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Immune mechanisms in *Borrelia burgdorferi sensu lato* infection in relation to clinical outcome

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True knowledge exists in knowing that you know nothing.

/Socrates

To my delight!

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Abstract

Lyme borreliosis (LB) is the most common tick-borne disease in the northern hemisphere. The infection is caused by spirochaetes from the *Borrelia* (*B.*) *burgdorferi sensu lato* (*s.l.*) group. The clinical outcome after *B. burgdorferi s.l.* infection differs between individuals from asymptomatic infection without history of LB to individuals who experience persistent symptoms post-treatment for more than six months after treatment. The difference in clinical outcome is not thought to be associated with persistent infection, but could instead be affected by the host's ability to mount an optimal immune response to the spirochaete.

The hypothesis of this thesis was that a strong inflammatory Th1-like immune response is required in the early stage of infection in order to achieve both an optimal eradication of the *B. burgdorferi s.l.* bacteria and a good clinical outcome. The inflammatory response must be down-regulated by an anti-inflammatory response in order to avoid excessive immune responses that will end in tissue injury. The proper down-regulation will also protect against development of a chronic Th1-like inflammatory response, with activated cytotoxic cells, which may lead to LB with persistent symptoms post-treatment.

The thesis aimed to investigate the immunological mechanisms behind the optimal resolution of human *B. burgdorferi s.l.* infection and to define the aberrant mechanisms leading to development of persisting symptoms.

A prospective study on newly tick-bitten individuals showed that although 25% of the collected ticks were infected with *B. burgdorferi s.l.* very few individuals bitten by infected ticks developed LB (3.7%). In addition, 4.9% of the individuals bitten by infected ticks developed asymptomatic infection, *i.e.* *B. burgdorferi s.l.*-specific antibody seroconversion without LB. Approximately one third of all tick-bitten study subjects reported self-experienced symptoms possibly associated with LB. Individuals bitten by infected ticks were more likely to report experience of symptoms than those bitten by uninfected ticks. Thus, only 8.6% of the individuals bitten by *B. burgdorferi s.l.*-infected ticks were infected, verified by seroconversion, and out of them 57% were asymptomatic.

A prospective study on EM patients showed that a good clinical outcome was associated with a strong early Th1 immune response since EM patients with persistent symptoms six months after treatment had reduced expression of Th1 cytokines in their EM lesions compared with EM patients without symptoms.

The investigation of blood samples from newly tick-bitten individuals, for detection of possible early immune biomarkers indicating good clinical outcome of LB, showed that none of the investigated markers clearly discriminated between the individuals who developed LB, asymptomatic individuals, or non-infected individuals. However,

tick-bitten individuals who developed asymptomatic infection showed an increase of early Th1-associated biomarkers in blood compared to individuals who developed clinical LB.

In an experimental study, Th2-immune-deviated mice had more pronounced clinical signs of infection and could not eradicate the spirochaete as efficiently as non-deviated *B. burgdorferi sensu stricto* (s.s.)-infected mice. Non-deviated *B. burgdorferi* s.s.-infected mice showed a decrease of mRNA expression associated with Th2, anti-inflammatory and Treg/Th1 responses during the course of infection, which suggested a termination of the inflammatory response – something that was not seen in the immune-deviated mice. Trends for increased expression of pro-inflammatory GM-CSF and Treg marker Foxp3 in immune-deviated mice suggested on-going inflammation. Non-deviated *B. burgdorferi* s.s.-infected mice showed increased systemic expression of the Th1-associated CXCL9 and CXCL10 during the course of infection, while immune-deviated mice showed an initial decrease in both chemokines at day 15 p.i. compared with day 0 p.i.

In conclusion, the risk of developing LB after a tick bite is low, and no infection or asymptomatic infection are the most common outcomes after a tick bite. The early immune response in humans and the immune response towards *B. burgdorferi* s.s. infection in mice support the hypothesis that a strong pro-inflammatory Th1 response is needed for an optimal clinical outcome and eradication of bacteria.

Original publications

I. Linda Fryland, Peter Wilhelmsson, Per-Eric Lindgren, Dag Nyman, Christina Ekerfelt, Pia Forsberg

Low risk of developing *Borrelia burgdorferi* infection in the south-east of Sweden after being bitten by a *Borrelia burgdorferi*-infected tick

International Journal of Infectious Diseases, 2011, 15, e174–e181.

II. Johanna Sjöwall, Linda Fryland, Marika Nordberg, Florence Sjögren, Ulf Garpmo, Christian Jansson, Sten-Anders Carlsson, Sven Bergström, Jan Ernerudh, Dag Nyman, Pia Forsberg, Christina Ekerfelt

Decreased Th1-type inflammatory cytokine expression in the skin is associated with persisting symptoms after treatment of erythema migrans

PLoS ONE 2011, 6, e18220.

III. Linda Fryland, Said Havarinasab, Tobias Jakobsson, Sven Bergström, Per Hultman, Christina Ekerfelt

Mapping of T-cell subsets in relation to disease course in experimental *Borrelia burgdorferi* infection

Manuscript submitted

IV. Linda Fryland, Pia Forsberg, Linnea Sandin, Peter Wilhelmsson, Pontus Lindblom, Dag Nyman, Per-Eric Lindgren, Jan Ernerudh, Christina Ekerfelt, Tick-Borne Diseases Sting study group

Increase of Th1-associated biomarkers a few days after a bite by a *Borrelia burgdorferi*-infected tick in blood from asymptomatic *Borrelia burgdorferi*-infected subjects compared to subjects who later develop Lyme borreliosis

Manuscript

Abbreviations

ACA	Acrodermatitis chronica atrophicans
APC	Antigen presenting cell
<i>B.</i>	<i>Borrelia</i>
Bb	<i>B. burgdorferi</i> s.s.-infected
BbId	Immune-deviated <i>B. burgdorferi</i> s.s.-infected
BCR	B cell receptor
BSKII	Barbour Stoenner Kelley II medium
cDNA	Complementary DNA
CD	Cluster differentiation
CR	Complement receptor
CRASP	complement regulator-acquiring surface protein
CSF	Cerebrospinal fluid
CTLA-4	Cytotoxic T-lymphocyte antigen 4
Cy3	Cyanine dye
DAMP	Damage-associated molecular pattern
DNA	Deoxyribonucleic acid
DC	Dendritic cell
EBI-3	Epstein-Barr virus induced gene 3
ECM	extra cellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EM	Erythema migrans
Erp	OspE-related protein
Fc	Fragment crystallizable region
FHL-1	Factor H-like-1
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead box p3
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HRP	Horseradish peroxidase
<i>I.</i>	<i>Ixodes</i>

IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
ILC	Innate lymphoid cells
Isac	Ixodes scapularis anti-complement protein
iTreg	induced Treg
LA	Lyme arthritis
LB	Lyme borreliosis
LPS	Lipopolysaccharides
MFI	Mean fluorescence intensity
MHC	Major histocompatibility
MIG	CXCL9 chemokine
MMP	Matrix metalloproteinase
mRNA	messenger RNA
NB	Neuroborreliosis
NFκB	Nuclear factor κB
NK	Natural killer cell
NKT	Natural killer T cell
NLRP	NOD-like receptor
NOD1/2	Nucleotide-binding oligomerization domain-containing protein 1/2
nTreg	natural Treg
OD	Optical density
Osp	Outer surface protein
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PE	Phycoerythrin
PHC	Primary health care
p.i.	Post-infection with <i>B. burgdorferi</i> s.s.
PRR	Pattern recognition receptor
RNA	Ribonucleic acid
RORC	retinoic acid-related orphan receptor C
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
RT	Room temperature

RT-PCR	Reverse transcriptase PCR
Salp	Salivary protein
<i>s.l.</i>	<i>sensu lato</i>
<i>s.s.</i>	<i>sensu stricto</i>
STAT	Signal transducers and activators of transcription
TBD	Tick-borne diseases
T-bet	T-box expressed in T cells
TCR	T cell receptor
Tfh	Follicular helper T cell
TGF	transforming growth factor
Th	T helper cell
TLR	Toll-like receptor
TMB	3,3'-5,5'-tetramethylbenzidine
TNF	Tumour necrosis factor
Treg	T regulatory cell
tRNA	Transfer RNA
VlsE	Variable major protein-like sequenced expressed

Introduction

Lyme borreliosis

History of Lyme borreliosis

Clinical manifestations of Lyme borreliosis (LB) were reported in Europe as early as the end of the 1800s and the beginning of the 1900s (reviewed in (1)), including the Swedish dermatologist Afzelius, who were the first to report about erythema migrans (EM) (2). During the following decades the different clinical manifestations of LB were linked with each other and were associated with an unknown bacterial agent transmitted by *Ixodes* (*I.*) ticks (1). However, the research into the spirochaete family of *Borrelia* (*B.*) *burgdorferi sensu lato* (*s.l.*) really took off in the 1970s, when reports were made to the State Health Department about the occurrence of a disease, misdiagnosed as juvenile rheumatoid arthritis, in several families in the county of Lyme in the north-eastern USA (3). Steere et al. investigated 51 afflicted children and adults and concluded after thorough investigations that the patients were all suffering from an epidemic form of arthritis, “Lyme arthritis”, with a suggested infectious aetiology (3). Characteristic of many of the patients was a preceding EM several weeks before the onset of the arthritis. Soon afterwards, Steere et al. were able to show that EM and Lyme arthritis (LA) were associated with bites from ticks (4). However, the infectious agent was first discovered in 1982 by Burgdorfer et al. (5), who successfully isolated *B. burgdorferi s.l.* spirochaetes from field-collected ticks. Furthermore, they also suggested that this “new” bacteria was involved in the aetiology of LB due to the bacteria’s ability to bind to immunoglobulins of LB patients (5). Soon after the discovery, several groups from both North America and Europe showed that the spirochaete could be isolated from field-collected ticks as well as from blood, EM lesions, and cerebrospinal fluid (CSF) samples of LB patients (6–10). This further strengthened the finding that the tick-borne spirochaete *B. burgdorferi s.l.* group transmitted the LB disease during a tick bite.

