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Bacterial dissemination and immuno-transcriptomics

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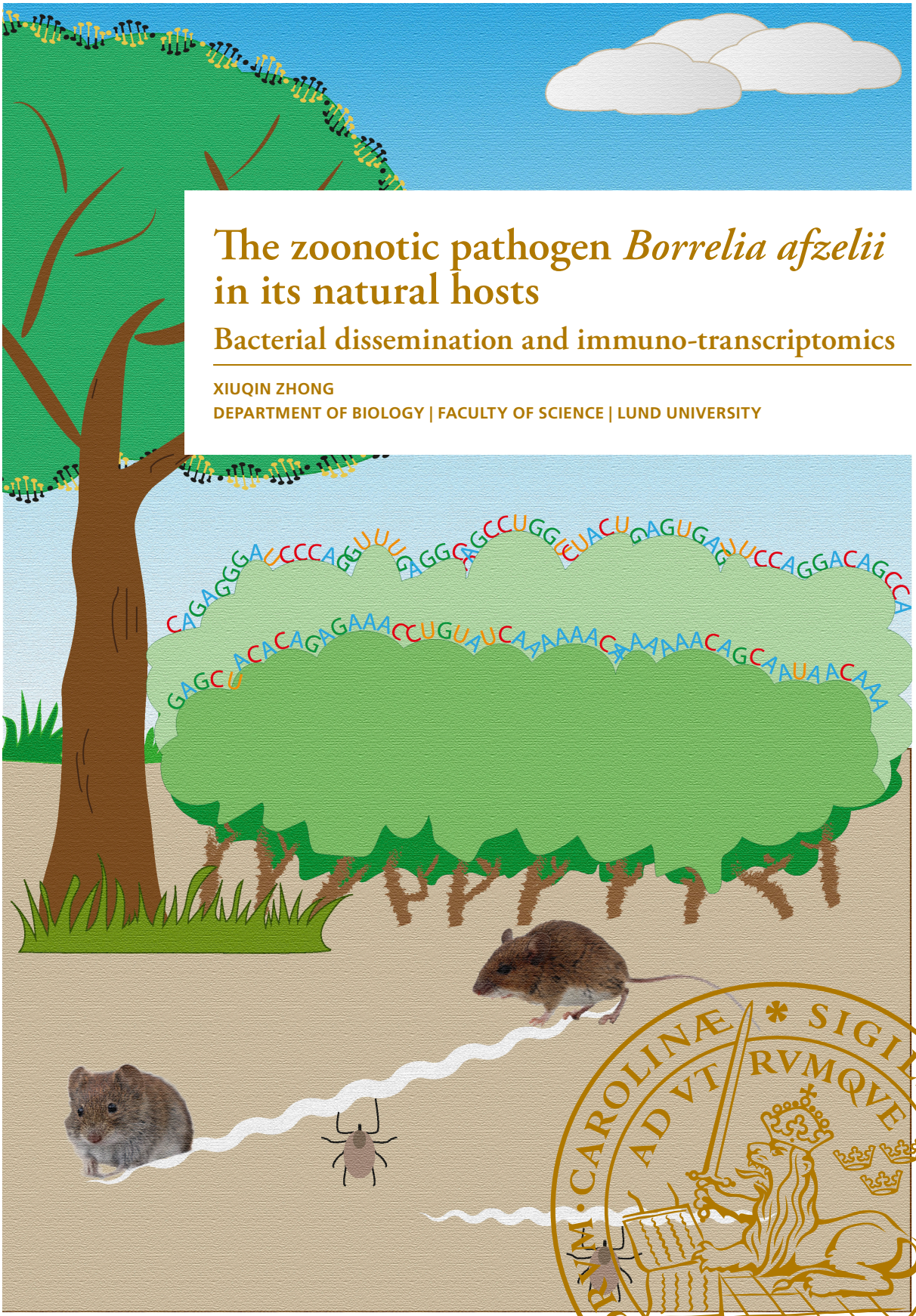

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Bacterial dissemination and immuno-transcriptomics

XIUQIN ZHONG
DEPARTMENT OF BIOLOGY | FACULTY OF SCIENCE | LUND UNIVERSITY

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LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Title and subtitle The zoonotic pathogen *Borrelia afzelii* in its natural hosts - Bacterial dissemination and immuno-transcriptomics

Abstract

Most pathogens can infect a number of different host species, but host species often differ considerably in susceptibility to a given pathogen. In the case of zoonotic pathogens, natural hosts typically present little or no disease symptoms, while humans and other “spill-over” hosts often present severe symptoms. Differences in susceptibility do not only occur between natural and non-natural hosts, though, but also among natural hosts.

The natural hosts of the tick-transmitted bacterium *Borrelia afzelii*, which is one of three causative agents of Lyme borreliosis in humans, include a variety of small mammals like voles and mice. Studies of *Borrelia* infection in laboratory mice and humans have revealed mechanisms behind susceptibility to disease. However, less is known about colonization, pathology, and host immune responses to Lyme borreliosis spirochetes in their natural hosts.

In this thesis, I used *B. afzelii* and its natural hosts (primarily yellow-necked mouse, *Apodemus flavicollis*; and bank vole, *Myodes glareolus*) as study system to investigate the colonization of a zoonotic pathogen in different natural host species, and the possible factors that cause inter- and intra-specific variation in susceptibility.

In papers I-III, I focused on interspecific variation in susceptibility to *B. afzelii.* In **Paper I**, I found that *B. afzelii* disseminates from the site of infection (skin) to internal tissues in natural hosts, like spirochetes do in non-natural hosts, but levels of colonization vary between both species and tissues. Significantly higher bacterial loads were seen in bank voles than in yellow-necked mice. However, none of the natural hosts showed signs of pathological effects of *B. afzelii* infection. In **Paper II,** species-specific regulation of immune responses (IFNα response, IL6 signaling and the complement pathway) associated with *B. afzelii* resistance was demonstrated by using RNA-sequencing to quantify gene expression in the spleen of voles and mice. Further analyses of the RNA-seq data set indicated that interspecific divergence in expression of pattern recognition receptors (PRRs), and cyto- and chemokines contribute disproportionately to interspecific variation in immune function in general (**Paper III**). In **Paper IV,** I focused on intra-specific variation in resistance in the bank vole, and showed that the previously demonstrated effect of variation in the PRR *TLR2* on resistance to *B. afzelii* could be a result of polymorphisms in the coding sequence, and/or polymorphisms in non-coding sequences affecting expression of *TLR2*.

In summary, the findings in this study have provided new insights to understand the causes of inter- and intraspecific variation in susceptibility to a zoonotic pathogen in its natural host species.

Key words: *Borrelia afzelii,* natural hosts, bacterial load, immune response, RNA-seq

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我非生而之知者，好古，敏以求知者也。

——《论语.述而》

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Author contributions

I. XZ and LR designed the study. XZ and LR performed field work. XZ performed the lab work for measuring bacterial loads and MN performed the histological work. XZ carried out the statistical analysis with help from LR. XZ wrote paper with input from LR and MN.

II. XZ and LR conceived and designed the study. XZ and LR performed field work. XZ performed the lab work. XZ performed bioinformatic analyses with help from ML. XZ wrote paper with input from LR and ML.

III. XZ and LR conceived and designed the study. XZ and LR performed field work. XZ performed the lab work. XZ performed bioinformatic analyses with help from ML. LR and XZ performed statistical analyses. LR and XZ wrote paper with input from ML.

IV. XZ and LR designed the study. XZ and LR performed field work. XZ performed Sanger sequencing and associated analyses. ML performed bioinformatic analyses of genome re-sequencing data. XZ performed analyses of LD and gene expression. LR performed other population genetic analyses. LR wrote paper with input from all authors.

Abstract

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Popular science summary

Every year there are about one billion cases of illness and millions of deaths caused by zoonotic diseases. Zoonotic means infectious diseases spread from animals to humans, and zoonotic pathogens include for example, Ebola virus from bats, and Lyme borreliosis bacteria from rodents. These animals, which are called natural host, typically do not get sick when they are infected by these zoonotic pathogens; why is that?

The immune system protects the host against infections and diseases. If any pathogen invades the body, immune cells and other components attack it. So, the immune system of hosts plays an important role in determining the outcome of an infection.

In my thesis, I used one of the causative pathogens of human Lyme borreliosis to study variation in susceptibility between and within its natural host species, and the possible causes of such variation.

Bank voles and yellow-necked mice are common small mammals and widespread in Europe. They are natural hosts of *Borrelia afzelii*, which is a zoonotic pathogen transmitted to humans by ticks. An expanding rash at the tick bitten site is a typical early clinical symptom of human Lyme borreliosis. In the late infection stage, bacteria disseminate to internal organs and induce various symptoms, like arthritis (inflammation in joints).

Voles and mice were caught in the wild, and we quantified how many bacteria that were present in different tissues. High loads of bacteria were generally found in skin (that is the site where the infection starts). However, there were also high loads in joints, showing that the infection spreads to other tissues also in natural hosts. Moreover, infected voles had ten times higher bacterial loads than infected mice. However, bacteria did not cause any pathological effects in the infected joints of these two species.

