-6 and TNF-α mRNA decay, and impairs nucleosome remodeling at the *IL-12p35* promoter. This is in contrast to pathogens such as mycobacteria and HIV-1 that interact with DC-SIGN to increase the transcription rate and prolong transcription activity through acetylation of p65 [9]. These data further demonstrate that pathogen binding to DC-SIGN might lead to different ligand-specific signaling cascades that regulate distinct adaptive immune responses. Although our data suggest that Raf-1 activation might play a central role in these immune responses, the downstream effectors of Raf-1 regulate the subsequent cytokine responses. Future research on the molecular mechanism of Salp15-induced DC immunosuppression will have to elucidate how MEK and downstream effectors affect mRNA stability and nucleosome remodeling to inhibit pro-inflammatory cytokine expression. This might be due to post-translational modifications of the proteins involved in nucleosome remodeling and mRNA decay [34,35]. Further characterization of the Salp15/DC-SIGN-induced signaling pathway could lead to the identification of new anti-inflammatory or immunosuppressive agents. TLR-mediated immune responses play an important role in a variety of diseases including infectious diseases, autoimmune diseases, and atherosclerosis. Therefore manipulation of TLR-triggered signaling is of wide clinical interest and the Salp15/DC-SIGN-induced signaling pathway described in the current study might prove to be important in the development of novel immunotherapies [36]. In addition, interfering with Salp15-induced signaling could potentially enhance anti-*Borrelia* immune responses and might be an alternative novel intervention strategy to prevent or potentially even treat Lyme borreliosis. Furthermore, efforts are being made to target biologically important vector proteins to prevent pathogen transmission from the tick to the host [37,38].

In summary, the salivary gland protein Salp15 is a tick protein exerting various activities at the tick-host-pathogen interface. We here report that Salp15 modulates TLR-induced DC pro-inflammatory cytokine production and renders DCs less capable of activating T lymphocytes. Salp15 binds to the surface of immature DCs through the C-type lectin receptor DC-SIGN, which results in the

phosphorylation of the serine/threonine kinase Raf-1 and subsequent MEK activation. Silencing or inhibition of Raf-1 as well as inhibition of MEK abrogates the effects of Salp15. Both mRNA destabilization and impairment of nucleosome remodeling are responsible for the decreased pro-inflammatory cytokine production observed after DC-SIGN/Salp15 ligation. Immunosuppression of the host by Salp15 could be advantageous for both the arthropod vector as well as the spirochete, since it could impair adaptive immune responses against tick and/or *B. burgdorferi* antigens.

Materials and Methods

Salp15 purification and tick saliva collection

Salp15 (GenBank accession number: AAK97817) was isolated from cultured *Drosophila* S2 cells as described previously [4]. Briefly, *Drosophila* S2 cells (Invitrogen), cotransfected with the recombinant pMT/BiP/V5-HisA-*salp15* vector and the hygromycin selection vector, pCOHYGRO (Invitrogen), were grown as large cultures in DES serum-free medium and induced with copper sulphate. The supernatant was used to purify recombinant Salp15 containing the V5 epitope and HIS-tag by using 5-ml pre-packed nickel charged HisTrap FF columns (GE Healthcare). The protein was eluted using 100 mM imidazole, extensively dialyzed against PBS (pH 7.4), and concentrated by centrifugal filtration through a 5-kDa filter (Vivascience). Tick saliva was collected from fed ticks as described previously [4]. Briefly, adult ticks were allowed to feed on rabbits, removed ticks were immobilized, and a capillary tube was fitted over the mouthparts. Two µl of 5% pilocarpine (Sigma-Aldrich) in methanol was applied topically to the dorsa, and saliva was collected and stored at −80 °C until use.

Borrelia burgdorferi cultures

B. burgdorferi sensu stricto strain B31 clone 5A11 [39], referred to as *B. burgdorferi* throughout the paper, was cultured in Barbour-Stoenner-Kelly (BSK)-H medium (Sigma-Aldrich). Spirochetes were grown to approximately 5 $\times 10^7$/ml (enumerated using a Petroff-Hausser counting chamber as described previously [40]), pelleted by centrifugation at 2,000 x *g* for 10 min, and resuspended in RPMI medium without antibiotics.

Cell culture and dendritic cell stimulation

Immature DCs were cultured as described before [41]. Immature DCs were used for experiments at day 6. A total of 100,000 DCs were stimulated with Salp15 (10–50 µg/ml) or tick saliva (diluted 1:75–1:300) in the presence or absence of *Samonella typhosa* LPS (10 ng/ml, Sigma-Aldrich) or LTA (10 µg/ml). For isolation of mRNA, cells were lysed after 6 h of incubation. To determine cytokine production and expression of cell surface markers, cells were incubated for 18 h. Supernatants were harvested to determine cytokine production, whereas the cells were analysed by flow cytometry analysis (FACS) for surface expression of CD80, CD86, or CD83. Cells were incubated at 4 °C for 30 min with PE-labeled antibodies (CD80-PE, CD86-PE, (Pharmingen); and CD83-PE (Immunotech). In addition, a staining with 7-amino-actinomycin D (7-AAD,

Molecular Probes) was performed. Spirochetes (1×10^5 or 1×10^6) were pre-incubated with Salp15 (25 μg/ml final concentration) for 30 min before addition to 100,000 DCs. After 18 h of incubation supernatant was harvested, and cells were fixed in 2% PFA before performing FACS analysis. For binding experiments, we used Raji cells and Raji transfectants expressing wild-type DC-SIGN (Raji-DC-SIGN). Raji-DC-SIGN was generated as previously described [27,42]. All cells used in these studies were cultured in RPMI containing 10% fetal calf serum.

T lymphocyte proliferation (mixed lymphocyte reaction)

After 18 h of stimulation, DCs were washed extensively with medium. Next, DCs were cultured with allogenic PBLs (1×10^5) at different ratios (1:50–1:500) for 4 d at 37 °C. T lymphocyte proliferation was assessed by measuring the overnight incorporation of [methyl-^{3}H] Thymidine (Amersham Biosciences).

Cytokine measurements.

For detection of cytokines, supernatants were harvested 18 h after DC activation and stored at −20 °C until further analysis. TNF-α, IL-10, IL-6, IL-12 p70, IL-8, and IL-1β were measured using cytometric bead array kits (BD Biosciences) according to the manufacturer's recommendations.

RNA extraction, mRNA half-life determination, and quantitative real-time PCR

mRNA was specifically isolated with the mRNA capture kit (Roche) and cDNA was synthesized with the reverse transcriptase kit (Promega). For real-time PCR analysis, PCR amplification was performed in the presence of SYBR green, as previously described [43]. Specific primers for IL-12p35, IL-6, TNF-α, IL-8, IL-10, and GAPDH were designed by Primer Express 2.0 (Applied Biosystems) [9]. IL-12p35, IL-6, TNF-α, IL-8, and IL-10 transcription was adjusted for GAPDH transcription. For the determination of mRNA half-life, DCs were stimulated with LPS for 6 h prior to the addition of 10 μg/ml actinomycin D (Sigma-Aldrich) to block transcription; mRNA was then isolated at 20-min time periods. We calculated the half-life of the different mRNA transcripts by applying non-linear fitting according to the one phase exponential decay model (Graphpad Prism Software version 4.0).

Fluorescent bead adhesion assays

Fluorescent bead adhesion assays were performed as described previously [27]. Briefly, streptavidin-coated TransFluorSpheres (488/645 nm, 1.0 μm; Molecular Probes) beads were incubated with biotinylated goat anti-mouse F(ab)2 fragments (6 μg/ml; Jackson Immunoresearch), followed by overnight incubation with mouse anti-V5 (Invitrogen) (3 μg), and overnight incubation with Salp15 (5 μg), or PBS with 1% BSA for the generation of Salp15-, or negative-control beads, respectively. As a positive control fluorescent beads coated with the HIV protein gp120 were used [27]. 50,000 cells were incubated with beads for 45 min at 37 °C. When indicated cells were pre-treated with mannan (1 mg/ml), EDTA (10mM), free sugars N-acetylgalactosamine (GalNac), GlcNac, galactose, fucose; all 50 mM) or biotinylated LeX/LeY- (20 μg /ml) for 15 min at 37 °C. Bead adhesion to the cells was measured by FACS analysis.

Plant lectin ELISA

Salp15 (5 µg/ml) was coated onto maxisorb ELISA plates (NUNC) for 18 h at room temperature (RT). The plate was blocked by incubating with 1% BSA for 1 h at 37 °C. Biotinylated lectins Concanavalin A (Con A), GNA (*Galanthus nivalis* agglutinin), NPA (*Narcissus pseudonarcissus* agglutinin), PSA (*Pisum sativum* agglutinin), LTA (*Lotus tetragonolobus* agglutinin), UEA (*Ulex europaeus* agglutinin), or PNA (peanut agglutinin; Sigma-Aldrich) were added for 2 h at a concentration of 5 µg/ml. Binding of biotinylated lectins was detected using peroxidase-labeled strepavidin and absorbance was read at 450 nm.

DC-SIGN-Fc binding ELISA

The soluble DC-SIGN-Fc binding ELISA was performed as previously described [44]. Briefly, 5 µg/ml Salp15 or mannan was coated onto maxisorb ELISA plates (NUNC) for 18 h at RT. Unspecific binding was blocked by incubating the plate with 1% BSA for 1 h at RT. Soluble DC-SIGN-Fc was added for 1 h at RT. Specificity was determined (unless indicated otherwise) by blocking with mannan (1 mg/ml). Unbound DC-SIGN-Fc was washed away and binding was determined using a peroxidase-conjugated goat anti-human Fc antibody (Jackson Immunoresearch). Peroxidase-labeled strepavidin was used to detect DC-SIGN-Fc binding. Absorbance was read at 450 nm. To assess whether carbohydrate structures on Salp15 were involved in binding to DC-SIGN Salp15 was deglycosylated using PGNaseF under non-denaturing conditions according to the manufacturer's instructions (New England Biolabs) and was coated onto maxisorb ELISA plates (NUNC).

Immunoprecipitation and immunoblot analysis

The surface of DCs was biotinylated for 30 min at 4 °C with 0.5 mg/ml of sulfo-NHS-biotin (Pierce) in PBS (pH 7,4) and cells were then lysed for 1 h at 4 °C in lysis buffer (10 mM triethanolamine [pH 8.2], 150 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, and 1% [volume/volume] Triton X-100, containing EDTA-free protease inhibitors) (Roche Diagnotics). Salp15 ligands were immunoprecipitated with Salp15- (via anti-V5) coupled protein A–Sepharose beads (CL-4B; Pharmacia). As a positive and negative control, we used anti-DC-SIGN (AZN-D2) [27], and anti-V5-coupled protein A–Sepharose beads respectively. Immunoprecipitation products were separated by SDS-PAGE and transferred to nitrocellulose membranes. A Page Ruler Protein ladder (Fermentas) was run adjacently. Blots were blocked with 5% BSA in PBS followed by immunoblot analysis with streptavidin-coupled peroxidase (Vector Laboratories). To immunoprecipitate Salp15 ligands from non-biotinylated DC lysate we performed a similar assay. However, the blot was stained with specific goat antibodies against DC-SIGN (Santa Cruz Biotechnology), followed by secondary peroxidase-conjugated swine anti-goat (Tago). Blots were developed by enhanced chemiluminescence.

Phosphorylation of Raf-1 and ERK, and inhibition of Raf-1, MEK, and p300/CBP-mediated acetylation of the NF-κB subunit p65

DCs were stimulated for 15 min with Salp15 (25 μg /ml) or controls. When indicated, cells were stimulated with LPS (10 ng/ml) or PMA (150 ng/ml) plus ionomycin (5 μg /ml) (positive control for ERK phosphorylation, Sigma-Aldrich) for 15 min, or pre-incubated with blocking anti-DC-SIGN antibodies (AZN-D2) [27] for 30 min. Subsequently, cells were fixed in 3% para-formaldehyde for 10 min and permeabilized in 90% methanol at 4 °C for 10 (Raf-1) or 30 (ERK) min. To assess phosphorylation of Raf-1 we used a rabbit anti-phospho-c-Raf (Ser338) mAb (Cell Signaling) and a rabbit anti-c-Raf (pTyr340, Tyr341) pAb (Calbiochem) and to assess phosphorylation of ERK a rabbit-anti-phospho-p44/42 MAPK (Thr202/Tyr204) mAb (Cell Signaling). Phosphorylation of Raf-1 and ERK was measured by flow cytometry after incubation with PE-conjugated donkey anti-rabbit antibodies as described [9]. When indicated, DCs were pre-incubated for 2 h with 1 μM GW5074 (Calbiochem), a Raf inhibitor, 4 μM U0126 (LC Laboratories), an inhibitor of MEK1 and MEK2, or 30 μM anacardic acid (AA) (Calbiochem), an inhibitor of the histone acetyltransferases p300 and CBP, respectively. Thereafter, cells were stimulated for 6 h with LPS as previously described, and mRNA was isolated for the generation of cDNA and real time PCR analysis.

RNA interference

DCs were transfected with 100 nM siRNA using transfection reagens DF4 (Dharmacon), according to the manufacturer's protocol. The siRNAs used were: Raf-1 SMARTpool (M-003601–00) and non-targeting siRNA pool (D-001206–13) as a control (Dharmacon). This protocol resulted in a nearly 100% transfection efficiency as determined by flow cytometry of cells transfected with siGLO-RISC free-siRNA (D-001600–01). At 72 h after transfection, cells were used for experiments as described above. Silencing of *Raf-1* transcription was confirmed by quantitative real-time PCR.

Chromatin accessibility measured by real-time PCR (ChART) assay

To quantify nucleosome remodeling at the *IL-12p35* promoter, chromatin accessibility was measured by a real-time PCR (ChART) assay. Nuclei were prepared from unstimulated cells, or cells stimulated as indicated, with lysis buffer (10 mM Tris-HCl [pH 7.5], 15 mM NaCl, 3 mM MgCl2, 0.5 mM spermidine, 1 mM PMSF, 0.5% Nonidet P-40). Digestion reactions were performed with 50 U BstIXI or 50 U EcoRI for 1 h at 37 °C. After proteinase K and RNase A treatment, DNA was purified using the QIAamp DNA blood kit (Qiagen). Real-time PCR reactions were then performed as described above. Amplification with primer set A (encompassing BstXI site located at nucleotide −298) is sensitive to remodeling of nuc-2 [16]. Increased accessibility of the region results in reduced amplification in the real-time PCR. Amplification with primer set B (encompassing BstXI site located at nt 456) was performed as an internal control to test for the efficiency of BstXI digestion as the accessibility of this locus is not subject to changes in the chromatin structure [16]. To normalize for DNA input amounts, each sample was analyzed with primer set C for GAPDH. Results are expressed as a percentage of the remodeling observed in the EcoRI-digested sample for each cell treatment using the formula (Nt*Eco*RI – Nt*Bst*XI/

Nt*Eco*RI) × 100%, with Nt = 2Ct(primer set C)-Ct(primer set A). The following primer sequences were used: set A (IL-12p35 promoter): forward 5' GCGGGGTAGCTTAGACACG 3', reverse 5' CCCAAAATGAAAGCGAAATG 3'; set B (BstXI control): forward 5' TCTAAAGTCAGGCTTGGCCG 3', reverse 5' GGTTTCACCATGTTGGTCAGG 3'; set C (GAPDH promoter): forward 5' TACTAGCGGTTTTACGGGCG 3', reverse 5' TCGAACAGGAGGAGCAGAGAGCGA 3'.

Statistical analysis

Statistical analysis was performed using parametric tests (Graphpad Prism Software version). When multiple conditions were compared a Dunnett multiple comparisons test was performed. Statistical significance of the data was set at $P < 0.05$, with one asterisk (*) representing $0.01 < P < 0.05$; two asterisks (**) $0.001 < P < 0.01$.

Author contributions. JWRH and MAWPJ conceived and designed the experiments, performed the experiments, analyzed the data, and wrote the paper. JD and ML performed experiments. EF and TVP contributed reagents/materials/analysis tools. SIG and TBHG conceived and designed the experiments, analyzed data, contributed reagents/materials/analysis tools, and wrote the paper.

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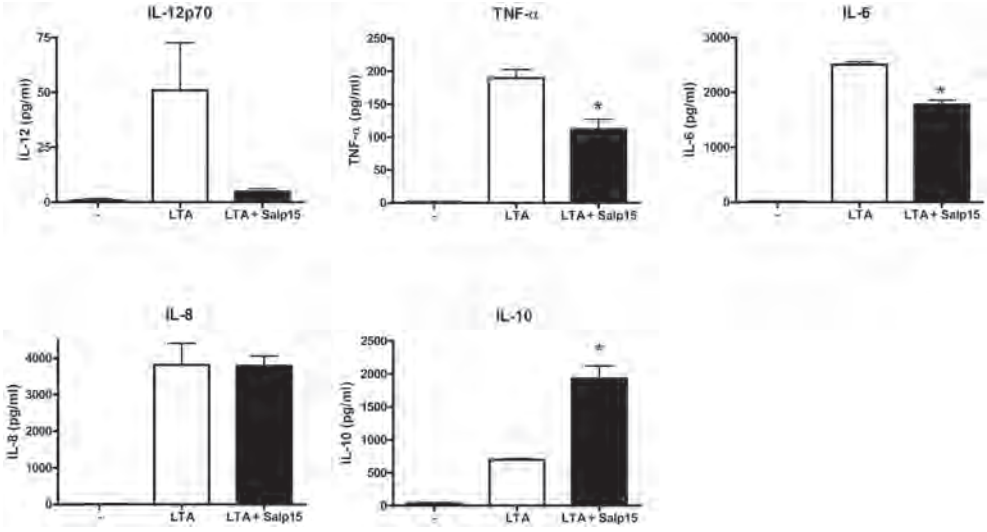
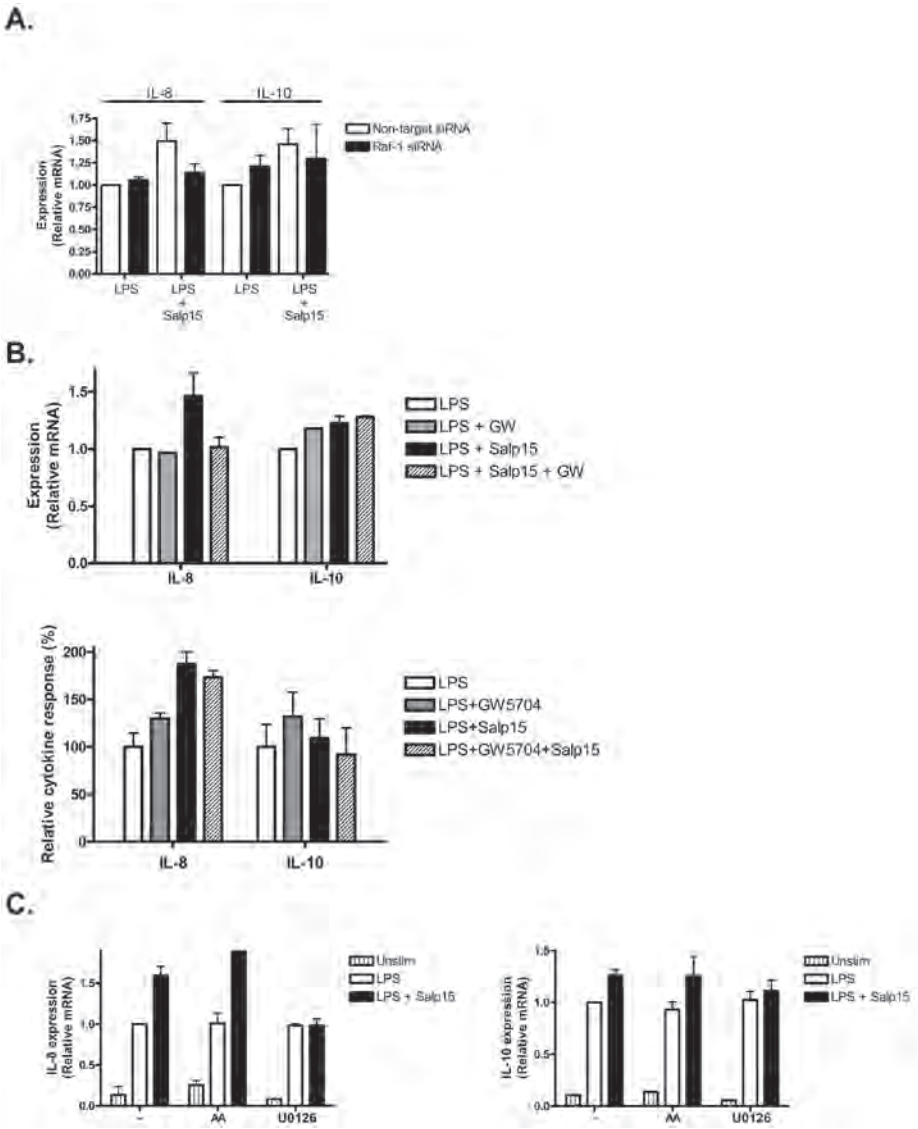
Competing interests. The authors have declared that no competing interests exist.

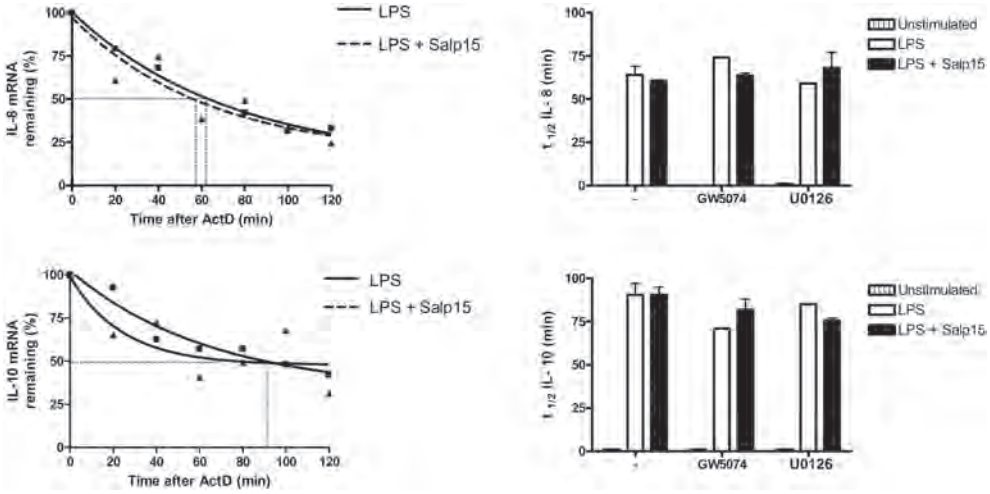
Supporting information



Supplemental figure 1. Salp15 inhibits IL-12, IL-6, and TNF-α production by DCs stimulated with LTA. Immature human DCs were stimulated with control medium (grey bars), or with 10 μg/ml LTA in the presence (black bars) or absence (white bars) of 25 μg/ml Salp15. Supernatants were analyzed for cytokine production after 18 h of stimulation. Bars represent triplicates within one experiment ± SE. The graphs are representative of two independent experiments with two independent donors. For a more detailed description of the experiment see legend Figure 1.



Supplemental figure 2. The Salp15 effect on DC cytokine production is Raf-1-fependent.
(A) RNAi-mediated silencing of Raf-1 abrogates the effect of Salp15 on DC cytokine production. siRNA-treated cells were stimulated for 6 h with LPS in the presence or absence of 25 μg/ml Salp15. Relative mRNA expression of LPS-stimulated non-targeting siRNA-treated cells was set at 1.
(B) Raf-1 inhibition by GW5704 abrogates the Salp15-induced effects on DC cytokine production. DCs were incubated with 1 μM GW5704 for 2 h before stimulation with LPS for 6 h (mRNA) or 18 h (protein) in the presence or absence of 25 μg/ml Salp15. Relative mRNA expression of LPS-stimulated cells was set at 1. For protein levels, protein levels in DCs stimulated with LPS was set at 100%, which is representative of 2,407 ± 355 pg/ml IL-8 and 6,194 ± 1,934 pg/ml IL-10.
(C) MEK1/2-inhibitor U0126 abrogates the Salp15-induced effects on cytokine production. DCs were preincubated with anacardic acid (AA), an inhibitor of the histone acetyltransferases p300 and CBP, or U0126, an inhibitor of MEK1 and MEK2, or DMSO as a control. Relative mRNA expression of LPS-stimulated cells was set at 1.
For a more detailed description of the experiments see legend Figure 3.



Supplemental figure 3. Salp15 does not destabilize IL-8 and IL-10 mRNA.
DCs were stimulated with Salp15 and LPS for 6 h. Actinomycin D was added to block new mRNA synthesis. Cells were harvested for 2 h to determine mRNA half-life. For a more detailed description of the experiment see legend Figure 4.

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PART II

TICK-PATHOGEN INTERACTIONS

Identification of Salp15 homologues in *Ixodes ricinus* ticks

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Chapter 4

Abstract

The 15-kDa *Ixodes scapularis* salivary gland protein Salp15 protects *Borrelia burgdorferi* sensu stricto from antibody-mediated killing and facilitates infection of the mammalian host. In addition, Salp 15 has been shown to inhibit T-cell activation. We determined whether *Ixodes ricinus*, the major vector for Lyme borreliosis in Western Europe, also express *salp15*-related genes. We show that engorged *I. ricinus* express *salp15* and we have identified three Salp15 homologues within these ticks by reverse transcriptase–polymerase chain reaction (RT-PCR). One of the predicted proteins showed 80% similarity to *I. scapularis* Salp15, evenly distributed over the entire amino acid sequence, whereas the two other predicted proteins showed approximately 60% similarity, mainly confined to the signal sequence and C-terminus. Comparison of the DNA and protein sequences with those deposited in several databases indicates that these proteins are part of a Salp15 family of which members are conserved among different *Ixodes* species, all capable of transmitting *B. burgdorferi* sensu lato. This suggests that these Salp15 homologues could also play a role in the transmission of diverse *Borrelia* species and in inhibition of T-cell activation.

Introduction

Ixodes ticks are present worldwide and can transmit numerous pathogens, including *Borrelia*, *Anaplasma*, *Babesia*, and flaviviral species (de la Fuente et al. 2006, Jongejan et al. 2004). A major tick-borne illness is Lyme borreliosis, which is common in many parts of Asia, Europe, and the United States. Human Lyme borreliosis is often characterized by an expanding red cutaneous lesion, *erythema migrans*. Moreover, cardiac conduction system abnormalities, arthritis, neurologic symptoms, and later during the course of the infection, cutaneous manifestations, such as *acrodermatitis chronica atrophicans* (ACA) can occur (van Dam et al. 1993). Joint disease is associated with *B. burgdorferi* sensu stricto infection, while neuroborreliosis and ACA are predominantly caused by *B. garinii* and *B. afzelii* infection, respectively (Steere 2001, van Dam et al. 1993). *B. burgdorferi* sensu stricto is prevalent in the United States, whereas all three pathogenic *Borrelia* species are found in Europe.

The main vector for Lyme borreliosis in Europe is *I. ricinus,* whereas in the United States *B. burgdorferi* sensu stricto is mainly transmitted by *I. scapularis*. Another *Ixodes* tick, *I. pacificus*, which is prevalent in the Western United States, can also transmit *Borrelia* spp. (Peavey et al. 1995). In Eurasia, *B. burgdorferi* sensu lato can also be transmitted by *Ixodes persulcatus. B. burgdorferi* sensu stricto has been shown to influence transcription of certain tick genes and to interact with *I. scapularis* proteins. For example, an *I. scapularis* receptor for *B. burgdorferi* outer surface protein (Osp) A, named the Tick Receptor for OspA (TROSPA), is critical for spirochete adherence to the tick gut (Pal et al. 2004). Moreover, a 15-kDa salivary gland protein, Salp15, facilitates the transmission of *B. burgdorferi* from the vector to the host (Ramamoorthi et al. 2005). Salp15 is secreted in the salivary glands of feeding *I. scapularis* ticks and binds to *B. burgdorferi* OspC. This interaction protects the spirochete from antibody-mediated killing in immune mice and also provides a survival advantage for *B. burgdorferi* in naive mice. Like *TROSPA*, *salp15* is expressed to a higher extent, when ticks are infected with *B. burgdorferi*. Salp15 also has been shown to inhibit T cell activation, and it is likely that ticks use Salp15 to more efficiently feed on a host (Anguita et al. 2002). Therefore, in nature, increased Salp15 levels could be beneficial for both vector and spirochete. To date there is very little information on similar proteins in *I. ricinus*, and we therefore determined whether Salp15 is present in this tick species.

Methods

Purification of *I. ricinus* RNA and RT-PCR

The *I. ricinus* ticks belonged to the fourth laboratory-bred generation of a colony that originated from Sweden. We fed adult female *I. ricinus* on rabbit ears in the presence of adult males. After 3 days of feeding, semi-engorged female *I. ricinus* ticks were removed and salivary glands were dissected under a microscope as described previously (Das et al. 2001). Three to four salivary glands were pooled. A similar approach was used for other tick species; however, these were fed on calves instead of rabbits. *I. hexagonus*, *Hyalomma detritum*, *Rhipicephalus evertsi evertsi*, *Boophilus annulatus*, and *Amblyomma variegatum* were all laboratory bred and maintained in tick feeding facilities at Utrecht

University. RNA was isolated using Tryzol reagent (Invitrogen, Carlsbad, CA). RNA was treated with DNAse to remove contaminating DNA (Invitrogen). Complementary DNA was made using a cDNA synthesis KIT in accordance with the manufacturer's recommendations (Stratagene, La Jolla, CA). Reverse transcriptase–polymerase chain reaction (RT-PCR) on *I. ricinus* salivary gland cDNA, or cDNA from other tick species, was performed using different sets of primers. We used primers designed from the 3'- and 5'- end of *I. scapularis salp15* (forward: ATGGAATCTTTCG-TCGCAATG, reverse: CTAACATCCGGGAATGTGC); primers targeting tick *beta-actin* (forward: GATCATGTTCGAGACCTTCA, reverse: AGAGCTTCTCCTTGATGTCG); or degenerate primers targeting conserved domains of Salp15, as determined by aligning sequences found by the PSI-BLAST search method (forward: GTNGCIATGAARGTIGTITG, reverse: CAIWIRAAAGTR-CARTYYTTRAA). RT-PCR conditions were: 5' 95°C; 30" 95°C, 30" 52°C, 30" 68°C for 35 cycles followed by 7' of extension at 68°C using High Fidelity Taq Polymerase (Invitrogen). Thus, three nucleotide sequences were identified that shared homology to *I. scapularis salp15*. The cDNA sequences were deposited in GenBank and designated *salp15 Iric-1* (EU128526), *Iric-2* (EU128527), and *Iric-3* (EU128528).

3'- rapid amplification of cDNA ends (RACE)

The RLM-RACE kit was used to identify the sequence at the 3'-end according to the manufacturer's instructions (Ambion, TX). First strand cDNA was synthesized from total *I. ricinus* salivary gland RNA using the 3'-RACE Adapter. The cDNA was then subjected to a PCR using the outer 3'-RACE primer, which is complementary to the outer part of the anchored adapter and a gene specific primer, i.e., GGAAGGAAGGGAGTTACGAGAT, AGGTGGTGTGCATAGTCCTTTT, and ATTTCCCCCGTATGTTCCTAAC for *salp15 Iric-1*, *2*, and *3*, respectively. For *salp15 Iric-2*, we also performed a nested PCR with a gene-specific primer - TCCCTATGTTCCTAACCACCAC - and an inner 3'-RACE primer that is complementary to the inner part of the anchored adapter (without overlap with the outer adapter primer). PCR conditions were similar to those described above; however, an annealing temperature of 60°C was used.

Sequencing

RT-PCR and PCR products were ligated into the pGEM-T easy sequencing vector according to the manufacturer's instructions (Promega, Madison, WI), cloned in DH5-alpha (Invitrogen) and plated on LB-ampicillin plates. Single colonies were cultured into LB-ampicillin (50 μg/mL) and recombinant DNA was isolated using the Mini-prep KIT (Qiagen, Valencia, CA). Inserts were sequenced using Big Dye Terminator mix, M13 forward or reverse primers, and an automated sequencer (3730 DNA analyzer; all from AB Applied Biosystems, Foster City, CA).

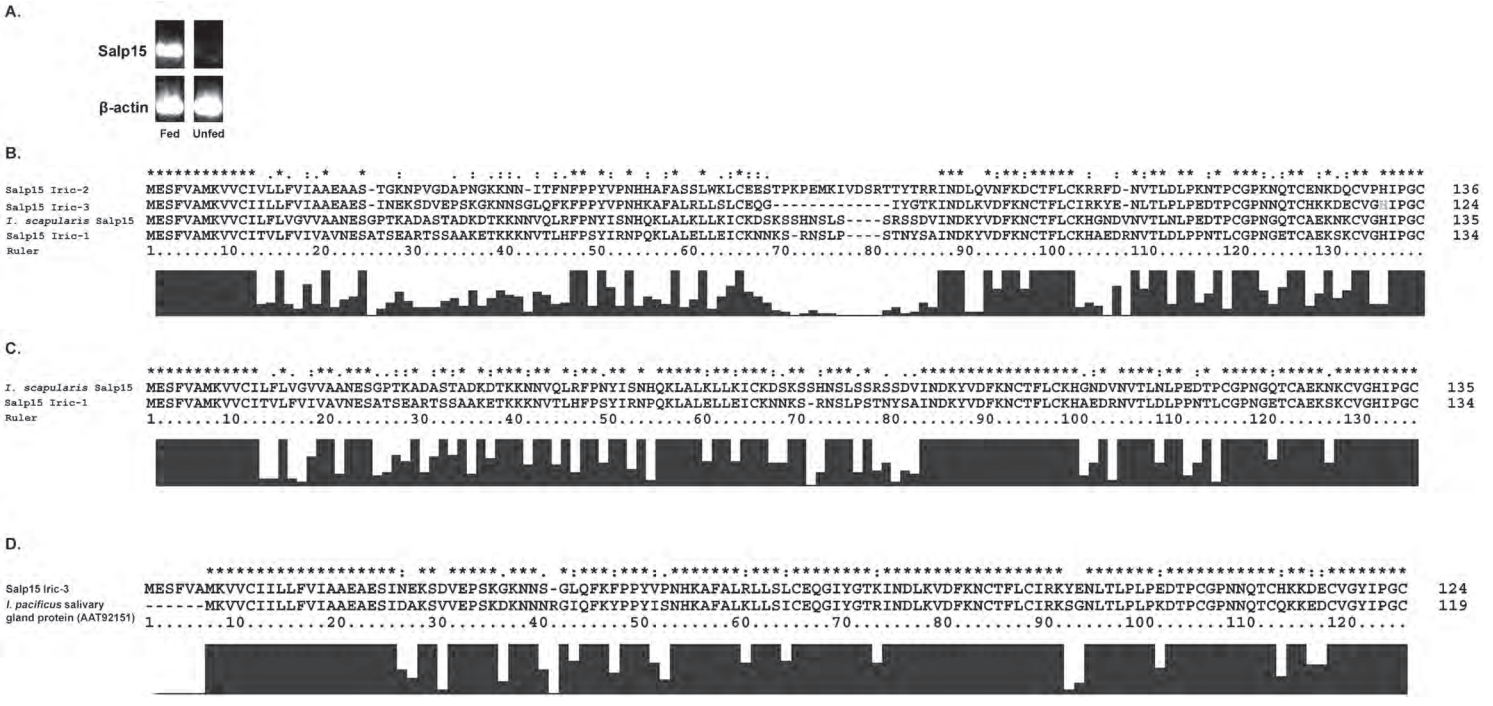

Figure 1. Salp15 homologues in *Ixodes ricinus*. (A) Salivary glands from fed (semi-engorged) and unfed adult female *I. ricinus* ticks were dissected, and RNA was extracted. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using primers based on the 3'- and 5'-end of *I. scapularis salp15* and primers directed against tick *β-actin*. The PCR product was cloned into a sequencing vector and sequenced. Over 60 sequences were retrieved, representing three assembled contigs (Codon Code Aligner 1.5.2) (B,C). We here show the alignment of the predicted proteins sequences with *I. scapularis* Salp15. Because of the similarity to *I. scapularis* Salp15, the predicted proteins were named *I. ricinus* Salp15 Iric-1 (ABU93613), 2 (ABU93614), and 3 (ABU93615) (B). For Salp15 Iric-3, the Histidine (H) is depicted in gray; it was shown by 3'-RACE (Fig. 2) that the actual amino acid should be a Tyrosine (Y). Salp15 Iric-1 was most similar to *I. scapularis* Salp15 and therefore is shown separately (C). Salp15 Iric-3 was highly similar to a putative secreted *I. pacificus* salivary gland protein (AAT92151) (D). Alignment was performed using the ClustalX 1.8.1 software. *, Complete conserved residue. :, a conserved residue from the "stronger similarity group" (S/T/A, N/E/Q/K, N/H/Q/K, N/D/E/Q, Q/H/R/K, M/I/L/V, M/I/L/F, H/Y, F/Y/W). ., conserved residue from the "weaker similarity group" (C/S/A/, A/T/V/, S/A/G/, S/T/N/K, S/T/P/A, S/G/N/D, S/N/D/E/Q/K, N/D/E/Q/H/K, N/E/Q/H/R/K, F/V/L/I/M, H/F/Y) as defined in the ClustalX 1.8.1 software that uses the PAM250-Gonnet matrix (Dayhoff et al. 1978, and Gonnet et al. 1992).

Phylogentic analysis

Sequences were aligned using the ClustalX 1.8.1 software. An unrooted Neighbor-Joining tree (Saitou et al. 1987) was constructed with the software Mega 3.1 (Kumar et al. 2004) using the Jones-Thorton-Taylor substitution matrix (Jones et al. 1992). Bootstrap sampling was reiterated 1000 times. Trees were visualized with Mega 3 Tree Explorer (Kumar et al. 2004).

Results and discussion

I. ricinus Salp15 homologues

By RT-PCR, using primers designed from the 3' and 5'-end of the *I. scapularis salp15* sequence, an approximately 400-bp amplification product was detected in cDNA obtained from salivary glands from fed – but not unfed – adult female *I. ricinus* ticks (Fig. 1A). This is similar to what has been shown for *I. scapularis* (Anguita et al. 2002). PCR products we-re then cloned into the pGEM-T easy sequencing vector and sequenced. More than 60 clones were analyzed, and three different assembled contigs were found (Codon Code Aligner Version 1.5.2). All three sequences consisted of a full-length open reading frame (ORF) and coded for predicted proteins that, because of the similarity to *I. scapularis* Salp15, were named Salp15 Iric-1, 2, and 3 and were assigned the GenBank accession number ABU93613, ABU93614, and ABU93615, respectively (Fig. 1B).

I. scapularis Salp15 has a 21–amino acid N-terminal signal peptide that is cleaved during protein expression (Anguita et al. 2002). Analysis using software to predict signal sequences (Signal IP 3.0, www.cbs.dtu.dk/ services/SignalP/) predicted signal peptides of 27 and 22 amino acids for Salp15 Iric-1, and Salp15 Iric-2 and 3, respectively (data not shown). Based on the complete identity of the first 12 amino acids of these proteins with the N-terminus from *I. scapularis* Salp15, we assume that we identified the start codon. Our approach did not allow us to identify upstream regions of the genes. Since the C-terminus is part of the mature protein, we determined whether the *I. ricinus salp15* sequences found by RT-PCR, using the primers designed based on the *I. scapularis salp15* sequence, were correct at the 3'-end. Using 3'-RACE, we confirmed that the 3'-ends of *salp15 Iric-1*, and *salp15 Iric-3* were correct. For *salp15 Iric-1,* there was only one, and for *salp15 Iric-3*, there were two basepair mutations, of which only one (in *salp15 Iric-3*) resulted in an altered, but similar, amino acid, i.e., Tyrosine instead of Histidine at the 120th amino acid position: H120Y (Fig. 2). We were unable to determine the 3'-end of *I. ricinus Iric-2*. Despite more stringent PCR conditions and a nested PCR, with an inner gene specific primer and a primer complementary to the inner part of the adaptor, we did not get a single amplicon, like we amplified for the other two sequences (data not shown). This is likely due to amplification of aspecific cDNA interfering with amplification of *I. ricinus Iric-2*. RT-PCR using degenerate primers directed against conserved regions of Salp15 confirmed the presence of the exact same three Salp15 homologues in fed *I. ricinus* female adult ticks (Fig. 3). Alignment of the three different full-length *I. ricinus salp15* sequences, and the different *I. ricinus* Salp15-like proteins, with *I. scapularis* Salp15, using the web-based NCBI software (www.ncbi.nlm.nih.gov/BLAST/) as described by Tatsuova et al. (1999), revealed

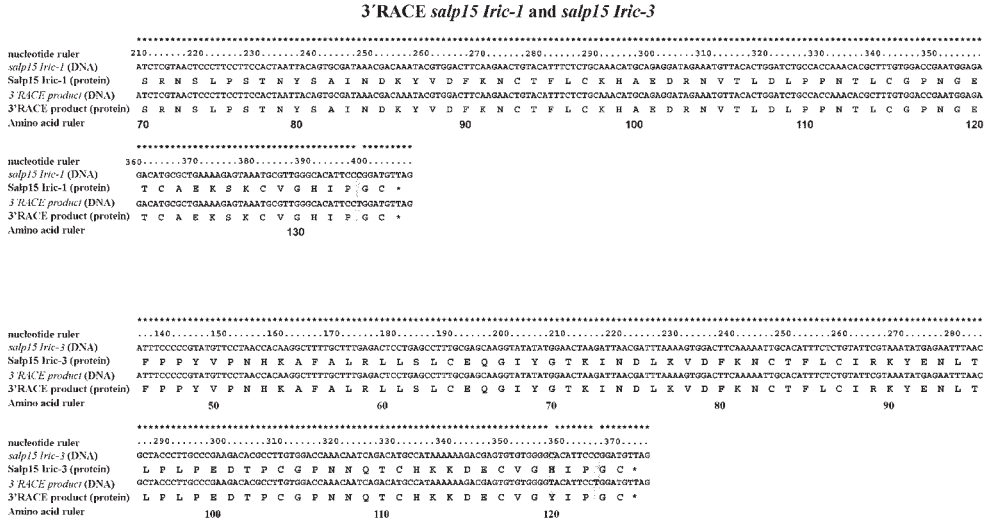

Figure 2. 3'-Rapid amplification of cDNA ends (RACE) of *Ixodes ricinus salp15* sequences. To verify the 3'-end of our sequences, we performed a 3'-RACE. Alignment of the original sequences with the RACE products showed only subtle differences. The nucleotide (DNA) and amino acid (protein) sequences are shown. In *I. ricinus salp15 Iric-1*, the change in nucleotide, T395C, did not result in a different amino acid. In *I. ricinus salp15 Iric-3*, in addition to a similar nucleotide change, there was another nucleotide change, T358C, which did result in an altered, but similar, amino acid, i.e., Tyrosine (Y) instead of Histidine (H); H120Y. These changes are highlighted in gray. Alignment was performed using the ClustalX 1.8.1 software. *Matching nucleotide.

different levels of identity and similarity (Table 1). Of the three *I. ricinus* Salp15 homologues, Salp15 Iric-1 was most similar to *I. scapularis* Salp15, with 80% similarity at the amino acid level (Table 1 and Fig. 1C). The other two predicted proteins, Salp15 Iric-2 and Salp15 Iric-3, were 62% and 63% similar to *I. scapularis* Salp15, respectively (Table 1 and Fig. 1B). In contrast, Salp15 Iric-3 was highly similar (94%) to a putative *I. pacificus* secreted salivary gland protein (Fig. 1D). Orthologues are homologous genes that result from a speciation event, whereas paralogues are

Table 1. Alignment of *Ixodes scapularis* Salp15 with the three *I. ricinus* Salp15 homologues.

			nucleotide level →		
		I. scapularis Salp15	Salp15 Iric -1	Salp15 Iric -2	Salp 15 Iric -3
	I. scapularis Salp15	–	82%	67%	66%
amino acid level ↓	Salp15 Iric -1	68% *80%*	–	68%	66%
	Salp15 Iric -2	50% *63%*	48% *61%*	–	72%
	Salp15 Iric -3	49% *62%*	47% *58%*	56% *69%*	–

Note. Alignment of the nucleotide sequences and amino acid sequences was performed using the web based Software from NCBI as described by Tatusova et al (Tatusova et al. 1999). % Identity *% similarity*

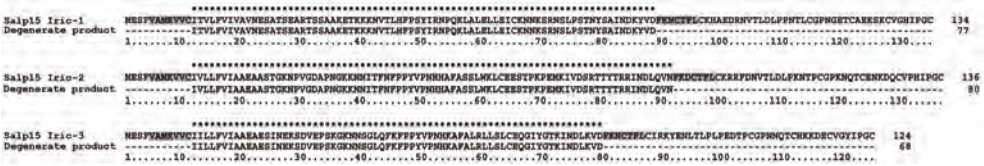

Figure 3. Reverse transcriptase-polymerase chain reaction (RT-PCR) with degenerate primers confirms the presence of three *Ixodes ricinus* Salp15 homologues. Salivary glands from fed adult female *I. ricinus* ticks were dissected, and RNA was extracted. RT-PCR was performed using primers targeting nucleotide sequences that coded for conserved domains (gray highlights) of Salp15 and similar proteins that were identified by PSI-BLAST search. The products we obtained are referred to as degenerate products. By this method, we confirmed the presence of three Salp15 homologues in *I. ricinus*. The alignment of predicted protein sequences of the *I. ricinus* Salp15 homologues and the corresponding degenerate products is depicted. Alignment was performed using the ClustalX 1.8.1 software. *Complete conserved residue.

homologous genes that result from a duplication event within a lineage. Therefore, *salp15 Iric-1* is probably an orthologue of *I. scapularis salp15*, and the other two could be paralogues of *salp15 Iric-1*. To obtain RNA for RT-PCR, we pooled salivary glands from three to four ticks. Theoretically, there could be a chance that, although these ticks are all part of the same inbred colony, the gene products we identified are polymorphic genes from individual ticks within this pool. However, we performed RT-PCR on several different pools and always amplified the same three gene products. Moreover, the diversity in the nucleotide sequence of these genes makes it even more unlikely that these genes are polymorphic genes from individual ticks.

I. scapularis Salp15 and Salp15 Iric-1 share homology over the entire amino acid sequence, whereas *I. scapularis* Salp15 and Salp15 Iric-2 and Iric-3 share homology that is confined to the N-terminal signal sequence and the C-terminus. Thus, our three clones all share great homology to *I. scapularis* Salp15 at the C-terminal part. Interestingly, Garg et al. (2006) have recently shown

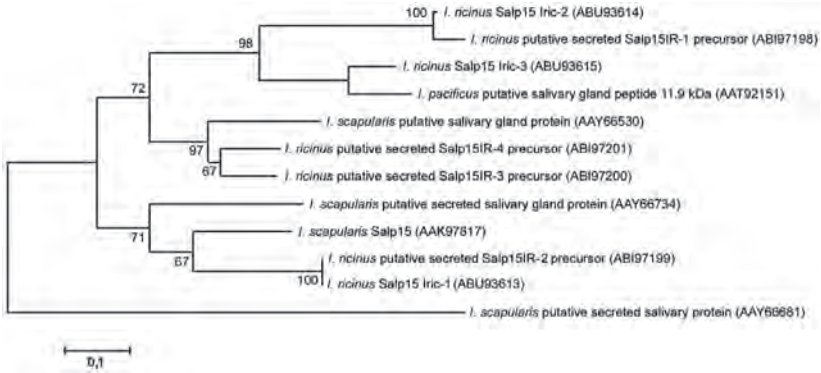

Figure 4. Salp15 is conserved between different *Ixodes* species. Using the PSI-BLAST search method searching the GenBank database, we found several known Salp15 homologues. Amino acid sequences were aligned using the ClustalX 1.8.1 software. An unrooted Neighbour-Joining tree (Saitou et al. 1987) was constructed with the software Mega 3.1 (Kumar et al. 2004) using the Jones-Thorton-Taylor substitution matrix (Jones et al. 1992). Bootstrap sampling was reiterated 1000 times, and the numbers represent the percentage of 1000 replicants (bootstrap support) for which the same branching patterns were obtained. Trees were visualized with Mega 3 Tree Explorer (Kumar et al. 2004). Scale bar = 10% diversity.

that the C-terminal part of *I. scapularis* Salp15 binds to murine $CD4^+$ T cells by binding to the CD4 coreceptor, thereby inhibiting T cell activation. In contrast, it is not yet known which part of Salp15 binds *Borrelia* OspC, and therefore these data could contribute to the prediction of the functional characteristics of Salp15.

The Salp15 family is conserved between different *Ixodes* species

Functional characterization of novel proteins in the post-genomic era can be done *in silico*, allowing for identification of proteins that potentially share biologically relevant homology. We therefore performed a Position Specific Iterated Search (PSI-BLAST) using the webbased software available at the NCBI website to search for proteins similar to *I. scapularis* Salp15 (Wu et al. 2006). Several known *I. scapularis*, *I. ricinus*, and *I. pacificus* salivary gland proteins with conserved domains were identified (Fig. 4), suggesting that these proteins are part of a Salp15 family of which members are conserved among different *Ixodes* ticks, all capable of transmitting *Borrelia* species. The *I. ricinus* Salp15 homologues identified in this study are also part of this same family (Fig. 4). By RT-PCR on salivary gland cDNA with both primers based on the *I. scapularis salp15* sequence, as well as degenerate primers, we were unable to identify Salp15 homologues in other tick species, including *I. hexagonus*, *Hyalomma detritum*, *Rhipicephalus evertsi evertsi*, *Boophilus annulatus*, and *Amblyomma variegatum* semi-engorged adult female ticks (data not shown). Of these, only *I. hexagonus* is known to be able to transmit *B. burgdorferi* sensu lato (Gern et al. 1991).

Recently, four other unpublished nucleotide sequences coding for putative *I. ricinus* Salp15 homologues became available from the Gen-Bank database (ABI97198–ABI97201). Of these, Salp15 IR-2 appeared to be 100% identical to our Salp15 Iric-1 (ABU93613), yet missing six amino acids at the N-terminal part. Salp15 IR-1 was 94% identical to our Salp15 Iric-2 (ABU93614). The fact that these sequences were found by a different method, i.e. screening of salivary gland cDNA libraries derived from fed and unfed *I. ricinus* ticks, but that similar sequences were found, underscores the usefulness of our approach. The findings support our hypothesis that *salp15* belongs to a widely distributed gene family within (*Ixodes*) tick species.

Implications for the transmission of *Borrelia burgdorferi* sensu lato by *Ixodes ricinus*

B. burgdorferi sensu stricto binds to *I. scapularis* Salp15 and is thereby protected from antibody-mediated killing, both *in vitro* and *in vivo* (Ramamoorthi et al. 2005). *I. scapularis* Salp15 also inhibits T cell activation, which is potentially beneficial for the arthropod (Anguita et al. 2002). The fact that expression of a tick protein may be beneficial for both the vector and the pathogen implies a symbiotic relationship. The presence of Salp15 in *I. ricinus* suggests that European *Borrelia* spp. such as *B. afzelii* and *B. garinii* might have similar interactions with this protein. Since we identified more Salp15 homologues from *I. ricinus* cDNA, it could well be that each *Borrelia* spp. has a preferential interaction with one of the Salp15 homologues. From the vector's point of view, it could be that the different Salp15 homologues in *I. ricinus* have evolved to adapt to the different hosts it feeds on during its lifecycle. A comparable phenomenon is described for Osp E/F-related

proteins (Erps), a family of complement inhibitory proteins, in *B. burgdorferi* sensu stricto. The relative affinity for complement inhibitors of various potential hosts differs between the various Erps, allowing the bacterium to inhibit complement-mediated killing in the diverse hosts it may encounter during its enzootic lifecycle (Stevenson et al. 2002).

More insight in the interrelationship of the vector, pathogen, and the host might lead to development of new strategies to combat tick-borne diseases. Targeting the vector in order to prevent transmission of the pathogen from the tick to the host has been shown effective for several tick-borne pathogens (de la Fuente et al. 1998, Labuda et al. 2006). Applying similar strategies to prevent Lyme borreliosis in Europe as well as in the United States is challenging because of the different *Ixodes* species that are the vector for Lyme borreliosis. Identifying putative targets that are conserved between *I. ricinus* and *I. scapularis*, such as Salp15, could circumvent this problem.

Acknowledgments

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Preferential protection of *Borrelia burgdorferi* sensu stricto by a Salp15 homologue in *Ixodes ricinus* saliva

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Chapter 5

Abstract

Background. *Ixodes* ticks are the main vectors for *Borrelia burgdorferi* sensu lato. In the United States, *B. burgdorferi* is the sole causative agent of Lyme borreliosis and is transmitted by *Ixodes scapularis*. In Europe, 3 *Borrelia* species – *B. burgdorferi, B. garinii,* and *B. afzelii* – are prevalent, which are transmitted by *Ixodes ricinus*. The *I. scapularis* salivary protein Salp15 has been shown to bind to *B. burgdorferi* outer surface protein (Osp) C, protecting the spirochete from antibody-mediated killing. *Methods and results.* We recently identified a Salp15 homologue in *I. ricinus,* Salp15 Iric-1. Here, we have demonstrated, by solid-phase overlays, enzyme-linked immunosorbent assay, and surface plasmon resonance, that Salp15 Iric-1 binds to *B. burgdorferi* OspC. Importantly, this binding protected the spirochete from antibody-mediated killing in vitro and in vivo; immune mice rechallenged with *B. burgdorferi* preincubated with Salp15 Iric-1 displayed significantly higher *Borrelia* numbers and more severe carditis, compared with control mice. Furthermore, Salp15 Iric-1 was capable of binding to OspC from *B. garinii* and *B. afzelii,* but these *Borrelia* species were not protected from antibody-mediated killing. *Conclusions.* Salp15 Iric-1 interacts with all European *Borrelia* species but differentially protects *B. burgdorferi* from antibody-mediated killing, putatively giving this *Borrelia* species a survival advantage in nature.

Introduction

Ticks are ectoparasites that, while taking a blood meal, can transmit a variety of human pathogens. Lyme borreliosis is a common tickborne disease in the United States and Europe, where *Ixodes scapularis* and *Ixodes ricinus* are the most important vectors, respectively. In the United States, *Borrelia burgdorferi* sensu stricto *(B. burgdorferi)* is the only prevalent *Borrelia* species and is transmitted by *I. scapularis,* whereas in Europe 3 *Borrelia* species – *B. burgdorferi, B. garinii,* and *B. afzelii* – can cause Lyme borreliosis and are transmitted by *I. ricinus*. In humans, all 3 species frequently cause an erythematous cutaneous lesion, erythema migrans. In later stages of infection, spirochetes can disseminate and cause disease that affects the joints, cardiac conduction system, central nervous system, and skin [1, 2]. It has been demonstrated previously that each *Borrelia* species is associated with distinct clinical entities [1, 3, 4].