The vector

Four hard tick (*Ixodidae*) species transmit *B. burgdorferi s.l.* spirochaetes to mammal hosts. The ticks *I. scapularis* and *I. pacificus* are present in eastern and western North America, respectively. *I. persulcatus* is present in a wide area stretching from Japan to the Baltic Sea (11) while *I. ricinus* is present in Europe, from Northern Scandinavia to the Maghreb (North Africa) and from Ireland to European Russia (12).



Figure 1. An adult female *Ixodes ricinus* tick. (Courtesy of Pontus Lindblom, Linköping University, Sweden).

The four tick species live on the forest floor because of the humidity and the low risk of desiccation. The ticks climb onto the low lying vegetation, *e.g.* grass, and quest for a host for their blood meal (9). *I. ricinus* ticks can use over 300 small and large mammals, birds and reptile species for their blood meal (9). Larvae and nymphs tend to infest smaller hosts (*e.g.* rodents, birds, insectivores, reptiles, lagomorphs) for blood meal while the adult ticks quest on higher vegetation and tend to infest larger hosts (deer, sheep, wild boar) (12). Humans, on the other hand, are dead-end hosts for spirochaetes.

The ticks have four life cycle stages (egg, larva, nymph and adult) during their approximately 2 year life cycle and need a blood meal between each stage in order to moult into the next developmental stage. The female adult tick needs a blood meal in order to lay eggs before dying (9). Transmission of *B. burgdorferi s.l.* occurs during the blood meal (11). Hatched larvae are *B. burgdorferi s.l.*-uninfected, or at least transovarial transmission occurs very rarely, except for the relapsing fever-associated *B. myamotoi* that can be found in newly hatched larva (13). Experimental studies have shown that the duration of attachment required for efficient transmission of spirochaetes to the host differs between the genotypes of *B. burgdorferi s.l.* (14). *B. burgdorferi s.s.* need at least 48 h of *I. ricinus* or *I. scapularis* attachment to the host for transmission (14–16) compared to *B. afzelii* that needs *I. ricinus* attachment to host for 17–48 h before transmission occurs (14,17).

Borrelia burgdorferi sensu lato

The *Borrelia* spp. consists of two phylogenetic groups - the group of bacteria causing relapsing fever, and the *B. burgdorferi s.l.* group causing LB (12).

B. burgdorferi s.l. is gram negative-like bacteria, 10–30 µm in length and 2–0.3 µm in diameter (5,18). A major difference between gram negative bacteria and *B. burgdorferi s.l.* is that the latter express a great quantity of lipoproteins on its outer cell

membrane instead of the gram negative lipopolysaccharide (LPS) (19,20). The bacteria have 7-11 flagella located in the periplasmic space, in contrast to other bacterial flagella, and attached to the bacteria's ends (12). The *B. burgdorferi s.l.* genome is unique amongst bacteria species with its relatively small linear chromosome (approximately 900 kbp) and numerous linear and circular plasmids (9-62 kbp). The chromosomes encode proteins involved in protein synthesis, energy metabolism and transport across the membranes, while the plasmids contain genes for most of the surface lipoproteins, many of which are involved in the interaction with vector but also involved in infection and immune evasion (9).

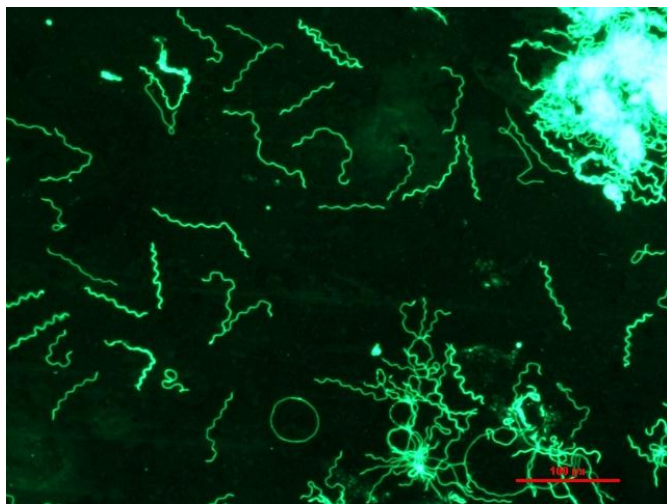


Figure 2. *Borrelia burgdorferi sensu lato* spirochaetes dyed with FITC. (Courtesy of Kristina Nilsson-Ekdahl, Linnéuniversitetet, Kalmar, Sweden).

The *B. burgdorferi s.l.* group consists of 18 different species but only five has been shown to be human pathogenic; *B. afzelii*, *B. garinii*, *B. burgdorferi sensu stricto* (*s.s.*) (the only pathogenic species in North America), *B. bavariensis* (former *B. garinii* OspA type 4) (21) and *B. spielmanii* (22–24). There are also a few reports that suggest *B. valaisiana* (25–27), *B. bissettii* (27–30) and *B. lusitaniae* (31) could be pathogenic for human. Although the distributions of *B. burgdorferi s.l.* genospecies vary in different geographical areas in Europe, *B. afzelii* and *B. garinii* are the two most common and widely spread species (32,33). Four of the pathogenic species are associated with different clinical manifestations. *B. afzelii* is usually found in skin-associated manifestations, while *B. garinii* and *B. bavariensis* usually cause NB and *B. burgdorferi s.s.* often is associated with LA (21,25,34–36). Rodents are the reservoir for *B. afzelii* and *B. bavariensis* while birds are reservoirs for *B. garinii* and *B. valaisiana* (6–9,37,38). Both rodents and birds are reservoirs for *B. burgdorferi s.s.*. One explanation for this division of the genotypes was shown by Kurtenbach et al.

(10) where rodent sera were borreliacidal for *B. garinii* while avian sera were borreliacidal for *B. afzelii*.

Epidemiology

LB, Lyme disease in North America, is the most common tick-borne infection in Europe and North America (39). The estimated mean annual number of LB cases per 100,000 (both notified cases and qualified estimations) are > 65,400 cases in Europe, >16,300 in North America, >3,400 in Asia and seven in Maghreb (North Africa) (40). These numbers are not exact due to that several countries, including Sweden, lack mandatory notification of LB cases, and due to differences in definition of both LB cases and LB diagnostics. There are some hyperendemic areas with annual incidence of LB > 100 cases per 100,000 inhabitants in Europe. The hyperendemic areas in northern Europe are the Swedish southern County of Blekinge with mean annual incidence of 464 EM cases (41) and the Åland Islands with an annual incidence of >1,100 LB cases each year since 2004 (42). Other hyperendemic areas are several north-eastern counties in Germany with annual incidences of approximately 300 LB cases and Slovenia as a whole with annual incidence of >200 LB cases (40).

The majority of annual LB cases in Europe occur from June to July which suggest that most infections are transmitted to the human host during late May and June (40). Few LB cases occur from January through April in Sweden (41,43). The EM cases in southern Sweden peak in July-September (41,43) while the other LB manifestations peaked in September (43). LB can affect all ages but the incidence of LB usually have a bimodal age distribution, with highest incidence in children 5-9 years old (40,43) and adults 60-74 years old (43). No significant difference in LB incidence were found between men and women in the Berglund et al. study, however Bennet et al. reported that the incidence of EM cases were higher in women than men (41), and reinfection seem to occur more often in women (44,45).

The reported prevalence of *B. burgdorferi* s.l. in ticks that have bitten humans is 16-32.8% (46,47). However, the risk of developing symptoms possibly associated with LB is 4.1% (47) and to develop asymptomatic infection or clinical LB is 8.2% (46).

Clinical manifestations and diagnosis

The clinical manifestations of LB can be divided into early and late stages of disease. Early localised infection is recognised by the presence of either EM or Borrelial lymphocytoma while early disseminated infection is characterised by multiple EM, neuroborreliosis (NB) or LA. The late stage of LB is characterised by late NB, LA or development of Acrodermatitis chronica atrophicans (ACA) in the skin (39).