To find the reason for the difference in bacterial load between voles and mice, we started to investigate their immune systems. The spleen is a key immune organ in mammals, and contains large numbers of immune cells. When pathogens enter the host body, immune cells will react. The amount of mRNA from a gene is a representative measure of how strong this gene reacts to pathogens. We measured the amount of mRNA of immune genes expressed in the spleen, and compared expression between infected and uninfected voles or mice. We looked at groups of genes that are connected with each other (known as “gene sets”). Three immunological gene sets were regulated in different ways during *Borrelia* infection in voles and mice. These differences could contribute to the difference in resistance to *Borrelia* between the host species. In a further analysis we divided immune genes into different categories based on their function. Here, we found that expression of pattern recognition receptors (PRRs), which recognize

pathogens and induce the immune response, were the most divergent gene category between species.

There is not only variation in susceptibility between host species, but also among individuals of a given species. To investigate possible causes of such variation, we paid attention to toll-like receptor 2 (*TLR2*), which is a key PRR for recognition of *Borrelia*. Previous studies of bank voles have shown that mutations in the *TLR2* gene are associated with resistance to *Borrelia.* A gene contains a coding region that is translated to amino acids for protein construction, and a non-coding region that includes elements responsible for the regulation of gene expression (e.g. promoter). Mutations in *TLR2* were found both in the coding and non-coding region, and these mutations were linked, meaning that they are inherited together. This means that the association between variation in the *TLR2* and resistance to *Borrelia* could be due to mutations in the coding sequence, and/or mutations in non-coding sequences affecting expression of *TLR2*.

To sum up, the results in this thesis demonstrate that susceptibility to a zoonotic pathogen varies among and within natural host species, and the thesis also identifies possible causes of this variation. In order to better understand the difference in susceptibility between natural and non-natural hosts, further studies covering more species are needed.

Introduction

Zoonotic diseases

Emerging infectious diseases (EIDs) — that is, infections that have increased in occurrence recently within a population or threatens to increase in the near future — are a significant and rapidly growing threat to public health and global economy. A review paper published in 2008 reported 335 EID "events" between 1940 and 2004. Among them, 60.3% of EIDs were zoonotic diseases, that is diseases caused by pathogens transmitted from animal reservoir hosts to humans. The majority of zoonoses are caused by pathogens (e.g. bacteria, viruses, parasites, protozoa, etc) with a wildlife origin, such as bats and rodents (Jones et al., 2008). Profound pathology and high morbidity are often observed in humans infected with zoonotic pathogens, such as Ebola viruses, Nipah viruses and severe acute respiratory syndrome coronavirus, but pathology in natural hosts seems to be more limited (Bean et al., 2013; Mandl et al., 2014). These hosts are called reservoirs and they get signs of clinical disease, but rather carry it seldom as a subclinical infection. For example, simian immunodeficiency virus, a close relative of HIV-1 in humans, has no detectable impact on the survival or health in its natural hosts, the sooty mangabeys and African green monkeys (Keele et al., 2009). Similarly, Sin Nombre virus, the etiologic agent of most hantavirus cardiopulmonary syndrome cases, showed persistent infection without signs of clinical disease in deer mice after inoculation (Schountz et al., 2012).

Studying the pathology and immunology of zoonotic diseases in natural host species can help to reveal key processes in disease susceptibility and transmission (Bean et al., 2013). The rapid development of DNA and RNA sequencing technologies has greatly facilitated such studies. Here, I used the tick-transmitted bacterium *Borrelia afzelii* and its natural hosts (small mammals) to bring some insights into the causes of inter- and intra-specific variation in immune response to a zoonotic pathogen in the wild.

Lyme borreliosis

Lyme borreliosis, which is a tick-borne infectious disease caused by the spirochete bacterium *Borrelia burgdorferi sensu lato* (s.l.), is common in North America and Europe, but is also reported in parts of Asia (Mead, 2015). The disease was first observed and associated with arthritis in the communities of Old Lyme in Connecticut, USA, in the 1970s (Steere et al., 1977). Today, Lyme borreliosis has been reported in more than 80 countries and the incidence is increasing. In the United States, around 3,000,000 cases of the disease are estimated to occur each year (Hinckley et al., 2014).

Borrelia burgdorferi s.l. is divided into 22 genospecies, of which at least three (*B. burgdorferi sensu stricto* (s.s.), *B. afzelii* and *B. garinii*) cause Lyme borreliosis in humans (Waindok et al., 2017). These genospecies cause Lyme borreliosis in different geographical regions, *B. burgdorferi* (that is, *B. burgdorferi* s.s.) is the primary causative pathogen of Lyme borreliosis in the United States, whereas *B. afzelii* and *B. garinii* dominate in Europe (Steere, 2001). Hard ticks of the genus *Ixodes* act as vectors that transmit the spirochetes between hosts, including humans. Different *Borrelia* genospecies are transmitted by different species of ticks, and the distribution of ticks limits the geographic distribution of Lyme borreliosis (Piesman and Gern, 2008).

Ticks have three developmental stages — larva, nymph and adult — and take a single blood meal during each stage. After feeding on an infected animal, larva or nymph drop off the animal and develop into the next stage (i.e. nymph or adult) (**Figure 1**) (Stanek et al., 2012). In the United States, various rodents, such as white-footed mice, are the main vertebrate reservoir hosts for *B. burgdorferi* (LoGiudice et al., 2003). In Europe, rodents and birds are the principal reservoirs for *B. afzelii* and *B. garinii*, respectively (Piesman and Gern, 2008). Deer are not a competent reservoir for *Borrelia* spirochetes, but are essential for the maintenance of a tick population in an area (Rand et al., 2009).

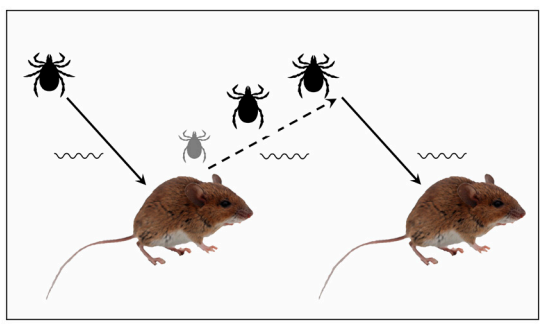

Figure 1 Life cycle of *B.afzelii* in the wild.
An infected nymph (eight legs) transmits spirochetes to a naïve host (yellow-necked mouse in the figure); uninfected larva (six legs) takes blood meal from infected animal, develops into nymph with spirochetes, and becomes the source of infection for other naïve animals. Here is no adult tick in the figure.

Lyme borreliosis is a disease with diverse clinical symptoms in humans. The skin is the most frequently involved tissue in Lyme borreliosis, and an expanding erythematous lesion known as erythema migrans (EM) is a typical early clinical symptom at the tick bite site (Nadelman and Wormser, 1995). Lyme neuroborreliois and Lyme carditis may also develop during the early disseminated disease, but the frequency is much lower than EM (Stanek and Strle, 2018). The principal symptom of late Lyme borreliosis is Lyme arthritis; approximately 60% of infected patients without antibiotics treatment develop arthritis (Steere et al., 1987). Different *B. burgdorferi* s.l. genospecies cause different symptoms: *B. burgdorferi* infection is often associated with Lyme arthritis, whereas *B. garinii* cause neuroborreliosis and *B. afzelii* primarily induce skin infection (Steere, 2001). As a result, there are regional differences in the clinical symptoms of Lyme borreliosis, with neuroborreliois predominantly in Europe, and arthritis primarily in the United States.

Colonization and dissemination of *Borrelia* spirochetes in the host

The feeding period of a tick upon a host required for spirochete transmission varies between borrelial genospecies; more than 48 hours are usually needed for *B. burgdorferi*, while *B. afzelii* transmits more rapidly (Strle et al., 1996; des Vignes et al., 2001). During the blood meal, spirochetes migrate from the midgut of the tick to the salivary gland, and are then transmitted to the host with the saliva. Transition from tick to vertebrate host involves changes in expression of surface proteins of the spirochete, for example, upregulation of OspC, DbpA and DbpB, and downregulation of OspA and OspB (Stanek and Strle, 2018). Additionally, tick salivary gland proteins, such as Salp15, enhance spirochete colonization through inducing immunosuppressive activity at the bite site (Rosa, 2005).

Dissemination from the tick bite site to other tissues or organs occurs via the blood and lymphatic system, or via direct spread through the tissues. Hematogenous dissemination seems to be the more common and is the most well understood (Wormser, 2006; Hyde, 2017). Spirochetes disseminate to the heart, joints and nervous system, and cause diverse symptoms of Lyme borreliosis. In addition, organs such as liver, lung, spleen, eye, and bladder may also be colonized by spirochetes (Humair and Gern, 2000).

The capacity of spirochetes for hematogenous dissemination varies between genotypes of a given *B. burgdorferi s.l.* genospecies, as determined by for example 16S–23S ribosomal RNA spacer or Ospc (Wormser et al., 2008). Many of the spirochete surface proteins upregulated during infection of vertebrate host (e.g. OspC, DbpA and DbpB) play key roles in colonization and dissemination. For

example, they interact with plasminogen in the blood to facilitate movement to distant sites, with complement regulatory proteins to evade immune responses, and with tissue-specific endothelial cell (EC) surface receptors to colonize new sites (Caine and Coburn, 2016).