During its enzootic life cycle, *B. burgdorferi* exploits tick salivary proteins [5]. These vector molecules are important for *B. burgdorferi* survival within the tick (e.g., TROSPA [6]), for transmission from the host to the tick (e.g., Salp25D [7]), and for transmission from the tick to the host (e.g., Salp15 [8]). Salp15 is a 15-kDa *I. scapularis* feeding-induced salivary protein [9] and has been shown to bind to *B. burgdorferi* outer surface protein (Osp) C [8]. *B. burgdorferi* expresses OspC in the tick salivary glands and during the early stages of mammalian infection [10]. Binding of Salp15 to OspC protects the spirochete from antibody-mediated killing by the host. In nature, the ability of *B. burgdorferi,* assisted by *I. scapularis* Salp15, to reinfect immune reservoir hosts could be an important factor in the continuation of the complex enzootic life cycle of the spirochete.

In addition to *B. burgdorferi, I. ricinus* is also able to transmit 2 other *Borrelia* species that cause Lyme borreliosis, *B. garinii* and *B. afzelii*. Recently, we identified 3 Salp15 homologues in *I. ricinus,* Salp15 Iric-1, -2, and -3, of which Salp15 Iric-1 is most similar to *I. scapularis* Salp15 [11]. In the present study, we describe the interaction of Salp15 Iric-1 with its presumed natural ligand, OspC from *Borrelia burgdorferi* sensu lato strains representing the 3 pathogenic *Borrelia* species in Europe.

Methods

***I. ricinus* Salp15 homologue–specific reverse-transcription polymerase chain reaction (RT-PCR).** Adult female *I. ricinus* ticks were fed on rabbits. After 3 days, semiengorged ticks were removed, salivary glands were dissected, and RNA was isolated and cDNA generated as described elsewhere [11]. Quantitative RT-PCR on *I. ricinus* salivary gland cDNA was performed using different sets of primers specific for the 3 Salp15 variants (table 1). Amplification of cDNA- and gene-specific standards was visualized and quantified using LightCycler software (Roche Diagnostics).

Cloning and expression of Salp15 Iric-11. *Salp15 Iric-1* (GenBank accession number EU128526) was amplified from a recombinant pGEM-T Easy vector (Promega) (table 1), inserted into the

Table 1. Primers and probe used.

Target	Forward	Reverse	Probe
Iric-1 specific	TAACAAATCTCGTAACTCCCTTC	ATCCAGGAATGTGCCCAA	–
Iric-2 specific	ATGAAAATTGTTGATAGCCGA	CGGAACGCATTGGTCTTT	–
Iric-3 specific	TGCGAGCAAGGTATATATGG	TACCCCACACACTCGTCTTT	–
Iric-1 cloning	AAAAAAAACCATGGAATGAAA-GCGCCACAAGCGAA	AAAAAAAAGCGGCCGCACA-TCCAGGAATGTGCCC	–
N40-*ospC* cloning	AAAAAAAAGGATCCGGAAAAG-ATGGGAATGC	AAAAAAAACTCGAGCTAAG-GTTTTTTTGGACTTTCTGC	–
PBi-*ospC* cloning	AAAAAAAAGGATCCGGTGGGG-ATTCTGCATC	AAAAAAAACTCGAGCTAAG-GTTTTTTTGGAGTTTCTGC	–
pKo-*ospC* cloning	AAAAAAAAGGATCCGGGAAAG-GTGGGGATTC	AAAAAAAACTCGAGCTAAG-GTTTTTTTGGACTTTCTGC	–
Borrelia flaB	GCTTCTGATGATGCTGCTG	TCGTCTGTAAGTTGCTCTATTTC	GAATTRGC AGTAACGG
Mouse *β-actin*	GGGACCTGACAGACTACCTC	AAGAAGGAAGGCTGGAAAAG	–

pMT/BiP/V5–His C vector (Invitrogen), transformed into DH5-α cells that were subjected to plasmid isolation (Miniprep Kit; Qiagen), and sequenced as described elsewhere [11]. *Drosophila* S2 cells (Invitrogen) were cotransfected with a blasticidin selection vector, pCOBlast (Invitrogen). Stably transfected cells were grown and induced and recombinant protein was purified as described elsewhere [8]. *I. scapularis* Salp15 (GenBank accession number AF209914) was generated in a similar fashion. Protein concentrations were determined using a standard Bradford assay, Coomassie staining, and Western blot analysis with anti-V5–horseradish peroxidase (HRP) antibody. Mock-transfected *Drosophila* S2 cells were grown and induced, and the supernatant was run over a purification column to generate a "vehicle control" to control for impurities (i.e., *Drosophila* background proteins) in the recombinant protein fractions. Where indicated, deglycosylation was performed by use of *N*-glycosidase F (New England Biolabs) under denaturing conditions.

***Borrelia burgdorferi* sensu lato.** *B. burgdorferi* sensu lato strains representing the 3 pathogenic *Borrelia* species that exist in Europe - *B. burgdorferi* strains N40 and B31 clone 5A11 [12], *B. garinii* strain PBi, and *B. afzelii* strain pKo - were cultured in Barbour-Stoenner-Kelly (BSK)-H medium (Sigma-Aldrich). Low-passage spirochetes were grown to 5 x 10^7 organisms/mL (enumerated by use of a Petroff-Hausser counting chamber, as described elsewhere [13]) and diluted to the indicated concentrations.

Solid-phase overlay. *Borrelia* lysates were separated by 12.5% SDS-PAGE and blotted onto an Immobilon-P membrane (Millipore) to have approximately similar amounts of OspC. As a negative control, we used lysate from *ospC*-deficient *B. burgdorferi* strain 297 [14]. Solid-phase overlays were performed as described elsewhere [8]. In addition, Western blot analysis was performed using a monoclonal antibody, L22 1F8, recognizing OspC from *B. burgdorferi* sensu lato [15].

Cloning and expression of recombinant *B. burgdorferi* sensu lato OspC. OspC was amplified from genomic DNA from *Borrelia* strains N40, PBi, and pKo (table 1) and inserted into the pGEX-

6p-2 vector (Amersham Biosciences). Recombinant plasmids were cloned into DH5-α cells, and inserts were sequenced as described elsewhere [11]. Large cultures were induced by use of isopropyl β-D-1-thiogalactopyranoside (final concentration, 1 mmol/L). Recombinant glutathione S-transferase (GST)–fused OspC proteins were harvested, purified, and cleaved from GST in accordance with the manufacturers' recommendations. Purity was checked using SDS-PAGE, and protein concentrations were measured using a Bradford assay.

OspC-Salp15 ELISA. Microlon ELISA plates (Greiner) were coated with recombinant OspC from the 3 *Borrelia* strains (400 ng/well) or with bovine serum albumin (BSA) as a control in 100 µL/well coating buffer (0.1 mol/L Na_2CO_3 [pH 9.6]) overnight at 4°C. Subsequently, wells were washed (wash buffer, PBS plus 0.05% Tween) and incubated with 200 µL of blocking buffer (10% fetal bovine serum in PBS) for 2 h. Subsequently, 50 µL of blocking buffer with *I. scapularis* Salp15 or Salp15 Iric-1 (0–2000 ng/well) was added, incubated for 90 min, and washed, followed by incubation with anti-V5 antibody (Invitrogen) in 50 µL of blocking buffer for 45 min and 6 washes. Anti-IgG-HRP antibodies were incubated in 50 µL of blocking buffer for 45 min followed by 8 washes. Finally, tetramethylbenzidine substrate was added, the reaction was stopped, and absorbance was measured at 450 nm.

Surface plasmon resonance (SPR). The 3 OspCs were coupled to a CM5 sensor chip. A control channel on each sensor chip was coated with BSA or treated with ethanolamine, to determine the background binding signal. Specific binding of *I. scapularis* Salp15 and Salp15 Iric-1 to the different OspCs was corrected for nonspecific binding to the control channel. Different molar concentrations (range, 0–4500 nmol/L) of *I. scapularis* Salp15 and Salp15 Iric-1 in PBS–Tween 0.005% were injected for 300 s at a flow rate of 5 µL/min, and binding was monitored in real time. Regeneration of the sensor chip was achieved by a 30-min wash with a buffer containing 0.08 mol/L NaSCN, 0.31 mol/L $MgCl_2$, 0.15 mol/L urea, and 0.31 mol/L guanidine-HCl, followed by equilibration with PBS-Tween. Binding kinetics were analyzed with BIAevaluation software (version 3.2). Control experiments with recombinant *Escherichia coli*-expressed human tumor necrosis factor (TNF)–α with a C-terminal His-tag and a vehicle control were also performed.

In vitro protection assays. To each well (Greiner Bio 96 U-well plate), 50 µL of BSK-H medium containing 1 x 10^8 spirochetes/mL and 4 µg of Salp15 (1µg/µl or a corresponding volume of PBS) were added. After 1 h, 46 µL of serum or antiserum (diluted in PBS) was added and incubated for 16 h at 33°C. Antiserum was derived from rabbits immunized with the indicated *Borrelia* species, and normal rabbit serum was derived from preimmune (naive) rabbits. Antisera recognized comparable antigen patterns (Supplemental figure 1), and titration experiments showed that the dilution of the antisera to reduce the numbers of viable spirochetes to approximately 30% was 1:200 for anti-N40 and 1:300 for anti-PBi and anti-pKo (data not shown). Normal rabbit serum was diluted accordingly. Experiments were performed in duplicate, a 25-µL aliquot from each well was diluted 5 times, and the number of live (i.e., motile) spirochetes was counted using dark-field microscopy (magnification, × 250; 10 random fields per well).

In vivo experiments. The generation of immune mice has been described elsewhere [8]. Briefly, pathogen-free C3H/HeN mice (8 mice/group) were infected with *Borrelia* (1 x 10^5 spirochetes/mouse) by needle inoculation, tested for antibodies against *B. burgdorferi* by ELISA 2 weeks after infection, and treated with intraperitoneal ceftriaxone. Absence of spirochetal DNA was confirmed by PCR on tissue derived from ear biopsies. Mice were reinfected with the indicated *Borrelia* species (1 x 10^5 spirochetes/mouse) preincubated with Salp15 Iric-1 (30 µg/1 x 10^5 spirochetes) or BSA. Tissue samples were collected 14 days after (re-)infection.

Quantitative PCR and histopathology. DNA from murine tissues was obtained with the DNeasy Kit (Qiagen). Quantitative PCR detecting *Borrelia flaB* and the gene for mouse β-actin *(Actb)* was performed as described elsewhere [8, 13]. Standards consisted of dilutions of genomic DNA from the indicated *Borrelia* species or mouse *Actb* (252 bp) cloned into the PCR2.1-TOPO vector (Invitrogen) as described elsewhere [13]. Histopathological changes in tibiotarsi and heart tissue were assessed as described elsewhere [13, 16, 17]. Infection appeared to be too short to induce arthritis, since no arthritis was observed (data not shown). Also, 4-µm-thick paraffin-embedded sections of sagittally dissected hearts were processed and stained with hematoxylin-eosin by routine histological techniques. Carditis was scored by an independent pathologist blinded to the experimental design on a scale from 0 to 3 (0, no inflammation; 1, mild inflammation with <2 foci; 2, moderate inflammation with ≥2 foci; 3, severe inflammation with focal as well as diffuse inflammation covering a large area) [16, 17]. As described elsewhere, inflammatory infiltrates were localized almost exclusively at the basis of the aorta and the adjacent ventricular walls [18].

Statistical analysis. Differences between the groups were analyzed using 2-sided nonparametric tests, and, whenever appropriate, multiple-comparison correction was applied (version 4.0; GraphPad Prism Software). Data are presented as means ± SEs.

Results

Identification and cloning of Salp15 Iric-1. Recently we identified 3 homologues of *I. scapularis* Salp15 in salivary glands from fed adult *I. ricinus* ticks [11]. One of these homologues shared 82% homology with *I. scapularis* Salp15 at the DNA level and was designated Salp15 Iric-1. To assess the role played by the Salp15 homologues in *I. ricinus* in the pathogenesis of Lyme borreliosis, we first defined their relative expression in salivary glands 3 days after engorgement (figure 1*A*). Both Salp-15 Iric-1 and Iric-2 were shown to be highly expressed. Because Salp15 Iric-1 was most similar to *I. scapularis* Salp15, we expressed this protein in a *Drosophila* expression system (figure 1*B*). Salp15 Iric-1 and *I. scapularis* Salp15 consist of 134 and 135 aa, respectively. However, the apparent molecular weight of Salp15 Iric-1 is 5 kDa greater than that of *I. scapularis* Salp15. Analysis of the amino acid sequences of the 2 proteins (http://www.cbs.dtu.dk/services/NetNGlyc) revealed that Salp15 Iric-1 has 4 and that *I. scapularis* Salp15 has 2 predicted *N*-glycosylation sites (figure

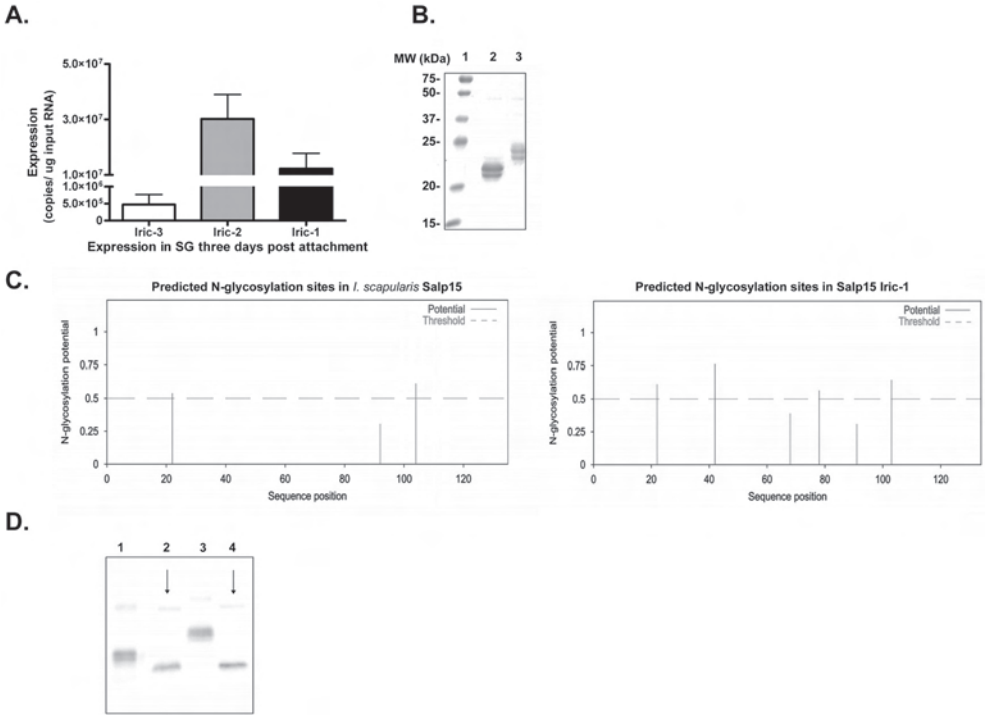


Figure 1. Characterization of (recombinant) Salp15 Iric-1. *A,* Quantitative reverse-transcription polymerase chain reaction (PCR) performed on RNA obtained from salivary glands (SG) from semi-engorged adult female *Ixodes ricinus* ticks with primers specific for Salp15 Iric-1, -2, and -3. The no. of Salp15 Iric-1, -2, and -3 copies was determined from the same cDNA sample in a single PCR run. Bars represent means ± SEs for 3 independent experiments. The no. of copies is normalized to the amount of input RNA. *B,* Salp15 Iric-1 cloned into the pMT/BiP/V5–His C expression vector and stably transfected into S2 *Drosophila* cells. Recombinant protein with a C-terminal His-tag and V5 epitope was purified using nickel-charged columns. Recombinant *Ixodes scapularis* Salp15 was purified similarly to Salp15 Iric-1. Recombinant proteins were separated by SDS-PAGE, and the gel was subjected to Coomassie brilliant blue staining; *lane 1,* molecular weight (MW) marker; *lane 2, I. scapularis* Salp15; *lane 3,* Salp15 Iric-1. *C,* Investigation of the difference in apparent molecular weight. We determined whether the 2 proteins were differentially modified at the posttranslational level by assessing potential *N*-glycosylation sites using the Web-based software available at http://www.cbs.dtu.dk/services/NetNGlyc. The *N*-glycosylation potential threshold was set at 0.5. *D, I. scapularis* Salp15 and Salp15 Iric-1 deglycosylated by *N*-glycosidase F (New England Biolabs) in accordance with the manufacturer's instructions under denaturing conditions, subjected to SDS-PAGE, and blotted onto an Immobilon-P membrane. Proteins were visualized with anti-V5–horseradish peroxidase antibody; *lanes 1* and *2, I. scapularis* Salp15; *lanes 3* and *4,* Salp15 Iric-1. Arrows indicate that samples were treated with *N*-glycosidase F.

1*C*). Indeed, deglycosylation of *I. ricinus* and *I. scapularis* Salp15 by *N*-glycosidase F (PGNaseF) resulted in proteins of comparable molecular weight (figure 1*D*).

Interaction of Salp15 Iric-1 with *B. burgdorferi* sensu stricto. We first demonstrated that, similar to *I. scapularis* Salp15, Salp15 Iric-1 binds to OspC in lysates from *B. burgdorferi* strain N40 in a solid-phase overlay (figure 2*A*). Salp15 Iric-1 also bound to OspC from *B. burgdorferi* strain B31 (Supplemental figure 2) but did not bind to a mutant strain lacking OspC (figure 2*A*). In addition, when overlays were performed with vehicle control instead of Salp15 proteins, similar background

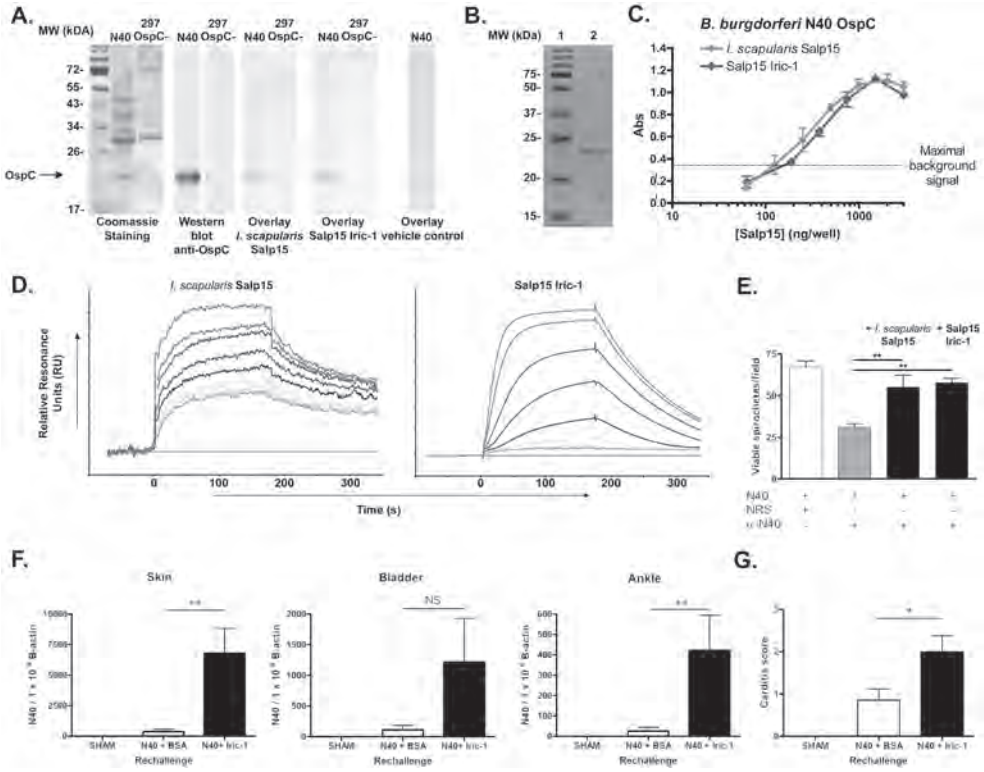


Figure 2. Interaction of Salp15 Iric-1 with *Borrelia burgdorferi* strain N40. *A,* Solid-phase overlays with *B. burgdorferi* strain N40 and *B. burgdorferi* strain 297 deficient for *ospC* for *Ixodes scapularis* Salp15 and Salp15 Iric-1. The left panel shows Coomassie blue staining of lysates from *B. burgdorferi* strain N40 (7.2 μg) and strain 297 deficient for *ospC* (5.1 μg), separated by SDS-PAGE and blotted onto an Immobilon-P membrane. The molecular weight (MW) was determined using a molecular weight marker. Western blot analysis using a monoclonal antibody, L22 1F8, recognizing outer surface protein (Osp) C from *B. burgdorferi* sensu lato confirmed that the 22-kDa band was indeed OspC. Binding of recombinant *I. scapularis* Salp15 or Salp15 Iric-1 to native OspC was assessed by a solid-phase overlay with 1 μg/mL *I. scapularis* Salp15, 1 μg/mL Salp15 Iric-1, or vehicle control as a negative control, and binding was visualized using mouse anti-V5 and rabbit anti–mouse IgG–horseradish peroxidase (HRP) antibodies. *B,* Purified *Escherichia coli*–expressed recombinant OspC from *B. burgdorferi* strain N40, separated by SDS-PAGE and visualized by Coomassie blue staining. *Lane 1,* Molecular weight marker; *lane 2,* N40-OspC. *C,* OspC-Salp15 ELISA. N40-OspC (400 ng/well) was coated onto ELISA plates and incubated with different concentrations of *I. scapularis* Salp15 *(black line)* and Salp15 Iric-1 *(gray line).* Binding was visualized by incubation with a secondary and tertiary antibody, anti-V5-HRP and anti–mouse IgG–HRP antibodies, respectively. After addition of the substrate, absorbance was read at 450 nm. As a control, bovine serum albumin (BSA) was coated, and background signals did not exceed the indicated dashed line. This graph is representative of 5 independent experiments. Error bars represent SEs of triplicates from a single experiment. *D,* Surface plasmon resonance N40-OspC and *I. scapularis* Salp15 and Salp15 Iric-1. N40-OspC was bound to a CM5 sensor chip and different molar concentrations (ranging from 0 to 4500 nmol/L, represented by individual lines, with the highest line representing 4500 and the lowest line representing 0 nmol/L) of *I. scapularis* Salp-15 or Salp15 Iric-1 were injected for 300 s. Resonance units (RUs) depicted are after subtraction of the RUs from the BSA reference channel. Binding constants were calculated by 1:1 Langmuir fitting and confirmed by steady-state affinity plotting (see table 2). In addition, *E. coli*–expressed tumor necrosis factor–α with a C-terminal His tag and vehicle control did not show any binding (data not shown). *E,* In vitro protection assay in which spirochetes were preincubated with *I. scapularis* Salp15, Salp15 Iric-1 (final concentration, 40 μg/mL), or BSA as a control and then subjected to serum (diluted 1:200) from an N40-immunized rabbit (α-N40) for 16 h. Viable spirochetes were visualized by dark-field microscopy. Bars represent means ± SEs for 3 independent expe-

riments. NRS, normal rabbit serum. *F,* In vivo experiment showing N40 burden in mice immune to N40 that were reinfected with Barbour-Stoenner-Kelly medium (SHAM) (2 mice), N40 preincubated with BSA (N40 plus BSA) (8 mice), or Salp15 Iric-1 (N40 plus Iric-1) (7 mice) and killed 2 weeks after reinfection. N40 burden was determined by quantitative polymerase chain reaction and was corrected for the amount of β-actin. Bars represent means ± SEs. As a positive control, naive mice (n = 3) were infected with N40. The N40 burdens in different tissues in these mice were comparable to those in immune mice rechallenged with N40 preincubated with Salp15 Iric-1 (data not shown). *G,* Carditis severity scores in hematoxylin-eosin–stained sagittal sections of mouse hearts. In comparison, 3 naive mice infected with N40 and killed 2 weeks after infection had an average carditis score of 2.7 ± 0.5 data not shown). A 2-sided Mann-Whitney *U* test was performed to determine statistical differences between the groups. * $P < .05$ and ** $P < .01$. NS, not significant.

signals were observed compared with the overlays performed with the Salp15 proteins, further indicating that the binding was specific to OspC (figure 2*A*). To characterize further the binding of Salp15 Iric-1 to *B. burgdorferi* OspC, we generated soluble recombinant OspC derived from *B. burgdorferi* strain N40 (N40-OspC) (figure 2*B*) and performed an OspC-Salp15 ELISA. Similar to *I. scapularis* Salp15, Salp15 Iric-1 bound to plate-bound N40-OspC (figure 2*C*). To determine more specifically the affinity of the 2 proteins for N40-OspC, we performed SPR and demonstrated that the binding constant for Salp15 Iric-1 and N40-OspC was $2{,}23 \times 10^{-6} \pm 0{,}15 \times 10^{-6}$ mol/L; for *I. scapularis* Salp15 and N40-OspC it was $0{,}78 \times 10^{-6} \pm 0{,}14 \times 10^{-6}$ mol/L (figure 2*D* and table 2). Control runs with vehicle control or equimolar amount of recombinant TNF-α with a C-terminal His-tag did not show any binding (data not shown). To investigate whether the subtle difference in affinity had implications for the extent of protection from antibody-mediated killing, we performed in vitro protection assays with rabbit N40-immune serum. N40 spirochetes preincubated with either *I. scapularis* Salp15 or Salp15 Iric-1 appeared to be equally protected from N40-specific antibodies (figure 2*E*), also when lower concentrations of the Salp15 proteins were used (Supplemental figure 3). *I. scapularis* Salp15 has also been shown to protect *B. burgdorferi* strain N40 from antibody-mediated killing in vivo; N40 by itself could not reinfect N40-immune mice, but N40 preincubated with *I. scapularis* Salp15 could [8]. We performed a similar experiment with Salp15 Iric-1 and N40. N40-immune mice rechallenged with 1×10^5 N40 preincubated with 30 μg of *I. ricinus* were more readily reinfected (table 3) and were found to have significantly higher spirochete loads in several tissues 2 weeks after infection than N40-immune mice rechallenged with N40 preincubated with 30 μg of BSA (figure 2*F*), resulting in more severe carditis (figure 2*G*).

Interaction of Salp15 Iric-1 with *B. garinii* and *B. afzelii*

In Europe, Lyme borreliosis is caused by 3 *Borrelia* species, *B. burgdorferi, B. garinii,* and *B. afzelii*. Therefore, we investigated the interaction of *B. garinii* strain PBi and *B. afzelii* strain pKo with Salp15 Iric-1. By solid-phase overlay, we demonstrated that Salp15 Iric-1 was able to bind to OspC from all 3 *Borrelia* species (figure 3*A*). To investigate the interaction further, we also produced soluble recombinant OspC derived from *B. garinii* strain PBi (PBi-OspC) and *B. afzelii* (pKo-OspC) (figure 3*B*). In both Salp15 Iric-1–OspC ELISA (figure 3*C*) and SPR (figure 3*D* and table 2), we showed that the affinity of Salp15 Iric-1 for OspC from PBi and pKo was comparable to the affinity of Salp15 Iric-1 for N40-OspC. Notably, Salp15 Iric-1 did not protect PBi and pKo from killing by specific antibodies in vitro (figure 3*E*), not even when higher amounts (i.e., 8 μg/well

Table 2. Binding kinetics of *I. scapularis* Salp15 and Salp15 Iric-1 for OspC from the different *B. burgdorferi* sensu lato strains.

Salp15 protein	OspC	Ka (1/MS)	Kd (1/s)	KD (M)
I. scapularis Salp15	N40-OspC	4,9E+03 ± 5	3,8E-03 ± 0,7E-03	0,78E-06 ± 0,14E-06
Salp15 Iric-1	N40-OspC	4,6E+03 ± 896	10,2E-03 ± 2,5E-03	2,23E-06 ± 0,15E-06
Salp15 Iric-1	PBi-OspC	3,7E+03 ± 412	8,4E-03 ± 1,0E-03	2,27E-06 ± 0,25E-06
Salp15 Iric-1	pKo-OspC	4,0E+03 ± 1,0E+03	9,7E-03 ± 2,5E-03	2,50E-06 ± 0,4E-06

Note. Binding curves of different molar concentrations of *I. scapularis* Salp15 and Salp15 Iric-1 to OspC from the different *Borrelia* species are shown in Figure 2 and 3 D. These data were analyzed with the BIA evaluation software (version 3.2) using 1:1 Langmuir fitting to calculate the KD. Results were confirmed by steady state affinity plotting (data not shown).
Ka: association constant, K_d: dissociation constant, K_D: binding constant, data represent the mean of three independent experiments ± SE.

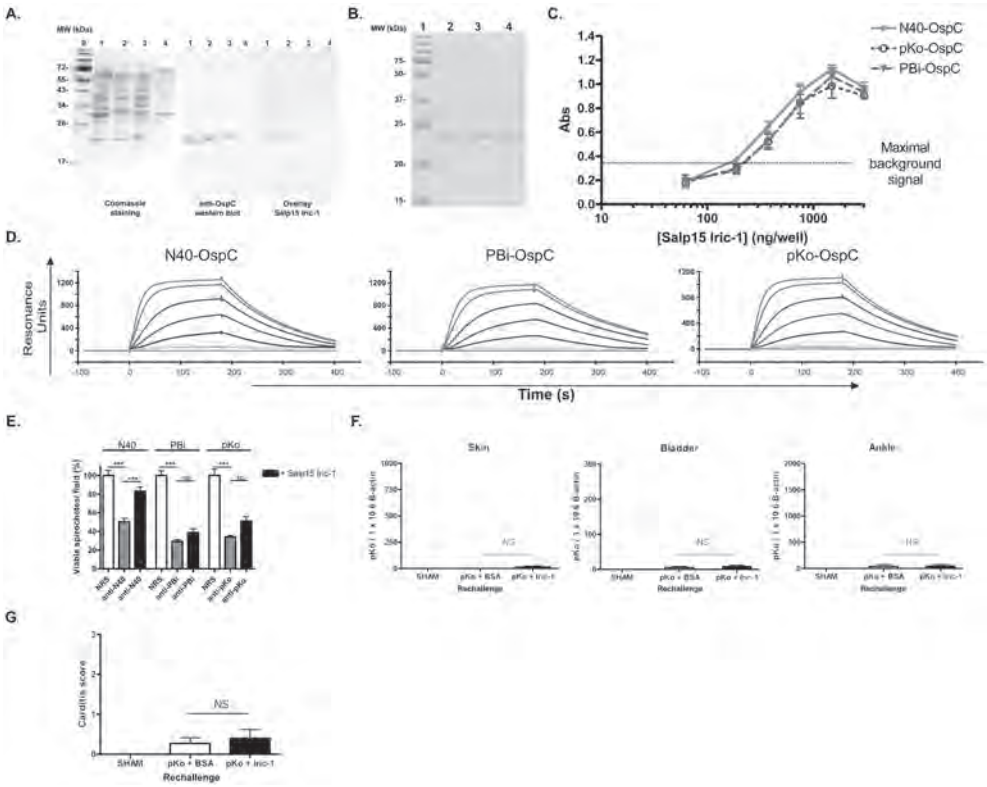


Figure 3. Interaction of Salp15 Iric-1 with *Borrelia garinii* strain PBi and *Borrelia afzelii* strain pKo. A, Solid-phase overlay with the different *Borrelia* species. Lane 0, molecular weight (MW) marker; lane 1, *Borrelia burgdorferi* strain N40; lane 2, *B. garinii* strain PBi; lane 3, *B. afzelii* strain pKo; lane 4, ospC-deficient strain 297. The amounts loaded on SDS-PAGE gel for *B. burgdorferi* strain N40, *B. garinii* strain PBi, *B. afzelii* strain pKo, and the outer surface protein (Osp) C mutant *B. burgdorferi* strain 297 were 7.2, 8.14, 8.62, and 5.1 µg, respectively. After transfer to an Immobiolon-P membrane, Coomassie blue staining, Western blot, and solid-phase overlay using 1 µg/mL Salp15 Iric-1 were performed. B, Coomassie blue staining of recombinant N40-OspC (lane 2), PBi-OspC (lane 3), and pKo-OspC (lane 4). Lane 1, molecular weight marker. Western blot analysis using monoclonal antibody L22 1F8, recognizing OspC from *B. burgdorferi* sensu lato, confirmed that the 22-kDa recombinant proteins were indeed OspC. The affinity of Salp15 Iric-1 for the different OspCs was assessed by OspC ELISA (C) and by surface plasmon resonance

(D). E, In vitro protection assay in which N40, PBi, or pKo spirochetes were preincubated with Salp15 Iric-1 (final concentration, 40 μg/mL) or with bovine serum albumin (BSA) as a control and then subjected to serum from a rabbit immunized with the indicated *Borrelia* species for 16 h (anti-N40, diluted 1:200; anti-PBi and anti-pKo, diluted 1:300). Bars represent means ± SEs for 3 independent experiments. The average amount of viable spirochetes per dark-field microscope field in the well incubated with normal rabbit serum (NRS) was set at 100%. F, *Borrelia* burden determined by quantitative polymerase chain reaction in tissues from an in vivo experiment with *B. afzelii* strain pKo as described for N40 in the legend to figure 3. Eight to 9 mice per group were used. As a positive control, naive mice (n=3) were infected with pKo. These mice had high spirochete numbers in skin (688 ± 491) spirochetes/1 x 10^6 *β-actin* DNA copies), bladder (288 ± 100 spirochetes/1 x 10^6 *β-actin* DNA copies), and ankle tissue (1210 ± 813 spirochetes/1 x 10^6 *β-actin* DNA copies). These data are not depicted in the graphs, but the Y-axis of the graphs is based on these values. Both immune and naive mice were killed 2 weeks after (re)infection. G, Carditis severity scores in hematoxylin-eosin-stained sagittal sections of mouse hearts. In comparison, 3 naive mice infected with pKo and killed 2 weeks after infection had an average carditis score of 3 ± 0 (data not shown). A 2-sided Mann-Whitney U test was performed to determine statistical differences between the groups. * $P < 0,05$, ** $P < 0,01$, and *** $P < 0,001$. NS, not significant.

Salp15 Iric-1) were used (data not shown). In concordance with this finding, we demonstrated that the presence of Salp15 Iric-1 did not enhance the ability of pKo to reinfect pKo-immune mice (figure 3*F* and table 3), and there was no difference in carditis scores between pKo-immune mice rechallenged with strain pKo preincubated with Salp15 Iric-1 and those rechallenged with strain pKo preincubated with BSA (figure 3*G*). It proved not to be possible to reinfect PBi-immune mice with strain PBi, regardless of the presence or absence of Salp15 Iric-1, although the strain was clearly infectious in naive mice (table 3).

Table 3. PCR positivity of tissues from naive or immune mice.

Mice	Inoculum	Ear (pos/total)	Skin (pos/total)	Ankle (pos/total)	Bladder (pos/total)	Any organ (pos/total) and (%)
Naive	N40	ND	3/3	3/3	3/3	(9/9) 100%
Naive	pKo	ND	3/3	3/3	3/3	(9/9) 100%
Naive	PBi	6/9	2/3	ND	2/3	(10/15) 66%
Immune	SHAM *	0/2	0/2	0/2	0/2	(0/8) 0%
Immune	N40 + BSA	ND	4/8	3/8	4/8	(11/24) 46%[a]
	N40 + Iric-1	ND	7/7	6/7	6/7	(19/21) 90%[b]
Immune	pKo + BSA	ND	0/9	2/9	2/9	(4/27) 15%
	pKo + Iric-1	ND	1/9	3/9	4/9	(8/27) 30%[c]
Immune	PBi + BSA	ND	0/8	0/8	0/8	(0/24) 0%[d]
	PBi + Iric-1	ND	0/8	0/8	0/8	(0/24) 0%

Note. PCR positivity of tissues from naive or immune mice inoculated with 1 x 10^5 spirochetes. Tissue were analyzed two weeks post (re-)infection.

* This was demonstrated for N40-, PBi, and pKo-immune mice.

[a] In addition, in these mice low spirochetal numbers were detected by q-PCR (Fig 2).

[b] Statistically more tissues from N40-immune mice were re-infected with *B. burgdorferi* strain N40 in the presence of Salp15 Iric-1 compared to BSA, p=0, 0018 (Two-sided chi-square test).

[c] This difference was not statistically significant for *B. afzelii* strain pKo.

[d] PBi-immune mice could not be re-infected with *B. garinii* strain PBi regardless of the presence or absence of Salp15 Iric-1, not even when a higher inoculum, 1 x 10^7 spirochetes was used (data not shown).

Discussion

The *I. scapularis* 15-kDa salivary protein Salp15 binds to OspC from *B. burgdorferi,* protecting the spirochete from antibody-mediated killing by the host [8]. Because we recently identified a Salp15 homologue, Salp15 Iric-1, in the European vector for Lyme borreliosis, *I. ricinus,* we hypothesized that this protein would also bind to OspC [11]. To test our hypothesis, we investigated whether Salp15 Iric-1 interacts with OspC from *Borrelia* strains representing the 3 major pathogenic *Borrelia* species in Europe. Therefore, we expressed Salp15 Iric-1 in a *Drosophila* expression system, similar to other tick proteins [19]; with SDS-PAGE, Salp15 Iric-1 appeared larger than the predicted molecular mass but also larger than *I. scapularis* Salp15. We demonstrated that these differences are due to differences in glycosylation.

Although the OspC sequences from the different *Borrelia* strains are only 70% homologous, Salp15 Iric-1 bound equally well to OspC from *B. burgdorferi* strain N40, *B. garinii* strain PBi, and *B. afzelii* strain pKo, as determined by ELISA and SPR. This suggests that the binding site of OspC for Salp15 Iric-1 is conserved between the 3 different *Borrelia* species. For ELISA and SPR we used truncated OspC, that is, mature OspC lacking the 20-aa leader sequence, because of unpublished observations indicating that *I. scapularis* Salp15 was unable to bind to full-length *B. burgdorferi* OspC. By SPR, we showed that *I. scapularis* Salp15 has a higher affinity for *B. burgdorferi* OspC than Salp15 Iric-1. The difference in affinity between the 2 proteins did not result in a difference in protection in the in vitro protection assays with *B. burgdorferi*. However, the amounts of Salp15 in both in vitro and in vivo experiments were based on previous research [8] and are estimated to be a surplus. We did not report the interaction between *I. scapularis* Salp15 and *B. garinii* or *B. afzelii* OspC because these spirochetes are not transmitted by *I. scapularis* in nature. Notably, the OspC sequence from *B. burgdorferi* strain N40 is 84% similar to the sequence of OspC from the *B. burgdorferi* reference strain B31, which is 100% identical to several European clinical isolates (PTa, Pho, Pboe, and PAIi).

Strikingly, Salp15 Iric-1 protected only *B. burgdorferi* strain N40 from antibody-mediated killing, resulting in significantly higher *B. burgdorferi* numbers and more severe carditis, compared with controls, in the in vivo experiments. Neither the *B. garinii* strain PBi nor the *B. afzelii* strain pKo was protected in the in vitro assays or the in vivo experiments with immune mice. This differential protection of *B. burgdorferi* by Salp15 Iric-1 could give *B. burgdorferi* a survival advantage over the 2 other *Borrelia* species in nature. Previously, we also demonstrated that *B. burgdorferi* outcompetes *B. garinii* in mice simultaneously inoculated with both *Borrelia* species through syringe inoculation [13].

The reason for the differential protection of *B. burgdorferi,* despite the ability of Salp15 Iric-1 to bind to OspC from all 3 pathogenic *Borrelia* species, can be explained in various ways. It could be that *B. burgdorferi* strain N40 expresses more OspC that the 2 European *Borrelia* strains. However,

we have previously shown that *B. afzelii* strain pKo expresses high amounts of OspC under in vitro conditions [20]. A different explanation would be that OspC, or the *I. scapularis* Salp15 or Salp15 Iric-1 binding site on OspC, is more exposed in *B. burgdorferi* than in *B. afzelii* and *B. garinii*. Whether this would be due to structural differences in the OspC proteins or to surrounding surface proteins remains an interesting topic for future research. Alternatively, the OspC surface consists of alternating positively and negatively charged areas that are important in the formation of OspC multimers or lattices on the surface of the spirochete [21]. Therefore, Salp15 binding to OspC could form a coating, which would explain the fact that the spirochete is protected not only from killing induced by anti-OspC antibodies but also from killing induced by *B. burgdorferi* antiserum [8]. Differences in OspC structure for *B. burgdorferi* compared with *B. garinii* and *B. afzelii* could affect the ability of the OspC dimers to form multimers or lattices and thereby expose other surface proteins, making the spirochete more susceptible to antibody-mediated killing. Last, it could be that other protein targets for bactericidal antibodies are more readily available in *B. garinii* or *B. afzelii*.

Interestingly, the OspC surface interface consists of a highly negatively charged cavity, suggested to be involved in protein-protein interactions [21, 22]. It is tempting to speculate that this site might be the binding site not only for Salp15 and Salp15 Iric-1 but also for the Salp15 paralogues in *I. ricinus,* Salp15 Iric-2 and -3. Therefore, if in vivo protection is dependent on the accessibility of this binding pocket, it seems unlikely that these Salp15 paralogues would protect *B. garinii* or *B. afzelii* against antibody-mediated killing. Another function of *I. scapularis* Salp15 is inhibition of $CD4^+$ T cells by binding to CD4 [23–25]. It will be interesting to see whether the recently identified variants of Salp15 in *I. ricinus,* which share high sequence homology with *I. scapularis* Salp15 at the C-terminus, are able to inhibit T cell activation.

Currently, no vaccine is available to prevent *B. burgdorferi* sensu lato infection. Anti-tick vaccination strategies have been shown to prevent transmission of tick-borne pathogens [5]. Applying similar strategies to prevent Lyme borreliosis in Europe as well as in the United States is challenging because of the different *Ixodes* species that are the vectors for Lyme borreliosis. We have hypothesized elsewhere that Salp15 could serve as a candidate target [5, 11]. However, our present findings show that a Salp15 vaccine (targeting *I. scapularis* Salp15 as well as Salp15 Iric-1) would not be effective in Europe, because although it might prevent transmission of *B. burgdorferi* from the tick to the host, it probably would not prevent transmission of *B. afzelii* and *B. garinii*.

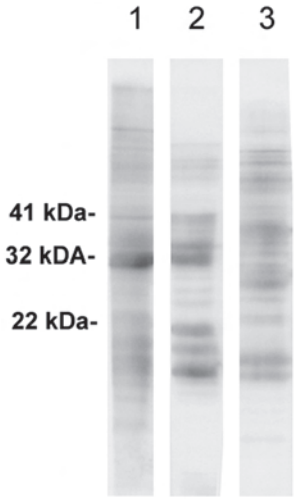
In summary, we have shown that a newly identified *I. ricinus* salivary protein, Salp15 Iric-1, binds to *B. burgdorferi* OspC, protecting the spirochete from antibody-mediated killing. In addition, we have demonstrated that Salp15 Iric-1 binds equally well to OspC from *B. garinii* and *B. afzelii* but that these *Borrelia* species are not protected. Our findings underscore the great complexity of tick-host-pathogen interactions in Lyme borreliosis in Europe.

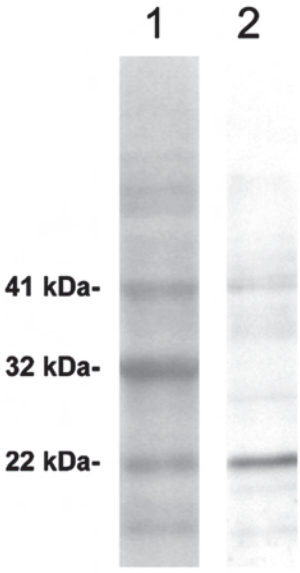
Acknowledgments

We are grateful to Dr. Utpal Pal and X. Frank Yang for the donation of the lysate from the *ospC*-deficient *Borrelia burgdorferi* strain, Dr. Volker Fingerle (Max Planck Institute, Munich, Germany) for the donation of the anti-pKo and anti-PBi rabbit serum, and Dr. Martin Sprick (Academic Medical Center, Amsterdam) for the donation of the *Escherichia coli*–expressed recombinant human His-tagged tumor necrosis factor–α. We also thank Robert Evers for his technical assistance with processing materials for histology.

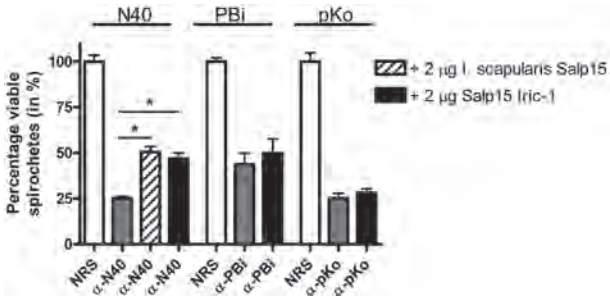
Supporting information

Supplemental figure 1. *Borrelia antigens* recognized by rabbit antisera. Shown are Western blot results for different antisera from rabbits immunized with a specific *Borrelia burgdorferi* sensu lato strain. Lane 1, *B. burgdorferi* strain N40 lysate probed with anti-N40 rabbit serum; lane 2, *Borrelia garinii* strain PBi lysate probed with anti-PBi rabbit serum; lane 3, *Borrelia afzelii* strain pKo lysate probed with anti-pKo rabbit serum. The dilution of antiserum was 1:200, and the dilution of the secondary antibody (goat anti–rabbit–horseradish peroxidase) was 1:1000.





Supplemental figure 2. Binding of Salp15 Iric-1 to outer surface protein (Osp) C from *Borrelia burgdorferi* strain B31. Shown is a solid-phase overlay of *B. burgdorferi* sensu stricto strain B31 and Salp15 Iric-1. Lane 1, Coomassie staining of *B. burgdorferi* sensu stricto strain B31 lysate; lane 2, *B. burgdorferi* sensu stricto strain B31 lysate probed with 1 μg/mL Salp15 Iric-1. Binding of the tick protein to the *Borrelia* lysate was visualized using mouse anti-V5 antibodies and rabbit anti–mouse IgG–horseradish peroxidase antibodies, as described in figures 3 and 6.



Supplemental figure 3. Protection of *Borrelia* strains by Salp15 proteins at lower concentrations, as shown by protection assays using the different Borrelia strains. Spirochetes were incubated overnight with normal rabbit serum (NRS) or specific rabbit anti-*Borrelia* serum in the absence or presence of 20 µg/mL Salp15 Iric-1, *Ixodes scapularis* Salp15, or bovine serum albumin as a control in a total volume of 100 µL. Anti-N40 antiserum was diluted 1:200, whereas anti-PBi and anti-pKo were diluted 1:300. NRS was diluted accordingly. Viable spirochetes were enumerated by dark-field microscopy, as described in figures 3 and 6. A 2-sided Mann-Whitney U test was performed to determine statistical differences between the groups. $P < .05$.

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The tick salivary protein Salp15 inhibits the killing of serum-sensitive *Borrelia burgdorferi* sensu lato isolates

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Chapter 6

Abstract

Borrelia burgdorferi, the causative agent of Lyme disease, is transmitted by ticks. During transmission from the tick to the host, spirochetes are delivered with tick saliva, which contains the salivary protein Salp15. Salp15 has been shown to protect spirochetes against *B. burgdorferi*-specific antibodies. We now show that Salp15 from both *Ixodes ricinus* and *Ixodes scapularis* protects serum-sensitive isolates of *Borrelia burgdorferi* sensu lato against complement-mediated killing. *I. ricinus* Salp15 showed strong protective effects compared to those of *I. scapularis* Salp15. Deposition of terminal C5b to C9 complement complexes, part of the membrane attack complex, on the surface of *B. burgdorferi* was inhibited in the presence of Salp15. In the presence of normal human serum, serum-sensitive *Borrelia burgdorferi* requires protection against complement-mediated killing, which is provided, at least in part, by the binding to the tick salivary protein Salp15.

Introduction

The causative agent of Lyme disease, *Borrelia burgdorferi*, survives in a tick-mouse cycle. In the United States, *B. burgdorferi* sensu stricto is maintained primarily in *Ixodes scapularis* ticks, while the European vector of *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* strains are generally *Ixodes ricinus* ticks. Feeding of ixodid ticks normally takes several days (2), which gives the host immune system time to react to the arthropod. Ticks have developed several mechanisms to evade both innate and adaptive host responses, which enable them to take an effective blood meal. Tick saliva possesses proteins with immunosuppressive (14, 18), anticomplement (5, 19, 27), and antihemostatic (21, 22) activity. Salp15, a feeding-induced tick salivary protein, is known to inhibit $CD4^+$ T-cell activation and proliferation by specifically binding to the CD4 co-receptor of the T cells (1, 6, 13). Also, Salp15 enhanced the survival of *B. burgdorferi* in the host after transmission by the tick by specifically interacting with *B. burgdorferi* outer surface protein C (OspC) and providing protection against borreliacidal antibodies (25). Recently we identified three Salp15 homologues in *I. ricinus* ticks (12), and one of these homologues, Salp15 Iric-1, showed 80% similarity to *I. scapularis* Salp15 (Iscap Salp15) at the DNA level.

The innate immune response, the complement system in particular, plays a crucial role in the eradication of invading pathogens. The complement system is also important in the initiation of an immune response against *B. burgdorferi*. The spirochetes are opsonized and also directly killed by the formation of the lytic pore-forming membrane attack complex (MAC) (3, 23). *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* isolates activate the complement cascade both by the classical pathway and by the alternative pathway in nonimmune human serum (NHS) in the absence of specific antibodies, but they differ in susceptibility to complement-mediated killing (28). Serum-resistant *Borrelia* strains are able to evade complement-mediated killing by binding to complement regulators of the alternative complement pathway, i.e., factor H and factor H-like protein-1 (FHL-1), through CRASP-1Bb (15), CRASP-2Bb (9), OspE (10), and/or CRASP-3Bb (16) proteins, or by expressing a CD59-like complement inhibitory molecule (24). The split products after complement activation are also important because of chemotaxis and the infiltration of immune cells in the *Borrelia*-infected tissue. Altogether, there are several reasons for the spirochetes to protect themselves against complement activation. In this study, we show that the tick salivary protein Salp15 plays a role in the protection of serum-sensitive *B. garinii* strains and intermediately resistant *B. burgdorferi* strains against direct killing by the complement system.

Materials and methods

Borrelia isolates and growth conditions.

Serum-sensitive strains *B. garinii* A87S and VSBP and intermediately resistant strains *B. burgdorferi* VS215 and B31 were used in this study. Both *B. garinii* strains are human isolates, while both *B. burgdorferi* strains are tick isolates. Spirochetes were cultivated at 33°C in Barbour-Stoenner-

Kelley medium plus sodiumbicarbonate (BSK-H medium) supplemented with 6% rabbit serum (Sigma).

Purification of recombinant *I. scapularis* and *I. ricinus* Salp15

For the purification of Iscap Salp15 (GenBank accession number AAK97817), *salp15* was cloned in frame in *Drosophila melanogaster* cells in conjunction with a His-tag, a V5 epitope, and a resistance gene for hygromycin as described previously (1). Salp15 Iric-1 (GenBank accession number ABU93613) was purified from *Drosophila* cells stably transfected with *salp15 Iric-1* and a plasmid containing a blastomycin resistance gene (J. W. Hovius, T. J. Schuijt, K. A. de Groot, J. T. T. H. Roelofs, A. Oei, J. A. Marquart, C. van 't Veer, T. van der Poll, N. Ramamoorthi, E. Fikrig, and A. P. van Dam, J. Infect. Dis. 2008;198: 1189). The Schneider *Drosophila* cells expressing the *salp15* gene from *I. scapularis* or *I. ricinus* were selected with hygromycin (500 μg ml–1) or blastomycin (25 μg ml–1), respectively, and were grown in large spinner flasks together with penicillin and streptomycin (Invitrogen) for 3 days. *Drosophila* cells were subsequently induced with copper sulfate with a final concentration of 500 mM for 4 days and centrifuged at 1,000 x *g* for 15 min. The supernatant was filtered using a 0.22-μm filter (Millipore). Both Salp15 Iric-1 and Iscap Salp15 were purified from the supernatant by use of the HisTrap Ni^{2+} column (GE Healthcare) and eluted with 100 mM imidazole. The eluted fractions were filtered through a 0.22-μm filter and concentrated with a 5-kDa concentrator (Vivascience) through centrifugal concentration. The purity of the purified Salp15 was checked by silver staining (Bio-Rad) according to the manufacturer's recommendations, and the concentration was determined with the Bradford assay.

NHS

Serum samples used were derived from one donor and were checked for the absence of antibodies against *B. burgdorferi* by Western blot analysis. Heat inactivation of NHS was achieved by incubation of the serum samples at 56°C for 30 min.

Assays for detection of complement-mediated killing of spirochetes and Salp15 protection

Two serum-sensitive *B. garinii* strains, the A87S and the VSBP strains, and two intermediately resistant *B. burgdorferi* sensu stricto strains, VS215 and B31, were used (10^7 spirochetes ml–1). Spirochetes (2.5 x 10^5) were preincubated with bovine serum albumin (BSA), Salp15 Iric-1, or Iscap Salp15 (80 μg/ml) for 30 min at 33°C. They were then incubated with NHS or heat-inactivated NHS and examined after 1.5 h, 4.5 h, and 24 h. The two parameters of borreliacidal effect that were recorded are immobilization and bleb formation of the spirochetes. Immotile spirochetes were considered dead (28). The percentages of immotile spirochetes for 200 spirochetes per well were assessed. In a separate titration experiment, different Salp15 concentrations, ranging from 5 μg/ml to 160 μg/ml, were also tested in the same way. To investigate whether membrane-bound Salp15 protects the spirochetes against antibody-independent complement-mediated killing, the spirochetes were washed twice with BSK-H medium (4,000 x *g*, 10 min) after incubation with

Salp15 Iric-1. After removal of unbound Salp15 by washing, the spirochetes were subjected to 12.5% NHS and examined for borreliacidal effect after 1.5 h, 4.5 h, and 24 h of incubation.

Subculture of *B. garinii* VSBP after incubation with *I. ricinus* Salp15

The serum-sensitive *B. garinii* VSBP strain was preincubated with Salp15 Iric-1 for 60 min at 33°C. Then, spirochetes were exposed to 50% NHS for 24 h. As described above, the surviving spirochetes were subcultured in BSK-H medium for 7 days. This selection process was repeated twice.