EM is the most common clinical manifestation of LB and occurs in 70-90% of the LB cases (21,43,48). The skin lesion occurs days to weeks after a tick bite and is

characterized by a red or bluish-red expanding rash, at least five centimetres in diameter, with or without a central clearing. This rash expands over days or weeks. EM is diagnosed only by the occurrence of an expanding EM at the site of the tick bite. EM patients may also have non-specific symptoms such as fatigue, fever, headache, stiff neck, arthralgia and myalgia (49).

Borreliolymphocytoma is a rare, early, and clinical manifestation characterised by a painless bluish-red nodule containing an intense B cell infiltrate. The lymphocytoma is usually found on the ear lobe, nipple or scrotum. The borreliolymphocytoma is more frequent in children than in adults. The lymphocytoma is diagnosed by occurrence of lymphocytoma on ear lobe, nipple or scrotum, supported by a history of EM or other LB manifestation or by positive serology. Histological examination is needed in some cases, especially when lymphocytoma occurs on locations other than ear lobe, to rule out malignancy (21).

ACA is a chronic, long-lasting and often progressive skin condition that occurs months to years after infection and is characterised by red or bluish-red lesions on outer extremities. The skin is discoloured and swollen during the early stage of ACA but changes to become permanently atrophic, often accompanied with sclerodermic changes. Bones and peripheral nerves below the atrophic skin may also be affected. High concentrations of *B. burgdorferi* s.l.-specific IgG antibodies are found in patients with ACA. (49) ACA is diagnosed by histological examination together with positive serology and is supported by isolation of spirochaetes from the involved skin (21).

Early neurological manifestations of NB occur weeks to months after infection and manifest as meningitis, facial palsy, cranial neuritis and radiculitis. More long-lasting NB manifestations are encephalomyelitis, radiculitis and chronic meningitis. Untreated early NB can persist for months and may result in sequelae after resolved infection. The diagnosis of NB is based on the presence of symptoms associated with NB (such as meningitis, facial palsy, cranial neuritis and radiculoneuritis), mononuclear pleocytosis in CSF and intrathecal production of *B. burgdorferi* s.l.-specific antibodies (49).

LA is characterised by acute and recurrent but brief attacks of synovitis in one or several joints, often the knees. High levels of *B. burgdorferi* s.l.-specific IgG antibodies are present in the sera and the synovial fluid in LA patients (49,50). Diagnosis of LA is based on medical history and clinical features, exclusion of other causes of arthritis and positive serum *B. burgdorferi* s.l.-specific IgG antibodies. LA diagnosis is supported by detection of *B. burgdorferi* s.l. DNA in synovial fluid or tissue (21).

Asymptomatic infections

Asymptomatic *B. burgdorferi s.l.* infection, *i.e.* infection without development of LB, seems to occur quite frequently (46,51–53). The mechanisms behind whether *B. burgdorferi s.l.* infections will develop into asymptomatic infection or into LB are mostly unknown. A proposed explanation is that non-invasive *B. burgdorferi s.l.* strains may not be able to induce persist long enough in the host to cause disease (54).

Persistent symptoms post-treatment

LB is treated successfully with antibiotics in the large majority of cases (39,55–58). However, patients with late LB manifestations (NB, LA and ACA) can recover slowly, weeks to months after treatment. Some patients may have non-specific persistent symptoms after LB (59–66) due to unknown mechanisms. Such persistent symptoms are called post-Lyme syndrome and can be defined as LB that is treated with antibiotics and ended with resolution or stabilisation of clinical manifestations, but non-specific symptoms are present more than six months after LB diagnosis. The most common non-specific symptoms are fatigue, myalgia, arthralgia, head ache, cognitive problems and decreased functional activity. However, post-Lyme syndrome does not include patients with active untreated co-infections, active untreated LB or presence of other diseases/conditions such as fibromyalgia and chronic fatigue syndrome (67,68).

Possible explanations for the unknown mechanisms behind post-Lyme syndrome are other tick-borne infections, natural resolution of symptoms after treatment, irreversible tissue damage, post-infective fatigue syndrome, autoimmune mechanisms or caused by other conditions/diseases (64,65,68). However, one should bear in mind that the non-specific symptoms that are present during post-Lyme syndrome are quite common in the general population too (56,64,68).

Persistent *B. burgdorferi s.l.* infection seems not to be the explanation for the persistent symptoms (60,64,68). Further supporting the non-existence of persistent infections are several studies on duration of antibiotic treatments that showed that prolonged antibiotic treatments did not show any beneficial outcomes compared to the standard antibiotic regimens (39,57,60).

Treatment

All LB cases, irrespective of which sort of LB manifestation occurs, are treated with β -lactam or tetracycline antibiotics. The β -lactam groups (*e.g.* penicillin V and cephalosporines) kill bacteria by binding to and inhibiting enzymes that are important for the bacteria's peptidoglycan synthesis, and thus for the bacteria's building and maintenance of its cell wall. This inhibition of peptidoglycan synthesis will ultimately result in cell death. Tetracyclines (*e.g.* doxycycline), on the other hand, reversibly inhibit bacterial synthesis in general by binding to the 30S ribosome which blocks the binding of tRNA to the ribosome (69). The Swedish recommendations for treatment

regimens for adult EM patients are penicillin V (or doxycycline in case of penicillin allergy or suspected co-infection) treatment for 10 days, for NB and LA patients 14 days of doxycycline or ceftriaxone (cephalosporine) treatment, and for ACA patients 21 days of doxycycline or penicillin V treatment (50).

Co-infection

In addition to *B. burgdorferi s.l.*, ticks transmit several other tick-borne bacteria, as well as tick-borne parasites and viruses, which all can cause disease in the host (e.g. humans). Humans can be infected by tick-borne pathogens such as the bacteria belonging to the *Rickettsia* group, *Anaplasma phagocytophilum*, *Francisella tularensis* (70), the protozoan parasite *Babesia* (71) and the tick-borne encephalitis (TBE) virus (72). Common for all these tick-borne pathogens are the similarities in the symptomatic infections. They can all manifest with non-specific symptoms such as fever, fatigue, general malaise, headache, myalgia and arthralgia (70–72). These symptoms may also occur in LB patients (71). Ticks can be coinfecting with different pathogens and coinfection in humans with one or more tick-borne pathogens have been reported (reviewed in (71)). The similar non-specific symptoms that may occur during LB and may occur during other tick-borne infections could probably affect the clinical outcome of LB if the non-specific symptoms in a coinfecting patient are only associated with LB.

Detection methods

There are several laboratory techniques that can be used for detecting *B. burgdorferi s.l.* in specimens from LB patients. The clinical laboratory use four different approaches for direct detection i.e. detecting presence of intact *B. burgdorferi s.l.* spirochaetes or spirochaete DNA or proteins. The approaches are culture, microscope-based assays, detection of *B. burgdorferi s.l.*-specific proteins or nucleic acids (73). *B. burgdorferi s.l.* can be isolated and cultured from tissues and body fluids from LB patients ranging from tissue biopsies to CSF, blood and synovial fluids (reviewed further in (73)). Clinical specimens from LB patients can be analysed either qualitatively or quantitatively by detecting *B. burgdorferi s.l.* nucleic acid with polymerase chain reaction (PCR) assays. Microscope-based assay are not optimal for clinical laboratory use due to that some assays require access to fluorescence microscope and due to the subjective reading and interpretation. Serology assays is an indirect detection of *B. burgdorferi s.l.* by detection of *B. burgdorferi s.l.*-specific antibodies in blood, synovial or CSF fluids. It is the most commonly used detection method for *B. burgdorferi s.l.* in clinical laboratories. Culture is not used in the clinical routine due to the labour-intensive and slow-growing culture. Due to the low sensitivity of PCR assays in detection of spirochaete DNA in CSF and blood samples PCR assays are not commonly used in the clinical laboratory. Finally, the serology also has its limitations due to lack of standardisation and a large amount of available assays with different antigenic compositions and different detection of immunoglobulin classes (73).

Immunology

Pathogenic bacteria, viruses and other microorganisms are a constant threat to humans and other mammals. The immune system has evolved to combat these threats and uses its ability to discriminate between self and non-self to destroy infectious microbes but avoid damaging the host's tissues. The human immune system consists of three parts. The first parts are the anatomical (skin, mucosal epithelia) and physiological barriers (low stomach pH and bacteriolytic lysozymes in body secretions) of the host (74). The second part is the innate system that responds within hours to foreign antigens, and the third part is the adaptive system that needs approximately a week to respond properly to infectious microbes due to the time consuming production of antigen-specific cell populations. Another difference between the innate and adaptive immune systems is that the innate system recognises a limited set of conserved common structures in microbes since their recognition mechanisms are germ line-encoded, while the adaptive system uses a somatic recombination of gene segments in order to utilise the vast diversity of antigen-specificity for its recognition of pathogenic antigens. The cells of the adaptive system can also develop into memory cells after resolution of inflammation. These memory cells “remember” and respond more rapidly and stronger to repeated exposures to the same microbe (74,75).