The host immune response

Innate immune response against *Borrelia*

The initial host innate immune response is mediated by recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) expressed on cells involved in innate immunity, such as macrophages, dendritic cells etc (Abbas et al., 2012). Some receptors are present on the cell surface, others are present in the endosome or cytosol. Toll-like receptors (TLRs) are one important type of PRRs, and several TLRs contribute to the host immune response to *Borrelia*. For example, TLR1 and TLR2 recognize lipoproteins, and TLR5 recognize flagellin (Hirschfeld et al., 1999; Shin et al., 2008; Salazar et al., 2009). After phagocytosis, TLR7 and TLR8 recognize bacterial RNA, and TLR9 recognize CpG sites in DNA (Shin et al., 2008; Petzke et al., 2009; Cervantes et al., 2011). NOD2 (Nucleotide-binding oligomerization domain-containing protein 2) and its adaptor RICK (receptor-interacting serine/threonine kinase), are also crucial for the recognition of spirochetes (Oosting et al., 2010).

The TLR2 pathway is essential for inducing inflammatory cytokines. Deficiency of TLR2 or one of its adaptors, such as MyD88 (myeloid differentiation primary response 88) or TRIF (TIR-domain-containing adaptor molecule 1), leads to higher spirochete loads and increase Lyme arthritis severity in laboratory mice (Liu et al., 2004; Wang et al., 2004; Petnicki-Ocwieja et al., 2013; Lasky et al., 2016).

Pro-inflammatory cytokines with increased expression levels after recognition of spirochetes include interleukin 1 beta (IL-1β), Il2, IL6 and tumour necrosis factor-alpha (TNF-α), which are known to be involved in the pathogenesis of Lyme borreliosis (Jablonska and Marcinczyk, 2006; Jones et al., 2008; Strle et al., 2009; Buffen et al., 2013). In addition, Type I interferons, including IFN-α and IFN-β, are induced by *B. burgdorferi* in human peripheral blood mononuclear cells (PBMCs; i.e. lymphocytes and monocytes) and laboratory mice (Petzke et al., 2009; Cervantes et al., 2011; Hastey et al., 2014; Love et al., 2014). Type I interferons induce resistance to viral infection, but have also been found to be involved in response to bacteria, with detrimental effects for the host in certain bacterial infections, while in others they are critical for host defense (Boxx and Cheng, 2016). In the case of *Borrelia*, type I IFNs are involved in the development

of neuropsychiatric symptoms and Lyme arthritis (Jacek et al., 2013; Paquette et al., 2017), and increased IFNs and IFN-responsive gene expression were found in *Borrelia* infection associated with disseminated Lyme borreliosis (Krupna-Gaylord et al., 2014; Petzke et al., 2016). *Borrelia* infection also induces expression of various chemokines. For example, the levels of neutrophil chemoattractant CXCL1 (CXC-chemokine ligand 1), macrophage chemoattractant CCL2 (CC-chemokine ligand 2), dendritic cell chemoattractant CCL20 and T-cell-active chemokines CXCL9 and CXCL10 were increased in the skin samples from patients with erythema migrans (Müllegger et al., 2007).

The complement cascade is another major innate immune component. The complement cascade may be activated by any of three pathways — the classical, lectin, or alternative pathway — all of which lead to the production of C3b. C3b is deposited on microbes, and initiates the late steps of complement activation, including the formation of a membrane attack complex (MAC), which is the key component causing lysis of microbes, such as *Borrelia* (Merle et al., 2015; Zhi et al., 2018). During the dissemination process in the host blood and other tissues, borrelial spirochetes are exposed to these complement proteins. To survive and establish a persistent infection in the host, spirochetes must resist killing by complement in the host blood and tissues. The interaction between borrelial proteins and host complement proteins has been investigated by a number of studies (Kurtenbach et al., 2002; de Taeye et al., 2013; Kraiczy, 2016). Serum-resistant *Borrelia* isolates can express CRASPs (complement regulator acquiring surface proteins) on their outer membrane to recruit host complement regulators that are part of the factor H family for complement evasion in host (Bykowski et al., 2008; Kraiczy et al., 2001). The CD59-like protein expressed by *Borrelia* is also the key protein to inhibit the formation of the MAC (Pausa et al., 2003). During the tick feeding, the *Borrelia* spirochetes are protected from the complement attack by the tick salivary proteins. The TSLPI (tick salivary lectin pathway inhibitor) protein inhibit the lectin pathway of complement activation (Schuijt et al., 2011), while Salp20 mediates complement inhibition by destabilizing the C3 convertase through displacement of properdin from the convertase complex (Tyson et al., 2007, 2008), and Salp15 binds to outer surface protein C (OspC) to protect *Borrelia* against MAC formation and lysis of the spirochetes (Schuijt et al., 2008).

Adaptive immune response against *Borrelia*

Mice infected with *B. burgdorferi* s.l. and cured by antibiotics were resistant to challenge with same genospecies for up to one year (Barthold, 1999), showing that hosts develop acquired immunity. However, the antibody response does not eliminate borrelial spirochetes from tissues (Hodzic et al., 2003). The antibody

response to *B. burgdorferi* infection is complex, and both T-cell independent and T-cell dependent antigens are targeted (LaRocca and Benach, 2008). Antigenic proteins include Arp, FlaB, BmpA, DbpB, OspC, VlsE, BBK07, and BBK12 (Baum et al., 2012; Elsner et al., 2015).

T-cell dependent responses involve different types of T helper cells, including Th1, Th2 and Th17. Several studies of experimental animal models of Lyme borreliosis have explored the Th1/Th2 immune balance in this disease, aiming to reveal which type of immune response is beneficial for efficient elimination of spirochetes. IFN-γ, a powerful pro-inflammatory cytokine, is the key mediator of Th1 responses and has been shown to drive inflammation upon borrelial infection (Moore et al., 2007; Oosting et al., 2016). IFN-γ activates macrophages to become more effective at killing phagocytosed microbes. The adaptive immune response in humans with *Borrelia* infection is characterized by a Th1 response with high IFN-γ production both within the central nervous system of neuroborreliosis patients and the joints of Lyme arthritis patients (Ekerfelt et al., 1997; Yin et al., 1997; Gross et al., 1998; Shin et al., 2007; Henningsson et al., 2011). However, increased IL-4, a key Th2 cytokine, was observed in patients with non-chronic neuroborreliosis after clearance of spirochetes, suggesting that a Th2 response modulates the resolution of inflammation induced by Th1 cells (Widhe et al., 2004). Murine models of Lyme borreliosis have shown varying results depending on infection route. With needle inoculation of *Borrelia*, C3H/HeN mice produced more IFN-γ than BALB/c mice, and lower or no IL-4 were detected during the Lyme arthritis development (Matyniak and Reiner, 1995; Kang et al., 1997). By contrast, C3H/HeN mice produced a polarized Th2 response after getting infected by tick bite (Zeidner et al., 1997). In one word, the Th1/Th2 immune balance in this disease is still unclear.

Increasing evidence shows that Th17 cells and their associated cytokines are involved in the pathogenesis of Lyme arthritis (Burchill et al., 2003; Codolo et al., 2008; Oosting et al., 2011). However, IL-17 is not solely responsible for development of severe pathology in response to infection with *B. burgdorferi*; it may contribute to disease through an interaction with IFN-γ (Kuo et al., 2016). Early neuroborreliosis is dominated by a Th1 response, but Th17-associated immune responses in Lyme neuroborreliosis pathogenesis were also reported (Henningsson et al., 2011; Gyllemark et al., 2017). By stimulating human PBMCs with live *Borrelia*, researchers found that the role of Th17 cytokines seems to differ depending on the clinical stage of Lyme borreliosis and on the *B. burgdorferi* genospecies (Bachmann et al., 2010; Grygorczuk et al., 2016).

Pathology

Erythema migrans (EM) is the most frequent manifestation of Lyme borreliosis in Europe. Biopsies from the centre of EM lesions reveal mild superficial perivascular infiltration by lymphocytes and some histiocytes, sometimes accompanied by plasma cells. Neutrophils may also be present around the blood vessels. Involvement of cell-mediated immune mechanisms in the initial host response to borrelial spirochetes is indicated by the presence of T cells and increased numbers of Langerhans cells (De Koning, 1993). Arthritis is the most common late disease manifestation, affecting up to 60% of patients (Steere et al., 1987). Since the spirochete does not express toxins, the tissue damage is caused by a strong inflammatory response to borrelial spirochetes. Lymphocytes, macrophages and scattered mast cells in the interstitium are accumulated around the blood vessels in patients with severe Lyme arthritis (Johnston et al., 1985). In synovial fluid, neutrophils often represent the majority of cells, producing a variety of inflammatory cytokines to mediate pathogenesis of Lyme arthritis (Gerber et al., 1998; Deanehan et al., 2014).