Binding of *I. ricinus* Salp15 to *B. garinii* VSBP in overlay assay

B. garinii VSBP lysates were obtained from 100-ml cultures that were grown to a density of 1 x 10^8 spirochetes/ml. Lysates were separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto an Immobilon-P membrane (Millipore) to have approximately similar amounts of OspC, as determined by Coomassie staining. After the membrane was incubated overnight in blocking buffer (1% BSA, 3% milk in TBS-0.05% Tween), it was incubated with 1 µg/ml purified Salp15 Iric-1 in blocking buffer for 1.5 h at room temperature, washed, and consequently incubated with a 1:5,000 dilution of horseradish peroxidase-conjugated V5 antibody (Invitrogen) in blocking buffer. Blots were developed by enhanced chemiluminescence.

Binding assay of Salp15 Iric-1 to *B. garinii* VSBP by immunofluorescence

Purified Salp15 Iric-1 was biotinylated by incubating 1 mg/ml Salp15 with 0.25 mg/ml sulfo-NHS-biotin (Pierce) for 45 min at 4°C. Unbound sulfo-NHS-biotin was removed by dialysis against 25 mM lysine in phosphate-buffered saline (PBS) overnight and four times against PBS for 15 min at 4°C. Biotinylated purified Salp15 Iric-1 was incubated with *B. garinii* VSBP spirochetes for 30 min at 33°C. The spirochetes were washed twice with PBS-1% BSA and were resuspended in PBS-1% BSA, air dried on microscope slides overnight, and fixed in 100% methanol. Slides were incubated with bisbenzimide and streptavidine-Cy3 (Sigma) and examined with a fluorescence microscope (Axioscop 2 mot plus; Carl Zeiss).

Detection of terminal C5b-9 complement complexes

B. garinii strain VSBP was preincubated with either BSA, Iscap Salp15, or Salp15 Iric-1 for 30 min at 33°C. Then, the spirochetes were incubated in BSK-H medium containing 12.5% NHS for 30 min. The spirochetes were washed twice with PBS-1% BSA. They were resuspended in PBS-1% BSA, air dried on microscope slides overnight, and fixed in 100% methanol. Spirochetes were detected by incubation with human serum containing antibodies against *B. burgdorferi*, and the C5b to C9 (C5b-9; one molecule each of C5b, C6, C7, and C8 and one or more molecules of C9) complement complexes were indicated with monoclonal mouse C5b-9 antibodies (Dako). Slides were washed with PBS-1% BSA and incubated with an anti-human immunoglobulin G-fluorescein isothiocyanate-labeled antibody (BioMerieux) and an anti-mouse Cy3 antibody (Jackson). After slides were washed and were mounted with Mowiol, they were visualized by confocal microscopy using a fluorescence microscope (Axioscop 2 mot plus; Carl Zeiss). At least 100 spirochetes were counted, and the experiment was performed two times.

Statistical analysis

The protection of spirochetes against complement-mediated killing by Salp15 Iric-1 or Iscap Salp15 was compared to the protection by the control protein BSA. The chi-square test was used for the analysis of proportions, where absolute numbers of spirochetes were used in cross tabulations. Crude relative risks for surviving different circumstances were estimated as odds ratios (OR) and presented with both 95% confidence intervals (95% CI) and *P* values. Tests were performed using SPSS 14 software. Calculated *P* values of < 0.05 were considered significant.

Results

Salp15 protects serum-sensitive *B. burgdorferi* sensu lato isolates against complement-mediated killing

As expected, *B. burgdorferi* sensu lato isolates differed in their sensitivities to NHS. No motile *B. garinii* A87S or *B. garinii* VSBP organisms were seen when incubated for 24 h in 12.5% NHS (Fig. 1). In contrast, other spirochetes required greater amounts of NHS to induce partial killing: *B.*

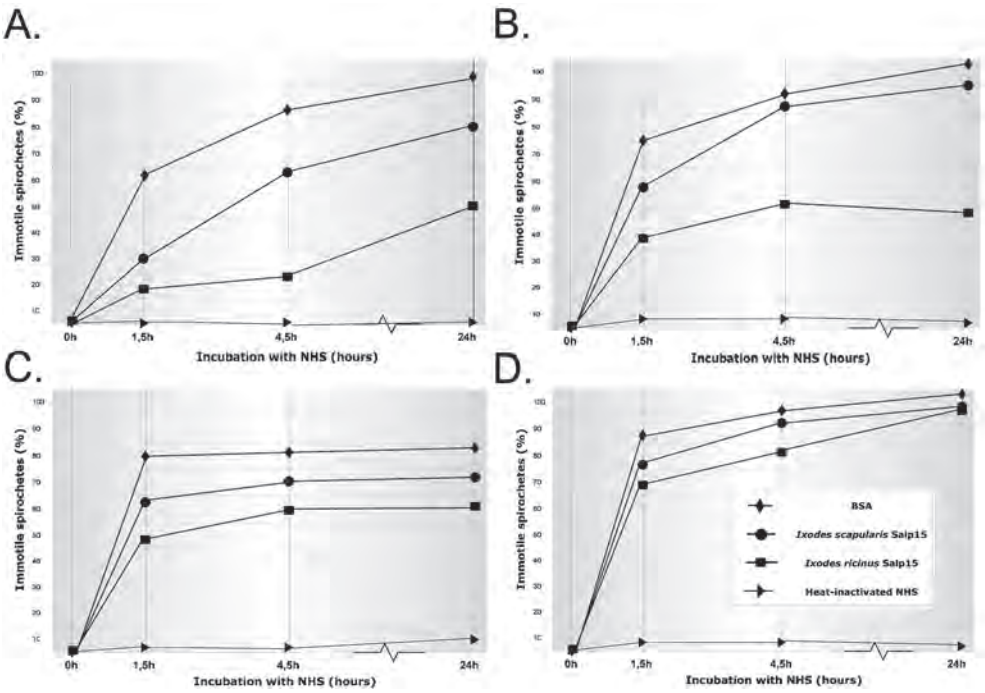

Figure 1. Spirochetes are protected against complement-mediated killing in the presence of Salp15 Iric-1 or Iscap Salp15. Serum-sensitive *B. garinii* strains VSBP (A) and A87S (B) were incubated with 12.5% NHS, while intermediately resistant *B. burgdorferi* strains VS215 (C) and B31 (D) were incubated with 50% NHS, after they had been preincubated for 30 min with BSA, Iscap Salp15, or Salp15 Iric-1. As a control, spirochetes were also incubated with heat-inactivated NHS. After 1.5 h, 4.5 h, and 24 h of incubation with serum, the percentages of immotile spirochetes were determined. Two hundred spirochetes were counted.

Table 1. Protective effect of Salp15 Iric-1 or Iscap Salp15 compared to that of BSA.

		Protection agains immobilization after 1,5 hours[a]				Protection against immabilization after 4,5 hours[a]				Protection against immobilization after 24 hours[a]			
Isolate	Salp15[b]	% Dead[c]	Odds ratio	95% . C.I	*P* value[d]	% Dead	Odds ratio	95% C.I.	*P* value[d]	% Dead	Odds ratio	95% C.I.	*P* value[d]
B. gar	BSA	57%/43%				82%/62%				93%/88%			
VSBP	Iric-1	13%	*8.9*	5.4-14.6	<0.0001	19%	19.4	11.7-32.2	<0.0001	48%	14.7	8.0-27.0	<0.0001
		9%	*7.4*	*6.0-9.8*	*<0.0001*	*22%*	*5.9*	*4.9-7.2*	*<0.0001*	*46%*	*8.2*	*6.6-10.3*	*<0.0001*
	Iscap	24%	4.2	1.7-6.4	<0.0001	59%	3.1	2.0-4.9	<0.0001	88%	1.9	.99-3.8	N.S.
		25%	*2.3*	*1.9-2.8*	*<0.0001*	*49%*	*1.7*	*1.4-2.0*	*<0.0001*	*83%*	*1.5*	*1.2-1.9*	*0.001*
B. gar	BSA	70%/71%				87%/91%				97%/94%			
A87S	Iric-1	34%	4.6	3.0-7.1	<0.0001	47%	7.6	4.6-12.4	<0.0001	44%	35.1	15.7-78.4	<0.0001
		33%	*5.0*	*4.2-6.1*	*<0.0001*	*64%*	*5.5*	*4.3-7.0*	*<0.0001*	*60%*	*10.9*	*8.1-14.5*	*<0.0001*
	Iscap	54%	2.0	1.4-3.1	0.001	84%	1.3	.73-2.2	N.S.	90%	3.1	1.3-7.4	0.01
		51%	*2.4*	*2.0-2.9*	*<0.0001*	*89%*	*1.2*	*.93-1.7*	*N.S.*	*89%*	*2.1*	*1.5-2.9*	*<0.0001*
B. burg	BSA	75%/79%				77%/80%				79%/83%			
VS215	Iric-1	44%	3.8	2.5-5.8	<0.0001	58%	2.6	1.7-3.9	<0.0001	55%	3.1	2.0-4.7	<0.0001
		53%	*2.7*	*2.2-3.3*	*<0.0001*	*59%*	*2.7*	*2.2-3.3*	*<0.0001*	*61%*	*3.2*	*2.6-4.0*	*<0.0001*
	Iscap	59%	2.1	1.4-3.3	<0.0001	65%	1.8	1.1-2.7	0.01	68%	1.8	1.1-2.8	0.01
		58%	*2.3*	*1.9-2.7*	*<0.0001*	*66%*	*2.0*	*1.6-2.4*	*<0.0001*	*61%*	*3.1*	*2.5-3.9*	*<0.0001*
B.burg	BSA	81%/89%				82%/90%				98%/89%			
B31	Iric-1	60%	2.2	1.4-3.5	<0.0001	75%	3.0	1.6-5.4	<0.0001	93%	2.9	1.0-8.3	0.03
		74%	*2.2*	*1.7-2.7*	*<0.0001*	*79%*	*2.3*	*1.8-3.0*	*<0.0001*	*77%*	*2.6*	*2.0-3.3*	*<0.0001*
	Iscap	71%	1.7	1.1-2.7	0.03	72%	1.5	.76-2.8	N.S.	93%	2.9	1.0-8.3	0.03
		79%	*1.6*	*1.3-2.1*	*<0.0001*	*83%*	*1.8*	*1.4-2.4*	*<0.0001*	*78%*	*2.4*	*1.9-3.1*	*<0.0001*

[a]Spirochetes were incubated for 1.5, 4.5 or 24 hours with 12.5% NHS in the case of *B. garinii* VSBP and *B. garinii* A87S or 50% NHS in the case of *B. burgdorferi* B31 and VS215.

[b]Spirochetes were pre-incubated with *I. ricinus* (Iric-1), *I. scapularis* Salp15 (Iscap) or BSA for 30 minutes before incubation with NHS.

[c]Percentage of dead spirochetes. One hundred spirochetes were counted and the experiment was performed two times. Non-italic values show the percentage of dead spirochetes treated with Iric-1 Salp15 while italic values indicate the percentage of dead spirochetes treated with Iscap Salp15

[d]The protective effect of Salp15 compared to BSA was calculated using the chi-square test. Non-italic values represent experiments with 200 spirochetes, while italic values indicate experiments with 1000 spirochetes.

burgdorferi B31 was killed at 24 h by 50% NHS (Fig. 1) and *B. burgdorferi* VS215 was killed by 75% NHS at 24 h (data not shown). All these *Borrelia* isolates survived incubation for 1.5, 4.5, or 24 h with heat-inactivated NHS, demonstrating the importance of complement in serum sensitivity.

Since Salp15 has previously been shown to enhance the capacity of spirochetes to survive in naive mice (25), we determined whether Salp15 could alter the serum sensitivity of these *B. burgdorferi* isolates. Indeed, Salp15 from *I. scapularis* ticks, Iscap Salp15, altered the serum sensitivity of *Borrelia* (Fig. 1). The percentage of dead spirochetes significantly decreased when *Borrelia* isolates were preincubated with Iscap Salp15 (Table 1). The strongest protective effect was seen with *B. garinii* VSBP. We initially used Salp15 from *I. scapularis* ticks, but since the serum-sensitive *Borrelia* are transmitted predominantly by *I. ricinus* ticks, we determined whether Salp15 from these ticks was as potent as or more potent than Salp15 from *I. scapularis* for selected *Borrelia* isolates. We therefore cloned, expressed, and purified Salp15 from *I. ricinus*, Salp15 Iric-1, as described in Materials and Methods. Salp15 Iric-1 afforded protection against complement-mediated killing (Fig. 1; Table 1). The protective effect of Salp15 Iric-1 for all four *Borrelia* isolates was also greater

than that of Iscap Salp15 (Table 1) and the difference in protection of serum-sensitive *B. garinii* spirochetes by Salp15 Iric-1 compared to that by Iscap Salp15 was most apparent (Table 2). A titration of Salp15 showed that both Iscap Salp15 and Salp15 Iric-1 had a dose-dependent protective effect (data not shown).

Salp15 Iric-1 binds to the surface of *B. garinii* VSBP

B. garinii VSBP was preincubated with biotinylated Salp15 Iric-1 and membrane-bound Salp15 Iric-1 was detected using streptavidin-Cy3 in the immunofluorescence assay. It has previously been shown that Iscap Salp15 binds on the surface of *B. burgdorferi* 297 (25) and we now show that Salp15 Iric-1 also specifically binds to the surface of *B. garinii* VSBP (Fig. 2A) by immunofluorescence

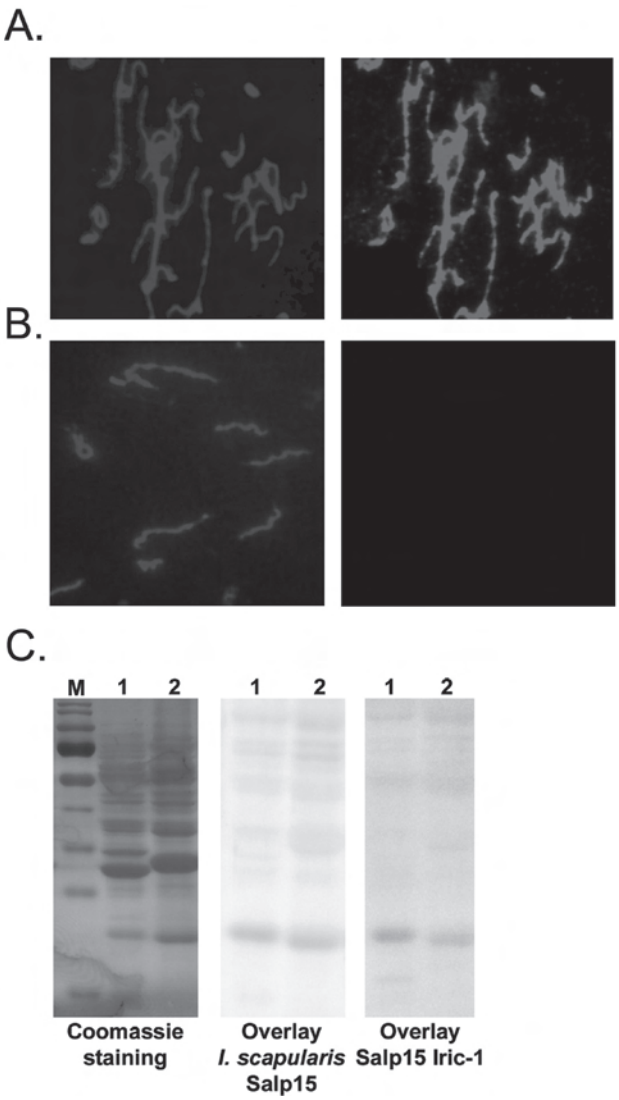

Figure 2. Salp15 Iric-1 binds to the surfaces of *B. garinii* VSBP spirochetes. Spirochetes were preincubated with biotinylated Salp15 Iric-1 (A) or biotinylated BSA (B). Spirochetes were detected with bisbenzimide (blue) and bound Salp15 or BSA was detected using streptavidin-Cy3 (red). (C) Both Iscap Salp15 and Salp15 Iric-1 bind *B. burgdorferi* N40 (1) and *B. garinii* VSBP (2) in the overlay binding assay. The arrow indicates Salp15 bound to OspC. M, molecular mass. For color figure see page 259.

Table 2. Protective effect of Salp15 Iric-1 compared to that of Iscap Salp15.

	Protection against immobilisation after 1,5 hours[a]				Protection against immobilisation after 4,5 hours[a]				Protection against immobilization after 24 hours[a]			
Isolate	% Dead[b]	Odds ratio	95% C.I.	*P* value[c]	% Dead[b]	Odds ratio	95% C.I.	*P* value[c]	% Dead[b]	Odds ratio	95% C.I.	*P* value[c]
B. gar VSBP	9%/25%	3.3	2.6-4.3	<0.0001	22%/*49%*	3.5	2.9-4.2	<0.0001	46%/*83%*	5.6	4.6-6.9	<0.0001
B. gar A87S	33%/51%	2.1	1.8-2.5	<0.0001	64%/*89%*	4.4	3.5-5.6	<0.0001	60%/*89%*	5.2	4.2-6.6	<0.0001

[a]Spirochetes were incubated for 1.5, 4.5 or 24 hours with 12.5% NHS.
[b]Percentage of dead spirochetes. One hundred spirochetes were counted and the experiment was performed two times. Non-italic values show the percentage of dead spirochetes treated with Iric-1 Salp15, while italic values indicate the percentage of dead spirochetes treated with Iscap Salp15.
[c]The protective effect of Salp15 Iric-1 versus Iscap Salp15 against complement-mediated killing was calculated using the chi-square test.

assay. All spirochetes were found to bind Salp15 Iric-1 on their surfaces. By solid-phase overlay, we demonstrated that both Iscap Salp15 and Salp15 Iric-1 are able to bind to OspC of *B. garinii* VSBP and *B. burgdorferi* N40 (Fig. 2C).

Surface-bound Salp15 protects spirochetes against complement-mediated killing

To study whether bound Salp15 protects spirochetes against complement-mediated killing, *B. garinii* was preincubated with Salp15 Iric-1, after which unbound Salp15 was washed away. BSA was used as a control. Spirochetes to which Salp15 had bound were still protected against complement-mediated killing (Fig. 3), and there was a significant difference between the survival of spirochetes after incubation with either BSA or Salp15 and subsequent washing. Notably fewer spirochetes were initially killed in the control group after washing. This could be caused by the loss of less viable spirochetes in the washing procedure. The fact that this difference is not observed among spirochetes preincubated with Salp15 suggests that all spirochetes, independently of initial viability, are potentially protected by Salp15.

Spirochetes that survive in the presence of Salp15 do nothave an increased capacity to bind Salp15

In the presence of Salp15 Iric-1, 2% of the *B. garinii* VSBP spirochetes survived incubation with 50% NHS for 24 h. The surviving spirochetes were reisolated to determine whether the "survival" phenotype could be enriched via selection. After repeating the selection assay two times, the survival rate of the VSBP subculture was compared to that of the original VSBP isolate after incubation with Salp15 and exposure to 12.5% NHS. The *B. garinii* VSBP subculture was not more protected by Salp15 Iric-1 than the original *B. garinii* VSBP culture (data not shown).

C5b-9 Deposition is Inhibited by Salp15 Iric-1 and Iscap Salp15

To examine the influence of Salp15 against complement-mediated killing, we investigated, by use of an immunofluorescence assay, the differences in deposition of C5b-9 terminal complement

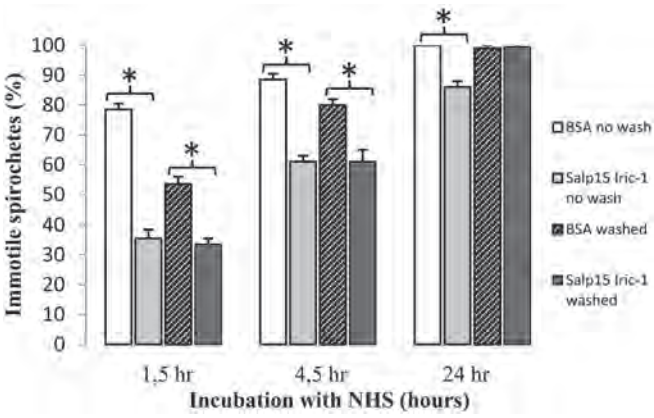

Figure 3. Membrane-bound Salp15 Iric-1 protects *B. garinii* VSBP against complement-mediated killing. Spirochetes were preincubated with BSA or Salp15 for 60 min. Subsequently, BSA or Salp15 was removed by washing as indicated in Materials and Methods, and spirochetes were exposed to 12.5% NHS. Percentages of immotile spirochetes were determined for samples and for controls in which Salp15 was still present. Standard deviations, based on triplicate countings of two independent experiments, are included. The asterisks indicate a statistically significant difference between the groups ($P < 0.0001$).

complexes between spirochetes that had been incubated with Salp15 Iric-1, Iscap Salp15, or BSA (control) and then exposed to NHS. When *B. garinii* VSBP was first incubated with Salp15 Iric-1 or Iscap Salp15, significantly fewer ($P < 0.0001$) C5b-9 complement complexes were found on the membranes of the spirochetes (Fig. 4A to C). Of the spirochetes that had been incubated with BSA and 12.5% NHS, 90% had C5b-9 complement complexes on their membrane, while 52% and 15% of the spirochetes showed C5b-9 deposition after incubation with Iscap Salp15 and Salp15 Iric-1, respectively (Fig. 4D).

Discussion

In the present study, we investigated the role of the tick salivary protein Salp15 in the protection of *B. burgdorferi* sensu lato strains against the complement system. Previously, we have shown that there is a great diversity in serum sensitivity among *B. burgdorferi* sensu lato strains (28). Many *B. garinii* strains are serum sensitive, while *B. burgdorferi* and *B. afzelii* are commonly resistant or intermediately resistant to serum. We now show that both serum-sensitive *B. garinii* and intermediately resistant *B. burgdorferi* strains are protected against complement-mediated killing when coincubated with sera and Salp15 from either *I. scapularis* or *I. ricinus* (Fig. 1; Table 1). Both Salp15 Iric-1 and Iscap Salp15 appeared to be protective, although Salp15 Iric-1 gave significantly more protection against complement-mediated killing than Iscap Salp15 (Table 2). We were not able to select for a more resistant subpopulation by subculturing spirochetes surviving incubation with 50% NHS and Salp15. When a lysate of the original spirochetes was compared to a lysate of the subcultured spirochetes on a Coomassie-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel, no differences in the expression of OspC, which is the natural ligand of Salp15, or of other proteins were found (data not shown).

Serum-resistant strains use CRASPs and Erps to bind plasma proteins factor H and FHL-1, which enables the spirochetes to inactivate the C3 convertase complex (9, 15, 16). Because serum-sensitive

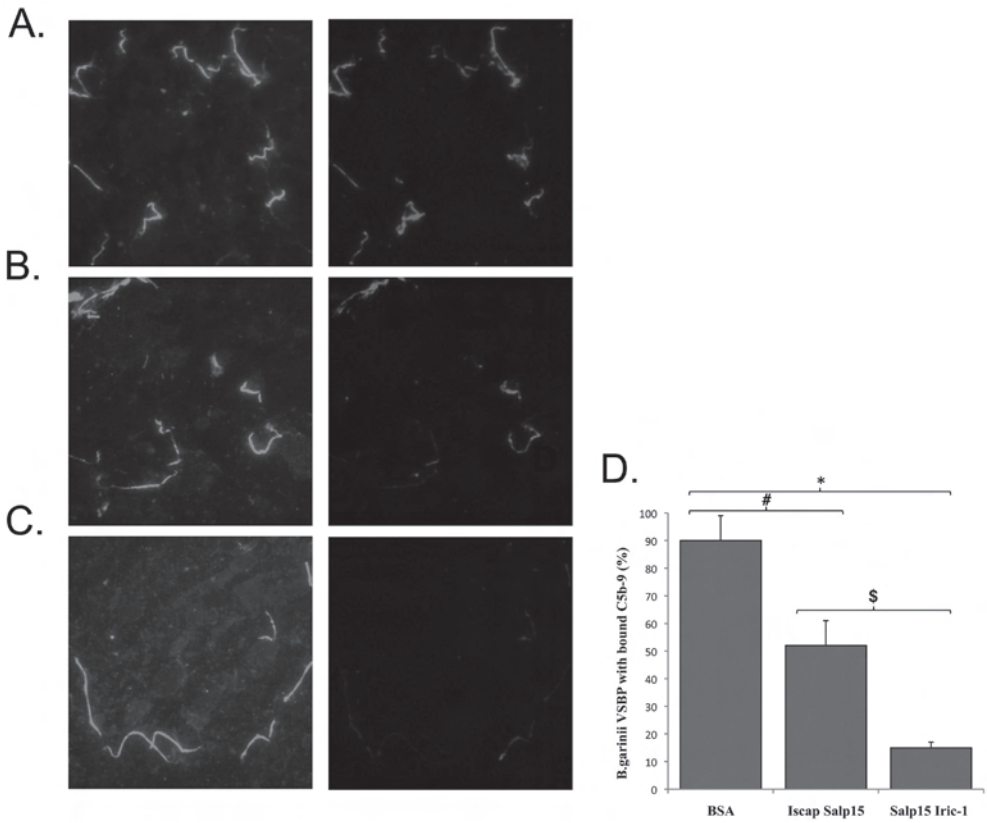

Figure 4. Inhibition of C5b-9 deposition by Salp15. *B. garinii* VSBP was preincubated with BSA (A), Iscap Salp15 (B), or Salp15 Iric-1 (C) before being subjected to 12.5% NHS. Spirochetes were double labeled with antibodies specific for *Borrelia* spirochetes (green) and specific for C5b-9 complement complexes (red). (D) Deposition of C5b-9 complexes was strongly inhibited in the presence of Iscap Salp15 and even more by Salp15 Iric-1. Bars indicate standard deviations, based on duplicate countings of two independent experiments. One hundred spirochetes were counted, and the experiment was performed two times. * indicates a statistically significant difference ($P < 0.0001$) between the BSA- and Salp15 Iric-1-treated groups, # shows a significant difference ($P < 0.0001$) between the BSA- and Iscap Salp15-treated groups, and $ indicates a significant difference ($P < 0.0001$) between the Iscap Salp15- and Salp15 Iric-1-treated groups. For color figure see page 260.

strains do not have their own mechanism to protect themselves against complement-mediated killing, they might benefit from binding proteins from their environment that protect them against this part of the innate immune system, which could explain why serum-sensitive *B. garinii* strains are protected to a great extent in comparison to intermediately resistant *B. burgdorferi* strains (Fig. 1; Table 1). During the blood meal of ticks, Salp15 is secreted by the tick salivary glands, and it was previously shown that Salp15 mRNA levels were 13-fold higher, and Salp15 protein levels were 1.6-fold higher, in salivary glands from engorged ticks infected with *B. burgdorferi* (25). Not only for spirochetes, but also for ticks it is important that the immune system of the host is suppressed. When complement is activated, anaphylatoxins and other proinflammatory mediators are able to trigger degranulation of mast cells and attract phagocytes.

Ticks have a cocktail of salivary proteins which are necessary to take an effective blood meal. It has already been shown that they use Salp15 to inhibit the activation and proliferation of $CD4^+$ T cells by binding to its CD4 receptor (1, 6, 13). In addition, tick salivary proteins have been found to inhibit B cells (8), dendritic cells (4, 11), NK cells (17), neutrophils (20), and macrophages (7). Isac (5, 27) and Salp20 (26) are two salivary proteins that were shown to inhibit the alternative pathway of the complement system, We here describe that a microorganism binding a protein from the vector is protected against killing by the complement system (Fig. 3). Since surface-bound Salp15 was able to protect *Borrelia* even in the absence of free unbound Salp15, it appeared that the protective effect was not caused by the neutralization of C5b-9 formation, at least not completely. When spirochetes were preincubated with Salp15, they were all found to bind Salp15 Iric-1 (Fig. 2) and Iscap Salp15 on their surfaces. For spirochetes, this protection is crucial for survival, since activation of the complement system also initiates the membrane attack pathway, which results in the formation of the MAC consisting of one molecule each of C5b, C6, C7, and C8 and one or more molecules of C9 (Fig. 4). We here demonstrate that Salp15 protected the spirochetes against the formation of MAC complexes. When spirochetes were incubated with Iscap Salp15, deposition of the terminal C5b-9 complement complexes was reduced by 38% compared to what was observed for spirochetes that were incubated with BSA. The effect of Salp15 Iric-1 was even more evident and reduced deposition of C5b-9 by 75% (Fig. 4D). These findings show that Salp15 gives protection not only against bactericidal antibodies but also against the complement system, an important part of the innate immune system. These results could help to explain the higher spirochetal loads in organs of naive mice when inoculated with spirochetes preincubated with Salp15 (25).

In summary, both *I. ricinus* Salp15 and Iscap Salp15 protect *Borrelia* against complement-mediated killing when bound to the membranes of the spirochetes. Protection against the complement system is possibly crucial for the establishment of *B. burgdorferi* infection of the vertebrate host. These findings make it even more interesting to target Salp15 for a vaccine, especially for the prevention of transmission of serum-sensitive *Borrelia* strains.

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PART III

HOST-PATHOGEN INTERACTIONS

Antibodies against specific proteins of and immobilizing activity against three strains of *Borrelia burgdorferi* sensu lato can be found in symptomatic but not in infected asymptomatic dogs

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Chapter 7

Abstract

In an area where Lyme disease is endemic in The Netherlands all dogs had positive titers by whole-cell enzyme-linked immunosorbent assay and appeared to be naturally infected by *Borrelia burgdorferi* sensu lato. To compare the antibody responses of symptomatic dogs and asymptomatic controls, we performed Western blots and in vitro immobilization assays to study antibody-dependent bactericidal activity. Strains from three different genospecies were employed as the antigen source: *B. burgdorferi* strain B31, *Borrelia garinii* strain A87S, and *Borrelia afzelii* strain pKo. Antibodies against flagellin (p41) and p39 for three strains were found in sera from both symptomatic and asymptomatic dogs and were therefore considered to be markers of exposure. Antibodies against p56 and p30 of strain B31, against p75, p58, p50, OspC, and p<19 of strain A87S, and against p56, p54, p45, OspB, p31, p26, and p<19 of strain pKo were found significantly more frequently in sera from symptomatic dogs younger than 8 years when the first symptoms were observed than in those from age-matched controls ($P < 0.01$). These antibodies were not found in preclinical sera and appeared during development of disease. Antibodies against OspA of strains B31 and A87S were only seen in acute-phase and convalescent sera from three dogs that recovered from disease. Seven of 15 sera from symptomatic dogs but none of the sera from 11 asymptomatic dogs had antibody-dependent immobilizing activity against one of the strains. Antibody-mediated bactericidal serum was not seen before onset of disease, was strongest in the acute phase of disease, and fluctuated during chronic disease. From seven out of eight symptomatic dogs *Borrelia* DNA was amplified by PCR; in three of them the bactericidal activity was directed against one of the genospecies amplified from that dog; however, four PCR-positive dogs lacked bactericidal activity. In conclusion, dogs with symptomatic canine borreliosis have more-extensive antibody reactivity against *Borrelia*, as shown by both Western blotting and immobilization assays.

Introduction

Borreliosis, a multisystemic infectious disease of humans and some animal species is caused by spirochetes of the *Borrelia burgdorferi* sensu lato group. The three pathogenic genospecies known to occur in Europe are *B. burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia afzelii* (4, 46, 49). A fourth genospecies, *Borrelia valaisiana* (former group VS 116), is widely distributed in Europe but its pathogenicity is not yet clear (35). In humans, Lyme borreliosis (LB) can be recognized by an expanding, sometimes migrating erythematous lesion (EM). Simultaneously with the EM, immunoglobulin M (IgM) and often IgG antibodies against specific antigens of *B. burgdorferi* sensu lato develop (1, 35, 43). In early LB, a response against the 41-kDa flagellin and the 21- to 23-kDa outer surface protein, OspC, is mounted, and later in the course of disease responses against an expanding number of proteins can be measured by immunoblotting (2, 8, 11, 18, 19, 51). Antibodies against the 31- to 34-kDa OspA, the major protein expressed when the spirochete inhabits the tick midgut, only develop in late LB with chronic often antibiotic-resistant arthritis (2, 24, 25). OspA is downregulated and OspC is expressed when spirochetes migrate from the midgut to the salivary gland of the tick and are subsequently transmitted to the host (10, 15, 37). In humans and dogs vaccinated with a recombinant OspA, bactericidal antibodies which are protective against infection develop (30, 34, 45). Paradoxically, in symptomatic humans and hamsters this naturally occurring bactericidal activity apparently does not resolve the disease (5, 13). In the hamster model the three genospecies are able to cause infection separately and at the same time elicit non-cross-reactive protective bactericidal activity (27).

Borreliosis can also occur in dogs, for which clinical symptoms were defined as malaise (caused by fever and showing as inappetence) and lameness (23). Apart from antibodies against the 41-kDa flagellin protein, which can be cross-reactive, antibodies against 39-, 30-, 28-, 26-, 25-, and 19-kDa proteins are frequently seen in *Borrelia*-exposed dogs (16, 23). In a wooded area in The Netherlands where Lyme borreliosis is endemic all household dogs developed antibodies against *Borrelia*, whereas a control group of dogs living in an area where the disease is not endemic did not show such antibodies (21). Therefore, it was concluded that all these Dutch dogs had *Borrelia* infections. By PCR we found that in dogs clinically suspected of having borreliosis the frequency of infection by *Borrelia*, often by more than one species at the same time, was much higher than in dogs that remained asymptomatic (22). Diseased dogs with clinical symptoms such as malaise (in most cases accompanied by fever) and lameness had a very high titer during the symptomatic period, which persisted in chronically diseased dogs or diminished when dogs recovered (21). Serum can exert antibody-independent borreliacidal activity through complement. In human sera, the intensity of this bactericidal activity differs between strains from the three pathogenic European species (47). Canine bactericidal activity through complement has not yet been tested and may exert differential protection against the different genospecies. The goal of this study is to characterize the specific immune responses of Dutch dogs against infection by one or several species of the *B. burgdorferi* sensu lato group. We determined antibody-independent and antibody-

dependent bactericidal activities in sera of symptomatic and asymptomatic dogs and investigated the expansion of the antibody response in the course of symptomatic infection. Sera were tested for bactericidal activity and specific antibodies against *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* by in vitro bactericidal assays and by Western blotting.

Materials and methods

Borrelia isolates

Three *Borrelia* strains representing the three major pathogenic genospecies were examined in this study. The specific isolates studied were B31 (*B. burgdorferi* sensu stricto), A87S (*B. garinii*), and pKo (*B. afzelii*). Strains B31 and pKo were both high-passage reference strains, but A87S was passaged less than 15 times. The isolates were stored at -70°C in 50% glycerol peptone and cultured in modified Barbour-Stoenner-Kelly (BSK) medium at 33°C. These isolates were used for the immobilization assays as well as for the preparation of antigen for immunoblots.

Dogs studied and serum samples

Dogs living in a wooded environment in the south of The Netherlands are all heavily infested by naturally occurring ticks during consecutive tick seasons, especially in May and June (20). These dogs were monitored in a local veterinary clinic for at least 5 years, and some of them developed symptoms compatible with canine LB as described by Jacobson et al. (23). Dogs were not vaccinated against borreliosis. The diagnosis of symptomatic borreliosis was made when dogs had a period of symptoms in which malaise (listlessness or inappetance) was followed by a period of lameness. Dogs without this combination of symptoms were referred to as asymptomatic. Results of the concurrent serological monitoring by whole-cell enzyme-linked immunosorbent assay (ELISA) were not used as an entry criterion. In the present study, 15 sera from dogs symptomatic for borreliosis were further analyzed by Western blotting and immobilization assays and compared with sera from 15 asymptomatic age-matched controls. For the symptomatic dogs we recognized three patterns in the course of the disease: dogs that recovered from disease, dogs with intermittent recurring disease, and dogs with progressive disease (Table 1). The age at which first symptoms were observed for symptomatic dogs is indicated, as is the age of occurrence of a high peak titer in asymptomatic control dogs. Ten dogs showed symptoms before their 8th year of life. In 13 of 15 symptomatic dogs malaise was accompanied by fever (>39.0°C). Only one of the asymptomatic dogs (dog 39) developed fever, which was explained by another infectious disease. Several organ systems were involved in most of the symptomatic dogs. In 13 of the symptomatic dogs one or several treatments with antibiotics were given (amoxicillin at 10 mg/kg of body weight twice daily, orally for 14 days) in at least one of the disease episodes. Treatment was usually given when very high fever was noticed and may have influenced the course of the disease, especially in three younger dogs that were treated during first symptoms and that completely recovered. However, in the other dogs, recovery from disease episodes occurred with and without treatment, and under both conditions episodes recurred. To exclude other causes of fever of undetermined origin, malaise, and

Table 1. Clinical history and dynamics of whole-cell ELISA antibody response for symptomatic and asymptomatic dogs.

Dog or serum	Age (yr)[a]	Breed	Titer[b]		Max temp (°C)[d]	No. of disease episodes[e]		Episodes with antibiotic therapy[c]	Organ system involved				Diagnosis	Out-come[i]
			Peak	1-5 yrs*		Total	With lame-ness		Urologic	Hepa-tol	Cardio resp[f]	Neuro		
Symptomatic dogs														
28	4	Golden retriever	2,560	80	40.0	1	1	1			Yes		Borreliosis	C
47	6	Scottish terrier	2,560	40	40.9	1	1	1	Yes	Yes	Yes		Borreliosis	C
63	7	Labrador retriever	2,560	320	39.8	4	3	1	Yes				Borreliosis	C
14	2	Siberian husky	2,560	160	38.8	5	2	2	Yes			Yes	Borreliosis	Int
40	2	Tatra mountain dog	2,560	640	39.6	4	2	3	Yes			Yes	Borreliosis	Int
58	4	Munsterlander	1,280	640	40.6	3	1	2	Yes	Yes	a	Yes	Borreliosis	Int
72	8	Labrador crossbreed	10,2400	1,280	40.7	8	3	2, 4	Yes	Yes		Yes	Borreliosis	Int
107	8	Bouvier	2,560	1,280	39.0	2	1	2	Yes	Yes		Yes	Borreliosis	Int
48	2	Bernese mountain dog	5,120	2,560	40.2	11	5	4, 5, 6, 8	Yes			Yes	Borreliosis	Prg
33	4	Bernese mountain dog	10,2400	2,560	38.8	8	5		Yes			Yes	Borreliosis	Prg
75	9	Bouvier	10,2400	2,560	39.5	8	4	2, 3	Yes				Borreliosis	Prg
77	9	Bernese mountain dog	10,2400	2,560	40.9	12	4	3, 8, 9	Yes	Yes	Yes	Yes	Borreliosis	Prg
79	12	Irish terrier	2,560	2,560	39.8	6	4	2, 3, 6	Yes	Yes	Yes		Borreliosis	Prg
78	13	Irish terrier	2,560	2,560	39.3	9	2	3, 4, 5	Yes	Yes	Yes	Yes	Borreliosis	Prg
83	13	Dachshund	2,560	640	40.1	9	2		Yes	Yes	Yes	Yes	Borreliosis	Prg
Asymtomatic dogs														
501	2	Crossbreed	640	320	38.8									
10	3	Bernese mountain dog	2,560	640	38.8		1						Osteochondritis	
22	3	Crossbreed	640	320	38.8									
34	5	Golden retriever	640	320	38.7									
35	5	Crossbreed	640	320	38.8									
39	5	American cocker spaniel	1,280	640	39.7	1		1	Yes				Urinary tract inf.	
54	7	Cairn terrier	1,280	320	38.8									
57	8	Golden retriever	640	320	38.4	1	1	1	Yes				Nefritis/ osteochondritis	
59	8	Bouvier	640	160	38.8		1						Spondylosis	
94	8	Yorkshire t\ier	320	80	38.5	1			Yes				Cystic calculi	
62	10	West Highland white terrier	640	320	38.4	1		1	Yes				Endometritis	
67	10	Cairn terrier	1,280	320	39.0								Tumor	
68	11	Golden retriever	2,560	320	38.8									
76	13	Dutch lure dog	1,280	640	38.8									
511	13	Greenland dog	640	320	38.8									
Pos. controls														
A92 1/2[g]	1	Beagle	2,560	2,560		1	1							
Neg controls[h]														
Lep. vac		Beagle	0											
NCS		Beagle	0											

[a] For symptomatic dogs, age when first disease episode was observed; for asymptomatic dogs, age when a peak titer was observed. [b] Reciprocal antibody titer by whole-cell ELISA with strain B31 as the antigen source. [c] Numbers are the episode designations (e.g., 1 refers to the first episode). [d] Maximum temperature of a dog during any of the symptomatic episodes. A temperature between 38.2 and 38.8°C is considered normal; a temperature >39.0°C is considered a fever. [e] Clinically relevant. [f] Cardioresp., cardiorespiratory. [g] Dog experimentally infected with *B. burgdorferi* sensu stricto. [h] Lep. vac., dog vaccinated against leptospirosis ($n = 1$). For NCS, $n = 4$. [i] C, convalescence; Int, intermittent disease; Prg, progressive disease. *Persisting for 1-5 years.

lameness, the clinical workup when appropriate included radiology, laboratory work in search of immune-mediated diseases (determination of antinuclear antibody and rheumatoid factor), and histology on biopsies. Moreover, from all symptomatic dogs complete hematological (complete and differential red and white blood cell and platelet counts) and serum biochemistry profiles (blood urea nitrogen, creatinine, glucose, electrolytes, liver enzymes, bilirubin, protein electrophoresis) were obtained at several time points in the course of disease (during the 5 years of monitoring). For most symptomatic dogs the test results suggested acute bacterial infection (neutrophilia with left shift) or prolonged antigenic stimulation (mild lymphocytosis and eosinophilia and polyclonal gammopathy) as the cause of disease. Asymptomatic dogs were not treated with antibiotics unless another infectious disease was diagnosed (Table 1).

From symptomatic and asymptomatic dogs we obtained sera at least twice a year or in the course of the development of clinical manifestations. We attempted to obtain preclinical sera, sera after first infection before development of clinical signs, acute-phase sera during or just after the start of clinical manifestations to several months after the clinical manifestations, chronic-phase sera, and convalescent sera several months to a year after seroconversion and recovery. Control dog sera for the immunoblots were kindly provided by others. Serum of a positive-control dog (A92 1/2), infected with *B. burgdorferi* sensu stricto, was kindly provided by M. Appel, New York, N.Y. This dog was infected through tick bite, and blood samples were taken after 4 months. Sera of negative-control dogs, leptospiral vaccinated and specific-pathogen-free (SPF) dogs, were kindly provided by A. Mollema, Fort Dodge Animal Health, Weesp, The Netherlands.

Antigen preparation

Spirochetes were grown in 50 ml of BSK medium at 33°C until the stationary phase was reached and the concentration of the spirochetes was approximately 5×10^7/ml. Cells were harvested by centrifugation at 5,000 × *g* for 20 min and washed three times with 50 mM Tris-HCl (pH 7.4). Protein concentrations were determined as described by Lowry et al., and the preparations were stored at -20°C (28). The amount of protein used for the gel electrophoresis was the same for each gel.

Whole-cell ELISA

Whole-cell antigen was prepared from *B. burgdorferi* sensu stricto strain B31 by sonication as described previously (21). After the incubation with serum, horseradish peroxidase-conjugated anti-dog IgG (1/3,000 dilution; Organon Teknika, Turnhout, Belgium) was used in combination with the chromogenic agent (ABTS [2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid]; Sigma Chemical Co., St. Louis, Mo.). Optical density at 405 nm was measured using a Titertek Multiscan ELISA reader. Antibodies were determined by end point titration; each serum was tested in a dilution range from 1/20 to 1/2,560. Higher dilution titers were extrapolated from the optical density values. Cutoff values were calculated on the basis of the results of the pre-tick-bite sera of 12 young dogs. Samples were considered positive if they had an end point titer of 1/320 (21). Sera

from 52 dogs in an area where the disease is not endemic (New Zealand) tested negative, as did sera from 16 SPF dogs vaccinated and challenged with *Leptospira icterohemorrhagiae* (21). Sera from all dogs in this study from the area of endemicity in the south of The Netherlands showed seroconversion to titers of 1/320 and higher in the first or second tick season. Dogs remaining asymptomatic were seen to reach persistent titers of 1/320 and 1/640; only occasionally was a higher titer observed (21). However, the symptomatic dogs showed a steep rise in titers to 1/2,560 or higher before or concurrent with the development of symptoms. In most of the symptomatic dogs these high titers persisted for 1 year or longer (Table 1).

SDS-PAGE and Western blots

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on whole-cell lysates of *B. burgdorferi* sensu lato by a modification of the methods described by van Dam et al. (46). Briefly, the antigen was diluted 1:1 with 2% SDS sample buffer and was boiled for 5 min. This suspension was electrophoresed on 13% polyacrylamide gels (15 by 10 cm). The gels were run at 50 mA for 3 to 4 h. Adjacent to the cell lysate, prestained low-molecular-mass markers (Bio-Rad, Münich, Germany) were applied in an extra lane. The separated proteins were blotted overnight onto nitrocellulose at 50 mA in a carbonate buffer (10 mM $NaHCO_3$ and 3 mM Na_2CO_3 containing 20% methanol). Blots were blocked with nonfat dried milk in an incubation buffer (10 mM Tris-HCl, 500 mM NaCl, 0.5% Tween 20; pH 7.5) for 1 h at 22°C and cut into 4-mm strips. Antigen strips were incubated with 1:100 dilutions of test serum for 120 min, washed three times for 5 min each time (with 10 mM Tris-HCl, 500 mM NaCl, 0.5% Tween 20; pH 7.5), and incubated with horseradish peroxidase-conjugated goat anti-dog IgG antibodies (Nordic, Breda, The Netherlands) diluted 1:1,000 in phosphate-buffered saline (PBS). After three washes, two times as described above and one time with PBS, the antibody reactivity was visualized by incubation with 4-chloro-1-naphthol-H2O2 for 15 min. When immunoblots were performed with monoclonal antibodies (MAb), 1 ml of a 1:50 dilution of the culture supernatant was incubated instead of the test serum and 1 ml of a 1:7,500 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG antibodies was used as a second antibody (Promega, Leiden, The Netherlands). The reactive protein bands were visualized with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Promega). Protein bands found by immunoblotting were scored at their molecular masses and intensities for further evaluation. Six monoclonal antibodies (MAb)s were used to locate the major proteins on the Western blots. H9724 recognizes a 41-kDa flagellar protein (p41) in all three species tested (19). LA 26 is directed to the 31-kDa outer surface protein A (OspA) of *B. burgdorferi* sensu stricto and *B. afzelii*, whereas LA 31 is directed to OspA of *B. burgdorferi* sensu stricto and *B. garinii* (46). OspB was located with MAb 84C in all three strains tested (40). Finally L22 1F8 and L22 C11 were used to locate a 21- to 23-kDa protein (OspC); L22 1F8 reacts with OspC of all three *Borrelia* species (48, 50), whereas L22 C11 is directed to OspC of *B. garinii* and *B. afzelii* (48). For the outer surface proteins the apparent molecular masses differed for the three strains. OspB differed in apparent molecular mass between 33 kDa for strain A87S and 34 kDa for strains B31 and pKo. OspA had an apparent molecular mass of 31 kDa in strains B31 and A87S. Strain pKo

expressed a 31-kDa band in the immunoblot, which showed no reactivity to a *B. afzelii*-specific anti-OspA MAb. OspC MAb to strains A87S and pKo reacted with protein bands with an apparent molecular mass of 21 kDa. Strain B31 expressed a 21-kDa protein that did not show reactivity with the specific MAb.

Bactericidal assays

Borrelia isolates were thawed and grown to a density of approximately 1 x 10^7 spirochetes per ml of BSK, as judged by dark-field microscopy. An aliquot of this suspension was added to an aliquot of heat-inactivated test serum and an aliquot of serum of SPF dogs, referred to as normal canine serum (NCS), as a complement source, to give a final volume of 100 µl. To assess the bactericidal activity of NCS (i.e., antibody-independent killing of spirochetes), an aliquot of heat-inactivated NCS and the active NCS was added to the spirochetes, as described previously for human normal serum (47). The concentrations of NCS used in the final assays differed for the three genospecies because of their different sensitivities to the bactericidal activity of the complement source. To assess the bactericidal activity of the serum of dogs attending the clinic (i.e., antibody-dependent killing of spirochetes), 15% NCS was added for strain A87S and 25% SPF serum was added for B31 and pKo. To avoid the presence of particles that could diminish the visibility of the spirochetes, all sera were centrifuged for 5 min at 14,000 × *g* and 4°C before use. Experiments were performed in duplicate in a 96-well microtiter plate. The plate was sealed and incubated at 33°C. After 0, 1, 3, and 5 h of incubation an aliquot of 5 µl was drawn from each well to assess the mobility and the extent of bleb formation of the spirochetes by dark-field microscopy. Immobilized, blebbed spirochetes were considered nonviable (47). In negative-control experiments heat-inactivated SPF serum was added to the suspension containing spirochetes with or without test serum. Borreliacidal activity of the test serum was corrected according to the formula corrected immobilization (CIM) = (the percentage of immotile spirochetes in test serum and NCS minus the percentage of immotile spirochetes in NCS only)/(100% minus the percentage of immotile spirochetes in NCS only). A CIM of 20% or more was considered significant immobilizing activity of the test serum.

Detection of bacterial DNA by PCR

From eight symptomatic dogs and four asymptomatic dogs tissue biopsies could be tested for the presence of *Borrelia* DNA. Positive tissue biopsies included skin, synovial tissue, heart, liver, bladder wall, and bone marrow tissue, and cerebrospinal fluid. All specimens were included in a study described elsewhere (22). After homogenization of tissues and DNA extraction, part of the 5S-to-23S rRNA spacer region was amplified by PCR. Amplification products were hybridized with specific probes for *B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, and *B. valaisiana*. Details of all procedures have been described earlier (22).

Results

Immunoblots

The 15 sera of symptomatic dogs and 15 sera of asymptomatic dogs were compared by Western blotting with three strains providing the antigens. A total of 40 antigens of *B. burgdorferi* sensu stricto, 41 antigens of *B. garinii*, and 39 antigens of *B. afzelii* reacted with antibodies from at least one of the sera. More bands were detected on immunoblots with sera of symptomatic dogs that were under 8 years of age when the first symptoms occurred than with sera of older symptomatic dogs or with sera of asymptomatic dogs (Table 2). The sera of the younger dogs were mostly sampled during early, and for some dogs temporary, stages of the disease; the sera of the older dogs were sampled during later stages of disease, and these dogs had progressive disease (Table 1).

For all groups of naturally infected dogs, the most reactivity was observed with *B. afzelii* strain pKo, followed by *B. garinii* strain A87S. The lowest reactivity was found with *B. burgdorferi* sensu stricto strain B31. In contrast, serum from a control dog experimentally infected with *B. burgdorferi* sensu stricto showed the most reactivity (14 bands) with strain B31. However, this serum also cross-reacted with 12 bands of *B. afzelii* strain pKo. The prevalences of antibodies against p41 (flagellin), p39, OspB, OspA (for strains B31 and A87S), p31 (strain pKo), OspC (strains A87S and pKo), and p21 (strain B31), which are antibodies widely used in the diagnosis of borreliosis, as well as of antibodies reacting with 16 other protein bands frequently present in acute-phase sera of young symptomatic dogs (younger than 8 years when first symptoms were observed) and less frequently present or not present in sera of young asymptomatic dogs (younger than 8 years when peak titer was observed) were statistically compared (chi-square test; $P < 0.01$). Statistically, antibodies against p56 and p30 of *B. burgdorferi* sensu stricto strain B31, against p75, p58, p50, OspC, and p<19 of *B. garinii* strain A87S, and against p56, p54, p45, OspB, p31, p26, and p<19 of *B. afzelii* strain pKo were associated with the presence of acute disease in dogs under 8 years of age when first symptoms were observed (Table 3). Proteins in the 60- to 66-kDa molecular mass range were statistically more often present in symptomatic dogs but were not considered markers of borreliosis because they may be related to heat shock proteins present in many bacterial species (7, 19, 32, 51).

Table 2. Average number of protein bands on immunoblots with sera from symptomatic and asymptomatic dogs using different *Borrelia* strains.

	Average no. of bands for sera from				
Borrelia strain	Symptomatic dogs ≤ 8 yr on first symptoms (n = 10)	> 8 yr on first symptoms (n = 5)	Asymptomatic dogs ≤ 8 yr on peak titers (n = 10)	> 8 yr on peak titers (n = 5)	Pos dog n = 1[a]
B31	7.2	1.4	2.9	2.1	14
A87S	10.8	1.6	3.0	2.9	8
pKo	13.1	3.6	1.9	2.0	12

[a] Experimentally infected with *B. burgdorferi* sensu stricto.

Furthermore, presymptomatic sera from seven dogs tested by immunoblotting did not show reactivity with the bands described above as being associated with acute disease. This is demonstrated for two dogs. Dog 33 had a persistently high titer by whole-cell ELISA after an episode of major clinical manifestations, and the same bands found on the immunoblot with acute-phase serum persisted on the immunoblot with serum sampled 2 years later when symptoms had relapsed (Fig. 1). None of these bands were found on immunoblots with preclinical serum (serum 1 year prior to and serum a few months prior to the major clinical manifestations). For dog 28, a dog that recovered from disease and for which the ELISA titer declined after one episode of major clinical symptoms, almost no bands were seen on immunoblots with preclinical serum (Fig. 1B). In the acute-phase serum of this dog, which showed a high ELISA titer, several antibodies that were associated with disease were detected. Observed were reactions against OspA and p30 and a very strong reaction against p28 on the immunoblots of strain B31. On immunoblots with a convalescent serum, showing low reactivity in the ELISA, the intensity of the p28 band waned, whereas the p30 band totally disappeared and a strong band, which was only weakly present on the immunoblots with acute-phase serum, was detected in the OspA region. The other two dogs (dogs 47 and 63) from this study that recovered from disease also had antibodies against OspA in their serum, one of strain B31 of *B. burgdorferi* sensu stricto and the other against strain A87S of *B. garinii* in their serum (Table 3).