The innate immune system

The innate immune system consists of immune cells such as phagocytes (neutrophils and macrophages), dendritic cells (DC) and natural killer (NK) cells. Complement proteins, natural antibodies and acute-phase reactants are all blood proteins that have important functions in the immune response. Another group of proteins, called cytokines, are also of great importance since the immune system uses them as a signal substance between both immune and tissue cells to regulate and coordinate the immune response (75).

The innate immune response

Tissue resident immune cells (macrophages, dendritic cells, mast cells and endothelial cells) become activated by their membrane bound or cytoplasmic pattern recognition receptors (PRR), such as Toll-like receptors (TLRs), scavenger receptors, NOD1/2 and NLRPs. Common for all these receptors is that they recognise conserved common microbial structures and products, *i.e.* pathogen-associated molecular patterns (PAMPs), such as nucleic acid, proteins (flagellin), cell wall lipids (LPS and lipoteichoic acid) and carbohydrates (mannan). The host's own damage signals, damage-associated molecular patterns (DAMPs) from the infection site or from sterile inflammation (caused by *e.g.* burns, chemical toxins and trauma) are also recognised by the innate immune cells. These DAMPs can be stress-induced proteins such as heat-shock proteins, crystals or nuclear proteins present outside of the nucleus.

The binding of PAMPs and DAMPs to the PRRs activates signal transduction and leads to intracellular signalling cascades that result in effector functions such as

phagocytosis and production of inflammatory mediators. The earliest inflammatory mediators that are produced are the cytokines tumour necrosis factor (TNF), interleukin (IL)-1 β and IL-6 and the chemokines CXCL1, CXCL8 and CCL2, which initiate the inflammatory response (75).

The inflammatory mediators activate the endothelial cells near the site of infection/tissue injury in order to change the expression of plasma membrane receptors on the endothelial cells. The changed expression of receptors will facilitate recruitment of circulating leukocytes to the infection/tissue injury. The cytokines from the tissue-resident cells also increase the permeability of the nearby blood vessels and the leukocytes can squeeze through the vessels and migrate chemotactically towards the site of infection/injury. Neutrophils are the first recruited leukocytes, and they are present hours before monocytes. This may be due to the expression of different chemokine receptors; neutrophils express the chemokine receptors CXCR1 and CXCR2 that bind *e.g.* the very early chemokine CXCL8, while inflammatory monocytes express the chemokine receptor CCR2 that binds other chemokines. Due to the permeability of the vessels, plasma proteins such as complement, antibodies, and acute phase reactants will also accumulate at the site of infection/injury (75).

Regulation of the innate immune response

The inflammatory mechanisms of the innate immune system can be harmful for the host's own tissues and must therefore be regulated when activated and shut off when no longer needed. Several regulating mechanisms are induced during, or shortly after, the induction of the inflammatory response. The cytokine IL-10 is an example of an immune regulator (75,76). IL-10 is expressed by macrophages and DCs both *in vitro* and *in vivo*, and by neutrophils *in vivo*. Its production is induced after TLR stimulation (77). The cytokine inhibit the production of several proinflammatory cytokines, including IL-1 β , IL-6 and TNF, by activated monocytes/macrophages and neutrophils (78). IL-10 also inhibit production of several CC and CXC chemokines that are all involved in recruitment of neutrophils, monocytes, dendritic cells and T cells. Monocytes/macrophages ability to produce MMPs and expression of both MHC class II antigens and costimulators are also inhibited by IL-10. The latter effects affect the cell's ability to function as T cell-activating APC. Upregulation of Fc γ R expression on monocytes correlates with enhanced phagocytizing ability although IL-10 decreases the ability to generate oxidative burst. IL-10 induces differentiation of immature DCs into macrophage-like cells that exhibit an enhanced ability to phagocytize but reduced ability to activate T cells.

In addition, IL-10 enhances the production of IL-1 receptor antagonist (IL-1RA) in activated macrophages and neutrophils. The IL-1RA is a homolog to IL-1 β but inactive (78). IL-1RA competitively inhibits IL-1 β by binding to the type I IL-1 receptor. In addition, the type II IL-1 receptor, which cannot transduce signals, may

also function as a negative regulator by competitively binding up the secreted IL-1 β . Another example of negative regulators is the cellular mechanism of autophagy, which is a process of organelle degradation by engulfing cytoplasmic contents within membrane-bound vesicles that fuse with lysosomes in order to degrade the vesicle contents. In this way, the autophagy process can regulate the amount of secreted IL-1 β and IL-18 by regulating the amount of cytosolic PRRs (75,76).

The adaptive immune system

The adaptive immune system consists of the T cell- and the B cell-mediated immunity. In addition, soluble molecules such as antibodies and cytokines are also involved in the adaptive immune response. The T and B cells undergo somatic recombination of their receptor genes during cell development which generates T and B cells that all have unique variations of the receptors (TCR and BCR, respectively), and therefore each cell is highly antigen-specific. The adaptive immune responses are delayed compared to the innate responses due to the requirement of clonal expansion of the activated cell in order to be able to mount an adequate adaptive response towards the pathogen (75,79).

T cell activation

Mature naive T cells circulate through the body, passing through lymphoid organs. The activated and mature antigen-presenting cells (APCs), mainly dendritic cells (DCs), from the site of infection migrate to the T cell zones of draining lymph nodes where they present a processed peptide from cytosolic antigens on their major histocompatibility (MHC) class I complexes and endocytosed antigens on their MHC class II complexes for recognition by CD8⁺ T cells and CD4⁺ T cells, respectively. The interaction between the TCR and the antigen-MHC complex is the first activation signal for T cells, while co-stimulators (*e.g.* CD3, CD4/CD8 and CD28) and integrins act as the second activation signal by stabilising the interaction between the TCR and the MHC complex and enhancing the signal transduction. There are also inhibitory receptors (*e.g.* CTLA-4) present on the T cell membrane. These receptors regulate the activation of the cell (75,79). The cell activation gives rise to an intracellular signal cascade, which results in the activation of the transcription factors NF κ B, NFAT and AP-1 (80). Together the transcription factors regulate genes involved in the expression of cytokines, cytokine receptors, and survival proteins, and they also promote cell proliferation (clonal expansion) and induce differentiation to effector and memory cells (75,79).

T cell immunity

The T cell immunity is mediated by the CD4⁺ and CD8⁺ T cells. CD4⁺ T cells activate other leukocytes or enhance their effector function while CD8⁺ T cells kill infected cells in a cytotoxic manner (75).

B cell activation

B cells can be activated in a T cell-dependent or -independent way. The BCR is a complex of immunoglobulin (Ig) molecules that binds antigens (proteins, polysaccharides and lipids) which activates the cell. In T cell-dependant B cell activation, follicular B cells present antigen peptides on their MHC II complex to activated Th cells. The activation of B cell occurs when CD40 ligand on T cells bind to CD40 on B cells and T cell-derived cytokines binds to their receptors on the B cell. The activated B cells differentiate into short-lived plasma cells, which have not gone through somatic mutation and therefore will secrete low-affinity antibodies. Other B cells will, in the presence of follicular helper T (Tfh) cells, differentiate into memory B cells and long-lived plasma cells that goes through somatic mutation in order to isotype-switch into production of high-affinity IgA, IgG or IgE antibodies. The T cell-independent B cell activation, on the other hand, occurs when antigen polysaccharides or lipids bind to the BCRs on the marginal zone or on the tissue resident B-1 B cells. These B cells also need a second signal, such as TLR signals or complement fragment C3d on opsonised microbes, for optimal activation. C3d bind to the coreceptor CR2 on B cells (75,79).

B cell immunity

The B cell immunity is mediated by secreted antibodies and their interactions within the immune system. The effector functions of antibodies are to bind to the surfaces of extracellular microbes and toxins in order to cover them and neutralise them, resulting in blocking the ability of the pathogen to bind to the host's cellular receptors. Another effector mechanism of antibodies is the activation of the classical pathway of the complement system. Antibodies can also opsonise microbes in order to enhance phagocytosis by phagocytes (75,81).

Regulation of the innate immune response

IL-10 can be produced by all four major Th cell subtypes (76,82–84) and by CD8⁺ T cells (77). The IL-10 production is induced by the same signals that occur in the Th cells during Th differentiation and in the CD8⁺ T cell after TCR activation, respectively. In addition, both IL-21 and IL-27 can enhance the IL-10 expression by Th cells, while IL-21 enhances the IL-10 production in CD8⁺ T cells (77). T cells are strongly affected by IL-10 due to the cytokines' downregulatory effects on APCs. But IL-10 also directly affects T cells, *e.g.* T cells activated in the presence of IL-10 can be induced to enter an anergic state. However, IL-10 stimulates CD8⁺ T cells and induces their recruitment, proliferation and cytotoxic activity (78).