Inbred strains of the laboratory mouse *Mus musculus* infected with *B. burgdorferi* have been found to show similar symptoms to those of human Lyme borreliosis. However, disease severity differed between mouse strains. For example, when C3H and BALB/c mice are infected with *B. burgdorferi*, they develop both ankle joint arthritis and carditis, whereas C57BL/6 and DBA mice display minimal inflammation in the joint tissue, despite harbouring high levels of spirochetes (Wooten and Weis, 2001; Radolf et al., 2012). Studies of *B. burgdorferi* infections in the natural host *Peromyscus leucopus* in the field and laboratory showed that spirochetes disseminate to joint and heart, but there is no observable pathology in adult mice, while juveniles develop carditis and multifocal arthritis, similar to what was observed in *M. musculus* (Barbour, 2017).

Variation in susceptibility between and within host species

Studies of wild animals have indicated that reservoir hosts differ in resistance to *Borrelia. B. afzelii* is mainly distributed throughout the temperate regions of Eurasia and it has multiple reservoir hosts in wild, including a wide range of small mammals, such as voles, mice and shrew (Hellgren et al., 2011). Previous studies have found that *B. afzelii* bacterial load (number of borrelial spirochetes/unit host tissue) as measured in skin biopsies from ears differ considerably between natural host species, with bank voles and common shrews having ten-fold higher bacterial

load than yellow-necked mice (Råberg, 2012; Strandh and Råberg, 2015), indicating that host species differ in resistance. This interpretation is consistent with the previous finding that *Apodemus* mice have stronger antibody responses to experimental *B. burgdorferi* s.s. infection than bank voles (Kurtenbach et al., 1994).

There is also evidence that genetic variation within host species affect susceptibility to *Borrelia*. An association between *TLR2* genotype and *Borrelia*-susceptibility has been found in humans, where a missense mutation (Arg753Gln) is associated with reduced susceptibility to Lyme borreliosis (Schröder et al., 2005). Similarly, an association between *TLR2* genotype and resistance to *B. afzelii* was found in bank voles (Tschirren et al., 2013). Polymorphism in *TLR1* was associated with cytokine and chemokine production induced by spirochetes and outcomes of Lyme borreliosis in humans (Strle et al., 2012). A polymorphism in the IL23 receptor (*IL23R;* Arg381Gln) leads to less IL17 production in PBMCs isolated from patients after exposure to *B. burgdorferi*, but does not influence clinical signs of chronic Lyme borreliosis; IL23 is responsible for the maintenance of Th17 helper cell population and production of IL17 (Oosting et al., 2011).

Aims of thesis

In this thesis, I used the study system of small mammals and *B. afzelii* to address the following questions:

1. Does *B. afzelii* disseminate to other tissues and organs than skin in natural hosts, like observed in infection experiments with laboratory mice? Does the degree of dissemination to internal tissues differ between host species? Does *B. afzelii* cause any pathology in natural hosts? (Paper I)
2. Which genes and pathways are up- or down-regulated in natural hosts with *B. afzelii* infection in the wild? What is the immunological basis of differences in resistance to *B. afzelii* between natural host species? (Paper II)
3. Does the degree of expression divergence between bank voles and yellow-necked mouse differ between different categories of immune genes? (Paper III).
4. What is the molecular basis of the association between *TLR2* genotype and susceptibility to *B. afzelii* in bank voles? Does *TLR2* genotype affect *TLR2* gene expression? (Paper IV)

Methodology

Study species and field work

My studies focused on four small mammal species, including bank voles (*Myodes glareolus*), yellow-necked mice (*Apodemus flavicollis*), common shrews (*Sorex araneus*) and pygmy shrews (*Sorex minutus*) (**Figure 2**). All these species are known hosts of *B. afzelii* (Hellgren et al., 2011). Animals were trapped at one locality (Stensoffa) in the Revinge area, 20 km east of Lund, southern Sweden, using live traps baited with grains and apple. The habitat at the trapping locality is mixed deciduous forest dominated by beech (*Fagus sylvatica*). Traps were left over night and checked the following morning.

Figure 2 Three small mammal species studied in this thesis.
Bank vole (left), yellow-necked mouse (middle), common shrew (right). Photos by Lars Råberg.

We collected tissue samples for analysis of infection status, and genetic and transcriptomic analyses. From all trapped animals, a biopsy from each ear was sampled and stored in 70% ethanol. During 2015 and 2016, the whole heart and two rear ankles were also collected for measuring the bacterial load of *B. afzelii*, and histological analysis. From animals trapped during 2016, spleens were collected for RNA extraction and to analyse gene expression.

Real time quantitative PCR

The bacterial load of *B. afzelii* was measured by real time quantitative PCR (qPCR) with *B. afzelii*-specific primers. DNA was extracted from ear biopsies, hearts and joints. Two samples of each tissue from individuals were prepared for DNA extraction to minimize measurement error. All reactions were performed in a Mx3000 instrument (Stratagene) with SYBR Green detection method (Platinum SYBR Green qPCR SuperMIX-UDG; Invitrogen, Carlsbad, CA) as described in Råberg (2012). Further details can be found in the method section in Paper I.

Histological analysis

To investigate if *B. afzelii* infection causes pathology in natural hosts, we performed histological evaluation of joints. One rear ankle from each animal was collected and fixed in 4% phosphate buffered formaldehyde, demineralized and processed for Hematoxylin and Eosin staining by routine histologic techniques. Joint tissue sections were blindly examined without knowledge of infection. Assessment of histological parameters were performed as described in Lin *et al.* (2014).

Transcriptomics

RNA sequencing

To investigate the immune responses of wild animals, I used RNA-sequencing of spleen transcriptomes. RNA sequencing (RNA-seq) is a technique that can examine the quantity and sequences of RNA in a sample using next generation sequencing (Wang et al., 2009). It is rapidly replacing gene expression microarrays in many labs. RNA-seq allows the entire transcriptome to be surveyed in a high-throughput and quantitative manner. Following sequencing, raw reads are either aligned to a reference genome or assembled *de novo*, followed by quantification of the level of expression for each gene.

The spleen is an important immune organ, containing large numbers of immune cells like phagocytes and lymphocytes. The spleen is often enlarged during infection due to proliferation of lymphocytes. Gene expression in the spleen should therefore reflect the overall activity of the immune system in an individual.

Collected spleen samples were stored in RNAlater RNA Stabilization Reagent (Qiagen) and subsequently extracted to retrieve RNA for transcriptome

sequencing. To generate transcriptome-wide gene expression data, samples were sent to SciLifeLab (Stockholm, Sweden) for paired-end Illumina HiSeq 2500 RNA Sequencing.

Mapping approaches

To obtain estimates of gene expression that were comparable between species, we wanted to use similar approaches for both species. An annotated reference genome is available for the bank vole (Lundberg *et al.* submitted) but not for the yellow-necked mouse, so mapping reads from each species to its reference genome was not possible. Instead, we considered two other mapping approaches: (i) mapping reads from both voles and mice to the house mouse genome, and (ii) mapping reads to *de novo* transcriptomes for each species.

To test the first approach, trimmed bank vole and yellow necked mouse reads were mapped to the house mouse genome. As could be expected, mapping rates were considerably higher for yellow-necked mouse than for bank vole. To avoid biased results because of differences in evolutionary distance between our two species and the reference genome, we therefore decided to map reads from each species to its *de novo* transcriptome instead. For details of *de novo* transcriptome assembly and annotation, see the Paper II. We evaluated this approach by comparing gene expression in bank voles when mapping to the reference genome and the *de novo* transcriptome (see paper II for details). Expression values from mapping to the reference genome and the *de novo* transcriptome were strongly correlated in each vole ($r = 0.91 \sim 0.93$; **Figure 3**), showing that mapping to the *de novo* transcriptome assembly was a valid approach.

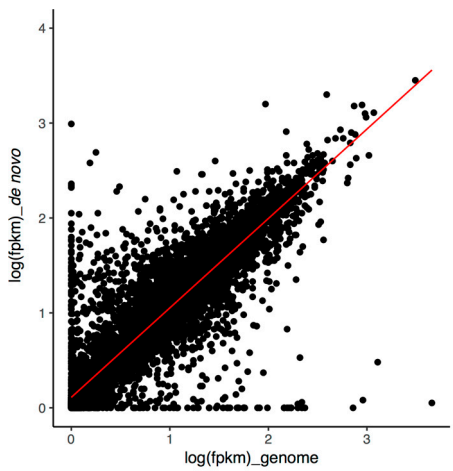

Figure 3 Example of a correlation between gene expression levels (log10-transformed FPKM) produced from *de novo* transcriptome mapping and reference genome mapping for one bank vole individual.

Effects of *B. afzelii* infection on gene expression

To investigate the immune response to *B. afzelii*, differential expression between *B. afzelii*-infected and uninfected animals was calculated using edgeR (Robinson et al., 2009) for bank voles and yellow-necked mice separately. Genes were considered as significantly differentially expressed between groups when the $\log_2$ fold change was greater than ± 1 and the false discovery rate (FDR) value less than 0.05.