Bactericidal activity of complement

Immobilizing activity against three spirochetal strains was determined with NCS. NCS in a concentration of 25% in the test medium had little immobilizing activity against strain B31 of

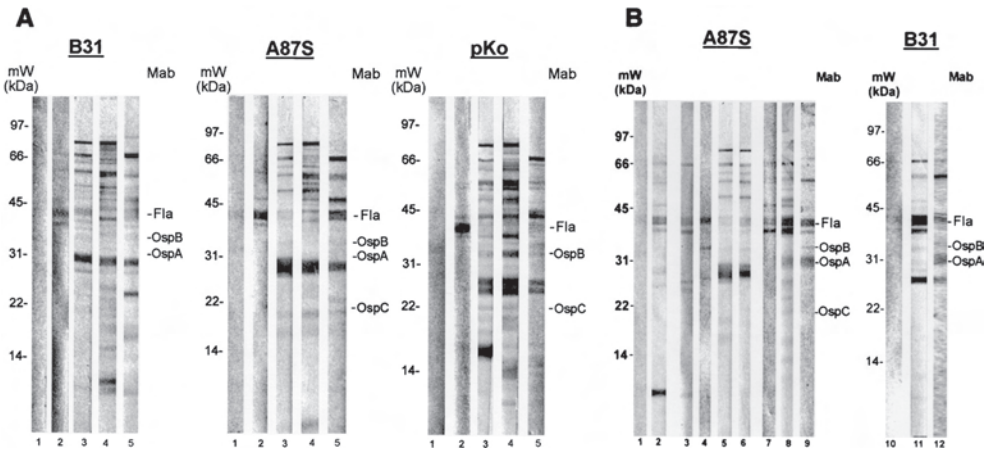

Figure 1. (A) Immunoblots with dog sera using different *Borrelia* strains as sources of antigens. Lane 1, negative-control dog; lane 2, asymptomatic dog 35; lane 3, symptomatic dog 33; lane 4, symptomatic dog 48; lane 5, positive-control dog. The molecular masses of the proteins were assessed by running a prestained low-molecular-mass marker adjacent to the cell lysate. All three strains were tested for the presence of OspA, OspB, OspC, and flagellin with MAb. (B) Immunoblots with dog sera using strains A87S and B31 as sources of antigens. Lane 1, negative-control dog; lane 2, asymptomatic dog 57; lanes 3 and 4, preclinical sera from dog 33; lane 5, acute-phase serum from dog 33; lane 6, chronic-phase serum from dog 33; lanes 7 and 10, preclinical serum from dog 28; lanes 8 and 11, acute-phase serum from dog 28; lanes 9 and 12, convalescent serum from dog 28. Identification of the proteins was as for panel A.

Table 3. Serum antibodies against strains of *B. burgdorferi* sensu lato as detected on immunoblots, serum bactericidal activity, and DNA detection by PCR in dogs.

Dog or serum	Age (yr)[a]	Strains[i] for which antibodies against bands with the indicated molecular masses (kDa) were detected																				Bact Act[e]	PCR[f]
		75	**66**	**62**	**60**	**58**	**56**	**54**	**50**	**48**	**45**	**43**	**41**	**39**	**33-34[b]**	**31[c]**	**30**	**28**	**26**	**25**	**21[d]**	**<19**	
Symptomatic dogs																							
28	4	g	bga	g	a	bg	a	a	g	g	a	a	bga	bga	ba	bga	b	b	ga		g	ga	None	ND[j]
47	6	g	bg	g	a	bg	a		g	g	a		bga	bga	bga	ba			a		a	a	None	b
63	7		bga	g		ga	ba	b	g	g		a	bga	bg	ba	g	b	bg			g		None	ND
14	2		a		g	a	b				b		bga	bga	a			b			g		g	bg
40	2	ga	bg	g	a	bg	a	a	g	g		a	bga	ga	ba		b				bg	g	g	ND
58	4	bg	bga	g	a	bg	a		g	g	a	a	bga	bga	ga	a			a		g	a	g	bgav
72	8		b	g					g		a	a	bga	b	b								bg	ND
107	8	g	bga	b	ba	bg	ba	a			ba	a	ba	ga	ga	a	b			a		bga	None	b
48	2	bga	bga	bga	bga	g	ba	a	a	g	ga	a	bg	ba	ba	a	b	bg	a	ba	ba	bga	a	ND
33	4	bga	bga	ga	b	g	ba	a	a			a	bg	b	bga	a	b	bg	a	a	ba	ga	a	ND
75	9		b										ga		a								None	Neg.
77	9			a			a	a				a	bga	bga	a				a	a			None	ND
79	12						a						bga	b	a								b	b
78	13			b									b										None	b
83	13									g		a	ga	ga	a	a							None	bga
Asymptomatic dogs																								
501	2																						None	g
10	3		b										bg	bg									None	ND
22	3		g			b							bg	g				g					ND	ND
34	5		b			b							bga	bg	bg			b					ND	ND
35	5									g		a	bga	bga									None	ND
39	5		g		ba					g			bga	ga									None	ND
54	7		bga			b							bga	ga	a								None	ND
57	8		bg							g		a	bga	bga	b			g		g			None	ND
59	8		b									a	bga	ba									None	ND
94	8		g										ba	bga									None	v
62	10						a						bga	bga	a								None	ND
67	10												bga	g	a								None	ND
68	11		g										bga		bg								ND	ND
76	13					b							bga	bga	ga			g					ND	Neg.
511	13		b										bg	g	g								None	a
Pos control																								
A92 1/2[g]	1		bga			g	a	ba		g	ga	a	bga	ba	ba		b	bg	a	a		bg		
Neg control[h]																								
Lep vac.		a				g							ba											
NCS (n = 1)													bga											
NCS (n = 3)																								

[a] As defined for Table 1. [b] Strains B31 and pKo expressed a 34-kDa protein, and strain A87S expressed a 33-kDa protein, both of which reacted with MAb for OspB. [c] Strains B31 and A87S expressed a 31-kDa protein that reacted with MAb for OspA, whereas strain pKo expressed a 31-kDa protein that did not react with the MAb for OspA. [d] Strains A87S and pKo expressed a 21-kDa protein that reacted with the MAb for OspC, whereas strain B31 expressed a 21-kDa protein that showed no reactivity to the OspC MAb. [e] Bactericidal activity, results from immobilization assays. See footnote i for strain abbreviations. None, no immobilization found. [f] For some dogs, tissue biopsies for the detection of specific *Borrelia* DNA, which was determined with species-specific probes, were available. b, *B. burgdorferi* sensu stricto; g, *B. garinii*; a, *B. afzelii*; v, *B. valaisiana*. [g] As defined for Table 1. [h] As defined for Table 1. [i] b, *B. burgdorferi* sensu stricto strain B31; g, *B. garinii* strain A87S; a, *B. afzelii* strain pKo. [j] ND, not done.

B. burgdorferi sensu stricto (mean ± SD, 4% ± 2%) and against *B. afzelii* strain pKo (6% ± 4%). Strain A87S was more sensitive to NCS, and incubation of strain A87S with 25% NCS resulted in high levels of immobilization (ranging from 31 to 95% in seven experiments with an average of 61% ± 24%). Thus *B. burgdorferi* strain B31 and *B. afzelii* strain pKo were resistant to the bactericidal activity of dog complement. In contrast, *B. garinii* strain A87S was susceptible to the bactericidal activity of NCS. With human sera and sera from other mammals also, differences in the complement susceptibilities of the genospecies have been described (26, 47). Complement-mediated killing in natural hosts could have ecological implications for the *Borrelia* species as it might determine the reservoir competence (26). In this respect, it is interesting to note that the dog is a competent reservoir for *B. burgdorferi* sensu stricto (31).

Low-passage isolates of strain A87S (passage 6 [P6]) were almost maximally immobilized at 1 h of incubation, whereas high-passage isolates (P13) were maximally immobilized at 3 h of incubation. However, with a lower concentration of complement (15% NCS) and with a higher passage of strain A87S (between P11 and P14), the immobilization was less (varying from 2 to 28% in 10 experiments with an average of 14% ± 8%). This last condition was employed to measure the antibody-dependent immobilization of this *B. garinii* strain in symptomatic and asymptomatic dogs.

Antibody-mediated bactericidal activity

Sera from all 15 symptomatic and 11 of the 15 asymptomatic dogs were tested in an immobilization assay with representative strains of the three different *Borrelia* species (Fig. 2 and Table 3). The immobilization by antibodies was corrected for the immobilization by NCS. A CIM of >20% was considered significant. Two sera from dogs 33 and 48, which are both Bernese mountain dogs, immobilized nearly all spirochetes of strain pKo (CIM of 80 to 100%) in the assay and had reactivity against many bands of this same strain on immunoblots (Table 3). The serum of four dogs (dogs 14, 40, 58, and 72) immobilized strain A87S. Two dogs (dogs 72 and 79) had serum that immobilized *B. burgdorferi* sensu stricto strain B31. The serum of dog 72 had a CIM against A87S (60 to 80%), but not against B31, during high peak titers and symptoms. When serum was tested 2 years later during one of the intermittent episodes with symptoms, the CIM against A87S was less (20 to 40%) while a CIM against strain B31 had developed (60 to 80%). In total, sera from 7 of the 15 symptomatic dogs immobilized one or two strains and none of the 11 asymptomatic dogs had serum that immobilized one of the three strains ($P = 0.0080$ by chi-square test).

To investigate the development of bactericidal activity in relation to the development of disease, consecutive sera from symptomatic dogs were tested for bactericidal activity. Development of immobilization activity always occurred after development of symptoms and a rise in titers as measured by whole-cell ELISA (with antigens from B31). The dynamics of the antibody response in relation to the course of disease are exemplified in Fig. 3 for three dogs with differing courses of disease (Table 1). Dog 28 developed a very high titer in its serum along with symptoms in its fourth

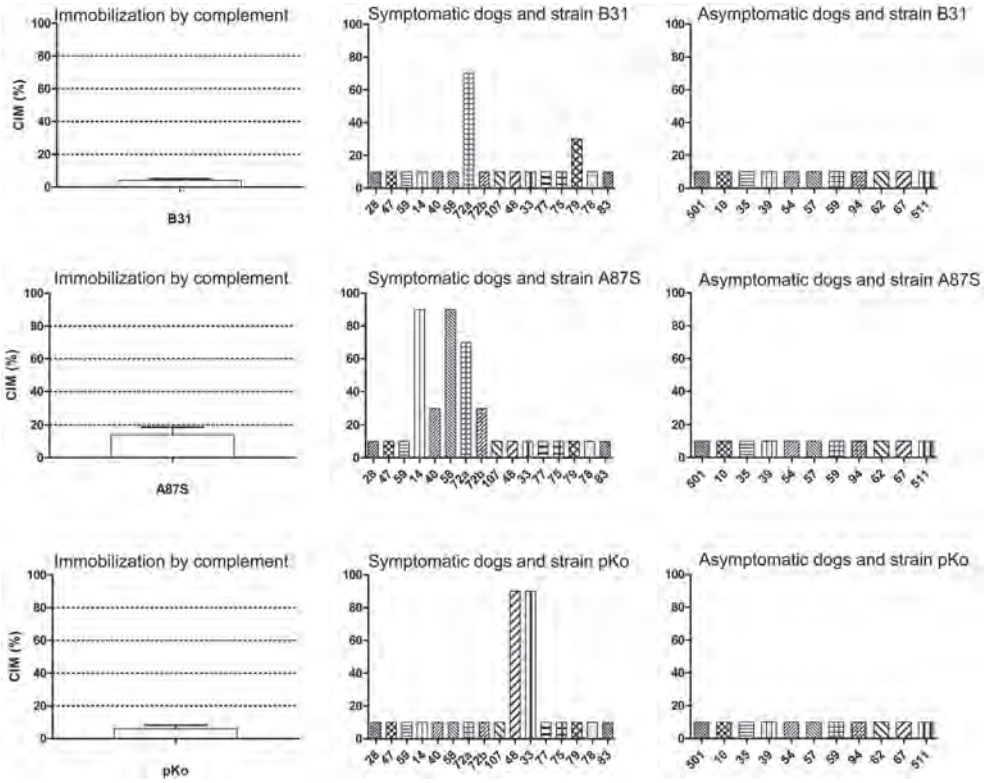

Figure 2. Antibody-dependent immobilizing activities of sera from symptomatic and asymptomatic dogs and antibody-independent immobilizing activities against three *Borrelia* strains after 5 h of incubation at 33°C. Immobilizing activities of sera from symptomatic and asymptomatic dogs were corrected for the immobilizing activity of complement, according to the formula CIM = (the percentage of immotile spirochetes in test serum and NCS minus the percentage of immotile spirochetes in NCS only)/(100% minus the percentage of immotile spirochetes in NCS only). Open bars, CIM < 20% (i.e., negative); hatched bars, CIM = 20 to 40% or 40 to 60% (i.e., positive) or 60 to 80% or 80 to 100% (i.e., strongly positive). Multiple sera from dogs in different stages of disease were tested. All dogs, except one, with a positive test result reacted against one of the strains in single or in consecutive sera. Dog 72 had immobilizing activity against strain A87S in one serum sample (72a) and against strain B31 and strain A87S in a consecutive serum sample (72b).

transmission season and completely recovered thereafter. This dog did not develop immobilization activity against any of the strains (Fig. 3A). Dog 14 had recurring symptoms, and its serum had whole-cell ELISA titers and fluctuating immobilization activity against strain A87S (Fig. 3B). Dog 33 developed a persistent immobilization activity of its persistently high-titered serum against strain pKo and had progressive symptoms (Fig. 3C).

Detection of *Borrelia* DNA in tissue biopsies

From 12 dogs tissue specimens were available. Seven out of eight symptomatic dogs and three out of four asymptomatic dogs were tested positive for *Borrelia* by PCR (Table 3). From all seven symptomatic dogs *B. burgdorferi* sensu stricto was amplified. Three of these dogs also contained *B.*

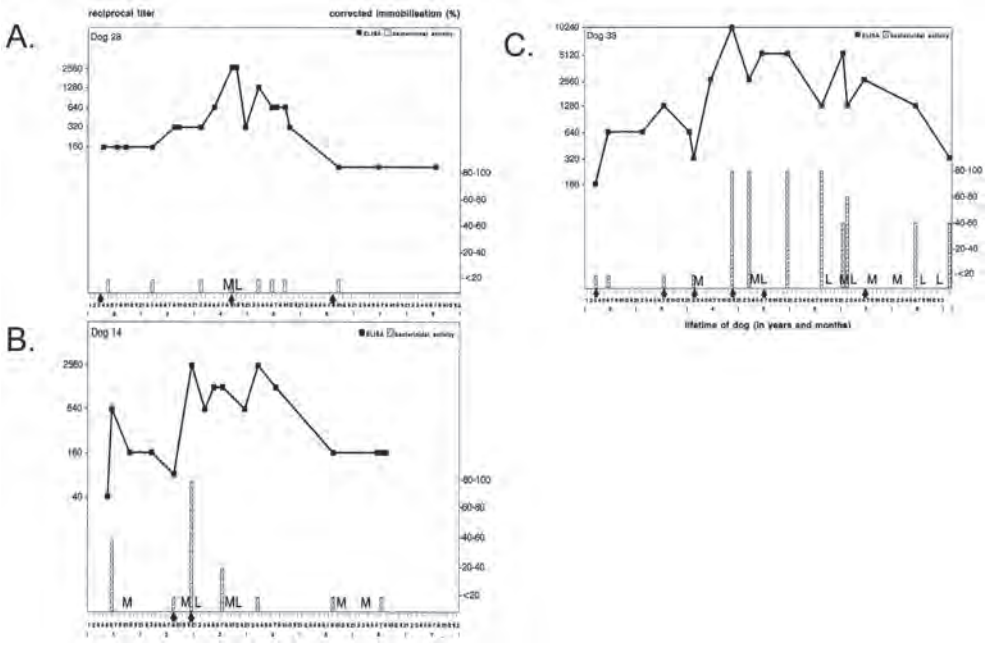

Figure 3. Whole-cell ELISA, as reciprocal titer, and antibody-dependent immobilizing activity, as CIM (in percent; presented as in Fig. 2), of consecutive sera from three dogs sampled before, during, and after disease. Symptomatic periods are represented by M (malaise) and L (lameness), and the arrows indicate when the serum used in immunoblots was obtained (see Fig. 1). (A) Dog 28. No immobilizing activity against any of the three strains was seen. This dog was one of the three dogs in this study that completely recovered from disease. (B) Dog 14. Bactericidal activity was only directed against *B. garinii* strain A87S. (C) Dog 33. Bactericidal activity was only directed against B. afzelii strain pKo.

garinii DNA, and two of these dogs also contained *B. afzelii* DNA. *B. burgdorferi* sensu stricto DNA was not amplified from any of the asymptomatic dogs. *B. garinii*, *B. afzelii*, and *B. valaisiana* were each detected once among asymptomatic dogs.

From dog 79, showing bactericidal activity against *B. burgdorferi* sensu stricto in its serum, DNA from the same species was amplified. From dogs 14 and 58, both showing bactericidal activity against *B. garinii*, *B. garinii* DNA was amplified. From the other four dogs (dogs 40, 72, 48, and 33) whose serum had bactericidal activity, it could not be confirmed whether the species against which reactivity was directed were indeed present, since no tissue specimens were available. In contrast, from six symptomatic dogs (dogs 47, 14, 58, 107, 78, and 83) *B. burgdorferi* sensu stricto DNA was amplified, but no bactericidal antibodies against the *B. burgdorferi* sensu stricto strain were detected in spite of high antibody titers by ELISA.

Discussion

Sera from pet dogs which were symptomatic or asymptomatic for borreliosis and which were monitored clinically and serologically during a 5-year period were sequentially collected. All dogs were exposed to *Ixodes ricinus* ticks and developed an antibody response in whole-cell ELISA employing *B. burgdorferi* sensu stricto antigens in contrast to a control group of dogs from an area where the disease is not endemic (21). In the present study, sera from 15 dogs symptomatic for borreliosis and sera from a comparable control group of 15 asymptomatic dogs were further analyzed by Western blot and immobilization assays.

All sera contained multiple antibodies against *Borrelia* as detected by Western blotting, whereas sera from only one out of four of the negative-control dogs showed a sole band against the 41-kDa flagellin protein. The reactivity in the sera from asymptomatic dogs was usually limited to the 41-, 39-, and, to a lesser extent, the 66- to 60-kDa regions. Therefore we regard antibodies against these proteins in dogs as markers of exposure to *B. burgdorferi* sensu lato. Sera from symptomatic dogs had a broader spectrum of reactivity, especially sera from dogs with occurrence of the first symptoms before the 8th year of life (Table 3). In preclinical sera from symptomatic dogs antibodies against p41 and p39 were already present long before the onset of disease. This is in accordance with our hypothesis that these antibodies are a consequence of exposure to spirochetes but that they are not necessarily related to clinical disease. Antibodies against the 41-kDa flagellin protein may be due to cross-reactivity of antibodies directed at the flagella of other bacteria (29, 39). However, the 39-kDa protein is *Borrelia* genus specific, and no cross-reactivity of antibodies against this protein has been demonstrated (29, 38, 41). The 66-kDa protein is in the range of the cross-reacting heat shock proteins, and three strains reacted with the serum of the positive-control dog, exclusively infected with *B. burgdorferi* sensu stricto. Therefore, this protein is probably not a recently described outer membrane protein which is species specific within the *B. burgdorferi* sensu lato group (3).

In sera from symptomatic dogs that were older than 8 years of age when the first symptoms occurred, the immune reactivity on Western blots was diminished. These dogs fulfilled the clinical entry criteria for borreliosis, and in three out of four tested by PCR, *B. burgdorferi* sensu lato DNA was detected in organ tissues (22). Moreover, they exhibited a strong rise in whole-cell ELISA titer before or during the onset of clinical manifestations, and therefore another disease as the cause of symptoms is not likely, although it is not excluded. It is known that the immune response can change during old age (36). Alternatively, frequent reinfections together with persistent infection may cause antigenic changes in the spirochete, resulting in a shift to in vivo-expressed antigens which we could not measure. Antigenic shifts may be part of the immune evasion strategies of the spirochete as determined in persistently infected laboratory mice (9).

In the acute-phase sera from young dogs (under 8 years on occurrence of the first symptoms), reactivity with seven proteins of *B. afzelii*, five proteins of *B. garinii*, and two proteins of *B.*

burgdorferi sensu stricto was associated with disease. Investigators studying European human sera found more reactions with the *B. afzelii* and *B. garinii* reference strains than with the *B. burgdorferi* sensu stricto strains (18, 19, 33). We found strong cross-reactivity of specific antigens on *B. afzelii* immunoblots with serum from the control dog infected with *B. burgdorferi* sensu stricto. Although cross-reactivity with homologous antigens of *B. afzelii* must be accounted for in the evaluation of the immunoblots (33), more probably reinfections and mixed infections with various *Borrelia* genospecies account for the discrepancy between the seroreactivities of the dogs and the PCR results showing most frequently *B. burgdorferi* sensu stricto in these dogs (Table 3) (22).

Bactericidal antibodies were found in six acute-phase sera from 10 symptomatic dogs that were younger than 8 years on occurrence of the first symptoms but in none of those from the 11 asymptomatic dogs tested. In one dog the target of these bactericidal antibodies changed over time from *B. garinii* to *B. burgdorferi* sensu stricto. In the other five dogs consecutive sera were bactericidal only against one bacterial genospecies, *B. garinii* or *B. afzelii*. It is remarkable that *B. burgdorferi* sensu stricto DNA was amplified from tissue biopsies from symptomatic dogs and not from asymptomatic dogs. This may indicate that *B. burgdorferi* sensu stricto strains are more virulent in dogs and that a protective immune response is more difficult to elicit. So far, only *B. burgdorferi* sensu stricto has been shown to be virulent in dogs in an animal model (44). Alternatively, the species that elicits bactericidal activity may be the cause of disease, as has been shown in the hamster model (27).

Three young dogs (dogs 28, 47, and 63; Table 1 and Table 3) without bactericidal activity had OspA reactivity on immunoblots using strains B31 and A87S with acute-phase and convalescent sera (Fig. 1B; dog 28). Interestingly, these three dogs recovered from disease and had low convalescent whole-cell ELISA titers (Fig. 3A; dog 28). In the mouse model the presence of anti-OspA antibodies in late disease was associated with accelerated resolution of disease (12, 42). For these three dogs the disease was suspected upon occurrence of the first symptoms and treatment was immediately initiated (Table 1), which could have facilitated recovery and may have influenced the antibody spectrum. Four of the six young dogs with bactericidal activity had bactericidal antibodies against *B. garinii* in their serum. Three of these had a preferential reactivity against *B. garinii*-specific OspC, which is in line with studies that report that this protein is capable of inducing the production of highly specific borreliacidal antibodies shortly after natural infection (6). Two of these dogs were investigated for the presence of spirochetal DNA in their tissues, and both were found to be infected with *B. burgdorferi* sensu stricto and *B. garinii*. This may be in line with the hypothesis that a heterogeneous population of spirochetes is delivered to the host, which would result in changes of the immune response over time enabling the infection to persist (14). Two other young dogs, which were both Bernese mountain dogs, developed a marked and persistent bactericidal antibody response against strain pKo (*B. afzelii*) as well as a preferential response against this strain, including a response against OspC, as detected with immunoblots. Subsequently

to the development of borreliacidal antibodies both Bernese mountain dogs developed a lifetime progressive disease, apparently not prevented by these antibodies.

Downregulation of antigens recognized by bactericidal antibodies and coinfection with a different strain not recognized by bactericidal antibodies could both be involved in the persistence of infection. Alternatively, persistent and recurring symptoms could be caused by autoreactive antibodies. Probably, there are different mechanisms for disease, which may have different outcomes depending on the host immune system idiosyncrasies.

In conclusion, although both naturally exposed and infected dogs have moderately titered to high-titered antibodies as measured by whole-cell ELISA, symptomatic dogs produce a much wider spectrum of antibodies, including immobilizing antibodies. Western blots especially may be helpful in confirming the diagnosis of canine borreliosis.

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Coinfection with *Borrelia burgdorferi* sensu stricto and *Borrelia garinii* alters the course of murine Lyme borreliosis

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Chapter 8

Abstract

Ixodes ricinus ticks and mice can be infected with both *Borrelia burgdorferi* sensu stricto and *Borrelia garinii*. The effect of coinfection with these two *Borrelia* species on the development of murine Lyme borreliosis is unknown. Therefore, we investigated whether coinfection with the nonarthritogenic *B. garinii* strain PBi and the arthritogenic *B. burgdorferi* sensu stricto strain B31 alters murine Lyme borreliosis. Mice simultaneously infected with PBi and B31 showed significantly more paw swelling and arthritis, long-standing spirochetemia, and significantly higher numbers of B31 spirochetes than did mice infected with B31 alone. However, the number of PBi spirochetes was significantly lower in coinfected mice than in mice infected with PBi alone. In conclusion, simultaneous infection with *B. garinii* and *B. burgdorferi* sensu stricto results in more severe Lyme borreliosis. Moreover, we suggest that competition of the two *Borrelia* species within the reservoir host could have led to preferential maintenance, and a rising prevalence, of *B. burgdorferi* sensu stricto in European *I. ricinus* populations.

Introduction

Lyme borreliosis is a common tick-borne zoonosis in many parts of Asia, Europe and the United States. A main clinical feature of early human Lyme borreliosis is an expanding red cutaneous lesion called erythema migrans. Later manifestations of Lyme borreliosis include cardiac conduction system delays, arthritis, neurologic symptoms, and cutaneous manifestations, such as acrodermatitis chronica atrophicans (ACA) (Steere, 2001; Steere et al., 2004). Joint disease has been associated with *Borrelia burgdorferi* sensu stricto infection, while neuroborreliosis and ACA are associated with *Borrelia garinii* and *Borrelia afzelii* infection, respectively (van Dam et al., 1993; Busch et al., 1996). *Borrelia burgdorferi* sensu stricto is prevalent in the United States, whereas all three pathogenic *Borrelia* species are encountered in Europe. The European vector for Lyme borreliosis, *Ixodes ricinus*, can be simultaneously infected with more than one *Borrelia* species (Rijpkema et al., 1995; Hovius et al., 1998; Misonne et al., 1998; Rauter et al., 2002). Depending on the area, up to 45% of infected ticks harbour more than one *Borrelia* species and, in a study by Misonne and colleagues, the majority of coinfected ticks were simultaneously infected with *B. burgdorferi* sensu stricto and *B. garinii* (Misonne et al., 1998).

The development of Lyme borreliosis is dependent on many factors, including pathogen burden (Yang et al., 1994), spirochetal virulence factors (Hughes et al., 1993; Carroll et al., 1996; Sellek et al., 2002; Yang et al., 2004), *Borrelia*-arthropod-interactions (Ramamoorthi et al., 2005), and host innate and adaptive immune responses (Matyniak and Reiner, 1995; Kang et al., 1997; Gross et al., 1998; Hirschfeld et al., 1999; Potter et al., 2000; McKisic and Barthold, 2000a; McKisic et al., 2000b; Anguita et al., 2002; Wooten et al., 2002; Wang et al., 2004; Guerau-de-Arellano et al., 2005). Moreover, coinfection of *B. burgdorferi* sensu stricto with other tick-borne pathogens, such as *Anaplasma phagocytophilum* (Thomas et al., 2001), enhances the severity of murine Lyme arthritis and increases *Borrelia* burden. The effect of coinfection of *B. burgdorferi* sensu stricto with another species from the *B. burgdorferi* sensu lato group on the outcome of murine Lyme borreliosis has not been investigated. However, in Europe the simultaneous presence of multiple *Borrelia* genospecies has been observed in both human and canine Lyme borreliosis cases (Demaerschalck et al., 1995; Hovius et al., 1999; Ruzic-Sabljic et al., 2005). In a study by Demaerschalck and collaborators, *B. burgdorferi* sensu stricto and *B. garinii* DNA was simultaneously detected in six out of 18 individuals with neuroborreliosis (Demaerschalck et al., 1995). The course of disease in mice, with the development of arthritis and carditis, partially mimics human disease (Barthold et al., 1990). In addition, infection with *B. burgdorferi* sensu stricto (strain B31), but not with *B. garinii* (strain PBi), readily causes arthritis and carditis in mice (Barthold, 1999). Here we assessed the role of coinfection with *B. burgdorferi* sensu stricto and *B. garinii* in the development of murine Lyme borreliosis.

Materials and methods

Mice, spirochetes and infection

C3H/HeJ and C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained in filterframed cages. Six-week-old mice were infected by intradermal syringe inoculation with *B. burgdorferi* sensu stricto strain B31 clone 5A11 (Purser and Norris, 2000) and/or *B. garinii* strain PBi (Barthold, 1999). Spirochetes were cultured at 33 ºC in complete Barbour-Stoenner-Kelly (BSK)-H medium (Sigma-Aldrich, Saint Louis, MO) and grown to the stationary phase (Barthold et al., 1993). Mice were inoculated by a single injection in the midline of the back according to established protocols with 1 x 10^5 spirochetes of strain B31 or PBi alone, with 1 x 10^5 of each strain simultaneously, with 1 x 10^5 PBi spirochetes followed by 1 x 10^5 B31 spirochetes 4 days later, or with BSK-H medium as a control (Fikrig et al., 1992). Spirochetes were enumerated using a Petroff-Hauser bacterial counting chamber (Barthold et al., 1999). In a small control experiment we did not find a difference in pathogen burden, paw swelling, arthritis or immune responses when mice were simultaneously infected with 5 x 10^4 instead of 1 x 10^5 spirochetes of each strain (data not shown). Mice were euthanized by CO_2 inhalation followed by cardiac exsanguination. Sera were stored at -20 °C for future use. Skin (inoculation site), urinary bladder, spleen, spine, heart and tibiotarsi were saved for histopathological examination, species-specific quantitative PCR (q-PCR) or culture. We used C3H/HeJ mice to assess the effect of coinfection on the severity of Lyme borreliosis. Mice were therefore sacrificed 3 weeks postinfection, i.e. at the expected peak of arthritis. In C57BL/6 mice, arthritis usually peaks 4 weeks postinfection (Yang et al., 1994; Ma et al., 1998; Potter et al., 2000), and this strain is relatively more resistant to Lyme disease. C57BL/6 mice were sacrificed either at 2 weeks postinfection, to assess whether simultaneous or sequential infection with PBi and B31 altered the initial development of Lyme borreliosis, or late in the course of infection, i.e. at 6 weeks postinfection, to determine whether coinfection of PBi and B31 prolonged the manifestations of Lyme borreliosis.

Culture

Fifty microliter of blood and c. 20 mg of spleen, bladder and spinal cord were cultured in BSK-H medium containing rifampicin (50 mg/mL), amphotericin (2.5 mg/mL) and phosphomycin (2 mg/mL) (Sigma-Aldrich). Cultures were checked weekly for the presence of viable spirochetes using dark-field microscopy, as previously described (Barbour, 1984). The presence of at least one viable spirochete in 10 microscope fields indicated a positive culture.

Quantitative PCR

DNA was extracted from tissues using a DNeasy tissue kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Real-time PCR for B31 *ospC*, PBi *dbpB*, and mouse *ß-actin* was performed with the primers listed in Table 1. The *ospC* and *dbpB* primers were newly designed based on the sequence of *B. burgdorferi* B31 *ospC* and *B. garinii dbpB* and are species-specific: the *ospC* primers amplify from B31 but not from PBi DNA, whereas the *dbpB* primers amplify from

Table 1. Primers (and probes) used for real-time PCR.

PCR target	Forward 5'- 3'	Reverse 5'- 3'	Taqman probe 5'- 3'
B31 *ospC*	ATACCGAAAATAAT CACAATGGA	CTGAATTAGCAAG CATCTCTTTAG	
PBi *dbpB*	TGCTG GCAGCCTG TTAATTTG	TTAGCTTCCTCTG AAATGGAGCTT	
mouse *β-actin*	GGGACCTGACAGA CTACCTC	AAGAAGGAAGGC TGGAAAAG	
B. burg flaB	TTGCTGATCAAGC TCAATATAACCA	TTGAGACCCTGAA AGTGATGC	CAGCTGAAGAGCT TGGAATGCAGCCT

PBi but not from B31 DNA. Each 50-mL real-time PCR reaction contained a 1-mL DNA sample, 0.3 mL of each 50-mM primer, 23.4 mL of H2O, and 25 mL of Hot Star SYBR Green I PCR Master Mix (BioRadlaboratories, Richmond, CA). PCR was performed with an initial denaturing step of 5 min at 95 °C, followed by 50 cycles of three-step amplification consisting of 30 s at 95 °C, 30 s at 56 °C (ospC) or 60°C (*dbpB* and *β-actin*), and 45 s at 72 °C. Standards were produced by cloning B31 *ospC* (397 bp), PBi *dbpB* (376 bp) or mouse *β-actin* (252 bp) PCR products into the vector pCR2.1-TOPO (Invitrogen, Carlsbad, CA). Amplification of test samples and 10-fold dilutions of gene-specific standards were visualized and quantified using the software ICYCLER provided with the thermocycler (iCycler iQ real-time PCR detection system; Biorad Laboratories). To validate PCR products we assessed the melting temperature of the amplified fragment or performed electrophoresis on agarose gels. Similar PCR methods were used by others (Li et al., 2006). Copy numbers of B31 *ospC* and PBi *dbpB* were normalized to the copy number of *β-actin*. All measurements were performed in duplicate. In one separate experiment, *flaB* primers and a Taqman probe were used to amplify B31 DNA (Ramamoorthi et al., 2005). We did not use these primers in experiments in which we coinfected mice with both B31 and PBi: because *flaB* primers amplify from B31 as well as PBi DNA it would have been impossible to distinguish between B31 and PBi spirochete levels in coinfected animals.

Paw swelling, arthritis and carditis

Inflammation of tibiotarsi was evaluated by assessing the degree of oedematous swelling of the tibiotarsi. Visual inflammation scores were assessed and scored from 0 to 3, with 0 being no swelling and 3 maximal oedematous swelling (Barthold et al., 1990). Joints were scored by the same independent observer blinded to the experimental design and several times throughout the course of the infection. In addition, right tibiotarsi were fixed with formalin, embedded in paraffin, haematoxylin- and eosin-stained, and examined microscopically. Arthritis was assessed as described previously (Fikrig et al., 1992). Disease severity was scored as 0 (no disease), 1 (mild disease), 2 (moderate disease), or 3 (severe disease). Mild disease consisted of neutrophil infiltration. Moderate disease was marked by neutrophil infiltration and at least some evidence of fibrin exudation. Severe disease was marked by neutrophil infiltration with fibrin exudation and synovial hypertrophy or hyperplasia. All measurements were made in a blinded fashion. Half of

the sagitally dissected hearts were processed and stained with haematoxylin and eosin by routine histological techniques. Carditis was considered active when acute inflammatory cell infiltrates were seen in heart base tissues (Armstrong et al., 1992).

IgG enzyme-linked immunosorbent assay and cytokine analysis

Borrelia burgdorferi sensu stricto (strain B31) lysates were prepared from large quantities of spirochetes cultured for 14 days. Spirochetes were pelleted at 13 000 x *g*, followed by three washes with phosphate-buffered saline (PBS). The pellet was resuspended in 500 mL of H_2O and disrupted by sonication with five 10-s pulses. Insoluble material was pelleted at 16 000 x *g* for 1 min. The supernatant was collected and used for enzyme-linked immunosorbent assay (ELISA). *Borrelia burgdorferi* sensu stricto strain B31-specific total IgG and IgG subclasses were measured in sera from infected animals and controls by ELISA (Anguita et al., 1996; Thomas et al., 2001). *Borrelia burgdorferi* B31 lysate (50 ng) in 100 µL of coating buffer (0.1M sodium bicarbonate, pH 9.6) was added to 96-well ELISA plates (ICN Biochemicals Inc., Costa Mesa, CA) and kept overnight at 4 °C. Plates were washed three times with wash buffer (PBS with 0.05% Tween 20) and blocked with 200 µL of blocking buffer (10% fetal calf serum in PBS) for 2 h at room temperature. Blocking buffer was removed and 50 µL of sera (diluted 1: 100) was incubated for 1 h at room temperature. The plates were washed five times with wash buffer, followed by the addition of biotinylated IgG-, IgG1-, or IgG2a-specific antibodies (Pharmingen, San Diego, CA) at a 1: 2000 dilution in blocking buffer. Plates were then incubated at room temperature for 1 h and washed eight times with wash buffer. Streptavidin-conjugated horseradish peroxidase was then added to the wells and left for 45 min, followed by eight washes with wash buffer. The plates were tapped to remove excess solution, 100 µL of tetramethylbenzidine (TMB) solution (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added, and the reaction was stopped with 100 µL of TMB stop solution. Optical density (OD) was read at 450 nm. SHAM inoculated mouse serum was used as a negative control. Several pro-inflammatory and Th1/Th2 cytokines, namely IFN-γ, TNF-α, IL-10, IL-6, IL-12, MCP-1, IL-2, IL- 4 and IL-5, were measured in serum using cytometric bead array kits (CBA; BD Biosciences) according to the manufacturer's recommendations. Measurement was performed using a fluorescence activated cell sorting (FACS) flow cytometer (BD Biosciences) and data were acquired and analysed using the CBA software (BD Biosciences). All measurements were performed in duplicate.

Statistical analysis

Differences in spirochete levels between the groups were analysed using the two-sided Mann Whitney test. For other parameters, differences between groups were analysed by two-sided one-way ANOVA, implementing a one-way ANOVA with Tukey's multiple comparison test using GRAPHPAD PRISM software (GRAPHPAD PRISM Software version 4.0, San Diego, CA). For comparison of the number of bloodculture-positive mice between the various groups, a two-sided chi-square was applied. A P value of ≤ 0.05 was considered significant.

Results

Borrelia burgdorferi sensu stricto and *B. garinii* burden

All C3H/HeJ and C57BL/6 mice infected with B31 alone, or simultaneously or sequentially with PBi and B31 were culture-positive. Three weeks postinfection, 4/6 C3H/HeJ mice infected with PBi alone had at least one positive tissue culture, and 2 weeks postinfection all (6/6) C57BL/6 mice infected with PBi alone had at least one positive tissue culture.

Next, we assessed whether spirochetes were also present in the blood of the mice. Two weeks postinfection, 6/8 simultaneously infected C57BL/6 mice had positive blood cultures, whereas only 1/5 of mice infected with B31 alone and 0/6 of mice infected with PBi had positive blood cultures (Table 2). In 2/4 simultaneously infected C3H/HeJ mice blood cultures were positive, whereas none of the mice infected with either B31 or PBi alone had positive blood cultures (Table 2). In a separate experiment, mice syringe inoculated with 1 x 10^5 B31 alone had detectable spirochetes in their blood from day 4 until day 8 postinfection, as detected by culture (Fig. 1a). This indicates that, under normal circumstances, B31 spirochetes are only detectable in the blood early in infection. The time-points at which blood cultures were positive correlated with peak spirochete numbers as detected by q-PCR (Fig. 1b).

We also assessed spirochete burden in several organ tissues by q-PCR. Three weeks postinfection, C3H/HeJ mice simultaneously infected with PBi and B31 had higher levels of B31 spirochetes in several tissues than mice infected with B31 alone, as detected by B31-specific q-PCR (Fig. 2a). The difference was significant in bladder and skin ($P < 0.05$, Mann Whitney test). In C57BL/6 mice 2 weeks postinfection, B31 spirochete levels were higher in mice simultaneously infected with PBi and B31 in all tissues examined as compared with mice infected with B31 spirochetes alone (Fig. 2b). The differences were statistically significant for bladder and heart tissue ($P < 0.05$, Mann

Table 2. Blood cultures of mice infected with *Borrelia burgdorferi* B31, *Borrelia garinii* PBi, or both *Borrelia* strains simultaneously.

	Bloodcultures C3H/HeJ Pos/Tot	C57BL/6 Pos/Tot	Total Pos (%)
PBi	0/6	0/6	0%
B31	0/5	1/5	10%
Sim	2/4	6/8	67%*

C3H/HeJ and C57BL/6 mice were sacrificed three and two weeks after syringe inoculation, respectively. Mice were inoculated with PBi (PBi) or B31 (B31) alone, or simultaneously with PBi and B31 (Sim). 50 µL of blood was cultured in BSK-H medium containing antibiotics. Cultures were checked weekly for one month for viable and motile spirochetes using dark-field microscopy. The cumulative number of positive blood cultures in C3H/HeJ and C57BL/6 mice simultaneously infected with PBi and B31 was compared with the number of positive blood cultures in mice infected with B31 alone. Statistical analysis was performed using a two-sided chi-square test. * A *P*-value < 0.05 was considered significant

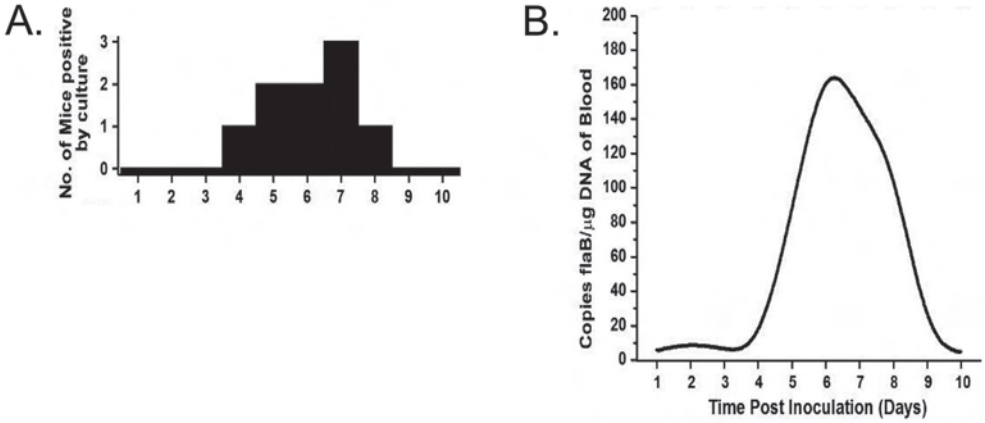

Figure 1. Spirochetes in blood of mice challenged with *Borrelia burgdorferi* B31. C3H/HeJ mice were syringe-inoculated with 1 x 10^5 *B. burgdorferi* sensu stricto strain B31. Three mice were sacrificed daily and blood was checked for the presence of B31 spirochetes by culture in BSK-H medium (a) and q-PCR (b). B31 was amplified using primers directed against strain B31 *flaB* and a B31 *flaB*-specific Taqman probe. Numbers are expressed as average numbers per µgram of DNA of mouse blood (b).

Whitney test). The same trend was observed in C3H/HeJ and C57BL/6 mice sequentially infected with PBi and B31 (data not shown). We observed a similar trend in other tissues examined, such as spleen and spine (data not shown). It should be noted that, in the spine, spirochetes were present in the surrounding structures such as vertebrae, muscles and ligaments, rather than in the spinal cord (data not shown).

In contrast, the levels of PBi spirochetes in several tissues from C3H/HeJ and C57BL/6 mice infected simultaneously with PBi and B31 were significantly lower compared with levels in mice infected with PBi alone (Fig. 2c and d; $P < 0.05$, Mann Whitney test). In contrast to simultaneously infected mice, sequentially infected mice had relatively high PBi levels in skin and bladder tissue (data not shown), indicating that the outcome of the competition between *B. burgdorferi* sensu stricto strain B31 and *B. garinii* strain PBi in this experimental setting is dependent on the timing of inoculation.

Paw swelling and arthritis

During the course of infection we evaluated swelling of the hind paws as a parameter for disease. As expected, C3H/HeJ and C57BL/6 mice infected with B31 spirochetes developed significant joint swelling (Fig. 3a and b). At early timepoints, i.e. 2 or 3 weeks postinfection, swelling was more pronounced in mice simultaneously, and also sequentially, infected with PBi and B31 as compared with mice infected with either PBi or B31 alone (Fig. 3a and b). At later timepoints, i.e. 6 weeks postinfection, swelling was more severe in simultaneously infected C57BL/6 mice than in sequentially infected animals. C3H/HeJ and C57BL/6 mice infected with PBi spirochetes alone did not develop, or only developed minor, swelling (Fig. 3a and b).We did not infect mice with B31 followed by PBi spirochetes. We speculate that this type of sequential infection does not influence

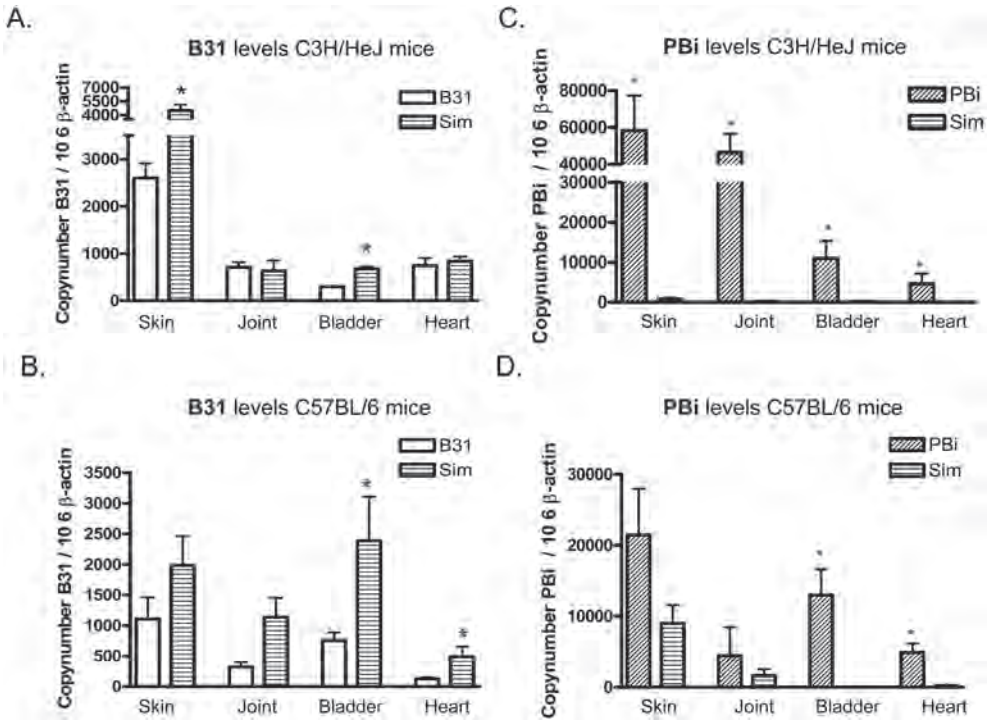

Figure 2. Pathogen burden in mice infected with *Borrelia burgdorferi* B31, *Borrelia garinii* PBi, or both *Borrelia* strains. C3H/HeJ (a and c) or C57BL/6 (b and d) mice were syringe-inoculated with 1 x 10^5 spirochetes per strain. Mice were infected with PBi (PBi), B31 (B31) or B31 and PBi simultaneously (Sim). Five to six mice per group were used. C3H/HeJ (a and c) mice were sacrificed three and C57BL/6 (b and d) mice 2 weeks postinfection. Specific primers targetted against B31 *ospC* (a and b) or PBi *dbpB* (c and d) were used to amplify either B31 or PBi DNA in combination with Sybr-Green I dye to quantify spirochete numbers. To standardize for the amount of tissue, mouse *β-actin* copies in each sample were determined. Experiments were performed in duplicate. Error bars represent SEs of the mean. Spirochete levels in mice infected simultaneously with B31 and PBi were compared with spirochete levels in mice infected with B31 or PBi alone with the two-sided Mann Whitney test (a P value < 0.05 was considered significant).

the outcome of Lyme borreliosis symptoms, because the host immune response against PBi evoked in the presence of B31, at least in simultaneous infection of these two *Borrelia* species, readily kills PBi. In the mouse model, B31 is arthritogenic and PBi is not.

In order to assess whether swelling could be correlated with disease we investigated haematoxylin- and eosin-stained sections of tibiotarsal joints microscopically. At three weeks postinfection, arthritis was significantly more severe in C3H/HeJ mice infected simultaneously with PBi and B31 than in mice infected with B31 alone (Fig. 4a; $P < 0.05$, one-way ANOVA with Tukey's multiple comparison test). At 6 weeks postinfection there was a trend towards more severe arthritis in C57BL/6 mice simultaneously infected with B31 and PBi as compared with mice infected with B31 alone, albeit not statistically significant (Fig. 4b; $P > 0.05$, one-way ANOVA with Tukey's multiple comparison test). Two weeks postinfection there was no arthritis in any group of infected C57BL/6

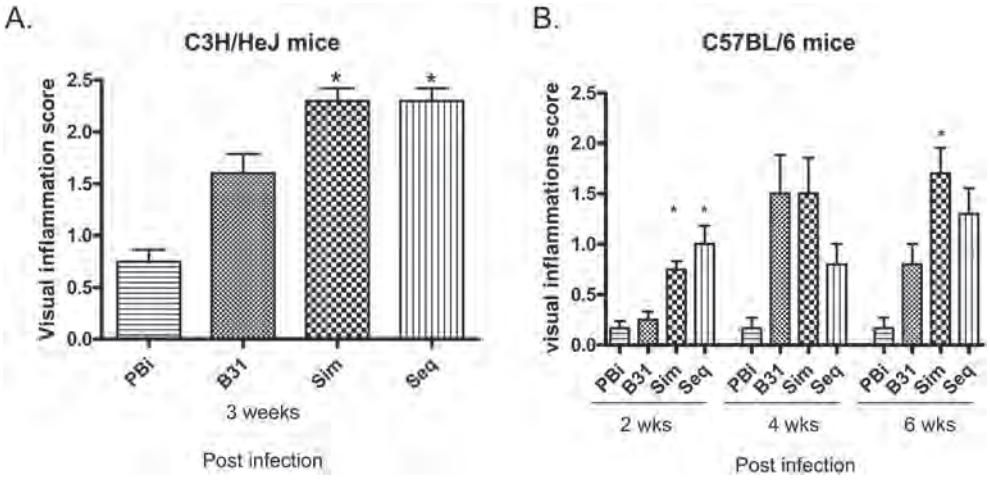

Figure 3. Paw swelling in mice infected with *Borrelia burgdorferi* B31, *Borrelia garinii* PBi, or both *Borrelia* strains. C3H/HeJ (a) and C57BL/6 (b, c and d) mice were syringe-inoculated with 1 x 10^5 spirochetes per strain. Mice were infected with PBi (PBi), B31 (B31), PBi and B31 simultaneously (Sim), or PBi followed by B31 4 days later (Seq). For C3H/HeJ mice visual inflammation of tibiotarsal joints, i.e. the degree of oedematous swelling, was assessed 3 weeks postinfection (a). For C57BL/6 mice tibiotarsal swelling was assessed 2, 4 and 6 weeks postinfection. Scores ranged from 0 to 3, with 0 denoting no swelling; 1, mild swelling; 2, moderate swelling; and 3, severe swelling. Scores were assessed in a blinded fashion by the same observer throughout the experiment. Five to six mice per group were used, and the data represent two independent experiments. Error bars represent SDs. Visual inflammation scores from mice infected simultaneously or sequentially with PBi and B31 were compared with inflammation in mice infected with B31 alone. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison test (a P value < 0.05 was considered significant).

mice (data not shown). In both C3H/HeJ and C57BL/6 mice, sequential infection with PBi and B31 did not result in more severe arthritis (Fig. 4a and b). Carditis was evident in C3H/HeJ mice infected with B31 (4/4), and in those simultaneously (3/4) or sequentially (3/5) infected with PBi and B31, but not in mice infected with PBi alone (0/6).

Immune responses

In search of an explanation for our findings we determined total IgG antibodies directed against *B. burgdorferi* sensu stricto strain B31 in mouse sera. The results were similar for all groups of C3H/HeJ and C57BL/6 mice (Table 3). However, in C57BL/6 mice simultaneously infected with PBi and B31, OD values of IgG1 directed against B31 were increased and OD values of IgG2a were decreased, as compared with mice infected with B31 alone (Table 3). Antibodies from PBi-infected animals also cross-reacted with B31 antigens (Table 3). Besides determining antibody responses we determined systemic cytokine responses, but we found no relevant differences in serum cytokine profiles between the various groups of mice (data not shown). Despite very sensitive detection methods (CBA), TNF-α, IL-4, IL-5, IL-6, IL-10 and IL-12 were below detection limits for all groups.

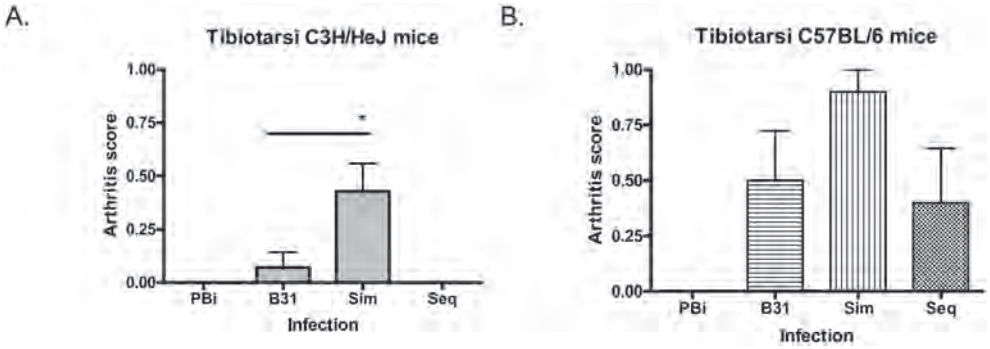

Figure 4. Arthritis in mice infected with *Borrelia burgdorferi* B31, *Borrelia garinii* PBi, or both *Borrelia* strains. C3H/HeJ (a) and C57BL/6 (b) mice were syringe-inoculated with 1 x 10^5 spirochetes per strain. Mice were infected with PBi (PBi), B31 (B31), PBi and B31 simultaneously (Sim), or PBi followed by B31 four days later (Seq). Five to six mice per group were used. Haematoxylin- and eosin-stained sections from tibiotarsi from C3H/HeJ mice 3 weeks postinfection and C57BL/6 mice 6 weeks postinfection were examined microscopically for disease. Disease severity was scored on a scale of 0-3 : 0, no disease; 1, mild disease; 2, moderate disease; and 3, severe disease. Error bars represent SDs. Arthritis from mice infected simultaneously or sequentially with PBi and B31 was compared with arthritis in mice infected with B31 alone. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison test (a P value < 0.05 was considered significant).

Discussion

The European Lyme borreliosis vector, *I. ricinus*, can transmit more than one *Borrelia* genospecies through a single tick bite. Misonne and colleagues found that the majority of coinfected ticks were simultaneously infected with *B. burgdorferi* sensu stricto and *B. garinii* (Misonne et al., 1998). Demaerschalck and collaborators showed that 33% of patients with neuroborreliosis were infected with both *B. burgdorferi* sensu stricto and *B. garinii* (Demaerschalck et al., 1995). Despite the frequent simultaneous occurrence of these two *Borrelia* species in ticks and human Lyme borreliosis cases, the role of coinfection with *B. garinii* and *B. burgdorferi* sensu stricto in the pathogenesis of Lyme borreliosis has never been investigated in an experimental setting.

In mice simultaneously infected with PBi and B31, there is long-lasting spirochetemia (Table 2). Interestingly, PCR analysis of in vitro positive blood cultures showed that, in simultaneously infected mice, the species identified was *B. burgdorferi* sensu stricto strain B31 (data not shown). This is unlikely to be the result of the preferential growth of B31 over PBi in vitro, because, when equal numbers, i.e. 1 x 10^3 spirochetes, of B31 and PBi were simultaneously cultured in BSK-H medium for two weeks, both strains grew to a density of c. 1 x 10^7 spirochetes as detected by q-PCR (data not shown). We have shown that in (C3H/HeJ) mice infected with B31 alone, spirochetes are only detectable in the blood during the initial stages of infection (Fig. 1a). However, in C57BL/6 mice simultaneously infected with PBi and B31, but not in mice infected with B31 alone, B31 spirochetes could be readily detected in the blood at 2 weeks postinfection (Table 2). Despite

Table 3. IgG antibodies against *Borrelia burgdorferi* sensu stricto strain B31 in C3H/HeJ and C57BL/6 mice.