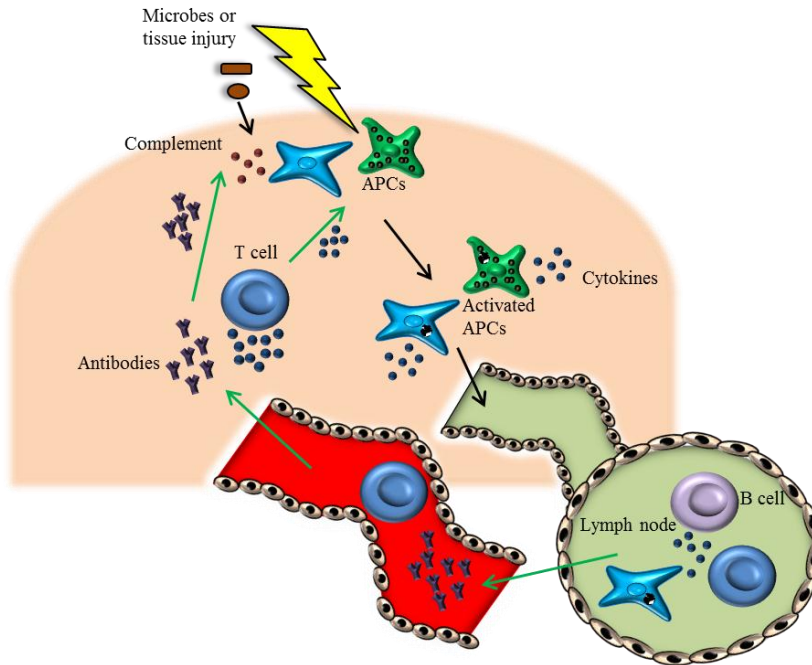


Figure 3. An illustration of the immune response against microbes or tissue injury. *Step 1.* Microbes or danger signals activate the APCs. *Step 2.* The activated APCs phagocytize the microbe and produce inflammatory mediators. *Step 3.* The antigens activate the complement. Activated APCs migrate to a lymph node, where it activates naïve T cells. The naïve B cells become activated when they recognise antigens. Both cell types differentiate and proliferate. *Step 4.* Mature T cells migrate out to the infected tissue, where it enhances the effector functions of APCs. Secreted antibodies also enter the site of infection, where they opsonise or neutralise antigens. APC: antigen presenting cell.

Neutrophils and macrophages

The function of neutrophils and macrophages are to recognise phagocytise and kill microbes.

Neutrophils are the most abundant but short-lived (about six hours) immune cells in the blood. The cells contain two different types of granule, the secretory specific granules that contain cytotoxic enzymes (lysozyme, collagenase and elastase) and azyrophilic granules that are lysosomes containing enzymes and microbicidal molecules (defensins and cathelicidin) (75).

Circulating monocytes finish their differentiation when they enter tissues and become mature, *i.e.* differentiate into macrophages. In addition to the killing of microbes, macrophages also clean up the environment in the tissues by ingesting dead host cells, such as dead neutrophils, after infection or tissue injury and apoptotic cells. Macrophages are one kind of antigen-presenting cells (APCs), *i.e.* the cell can activate

T cells by presenting microbial antigens. More specifically, macrophages activate T effector cells that have migrated into the site of infection. (75). Macrophages can be divided into two different subsets, the classically activated (M1) and the alternatively activated (M2) macrophages, due to the received stimulation. Macrophages that are activated by microbes, T helper (Th)1 cells and IFN- γ become M1 cells that are involved in inflammation and are microbicidal. Macrophages that are activated by the Th2 cytokines IL-4 and IL-13 become M2 cells that are involved in tissue remodelling and fibrosis but also are anti-inflammatory (75).

The neutrophils and macrophages eliminate the invading pathogens by ingestion via phagocytosis. The phagocytised microbes are destroyed in phagolysosomes and at the same time, peptides from the destroyed microbes will be presented on the MHC molecules for presentation to T cells in order to initiate the adaptive immune response. The phagocyte oxidase enzyme is induced and activated by IFN- γ and TLR signals, and assembles in the phagolysosome membrane, where it reduces O₂ into reactive oxygen species (ROS) during the respiratory burst which results in conversion of unreactive halide ions into reactive hypohalous acid (halogen oxyacid) that is toxic to microbes. Reactive nitrogen species, mainly nitric oxide (NO), are also produced in the cytosol after stimulation by IFN- γ and TLR signals. The released NO gas freely diffuses and can go in to phagolysosomes where, together with ROS, it kills the microbe. In addition, activated phagocytes produce several proteolytic enzymes in their phagolysosomes in order to destroy microbes (75).

Dendritic cells

DCs originate from the same precursor cells as monocytes and are widely distributed in all tissues. The cells, like phagocytes, express PRRs and respond after activation by microbes with secretion of cytokines. The majority of the DCs are conventional DCs, which just like other APCs, phagocytise recognised microbes and migrate to lymph nodes in order to activate naïve T cells. The smaller DC population is called plasmacytoid DCs and recognise viral nucleic acid and respond with production of type I IFNs, which result in an anti-viral immune response. DCs express a larger amount of and more different types of PRRs than other cells. The plasmacytoid DCs especially express an abundance of endosomal TLRs that recognise nucleic acid from viruses. Conventional DCs are the most important cells for naïve T cell activation. The DCs will, depending on microbe, stimulate T cell differentiation into different T effector cells, *e.g.* IFN- γ producing Th1 cells or IL-17 producing T helper (Th)17 cells (75).

NK cells

Originate from the same progenitor cells as T and B cells but express germ line-encoded receptors just like other innate cells. The NK cell has cytotoxic granules in the cytoplasm. The granules contain cytotoxic granzymes and pore-forming perforin,

which are both released on the cell surface on targeted cells resulting in apoptosis of the infected cell. NK cells recognise infected cells due to the latter's altered or absent expression of MHC class I but also due to activating signals from the NKG2D receptors bound to MICA/B on infected or tumour cells (81). The recognition of infected cells activates NK cells, which will produce and secrete cytokines (*e.g.* IFN- γ) and release cytoplasmic granule contents. The granule contents are the cytotoxic granzymes and pore-forming perforins that, when released on the cell surface on targeted cells, cause apoptosis of the infected cell. Infected cells can have an expression of antigen molecules on the cell surface, which are recognised by antibodies. These antibody-coated target cells activate the NK cell through the binding of the antibodies to the Fc receptors on the NK cell. This process is called antibody-dependent cellular cytotoxicity (81).

Innate lymphoid cells

Innate lymphoid cells (ILCs) are the name for several newly discovered cells that share common phenotypic and functional features. These cells are morphologically similar to lymphoid cells but lack the rearranged antigen receptors of the latter cells. There are different subtypes of ILCs, ranging from ordinary NK cells to ROR γ ⁺ ILCs that produce Th17-associated cytokines (IL-17 and IL-22) and ILC2 that produce Th2-associated cytokines (IL-5 and IL-13). NK cells are the cytotoxic ILCs, while ROR γ ⁺ ILCs are involved in lymphoid organogenesis, tissue repair and innate immunity against bacteria. The ILC2s are involved in the innate immunity against extracellular parasites. Both the ROR γ ⁺ ILCs and ILC2s are found in the intestinal mucosa where they are involved in both the intestinal immunity and the epithelial homeostasis (85).

T helper cell subsets

Th cells orchestrate the adaptive immune responses, mainly by their secretion of cytokines, which activate and recruit target cells (86). The Th cell population is divided into several T cell subtypes that are recognised by their characteristic cytokine profiles, and by their different expression of Th cell-associated transcription factors (figure 4). The differentiation into the different Th subsets depends on the cytokine milieu during the activation of the naïve CD4⁺ T cells. Innate cells, including APCs, produce different cytokines depending on the encountered PAMPs (87).

The Th1 cells are important during infections caused by intracellular pathogens and extracellular bacteria. The main cytokines that are produced by Th1 cells are IFN- γ , IL-2, Lymphotoxin and Lymphotoxin α . Th1 cells preferentially express the chemokine receptors CXCR3 and CCR5 (86). IL-12p70 produced by APCs stimulates activation of the transcription factor transducer and activator of transcription 4 (STAT4). STAT4 activates the expression of the transcription factor T-box expressed in T cells (T-bet). Activation of T-bet results in production of IFN- γ and IL-12R β 2, the

latter the prerequisite for the functional IL-12-receptor. IL-27 also activates T-bet resulting in increased T-bet and IFN- γ expression, while IL-18 upregulates IL-12R β 2 (88). APC-derived IL-12p70 will both induce further IFN- γ production and sustain the IL-12R β 2 expression in the Th1 cells. IFN- γ is also important for sustained expression of IL-12R β 2 (86). The produced IFN- γ will in turn stimulate enhancement of the effector functions of macrophages, neutrophils and NK cells (88).

The Th2 cells are involved in the immune response against extracellular parasites such as helminths, but are also important in allergic reactions. The cells produce, amongst others, the cytokines IL-4, IL-5, IL-9 and IL-13. CD25 (IL-2R α) is expressed in higher levels on Th2 cells compared to Th1 cells. Th2 cells express the chemokine receptors CCR3, CCR4, CCR8 and CRTh2. Newly activated naïve CD4⁺ T cells that are stimulated with IL-4 will start to differentiate into Th2 cells by activating STAT5, which upregulate the expression of both the transcription factor GATA-3 and the IL-4R α (86). The produced IL-4 and IL-13 induce IgE class switch in B cells. The produced IL-5 recruit, activate and enhance the function of eosinophils (88).