As a complement to the differential expression analyses, we performed Gene Set Enrichment Analyses (GSEA; Subramanian et al., 2005). All genes that were detected in the bank vole and yellow-necked mouse transcriptomes were ranked based on the differential expression analysis (i.e. the difference in expression between infected or uninfected animals within each species). GSEA uses *a priori* defined gene sets (such as the genes in a particular pathway) and tests whether the members of a gene set are randomly distributed throughout the ranked list of all expressed genes, or enriched at the top or bottom. If genes in a gene set are enriched at either the top (up-regulated) or bottom (down-regulated), the gene set is considered to be related to the phenotypic difference between samples (here infected vs uninfected). GSEA is considered a more powerful approach than ordinary differential expression analysis when the data is noisy (as in our case, where we compared individuals sampled in the wild, rather than from a controlled experiment).

Expression divergence between species

Besides comparing the response to *B. afzelii* infection between species, we also performed analyses to compare expression of immune genes in general, regardless of infection status. In particular, we were interested in testing if certain categories of immune genes have particularly high expression divergence.

To identify one-to-one orthologs between bank vole and yellow-necked mouse, reciprocal best hit BLASTp was performed using predicted amino acid sequences from *de novo* transcriptome assemblies. The annotation of putative orthologs was performed by using BLASTx against house mouse (*Mus musculus*) proteins.

The mapping of the RNA-seq reads to the annotated orthologs matrix and quantification of gene expression was performed with RSEM. Initial gene expression matrices were produced for the two species separately, including non-normalized expected counts for each individual within species. zFPKM values were computed across all individuals according to the procedure described in Hart *et al.* (2013).

We compiled a list of immune genes likely to be expressed in spleen based on KEGG immune system pathways (Kanehisa et al., 2017). In addition, we included all cytokines and their receptors listed in appendix IV in Janeway's

Immunobiology (Murphy and Weaver, 2017). This yielded in total 732 genes. Of these, 676 could be divided into four functional categories: (1) PRRs, which are involved in the initial detection of pathogens; (2) signal transduction proteins (adaptors, protein kinases, etc, henceforth signaling proteins), which mediate signaling downstream of PRRs and other receptors; (3) transcription factors, and (4) cyto-/chemokine or cyto-/chemokine receptor, which mediate intercellular communication (**Figure 4**). The remaining 56 did not easily fit into any of the above categories and they were therefore excluded from further analyses. The degree of interspecific expression divergence of the four categories of immune genes were compared to each other, and to all other genes, which include both non-immune genes and immune genes not assigned to any of the four categories.

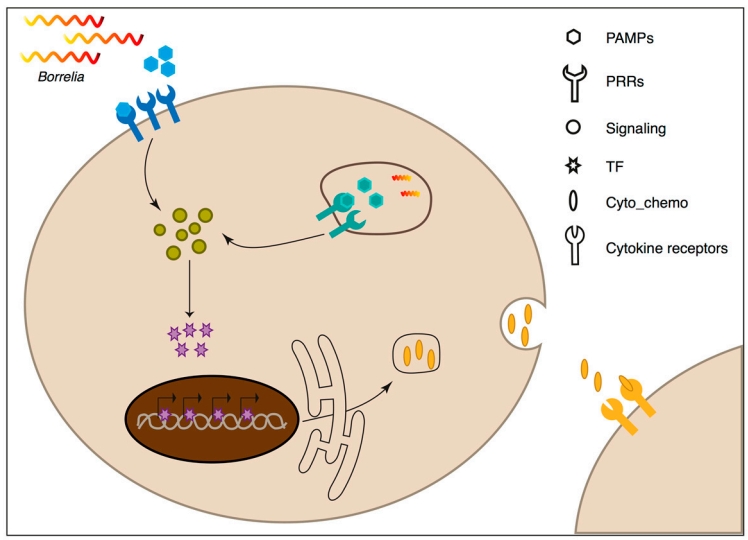

Figure 4 Overview of four functional immune gene categories involved in innnate immunity.
PRRs, pattern recognition receptors (which detect PAMPs, pathogen-associated molecular pattern molecules); Signaling, signal transduction proteins; TF, transcription factors; Cyto_chemo, cytokines and chemokines.

To estimate expression divergence between species, we used smatr3 (Warton et al., 2012) to extract residuals from a Standardized Major Axis regression (where residuals are orthogonal to the regression line; Warton *et al.* 2006) of expression in bank voles against expression in yellow-necked mice, and used the squared residuals as a measure of divergence (Chen et al., 2019)(see paper III for details).

Polymorphism and expression of TLR genes

TLRs play a crucial role to recognize borrelia spirochetes once they enter the host (Oosting et al., 2014). To investigate levels of polymorphism in bank vole *TLR1, TLR2* and *TLR6,* I used whole genome re-sequencing data from 31 bank voles from our study population (Lundberg *et al.* submitted). In addition, I performed Sanger sequencing of partial sequences in the coding region of *TLR1*, *TLR2* and *TLR6*, to increase sample sizes for analyses of haplotype structure. Further details can be found in the method section in Paper IV. Haploview (Barrett et al., 2005) was used to generate *TLR2* linkage disequilibrium (LD) plots based on the whole-genome re-sequencing data.

To examine the association between *TLR2* genotype and *TLR2* gene expression, zFPKM transformed expression values were used to represent the overall expression of *TLR2* in an individual. To test for allele-specific expression of *TLR2,* variants calling from RNA-seq was performed by the Genome Analysis Toolkit (GATK) Best Practice (Van der Auwera et al., 2013).

Results & Discussion

Bacterial dissemination

Here I investigated whether *B. afzelii* disseminates to other tissues and organs than skin in its natural hosts, and if the bacterial load differs between species and tissues. Furthermore, I also compared the degree of dissemination between host species, and investigated whether *B. afzelii* causes pathology in natural hosts. Four host species were included in this study: bank vole, yellow-necked mouse, common shrew and pygmy shrew.

There were no infections detected in the sample of pygmy shrews. Spirochetes can disseminate from the tick bite site to the heart and joint in bank vole, yellow-necked mouse and common shrew. The prevalence of infection was higher in ear than in heart and joint, but the prevalence in different tissues was similar across species. Thus, *B. afzelii* readily disseminates from the skin to joint and heart tissue in its main natural hosts, and there are no differences between host species in dissemination patterns.

For the subsample of infected individuals, the bacterial load differed between host species ($F_{2, 93} = 18.32$, $p < 0.0001$), and between tissues ($F_{2, 93} = 40.52$, $p < 0.0001$; **Figure 5**). Separate analyses for each tissue showed that the bacterial load in ear and joint were higher in voles and shrews than in mice, whereas the bacterial load in heart were lower in both shrews and mice as compared to voles. Voles and shrews had >10-fold higher spirochete load than mice in skin and joints.

To test if bacterial load in ear predicted bacterial load in heart and joint, we performed ANCOVAs with bacterial load in heart or joint against bacterial load in ear, species, and their interaction. There was a positive but not significant relationship between bacterial load in ear and heart (ear: F1, 48 = 2.70, p=0.11; species: F2, 48 = 2.66, p=0.08; the ear × species interaction was not significant and therefore excluded from the model: F2, 48 = 0.78, p=0.46) (**Figure 6a**). There was a significant positive relationship between bacterial load in ear and joint (ear: F1, 49 = 4.26, p=0.044; species: F2, 49 = 7.08, p=0.002; the ear × species interaction was not significant and therefore excluded from the model: ear × species: F2, 49 = 1.79, p=0.18, **Figure 6b**).

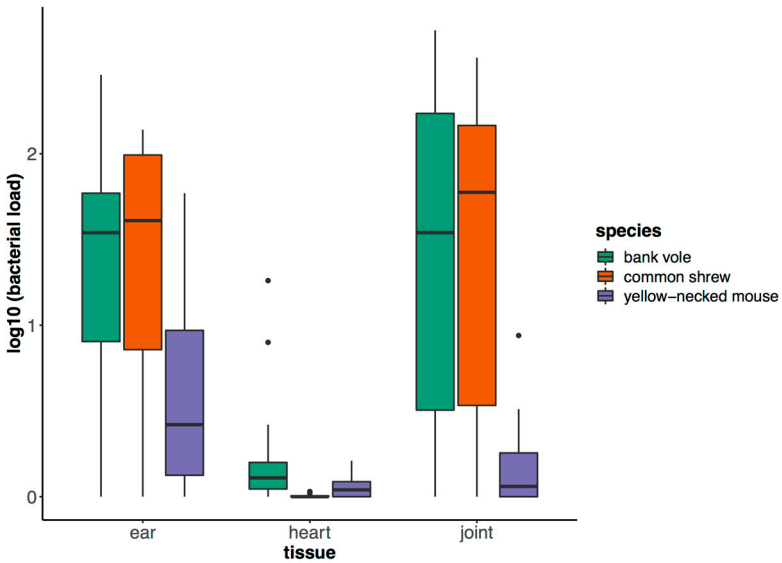

Figure 5 Box plot of bacterial load in different tissues in three small mammal species.
The box plots indicate the median, first and third quartiles, and range of the data. Bacterial load was estimated with qPCR of *B. afzelii flaB*. Reprinted from Paper I.