C3H/HeJ	SHAM		B31		Sim		Seq		PBi	
	Mean	*SD*	Mean	*SD*	Mean	*SD*	Mean	*SD*	Mean	*SD*
total IgG	0,15	*0,02*	0,38	*0,04*	0,37	*0,04*	0,40	*0,04*	0,33	*0,03*
IgG1	0,08	*0,00*	0,19	*0,05*	0,15	*0,03*	0,17	*0,02*	0,13	*0,01*
IgG2a	0,06	*0,00*	0,17	*0,03*	0,15	*0,04*	0,15	*0,03*	0,12	*0,01*
C57BL/6										
total IgG	0,07	*0,01*	0,42	*0,04*	0,39	*0,03*	0,38	*0,02*	0,30	*0,02*
IgG1	0,07	*0,00*	0,18	*0,01*	0,24*	*0,06*	0,19	*0,03*	0,13	*0,02*
IgG2a	0,07	*0,00*	0,30	*0,03*	0,23 *	*0,05*	0,20 *	*0,02*	0,14	*0,03*

Mice were infected with BSK-H-medium (SHAM), strain B31 (B31), strain PBi (PBi), strain PBi and B31 simultaneously (Sim), and strain PBi followed by strain B31 four days later (Seq). OD values of total IgG, IgG1 and IgG2a directed against B31 were determined in sera three (C3H/HeJ) or six (C57BL/6) weeks post-infection by enzyme-linked immunosorbent assay using whole B31 lysate as an antigen. Sera were diluted 1: 100 and OD was read at 450 nm. Antibody responses in mice infected simultaneously or sequentially with PBi and B31 were compared with antibody responses in mice infected with B31 alone. Statistical analysis was performed using one-way analysis of variance with Tukey's multiple comparison test. *A P value < 0.05 was considered significant.

the fact that C3H/HeJ mice infected simultaneously with PBi and B31 were sacrificed 3 weeks postinfection, we could still detect spirochetes in the blood (Table 2).

Coinfection of PBi and B31 results in higher B31 spirochete levels in several tissues of C3H/HeJ and C57BL/6 mice (Fig. 2). In contrast, PBi levels were significantly lower in mice coinfected with PBi and B31 than in mice infected with PBi alone. Thus, there appears to be competition of the two strains within the murine host, resulting in higher B31 spirochete levels and diminished PBi spirochete levels. It has previously been shown that nymphal *I. ricinus* ticks fed upon mice naturally infected with *B. garinii* and *B. burgdorferi* sensu stricto preferentially take up *B. burgdorferi* sensu stricto (Kurtenbach et al., 1998). Originally, this was explained by differential complement sensitivity of the two *Borrelia* species (Kurtenbach et al., 1998). However, competition between two complement-resistant *Borrelia* strains within the host, as in our study, could be an additional explanation for this phenomenon. This competition and preferential uptake of one *Borrelia* species over the other by ticks feeding on coinfected mice could contribute to the preferential maintenance of *B. burgdorferi* sensu stricto in European *I. ricinus* populations. This would increase the risk of human infection with this *Borrelia* species over time. Genomic analysis by Baranton and collaborators suggests that *B. burgdorferi* sensu stricto might have originated in the United States of America and migrated to Europe in the post-Columbian era (Marti et al., 1997; Farlow et al., 2002). Thus, sporadic introductions of *B. burgdorferi* sensu stricto into the 'Old World' are expected, and it is not remarkable that the prevalence of *B. burgdorferi* sensu stricto in Europe varies from 0% (Junttila et al., 1999) to c. 20% (Kirstein et al., 1997) of infected questing ticks. Furthermore, we suggest that local high prevalences of *B. burgdorferi* sensu stricto in Europe might be explained by competition of *B. burgdorferi* sensu stricto with other *Borrelia* species within the reservoir host followed by preferential uptake of *B. burgdorferi* sensu stricto by *I. ricinus* ticks. The

different strains of *B. burgdorferi* sensu stricto, *Borrelia afzelii* and *B. garinii* that exist in Europe and the timing of the coinfection may influence the final outcome of this competition.

Simultaneous infection with PBi and B31 spirochetes also alters systemic immune responses. C57BL/6 mice show higher OD values of IgG1 and lower OD values of IgG2a levels directed against B31 6 weeks after simultaneous infection with B31 and PBi, as compared with mice infected with B31 spirochetes alone (Table 3). However, the altered IgG responses do not seem to be important for the differences in disease severity between the different groups of infected mice, because no difference in IgG1 and IgG2a responses was observed in C3H/HeJ mice (Table 3). We could not find relevant differences in levels of several cytokines in sera from the different groups of infected mice (data not shown). It is noteworthy that MCP-1 levels in sera from C3H/HeJ mice simultaneously infected with PBi and B31 (74.4 pg/mL ± 16.9) were significantly higher than MCP-1 levels in mice infected with B31 alone (31.4 pg/ml ± 9.2). However, because PBi-infected mice that did not develop arthritis or carditis had similar MCP-1 levels (58.6 pg/mL ± 9.6) as compared with coinfected mice, we could not associate increased MCP-1 levels with arthritis or carditis. In contrast, Guerau-de-Arellano and collaborators have shown that MCP-1 production by dendritic cells following *B. burgdorferi* sensu stricto stimulation is likely to contribute to the development of Lyme carditis (Guerau-de-Arellano et al., 2005).

Thus, the exact mechanism of the competition between the two *Borrelia* species remains unknown. We show that in single infection, PBi and B31 have, at least in part, tropisms for similar tissues, i.e. skin, bladder, joints and heart tissue. However, when injected simultaneously, B31 dominates PBi resulting in higher B31 spirochete levels and in lower PBi spirochete levels. The competition of the two *Borrelia* species is unlikely to be caused by competition for host factors, such as receptors on synovial tissue such as decorin, fibronectin, or integrins, because these are abundantly present in the host (Coburn et al., 2002). However, the relative affinity for these receptors might differ between *Borrelia* species, but, to our knowledge, this is currently unknown (Heikkila et al., 2002). The competition might also be driven by the immune response of the host. We focussed on the adaptive host immune response and were unable to find relevant differences in systemic cytokine production and IgG antibody production. Perhaps a more local approach, i.e. restimulating local lymphocytes with *Borrelia* antigen, might have revealed differences in cytokine responses between the experimental groups. Alternatively, altered innate immune responses in coinfected animals might have been the cause of the competition between the two *Borrelia* species. An adequate innate immune response is essential for clearance of *B. burgdorferi* (Wang et al., 2004). In mice coinfected with PBi and B31, innate immune responses might be mostly triggered by, and directed at, PBi, resulting in clearance of PBi, and to a lesser extent at B31, allowing for higher B31 spirochete levels. Thus, PBi would serve as a decoy for B31. Another explanation could be differences in resistance to complement-mediated killing (Brooks et al., 2005). Although both *Borrelia* strains are known to be partially serum-resistant, coinfection could have changed complement activation in a way that favours the persistence of the more complement-resistant *B. burgdorferi* strain B31 (Breitner-

Ruddock et al., 1997; van Dam et al., 1997). Finally, Liang and collaborators have shown that *B. burgdorferi* changes its surface antigenic expression in response to host immune responses (Liang et al., 2004). It is possible that, in our studies, the altered immune response in coinfected mice changed the expression of proteins important for early infection of, and persistence in, the host, for example OspC, dbpA and VlsE in PBi and/or B31.

Regardless of the underlying mechanism, we have shown that simultaneous, and to a lesser extent sequential, infection with *B. burgdorferi* sensu stricto and *B. garinii* alters the course of murine Lyme borreliosis. Paw swelling was more pronounced in C3H/HeJ mice simultaneously and sequentially infected with B31 and PBi than in mice infected with B31 spirochetes alone (Fig. 3a). In C57BL/6 mice, paw swelling persisted longer in simultaneously infected mice (Fig. 3b). In C3H/HeJ mice, arthritis was significantly more severe in mice simultaneously infected with PBi and B31 than in mice infected with B31 alone (Fig. 4a). We have also shown that these coinfected mice have higher B31 spirochete levels in several tissues. This implies that higher overall B31 spirochete levels result in more local oedema formation and more neutrophil influx and inflammation in tibiotarsal joints. Apparently, oedema formation and immune cell influx are not caused solely by high levels of just any *Borrelia* strain, because we have clearly shown that mice infected with *B. garinii* strain PBi, despite high PBi spirochete levels in the joint, do not develop arthritis, a result that was to be expected (Barthold, 1999). In our studies, coinfected mice showed higher B31 spirochete levels in various tissues; however, B31 spirochete levels were not significantly higher in tibiotarsal joints. The increased inflammation we have observed in coinfected mice could be the result of an altered immune response by the host caused by coinfection of B31 and PBi. Others have postulated that in Lyme borreliosis, as in other systemic diseases, local precipitation of immune complexes in the joint contributes to inflammation (Hardin et al., 1984). In C3H/HeJ mice, sequential infection of PBi and B31 resulted in more pronounced paw swelling, but not arthritis. Others have previously shown that swelling and arthritis do not always coincide in murine Lyme borreliosis (Anguita et al., 1996).

In summary, this study shows that coinfection of *B. burgdorferi* sensu stricto and *B. garinii* alters the outcome of murine Lyme borreliosis. Coinfection is likely to occur in Europe and could be of medical importance because animals, and possibly also humans, simultaneously infected with *B. burgdorferi* sensu stricto and *B. garinii* could develop more severe Lyme borreliosis symptoms. Coinfection could also be of ecological importance, because, in nature, competition between *B. burgdorferi* sensu stricto and *B. garinii* within the animal host could lead to preferential maintenance of *B. burgdorferi* sensu stricto in European *I. ricinus* populations.

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The urokinase receptor (uPAR) facilitates clearance of *Borrelia burgdorferi*

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Chapter 9

Abstract

The causative agent of Lyme borreliosis, *Borrelia burgdorferi*, has been shown to induce expression of the urokinase receptor (uPAR); however the role of uPAR in the immune response against *Borrelia* has never been investigated. uPAR not only acts as a proteinase receptor, but can also, dependently or independently of ligation to uPA, directly affect leukocyte function. We here demonstrate that uPAR is upregulated on murine and human leukocytes upon exposure to *B. burgdorferi* both in vitro as well as in vivo. Notably, *B. burgdorferi*-inoculated C57BL/6 uPAR knock-out mice harbored significantly higher *Borrelia* numbers compared to WT controls. This was associated with impaired phagocytotic capacity of *B. burgdorferi* by uPAR knock-out leukocytes in vitro. *B. burgdorferi* numbers in vivo, and phagocytotic capacity in vitro, were unaltered in uPA, tPA (low fibrinolytic activity) and PAI-1 (high fibrinolytic activity) knock-out mice compared to WT controls. Strikingly, in uPAR knock-out mice partially backcrossed to a more *B. burgdorferi* susceptible C3H/HeN background, higher *B. burgdorferi* numbers were associated with more severe carditis and increased local TLR2 and IL-1β mRNA expression. In conclusion, in *B. burgdorferi* infection, uPAR is required for phagocytosis and adequate eradication of the spirochete from the heart by a mechanism that is independent of binding of uPAR to uPA or its role in the fibrinolytic system. A better understanding of the role of uPAR in the course of Lyme borreliosis is of interest since various uPA/uPAR agonists/antagonists have been tested in oncological trials and disseminated Lyme borreliosis can be difficult to treat. Therefore, compounds modulating host immune responses may prove to be a valuable asset in the treatment of Lyme borreliosis in adjunction to antibiotics.

Authors Summary

Lyme borreliosis is caused by *Borrelia burgdorferi* and is transmitted through ticks. Since its discovery approximately 30 years ago it has become the most important vector-borne disease in the Western world. The pathogenesis is still not entirely understood. We here demonstrate that the urokinase receptor (uPAR) is upregulated in mice and humans upon exposure to *B. burgdorferi* in vitro and in vivo. Importantly, we describe the function of uPAR in the immune response against the spirochete; using uPAR knock-out mice we show that uPAR plays an important role in phagocytosis of *B. burgdorferi* by leukocytes both in vitro as well as in vivo. In addition, we show that the mechanism by which uPAR is involved in the phagocytosis of *B. burgdorferi* is independent of ligation to its natural ligand uPA or uPAR's role in fibrinolysis. Understanding the role of uPAR in the course of Lyme borreliosis might be of clinical interest since various uPA/uPAR agonists/antagonists have been tested in oncological trials and disseminated Lyme borreliosis can be difficult to treat. Therefore, compounds modulating host immune responses may prove to be a valuable asset in the treatment of Lyme borreliosis in adjunction to antibiotics.

Introduction

Lyme borreliosis, an emerging tick-borne disease in both the New and Old world, is caused by spirochetes belonging to the *Borrelia burgdorferi* sensu lato group and is predominantly transmitted by *Ixodes* ticks [1]. In the United States *Borrelia burgdorferi* sensu stricto, from here on referred to as *B. burgdorferi*, is the only prevalent *Borrelia* species, whereas in Europe three *Borrelia* species - *B. burgdorferi*, *Borrelia garinii* and *Borrelia afzelii* – are able to cause Lyme borreliosis [2,3]. In humans, all three species frequently cause an erythematous cutaneous lesion, *erythema migrans*. In later stages of infection spirochetes can disseminate and cause disease that affects the joints, cardiac conduction system, central nervous system and the skin [4].

Borrelia has been shown to differentially express specific genes to inhibit, modulate or to bypass the host immune system [5] and to bind to host molecules in order to establish a persisting infection. In addition, *B. burgdorferi* can interact with the host fibrinolytic system [6]. *B. burgdorferi* abuses host plasminogen activators to activate plasminogen within the tick gut to facilitate migration through the arthropod vector [7]. However, plasminogen is not critical for transmission and infection, since plasminogen deficient mice do develop an infection after intradermal inoculation with *B. burgdorferi* [7]. In in vitro studies, the spirochete causes upregulation of the urokinase Plasminogen Activator (uPA) [8,9], the Plasminogen Activator Inhibitors (PAI)-1 and 2 [10,11], and the uPA Receptor (uPAR; CD87; PLAUR) [12,13]. uPAR is a multi-ligand receptor with a high affinity for uPA, but also vitronectin, many integrins and G-protein-coupled receptors, and is expressed by many different cell types, including leukocytes [14]. Binding of uPA to uPAR results in formation of plasmin at the leading edge of cells facilitating leukocyte migration by pericellular proteolysis of extracellular matrix proteins [14]. Besides functioning as a proteinase receptor, uPAR also affects leukocyte migration and adhesion [15–20], as well as phagocytosis [19,21], through intracellular signaling. This occurs, in part, independently of ligation of uPA by uPAR [22,23].

Importantly, uPAR has been shown to contribute to activation and mobilization of leukocytes in bacterial infections [14,15,19–24]. To elucidate the role and function of uPAR in the development of Lyme borreliosis in vivo we infected wildtype (WT) and uPAR knock-out C57BL/6 mice with *B. burgdorferi* sensu stricto and monitored *B. burgdorferi* numbers in multiple organs, histopathological changes of tibiotarsi and heart, and host immune responses. In addition, to investigate whether the observed phenotype in uPAR knock-out C57BL/6 mice was dependent on uPAR's role in the fibrinolytic system or dependent on the interaction with uPA we also investigated the course of Lyme borreliosis in tPA, PAI-1 and uPA knock-out C57BL/6 mice. Moreover, we investigated the course of *Borrelia* infection in uPAR knock-out mice partially backcrossed to a C3H/HeN genetic background to assess the role of uPAR in mice more susceptible for infection with *B. burgdorferi*.

Results

Borrelia burgdorferi upregulates uPAR expression in mice and humans

Previous reports have shown that uPAR is upregulated on both a monocytic cell line and primary monocytes upon activation with *B. burgdorferi* [12,13]. We here show that in vitro stimulation with different concentrations of viable *B. burgdorferi* resulted in significantly increased uPAR expression on both murine peritoneal macrophages and ex vivo generated – peripheral blood mononuclear cells-derived - human macrophages (Fig 1A and Supplemental fig 1A). In addition, using murine and human whole blood we observed similar results for granulocytes and monocytes (Fig 1B and supplemental B). By contrast, non-phagocytotic cells, i.e. T lymphocytes, did not upregulate uPAR upon ex vivo exposure to *B. burgdorferi* (Supplemental fig 1D). Other *Borrelia* species, such as *B. garinii* strain PBi and *B. afzelii* strain pKo - both able to cause Lyme borreliosis - also induced enhanced uPAR expression on leukocytes (data not shown). To determine whether uPAR is upregulated in humans upon *B. burgdorferi* infection, we quantified uPAR expression in transcutaneous skin biopsies from *B. burgdorferi* PCR and culture confirmed positive erythema migrans patients and healthy controls. We could not detect uPAR expression in control patients, where as we could easily detect uPAR expression in the diseased group (Fig 1C). Lastly, in WT C57BL/6 mice inoculated intraperitoneally with viable *B. burgdorferi* for 1 hour we observed a significant upregulation of uPAR on the surface of (F4/80 positive) macrophages (Supplemental fig 1C).

C57BL/6 uPAR knock-out mice exhibit increased *B. burgdorferi* numbers in vivo and impaired phagocytosis of *B. burgdorferi* in vitro

To assess the role of uPAR in the immune response against *B. burgdorferi* vivo, we infected C57BL/6 WT and uPAR knock-out mice with *B. burgdorferi* and sacrificed mice two and four weeks post infection. By quantitative PCR we assessed *B. burgdorferi* numbers in skin, bladder and tibiotarsi post mortem. C57BL/6 uPAR deficient mice harbored higher *B. burgdorferi* numbers compared to WT animals in all tissues examined. This was most pronounced, and statistically significant, four weeks post infection (Fig 2A). These data were underscored by the fact that two weeks post infection only 3/8 bladder tissue cultures were positive in WT mice versus 7/7 in uPAR knock-out mice (Chi-square p= 0,026). We did not determine *B. burgdorferi* numbers in cardiac tissue in these experiments since we used the heart in toto for histopathology. In line with higher systemic *B. burgdorferi* numbers in uPAR deficient mice a significant increase in total IgG against *B. burgdorferi* over time (Fig 2B), and significantly higher IgG1 antibody levels four week post infection, were observed (Fig 2C). We detected no differences in IgM and IgG2b subclass-levels four weeks post infection (data not shown). To obtain a first insight into the mechanism by which uPAR deficiency could impact pathogen burden after infection with *B. burgdorferi* we stimulated leukocytes with viable spirochetes in vitro. We harvested peritoneal macrophages from C57BL/6 WT and uPAR knock-out mice, which we stimulated with viable *B. burgdorferi* (Cell:*Borrelia* = 1:50) for 16 hours. We demonstrate that *Borrelia* induced similar cytokine levels in WT and

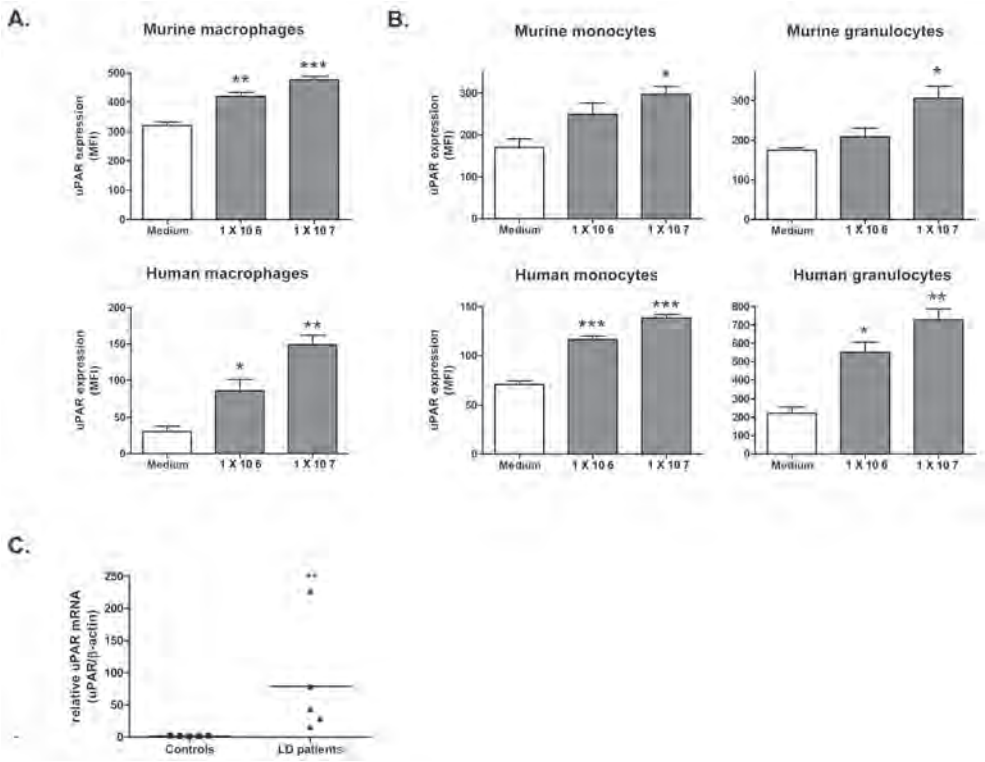

Figure 1. *Borrelia burgdorferi* induces upregulation of the urokinase receptor on murine and human leukocytes in vitro and in vivo.

A. Viable *B. burgdorferi* induces uPAR expression on murine and human macrophages. Murine peritoneal macrophages, and ex vivo generated human macrophages, (1 x 10^5) were stimulated with viable *Borrelia burgdorferi* (strain B31) for 16 hours (Cell:*B. burgdorferi* = 1:10 or 1:100). Cells were harvested and analyzed for uPAR expression by FACS analysis.

B. Viable *B. burgdorferi* induces uPAR expression on murine and human granulocytes and monocytes. Murine and human whole blood was incubated with viable *B. burgdorferi* for 16 hours. Erythrocytes were lysed and cells were costained for granulocyte or monocyte markers and uPAR and analyzed by FACS analysis.

C. Expression of uPAR is increased in skin biopsies from Lyme borreliosis patients. Total RNA was isolated from biopsies derived from culture and PCR confirmed *B. burgdorferi* positive erythema migrans lesions from Lyme borreliosis patients (n=5) or healthy controls (n=5) and subjected to quantitative uPAR and β-actin RT-PCR. We could not detect uPAR mRNA in healthy controls, for these samples the level of uPAR mRNA was set at the detection limit. Expression of uPAR mRNA was corrected for β-actin mRNA expression and depicted as a relative number. Expression of uPAR of one of the healthy controls was set at 1.

Graphs in panel A and B are representative of at least three independent experiments and error bars represent the mean of triplicates within one experiment ± SEM. A P value < 0.05 was considered statistically significant. * indicating $P < 0,05$; ** $P < 0,01$ and *** $P < 0,001$

uPAR deficient macrophages (Fig 2D). We obtained comparable results when we stimulated whole blood in a similar fashion (data not shown). Next, because uPAR has been shown to play a crucial role in phagocytosis of *Escherichia coli* by neutrophils [19,21,22], we investigated whether WT and uPAR knock-out neutrophils and macrophages differed in their capacity to phagocytose *B. burgdorferi*. In these assays extracellular bacteria were quenched by addition of a quenching dye

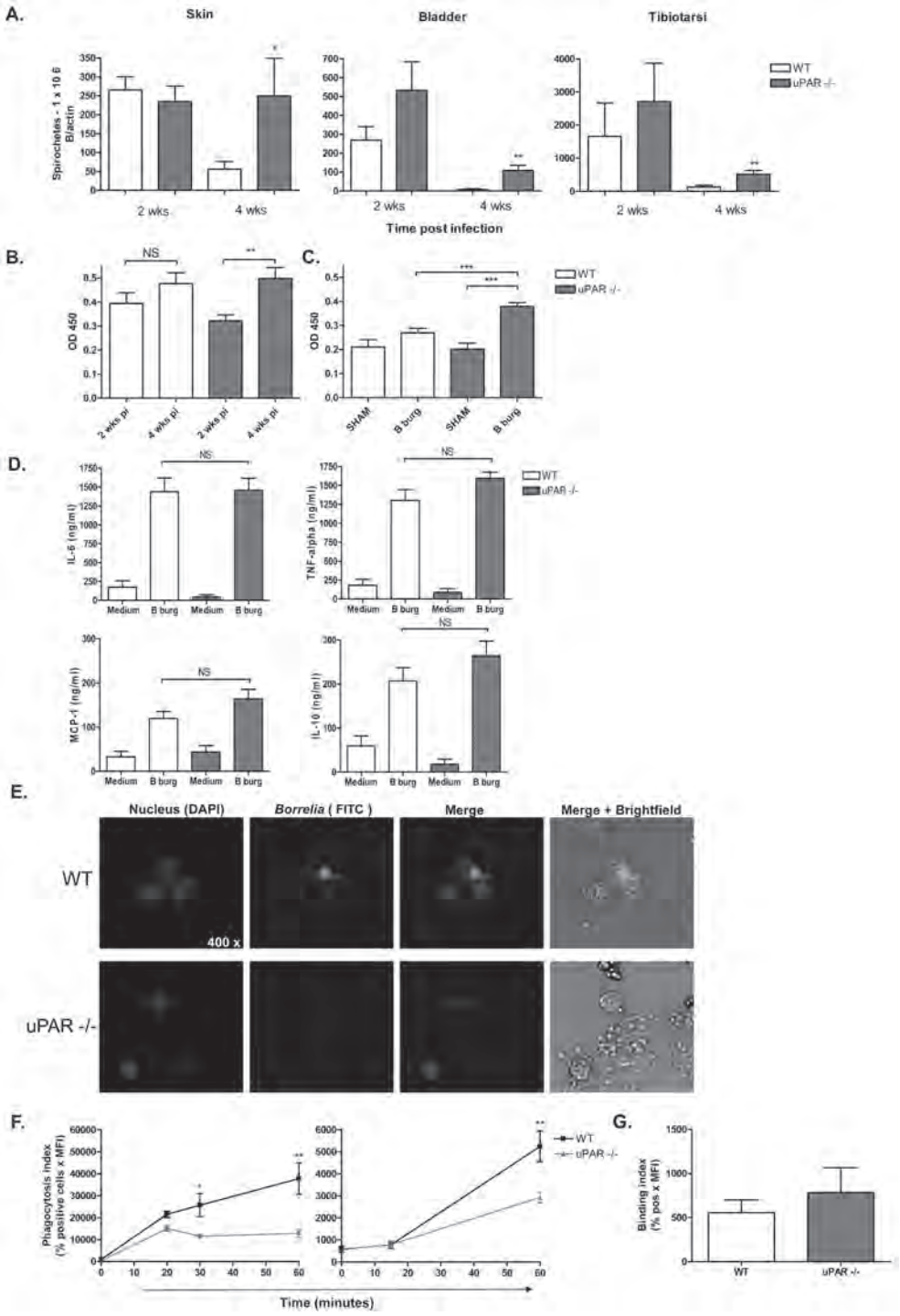
A.
Skin
Bladder
Tibiotarsi
Spirochetes - 1 x 10 6 B/actin
WT
uPAR -/-
2 wks
4 wks
Time post infection
B.
OD 450
NS
2 wks pi
4 wks pi
C.
SHAM
B burg
D.
IL-6 (ng/ml)
TNF-alpha (ng/ml)
MCP-1 (ng/ml)
IL-10 (ng/ml)
Medium
E.
Nucleus (DAPI)
Borrelia (FITC)
Merge
Merge + Brightfield
400 x
F.
Phagocytosis index (% positive cells x MFI)
Time (minutes)
G.
Binding index (% pos x MFI)

Figure 2. The urokinase receptor (uPAR) is involved in clearance of *B. burgdorferi*.
A. Urokinase receptor knock-out C57BL/6 mice display higher systemic *B. burgdorferi* numbers. WT and uPAR -/- mice were inoculated with *B. burgdorferi* and sacrificed two and four weeks post infection. DNA was extracted from the indicated tissues and subjected to quantitative *Borrelia flab* and mouse *β-actin* PCR. In SHAM inoculated mice (2 to 3 per group) we did not detect *B. burgdorferi* DNA. Six to eight mice per group were used and bars represent the mean ± SEM.
B and C. Urokinase receptor knock-out C57BL/6 mice develop more rigorous IgG responses. Sera from C57BL/6 WT and uPAR knock-out mice, 2 and 4 weeks post *B. burgdorferi* (B burg) or SHAM inoculation (SHAM) was used for whole cell *B. burgdorferi* ELISA. Thus, we determined total IgG directed against *B. burgdorferi* (B) and IgG subclasses, of which only IgG1 (C) is shown.
D. WT and uPAR -/- macrophages produce similar levels of pro-inflammatory cytokines when exposed to viable *B. burgdorferi* in vitro. Peritoneal macrophages were stimulated with control medium (medium) or *B. burgdorferi* (B burg) for 16 hours. The supernatant was analyzed for cytokine production using a mouse inflammation cytometric bead array.
E and F. Urokinase receptor deficient granulocytes and macrophages are incapable of adequately phagocytosing *B. burgdorferi*. Whole blood or peritoneal macrophages were incubated with CFSE labeled viable or heat-killed FITC-labeled *B. burgdorferi* at 37 °C or at 4 °C as a control. Phagocytosis was stopped by transferring the tubes to ice and extracellular bacteria were quenched by addition of a quenching dye containing Trypan blue. When whole blood was used erythrocytes were lysed before cells were DAPI stained and subjected to fluorescent microscopy (E) or stained for Gr-1 (granulocytes) and subjected to FACS analysis (F; left panel). Peritoneal macrophages were directly subjected to FACS analysis (F; right panel). Phagocytosis was depicted as the phagocytosis index [63,64]: mean fluorescence intensity (MFI) x percentage (%) positive cells) at 37°C minus (MFI x % positive cells at 4°C). Six to eight mice per group were used, graphs represent the mean ± SEM and are representative of three independent experiments.
G. *B. burgdorferi* binds equally well to WT and uPAR -/- macrophages. A similar experiment as described in F was performed, albeit at 4 °C and without the addition of quenching dye to determine binding of *B. burgdorferi* to peritoneal macrophages. Binding is expressed as the binding index: % CFSE positive cells x MFI. Four to six mice per group were used and bars represent the mean ± SEM. The experiment was repeated twice. A P value < 0.05 was considered statistically significant. * indicating $P < 0{,}05$; ** $P < 0{,}01$ and *** $P < 0{,}001$. For color figure see page 261.

containing Trypan blue. We demonstrate that both uPAR knock-out neutrophils (in whole blood) and uPAR knock-out peritoneal macrophages were significantly less capable of phagocytosing *B. burgdorferi*, using either heat-killed FITC-labeled or viable CFSE-labeled *B. burgdorferi* (Fig 2E and F and Supplemental fig 2). Confocal microscopy confirmed labeled bacteria were localized intracellularly (Supplemental fig 3A and B). To distinguish between binding and phagocytosis we performed similar experiments, but at 4 °C and without the addition of quenching solution. These experiments showed no difference in the capacity of WT and uPAR deficient leukocytes to bind *B. burgdorferi* (Fig 2G). In addition, binding experiments with recombinant human uPAR and viable *B. burgdorferi* failed to show direct binding of the spirochete to uPAR (data not shown). Since uPAR has been shown to be of importance in the migration of leukocytes, we also investigated whether there was impaired migration of leukocytes in *B. burgdorferi*-infected uPAR knock-out mice. We intradermally inoculated C57BL/6 WT and uPAR knock-out mice with *B. burgdorferi* or controls and harvested skin at 0, 6 or 32 hours post infection. We did not observe influx of immune cells at t=0 (data not shown). By H&E, Ly6G and F4/80 stainings on sagittal skin sections we did observe an evident influx of immune cells and inflammation at t=6 hours, however there were no differences between WT and uPAR knock-out mice (Fig 3). As has been shown by others [25], the predominant cells at this early time point were granulocytes (Fig 3). Importantly, these data show that the phenotype in uPAR knock-out mice is not explained by impaired influx of immune cells at

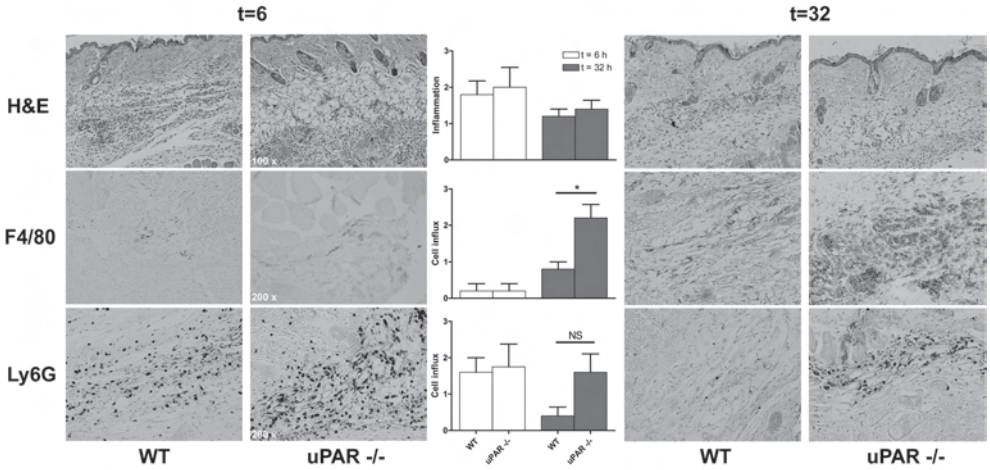

Figure 3. Leukocyte migration in uPAR knock-out mice in response to *B. burgdorferi* infection in vivo.
C57BL/6 WT and uPAR knock-out mice were intradermally injected with 1 x 10^6 *B. burgdorferi* in PBS in the midline of the neck and mice were sacrificed 6 or 32 hours post inoculation. Skin was harvested, formalin fixed and imbedded in paraffin. Five µm-thick sagittal skin sections were processed and H&E, Ly6G and F4/80 stained by routine histological techniques. Control animals injected with PBS alone did no display influx of leukocytes (data not shown). Slides were scored for influx of leukocytes by an independent pathologist who was blinded to the experimental design. Influx was semi-quantitatively scored on a scale from 0-3, with 0 being no, 1 mild, 2 moderate, and 3 being severe diffuse infiltration. Per group and time point 5 five mice were used, error bars represent SEM. Representative sections are depicted in the figure. A *P* value < 0.05 was considered statistically significant. * indicating *P* < 0,05; NS, not significant. For color figure see page 263.

the site of inoculation allowing for more dissemination of the spirochete. By contrast, later in the course of infection, at t=32 hours, we observed a more pronounced influx of macrophages in uPAR knock-out mice compared to WT controls, which probably is explained by the increased *Borrelia* burden in uPAR knock-out mice (Fig 3). In conclusion, higher *B. burgdorferi* numbers in C57BL/6 uPAR knock-out mice compared to WT mice could be explained by a decreased phagocytotic capacity of uPAR deficient leukocytes observed in vitro, and not by impaired migration of uPAR deficient leukocytes.

Higher *B. burgdorferi* numbers and impaired phagocytotic capacity in C57BL/6 uPAR knock-out mice are independent of ligation of uPA to uPAR

Since uPAR has been suggested to affect function of leukocytes in both an uPA-dependent as well as an uPA-independent fashion we also assessed the course of *B. burgdorferi* infection in C57BL/6 uPA knock-out mice. Both 2 and 4 weeks post *B. burgdorferi* infection, C57BL/6 WT and uPA deficient mice displayed similar *Borrelia* numbers in all tissues examined as detected by quantitative PCR (Fig 4A). In addition, compared to WT controls, uPA deficient neutrophils and peritoneal macrophages were equally capable of phagocytosing *B. burgdorferi* (Fig 4B). These data suggest that the phenotype observed in C57BL/6 uPAR knock-out mice was independent of ligation of uPA to uPAR.

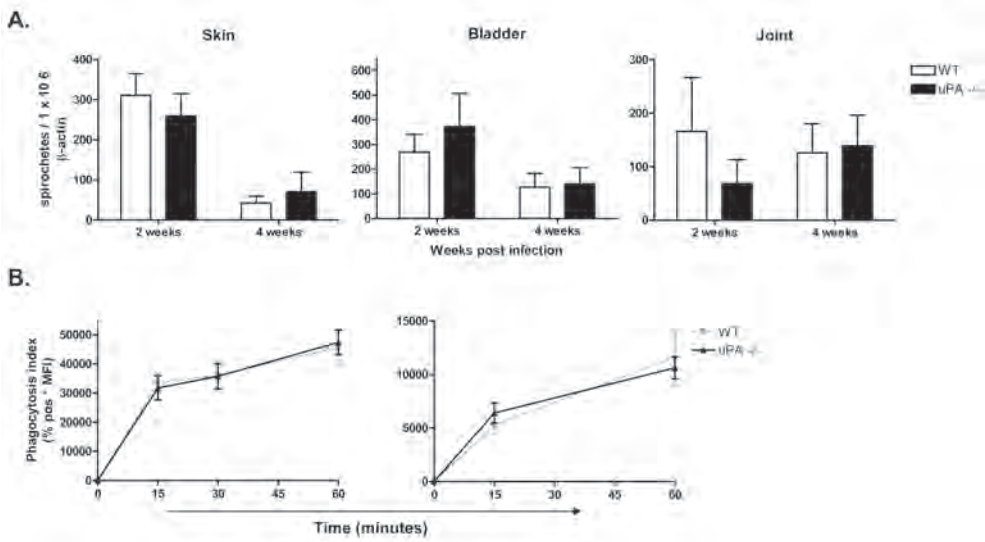

Figure 4. The urokinase activator (uPA) is not involved in clearance of the spirochete.
A. Urokinase activator knock-out C57BL/6 mice display similar systemic *B. burgdorferi* numbers compared to WT controls. WT and uPA -/- mice were inoculated with *B. burgdorferi* and sacrificed two and four weeks post infection. DNA was extracted from the indicated tissues and subjected to quantitative *Borrelia flab* and mouse *β-actin* PCR. Six to eight mice per group were used and *B. burgdorferi* numbers are depicted as described in figure 2A.
B. Urokinase activator deficient granulocytes and macrophages (solid lines) are just as capable as WT controls (dotted lines) of phagocytosing *B. burgdorferi*. Phagocytosis assays were performed as described in figure 2 F. Six to eight mice per group were used, error bars represent SEM and the graphs are representative of two independent experiments. A P value < 0.05 was considered statistically significant.

Higher *B. burgdorferi* numbers and impaired phagocytotic capacity in C57BL/6 uPAR knock-out mice are independent of uPAR's role in the fibrinolytic system

Next, since uPAR has been shown to affect function of leukocytes through its role in the fibrinolytic system [14], we infected mice in which the activity of the fibrinolytic system was either impaired, i.e. C57BL/6 tPA deficient mice, or enhanced, i.e. C57BL/6 PAI-1 knock-out mice. First we demonstrated that *B. burgdorferi* infection did not influence fibrinolytic activity in citrate plasma in either mouse strain, or WT controls, as measured by amidolytic plasminogen activator activity assays (Table 1). Next, we showed that, compared to C57BL/6 WT mice, both C57BL/6 tPA and as PAI-1 knock-out mice display normal *Borrelia* numbers in various tissues two week post infection, as detected by quantitative PCR (Table 1). In line with these data, phagocytotic capacity of C57BL/6 tPA and PAI-1 deficient neutrophils was comparable to that of WT mice (Table 1). Importantly, uPAR knock-out mice, regardless whether they were infected with *B. burgdorferi*, have comparable fibrinolytic activity to WT mice (data not shown). Together these data indicate that the impaired phagocytotic capacity of uPAR deficient mice, resulting in higher spirochete numbers upon *B. burgdorferi* infection in vivo, is not dependent on the role of uPAR in fibrinolysis.

Table 1. *B. burgdorferi* infection in WT, tPA -/- and PAI-1 -/- mice.

PA activity (in %)[#]	SHAM	*B. burgdorferi*
WT	91,2 ± 1,4	89,6 ± 0,8
tPA -/-	10,7 ± 0,9[a]	9,9 ± 2,1[c]
PAI-1 -/-	132,7 ± 4,6[b]	141,7 ± 3,9[d]
Pathogen numbers[##] (per 1 x 10 6 β-actin)	–	ankle
WT	–	1292 ± 473
tPA -/-	–	636 ± 366
PAI-1 -/-	–	560 ± 149
		skin
WT	–	872 ± 290
tPA -/-	–	724 ± 301
PAI-1 -/-	–	1463 ± 488
		bladder
WT	–	578 ± 173
tPA -/-	–	967 ± 310
PAI-1 -/-	–	367 ± 125
Phagocytosis index[$] (% pos * MFI)		
WT	ND	41015 ± 5826
tPA -/-	ND	39828 ± 3350
PAI-1 -/-	ND	49928 ± 2752

Note. C57BL/6 WT, tPA and PAI-1 knock-out mice (6-8 per group) were inoculated with *B. burgdorferi* strain B31 or SHAM and sacrificed two weeks later. [#] Plasminogen activity (PA) activity was measured in citrate plasma using amidolytic assays and expressed as a percentage.
[##] *B. burgdorferi* numbers were determined by quantitative PCR and expressed as described in figures 2 and 3.
[$] In addition, an in vitro phagocytosis assay was performed using naive mice (n = 6-8 per group) as described in figure 2. Whole blood was incubated with viable CFSE-labeled *B. burgdorferi* for 60 minutes at 37 or 4 °C as a control and phagocytosis was depicted as the phagocytosis index as described in figure 2.
[a,c] PA activity was significantly lower in tPA knock-out mice compared to WT controls ($P < 0,0001$), regardless whether mice were inoculated with *B. burgdorferi* or SHAM.
[b,d] PA activity was significantly higher in PAI-1 knock-out mice compared to WT controls ($P < 0,0001$), regardless whether mice were inoculated with *B. burgdorferi* or SHAM.
Results represent the mean ± SEM. Non-parametric statistical tests were used to analyze the differences between the groups. A P value < 0,05 was considered statistically significant.

The effect of uPAR deficiency on the development of Lyme carditis

We assessed carditis severity in *B. burgdorferi* inoculated C57BL/6 uPAR knock-out and WT mice two and four weeks post infection. Two weeks post infection, in hematoxylin and eosin (H&E) stained sagittal sections of mouse hearts, we found comparable carditis severity scores in C57BL/6 WT and uPAR knock-out mice (Supplemental fig 4A and B). The localization and severity of carditis in our experiments using C57BL/6 mice appeared to be similar to the localization and carditis severities reported by ourselves and others using the same, relatively resistant, mouse strain [26–29]. SHAM inoculated mice did not develop carditis (data not shown). We were unable to reliably score carditis four weeks post infection, since, as observed by others, at this stage, carditis was characterized by an organizing rather than ongoing inflammation (Supplemental fig 4A) [26].

However, in 4/8 uPAR deficient mice and 0/8 WT mice a mild active carditis, characterized by the presence of small cellular infiltrates at the aortic root, could still be observed 4 weeks post inoculation (Chi-square p= 0,021) (data not shown). By contrast, in 5/8 of WT mice and only in 2/8 uPAR deficient mice we observed organized inflammatory infiltrates, characterized by sharply delineated foci (Supplemental fig 4A) of mononuclear leukocytes situated in the atrial wall (Chi-square p = 0,0721). Together these findings suggest a difference with respect to the kinetics of the organization of carditis in C57BL/6 uPAR knock-out and WT mice. These data are in line with the previously described increased influx of macrophages in uPAR knock-out mice 32 hours after intradermal injection with *B. burgdorfer*i (Fig 3), which is likely to be caused by the increased pathogen burden in these mice. Although we observed ankle swelling in both WT and uPAR knock-out mice during the course of infection, histological examination of H&E stained section of tibiotarsi did not reveal any signs of arthritis (data not shown). Finally, in line with the observed normal *Borrelia* numbers, in uPA, tPA and PAI-1 knock-out mice severity of carditis was comparable to that in WT mice (Supplemental fig 4C and D). Together these data demonstrate that, despite higher *B. burgdorferi* numbers, C57BL/6 uPAR knock-out mice develop carditis with a similar severity, albeit for a prolonged period of time, compared to WT controls.

The course of *B. burgdorferi* infection in uPAR deficient mice on a *B. burgdorferi* susceptible genetic background

To further investigate the effect of uPAR deficiency on the development of Lyme borreliosis symptoms we generated uPAR deficient mice on a more *Borrelia* susceptible genetic background. It is well-known that C57BL/6 mice are relatively resistant to *B. burgdorferi* and develop less severe symptoms after infection with the spirochete, and that C3H/HeN mice are more susceptible and develop more severe symptoms after infection with *B. burgdorferi* [30]. In addition, it has been described that F1 of WT C57BL/6 crossed with (x) C3H/HeN mice are intermediately sensitive to *B. burgdorferi* infection [30]. Therefore we investigated the course of Lyme borreliosis in F2 of C57BL/6 x C3H/HeN uPAR knock-out mice and WT littermate controls. We first showed that, similar to uPAR knock-out mice on a pure C57BL/6 background, these mice harbor higher *Borrelia* numbers in multiple tissues compared to WT littermate controls two weeks post infection (Fig 5A), indicating that the lack of uPAR in these mice also resulted in impaired phagocytosis and increased pathogen burden. Indeed, in in vitro phagocytosis assays, compared to WT littermate controls, C57BL/6 x C3H/HeN uPAR deficient neutrophils were significantly less capable of phagocytosing *B. burgdorferi* (Fig 5B). Strikingly, compared to WT littermate controls (Fig 5C), C57BL/6 x C3H/HeN uPAR knock-out mice developed significantly more severe carditis (Fig 5D), reflected by influx of greater numbers of leukocytes in more and larger parts of cardiac tissue two weeks post infection (Fig 5E). As has been shown by others the main cells involved in inflammation were macrophages, as determined by F4/80 immunostaining (Fig 5F and G). By multiplex ligation-dependent probe amplification (MLPA), we detected significantly increased levels of interleukin (IL)-1β, IL-1 Receptor Associated Kinase (IRAK)-3, and toll-like receptor (TLR)2 mRNA in hearts from uPAR knock-out mice compared to WT littermate controls two weeks post infection (Fig 5H),

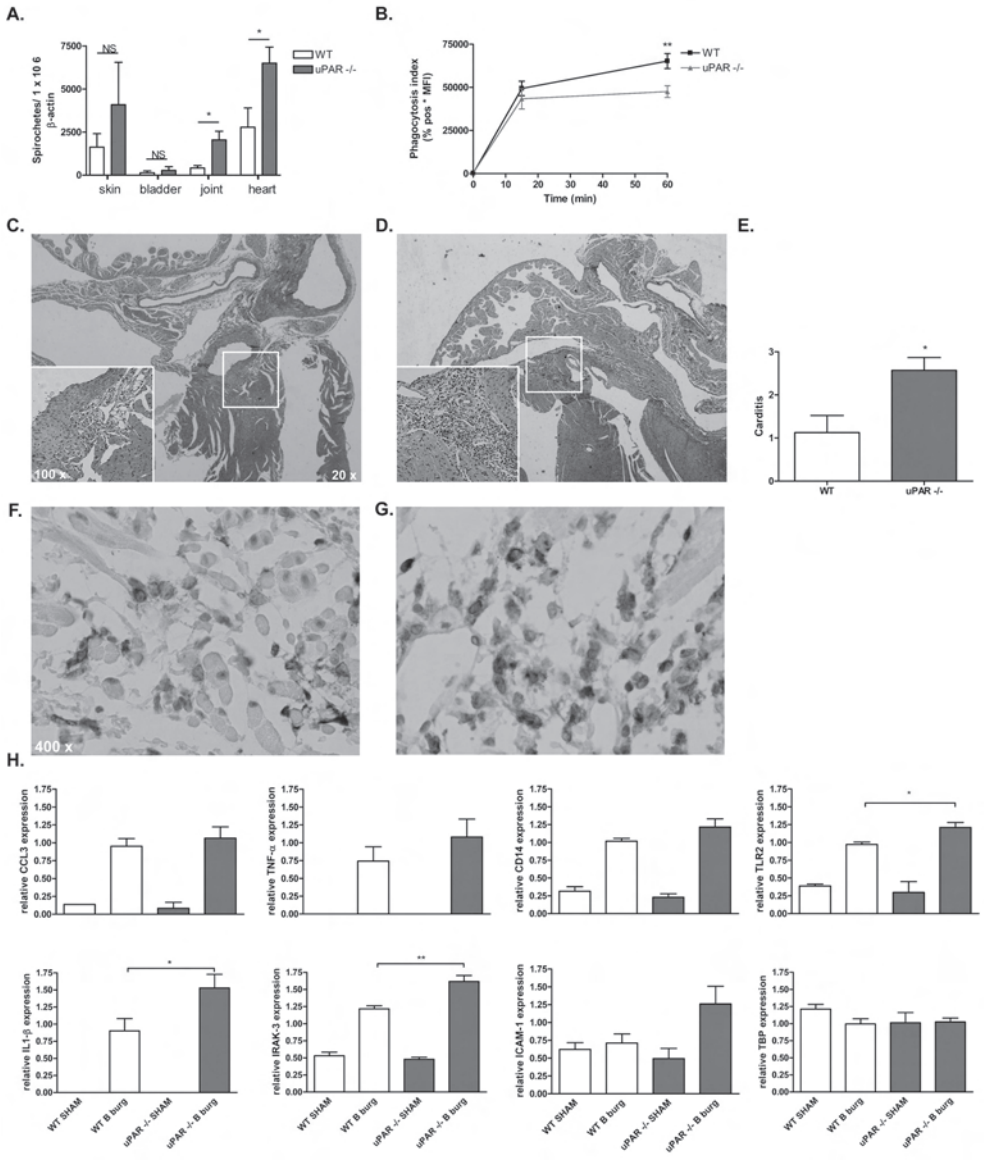

Figure 5. The course of Lyme borreliosis in uPAR knock-out mice on a *B. burgdorferi* susceptible mixed C57BL/6 x C3H/HeN genetic background.

A. Urokinase receptor deficient mice on the mixed genetic background also display higher *B. burgdorferi* numbers compared to WT littermate controls. C57BL/6 mice were backcrossed twice to a C3H/HeN background. We intercrossed F2 mice and used the homozygous and nullizygous offspring (F2 homozygous uPAR deficient C57BL/6 x C3H/HeN mice and WT littermate controls) for our experiments. Mice were inoculated with *B. burgdorferi* or SHAM and sacrificed two weeks post infection, DNA was extracted from the indicated tissues and samples were subjected to quantitative *Borrelia flab* and mouse *β-actin* PCR. *B. burgdorferi* numbers are depicted as described in figure 2. Six to eight mice per group were used.

B. Urokinase receptor deficient leukocytes from mice on the mixed genetic background are not as capable of phagocytosing *B. burgdorferi* as are granulocytes from WT littermate controls. Phagocytosis assays with whole blood were

performed as described in figure 2. Six to eight mice per group were used.
C, D and E. Peak carditis in these uPAR -/- mice (D) is more severe compared to carditis in WT littermate controls (C). Mice were inoculated with *B. burgdorferi* and sacrificed two weeks post infection. Pictures of hematoxyline and eosin stained sagittal sections depict representative sections. Carditis was scored as described in supplemental figure 4 within the same session (E). Six to eight mice per group were used.
F and G. The main cell involved in murine Lyme carditis is the macrophage. Representative pictures of F4/80 stained sagittal sections of hearts from *B. burgdorferi* infected uPAR deficient mice (G) and WT littermate controls (F).
H. More severe inflammation in *B. burgdorferi* infected uPAR deficient animals (n=7) compared to WT littermate controls (n=7) as measured by multiplex ligation-dependent probe amplification (MLPA). MLPA was performed on RNA obtained from half of sagittally dissected hearts from *B. burgdorferi* or SHAM inoculated mice. Depicted are mRNA expression of *TNF-α*, *CCL3, TLR2, CD14, IL1-β*, *IRAK3, ICAM1* and *TBP (housekeeping gene [69])*. Other genes included in the assay were *IL6, IL10, INF-γ, TFPI, F3,PROCR, SERPINE1P, PLAT, PLAUR, TLR4, TLR9 LY96, IRAK1, F2R, NFKB1a, NOS3, ITGA5,B2M, ITGAV, ITGAB3, TFRC, HIF1A, MMP2* and *HP.*
Bars represent the mean ± SEM. A *P* value < 0.05 was considered statistically significant.
* indicating $P < 0{,}05$; ** $P < 0{,}01$. For color figure see page 264.

consistent with the observed higher *B. burgdorferi* numbers and more severe cardiac inflammation in uPAR knock-out mice. Since, uPAR has also been shown to enhance migration of leukocytes towards the site of infection for some, but not all bacteria, in these mice we performed in vitro migration assays with WT and uPAR deficient macrophages (Supplemental fig 5A). We observed impaired migration of uPAR deficient macrophages to C5a (Supplemental fig 5B), however not to supernatant from a cardiomyoblastic rodent cell line (Supplemental fig 5C), compared to migration of WT macrophages, which is in line with our observations in the in vivo migration assays in WT C57BL/6 mice. In this in vitro setting, whether or not this cell line was stimulated with viable *B. burgdorferi* did not affect migration of WT and uPAR deficient macrophages. WT and uPAR deficient C57BL/6 x C3H/HeN mice developed comparable ankle swelling during the course of *B. burgdorferi* infection (Supplemental fig 5D), however despite the more susceptible phenotype of these mice compared to C57BL/6 mice, these mice did not develop any histological signs of arthritis, as determined by hematoxyline and eosin staining, but also Ly6G - a marker for granulocytes - immunostaining (data not shown). In line with these data, post mortem radiological examination of the hind limbs did not reveal any signs of arthritis (Supplemental fig 5E).

Discussion

Since its discovery approximately 30 years ago Lyme borreliosis has become the most important vector-borne disease in the Western world. We here demonstrate, to our knowledge for the first time, that uPAR plays an important role in the antibacterial innate immune response against *B. burgdorferi*. We show that uPAR expression is upregulated in response to *B. burgdorferi* on human and murine leukocytes both in vitro, as well in vivo. Importantly, we describe the role of uPAR in the immune response against *B. burgdorferi.* By using C57BL/6 WT and uPAR knock-out mice we show that uPAR plays an important role in phagocytosis of *B. burgdorferi* - a prerequisite for the eradication of the spirochete - by leukocytes. Moreover, experiments with C57BL/6 uPA, tPA and PAI-1 knock-out mice show that the mechanism by which uPAR is involved in the phagocytosis

of *B. burgdorferi* is independent of ligation to uPA or uPAR's role in fibrinolysis. Finally, we show that, in mice relatively susceptible to *Borrelia* infection - mice on a mixed C57BL/6 and C3H/HeN background - uPAR deficiency also impaired phagocytotic capacity in vitro, which was associated with higher *B. burgdorferi* numbers, more local inflammation and more severe carditis, compared to WT littermate control animals, further underscoring the in vivo relevance of our findings. Together these data demonstrate an important role for uPAR in the innate immune response against, and the clearance of, the causative agent of Lyme borreliosis.