Th17 play an important role in the immune response against extracellular bacteria and fungi and is involved in the induction of many organ-specific autoimmune diseases (86). The cells are enriched at epithelial barrier sites such as skin, lungs and intestine. The Th17 cells are found at inflamed sites early in the immune response where they orchestrate the innate response, *e.g.* recruit neutrophils (87). The Th17 cells produce the cytokines IL-17A, IL-17F, IL-21 and IL-22. Th17 cells express high levels of IL-23R and express the chemokine receptors CCR6 and CCR4 (86). Transforming growth factor (TGF)- β together with IL-6 induces Th17 differentiation of newly activated Th cells by activation of STAT3 (88), which is followed by the upregulated expression of both the retinoic acid receptor-related orphan receptor (ROR) γ C and IL-23R (89). IL-21 can replace IL-6 in the induction of RORC expression. The Th17 produced IL-23 is important for the Th17 survival and functions (86). IL-17A induces production of many proinflammatory cytokines, IL-6 and CXCL8. Both IL-17 cytokines recruits neutrophils. IL-21 stimulates Th17 differentiation (86).

The majority of the T regulatory cells (Tregs) mature in thymus, not in the peripheral tissues and lymphoid organs like the naïve CD4⁺ and CD8⁺ T cells. These thymus-derived Tregs are called natural Tregs (nTreg). However, some T effector T cells are induced to develop into Tregs in the peripheral tissues and are therefore called induced Tregs (iTregs). Both Tregs express TGF β and IL-10 (87). The nTregs also express high and constitutive levels of CD25 compared to other activated T cells (86). TGF β is important for the nTreg development and differentiation since it induce the expression of the important Treg transcription factor forkhead protein (Foxp) 3. TGF β induce iTreg differentiation in naïve mouse CD4⁺ T cells when the cytokine milieu is absent from proinflammatory cytokines (86). Tregs are thought to suppress effector cells in

four different ways, both with soluble molecules and with direct cell-cell contact. The Treg cells can use IL10 and TGF β to suppress cells or induce apoptosis in cells by secretion of granzyme and perforin. The Tregs can also disrupt the metabolism of target cells by *e.g.* CD25-dependent deprivation-mediated apoptosis, or target DCs and suppress their maturation by MHC class II-mediated suppression or CTLA4-mediated induction of IDO production in the DCs (90).

The different Th subsets cross-regulate each other by suppressive mechanisms. IFN- γ and IL-4 mutually suppress each other, and thereby suppress Th2 and Th1 differentiation, respectively, in CD4⁺ T cells. TGF β suppress both Th1 and Th2 differentiation, while both IFN- γ and IL-4 suppress the Th17 differentiation. Some of these cross-regulations occurs on the transcriptional level, *e.g.* T-bet (Th1) suppress GATA3 (Th2) function. Tregs express RORC due to TGF β stimulation, but the Foxp3 expression in Tregs seem to suppress RORC expression (86).

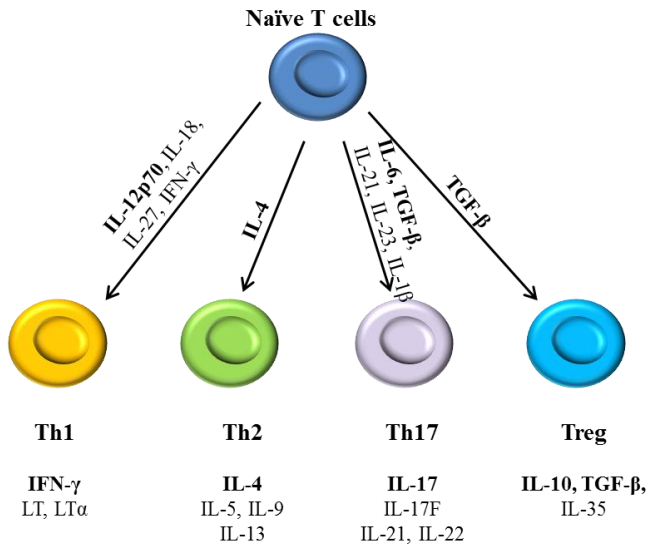


Figure 4. Simplified view of the cytokines involved in T helper cell differentiation. Each T helper cell expresses chemokine receptors and secretes cytokines.

Cytotoxic CD8⁺ cells

CD8⁺ T cells, like NK cells target and kill infected host cells. These T cells are activated in the lymph nodes by antigens presented on MHC class I molecules on APCs. The activation is followed by clonal expansion and differentiation before migration into the infected tissue. Mature CD8⁺ T cells are activated by binding to the

MHC class I presented antigen on infected cells (91). The CD8⁺ T cells will during this immune synapse secrete its granule contents. The secreted perforin and granzyme will induce apoptosis of the target cell in the same manner as with NK cells. CD8⁺ T cells can also kill target cells by binding of the CD8⁺ T cell's Fas ligand to the target cell's Fas receptor, which induce apoptosis. CD8⁺ T cells also express the cell activating NKG2D receptor that is expressed on all cytotoxic cells (75).

$\gamma\delta$ T cells

About 1-10% of the circulating T cells in a healthy adult are $\gamma\delta$ T cells, V γ 9V δ 2 T cells. $\gamma\delta$ T cells (V $\gamma\delta$ 1 T cells) are also present in other compartments, especially the epithelia in the small intestine (92). The germ line repertoire of the genes for the γ and δ chains of the T cell receptor is small, but the diversity of the $\gamma\delta$ TCR is at least as large as the $\alpha\beta$ TCR repertoire. Activation of $\gamma\delta$ T cells does not occur through presentation of antigen peptides by APCs' MHC class molecules. The cells are activated by binding of phosphorylated microbial metabolites to the TCR in a PRR-like manner (V γ 9V δ 2 T cells) or through binding of stress-induced molecules such as MICA/B and lipid antigens (V $\gamma\delta$ 1 T cells). The effector functions of $\gamma\delta$ T cells are similar to those of CD4⁺ and CD8⁺ T cells since the $\gamma\delta$ T cell can exert cytotoxic activity through death receptor/death ligand and perforin/granzyme pathways and secretion of cytokines (mainly proinflammatory Th1-like cytokines) on microbes and tumour cells. In addition, V γ 9V δ 2 T cells also express the cell activating NKG2D receptor (92).

B cells

B cells are lymphocytes that after recognition of extracellular antigens differentiate into plasma cells. The plasma cells produce and secrete antibodies against the recognised microbes. The antibodies can be of different Ig classes (IgD, IgA, IgE, IgG and IgM) but all Ig molecules have a common core structure with only the antigen-binding regions being highly variable (75).

Natural antibodies, mostly of the IgM subclass, are present in the circulation of normal individuals and are thought to be produced in a T-independent antibody response by B1 cells upon stimulation by bacteria that colonise the gastrointestinal tract, and by marginal zone B cells in lymphoid tissue. These natural antibodies recognise a limited number of specific common molecular carbohydrate and lipid patterns on microbes or stressed and dying cells (75).

Complement system

The complement system consists of more than 25 plasma, cell-surface proteins and complement regulatory proteins and its purpose is to target and mark pathogens for destruction. Many of the complement proteins are proteinases that are activated by proteolytic cleavage. Activation of the complement system can occur through the

classical pathway with antigen-antibody complexes on microbial surfaces binding to the complement protein C1q, through the alternative pathway by direct deposition of C3 on the microbial surface, or through the lectin (mannose binding) pathway by binding of mannose-binding lectin to the microbial surface. All three pathways result in a proteolytic activation cascade ending up with the formation of the membrane attack complex that kills microbes by osmotic lysis. Another function of the complement is C3b opsonisation of microbial cell surfaces, which result in enhanced neutrophil and macrophage phagocytosis. The C3a, C4a and C5a complement fragments induce acute inflammation by activating mast cells and neutrophils. The complement system causes intense local inflammation, and therefore need to be regulated in order to avoid harming the host. Several plasma proteins (*e.g.* factor H) and membrane proteins inhibit the complement activation to protect the host (75,80).

Cytokines

Cytokines are a large group of proteins that can be secreted by many different cell types. They play an important role in both the innate and adaptive immune responses. Cytokine production is initiated after cell activation and they are rapidly secreted, but since they are short lived, they are also rapidly degraded. The cytokines are used as a way for the cells' to communicate with each other, both near and far, without need of direct cell-cell contact. Cytokines can act on many different cell types or have many biological effects (pleiotropic), but many cytokines may have the same action (redundancy). Cytokines can also stimulate or inhibit production of other cytokines or they can have antagonising or additive (synergic) effects (75).

Table 1. Cytokines analysed in this thesis.