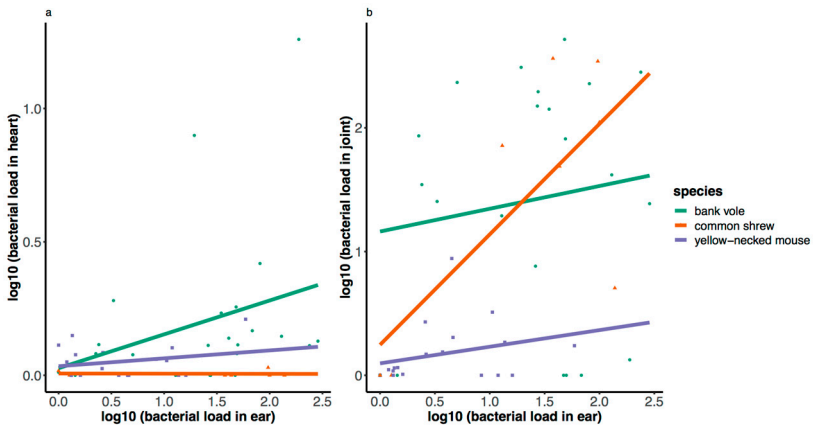

Figure 6 Correlation between tissue-specific bacterial loads in bank voles, yellow-necked mice and common shrews.
(a) Correlation of bacterial load between ear and heart; (b) correlation of bacterial load between ear and joint. Reprinted from Paper I.

Pathology was evaluated in tibio-tarsal joints from bank voles and yellow-necked mice. No inflammatory cells or other pathological signs were observed in surrounding tendons and in the synovium when comparing the infected and uninfected groups.

In conclusion, *B. afzelii* disseminates to internal tissues in natural hosts, but levels of colonization vary between both species and tissues. There is as yet little evidence for pathological effects in natural hosts.

Immuno-transcriptomics

Differential expression and GSEA

The results from Paper I showed that *B. afzelii* bacterial loads differ between host species, with voles and shrews having 10-fold higher level of spirochetes in ear and joints than mice. This result indicates that host species differ in resistance to *B. afzelii*. Here, I used RNA-sequencing of spleen transcriptomes to investigate if the difference in resistance is a result of that different immune pathways are activated during infection in the two rodent species. Eighteen bank voles and seventeen yellow-necked mice were included.

In total, 842,299 and 761,841 contigs (presumptive transcripts) in bank vole and yellow-necked mouse were assembled, respectively. The contig N50 score, which is a statistic representing a weighted average transcript length, was 1799 for bank vole and 2022 for yellow-necked mouse. When mapping back the reads used in the assembly, both of the assemblies showed a high proportion (>95%) of properly aligned read pairs.

Following the removal of redundant contigs and contigs with low expression levels (TPM < 1), the transcriptome assemblies consisted of 31,944 contigs for bank vole and 32,238 contigs for yellow-necked mouse. Selecting the contig with the highest bit score to a specific *M. musculus* gene resulted in the retention of 13,631 contigs in bank vole and 13,744 contigs yellow-necked mouse.

Trimmed reads from each individual were subjected to RSEM (Li and Dewey, 2011) for estimation of transcript abundance. A total of 51% and 54% reads from bank vole and yellow-necked mouse were successfully mapped, respectively. Estimated counts were used as input in edgeR (Robinson et al., 2009) for analysis of differential expression between *B. afzelii*-infected and uninfected groups. When setting the adjusted p-value at <0.05 and $\log_2$ fold change greater than ± 1, eight differentially expressed genes were found between bank vole groups, and five differentially expressed genes were found between yellow-necked mice groups (**Figure 7**). *CD209A* (up-regulated in infected mice) is a PRR expressed by innate immune cells; *CD177* (down-regulated in infected mice) is a surface glycoprotein that plays a role in activation of neutrophils; *MRGPRX2*, *CPA3* and *CMA1* (all up-regulated in infected voles) are primarily expressed in mast cells; and *MARCO* (down-regulated in infected voles) is a scavenger receptor expressed by innate immune cells and involved in recognition of bacteria (Bateman, 2019).

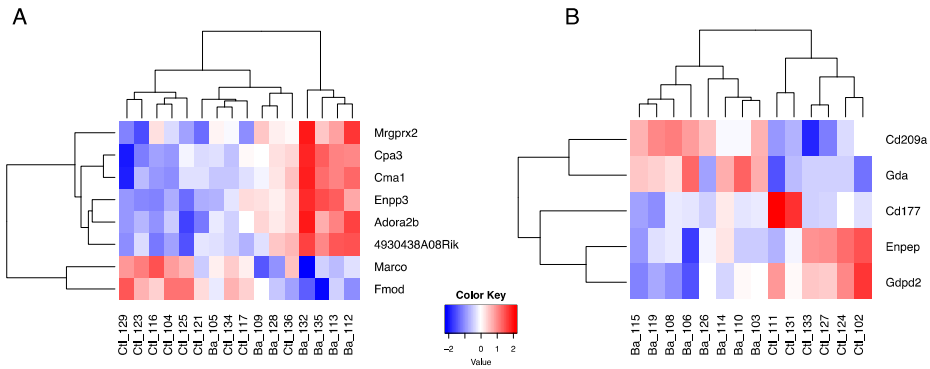

Figure 7 Heatmaps and hierarchical clustering of significantly differentially expressed genes between *B. afzelii*-infected and uninfected individuals.
(A) Bank vole; (B) Yellow-necked mouse. Ba_XXX are infected individuals, and Ctl_XXX are uninfected. Extracted from Paper II.

To further explore the effects of *Borrelia* infection on gene expression, I used Gene Set Enrichment Analysis (GSEA). We performed GSEA on all 50 "Hallmark gene sets", where each set represents a specific well-defined biological state or processes (e.g. a specific signaling pathway; Liberzon et al., 2015). In bank voles, 27 gene sets were significantly enriched, including 9 up-regulated and 19 down-regulated gene sets when comparing *B. afzelii*-infected individuals to uninfected. In the *B. afzelii*-infected yellow-necked mice, 8 gene sets were up-regulated and 11 were down-regulated. It should be noted that some of the gene sets that were up- or down regulated in infected vs uninfected individuals have no obvious function in the spleen (e.g. spermatogenesis, UV-response); those cases presumably reflect differential expression of highly pleiotropic genes that are also involved in pathways expressed in the spleen.

To better understand the immune response against *B. afzelii* in natural hosts, the seven immunological Hallmark gene sets were used for further analysis. Six of these gene sets were enriched in at least one of the species. Each of these six gene set consists of 87-200 genes involved in a specific immune pathway.

Three gene sets showed significant negative enrichment scores in both bank voles and yellow-necked mice (IL2_STAT5 signaling, TNFα_via_NFκB signaling and inflammatory response), indicating that these three gene sets were down-regulated in *B. afzelii*-infected individuals regardless of host species. Two gene sets were only enriched in bank voles with negative enrichment scores, while there was no difference in yellow-necked mice (IL6_JAK_STAT3 signaling, complement system). However, the gene set IFNα response was up-regulated in *B. afzelii*-infected yellow-necked mice, but not in bank voles (**Figure 8**).

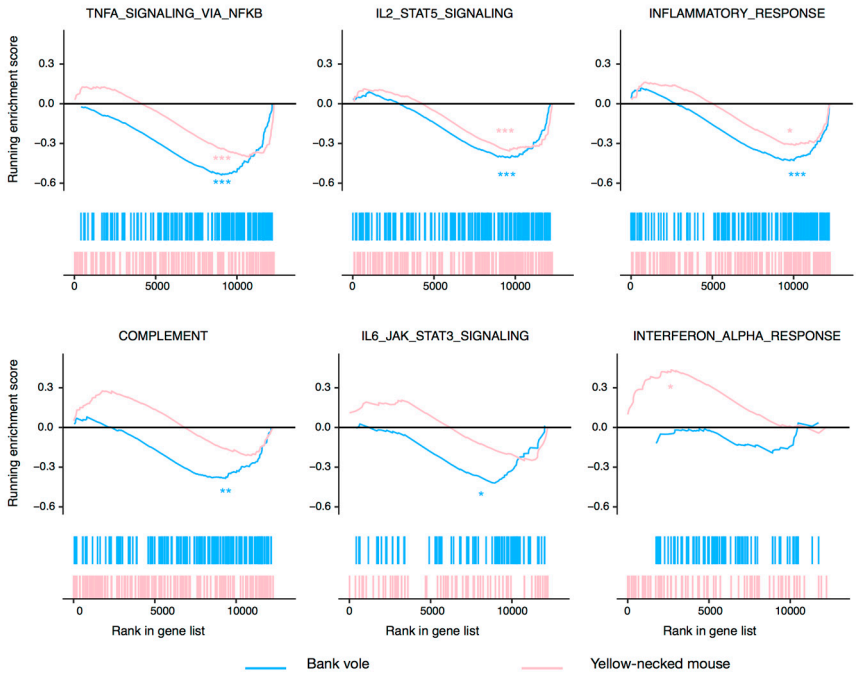

Figure 8 Gene set enrichment plots of immunological pathways involved in *B. afzelii* infection in two host species.
For each plot, the top portion shows the running enrichment score (ES) for the gene set as the analysis walks down the ranked list, the bottom portion shows where the members of the gene set appear in the ranked list of genes. In each enrichment plot, the score at the peak of the plot (the score furthest from 0.0) is the enrichment score for the gene set. * P < 0.05, ** P < 0.01, *** P < 0.001.Extracted from Paper II.