Earlier studies documented that membrane bound uPAR and uPAR mRNA are upregulated in human peripheral blood-derived monocytes and the human monocyte-like cell line U937 upon exposure to viable and heat-killed *B. burgdorferi* [12,13]. We here show that viable *B. burgdorferi* induces upregulation uPAR (Fig 1 and Supplemental fig 1), not only on murine and human monocytes, but also on macrophages and granulocytes in vitro. Notably, uPAR expression in response to *B. burgdorferi* in vivo has never been investigated. We here show that in skin from Lyme borreliosis patients with erythema migrans uPAR mRNA expression is significantly increased and could be readily detected by quantitative RT-PCR (Fig 1). In addition, in a small pilot experiment, intradermal syringe inoculation of C57BL/6 WT mice with viable *B. burgdorferi* (or PBS as a control) also appeared to increase uPAR mRNA levels at the site of inoculation 6 hours post injection, but - due to small group numbers - these differences were not statistically significant (data not shown). Increased levels of uPAR are likely to be caused by influx of leukocytes to the site of the tick-bite. Indeed, in preliminary experiments in which we inoculated human skin ex vivo with viable *B. burgdorferi* - a model in which there is no influx of leukocytes [31]- we did not observe an increase in uPAR expression as determined by uPAR immunostaining on snapfrozen sagittal skin sections (data not shown). Erythema migrans lesions are characterized by perivascular infiltrates in the dermis composed primarily of lymphocytes and macrophages [32]. We do not know which infiltrating cell type is responsible for the elevated uPAR levels, but based on our in vitro data we speculate that the macrophage is the most likely candidate. Indeed, macrophages from intraperitoneally *B. burgdorferi*-inoculated WT C57BL/6 mice did upregulate uPAR expression, further indicating that *Borrelia*-phagocyte interaction in vivo results in induction of uPAR expression (Supplemental fig 1). Upregulation of uPAR appeared not to be specific for *B. burgdorferi* since, in our in vitro experiments, other bacteria, i.e. *Klebsiella pneumoniae* and *Burkholderia pseudomallei*, also induce upregulation of uPAR to a similar extent (data not shown).

To investigate the role of uPAR in the immune response against *B. burgdorferi* and the course of murine Lyme borreliosis we inoculated C57BL/6 WT and uPAR knock-out mice with *B. burgdorferi*. We demonstrate by quantitative PCR and culture that mice lacking uPAR display significantly increased *B. burgdorferi* numbers in all tissue examined, indicative of a more disseminated infection (Fig 2), although also in these mice there appeared to be clearance of *B. burgdorferi*, as suggested by lower numbers 4 weeks compared to 2 weeks post infection. The increased *B. burgdorferi* burden in uPAR deficient mice was underscored by a more abundant, putatively reactive, IgG response (Fig 2). The role of uPAR in leukocyte adhesion and migration, leading to recruitment of these cells to

the site of infection, has been the topic of investigations for many years. Several in vivo studies show that migration of uPAR deficient leukocytes is impaired in response to, for example, *Pseudomonas aeruginosum* [22] and *Streptococcus pneumoniae* [23]. In other studies, e.g. in *E. coli*-induced peritonitis [20] and pyelonephritis [21] uPAR deficiency did not affect leukocyte recruitment, indicating that the role of uPAR in migration of leukocytes is dependent on the pathogen, the site of infection and the disease model. Interestingly, in the mouse model for Lyme borreliosis uPAR is not crucially involved in migration of leukocytes to *B. burgdorferi* infected tissues, as can be deducted from our in vivo migration experiments (Fig 3). Strikingly, the fact that we observed more macrophages 32 hours after injection with *B. burgdorferi* in uPAR knock-out skin compared to WT controls, but no differences in H&E staining, suggests that the quality of the inflammatory infiltrate is affected rather than the quantity; presumably due to higher *B. burgdorferi* numbers in the uPAR knock-out mice. Interestingly, recently it was shown that uPAR also facilitates phagocytosis of the gram-negative bacterium *E. coli* by neutrophils [19,21]. We here show, by fluorescent microscopic assays, and FACS-based phagocytosis assays, that both uPAR deficient granulocytes and macrophages are significantly less capable of phagocytosing viable spirochetes (Fig 2 and Supplemental fig 2 and 3). Importantly, uPAR deficiency did not affect binding of the spirochete to the surface of leukocytes (Fig 2). In addition, in an in vitro killing assay uPAR appeared not to be involved in killing of the spirochete following phagocytosis (data not shown), indicating that uPAR is involved strictly in the process of internalization of *B. burgdorferi* by leukocytes. Others have previously shown that phagocytosis of spirochetes by immune cells can be crucial for adequate cytokine induction and leukocyte activation [33–35]. We did not observe defects in pro-inflammatory cytokine production in uPAR deficient leukocytes when stimulated in vitro with *B. burgdorferi*. In contrast to the studies described above our results describe more subtle differences in phagocytotic capacity between WT and uPAR deficient leukocytes; we demonstrate diminished, but not absent, phagocytosis in uPAR deficient macrophages compared to WT controls. Thus, there could still be activation of intracellular signaling pathways and/or recognition by intracellular/internalized TLRs that could contribute to the production of normal pro-inflammatory cytokines in uPAR deficient leukocytes. Secondly, our phagocytosis assays clearly show that early phagocytosis is not affected by uPAR deficiency. Most likely early events are critical for the rapid induction of innate pro-inflammatory cytokines.

The role of uPAR in phagocytosis of *B. burgdorferi* appeared to be independent of uPA and uPAR's role in the fibrinolytic system, since in our phagocytosis assays uPA, tPA and PAI-1 knock-out mice all displayed normal phagocytotic capacity of the spirochete compared to WT mice (Fig 3 and table 1). In addition, in vivo experiments clearly show that when these mice were inoculated with *B. burgdorferi* and sacrificed two weeks post infection, normal *B. burgdorferi* numbers were detected (Fig 3 and table 1). There are numerous in vitro studies reporting that *B. burgdorferi* interacts with the fibrinolytic system (reviewed in [6]). Extrapolating these data to the in vivo situation, this interaction, mainly through binding to host derived plasminogen, was thought to enable the spirochete to penetrate tissues, the blood-brain barrier and migrate through the extracellular matrix [8,36–38]. Indeed, for the spirochetal causative agent of relapsing fever, using plasminogen

knock-out mice, it has been clearly shown that plasminogen is required for dissemination of the spirochete to the heart and brain in vivo [39]. To our knowledge, for *B. burgdorferi* however, there is only one previously published study that describes the effect of diminished fibrinolytic activity on the course of *B. burgdorferi* infection in vivo [7]. In this study, in which plasminogen deficient mice were used, plasminogen was shown to be important for dissemination of the spirochete within the feeding tick. Strikingly, despite a short-lived spirochetemia, all plasminogen deficient mice developed infection after intradermal inoculation with *B. burgdorferi*. Importantly, there were no differences in *B. burgdorferi* numbers in any of the tissues examined in plasminogen knock-out and WT mice at several time points post infection [7]. In line with these data, our results demonstrate that the fibrinolytic system per se does not affect the course of *B. burgdorferi* infection. Strikingly, we here show that one of the key players in the fibrinolytic system, uPAR, independently of ligation to uPA or its presumptive role in fibrinolysis, is importantly involved in the course of experimental murine Lyme borreliosis.

The fact that we show that the requirement of uPAR in phagocytosis of *B. burgdorferi* is independent of uPA or uPAR's role in the fibrinolytic system suggests that the requirement of uPAR in internalization of *Borrelia* is dependent on interaction of uPAR with other cell surface molecules. Indeed, uPAR has been shown to facilitate various leukocyte functions, among which adhesion, migration and phagocytosis through interaction with αβ-integrins and other cell surface molecules, but also vitronectin [14,15]. This implies a role for uPAR as a signaling receptor. However, because uPAR is a glycosyl-phosphatidylinositol linked receptor and lacks a cytosolic domain it needs to form functional transmembrane units with other molecules, such as multiple αβ-integrins, G-protein-coupled receptors, and caveolin in order to induce intracellular signaling events leading to cytoskeleton rearrangements and consequent cell movement [14,15]. Since both uPAR and *B. burgdorferi* share many molecules with which they can interact, for example αβ-integrins and vitronectin, it will be challenging to identify the surface molecule with which uPAR associates to facilitate phagocytosis of *B. burgdorferi*.

When we infected uPAR knock-out mice on a mixed C57BL/6 and C3H/HeN background with *B. burgdorferi* these mice exhibited higher *B. burgdorferi* numbers in cardiac tissue two weeks post infection compared to WT littermate controls, which was also associated with decreased phagocytosis of *B. burgdorferi*. Strikingly, in these mice we observed a significantly increased influx of leukocytes, predominantly macrophages, at the atrioventricular junction and at the aortic root compared to WT littermate controls (Fig 5). This further indicates that uPAR's role in migration of leukocytes towards *B. burgdorferi* infected and inflamed organs can be compensated for and is not of in vivo importance, since, otherwise we would have observed diminished influx of leukocytes in hearts from uPAR knock-out mice. In line with this hypothesis, in our vitro migration assays using cells from these mice, we observed impaired migration of uPAR deficient macrophages to C5a, but not to supernatant from a cardiomyoblastic rodent cell line (Supplemental Fig 5). The fact that cells did not migrate better to *B. burgdorferi* stimulated H9c2 cells could be due to production of both

stimulating and inhibitory chemotactic stimuli of these cardiomyoblastic cells upon exposure to *B. burgdorferi*, as has been recently shown for neutrophils [40]. Furthermore, our data indicate that, although the underlying mechanisms appear to be the same, the consequences of uPAR deficiency for the course of murine Lyme borreliosis are dependent on the genetic background of the host. Others have shown that C57BL/6 and C3H/HeN mice harbor similar *B. burgdorferi* numbers after infection, but the severity of symptoms was more pronounced in C3H/HeN mice [30], indicating that the extent of the immune response that is mounted against the spirochete is dependent on the genetic background of the host. Indeed, we have demonstrated that uPAR deficiency in *Borrelia* resistant C57BL/6 mice leads to higher *B. burgdorferi* loads, but to comparable, albeit longer-lived active carditis compared to WT controls. By contrast, uPAR deficient mice on a more susceptible mixed C57BL/6 x C3H/HeN background also exhibited higher *B. burgdorferi* numbers, but more pronounced influx of leukocytes and more severe (peak) carditis. Local cytokines and chemokines induced by *B. burgdorferi* are thought to mediate Lyme carditis. A cytokine that has been implicated to be of paramount importance for local inflammation and migration of leukocytes is IL-1β [41–43]. Also in *B. burgdorferi* infected mice and patients IL-1β has been shown to be upregulated in heart or joints [44–46]. Interestingly, by MLPA we found significantly higher levels of mRNA coding for IL-1β, IL-1 receptor associated kinase (IRAK)-3 (predominantly expressed in macrophages) and TLR2 (the TLR preferentially recognizing *B. burgdorferi* lipoproteins) in hearts from *B. burgdorferi* infected uPAR knock-out mice on the mixed genetic background compared to WT littermate controls (Fig 5). Interestingly, in previous studies we showed that (human) peripheral blood-derived dendritic cells stimulated with the TLR2 ligand lipoteichoic acid (LTA) or viable *B. burgdorferi* produced high levels of IL-1β [47]. By contrast, when the same cells were stimulated with the TLR4 ligand LPS IL-1β levels were undetectable. Macrophages have also been shown to be able to produce IL-1β upon *B. burgdorferi* stimulation [48]. We postulate that, in the in vivo infection experiments, decreased uptake of *B. burgdorferi* resulted in higher local and systemic *B. burgdorferi* numbers. These higher *Borrelia* numbers might have led to an increased influx of leukocytes, as we demonstrated by pathology in mouse hearts and mouse skin, in turn leading to an increased induction of pro-inflammatory molecules. One could argue against the use of F2 mice in our studies, however the fact that F1 WT C57BL/6 x WT C3H/HeN mice are already intermediate susceptible to *Borrelia* infection [30], encouraged us to perform our experiments with F2 mice. In these mice we also obtained surface ECGs during the course of *B. burgdorferi* infection. Using signal averaged ECG analysis with the digital acquisition and analysis Power Lab/4SP system (AD instruments, Oxfordshire, UK) we did not find statistical differences between *B. burgdorferi* infected uPAR deficient and WT animals, although we did observe tachycardia in both groups following *B. burgdorferi* infection (data not shown). On histological examination we did not observe arthritis in *B. burgdorferi* infected WT and uPAR knock-out C57BL/6 mice, not even when mice were sacrificed 6 weeks post infection (data not shown). By backcrossing uPAR deficient mice to the more *Borrelia* susceptible genetic C3H/HeN background, we speculated, based on finding by others [30], we would be able to observe the effect of uPAR deficiency on arthritis. We measured swelling of the hindpaws during the course of infection, however we did not find

significant differences in ankle swelling between the groups (Supplemental fig 5). Furthermore, two and three weeks post infection we could not detect histopathological changes suggestive of arthritis in the hindlimbs of C57BL/6 x C3H/HeN uPAR knock-out and WT littermate controls. The development of edema without histopathological changes has been described previously in response to *Borrelia* infection [29]. Alternatively, we might have sacrificed the mice too early in the course of infection, which could be underscored by the fact that the ankles had not reached maximal swelling yet (Supplemental fig 5). In line with the absence of histopathological changes, no signs of arthritis were observed in post mortem radiological examination of hindpaws three weeks post infection (Supplemental fig 5).

In conclusion, we here show that uPAR is importantly involved in the host defense against *B. burgdorferi* by facilitating leukocyte phagocytosis of *B. burgdorferi*. A better understanding of the role of uPAR in the course of Lyme borreliosis might be of clinical interest since several uPA/uPAR agonists and antagonists have already been tested in oncological trials in humans [49,50] and it has been well-established that late in the course of Lyme borreliosis the infection tends to be more difficult to treat [51]. Therefore compounds modulating the host immune response, in adjunction to antibiotics, may prove to be a valuable asset in both the treatment of early Lyme borreliosis, when the bacterium needs to be cleared, as well as the treatment of late Lyme borreliosis, when ongoing inflammation might be causative of persisting symptoms and when the immune response might need to be dampened.

Material and methods

Mice, spirochetes and infection. Specific pathogen-free wildtype C57BL/6 mice were purchased from Harlan Sprague Dawley Inc. (Horst, The Netherlands) and uPAR knock-out C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) [52]. In addition C57BL/6 uPAR knock-out mice were backcrossed twice to a C3H/HeN - purchased from Jackson Laboratories – background, generating F2 C57BL/6 x C3H/HeN heterozygous uPAR deficient mice. F2 mice were crossed among each other to generate homozygous C3H/HeN x C57BL/6 uPAR knock-out mice and WT littermate controls. uPA, tPA and PAI-1 knock-out mice were also purchased from Jackson Laboratories. All mice were bred in the animal facility of the Academic Medical Center (Amsterdam, The Netherlands). Age-and sex-matched animals were used in each experiment and the Animal Care and Use Committee of the University of Amsterdam approved all experiments. Six to eight-week old mice were infected by intradermal syringe inoculation with 1 x 10^6 *B. burgdorferi* sensu stricto strain B31 clone 5A11 [53], that had previously been recovered from an experimentally infected mouse [29]. Spirochetes were cultured in BSK-II medium, enumerated and inoculated in the midline of the back or with BSK-II medium as a control (SHAM), as described previously [29,54]. Mice were sacrificed by bleeding from the inferior vena cava at the indicated time points, i.e. 2, 4 (or 6 weeks) post infection. Heparin or citrate plasma was stored at - 20 °C for future

use. Skin (inoculation site), urinary bladder, heart and tibiotarsi were saved for histopathological examination, culture or quantitative Polymerase Chain Reaction (q-PCR).

Q-PCR. DNA from murine tissues was obtained with the DNeasy KIT (Qiagen, Venlo, The Netherlands) as previously described [29]. Quantitative PCR detecting *Borrelia flaB* and mouse *β-actin* was performed, as described previously [29]. Standards consisted of dilutions of genomic DNA from *B. burgdorferi* or mouse *ß-actin* (252 bp) cloned into the PCR2.1-TOPO vector (Invitrogen, Breda, The Netherlands), as described previously [29,54]

Arthritis, paw swelling and radiological examination. Histopathological changes in tibiotarsi were assessed as previously described [29,55]. We monitored ankle swelling of both tibiotarsal joints using a Mitutoyo pressure controlled microcaliper (Mitutoyo, Kanagawa, Japan). Measurements were performed several times throughout the course of the infection by the same observer blinded to the experimental design. Lastly, we performed post mortem radiological examination of formalin fixed right hind paws, as described previously [56].

Carditis. Five μm-thick paraffin embedded sections of sagittally dissected hearts were processed and H&E stained by routine histological techniques. Carditis was scored on a scale from 0 to 3 by a pathologist blinded to the experimental design, essentially as previously described [27,28,54], with 0: no carditis; 1: mild carditis; 2: moderate carditis and 3: severe carditis. As described previously [26], 2 weeks post infection, carditis was characterized by disperse inflammation at the atrioventricular junction and aortic root, where as four weeks post infection, organizing inflammation was characterized by the presence of sharply delineated foci in the atrial walls. An F4/80 immunostaining (BMA Biomedicals, Augst, Switzerland) was performed to detect influx of macrophages [57].

Multiplex ligation-dependent probe amplification. MLPA was performed in essence as described before [58]. The genes that were analyzed are listed in the figure legend of Supplemental Figure 3. Equal amounts of mRNA were included per reaction and all samples were tested in a single experiment using the same batch of reagents. The levels of mRNA for each gene were expressed as a normalized ratio of the peak area of the fluorescent intensity (in arbitrary units) and divided by the cumulative peak area of all genes in the assay, resulting in the relative abundances of mRNAs of the genes of interest [59].

Whole cell *B. burgdorferi* ELISA. *Borrelia burgdorferi* sensu stricto strain B31 specific total immunoglobulin (Ig)G and IgG subclasses were measured in heparin plasma from infected animals and controls by ELISA as described previously [29]. All measurements were performed in duplicate.

Amidolytic assays of PA activity. Plasminogen activator was measured as a measure for the activity of the fibrinolytic system using an amidolytic assay as described earlier [23,60]. Briefly, citrate plasma was incubated with S-2251 (Chromogenix, Mölndal, Sweden), plasminogen and cyanogen bromide fragments of fibrinogen (Chromogenix, Milano, Italy). Conversion of plasminogen to

plasmin was assessed by subsequent conversion of the chromogenic substrate S-2251 and was detected with a spectrophotometer.

Stimulation assays. Whole blood and peritoneal macrophages from three naive uPAR knock-out or WT mice were harvested as described [61]. Briefly, 1 x 10^5 adherent macrophages and heparinized whole blood were stimulated in duplo in 96-well microtiter plates (Greiner) with 1 x 10^6 or 1 x 10^7 viable *B. burgdorferi* suspended in Roswell Park Memorial Institute (RPMI) 1640 medium or medium as a negative control for 16h. Supernatants were collected and stored at -20°C until cytokine production was measured by CBA. For assessment of uPAR expression by fluorescence activated cell sorter (FACS), cells were harvested and stained with murine anti-CD87-Phycoerythrin (PE) (BD Pharmingen, Maarssen, The Netherlands). To assess uPAR expression on specific cells, cells were double-stained with anti-GR1-fluorescein isothiocyanate (FITC) (BD Pharmingen) (granulocytes) or F4/80-allophycocyanin (APC) (BD Pharmingen) (monocytes and macrophages). In addition, in non-phagocytosing cells, i.e. $CD4^+$ and $CD8^+$ T cells - stained with anti-CD3-APC (BD Pharmingen) and anti-CD4-FITC or anti-CD8-PerCP respectively (BD Pharmingen) - we also assessed uPAR expression by FACS analysis. Similarly, uPAR expression on human cells derived from heparinized whole blood was analyzed with a human biotin-labeled antibody against uPAR (R&D Systems, Minneapolis, MN) in combination with streptavidin conjugated to PE; these cells were triple-stained with anti-CD15-APC (BD Pharmingen) (granulocytes) and anti-CD14-Cy-Chrome 5 (Cy5) (BD Pharmigen) (monocytes) (BD Pharmingen). Human macrophages were generated as described previously [62]. Briefly, human peripheral blood derived mononuclear cells were isolated from buffy coats by centrifugation over a Ficoll-Paque gradient. Subsequently, adherent monocytes were cultured in X-VIVO medium (BioWhittaker, Walkersville, MD) with 1% heat-inactivated autologous plasma to allow for differentiation to human monocyte-derived macrophages in 7 days. Antibodies were used in concentrations recommended by the manufacturer and FACS analysis was performed using the BD FACScalibur (BD Biosciences, Breda, The Netherlands). Endotoxin concentration in the *B. burgdorferi* culture media was approximately 1 IU/ml, as determined by a Cambrex QCL LAL assay (Cambrex). We established that the maximal amount of LPS that could have possibly contaminated the final *Borrelia* preparation used for the in vitro stimulations - after extensive washing and resuspension in different cell culture media - was insufficient to influence uPAR expression (data not shown). In a separate experiment viable *B. burgdorferi* (1 x 10^8) were injected into the peritoneal cavity of C57BL/6 WT or uPAR knock-out mice for one hour. Hereafter cells were harvested, stained for F4/80, and CD87 (uPAR) expression was measured by FACS analysis

Detection of uPAR mRNA expression in human samples. Transcutaneous skin biopsies were collected from healthy volunteers, i.e. non-inflammed skin, or patients with active Lyme erythema migrans at the Academic Medical Center, Amsterdam, The Netherlands and New York Medical College, NY. IRB approval was obtained from both institutes. All Lyme patient skin samples were tested positive for *B. burgdorferi* spirochetes by in vitro culture and PCR. Skin samples were frozen-ground to fine powder using a china grinder and RNA was extracted using the TRIZOL reagent

from Invitrogen (Carlsbad, CA, U.S.A). RNA samples were treated with TURBO DNase (Applied Biosystems, Foster City, CA, U.S.A) to remove DNA contaminants. RNA was then converted to cDNA using an Affinity Script kit (Stratagene, La Jolla, CA, U.S.A). Quantification of uPAR was performed by Taqman PCR (Applied Biosystems) and normalized to β-actin (*ACTB*). The primers and probes used for uPAR were forward 5'AATCCTGGAGCTTTGAAAATCT 3', reverse 5'CCACTTTTAGTACAGCAGGAGA 3', and probe 5'6FAM-ACTGCCGAGGCCCCATGAATC 3'-TAMRA. Human β-actin primers and probe were inventoried products of Applied Biosystems.

Phagocytosis assays. Phagocytosis assays were performed in essence as described before [63–65]. Viable *B. burgdorferi* were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen) as described by others [66] or heat-inactivated (30 min at 56 °C) non-motile, but intact, *B. burgdorferi* were labeled with fluorescein isothiocyanate (FITC). Adhered peritoneal macrophages (derived from 6-8 mice per group) were incubated with CFSE-labeled *B. burgdorferi* (Cell:*Borrelia* = 1:50) in serum-free RPMI 1640 medium in 24-well microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands) for 0, 15 and 60 minutes at 37 °C. Phagocytosis was stopped by transferring the cells to 4 °C. Extracellular signal of *B. burgdorferi* was eliminated by addition of a quenching solution for one minute - containing Trypan blue that absorbs the fluorescence emission of both FITC and CFSE (Orpegen, Groningen, The Netherlands; [65]) - and three washes with ice-cold PBS. For each sample and each time point 4 °C controls were performed, however there was hardly any phagocytosis detectable under these conditions (data not shown). Cells were resuspended in FACS buffer (PBS supplemented with 0,5% bovine serum albumin (BSA), 0,01% NaN_3 and 0,35 mM EDTA) followed by FACS analysis. At 37 °C the majority of spirochetes was internalized as was determined by control experiments in which we did not add the quenching solution (data not shown). Similarly, to determine neutrophil phagocytosis capacity, 50 µl of whole blood was incubated with 2 x 10^6 viable CFSE-labeled *B. burgdorferi* for the indicated time, after which quenching solution was added for one minute and samples were washed twice with ice-cold FACS buffer. Thereafter cells were incubated with BD Lyse/Fix solution (BD Biosciences) and neutrophils were labeled using anti-Gr-1-PE (BD Pharmingen). Live cells were electronically gated and phagocytosis was determined using FACS. The phagocytosis index of each sample was calculated as previously, described: (mean fluorescence intensity (MFI) x percentage (%) positive cells) at 37°C minus (MFI x % positive cells) at 4°C [63,64].

Migration assays. In vitro migration experiments with murine peritoneal macrophages from WT and uPAR knock-out mice were performed essentially as described[67,68]. Prior to experimentation cells were labeled with CellTracker Green (Molecular Probes, Eugene, Or) in serum-free Dulbecco's modified Eagle's medium (DMEM). The dye was fixed by 1 h incubation in DMEM plus 10% FCS. Thereafter cells were washed and resuspended in serum-free medium and transferred to 3 µM pore size HTS FluoroBlok Cell Culture Inserts (BD Falcon) which were inserted in fitting 24-well plates containing various attractants (*B. burgdorferi*, activated complement factor 5 (C5a)) also in DMEM serum-free medium. Fluorescence, representing the number of cells on the bottom side of the insert, was read

every 2 min on a Series 4000 CytoFluor Multi-Well Plate Reader (Perseptive Biosystems, Framingham, MA). Raw fluorescence data were corrected for background fluorescence and no-attractants controls were subtracted at each measured time point to correct for random migration. Migration start points were set to zero. To mimic the in vivo situation more closely we also performed experiments with an embryonic rodent heart-derived cell line, H9c2 cells (CRL-1446, American Type Culture Collection, Queens Road, Teddington, UK). These cardiomyoblasts were maintained in DMEM with 10% foetal bovine serum (FBS). Prior to experimentation, cells were washed and resuspended in serum-free DMEM and incubated with viable *Borrelia* (Cell:*Borrelia* =1:50) or medium as a control for 16 h. The supernatants were centrifuged for 5 minutes at 1200 x *g* to remove cells and other particles, followed by centrifugation at 4000 x *g* for 15 minute to remove the spirochetes. Supernatants were used undiluted or diluted (data not shown) as chemoattractants in the indicated experiments. All experiments were performed in duplo or in triplo and repeated three times. In addition, we also assessed migration of leukocytes in skin from C57BL/6 WT and uPAR deficient mice in response to *B. burgdorferi* in vivo (n=5 per group). In these set of experiments we intradermally injected C57BL/6 WT mice with 1 x 10^6 *B. burgdorferi* in PBS in the midline of the neck and mice were sacrificed 0, 6 or 32 hours post inoculation. Control animals were injected with PBS. Skin was harvested, formalin fixed and imbedded in paraffin. Five µm-thick sagittal skin sections were processed and H&E, Ly6G and F4/80 stained by routine histological techniques [57]. The control animals did no display influx of leukocytes (data not shown). Slides were scored for influx of leukocytes by an independent pathologist who was blinded to the experimental design. Influx was semi-quantitatively scored on a scale from 0-3, with 0 being no, 1 mild, 2 moderate, and 3 being severe diffuse infiltration.

Statistical analysis. Differences between the groups were analyzed using the two-sided non-parametric Mann-Whitney U test (Graphpad Prism Software version 4.0, San Diego, CA). Where indicated a two-sided Chi-square indicated was applied. Data are presented as the mean ± standard errors of the mean (SEM). A P value of < 0.05 was considered significant, where * indicated $P < 0{,}05$, ** $P < 0{,}01$ and *** $P < 0{,}001$. For ECG data statistical analysis was performed using a multivariate repeated measurements model (SPSS statistics software 17.0).

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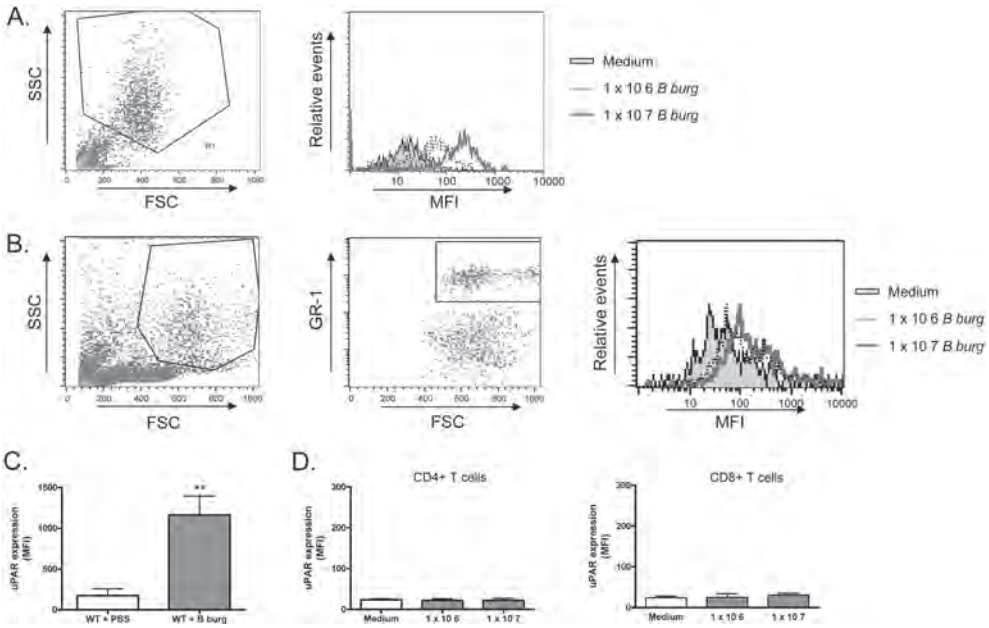
Competing interests. The authors declare that no competing interests exist.

Supporting information



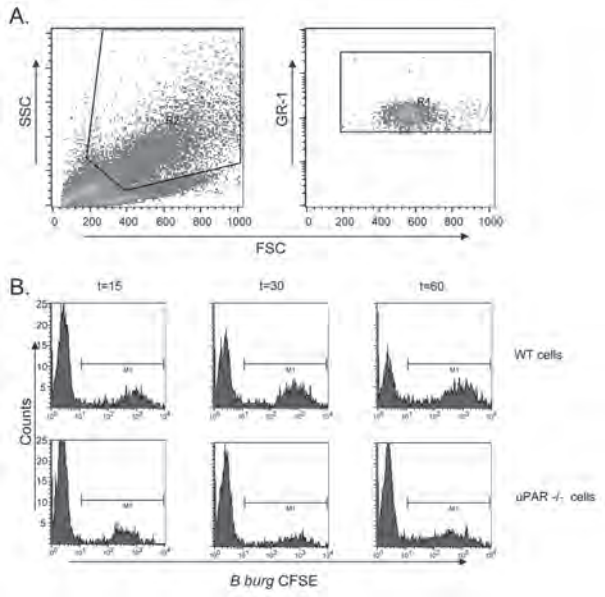
Supplemental figure 1. *Borrelia burgdorferi* induces upregulation of the urokinase receptor on leukocytes in vitro and in vivo.

A. Viable *B. burgdorferi* induces uPAR expression on ex vivo generated human macrophages. Cells were incubated with viable *B. burgdorferi* for 16 hours. Thereafter cells were stained with anti-CD87 (uPAR), electronically gated and analyzed by FACS analysis. Representative cytograms and histograms are shown.

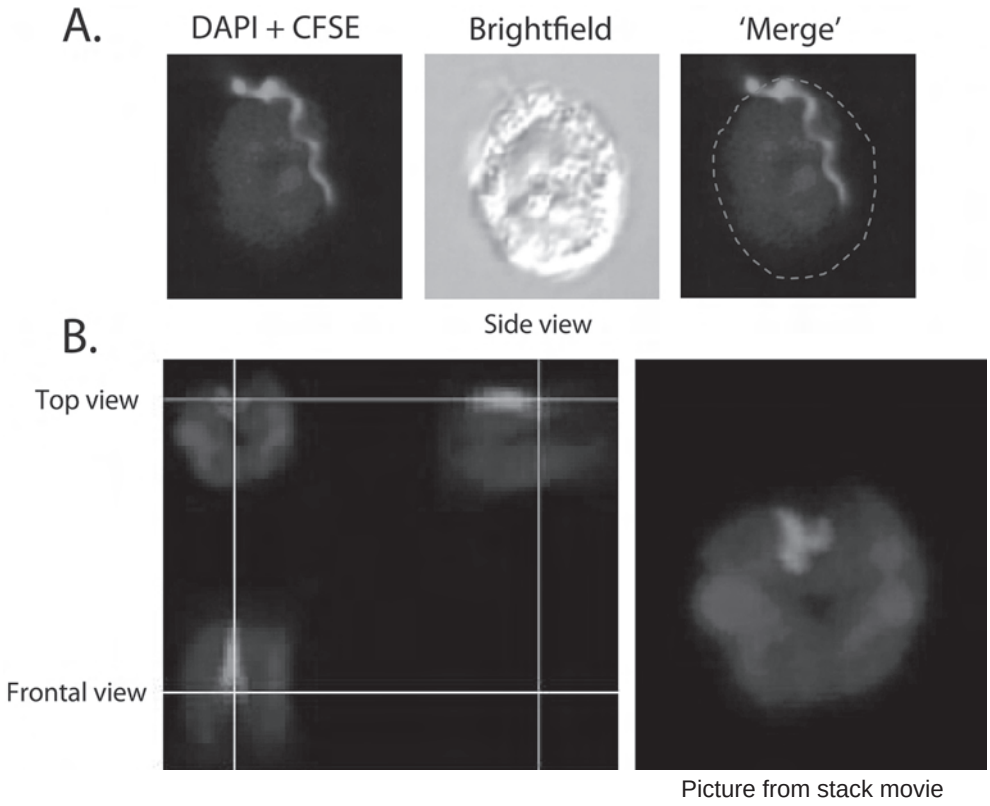
B. Viable *B. burgdorferi* induces uPAR expression on murine granulocytes. Whole blood was incubated with viable *B. burgdorferi* for 16 hours. Erythrocytes were lysed, cells were co-stained with anti-GR-1 and anti-CD87 (uPAR), electronically gated and analyzed by FACS analysis. Representative cytograms and histograms are shown.

C. Viable *B. burgdorferi* (1 x 10^8) were injected into the peritoneal cavity of C57BL/6 WT (n=6) or uPAR knock-out (n=4) mice for one hour. Hereafter cells were harvested, stained for F4/80, and CD87 (uPAR) expression was measured by FACS analysis. A P value < 0.05 was considered statistically significant. * indicating $P < 0{,}05$; ** $P < 0{,}01$

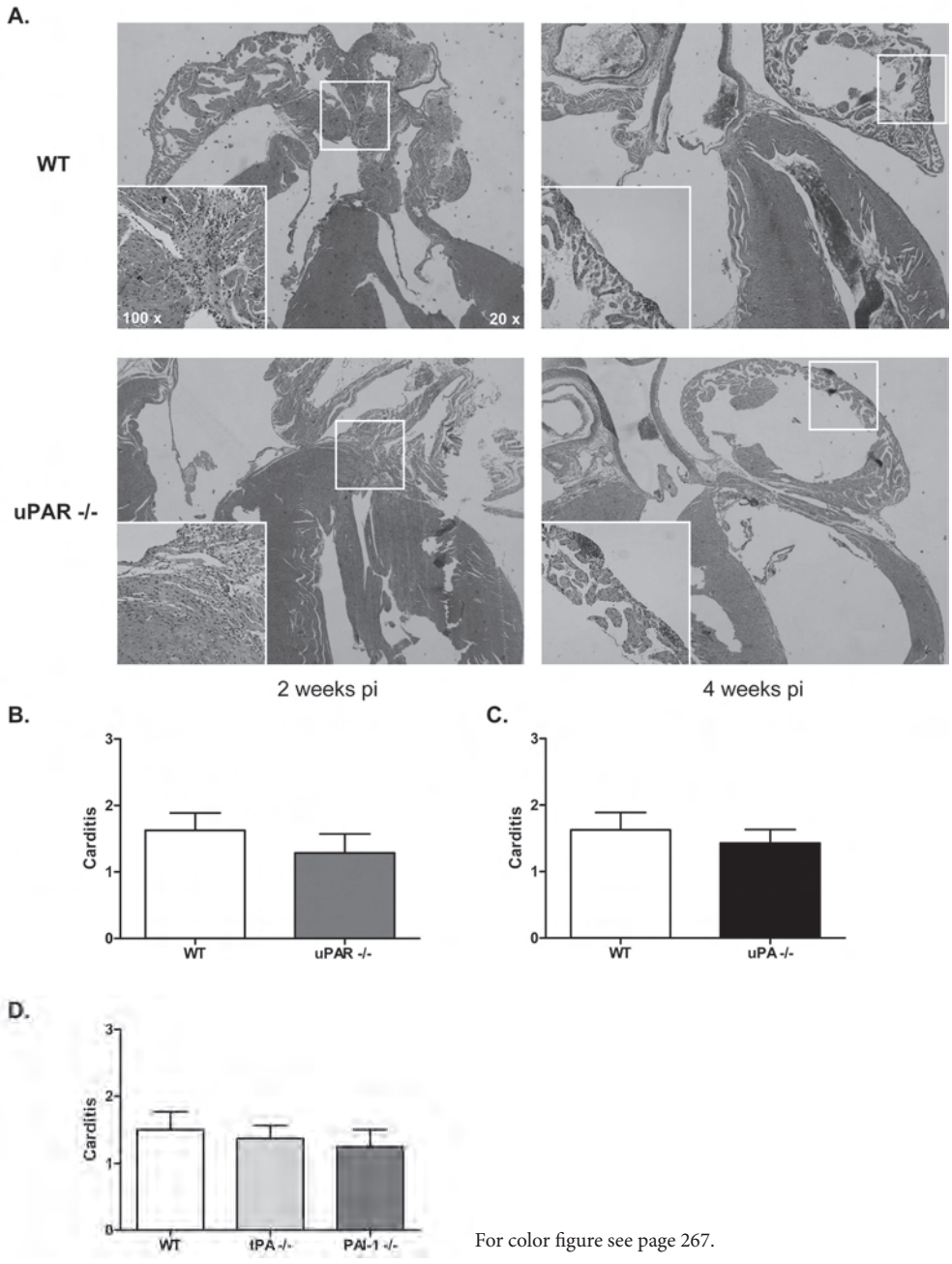
D. In non-phagocytosing cells, i.e. $CD4^+$ and $CD8^+$ T cells - doublestained with anti-CD3-APC (BD Pharmingen) and anti-CD4-FITC and anti-CD8-PerCP, respectively - we also assesses CD87 (uPAR) expression by FACS analysis. Error bars represent the mean of triplicates within one experiment ± SEM.



Supplemental figure 2. Impaired phagocytosis of *B. burgdorferi* by uPAR deficient leukocytes.
After the assays whole blood was lysed and stained with anti-GR-1 (granulocytes), viable cells were gated (left panel A) and GR-1 positive cells (right panel A) were analyzed for intracellular *B. burgdorferi*-CFSE signal over time (B): Assays were performed as described in figure 2. Marker (M)1 encompasses positive cells.



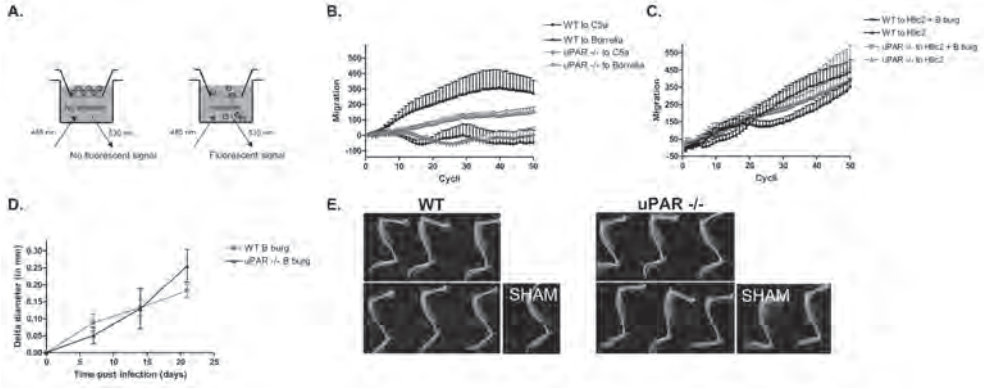
Supplemental figure 3. Confocal microscopy of *B. burgdorferi* phagocytosis.
A and B. Confocal microscopy confirmed that *B. burgdorferi* in in vitro phagocytosis assays were localized intracellularly. Cells incubated with CFSE-labeled *B. burgdorferi* were subjected to confocal microscopy. Nuclei of cells were stained with DAPI. In Panel A we depicted the widest transversal section of a segmented nucleus of a granulocyte stained with DAPI and a CFSE-labeled *B. burgdorferi* spirochete. Superimposing the brightfield image confirms the bacterium is localized intracellularly. Panel B shows another granulocyte and *B. burgdorferi* from different view points (left panel) and a picture from a stack movie (right panel) further verifying that we are assessing internalized bacteria in the in vitro phagocytosis assays. For color figure see page 266.



For color figure see page 267.

Supplemental figure 4. Carditis in WT, uPAR, uPA, tPA and PAI-1 knock-out mice.
A and B. Peak carditis in C57BL/6 uPAR -/- is of similar severity compared to WT controls, although active carditis persists longer in uPAR -/- mice. WT and uPAR -/- mice were inoculated with *B. burgdorferi* and sacrificed two or four week post infection. Sagittal sections of formalin fixed and paraffin embedded hearts were H&E stained. The severity two weeks post infection was scored by a pathologist blinded to the experimental design on a scale of 0-3, with 0: no carditis; 1: mild carditis; 2: moderate carditis and 3: severe carditis. SHAM inoculated mice did not develop

carditis (data not shown). Pictures depict representative sections.
C and D. Peak carditis in C57BL/6 uPA, tPA and PAI-1 knock-out mice is comparable to peak carditis in WT C57BL/6 mice. Carditis was scored as described above. Six to eight mice per group were used and bars represent the mean ± SEM. A *P* value < 0.05 was considered statistically significant.



For color figure see page 268.

Supplemental figure 5. Migration and arthritis in WT and uPAR knock-out mice on a *B. burgdorferi* susceptible genetic background.
A, B and C. Urokinase receptor deficient macrophages from mice on the mixed genetic background can migrate to cardiogenic stimuli just as well as macrophages from WT littermate controls. Migration of CellTracker Green labeled WT or uPAR deficient macrophages towards several chemotactic stimuli was investigated in vitro (A). As chemotactic stimuli we used *B. burgdorferi* or activated complement factor 5 (C5a) (B) and supernatant from the cardiomyoblastic rodent cell line H9c2 stimulated with *B. burgdorferi* or control medium for 16 hours prior to experimentation (C). All experiments were performed in duplo in serum free DMEM medium without the addition of antibiotics and migration was corrected for the no-attractant control. Graphs represent the mean of three independent experiments ± SEM. The fluorescent signal in the lower chamber (indicative of migration) was measured in real time every two minutes (cycli).
D and E. Only edema, no arthritis in uPAR knock-out mice (n=7) and WT littermate controls (n=8). Ankle swelling was measured using a microcaliper during the course of infection (D). In this particular experiment mice were monitored for three weeks. Post mortem, but before decalcification, radiological examination of the right hindlimb was performed (E). No differences between SHAM inoculated and *B. burgdorferi* infected animals were observed.
A *P* value < 0.05 was considered statistically significant. * indicating *P* < 0,05

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PART IV

SPIN-OFF OF RESEARCH ON TICK-HOST-PATHOGEN INTERACTIONS

Salivating for knowledge: potential pharmacological agents in tick saliva

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Chapter 10

Introduction

The incidence of tick-borne diseases has drastically increased over the past few years [1,2], resulting in a marked increase in research on tick–host–pathogen interactions. As a result, the knowledge on molecules present in tick saliva and their function has significantly expanded [3,4]. Ticks are obligate hematophagous ectoparasites, and hundreds of tick species are distributed worldwide. While taking a blood meal, ticks are attached to their host for several days and introduce saliva into the host skin. Like saliva from other hematophagous animals, such as mosquitoes, flies, leeches, and nematode species, tick saliva contains a wide range of physiologically active molecules that are crucial for attachment to the host or for the transmission of pathogens [5], and that interact with host processes, including coagulation and fibrinolysis, immunity and inflammation, and angiogenesis [3,6,7]. In this article, we discuss molecules in tick saliva that have been intensively studied in vitro or in animal models for human diseases, and that, due to their specificity, are potential future anticoagulant or immunosuppressive agents. We also discuss how immunologically targeting specific tick salivary proteins could prevent the transmission of tick-borne pathogens from the tick to the host.

Five key papers in the field

Hepburn et al., 2007 [40] After identification of a specific activated C5 inhibitor, OMCI, the authors showed how this protein can be used in an experimental animal model for myasthenia gravis.

Paveglio et al., 2007 [50] Showed that a T cell inhibitor from tick saliva, Salp15, is able to prevent the development of pathological features in an animal model for atopic asthma.

Labuda et al., 2006 [55] Showed that an anti-tick vaccine, directed against the 64TRP cement protein in tick saliva, prevented lethal infection of mice with the tick-borne encephalitis virus, indicating that anti-tick vaccines could be used to combat tick-borne pathogens.

Ramamoorthi et al., 2005 [5] Showed that *B. burgdorferi*, the causative agent of Lyme disease, uses a protein in tick saliva, Salp15, to establish an infection in the mammalian host, underscoring the complex tick–host–pathogen interactions that are involved in the development of Lyme disease.

Waxman et al., 1990 [9] Identified the first highly specific activated factor X inhibitor in tick saliva, TAP. This research has been the inspiration for numerous researchers working in the field of coagulation.

Anticoagulants

The hemostatic response enables mammals to control blood loss during vascular injury. Platelets adhere to macromolecules in exposed subendothelial tissue and aggregate to form a hemostatic plug, while local activation of plasma coagulation factors leads to generation of a fibrin clot that reinforces the platelet aggregate. The coagulation cascade starts when exposed subendothelial tissue factor (TF) binds to activated factor VII (FVIIa). This complex activates factor X (forming FXa), which mediates the formation of minute amounts of thrombin that activate other coagulation proteases and additional platelets. Subsequently, by means of two amplification loops (Figure 1), more thrombin is generated, which leads to fibrinogen-to-fibrin conversion and fibrin deposition [8].

Tick feeding is hampered by the hemostatic response of the host. Therefore tick saliva contains an extensive selection of molecules that counteract coagulation, enhance fibrinolysis, and inhibit platelet aggregation [7]. Traditional anticoagulant agents such as unfractionated heparin and vitamin K antagonists (e.g., warfarin) have a narrow therapeutic index, requiring frequent monitoring and dose adjustments [7]. Tick saliva presents a possible source of novel, and ideally more easily used, anticoagulant agents (Figure 1) [7].

FXa inhibitors

Saliva from the soft tick *Ornithodoros moubata* contains a serine protease inhibitor of FXa, tick anticoagulant peptide (TAP). TAP is a tight-binding specific FXa inhibitor that inhibits clotting of human plasma ex vivo [9]. The inhibitory characteristics and the high selectivity of recombinant forms of TAP (rTAP) for FXa are due to the interaction of rTAP with the active site as well as with regions remote from the active site pocket of FXa [10]. rTAP has been tested in a variety of animal models for both venous and arterial thrombosis [11–13]. A recent study showed that rTAP, when fused to a single-chain antibody specifically targeting activated platelets (through binding to the platelet receptor GPIIb/IIIa), had highly effective antithrombotic properties in comparison to enoxaparin in a murine carotid artery thrombosis model. In addition, in contrast to conventional anticoagulants tested, the TAP–antibody fusion protein did not prolong bleeding time [14]. Future research should reveal whether this or similar approaches are equally effective and safe in humans. Other FXa inhibitors characterized in tick saliva are shown in Table 1 [15,16].

Tissue factor pathway inhibitors

In view of the central role of TF in the initiation of coagulation in both physiological and pathological states, targeting TF may be an effective antithrombotic strategy. Tick saliva contains several TF pathway inhibitors (TFPIs) (Table 1) [7,17]. Recently, Ixolaris was identified in saliva from the deer tick *Ixodes scapularis* [17]. Ixolaris has two kunitz-like domains, a type of domain conserved in a wide family of serine protease inhibitors, and sequence homology to human TFPI [18]. In a rat model for venous thrombosis, administration of recombinant Ixolaris resulted in effective antithrombotic activity, without hemorrhage or bleeding [19]. Because of its fast and tight binding to FXa, giving

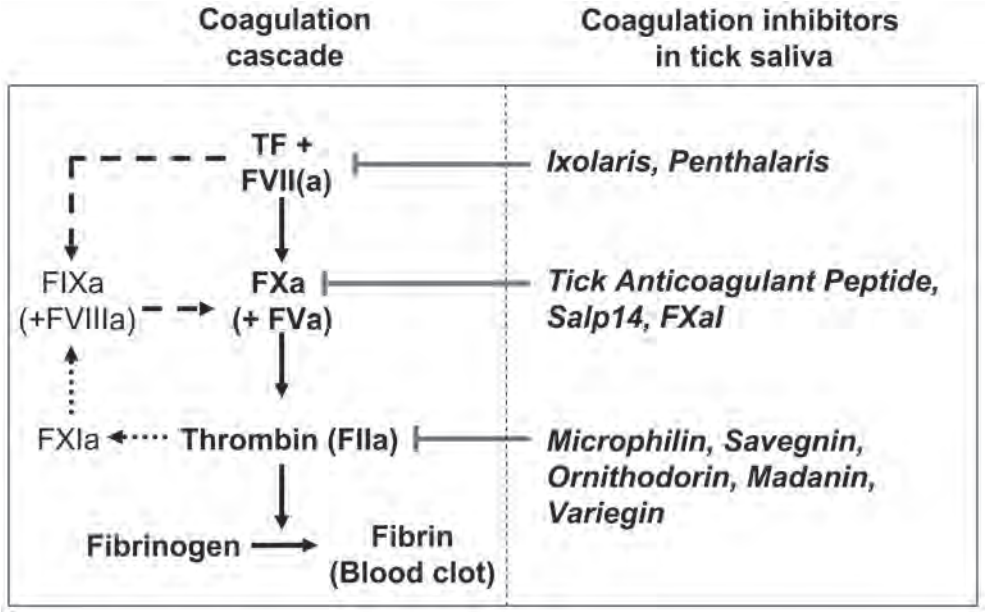

Figure 1. Schematic overview of the coagulation cascade. The two major amplification loops in the coagulation cascade are depicted. The first amplification loop consists of TF-FVIIa-mediated factor IX (FIX) activation, which leads to the generation of more FXa. A second amplification loop is formed by the activation of factor XI (FXIa) by thrombin, which results in more activated FIX (FIXa), and, subsequently, additional FXa generation. The right panel indicates how selected tick proteins exert their anticoagulant effect. FIIa, activated factor II; FVa, activated factor V; FVIIIa, activated factor VIII. For color figure see page 269.

rapid-acting, selective, and long-lasting effects, and the encouraging results in vivo, Ixolaris could serve as a template for potential new anticoagulant agents targeting the TF pathway.

Direct thrombin inhibitors

In comparison with heparin (derivatives), which act via antithrombin, direct thrombin inhibitors more effectively inhibit clot-bound thrombin, which is likely to result in a stronger antithrombotic effect [20]. Several specific direct thrombin inhibitors have been characterized in tick saliva (Table 1) [7,21–24], but most have not yet been tested in vivo. Recently a new direct thrombin inhibitor, variegin [25], was characterized from the tropical bont tick, *Amblyomma variegatum*, and shown to be structurally similar to, but much more potent than, hirulog, a 20-amino-acid synthetic thrombin inhibitor based on the natural leech peptide hirudin. Hirulog belongs to a class of drugs that have been approved for treatment of patients with acute coronary syndromes who are undergoing percutaneous coronary intervention [26].

Immunosuppressors

Cellular innate immune responses, depending on invariant receptors such as the Toll-like receptors, are one of the first lines of defence against invading microbes. Another important innate defence

Table 1. Anticoagulants and immunosuppressors in Tick Saliva.

Molecule	Accession number(s)	Tick species	Target(s)	Additional information	Type of experiments	Animal disease model(s)	Reference(s)
Anticoagulants							
Faxtor Xa inhibitors							
Tick Anticoagulant Peptide (TAP)	GI1421459	*Ornithodoros moubata*	FXa	Slow tight binding 60 amino acid serine protease	Human in vitro and animal in vivo studies	Arterial and venous thrombosis	9 11
Salp14	AAK97824	*Ixodes scapularis*	FXa	RNAi of Salp14 in *I. scapularis* resulted in 60-80% reduction of anti-FXa activity of *I. scapularis saliva*	Human in vitro studies	–	16
Factor Xa inhibitor (FXaI)	AAN76827	*Ornithodoros savignyi*	FXa	Recombinant FXaI consists of 60 amino acids and inhibits FXa by 91%. FXaI shares 78% homology to TAP.	Human in vitro studies	–	15
Tissue Factor Pathway Inhibitors							
Ixolaris	AAK83022	*Ixodes scapularis*	Inhibits TF/FVIIa - induced activation of FX	Sequence homology to human Tissue Factor Pathway Inhibitor (TFPI), 2 kunitz-like domains	Human in vitro studies and animal in vivo studies	Venous thrombosis	18 19
Penthalaris	AAM93638	*Ixodes scapularis*	Inhibits TF/FVIIa - induced activation of FX	Sequence homology to human Tissue Factor Pathway Inhibitor (TFPI), 5 tandem kunitz domains	Human in vitro studies		17
Direct thrombin inhibitors							
Microphilin	Not done	*Boophilus microplus*	Thrombin exosite 1	Small thrombin inhibitor (1,8 kDa)	Animal in vitro studies	-	21
Savignin	AAL37210	*Ornithodoros savignyi*	Thrombin active site and exosite 1	2 kunitz-like domains	Human in vitro studies	–	23
Ornithodorin	P56409	*Ornithodoros moubata*	Thrombin active site and exosite 1	2 kuntiz-like domains resembling the basic pancreatic trypsin inhibitor (BPTI)	In silico studies	-	24
Madanin 1 and 2	AAP04349, AAP04350	*Haemaphysalis longicornis*	Thrombin exosite 1	No homology to other direct thrombin inhibitors, estimated KD of 25 and 34.5 nM respectively	Human in vitro studies	–	22
Variegin	Described in the original paper not (yet) submitted	*Amblyomma variegatum*	Thrombin active site and exosite 1	A polypeptide of 32 amino acids that is a potent inhibitor of thrombin and is structurally and functionally similar to hirulog	Human in vitro studies	–	25
Immunosuppressors							
Complement inhibitors							
OMCI	AAT65682	*Ornithodoros moubata*	C5	A 16 kD protein with a lipocalin fold that interferes with C5 activation through prevention of interaction of C5 with C5 convertase	Human in vitro studies and animal in vivo studies	Myasthenia gravis	34 39

Table 1. Continued.