Cytokine	Mainly produced by	Actions	Main target	Paper	Reference
GM-CSF	Macrophages, T cells	Stimulate growth and differentiation of neutrophils, monocytes, dendritic cells	neutrophils, monocytes, DCs	III	(81)
IFN-γ	Macrophages, NK cells, NKT cells, Th1 cells, CD8 ⁺ T cells, B cells	Macrophage activation, increased expression of MHC molecules, Ig class switch, promote Th1 development, promote cytotoxic activity, suppresses Th2	Macrophages, T cells, B cells	II, III	(81,93)
IL-1β	Macrophages, epithelial cells	Fever, T cell and macrophage activation	Macrophages, T cells	IV	(81)

IL-2	Activated T cells, DCs, NK cells, NKT cells	Proliferation of effector B and T cells, development of Treg, differentiation and proliferation of NK cells, B cell growth factor	T cells, NK cells, B cells	III	(93)
IL-4	Th2 cells, basophils, eosinophils, mast cells, NKT cells, $\gamma\delta$ T cells	Induce Th2 differentiation, B cell activation IgE class switch	T and B cells	II, III	(93)
IL-6	Endothelial cells, fibroblasts, monocytes/macrophages, T cells	T and B cell growth and differentiation, fever, acute phase protein synthesis	T cells, B cells, hepatocytes	IV	(81,93)
IL-7	Epithelial cells, keratinocytes, DCs, B cells, monocytes/macrophages	Growth of pre-T and pre-B cells	B, T and NK cells	III	(81)
IL-10	T cells, APCs, NK cells	Immune suppression: downregulation of MHC class II and costimulators, inhibit expression of proinflammatory mediators, increase IgG ₄ production	T cells, APCs, NK cells, mast cells, granulocytes	II, III	(93)
IL-12p40/40	Monocytes, macrophages, DCs, neutrophils, microglia	Inhibition of Th1	T cells, NK cells	III	(94)
IL-12p70	Monocytes, macrophages, DCs, neutrophils, microglia	Th1 cell differentiation, cytotoxicity	Th cells, NK cells	II, III, IV	(93)
IL-15	Monocytes,	T cell and NK	T cells, NK	III	(81,93)

	keratinocytes, skeletal muscle cells	growth, cytotoxicity	cells, NKT cells, CD8 ⁺ T cells		
IL-17A	Th17 cells, CD8 ⁺ T cells, $\gamma\delta$ T cells, NK cells, neutrophils	Induce expression of proinflammatory cytokines, chemokines and metalloproteases, neutrophil attractant	Epithelial, endothelial cells, fibroblasts	III	(81,93)
IL-22	Th17 cells, NK cells	Induce acute-phase proteins, proinflammatory agents, epithelial barrier	Keratinocytes, hepatocytes	III, IV	(81,93)
IL-23	Macrophages, DCs	Induce proliferation of Th17 memory cells, increase IFN- γ production	Th17, macrophage	III	(81,93)
IL-27	Macrophages, monocytes, DCs, endothelial cells	Induce IL12R via T-bet induction, induce IL-10 production	T cells, NK cells	IV	(81,93)
IL-35	Treg cells	Immunosuppression	T cells	III	(93)
TNF	Neutrophil, T cells, NK cells, endothelium, mast cells	Promote inflammation, endothelial activation	Hepatocytes, endothelial cells, dendritic cells	IV	(81,88)

Chemokines

Chemokines are a family of over 50 human cytokines specialized in attracting and stimulating the movement of leukocytes. The chemokine family is divided into four groups according to their location and number of N-terminal cysteine residues. The largest groups of chemokines are the CC and the CXC groups. Both CC and CXC chemokines are produced by leukocytes, but also non-immune cells such as endothelial cells, epithelial cells and fibroblasts. Secretion of chemokines is often triggered by recognition of PAMPs on the cells' PRRs. Chemokine production is also stimulated by the inflammatory cytokines IL-1 β and TNF, and several CC chemokines are produced by T effector cells. The binding of chemokines to their receptors results in an intracellular signalling that causes the activation of protein kinase C and increased intracellular Ca²⁺. These changes induce both cytoskeletal changes, which increase the cell motility, and conformation changes of integrins in the plasma membrane (75).

Table 2. Chemokines analysed in this thesis.

Chemokine	Actions	Main target	Paper	Reference
CCL3	promotes Th1 immunity	Monocytes, macrophages, DCs, activated T cells (Th1), NK cells	III	(81,95,96)
CCL4	promotes Th1 immunity	Monocytes, macrophages, activated T cells (Th1), NK cells, DCs	III	(81,95,96)
CCL5	Promote Th1 immunity; attract and degranulation : eosinophils, basophils	Monocytes, macrophages, Th cells, NK cells, DCs, eosinophils, basophils	III	(81,95,96)
CCL18	Attract dendritic cells, naïve T cells	DCs, naïve T cells	IV	(95,97)
CCL20	Attract T and B cells	DCs, activated T cells, B cells	III, IV	(95,97)
CCL22	Attract T cell and basophil	DCs, Th2, NK, skin-homing T cells	IV	(75,95)
CXCL1	Activate neutrophils, fibroplasia, angiogenesis	Neutrophils, naïve T cells, fibroblasts	IV	(81,95)
CXCL8 (IL-8)	Attract, activate and degranulate neutrophils, angiogenesis	Neutrophils, naïve T cells	IV	(81,95,96)
CXCL9	Attract NK and Th1 cells	Th1, NK	III, IV	(95,98)
CXCL10	Attract NK and Th1 cells	Th1, NK	III, IV	(95,98)
CXCL11	Attract NK and Th1 cells	Th1, NK	IV	(95,98)

Other immune markers

Matrix metalloproteinases (MMPs) is a family of enzymes that regulates inflammatory and tissue repair processes. The MMPs are secreted or anchored to cell surfaces. One of MMPs functions is to degrade extra cellular matrix (ECM) (99).

Calprotectin is an antimicrobial protein found in large amounts in the cytoplasm of neutrophils. The protein can act chemotactic for neutrophils when secreted. Secreted calprotectin also have antimicrobial functions and can inhibit several MMPs (100).

Table 3. Other immune markers analysed in this thesis.

Immune marker	Mainly produced by	Actions	Targets	Paper	Reference
Calprotectin	Neutrophils, macrophages, monocytes	Sequester zinc, inhibits MMPs and microbial growth	Microbes, MMPs	IV	(101)
MMP-3	Keratinocytes, fibroblasts, chondrocytes	Degrade ECM	<i>E.g.</i> collagen III-V, IX-XI	IV	(102)
MMP-8	Macrophages, neutrophils, plasma and T cells, epithelial and endothelial cells etc.	Degrade ECM	<i>E.g.</i> collagen I-III, V, VII, VIII, X	IV	(102)
MMP-9	Macrophages, Neutrophils, DCs, T, B, NK epithelial cells, connective tissue cells	Degrade ECM	<i>E.g.</i> collagen I, III-V, VII, X, XII	IV	(102)

The immune response against *B. burgdorferi* s.l.

Transmission

The outer surface proteins (Osp) A and OspB on the spirochaete outer membrane are important for *B. burgdorferi* s.l. attachment to the tick receptor for OspA in the tick midgut (103,104). During tick feeding, the spirochaete, due to changes in pH and temperature to more “mammal-like” conditions, change its gene expression of surface molecules and OspA is downregulated while OspC expression becomes upregulated. These changes of expression result in migration of spirochaetes from the midgut to the tick salivary glands (105). OspC seems to be important for the spirochaete transmission since it has been shown that OspC-deficient spirochaetes were unable to colonize ticks and infect mice (104,106).

Tick saliva

The tick saliva that is injected into the host during tick feeding contains substances that impair haemostasis, host immunity and wound healing (12). Some of the salivary substances are protease inhibitors that impair neutrophil chemotaxis, integrin

expression and ROS generation (107,108). Other salivary substances impair macrophage activation or inhibit complement activation (109,110).

B. afzelii-stimulated mouse splenic DCs exposed to *I. ricinus* saliva showed decreased expression of TNF due to the saliva's ability to suppress TLR2 signalling pathways. In contrast, IL-10 expression was enhanced in these cells (111). DCs ability to phagocytise *B. afzelii* and to produce TNF, IL-6 and IL-10 (the latter first after 48h) were decreased when cells were exposed to saliva from *I. ricinus*. In addition, the induction of proliferation and IL-2 production in CD4⁺ cells by *B. afzelii*-exposed DCs were also decreased in presence of tick saliva (112). The mRNA expression of several antimicrobial peptides, TNF and the chemokine CCL2 in response to *B. burgdorferi* s.s. N40 strain inoculation were dampened when the spirochaetes were inoculated into mouse skin in presence of tick saliva (113). Tick saliva contains an IL-2-binding protein that inhibits T cell proliferation in vitro (114). Prostaglandin E₂ in the tick saliva reduces IL-12p70 and TNF production in DCs and suppress DC-induced Th cell proliferation and IL-2 production but increases the production of IL-10 (115).