In conclusion, the IFNα response was up-regulated in *B. afzelii*-infected yellow-necked mice, while IL6 signaling and the complement pathway were down-regulated in infected bank voles. Differences in regulation of these three pathways between bank voles and yellow-necked mice could thus contribute to the difference in resistance to *B. afzelii* between the species.

Expression divergence

A recent analysis of immune gene expression in cell cultures in response to stimulations showed that expression divergence between species was concentrated to a few functional categories, with high divergence in expression of PRRs and cytokines and chemokines, but little divergence of most signal transduction genes (Hagai et al., 2018).

To test if similar patterns occur in naturally infected wild animals, I analysed divergence in expression between bank voles and yellow-necked mice regardless of infection status, by using RNA-seq data.

One-to-one orthologs between bank vole and yellow-necked mouse were determined using reciprocal best hit (RBH) via BLASTp, which resulted in 8599 annotated orthologs. Of our list of 676 immune genes based on KEGG pathways (see Methodology), 315 were annotated in the set of 8599 orthologs. These were categorized into four groups: PRRs, signal transduction proteins, transcription factors, and cyto-/chemokine or cyto-/chemokine receptors.

Intraspecific expression variation was measured as the standard deviation (SD) of zFPKM values. In bank voles and yellow-necked mice, PRRs and cyto-/chemokine genes had higher variation (SD) than the category "all other", signaling and transcription factor genes (**Figure 9**).

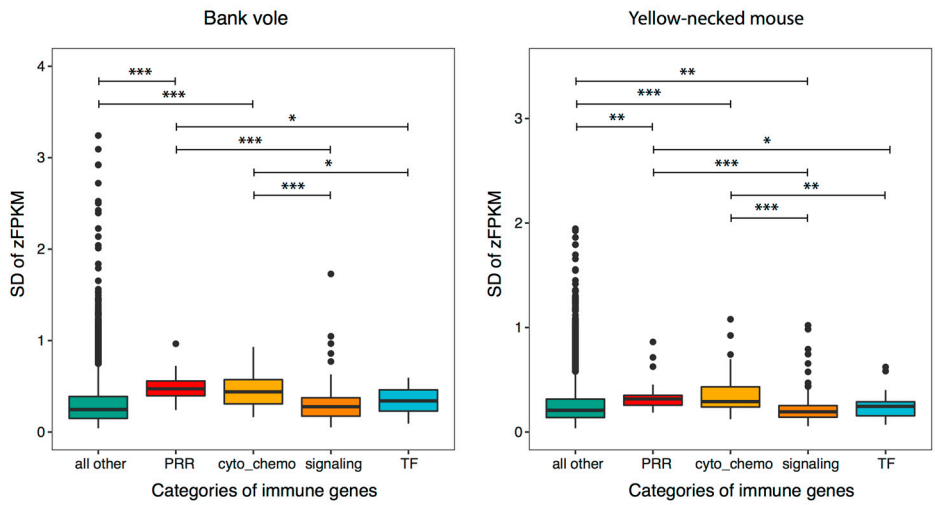

Figure 9 Intraspecific variation (SD of zFPKM) of different categories of genes in spleen transcriptomes in bank voles and yellow-necked mice.
Comparison of all five categories: BV: GLM, F 4, 8594=13.6, P<0.0001; YNM: GLM, F 4, 8594=11.9, P<0.0001. Comparison of the four immune gene categories (based on log10 transformed values): BV: GLM, F 3, 311=18.47, P<0.0001; YNM: GLM, F 3, 311=22.01, P<0.0001. Extracted from Paper III.

Interspecific expression divergence was measured as the squared residual deviation in a SMA regression of expression in bank voles against expression in yellow-necked mice (Chen et al., 2019). When comparing the four immune gene categories against all other genes, expression divergence did not differ between gene categories. However, a comparison of the immune gene categories against each other showed that PRRs had higher divergence than transcription factors and signaling genes (**Figure 10**).

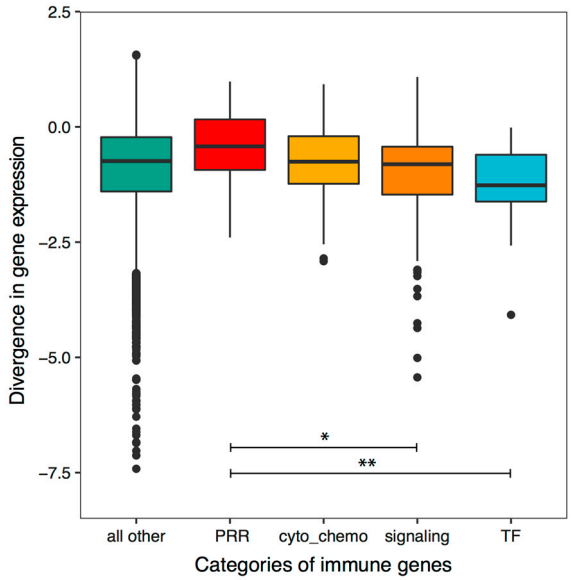

Figure 10 Divergence in gene expression between bank vole and yellow-necked mice for different categories of genes.
Divergence is measured as the squared residual deviation in a SMA regression of mean expression in bank voles against mean expression in yellow-necked mice. Comparison of all five categories: GLM, F 4, 8588=3.11, P<0.014. Comparison of immune gene categories: GLM, F 3, 311=3.04, P<0.029. Extracted from Paper III.

In conclusion, as in Hagai *et al.*(2018), PRRs had higher divergence in expression level than signaling genes and transcription factors. However, in contrast to Hagai *et al.,* cyto- and chemokines had unremarkable divergence in expression, but this is probably a result of high intraspecific variation of genes in this category. Indeed, further analyses of divergence in "expression variability" indicated that also expression of cyto- and chemokines had high interspecific divergence (see paper III for details). These results indicate that certain categories of immune genes, in particular PRRs, contribute disproportionately to interspecific variation in immune function.

TLR2 expression in the bank vole

Tschirren *et al* (2013) found that *TLR2* is highly polymorphic in bank voles and that this polymorphism is associated with *B. afzelii* infection status, indicating that variation at *TLR2* affects susceptibility to *B. afzelii.* Further population genetic analyses of genes in PRRs signaling pathways in the bank vole have shown that also *TLR1* and *TLR6,* which encode co-receptors of *TLR2,* also are highly polymorphic (Lundberg *et al.* submitted). To investigate how polymorphisms in

bank vole *TLR2* might affect susceptibility to *B. afzelii,* we performed more detailed analyses of the polymorphism of these TLR genes, and tested for associations between polymorphism in coding sequences and gene expression.

Analyses of a part of the coding regions of *TLR1*, *TLR2* and *TLR6* showed that all genes have high haplotype diversity in the studied bank vole population. As in the previous study of *TLR2* (Tschirren et al., 2013), *TLR2* haplotypes grouped into two major clusters (c1 and c2) and a rare one; the main difference between c1 and c2 was nine linked SNPs, of which four sites are non-synonymous (**Figure 11**). In *TLR1*, there were fifteen unique DNA haplotypes and eleven protein haplotypes (**Figure 11**). In *TLR6*, there were seventeen DNA haplotypes and eleven protein haplotypes (**Figure 11**). In the case of *TLR1* and *TLR6,* haplotypes did not group in a few discrete clusters (**Figure 11)**. To test for differences in gene expression between haplotypes, I therefore focused on *TLR2.*

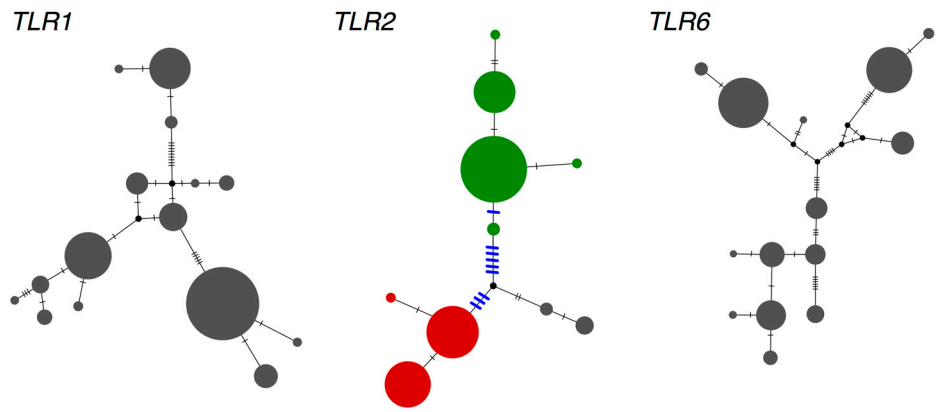

Figure 11 Haplotype networks of *TLR1*, *TLR2* and *TLR6* in bank voles.
Haplotype networks were constructed by sequencing the most polymorphic region of the coding sequence of each gene (TLR1: 531 bp, TLR2: 562 bp, TLR6: 784 bp). In the *TLR2* haplotype network, green haplotypes belong to cluster 1 (c1) and red haplotypes belong to cluster 2 (c2), where c2 is associated with reistance to *B. afzelii* in wild bank voles (Tschirren et al., 2013). Blue hatch marks are nine linked SNPs used to estimate haplotype-specific expression of *TLR2* (the rare c1 haplotype most closely related to c2 did not occur in our resequencing data). Extracted from Paper IV.