Molecule	Accession number(s)	Tick species	Target(s)	Additional information	Type of experiments	Animal disease model(s)	Reference(s)
Isac	AAF81253	*Ixodes scapularis*	C3 convertase	A 18.5 kDa protein which acts as a regulator of complement activation, similar to human factor H, by interacting with C3 convertase	Human in vitro studies	–	37
IRAC 1 and 2	AAX63389, AAX63390	*Ixodes ricinus*	-	Isac paralogues, also inhibiting the alternative complement pathway	Human in vitro studies	–	35
Salp20	AAK97820	Ixodes scapularis	C3 convertase	Like Isac, and IRAC 1 and 2, Salp20 and its homologues are part of a larger Isac protein family	Human in vitro studies	–	36
T-cell inhibitors							
Salp15	AAK97817 ABU93613	*Ixodes scapularis* *Ixodes ricinus*	CD4+ T-cells	Salp15 binds to CD4 impairing TCR-induced signaling resulting in impaired IL-2 production and T-cell proliferation	Animal in vitro and in vivo studies	Atopic asthma	45 46
			B. burgdorferi OspC	Salp15 also binds *B. burgdorferi* OspC protecting the spirochete from antibody-mediated killing			5
IL-2 binding protein	Not done	*Ixodes scapularis*	IL-2	A protein in tick saliva that inhibits proliferation of human T-cells and CTLL-2 cells	Animal and human in vitro studies	–	47
Iris	CAB55818	*Ixodes ricinus*	T lymphocytes and macrophages	Iris modulates T lymphocyte and macrophage responsiveness by inducing Th2 type responses and by inhibiting the production of pro-inflammatory cytokines	Animal and human in vitro studies	–	49
			Several serine proteases	Iris has also been shown to have anti-hemostatic responses Iris has been tested as an anti-tick vaccine candidate			51 57
Sialostatin L	GI22164282	*Ixodes scapularis*	Cytotoxic T lymphocyte	Sialostatin L specifically inhibits cathepsin L activity	Animal in vitro studies	–	48
B-cell inhibitors							
B-cell inhibitory protein (BIP)	Not done	*Ixodes ricinus*	B-cells	BIP inhibits *B. burgdorferi* antigen-induced proliferation of B-cells	Animal in vitro studies	–	42
B-cell inhibitory factor (BIF)	Described in the original paper, not (yet) submitted	*Hyalomma asiaticum asiaticum*	B-cells	BIF inhibits LPS-induced proliferation of B-cells	Animal in vitro studies	–	41

Note. RNAi: RNA interference, K_D: Binding constant, kD: kilo Dalton, TCR: T-cell receptor, IL-2: Interleukin 2, B. burgdorferi OspC: *Borrelia burgdorferi* outer surface protein C, CTLL-2: cytotoxic T-lymphocyte cell line 2

system is the complement cascade. Activation of the complement system leads to opsonization of an invading pathogen as well as formation of the membrane attack complex that can lyse invading bacteria. The more specific adaptive immune response, which responds against pathogens that bypass the innate immune response, is triggered when activated antigen-presenting cells migrate to lymphoid tissue. In lymph nodes, antigen-presenting cells present processed antigen to T cells, which, upon activation, play a central role in cellular immune responses at the site of infection, or assist in the activation of B cells for the generation of an antigen-specific humoral response.

Ticks acquire a blood meal over a period of days, allowing the host sufficient time to generate anti-tick immune responses. The tick, in turn, has developed mechanisms to protect itself against host inflammation and immune responses [4]. In light of the central role of the complement cascade and T and B cells in many human diseases, we focus on specific tick salivary molecules that target these responses.

Complement inhibitors

The complement system is involved in the pathogenesis of many autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus, and multiple sclerosis, and also in ischemia-reperfusion injury as observed in acute myocardial infarction or ischemic stroke [27–29]. Inhibitors of the complement cascade are therefore of potential clinical interest. Many agents inhibit complement factor 3 (C3) convertase early in the complement cascade, but this inhibition can result in immunosuppression, impairment of opsonization, or immune complex deposition. Novel complement inhibitors should therefore preferably inhibit the complement cascade downstream of complement factor 5 (C5), allowing the upstream cascade to proceed physiologically. Early randomized controlled clinical trials studying the effect of an antibody targeting C5 in acute myocardial infarction showed promising results [30,31], although a more recent randomized controlled trial showed no beneficial effect on all-cause mortality of a C5-antibody compared to placebo [32]. A similar antibody was shown to be effective in the treatment of autoimmune diseases [33].

Tick saliva contains many molecules that specifically inhibit complement activation (Table 1) [34–38]. A promising tick complement inhibitor is the C5 activation inhibitor from the soft tick *O. moubata*, OMCI [34,39]. OMCI inhibits C5 activation by interfering with C5 convertase [39], and has been shown to inhibit human complement hemolytic activity and the development of pathological features in a rodent model for autoimmune myasthenia gravis [40].

B cell inhibitors

The *I. ricinus* B cell inhibitory protein (BIP) is one of the tick salivary proteins that suppress proliferation of murine B cells (Table 1) [41,42]. Suppression of B cell responses benefits the tick by inhibiting specific anti-tick antibody responses that could lead to rejection by the host. In addition, B cells are unable to respond adequately to *Borrelia burgdorferi* antigens in the presence of BIP,

suggesting that *B. burgdorferi* might also benefit from BIP-mediated B cell suppression. Specific inhibition of B cells has been shown to be effective in clinical studies of lymphoproliferative disorders and autoimmune diseases, such as rheumatoid arthritis and multiple sclerosis [43,44]. In order to serve as a template for novel drugs specifically targeting B cells, tick B cell inhibitors need further characterization.

T cell inhibitors

The *I. scapularis* 15 kDa salivary protein, Salp15, is an example of a feeding-induced protein that inhibits the activation of T cells (Table 1) [45–49]. Salp15 specifically binds to the CD4 molecule on CD4+ T (helper) cells, which results in inhibition of T cell receptor–mediated signaling, leading to reduced interleukin-2 production and impaired T cell proliferation [46]. In an experimental mouse model of allergic airway disease, Salp15 prevented the development of atopic asthma [50], suggesting that Salp15 might be used to modulate atopic disease as well as T cell–driven autoimmune diseases. We have shown that Salp15 also inhibits inflammatory cytokine production by human monocyte-derived dendritic cells by interacting with the C-type lectin receptor DC-SIGN [51], indicating that Salp15 has the potential to modulate human adaptive immune responses. Iris, an immunosuppressive protein from *I. ricinus*, has been shown to modulate T cell responses through inhibition of interferon- and to inhibit interleukin-6 and tumor necrosis factor-a production by human macrophages [49]. In addition, Iris also has been shown to have anti-hemostatic effects by inhibiting several serine proteases involved in the coagulation cascade and fibrinolysis [52].

New strategies to prevent tick-borne diseases

Understanding the importance of specific tick salivary proteins for attachment to the host and for transmission of pathogens may permit us to develop new strategies (e.g., anti-tick vaccines) for preventing tick-borne diseases. The idea of a tick-antigen-based vaccine is supported by the observation that repeated exposure of certain animals to tick bites results in an inability of ticks to successfully take a blood meal [45]. These animals, as well as humans who develop hypersensitivity after repeated tick bites [49], are less likely to be infected by tick-borne pathogens [53]. Ideally, an anti-tick vaccine would protect against infestation by a wide range of tick species and prevent transmission of multiple tick-borne pathogens.

Discussing all tick antigens that have been assessed in vaccination trials is beyond the scope of this article. For an overview of the current stage of development of anti-tick vaccines, there is an excellent review available [54]. An interesting example of an anti-tick vaccine that also protects against the transmission of a tick-borne pathogen is a vaccine targeting the salivary cement protein, 64P, from the tick *Rhipicephalus appendiculatus* [55,56]. Tick feeding on animals immunized with truncated recombinant forms of 64P (64TRP) resulted in local inflammatory responses and protection against infestation by a wide range of tick species [56]. Importantly, 64TRP-vaccinated mice challenged with tick-borne encephalitis virus (the most important human vector-borne viral infection in Europe [57])

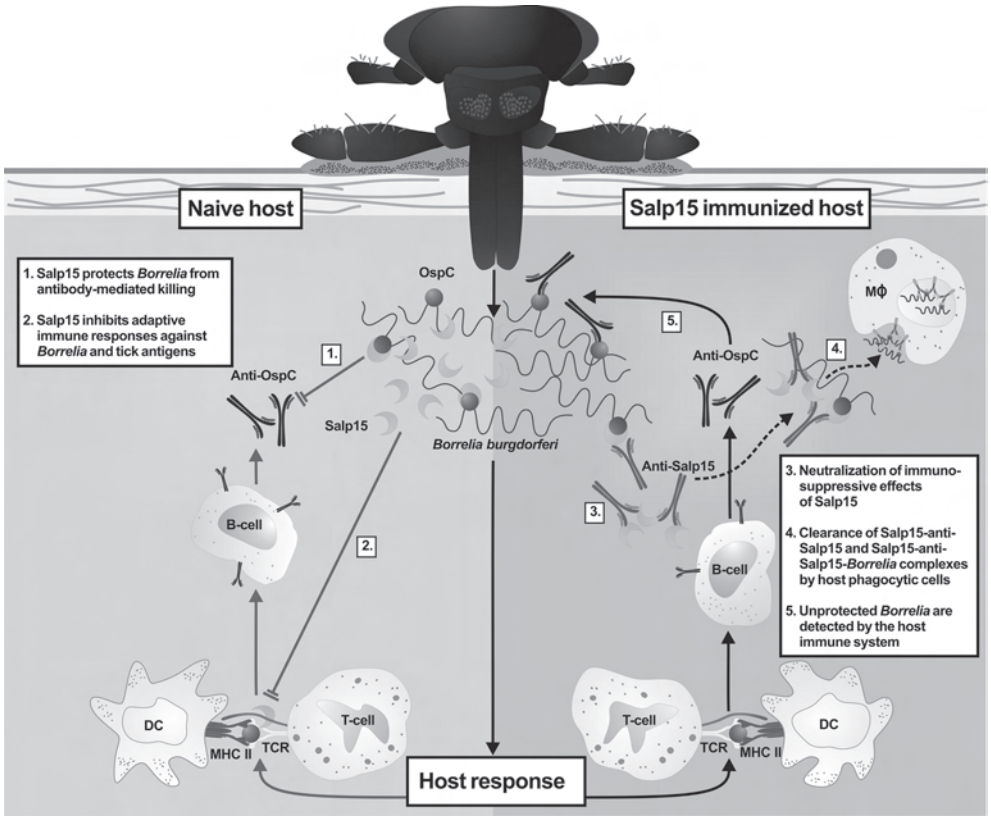

Figure 2. Diagram showing how an anti-Salp15 vaccine could prevent transmission of *B. burgdorferi*. During tick feeding and early mammalian infection, *B. burgdorferi* expresses OspC, which binds to Salp15 in tick saliva. This binding acts as a shield and protects the spirochete from killing by the host. In addition, Salp15 has been shown to directly inhibit dendritic cell and T cell activation, which could facilitate tick feeding. Salp15 antibodies are likely to bind to Salp15 that has previously bound to OspC on the surface of *B. burgdorferi* in the tick salivary gland and could thereby enhance clearance by host phagocytes. Obviously, the Salp15 antibodies would need to recognize a Salp15 epitope other than the epitope that is required for binding of Salp15 to OspC. Similarly, if anti-Salp15 antibodies were to bind to free Salp15, they could neutralize the immunosuppressive effects of Salp15, which could hamper tick feeding and thereby transmission of *B. burgdorferi* from the tick to the host. Lastly, if anti-Salp15 antibodies were to inhibit binding of Salp15 to *Borrelia* OspC, this would render the spirochete susceptible to pre-existing or newly generated immunoglobulins. Importantly, Salp15 was originally identified by screening of a tick salivary gland cDNA expression library with tick immune rabbit sera, suggesting that antibodies against Salp15 may participate in tick rejection. DC, dendritic cell; MHC, major histocompatibility complex; MF, macrophage; TCR, T cell receptor. For color figure see page 270.

through tick bite were protected from lethal encephalitis [55]. Proteins that enhance tick feeding may also modulate host immune responses to pathogens, thus playing a double role in transmission. For example, an *I. scapularis* tick can introduce both Salp15 and *B. burgdorferi* into the host skin. As described earlier, Salp15 may enhance tick feeding by inhibiting host immune responses to tick antigens. In addition, the *B. burgdorferi* outer surface protein C (OspC) has been shown to bind to Salp15 in tick saliva [5]. This binding acts as a shield that protects the spirochete against the host immune response (Figure 2). Salp15 would therefore be a candidate to consider for immunization

studies. Also, the pleiotropic protein Iris, that not only modulates T cell responses, but also specifically disrupts coagulation [52], could be an interesting candidate. Recently, it was shown that vaccinating rabbits with Iris partially protected these rabbits from tick infestations [58].

Conclusion

Tick saliva is a potential source for novel pharmacological agents that could be useful for clinical practice. Future research must confirm whether these specific and potent molecules, with promising results in animal models and in human ex vivo experiments, are effective in humans in vivo. The molecules discussed are only a selection of the many physiologically active molecules that have been identified and characterized. However, this selection illustrates the impressive resourcefulness that ticks display to modulate host processes, and demonstrates how we could use these molecules to our benefit. Undoubtedly, future research on tick–host and tick–host–pathogen interactions will reveal even more potential molecules that could be used in clinical practice.

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Summary and general discussion

Chapter 11

Lyme borreliosis is an emerging zoonosis caused by the spirochetal agent *Borrelia burgdorferi* sensu lato, which is primarily transmitted by *Ixodes* ticks [1–3]. Recently we have began to realize that the pathogenesis of Lyme borreliosis is dependent on a plethora of complex interactions between tick, host and pathogen [4], of which a selection is described in this thesis. We have divided this thesis in multiple sections and herein describe in molecular detail newly identified tick-host-pathogen interactions and how these could influence the course and outcome of *Borrelia* infection.

As an introduction to the thesis, in **chapter 1,** we review the literature on tick-host-pathogen interactions currently known to play a role in the pathogenesis of Lyme borreliosis [4]. We elaborate on how tick salivary proteins modulate host immune responses and how this could affect both the process of tick feeding and pathogen transmission from the tick to the host. In addition, we review *B. burgdorferi* genes that are preferentially expressed while *Borrelia* resides in the tick and the vertebrate host and describe how direct interaction of the gene products with either tick or host proteins facilitates spirochete survival throughout the enzootic life cycle.

PART I: Tick-host interactions

Ticks acquire a bloodmeal over a period of days, giving the host sufficient time to generate anti-tick immune responses [5]. However, the tick, in turn, has developed mechanisms to protect itself against host inflammation and immune responses. Tick saliva that is introduced in the host skin during tick feeding has multiple effects, including inhibition of the complement cascade [6], impairment of NK [7] and dendritic cell function [8], reduction of antibody titers [9], repression of production of cytokines, such as interleukin (IL)-2 and IFN-γ [10–14], IL-4 [15] and IL-10 [16], blocking of chemokine activity [17], and inhibition of T lymphocyte proliferation [13,18]. Importantly, immunosuppression by tick saliva may result in more efficient transmission of several tick-borne pathogens [19,20]. By screening a cDNA *I. scapularis* salivary gland expression library Das and colleagues identified several salivary gland proteins that were recognized by antibodies from so-called 'tick-immune' animals; rabbits that were repeatedly exposed to ticks and that developed antibodies against tick salivary gland components resulting in an acquired resistance to tick infestation [21,22]. One of these proteins was Salp15, a secreted 15 kDa salivary protein that was expressed in fed, but not in unfed, *I. scapularis* ticks. Other salivary proteins that were identified by this method include Salp14 and Salp9pac, which were later shown to possess anticoagulant activity [23–25]; Salp20, that inhibited the alternative complement cascade [26,27]; Salp16, that facilitated uptake from another tick-borne pathogen, *Anaplasma phagocytophilum*, by the tick [28]; and Salp25D, that inhibited the oxidative burst from neutrophils enabling acquisition of *B. burgdorferi* from the host by the tick [29], a prerequisite for the continuation of the enzootic life cycle of the pathogen. Salp15 showed weak homology to Inhibin A, a protein that is part of the transforming growth factor (TGF)-β superfamily, a family of proteins with immunosuppressive activity. Therefore, in this section of the thesis, we investigated the immunosuppressive activity of Salp15.

In **chapter 2** we describe how the feeding induced and secreted *I. scapularis* salivary protein, Salp15, directly inhibits T cell activation and proliferation. Salp15 interferes with early T cell receptor (TCR)-triggered interleukin (IL)-2 production by $CD4^+$ T cells *in vitro*, and also inhibits T cell-mediated immune responses *in vivo* [30]. Recently the research group of Anguita showed that the C-terminus of Salp15 binds to the CD4 co-receptor on T cells [31], interfering with early TCR-signaling events [32]. These data provide a molecular basis for understanding the immunosuppressive activity of *I. scapularis* saliva and vector-host interactions.

In **chapter 3** we further investigated the immunosuppressive effects of *I. scapularis* Salp15 and demonstrate that Salp15 is capable of inhibiting human dendritic cell (DC) function [33]. DCs are sentinel cells abundantly present in the dermis (the site of the tick-bite), and are of crucial importance for the initiation of an adequate host immune response [34]. We show that Salp15 inhibits production of the pro-inflammatory cytokines IL-6, TNF-α and IL-12p70 by DCs, as well as DC-induced T cell activation. Salp15 binds to the C-type lectin receptor DC-SIGN on the surface of DCs, which results in activation of the serine/threonine kinase Raf-1. Strikingly, Raf-1 activation by Salp15 leads to mitogen-activated protein kinase kinase (MEK)-dependent decrease of IL-6 and TNF-α mRNA stability and impaired nucleosome remodeling at the *IL-12p35* promoter.

Immunosuppression induced by a tick salivary protein is likely to be instrumental to both tick feeding and *B. burgdorferi* infection by making the tick-host-pathogen interface a less hostile environment for both the tick as well as the spirochete. This section of the thesis provides new insights into the molecular mechanism of immunosuppression by a specific tick salivary gland protein and might lead to the development of novel anti-inflammatory or immunosuppressive agents. Noteworthy, studies to investigate the potential application of tick salivary proteins as immunosuppressive agents are currently being performed at the Academic Medical Center and are part of future research projects.

PART II: Tick-pathogen interactions

The main vector for Lyme borreliosis in Europe is the tick *I. ricinus,* whereas in the United States *B. burgdorferi* sensu stricto is mainly transmitted by *I. scapularis*. In **chapter 4** we set out to identify whether *I. ricinus* also contained Salp15-like proteins. Using reverse transcriptase polymerase chain reaction (RT-PCR) techniques we show that indeed *I. ricinus* saliva contains at least three Salp15-like proteins, with one of the proteins, Salp15 Iric-1, sharing 80% similarity evenly distributed over the entire amino acid sequence [35]. Our findings were corroborated by others that identified principally similar Salp15-like proteins in fed *I. ricinus* salivary glands [36]. Comparison of our protein sequences with those deposited in several databases indicated that these proteins are part of a Salp15 family of which members are conserved among different *Ixodes* tick species, which are

all capable of transmitting *B. burgdorferi* sensu lato. This suggests that these proteins might have similar activities as *I. scapularis* Salp15.

Recently, it was shown that the interaction between tick, host and *B. burgdorferi* was even more complex than previously assumed. Ramamoorthi et al. showed that *I. scapularis* Salp15 binds to *B. burgdorferi* outer surface protein (Osp)C. This was shown to coat the outer surface of the spirochete with Salp15, protecting *B. burgdorferi* from antibody-mediated killing in mice immune to *B. burgdorferi* [37]. In Europe, apart from *B. burgdorferi*, *B. garinii* and *B. afzelii* are transmitted by *I. ricinus*. These *Borrelia* species can also cause Lyme borreliosis [38,39]. Therefore, we investigated the interaction of a Salp15 homologue in *I. ricinus*, Salp15 Iric-1, with *Borrelia* isolates representative of the three major European pathogenic *Borrelia* species. In **chapter 5** we demonstrate that Salp15 Iric-1 binds to OspC from all European *Borrelia* species in vitro. However, only *B. burgdorferi* was protected from antibody-mediated killing by Salp15 Iric-1 in vivo, putatively giving this *Borrelia* species a survival advantage in nature [40].

In the aforementioned study by Ramamoorthi and colleagues [37] it was shown that Salp15 also provided *B. burgdorferi* with a survival advantage in naive mice, i.e. mice not previously exposed to *B. burgdorferi*. Obviously, naive mice do not have specific antibodies against *B. burgdorferi*. In **chapter 6** we show that binding of *I. scapularis* Salp15, but also Salp15 Iric-1, to isolates from European *Borrelia* species that are sensitive to killing by the complement system provides protection against complement-mediated borreliacidal activity in vitro [41]. Others have shown that specific *B. burgdorferi* strains can also produce complement regulating-acquiring surface proteins (CRASPs) [42] and Osp E/F related proteins (Erps) [43–45] to bind factor H or factor H-like (FHL)-protein and consequently inhibit complement-mediated borreliacidal activity by itself.

In nature, the mechanisms described in **PART I and II** might contribute in parallel to the modulation of host immune responses by tick saliva and *B. burgdorferi* and collectively assist the spirochete in establishing an infection. Our research has delineated new molecular interactions that occur at the tick-host-pathogen interface and provide insight into the (early) pathogenesis of Lyme borreliosis and other tick-borne illnesses. Besides, research such as presented in **PART I and II** may lead to the identification of novel tick salivary protein candidates for anti-tick vaccines, since interfering with tick-host-pathogen interactions that are crucial for pathogen transmission, e.g. by an anti-tick vaccine based on specific tick salivary proteins, could prevent the transmission of *B. burgdorferi* from the tick to the host.

PART III: Host-pathogen interactions

A variety of interactions between host molecules and *B. burgdorferi* proteins determine the outcome of *B. burgdorferi* infection. During the initial stages of mammalian infection, when adhesion to extracellular host components may assist in survival of spirochetes that are transmitted

through a tick bite, *Borrelia* upregulates several proteins on its outer surface, for instance OspC [46–51], Decorin binding protein A and B [52] and the fibronectin binding protein BBK32 [53]. By contrast, most likely due to host immune pressure, during later stages of infection many exposed lipoproteins are downregulated [51,54]. Some *B. burgdorferi* genes are even either differentially up- or downregulated dependent on the tissue that is colonized [55,56]. In addition, as previously discussed, the spirochete attempts to evade the host immune system by inhibiting the complement cascade [42–45]. Importantly, during persistent infection, *B. burgdorferi* also tries to evade host immune responses by recombination at the variable major protein like sequence (*vls*) locus [57,58]. The *B. burgdorferi vls* locus consists of a *vls* expression site (*vlsE*) and 15 unexpressed upstream silent cassettes [59]. Segments of the silent *vls* cassettes randomly recombine into the *vlsE* expression site, resulting in multiple variations of the VlsE protein during the course of infection [59–61]. The examples detailed above underscore how well the spirochete is adapted to survive in the mammalian host.

The host in turn, upon infection with *B. burgdorferi*, mounts an immune response in an attempt to eradicate the spirochete. A great deal of our current knowledge on the host immune response against *B. burgdorferi* comes from the murine model for Lyme borreliosis [62]. As for all infections, the initial defense against *Borrelia* is formed by innate immune responses. A crucial line of defense in the innate immune response against *Borrelia* is the complement cascade, consisting of the classical, the lectin, and the alternative pathways. These three pathways merge at the common intersection of the complement factor C3 and can lead to formation of the membrane attack complex (MAC), which causes lysis of complement sensitive *Borrelia* isolates [63]. In addition, activation of the complement cascade can lead to opsonization followed by phagocytosis of *Borrelia* by, for instance, polymorphonuclear leukocytes, monocytes and macrophages [64–66]. These cells can also produce pro- or anti-inflammatory cytokines and chemokines upon recognition of Borrelial lipoproteins through a variety of invariant cell-surface receptors, among which Toll-like receptors (TLRs). *B. burgdorferi* is predominantly recognized by TLR2 [67]. Indeed, mice deficient for TLR2 have spectacularly increased systemic *Borrelia* numbers upon experimental infection [68,69]. Strikingly, patients with a polymorphism in the *TLR2* gene were less susceptible for developing symptoms of late Lyme borreliosis [70], indicating that ongoing inflammation could be responsible for disease manifestations.

The outcome of the clash between *B. burgdorferi* and the host immune system, i.e. clearance or persistence of the spirochete, is dependent on virulence factors of the spirochete and the efficacy of the immune response raised by the host. In most animal models, in which 100% infectivity is pursued, *B. burgdorferi* is able to cause a persistent infection despite the development of vigorous innate and adaptive immune responses against the pathogen. It is here to mention that, although *B. burgdorferi* has been isolated from patients many years after the initial infection, in general *B. burgdorferi* is incapable of causing a persisting infection in most individuals, even without

antibiotic treatment. In this section of the thesis we investigated novel host and pathogen factors that could influence the outcome of *Borrelia* infection.

Lyme borreliosis can also occur in naturally infected dogs, manifesting as malaise and lameness [71]. Hovius and Hovius investigated the course of infection and immune responses in a cohort of pet dogs, naturally infected through tick-bite [72]. In this study we show that all naturally exposed dogs have moderate to high antibody titers, but symptomatic dogs produce a much wider spectrum of antibodies, including immobilizing antibodies, compared to asymptomatic infected dogs (**Chapter 7**). This indicates that the magnitude of the immune response directed against the bacterium is associated with disease activity in dogs. In human clinical practice we do not yet have access to reliable (serological) diagnostic tests distinguishing between active infection, subclinical infection and *B. burgdorferi* infection in the past. Importantly, to some extent, these tests are available for syphilis, another spirochetal disease [73–75]. Possibly, the data as presented in **chapter 7** could contribute to the development of such tests for *B. burgdorferi*, and research on this complicated topic should have high priority on the research agenda [76].

In Europe, a considerable percentage of ticks is coinfected with multiple *Borrelia* species [77]. A tick can therefore transmit more than one *Borrelia* species to the host through a single tick-bite [78–80]. In **chapter 8** we assessed the effect of simultaneous infection with the non-arthritogenic *B. garinii* strain PBi and the arthritogenic *B. burgdorferi* strain B31 on the course of experimental murine Lyme borreliosis [81]. We demonstrate that mice co-infected with PBi and B31 show significantly more paw swelling and arthritis, long-standing spirochetemia, and significantly higher systemic numbers of B31 spirochetes than mice infected with B31 alone. Mice infected with *B. garinii* strain PBi alone did not develop any symptoms. These data indicate that coinfection of *B. garinii* and *B. burgdorferi*, which is not unlikely in nature, results in an aggravated course of Lyme borreliosis and might even play an important role in the pathogenesis of human Lyme borreliosis in Europe. Interestingly, the evolutionary origin of *B. burgdorferi* has been a topic of debate. Recently, it was argued that *B. burgdorferi* originates from Europe [82], where as earlier studies suggested that *B. burgdorferi* ancestors must have been located primarily in the USA and were introduced into Europe in the post-Columbian era [83]. Our findings in **chapter 8**, but also in **chapter 5**, suggest a preferential survival advantage for *B. burgdorferi* over the other European *Borrelia* species. Therefore, regardless of the origin of *B. burgdorferi*, our findings provide experimental evidence of how this preferential protection could have influenced, or will influence, the introduction and persistence of *B. burgdorferi* in tick populations.

In *in vitro* studies *B. burgdorferi* has been shown to upregulate expression of the urokinase Plasminogen Activator Receptor (uPAR; CD87; PLAUR) [84,85], a receptor that can bind urokinase Plasminogen Activator (uPA) and is expressed on a variety of immune cells [86]. The urokinase receptor also affects cellular migration, adhesion, but also phagocytosis by facilitating intracellular signaling in conjunction with other surface receptors, including many integrins. This

can occur independently of the proteolytic activity of uPA [87]. We here show that uPAR is not only upregulated on murine and human leukocytes upon *B. burgdorferi* exposure, but that uPAR is involved in phagocytosis of the spirochete both in vitro as well as in vivo in the mouse model of Lyme borreliosis (**Chapter 9**). Better understanding of how the immune system copes with acute and persistent *Borrelia* infection might lead to novel or additional treatment strategies in conjunction to antibiotic treatment. This could be specifically true for understanding the role of uPAR, since several antagonists or agonists of the uPA/uPAR system have already been tested in oncological clinical trials [88,89], paving the way for research on the effects of these molecules in other disease entities.

PART IV: Spin-off of research on tick-host-pathogen interactions

Research on tick-host-pathogen interactions assists in understanding the complex processes that occur at the site of the tick-bite and how these could affect the (early) pathogenesis of Lyme borreliosis. However, there is more to be learned from the data presented in this thesis. Tick saliva contains a wide range of physiologically active molecules that are crucial for attachment to the host and/or for the transmission of pathogens [37] and that interact with host processes, including coagulation and fibrinolysis, and immunity and inflammation [90,91]. In this section we speculate how immunologically targeting specific tick salivary proteins could prevent the transmission of tick-borne pathogens from the tick to the host [92]. As an example we discuss an anti-tick vaccine based on Salp15 (**Chapter 10**). Importantly, we also discuss molecules in tick saliva that have been extensively studied in vitro or in animal models for human diseases, and that, due to their specificity, are potential future anticoagulant or immunosuppressive agents (**Chapter 10**). The *I. scapularis* tick genome is currently being sequenced, an effort known as the *Ixodes* Genome Project [93,94]. Once the genome is known this might give an enormous additional impulse to research in this field.

Concluding remarks

Since its discovery approximately 30 years ago, Lyme borreliosis has become the most important vector-borne disease in the Western world. The pathogenesis of this tick-borne disease is influenced by a plethora of tick-host-pathogen interactions and is still not entirely understood. This thesis describes in molecular detail novel tick-host, tick-pathogen and host-pathogen interactions in Lyme borreliosis, contributing to the understanding of the pathogenesis of this emerging zoonotic disease.

We have focused on the interaction of the *Ixodes* tick salivary gland protein, Salp15, with both *B. burgdorferi* as well as the mammalian immune system. The inhibition of host immune responses on the one hand, and the protection of *B. burgdorferi* on the other hand, by this pleiotropic tick salivary protein are exemplary of the complexity of tick-host-pathogen interactions that collectively

determine the outcome of an infection with *B. burgdorferi*. Unquestionably, in the future we will be able to use tick salivary proteins, or compounds based thereon, to our own benefit, and develop new tools to combat or prevent tick-borne diseases. Noteworthy, we have some catching up to do, since the majority of the interactions described in this thesis are likely to be the result of millions of years of co-evolution.

In addition, in both the experimental murine model for Lyme borreliosis as well as in naturally *B. burgdorferi* infected dogs, we show a delicate role for the host immune response in the genesis of Lyme borreliosis symptoms. The mammalian immune system should not generate a weak immune response, since this may fail to eradicate the spirochete, however an excessive immune response will lead to (irreversible) tissue damage and clinical symptoms; this is not an enviable task with both the arthropod vector as well as the bacterium trying to tip the balance of this fragile equilibrium. Importantly, better understanding of the host immune response to *Borrelia* brings us closer to the development of clear-cut diagnostic tests and therapeutic compounds that can specifically and favorably target the immune response against the bacterium.

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Epilog

'Cruel and sudden, hast thou
purpled thy nail in blood of innocence?

Wherein could this creature guilty be,
except in that drop which it suck'd from thee?

Yet thou triumph'st, and say'st that thou
find'st not thyself the weaker now.

'Tis true; then learn how false fears be;
as this creature's death took life from thee.'

Adapted from
John Donne (1572-1631)

Over de teek voor de leek: Nederlandse samenvatting voor niet-ingewijden

Interacties tussen teek, gastheer en pathogeen in de ziekte van Lyme

Lyme borreliose, ofwel de ziekte van Lyme, is een ziekte die wordt veroorzaakt door de spiraalvormige bacterie *Borrelia (B.) burgdorferi*, die voornamelijk wordt overgebracht door *Ixodes* teken [1–3]. Het wel of niet ontstaan van ziekteverschijnselen na infectie met deze bacterie is afhankelijk van een aantal specifieke interacties tussen de teek, de gastheer en de bacterie (pathogeen) [4]. In dit proefschrift ontrafelen en beschrijven wij het moleculaire mechanisme achter enkele van dergelijke, nog niet eerder beschreven, interacties.

Hoofdstuk 1 dient als een inleiding tot dit proefschrift en beschrijft reeds bekende interacties tussen de teek, de gastheer en *B. burgdorferi* en hoe deze het beloop van de ziekte van Lyme zouden kunnen beïnvloeden. Wij bediscussiëren hoe eiwitten in tekenspeeksel het immuunsysteem van de gastheer kunnen aantasten en wat hiervan de gevolgen zijn voor, enerzijds het bloedmaal van de teek en anderzijds de overdracht van micro-organismen van de teek naar de gastheer. Ook behandelen wij de meest prominente specifieke directe interacties tussen *Borrelia* en de teek, maar ook tussen *Borrelia* en de gastheer, en de rol die deze interacties spelen in het beloop van *B. burgdorferi* infectie. Dit proefschrift is onderverdeeld in vier delen, waarbij in **deel I tot en met III** achtereenvolgens nieuwe interacties tussen teek en gastheer; teek en pathogeen; en pathogeen en gastheer worden besproken. In **deel IV** bediscussiëren wij eventuele afgeleiden van ons en verwant onderzoek en de mogelijke implicaties voor de dagelijkse klinische praktijk.

DEEL I: Interacties tussen teek en gastheer

Teken voeden meerdere dagen op hun gastheer om een bloedmaal te verkrijgen. Dit zou de gastheer ruim voldoende tijd moeten geven om een adequate afweerreactie tegen de teek te ontwikkelen [5]. Echter, de teek heeft op haar beurt verschillende mechanismen ontwikkeld om de afweerreactie van de gastheer te moduleren [6–13,13–17]. Opmerkelijk genoeg leidt het onderdrukken van de afweer van de gastheer door de teek tot een efficiëntere overdracht van verschillende pathogenen van de teek naar de gastheer [18,19].

De groep van professor Fikrig van *Yale University* heeft enkele functioneel belangrijke eiwitten geïdentificeerd in de speekselklier van de frequent in Amerika voorkomende teek, *Ixodes (I.) scapularis* [20,21]. Eén van deze eiwitten werd alleen geproduceerd door voedende en niet door vastende teken. Dit eiwit, dat vanwege zijn moleculaire grootte van 15 kilodalton (kDa) Salp15 (*sal*ivary *p*rotein of *15 kDa*) werd genoemd, vertoonde een milde gelijkenis met Inhibin A, een humaan eiwit dat tot een familie van afweeronderdrukkende eiwitten behoort. Dit bracht ons ertoe om in dit deel van het proefschrift de eventuele afweeronderdrukkende capaciteiten van dit tekenspeekseleiwit Salp15 te onderzoeken.

Als men het afweer- of immuunsysteem zou willen onderverdelen in twee onderdelen, dan zou dat kunnen door het onderscheiden van een snel, aangeboren aspecifiek afweersysteem aan de ene kant, en een trager, doch specifieker, verworven afweersysteem aan de andere kant. Eén van de belangrijkste cellen van het verworven immuunsysteem zijn de zogenaamde T-cellen. In **hoofdstuk 2** demonstreren wij hoe Salp15 de activatie van T-cellen inhibeert [22] en hiermee het ontwikkelen van een adequate immuunreactie tegen de teek zou kunnen voorkomen. Recentelijk heeft de groep van Professor Anguita van de *University of Massachusetts* deze bevinding uitgediept en het moleculaire mechanisme achter deze T-cel onderdrukking ontrafeld [23,24].

In **hoofdstuk 3** onderzochten wij het effect van Salp15 op een andere belangrijke cel van het immuunsysteem, de zogeheten dendritische cel [25]. Dendritische cellen zou men kunnen karakteriseren als de verkenners van het immuunsysteem. Deze cellen zijn onder andere gesitueerd in de huid, waar zij dienen als een brug tussen het aangeboren en het verworven immuunsysteem. Wij tonen in hoofdstuk 3 aan, dat Salp15 ook de activatie van dit type cel remt. Tevens beschrijven wij het mechanisme hierachter tot in de moleculaire details [25].

Onderdrukking van het afweersysteem van de gastheer door eiwitten in tekenspeeksel draagt niet alleen bij aan het verkrijgen van een succesvol bloedmaal door de teek maar ook aan de overdracht van *Borrelia* van de teek naar de gastheer. De reden hiervoor ligt in het feit dat locale onderdrukking van het afweersysteem door tekenspeeksel de plek van de tekenbeet een minder vijandige omgeving maakt voor zowel de teek als voor *B. burgdorferi*. Naast een beter begrip van de complexe interacties tussen teek en gastheer gedurende het bloedmaal van de teek, kan onderzoek als beschreven in dit deel van het proefschrift, ook leiden tot de ontdekking en ontwikkeling van nieuwe ontsteking- of afweeronderdrukkende middelen. Onderzoek naar een dergelijke toepassing wordt momenteel uitgevoerd in het Academisch Medisch Centrum (AMC).

DEEL II: Interacties tussen teek en pathogeen

In Europa wordt de ziekte van Lyme voornamelijk overgebracht door de schapenteek, *Ixodes (I.) ricinus*, terwijl in Amerika de ziekte voornamelijk wordt overgebracht door de hertenteek, *I. scapularis*. Het tekenspeekseleiwit Salp15 werd initieel geïdentificeerd in *I. scapularis*. In **hoofdstuk 4** tonen wij echter aan, dat de Europese teek vergelijkbare eiwitten in haar speeksel heeft. Wij doopten deze eiwitten Salp15 Iric-1, -2 en -3, waarbij Salp15 Iric-1 de meeste gelijkenis vertoonde met *I. scapularis* Salp15.

Recentelijk is door Ramamoorthi en collega's aangetoond dat de interactie tussen teek, gastheer en *B. burgdorferi* nog ingewikkelder is dan voorheen voor mogelijk werd gehouden. Zij lieten namelijk zien, dat Salp15 in de speekselklier van de *I. scapularis* teek, bindt aan een eiwit op het oppervlak van *B. burgdorferi*, het zogenaamde *Outer surface protein* (Osp) C [26]. Aangezien *Borrelia*, zowel in de speekselklier als vroeg in het beloop van de infectie, op zijn gehele oppervlak veel van deze OspC

moleculen heeft, zorgt deze binding voor een soort jasje van Salp15 - analoog aan Harry Potter´s *invisibility cloak* - waardoor antilichamen de bacterie niet meer kunnen herkennen en niet meer kunnen doden. Het fascinerende aan deze bevinding is, dat teken die *Borrelia* met zich meedragen meer Salp15 maken dan teken die dat niet doen. Er lijkt een wederzijds voordeel te bestaan, dat het gevolg is van miljoenen jaren van co-evolutie; blijkbaar induceert *Borrelia* de productie van Salp15 door de teek, wat enerzijds gunstig is voor de teek, omdat zij zo de afweer van de gastheer onderdrukt en onbezorgd een bloedmaal kan nuttigen, en anderzijds de overlevingskansen van *Borrelia* vergroot, doordat deze Salp15 kan gebruiken als schild tegen antilichamen van de gastheer.

Nu is de situatie hier in Europa nog een stukje ingewikkelder. Niet alleen hebben wij een andere teek die de verwekkers van de ziekte van Lyme kan overdragen, ook hebben wij drie verschillende *Borrelia* soorten die de ziekte van Lyme kunnen veroorzaken, naast *B. burgdorferi*, die ook in Amerika voorkomt, *Borrelia garinii* en *Borrelia afzelii* [27,28]. Daarom onderzochten wij in **hoofdstuk 5** de interactie tussen de Salp15 homoloog in het speeksel van de Europese teek, Salp15 Iric-1, en de drie verschillende *Borrelia* soorten. Wij laten zien dat Salp15 Iric-1 weliswaar aan alle drie de *Borrelia* soorten bindt, maar dat deze alleen *B. burgdorferi*, en dus niet *B. garinii* en *B. afzelii*, beschermt tegen doding door antilichamen van de gastheer. Dit zou betekenen dat Salp15 Iric-1 in de natuur *B. burgdorferi* een overlevingsvoordeel zou kunnen geven ten opzichte van de twee andere *Borrelia* soorten [29].

In de studie van Ramamoorthi en anderen werd ook aangetoond, dat *I. scapularis* Salp15 *Borrelia* een overlevingsvoordeel geeft in muizen zonder antilichamen tegen *B. burgdorferi*. Hoewel deze muizen na infectie met *B. burgdorferi* uiteraard wel antilichamen gaan ontwikkelen, lijkt deze bevinding niet goed te verklaren door bescherming tegen doding door antilichamen. Dit bracht ons ertoe om te onderzoeken of het 'jasje met Salp15' wellicht ook zou beschermen tegen een belangrijke component van de aangeboren afweer, namelijk het complementsysteem. Met behulp van proeven in het laboratorium tonen wij in **hoofdstuk 6** aan, dat binding van Salp15 aan *Borrelia* stammen die normaliter gedood worden door het complementsysteem, inderdaad complement-gemediëerde doding voorkomt.

In de natuurlijke situatie dragen de bevindingen zoals beschreven in **DEEL I en II** gezamenlijk mogelijk bij aan een succesvol bloedmaal van de teek en een succesvolle overdracht van *Borrelia* van de teek naar de gastheer. Onderzoek naar de moleculaire interactie tussen teek, gastheer en *Borrelia* leert ons echter niet alleen veel over de (vroege) ziekteontwikkeling van de ziekte van Lyme, het zou ook kunnen bijdragen aan de ontwikkeling van nieuwe strategieën voor preventie of behandeling van de ziekte van Lyme: In theorie kan een vaccin dat Salp15 wegvangt de teek een belangrijke afweeronderdrukker en *Borrelia* zijn *invisibility cloak* afpakken. Een dergelijk anti-Salp15 vaccin zou er om deze twee redenen voor kunnen zorgen, dat de overdracht van *Borrelia* naar de gastheer, in dit geval de mens, wordt voorkomen.

DEEL III: Interacties tussen gastheer en pathogeen

De kwantiteit en kwaliteit van de afweerreactie van een met *B. burgdorferi* geïnfecteerde gastheer is zeer bepalend voor het verdere beloop van de infectie. Het merendeel van de gastheren zal, na infectie met de bacterie, een zodanig hevige afweerreactie ontwikkelen, dat de bacterie wordt geklaard. Andere gastheren echter zullen een afweerreactie ontwikkelen die niet in staat blijkt de bacterie te vernietigen. Gedurende het beloop van de infectie probeert *B. burgdorferi* de gastheer op verschillende manieren te slim af te zijn: door het maken van eiwitten die kunnen binden aan bepaalde gastheercellen of -weefsels [30,31], door het meer of minder tot expressie brengen van eiwitten die respectievelijk een immuunreactie zouden kunnen remmen [32–35] dan wel provoceren [36–40] en door bepaalde eiwitten zodanig te veranderen dat het afweersysteem ze niet meer kan herkennen [41–45]. In dit derde deel van het proefschrift onderzochten wij de bijdrage van nog niet eerder onderzochte factoren van de gastheer en pathogeen die de ontwikkeling van de immuunreactie en daarmee het beloop van de infectie met *Borrelia* zouden kunnen beïnvloeden.

In **hoofdstuk 7** onderzochten mijn vader en ik de kwantiteit en kwaliteit van de immuunrespons in natuurlijk geïnfecteerde honden in de Brabantse Kempen. Honden kunnen namelijk ook de ziekte van Lyme krijgen, wat zich uit in algehele malaise en kreupelheid [46]. De ziekte lijkt vaker voor te komen bij jonge of juist oudere honden, of bij honden waarvan het afweersysteem niet (meer) goed functioneert. Ook lijken bepaalde hondenrassen ontvankelijker voor symptomatische infectie met *B. burgdorferi* dan andere. In hoofdstuk 7 tonen wij aan, dat honden die symptomen ontwikkelen die passen bij de ziekte van Lyme meer specifieke antilichamen maken tegen *B. burgdorferi* dan honden die ook geïnfecteerd zijn geraakt maar geen symptomen ontwikkelen. Paradoxaal genoeg bezitten juist de symptomatische honden antilichamen die de bacterie kunnen doden. Deze data suggereren, dat de reactie van de gastheer op de bacterie minstens net zo bepalend is voor het optreden van symptomen van de ziekte van Lyme als de aanwezigheid van de bacterie zelf. Het hierboven beschreven onderzoek vormde de basis van mijn promotieonderzoek. De gegevens die dit onderzoek hebben opgeleverd zouden kunnen bijdragen aan de ontwikkeling van diagnostische testen die kunnen differentiëren tussen actieve en persisterende *B. burgdorferi* infectie en een in het verleden doorgemaakte infectie. In de dagelijkse humane geneeskundige praktijk zijn dergelijke diagnostische assays tot op zekere hoogte reeds voorhanden voor syfilis, een andere door spirocheten veroorzaakte ziekte. Voor de ziekte van Lyme bestaan deze testen helaas nog niet [47].

In Europa is een hoog percentage van de teken gelijktijdig geïnfecteerd met meerdere *Borrelia* soorten [48,49]. Daarom kan een teek door middel van één beet meerdere *Borrelia* soorten overdragen [50–52]. Polymerase ketting reactie (PCR) op weefsels van de in hoofdstuk 7 beschreven symptomatische honden laat zien dat in meerdere van deze honden *B. burgdorferi* DNA aantoonbaar was in huid of gewricht en tegelijkertijd *B. garinii* DNA in de lever [51]. Dit deed ons vermoeden dat co-infectie met meerdere *Borrelia*-soorten het beloop van de infectie kan beïnvloeden. Daarom onderzochten wij in **hoofdstuk 8,** in een muizenmodel voor de ziekte

van Lyme, het effect van gelijktijdige infectie met *B. burgdorferi* en *B. garinii* op het beloop van de ziekte. In dit hoofdstuk laten wij zien, dat enkele weken na gelijktijdige infectie met beide *Borrelia* soorten, er langdurig bacteriën in het bloed circuleren, hogere *B. burgdorferi* aantallen zijn terug te vinden in de weefsels, en er een heftigere ontsteking van het hart en meer uitgesproken zwelling van de gewrichten optreedt, dan na infectie met *B. burgdorferi* alleen [53]. Muizen geïnfecteerd met alleen *B. garinii* ontwikkelen helemaal geen symptomen. Dit wijst erop dat co-infectie met meerdere *Borrelia* soorten, iets wat zeker niet ondenkbaar is in de natuurlijke humane situatie, bijdraagt aan de ontwikkeling van ziekte na een tekenbeet.

Eerdere studies hebben laten zien een bepaalde receptor, de urokinase plasminogen activator receptor (uPAR, CD87, PLAUR), op verschillende afweercellen verhoogd tot expressie wordt gebracht als deze worden blootgesteld aan *B. burgdorferi* [54,55]. De rol van uPAR in de pathogenese van Lyme is echter niet eerder onderzocht. Naast een bescheiden rol in het opruimen van bloedstolsels - het fibrinolytische systeem -, speelt uPAR vooral een belangrijke rol in het op gang komen van de afweerrespons [56]. Andere niet aan *Borrelia* onderzoek gerelateerde studies demonstreerden dat uPAR niet alleen betrokken is bij migratie en adhesie van ontstekingscellen, maar ook bij de capaciteit om bacteriën te verorberen, een proces dat bekend staat als fagocytose [57]. In **hoofdstuk 9** laten wij zien, dat uPAR verhoogd tot expressie werd gebracht in levende muizen en mensen in reactie op *B. burgdorferi* infectie. Ons onderzoek toont aan dat muizen zonder functioneel uPAR minder goed *Borrelia* kunnen fagocyteren dan muizen met uPAR, zogenaamde wildtype controle muizen; en dat uPAR dus ook een belangrijke rol speelt in het verorberen en klaren van *B. burgdorferi*. Ook tonen wij aan dat deze bevinding volledig los staat van zowel de rol van uPAR in het fibrinolytische systeem als van de binding van uPAR aan uPA.

Een beter begrip van de rol van het immuunsysteem in de ontwikkeling van symptomen tijdens een infectie met *B. burgdorferi* kan leiden tot nieuwe diagnostische testen of nieuwe en verbeterde behandelingsmogelijkheden van de ziekte van Lyme. Dit laatste geldt mogelijkerwijs in extremis voor het uPA/uPAR systeem, omdat in de kliniek en in het veld van het kankeronderzoek [58,59] reeds uitgebreid onderzoek is verricht met remmers en stimulatoren van dit systeem.

DEEL IV: Afgeleiden van onderzoek naar de interactie tussen de teek, gastheer en pathogeen

Onderzoek naar de moleculaire interacties tussen de teek, gastheer en *Borrelia* leert ons beter de complexe processen begrijpen die ten grondslag liggen aan het ontstaan van de ziekte van Lyme. Dat zal in de toekomst ongetwijfeld bijdragen aan nieuwe diagnostische tests en nieuwe methoden voor preventie of behandeling van de ziekte van Lyme. Speeksel van de teek bevat eiwitten met uiteenlopende functies, onder andere het remmen van de bloedstolling [60] en het afweersysteem van de gastheer [61]. In **hoofdstuk 10** bespreken wij, dat we in die zin veel van de teek kunnen leren en dat onderzoek van specifieke moleculen in tekenspeeksel zoals gepresenteerd in dit proefschrift,

kan leiden tot de ontdekking en ontwikkeling van nieuwe antistollingsmiddelen of medicijnen die specifiek een onderdeel van het afweersysteem onderdrukken [62]. Verder blijkt uit onderzoek van anderen en van ons, dat sommige eiwitten in het speeksel van de teek cruciaal zijn voor de overdracht van pathogenen van de teek naar de gastheer. Het immunologisch uitschakelen van deze eiwitten, door bijvoorbeeld een vaccin, zou derhalve verspreiding van door teken overdraagbare ziekten kunnen helpen voorkomen. Zoals eerder genoemd zijn onderzoeken naar deze beide afgeleide toepassingen van onderzoek naar de interactie tussen de teek, gastheer en *Borrelia*, onderdeel van onderzoeksprojecten aan de Universiteit van Amsterdam en *Yale University*.

Teek home messages

Circa dertig jaar na de ontdekking van de ziekte van Lyme, is de ziekte van Lyme de belangrijkste door teken overdraagbare ziekte geworden in de Westerse wereld. De pathogenese - het ontstaan/ontwikkelen van ziekte - van deze zoönose is afhankelijk van een enorme verscheidenheid aan interacties tussen de teek, gastheer en ziekteverwekker. Pas de laatste jaren beginnen wij deze complexe interacties tot in de moleculaire details te begrijpen. Dit proefschrift beschrijft een aantal niet eerder beschreven interacties tussen teek, gastheer en *Borrelia* en hun rol in de ontwikkeling van verschijnselen bij de ziekte van Lyme. Een belangrijk deel van het proefschrift bestaat uit de karakterisering van één specifiek eiwit uit tekenspeeksel, Salp15. De twee belangrijkste functies van dit multifunctionele eiwit zijn onderdrukking van het afweersysteem van de gastheer en bescherming van *B. burgdorferi,* de verwekker van de ziekte van Lyme, tegen antilichaamgemedieerde bactericide activiteit van de gastheer. Dit onderzoek geeft inzicht in de onvoorstelbare complexiteit van de in de loop der miljoenen jaren ontstane interacties tussen teek, gastheer en *Borrelia*. Het draagt niet alleen bij aan een beter begrip van de ontstaanswijze van de ziekte van Lyme, het laat ook zien, dat we wat kunnen leren van de teek en haar ingenieuze speekseleiwitten zouden kunnen gebruiken als basis voor nieuwe geneesmiddelen.

Het andere deel van het proefschrift behandelt de rol van het immuunsysteem in de ontwikkeling van ziekteverschijnselen tijdens een infectie met *B. burgdorferi*. Hiertoe hebben wij gebruik gemaakt van een experimenteel model, namelijk het muizenmodel voor de ziekte van Lyme, van natuurlijk met *B. burgdorferi* geïnfecteerde honden en van in het laboratorium aan *B. burgdorferi* blootgestelde humane cellen. Uit vorig onderzoek en uit onderzoeken beschreven in dit proefschrift wordt duidelijk, dat het immuunsysteem niet te hevig dient te reageren op een infectie met *Borrelia*, omdat dit zal leiden tot heftige klinische symptomen, maar ook niet te zwak, aangezien in dat geval er een langdurige en hevige infectie met *B. burgdorferi* kan optreden. Dergelijk onderzoek opent de deur naar vervolgonderzoek en eventuele ontwikkeling van nieuwe eenduidige diagnostische testen. Tevens kan het aan de wieg staan van de ontwikkeling van nieuwe middelen die specifiek de afweerrespons tegen *B. burgdorferi* zouden kunnen moduleren en die zouden kunnen worden ingezet naast de huidige antibiotische behandelingsmogelijkheden.

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Een woord van dank

Tot slot van dit proefschrift zou ik een ieder die zich heeft ingezet voor mijn promotie willen bedanken.

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Het Centrum voor Experimentele en Moleculaire Geneeskunde (CEMM). Collegae van de infectieziekten groep: oud en nieuw: Markie Dressing, Ilona, Goda, Jacobien, WJW, de Grote Steeg, Masja, Floor, Liesbeth, Miriam, Jolanda, Arjan, van Zoelen, maar ook Cathrien, Alex, Petra, Jennie en Daniëlle, dank voor jullie enorme steun. Andere CEMM-ers, analisten, biotechnici (Joost en Mariekske), secretariaat en ICT-personeel, dank voor jullie geduld en helpende hand. Speciaal wil ik nog even noemen; Marcello, jouw humor heeft mijn tijd in het lab zeer veraangenaamd. Gelukkig ga je ook inwendige geneeskunde doen, dus hoef ik je niet lang te missen. Hianni, ja duh, jij natuurlijk ook reuze bedankt voor alle phagocytose proeven. Regina, er zijn er maar weinig die zo veel coupes zouden hebben kunnen snijden èn kleuren! Ongelofelijk wat een werk. (MLP)Anita, er is er maar één die zo goed kan cloneren als jij. Cees, 'ouwe tekenpulker', ook al moet ik je ieder keer weer opnieuw uitleggen dat Outer surface protein (Osp) C op *Borrelia* zit en Salp15 uit de teek komt; je kan en kon als geen ander meedenken met mijn proeven. Uiteraard mag ik mijn (oud-) kamergenootjes niet vergeten: Angnieszka, Akwenie, Marleen, Carina, Jan-10 (leuke naam heeft je zoon!), Sylvia B., Wytske, Inge Huibregste (IL-H), Markie Löwenberg, Keren (j'aime parler Francais avec toi), Francesca 'Ciao Bella' Milano, Simoni (ook al heeft ze niet bij me op de kamer gezeten), en natuurlijk Esmeralda, De Dopstra en Maarten Fokke Bijlsma (ja, die van die publicatie in PLoS Biology, van wie ik ooit nog paranimf heb mogen zijn). Over paranimfen gesproken; Marcello, collega met een kritische blik, maar vooral nieuwe vriend, Tinus, amice, maar vooral vriend van het eerste uur; wat mooi dat jullie mij in de aanloop naar mijn promotie hebben willen bijstaan. Tinus, ik weet uit eerste hand dat je het kan!

Een promovendus kan niet promoveren zonder studenten. Karel, dank voor al je gekloneer en voor het feit dat je zelfs nog in Amerika met onze tekeneiwitten bent gaan stoeien. Ik vond het werkbezoek aan UMASS een onverdeeld succes! Rick, als mijn boekje gedrukt is, hoop ik dat het Pharmaceutisch weekblad ons stukje ook heeft uitgebracht. Jeroen, ik heb geteld; 185 tenen heb je gegenotypeerd en nog bleef je lachen! Je bent een mooie vent en hebt een gouden toekomst voor je.

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Toen één van mijn professoren zei dat ik Biacore moest gaan doen wist ik niet dat Arnoud Marquart bij het apparaat 'geleverd' zou worden. Arnoud, wat een kennis en passie heb jij voor dit fantastische apparaat. Dank voor alle prachtige curves en analyses.

Als laatste van de AMC afdelingen mag ik de afdeling Inwendige Geneeskunde niet over het hoofd zien. Marcel Levi, wat je allemaal achter de schermen voor me geregeld hebt heeft mijn promotie vele malen eenvoudiger gemaakt en ik ben je daar ontzettend dankbaar voor. Leuk dat we samen een stukje hebben kunnen schrijven.

Het zou niet goed zijn als een promovendus niet samen zou werken met afdelingen van andere instituten. Veruit de soepelste samenwerking die ik ooit heb gehad was die met het VUMC, afdeling Moleculaire Biologie, onder leiding van Theo Geijtenbeek. Marein, mede eerste auteur van het *PLoS Pathogens* stuk, wat een genot om met je samen te werken. Sonja, dank voor je input wat betreft de DC-SIGN signalering en Theo, wat heb ik een enorm respect voor het werk dat jij en jouw groep doen: absolute wereldklasse! Leuk dat onze samenwerking nog een staartje krijgt! It really gets under your skin, doesn't it?

Menigmaal reisde ik af naar het LUMC om daar mee te doen aan het *Borrelia* werkoverleg met Nathalie en Tim. Ik denk dat we zeker wat aan elkaar gehad hebben en ben blij dat ik menig eiwitpurificatie daar heb mogen uitvoeren. Tim, fantastisch dat onze samenwerking een vervolg krijgt nu jij ook AIO bent in het AMC.

Uiteraard mag ik Frans Jongejan, Ard Nijhof en Eric de Vries van het Utrecht Center for Tick-Borne Diseases niet vergeten. Dank voor jullie hulp met het isoleren van de tekenspeekselklieren. Ard, het was heel prettig met je samen te werken. Ik zal die foto's van mij als Boer Harm bij de koeien niet zo snel vergeten.

Part of my PhD studies was performed at Yale University, New Haven, Connecticut, USA. I owe you guys so much. Especially, Venetta, a.k.a V, whom I respect very much, Nandhini for helping me with the Salp15 work, Debby who is the fastest biotechnician I have ever seen and a huge soccer fan, Jono for all the beers and housing me for a short period of time and Xin for explaning me the quantitative PCR over and over again. Hope we will stay in touch.

Thanking all those people in the States brings me to Juan Anguita. Now associate professor at the University of Massachutes, but formely also a post-doc at Yale Universuty. Juan, it is you I should thank for my real introduction to science. I still think a lot of the time we spent at Yale trying to figure out how the tick protein Salp15 inhibited T cell responses, and of the many beers we drank to celebrate or forget our results. I don't exaggerate if I state that you are the best supervisor a student could wish for. We will prevail!

Ajax en Feyenoord, Amsterdam en Rotterdam hand in hand, is het mogelijk? Dr. Byron Martina en prof. dr. Thijs Kuiken van het Erasmus Medisch Centrum ik hoop dat onze eerste gesprekken zullen leiden tot een volwaardige en succesvolle samenwerking.

Iedere promovendus heeft mensen nodig die hem af en toe wegsleuren bij zijn onderzoek, want af is het toch nooit. Daarom ben ik mijn vrienden Stijn, Jesse, Lemon Tree, en Tinus, en uiteraard ook mijn familie, mam, pap, Taco en Wendy en Anna en Pien natuurlijk uiterst dankbaar, want zo gemakkelijk liet ik me nou ook weer niet altijd wegsleuren. Eigenlijk kan ik mijn vader hier dan helemaal niet bedanken, want die sleurde me juist weer terug naar mijn werk. De urenlange telefoongesprekken die we hebben gevoerd zijn niet alleen iets heel bijzonders, maar kunt u ook deels terugvinden in de discussies van de verschillende hoofdstukken in dit boekje, op mijn telefoonrekeningen en uiteraard integraal in hoofstuk 7. Zo vader zo zoon hebben wij wel erg letterlijk genomen.

Sjakkie, lieverd, vanaf de dag dat wij elkaar ontmoetten ben ik bevangen door draaiduizeligheid. Ik ben zo blij dat jij er altijd bent en dat je vol zit met initiatieven en wilde plannen. We gaan nog zo veel fijne dingen doen samen!

Tot slot, dank voor al diegenen die de moeite hebben genomen om dit boekje te lezen. Als ik jullie een tip had mogen geven: met de samenvatting was je der ook wel gekomen.

Dank

Publication list

1. **J Infect Dis. 2008 Oct 15;198(8):1189-97.** Preferential protection of *Borrelia burgdorferi* sensu stricto by a Salp15 homologue in *Ixodes ricinus* saliva. Hovius JW, Schuijt TJ, de Groot KA, Roelofs JJ, Oei GA, Marquart JA, de Beer R, van 't Veer C, van der Poll T, Ramamoorthi N, Fikrig E, van Dam AP.

2. **Infect Immun. 2008 Jul;76(7):2888-94.** The tick salivary protein Salp15 inhibits the killing of serum-sensitive *Borrelia burgdorferi* sensu lato isolates. Schuijt TJ, Hovius JW, van Burgel ND, Ramamoorthi N, Fikrig E, van Dam AP.

3. **PLoS Pathog. 2008 Feb 8;4(2):e31.** Salp15 binding to DC-SIGN inhibits cytokine expression by impairing both nucleosome remodeling and mRNA stabilization. Hovius JW, de Jong MA, den Dunnen J, Litjens M, Fikrig E, van der Poll T, Gringhuis SI, Geijtenbeek TB.

4. **PLoS Med. 2008 Feb;5(2):e43.** Salivating for knowledge: potential pharmacological agents in tick saliva. Hovius JW, Levi M, Fikrig E.

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6. **Trends Parasitol. 2007 Sep;23(9):434-8.** Tick-host-pathogen interactions in Lyme borreliosis. Hovius JW, van Dam AP, Fikrig E.

7. **FEMS Immunol Med Microbiol. 2007 Mar;49(2):224-34.** Coinfection with *Borrelia burgdorferi* sensu stricto and *Borrelia garinii* alters the course of murine Lyme borreliosis. Hovius JW, Li X, Ramamoorthi N, van Dam AP, Barthold SW, van der Poll T, Speelman P, Fikrig E.

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9. **J Clin Microbiol. 2000 Jul;38(7):2611-21.** Antibodies against specific proteins of and immobilizing activity against three strains of *Borrelia burgdorferi* sensu lato can be found in symptomatic but not in infected asymptomatic dogs. Hovius JW, Hovius KE, Oei A, Houwers DJ, van Dam AP.

Other:

10. The urokinase receptor (uPAR) facilitates clearance of *Borrelia burgdorferi*. Joppe W.R. Hovius, Maarten F. Bijlsma, Gerritje J.W. van der Windt, W. Joost Wiersinga, Bastiaan J. D. Boukens, Jeroen Coumou, Anneke Oei, Regina de Beer, Alex F. de Vos, Cornelis van 't Veer, Alje P. van Dam, Penghua Wang, Erol Fikrig, Marcel M. Levi, Joris J.T.H. Roelofs, Tom van der Poll. *Submitted for publication.*

11. Pharmaceutisch weekblad. De teek als apo-teek van de toekomst? Rick Henderik, G. Folkerts, J.W. Hovius. *In press.*

12. Ned Tijdschr Geneeskd. 2008 Jul 12;152(28):1594. Tick-borne disease or not? Do not let yourself be fooled. Hovius JW. *Comment.*

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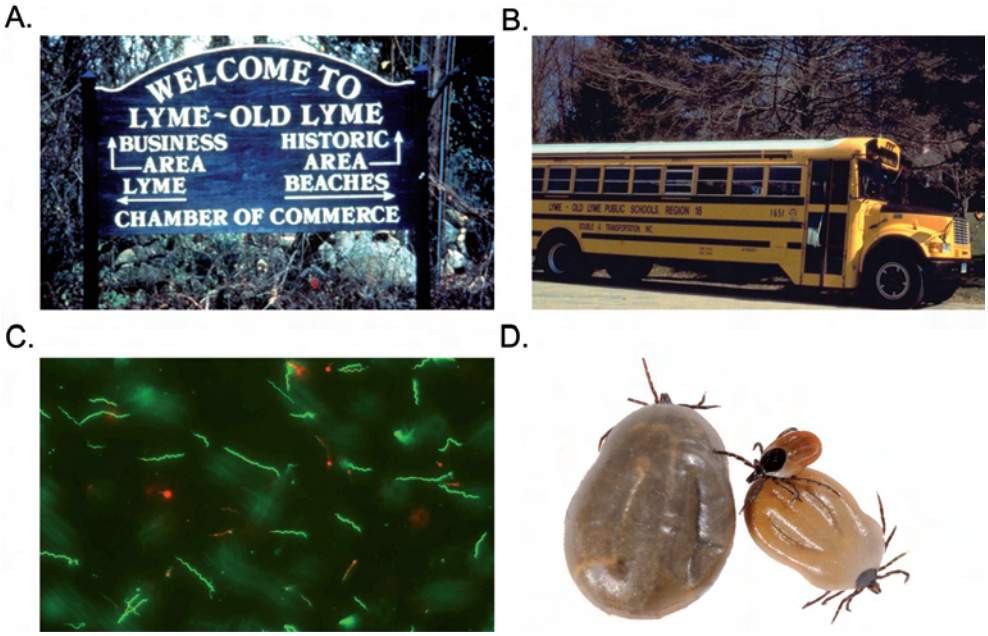
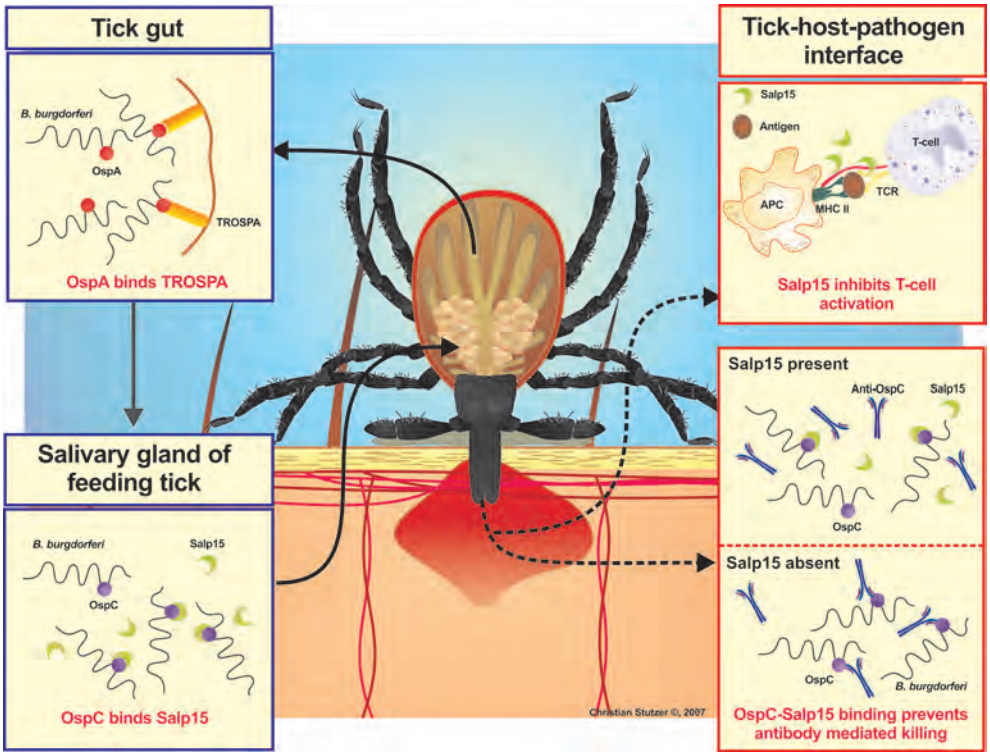
Curriculum vitae

Joppe Willem Robert Hovius was borne on the 14th of October 1976 in Hunsel, The Netherlands. He graduated from the Van Maerlant Lyceum, Eindhoven, The Netherlands in 1995 and started studying Medicine at the University of Amsterdam (UvA). For his scientific internship he and his dad studied the immune response of *Borrelia* infected dogs at the Department of Medical Microbiology, Academic Medical Center (AMC), Amsterdam, The Netherlands, under supervision of dr. Alje P. van Dam. Shortly thereafter, Hovius left for the United States and studied the effect of a tick salivary protein, Salp15, on T cell activation at Yale University, school of Medicine, New Haven, Connecticut, USA, under supervision of prof. dr. Erol Fikrig and dr. Juan Anguita. In 2001 he received his Masters of Science at the faculty of Medicine at the UvA and started his preclinical medical training. In 2003 he received his medical degree and continued as a resident Internal Medicine at the AMC. In 2005 he received a travel stipend form the Dutch Infectious Diseases Society and returned to Yale University to study the effect of coinfection with different *Borrelia* species on the course of Lyme borreliosis. That year Hovius also received an 'AGIKO stipend' from the Netherlands organization for health research and development to study the pathogenesis of Lyme borreliosis under supervision of prof. dr. Erol Fikrig, dr. Alje P. van Dam, prof. dr. Peter Speelman and prof. dr. Tom van der Poll. In October 2008 he resumed his residency in Internal Medicine. He will start specializing in Infectious Diseases in October 2010. Hovius has spent a great deal of time at the field hockey pitch, but has now exchanged his field hockey stick for a kite to kite-surf the North sea together with his one and only love Jacqueline Tromp.

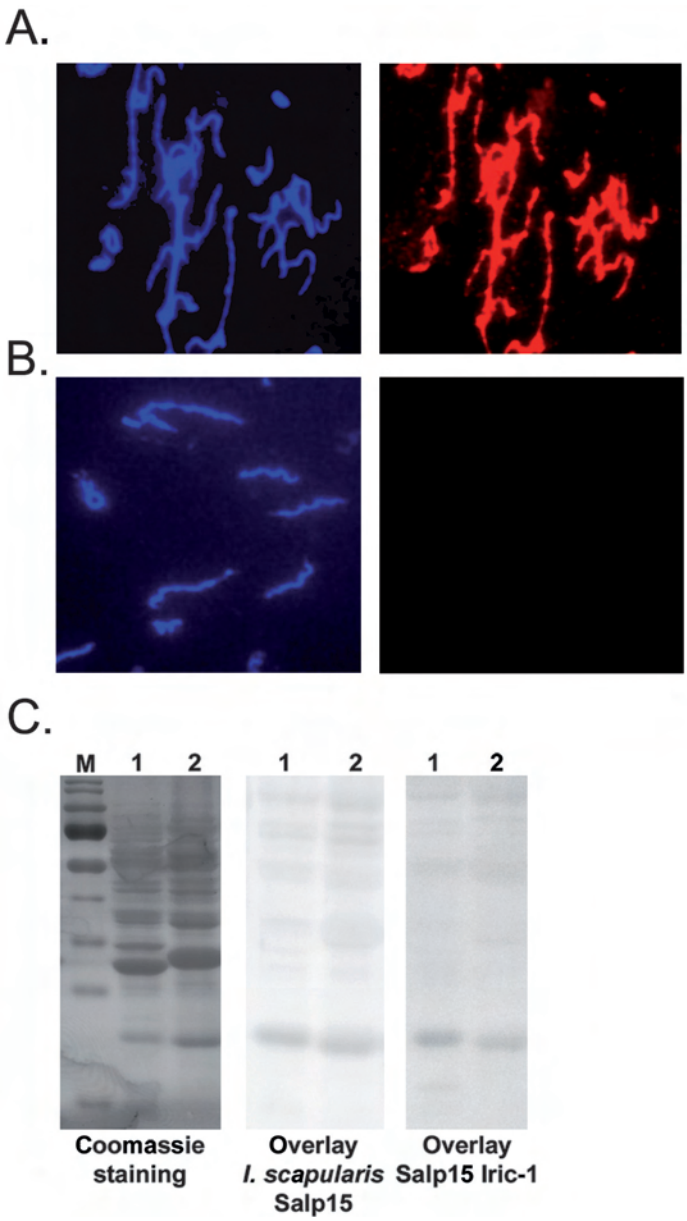
Color print section



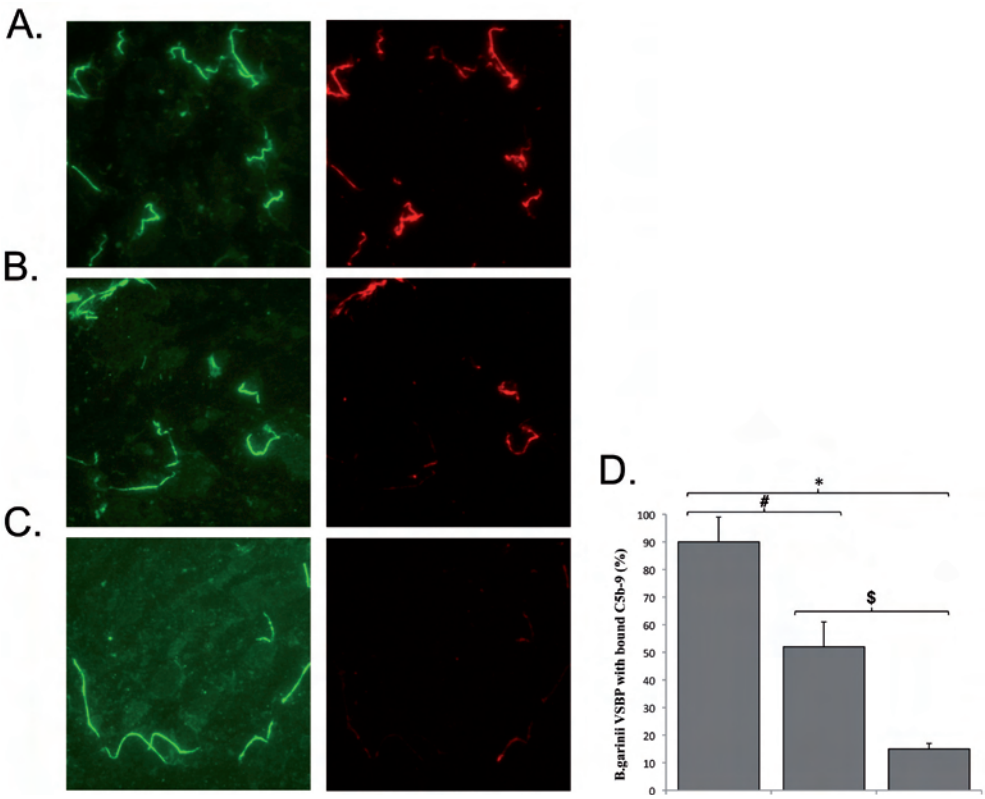
Prefaces Figure 1. Historical, ecological and biological aspects of Lyme borreliosis.
(A) Lyme disease was named after the town Old Lyme, Connecticut, USA.
(B) The Old Lyme public schoolbus. Picture: K.E. Hovius.
(C) *Borrelia burgdorferi* visualized by microscopy.
(D) *Ixodes* ticks are the main vector for Lyme borreliosis. Picture: G.A. Oei.



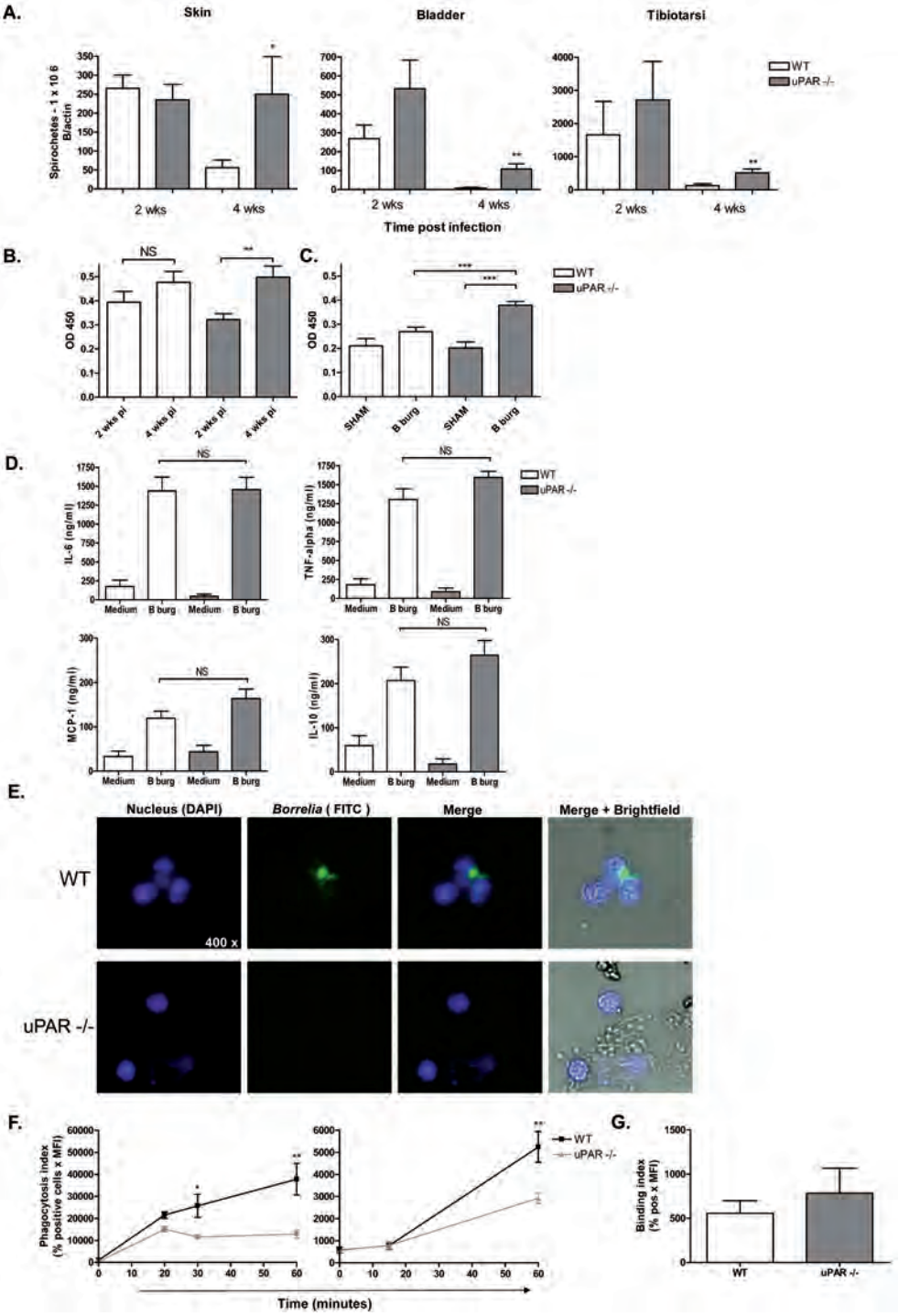
Chapter 1 Figure 1. Schematic overview of tick–host–pathogen interactions important in the development of Lyme borreliosis. In the gut of *Ixodes scapularis* ticks, *Borrelia burgdorferi* upregulates *ospA* expression. OspA binds to the tick receptor for OspA (TROSPA) [11]. During tick feeding, *B. burgdorferi* downregulates *ospA* expression, starts producing OspC and migrates to the salivary gland. OspC binds to a tick salivary protein, Salp15. Salp15 has immunosuppressive properties, such as inhibiting $CD4^+$ T-cell activation [48], and immunoprotective effects, such as inhibiting antibody-mediated killing of *B. burgdorferi* by the host [13]. For other tick–host–pathogen factors that are of importance in Lyme borreliosis see the text. Unbroken arrows represent processes that take place within the tick, and broken arrows represent processes that take place at the tick–host–pathogen interface. Abbreviations: APC, antigen presenting cell; MHC, major histocompatability complex; OspA and OspC, outer surface protein A and C; TCR, T cell receptor.



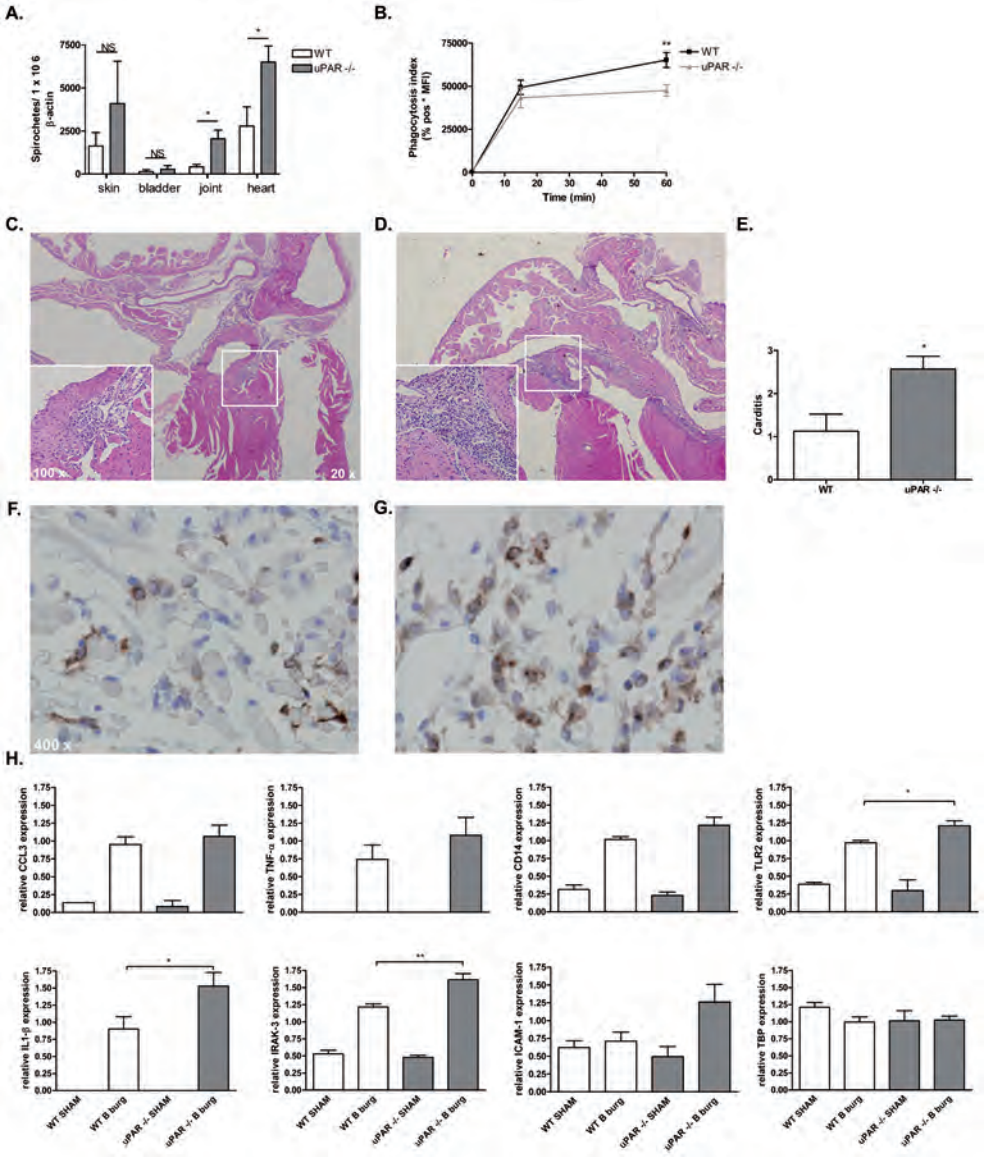
Chapter 6 Figure 2. Salp15 Iric-1 binds to the surfaces of *B. garinii* VSBP spirochetes. Spirochetes were preincubated with biotinylated Salp15 Iric-1 (A) or biotinylated BSA (B). Spirochetes were detected with bisbenzimide (blue) and bound Salp15 or BSA was detected using streptavidin-Cy3 (red). (C) Both Iscap Salp15 and Salp15 Iric-1 bind *B. burgdorferi* N40 (1) and *B. garinii* VSBP (2) in the overlay binding assay. The arrow indicates Salp15 bound to OspC. M, molecular mass.



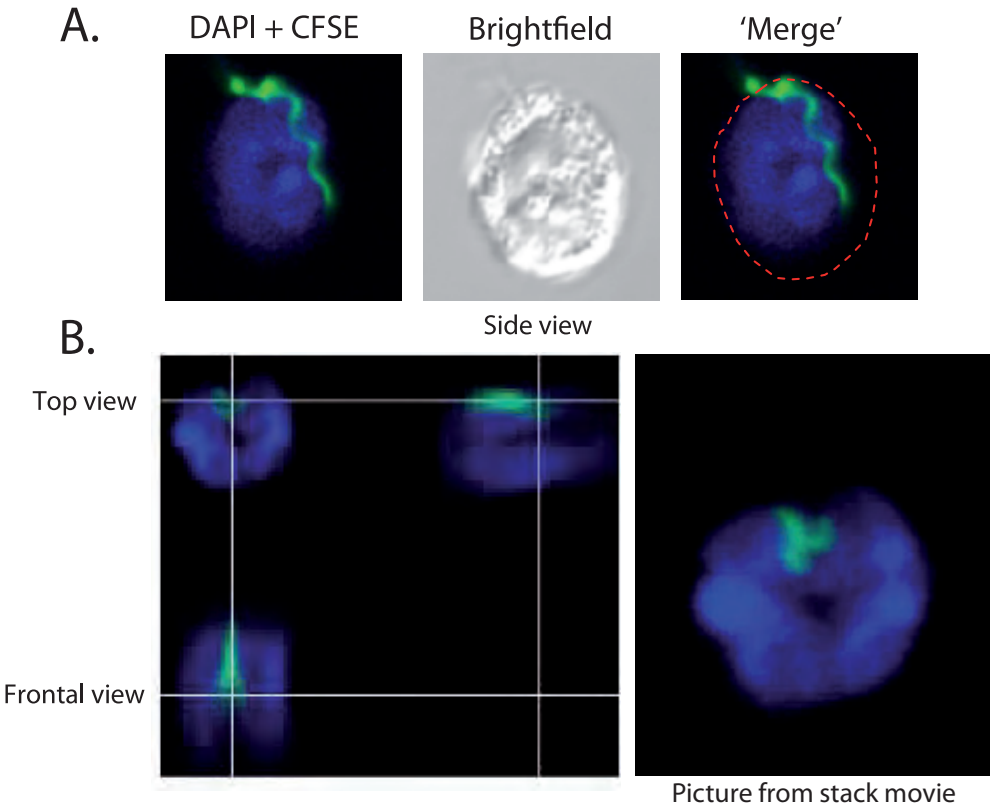
Chapter 6 Figure 4. Inhibition of C5b-9 deposition by Salp15. *B. garinii* VSBP was preincubated with BSA (A), Iscap Salp15 (B), or Salp15 Iric-1 (C) before being subjected to 12.5% NHS. Spirochetes were double labeled with antibodies specific for *Borrelia* spirochetes (green) and specific for C5b-9 complement complexes (red). (D) Deposition of C5b-9 complexes was strongly inhibited in the presence of Iscap Salp15 and even more by Salp15 Iric-1. Bars indicate standard deviations, based on duplicate countings of two independent experiments. One hundred spirochetes were counted, and the experiment was performed two times. * indicates a statistically significant difference ($P < 0.0001$) between the BSA- and Salp15 Iric-1-treated groups, # shows a significant difference ($P < 0.0001$) between the BSA- and Iscap Salp15-treated groups, and $ indicates a significant difference ($P < 0.0001$) between the Iscap Salp15- and Salp15 Iric-1-treated groups.


A.
Skin
Bladder
Tibiotarsi
Spirochetes - 1 x 10 6 B/actin
WT
uPAR -/-
2 wks
4 wks
Time post infection
B.
OD 450
NS
2 wks pi
4 wks pi
C.
SHAM
B burg
D.
IL-6 (ng/ml)
TNF-alpha (ng/ml)
MCP-1 (ng/ml)
IL-10 (ng/ml)
Medium
E.
Nucleus (DAPI)
Borrelia (FITC)
Merge
Merge + Brightfield
400 x
F.
Phagocytosis index (% positive cells x MFI)
Time (minutes)
G.
Binding index (% pos x MFI)

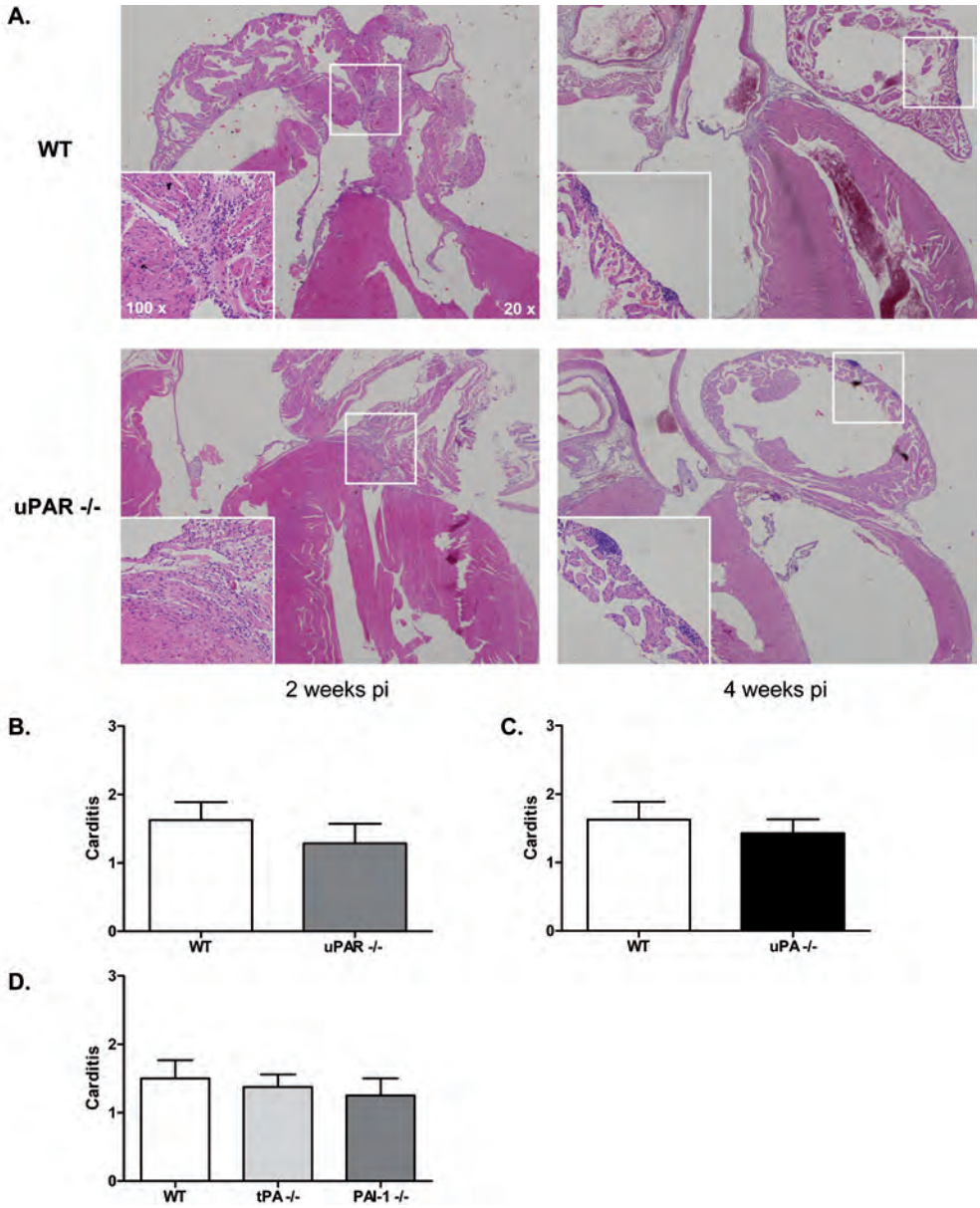
Chapter 9 Figure 2. The urokinase receptor (uPAR) is involved in clearance of *B. burgdorferi*.
A. Urokinase receptor knock-out C57BL/6 mice display higher systemic *B. burgdorferi* numbers. WT and uPAR -/- mice were inoculated with *B. burgdorferi* and sacrificed two and four weeks post infection. DNA was extracted from the indicated tissues and subjected to quantitative *Borrelia flab* and mouse *β-actin* PCR. In SHAM inoculated mice (2 to 3 per group) we did not detect *B. burgdorferi* DNA. Six to eight mice per group were used and bars represent the mean ± SEM.
B and C. Urokinase receptor knock-out C57BL/6 mice develop more rigorous IgG responses. Sera from C57BL/6 WT and uPAR knock-out mice, 2 and 4 weeks post *B. burgdorferi* (B burg) or SHAM inoculation (SHAM) was used for whole cell *B. burgdorferi* ELISA. Thus, we determined total IgG directed against *B. burgdorferi* (B) and IgG subclasses, of which only IgG1 (C) is shown.
D. WT and uPAR -/- macrophages produce similar levels of pro-inflammatory cytokines when exposed to viable *B. burgdorferi* in vitro. Peritoneal macrophages were stimulated with control medium (medium) or *B. burgdorferi* (B burg) for 16 hours. The supernatant was analyzed for cytokine production using a mouse inflammation cytometric bead array.
E and F. Urokinase receptor deficient granulocytes and macrophages are incapable of adequately phagocytosing *B. burgdorferi*. Whole blood or peritoneal macrophages were incubated with CFSE labeled viable or heat-killed FITC-labeled *B. burgdorferi* at 37 °C or at 4 °C as a control. Phagocytosis was stopped by transferring the tubes to ice and extracellular bacteria were quenched by addition of a quenching dye containing Trypan blue. When whole blood was used erythrocytes were lysed before cells were DAPI stained and subjected to fluorescent microscopy (E) or stained for Gr-1 (granulocytes) and subjected to FACS analysis (F; left panel). Peritoneal macrophages were directly subjected to FACS analysis (F; right panel). Phagocytosis was depicted as the phagocytosis index [63,64]: mean fluorescence intensity (MFI) x percentage (%) positive cells) at 37°C minus (MFI x % positive cells at 4°C). Six to eight mice per group were used, graphs represent the mean ± SEM and are representative of three independent experiments.
G. *B. burgdorferi* binds equally well to WT and uPAR -/- macrophages. A similar experiment as described in F was performed, albeit at 4 °C and without the addition of quenching dye to determine binding of *B. burgdorferi* to peritoneal macrophages. Binding is expressed as the binding index: % CFSE positive cells x MFI. Four to six mice per group were used and bars represent the mean ± SEM. The experiment was repeated twice. A P value < 0.05 was considered statistically significant. * indicating $P < 0{,}05$; ** $P < 0{,}01$ and *** $P < 0{,}001$.


A.
Spirochetes/ 1 x 10 6 β-actin
7500
5000
2500
0
NS
NS
*
*
WT
uPAR -/-
skin
bladder
joint
heart
B.
Phagocytosis index (% pos * MFI)
75000
50000
25000
0
**
WT
uPAR -/-
0
10
20
30
40
50
60
Time (min)
C.
100 x
20 x
D.
E.
Carditis
3
2
1
0
*
WT
uPAR -/-
F.
400 x
G.
H.
relative CCL3 expression
relative TNF-α expression
relative CD14 expression
relative TLR2 expression
relative IL-1β expression
relative IRAK-3 expression
relative ICAM-1 expression
relative TBP expression
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
*
**
WT SHAM
WT B burg
uPAR -/- SHAM
uPAR -/- B burg

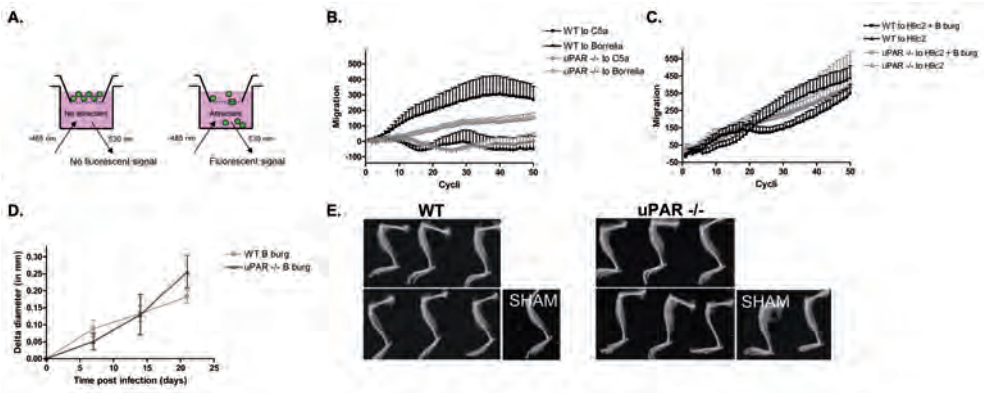
Chapter 9 Figure 5. The course of Lyme borreliosis in uPAR knock-out mice on a *B. burgdorferi* susceptible mixed C57BL/6 x C3H/HeN genetic background.
A. Urokinase receptor deficient mice on the mixed genetic background also display higher *B. burgdorferi* numbers compared to WT littermate controls. C57BL/6 mice were backcrossed twice to a C3H/HeN background. We intercrossed F2 mice and used the homozygous and nullizygous offspring (F2 homozygous uPAR deficient C57BL/6 x C3H/HeN mice and WT littermate controls) for our experiments. Mice were inoculated with *B. burgdorferi* or SHAM and sacrificed two weeks post infection, DNA was extracted from the indicated tissues and samples were subjected to quantitative *Borrelia flab* and mouse *β-actin* PCR. *B. burgdorferi* numbers are depicted as described in figure 2. Six to eight mice per group were used.
B. Urokinase receptor deficient leukocytes from mice on the mixed genetic background are not as capable of phagocytosing *B. burgdorferi* as are granulocytes from WT littermate controls. Phagocytosis assays with whole blood were performed as described in figure 2. Six to eight mice per group were used.
C, D and E. Peak carditis in these uPAR -/- mice (D) is more severe compared to carditis in WT littermate controls (C). Mice were inoculated with *B. burgdorferi* and sacrificed two weeks post infection. Pictures of hematoxyline and eosin stained sagittal sections depict representative sections. Carditis was scored as described in supplemental figure 4 within the same session (E). Six to eight mice per group were used.
F and G. The main cell involved in murine Lyme carditis is the macrophage. Representative pictures of F4/80 stained sagittal sections of hearts from *B. burgdorferi* infected uPAR deficient mice (G) and WT littermate controls (F).
H. More severe inflammation in *B. burgdorferi* infected uPAR deficient animals (n=7) compared to WT littermate controls (n=7) as measured by multiplex ligation-dependent probe amplification (MLPA). MLPA was performed on RNA obtained from half of sagittally dissected hearts from *B. burgdorferi* or SHAM inoculated mice. Depicted are mRNA expression of *TNF-α*, *CCL3, TLR2, CD14, IL1-β*, *IRAK3, ICAM1* and *TBP (housekeeping gene [69])*. Other genes included in the assay were *IL6, IL10, INF-γ, TFPI, F3,PROCR, SERPINE1P, PLAT, PLAUR, TLR4, TLR9 LY96, IRAK1, F2R, NFKB1a, NOS3, ITGA5,B2M, ITGAV, ITGAB3, TFRC, HIF1A, MMP2* and *HP.*
Bars represent the mean ± SEM. A *P* value < 0.05 was considered statistically significant.
* indicating $P < 0{,}05$; ** $P < 0{,}01$.



Chapter 9 Supplemental figure 3. Confocal microscopy of *B. burgdorferi* phagocytosis.
A and B. Confocal microscopy confirmed that *B. burgdorferi* in in vitro phagocytosis assays were localized intracellularly. Cells incubated with CFSE-labeled *B. burgdorferi* were subjected to confocal microscopy. Nuclei of cells were stained with DAPI. In Panel A we depicted the widest transversal section of a segmented nucleus of a granulocyte stained with DAPI and a CFSE-labeled *B. burgdorferi* spirochete. Superimposing the brightfield image confirms the bacterium is localized intracellularly. Panel B shows another granulocyte and *B. burgdorferi* from different view points (left panel) and a picture from a stack movie (right panel) further verifying that we are assessing internalized bacteria in the in vitro phagocytosis assays.



Chapter 9 Supplemental figure 4. Carditis in WT, uPAR, uPA, tPA and PAI-1 knock-out mice.
A and B. Peak carditis in C57BL/6 uPAR -/- is of similar severity compared to WT controls, although active carditis persists longer in uPAR -/- mice. WT and uPAR -/- mice were inoculated with *B. burgdorferi* and sacrificed two or four week post infection. Sagittal sections of formalin fixed and paraffin embedded hearts were H&E stained. The severity two weeks post infection was scored by a pathologist blinded to the experimental design on a scale of 0-3, with 0: no carditis; 1: mild carditis; 2: moderate carditis and 3: severe carditis. SHAM inoculated mice did not develop carditis (data not shown). Pictures depict representative sections.
C and D. Peak carditis in C57BL/6 uPA, tPA and PAI-1 knock-out mice is comparable to peak carditis in WT C57BL/6 mice. Carditis was scored as described above. Six to eight mice per group were used and bars represent the mean ± SEM. A P value < 0.05 was considered statistically significant.

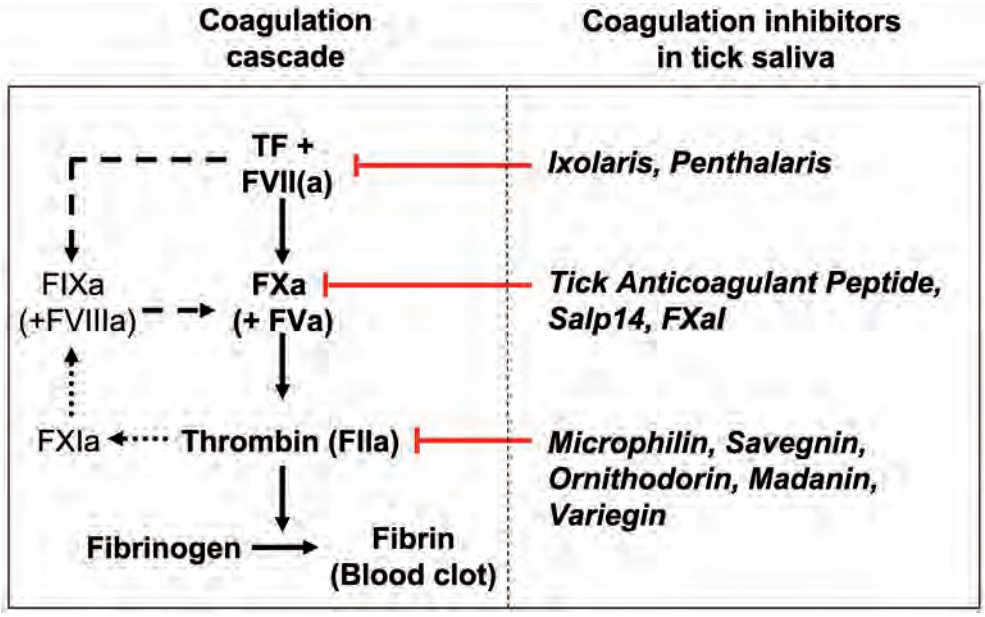


Chapter 9 Supplemental figure 5. Migration and arthritis in WT and uPAR knock-out mice on a *B. burgdorferi* susceptible genetic background.

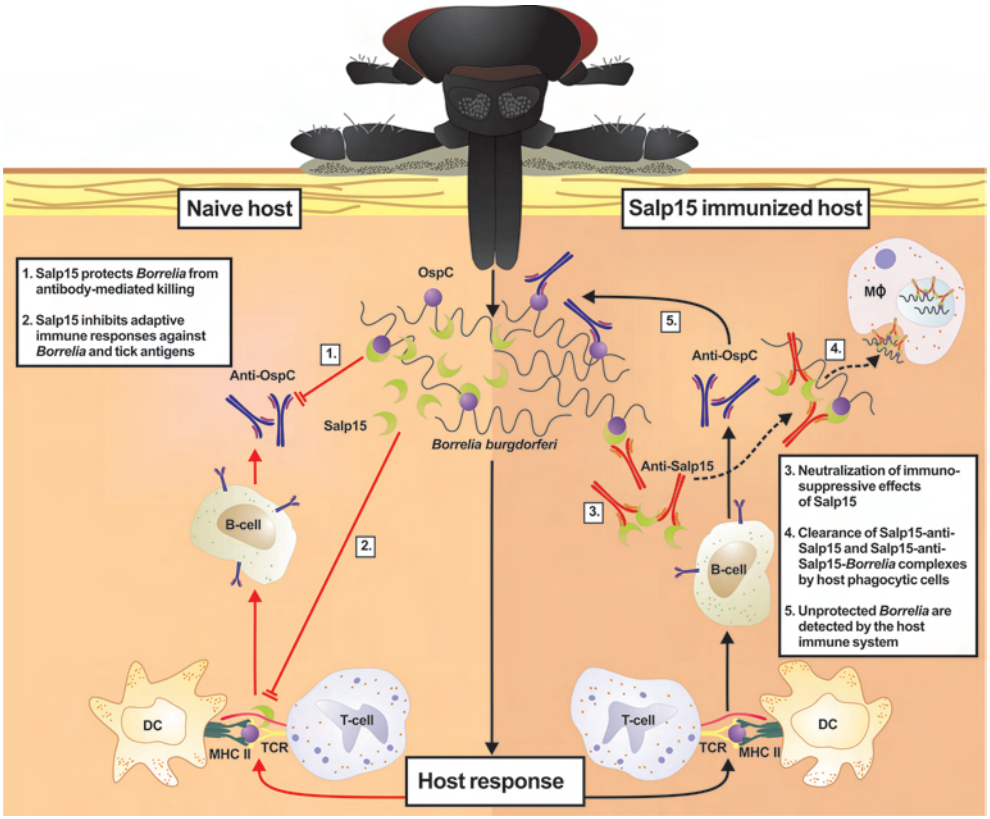
A, B and C. Urokinase receptor deficient macrophages from mice on the mixed genetic background can migrate to cardiogenic stimuli just as well as macrophages from WT littermate controls. Migration of CellTracker Green labeled WT or uPAR deficient macrophages towards several chemotactic stimuli was investigated in vitro (A). As chemotactic stimuli we used *B. burgdorferi* or activated complement factor 5 (C5a) (B) and supernatant from the cardiomyoblastic rodent cell line H9c2 stimulated with *B. burgdorferi* or control medium for 16 hours prior to experimentation (C). All experiments were performed in duplo in serum free DMEM medium without the addition of antibiotics and migration was corrected for the no-attractant control. Graphs represent the mean of three independent experiments ± SEM. The fluorescent signal in the lower chamber (indicative of migration) was measured in real time every two minutes (cycli).

D and E. Only edema, no arthritis in uPAR knock-out mice (n=7) and WT littermate controls (n=8). Ankle swelling was measured using a microcaliper during the course of infection (D). In this particular experiment mice were monitored for three weeks. Post mortem, but before decalcification, radiological examination of the right hindlimb was performed (E). No differences between SHAM inoculated and *B. burgdorferi* infected animals were observed.

A P value < 0.05 was considered statistically significant. * indicating $P < 0{,}05$



Chapter 10 figure 1. Schematic overview of the coagulation cascade. The two major amplification loops in the coagulation cascade are depicted. The first amplification loop consists of TF-FVIIa-mediated factor IX (FIX) activation, which leads to the generation of more FXa. A second amplification loop is formed by the activation of factor XI (FXIa) by thrombin, which results in more activated FIX (FIXa), and, subsequently, additional FXa generation. The right panel indicates how selected tick proteins exert their anticoagulant effect. FIIa, activated factor II; FVa, activated factor V; FVIIIa, activated factor VIII.



Chapter 10 Figure 2. Diagram showing how an anti-Salp15 vaccine could prevent transmission of *B. burgdorferi*. During tick feeding and early mammalian infection, *B. burgdorferi* expresses OspC, which binds to Salp15 in tick saliva. This binding acts as a shield and protects the spirochete from killing by the host. In addition, Salp15 has been shown to directly inhibit dendritic cell and T cell activation, which could facilitate tick feeding. Salp15 antibodies are likely to bind to Salp15 that has previously bound to OspC on the surface of *B. burgdorferi* in the tick salivary gland and could thereby enhance clearance by host phagocytes. Obviously, the Salp15 antibodies would need to recognize a Salp15 epitope other than the epitope that is required for binding of Salp15 to OspC. Similarly, if anti-Salp15 antibodies were to bind to free Salp15, they could neutralize the immunosuppressive effects of Salp15, which could hamper tick feeding and thereby transmission of *B. burgdorferi* from the tick to the host. Lastly, if anti-Salp15 antibodies were to inhibit binding of Salp15 to *Borrelia* OspC, this would render the spirochete susceptible to pre-existing or newly generated immunoglobulins. Importantly, Salp15 was originally identified by screening of a tick salivary gland cDNA expression library with tick immune rabbit sera, suggesting that antibodies against Salp15 may participate in tick rejection. DC, dendritic cell; MHC, major histocompatibility complex; MF, macrophage; TCR, T cell receptor.