Sliding window analyses of nucleotide diversity along the whole annotated gene interval of *TLR2* showed there was high diversity not only in the coding sequence, but also in the intron. Haploview was used to investigate if polymorphisms in the coding sequence were in linkage disequilibrium with polymorphisms in up-stream non-coding regions. Across the full length of the *TLR2* gene and ± 3 kb up- and down-stream, 47 SNPs spanning around 11 kb showed extensive linkage disequilibrium (**Figure 12B**). One haplotype block was formed by 31 SNPs, of which 22 are located in the coding region. Another haplotype block was formed by

12 SNPs, of which 10 were located near the predicted promoter region (**Figure 12C**). Because of the linkage between coding and promoter region, we tested for associations between polymorphisms in the coding sequence and gene expression.

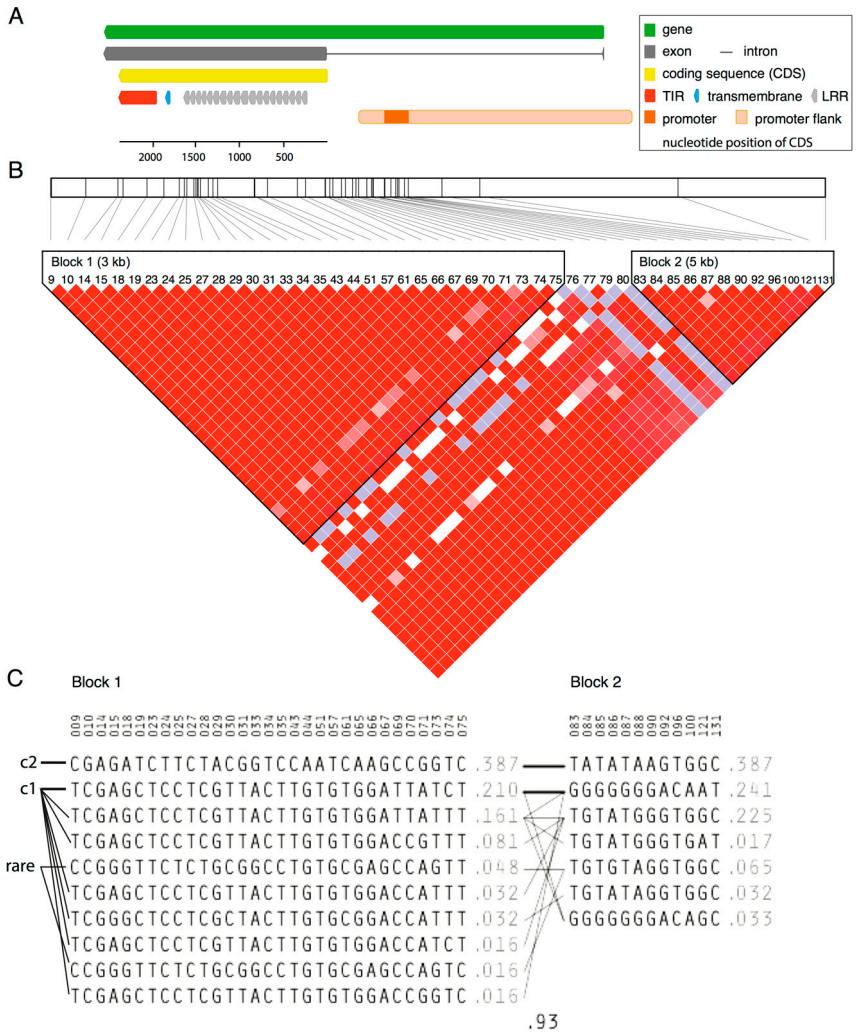

Figure 12 Strong linkage disequilibrium between coding and non-coding region in *TLR2* gene of bank vole.
A) Structure of the *TLR2* gene in bank vole and its predicted promoter region; B) Pairwise linkage disequilibrium (LD) among 47 SNPs in and surrounding the *TLR2*. LD was measured by the D' statistic (Barrett et al. 2005). C) Haplotypes and frequencies within two haplotype blocks. Extracted from Paper IV.

First, we tested for an association between *TLR2* genotype and overall expression of *TLR2*. Across all individuals (both homo- and heterozygotes), there was a trend that the *TLR2* genotype affects overall expression of *TLR2* ($F_{1,16} = 3.72$, $p = 0.074$, **Figure 13A**). Second, we tested for haplotype-specific expression by comparing expression of c1 and c2 in c1/c2 heterozygotes. We found that c2 had a significantly higher expression than c1 (paired t-test: $t(9) = -2.70$, $p = 0.025$, **Figure 13B**).

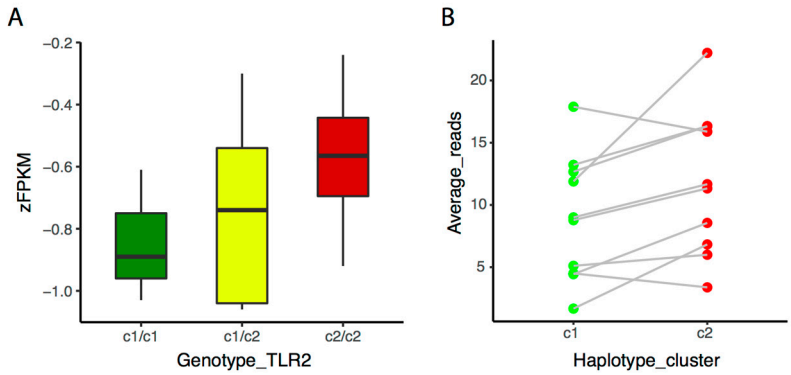

Figure 13 Association between coding sequence polymorphisms and expression of *TLR2*.
A) Overall expression of TLR2 in bank voles with different genotypes; B) Haplotype-specific expression of TLR2 in heterozygous (c1/c2) bank voles. Extracted from Paper IV.

In conclusion, our analyses indicate that the effect of *TLR2* genotype on *B. afzelii* infection status could be a result of combined effects of polymorphisms in the coding sequence and *cis*-regulatory variation causing haplotype-specific expression.

Conclusions and outlook

It is common that infection with a given pathogen has very different consequences in different host species, and even in different individuals of a given host species. As the immune system is the front line against pathogens, it makes sense to study variation in immune responses to uncover the basis of such variation in susceptibility. Traditionally, studies to explore immune responses were based on laboratory animals and performed under experimentally controlled conditions. However, analysis of "real" data from the wild is also needed and could provide a more complete understanding of interactions between pathogen and host.

In this thesis, I investigated the host factors that could contribute to differences in resistance within and between natural host species, using *B. afzelii* and its small mammal hosts as study system.

First of all, I showed that *B. afzelii* disseminates from skin (tick bite sites) to internal tissues (heart and joints) in natural hosts, but that levels of colonization vary between both species and tissues. Bank voles and common shrews have about 10-fold higher bacterial loads than yellow-necked mice in both skin and joints (but not in hearts), indicating that host species differ in resistance to *B. afzelii*. No inflammation was found in the infected joints; thus, there is as yet little evidence for pathological effects in natural hosts (**Paper I**).

By comparing transcriptomes of spleens between bank voles and yellow-necked mice, I identified possible species-specific regulation of immune responses associated with resistance. Up-regulation of IFNα response in *B. afzelii*-infected yellow-necked mice, and down-regulation IL6 signaling and the complement pathway in infected bank voles, could contribute to the difference in resistance to *B. afzelii* between the species (**Paper II**).

Analyses of general interspecific divergence in expression of immune genes indicated that certain categories of immune genes, in particular pattern recognition receptors, contribute disproportionately to interspecific variation in immune function (**Paper III**).

Analyses of genetic variation within bank voles showed that three toll-like receptors (*TLR1*, *TLR2*, *TLR6*) were highly polymorphic in the coding sequence. In addition, there was evidence of haplotype-specific expression of *TLR2.* Thus, the previously observed effect of *TLR2* genotype on *B. afzelii* infection status could be a result of combined effects of polymorphisms in the coding sequence

affecting dimerization, and cis-regulatory variation causing haplotype-specific expression (**Paper IV**).

As non-natural host of *Borrelia*, laboratory mice have similar symptoms as human Lyme borreliosis. It would be interesting to expand the comparison of immune response to *B. afzelii* to include wild rodents vs laboratory mice, to investigate why laboratory mice get sick while wild rodents do not. This could be done by stimulating spleen cells with live borrelial spirochetes *in vitro*, and then compare the regulation of gene expression between species. That would provide a greater understanding of how *Borrelia* induces variable outcomes and immune responses in different species. There are as yet relatively few studies on differences in immune responses to a given pathogen between natural and non-natural hosts (but see e.g. Palesch et al., 2018).

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