Dissemination from the site of tick bite

Spirochaete survival and replication in the host's dermis involves adaptation to the increased temperature, pH change, differences in nutrient composition and exposure to antibodies and complement proteins (12). After transmission into the mammal host *B. burgdorferi* s.l. multiply in the dermis (116) and start to express adhesins on its outer membrane in order to disseminate through the extracellular matrix (ECM) from the site of the tick bite. These adhesins binds to host decorin, fibronectin, glycosaminoglycans and plasminogen in the ECM, but can also bind to platelets and integrins on e.g. endothelial cells (reviewed in (117)). In addition, *B. burgdorferi* s.l. induces expression and release of matrix metalloproteinases (MMPs) *in vitro*, which may further facilitate dissemination through ECM, due to the degradation of the ECM (118). *In vivo*, MMP-9 was shown to be upregulated in EM lesions (119) and increased levels were found in CSF of NB patients (120).

Spirochaetes seem to remain locally in the skin for several days. The delayed dissemination is hypothesised to be due to a re-programming of the spirochaetal gene expression. Changed protein expression could facilitate nutrient acquisition, evasion of the immune response and upregulation of proteins that are required for dissemination in the host (12).

***B.burgdorferi* s.l. immune evasion**

OspC binds to the tick protein Salp15 in the tick salivary glands, which facilitates transmission into the host because of Salp15's ability to inhibit antibody-mediated killing and CD4⁺ T cell activation (121–124).

Another salivary protein in *I. scapularis* ticks is *I. scapularis* anti-complement (Isac), which inhibits the complement system (110). Vertebrates express complement regulatory proteins such as factor H and factor H-like protein (FHL-) 1. These proteins inactivate the C3b complement protein and thereby prevent C3b deposition on the cell membrane (125). *B. burgdorferi* s.l. can also by itself inactivate the C3b complement protein by binding of the spirochaete expressed complement regulatory-acquiring surface proteins (CRASPs) and OspE-related proteins (Erps) to the host complement regulators Factor H and Factor-H-like proteins (126–130). *B. burgdorferi* s.l. subtypes have different susceptibility to the host's complement. *B. afzelii* is complement-resistant, *B. garinii* is complement-sensitive and *B. burgdorferi* s.s. is intermediately sensitive (128,131,132).

B. burgdorferi s.l. also tries to evade the host's immune system by use of antigenic variation, i.e. changing the expression of membrane-bound proteins by up- and downregulation in order to avoid immune reactions. The lipoproteins variable major protein-like sequence expressed (VlsE) and Osp A-C go through antigenic variation during infection (133–136). The antigenic variation of the vlsE gene occurs during mammal infection through random recombination of the vlsE gene, which continually creates new vlsE gene variations (137,138).

B. burgdorferi s.l. requires zinc not iron for growth, which could help the spirochaete to avoid host immune mechanism of iron deprivation (139).

Co-cultures of macrophages and viable *B. burgdorferi* s.s. and *in vivo* analyses of IL-10 upregulation after *B. burgdorferi* s.s. inoculation suggest that the spirochaete can suppress early macrophage responses by stimulating the release of IL-10 from host cells (140).

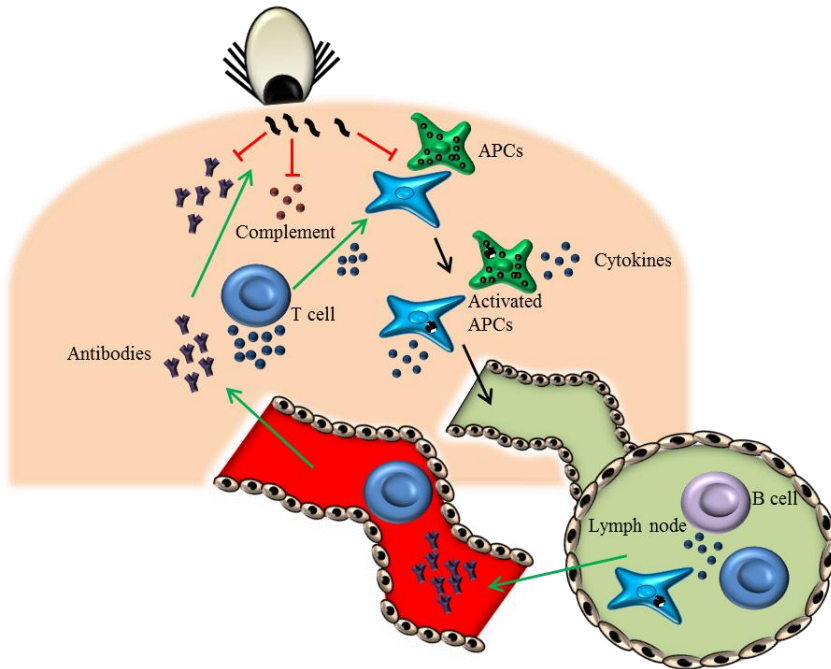


Figure 5. The interaction between *Borrelia burgdorferi sensu lato* and the host's immune responses. The spirochaete can block or inhibit immune components in order to protect itself from killing. Antigenic variation and binding to Salp15 (tick salivary protein) protect against antibodies, binding to complement regulatory components (e.g. Factor H) inhibit the complement system, and stimulation of IL-10 production suppress the APCs.

Immune response against *B. burgdorferi s.l.*

All *B. burgdorferi s.l.* subtypes activate both the classical and the alternative pathways of the complement system (128,132). The complement protein C5 is not essential for antibody-mediated protection from *B. burgdorferi s.l.* in mice (141). In contrast, C3 deficient mice had high spirochaetal burden in tissues (142,143). Taken together, these experimental studies suggest that complement opsonisation, which facilitates clearance of spirochaetes by phagocytes, play a role during *B. burgdorferi s.l.* infection, rather than complement-mediated lysis of spirochaetes.

B. burgdorferi s.l. spirochaetes tethered to platelets moved across platelets with the mean crossing speed 1.636 $\mu\text{m}/\text{min}$. The spirochaete moved the fastest when it reached an approximately speed of 2800 $\mu\text{m}/\text{min}$, which can be compared to the approximately speed of 20 $\mu\text{m}/\text{min}$ that neutrophils are moving (144–146). Neutrophils and monocytes seem to have a limited ability to ingest unopsonised *B. burgdorferi s.l.* *in vitro* and seem to only kill extracellular spirochaetes (147). Opsonised spirochaetes are

rapidly cleared by neutrophils (147,148). In contrast, mature macrophages phagocytise and kill both opsonised and unopsonised spirochaetes in phagolysosomes (147,149,150). Human peripheral blood mononuclear cells (PBMCs) recognise *B. burgdorferi* s.s. by the heterodimer TLR1/TLR2 (151). Mannose receptors on DCs have been shown to be highly upregulated after activation by spirochaetes. The mannose receptor may facilitate the phagocytosis of *B. burgdorferi* s.l. by monocytes and macrophages (152). PRR activation by *B. burgdorferi* s.l. induces intracellular signalling cascades that activates the cells, and activates transcription factors such as NF κ B that will start transcription of inflammatory genes (reviewed in (153)). *In vitro*, *B. burgdorferi* s.l. Osp proteins stimulate, through host PRRs, PBMC and mast cells to produce proinflammatory cytokines such as IL-1 β , IL-6; IL-12p70, TNF, IFN- γ (154–159), anti-inflammatory IL10 (160), chemokines CCL3, CCL4, CXCL8 (154) and induce production of several MMPs (161).

B. burgdorferi s.l. stimulates proliferation of B cells and production of antibodies (162). The opsonising and complement activating IgG1 and IgG3 dominate during *B. burgdorferi* s.l. (163,164).

Human V δ 1 $\gamma\delta$ T cells in synovial fluid respond to *B. burgdorferi* s.l. lipoproteins and lipopeptides and induce maturation of DCs, resulting in secretion of IL-12p70 and upregulation of costimulatory molecules (165–167). Synovial $\gamma\delta$ T cells also exhibit cytotoxic activity against synovial cell types in the presence of *B. burgdorferi* s.l., which help to reduce the inflammatory response (12). NKT cells can become activated *in vitro* by *B. burgdorferi* s.l. through CD1d binding to the spirochaete (168). The NKT cells can activate NK cells, DCs, T cells and B cells. Absence of CD1d and NKT cells result in higher spirochaetal burden and enhanced arthritis in mice during the early course of *B. burgdorferi* s.l. infection (169). Kinetic study of cytokine induction in mice showed that disease-resistant BALB/c mice produced IFN- γ earlier in the course of infection than disease-susceptible C3H mice and IL-4 was a sign of arthritis resolution (170).

Characterisation of EM lesions from LB patients showed infiltrate predominated by T cells, monocytes/macrophages, DCs, a few B cells and occasional neutrophils (171–173). The dominant cytokines in EM lesions were IFN- γ and IL-6 (172), while the dominating mRNA expression were proinflammatory with detection of IFN- γ , TNF, IL-1 β and IL-6 mRNA, but also anti-inflammatory IL-10 mRNA expression (173,174). In addition, mRNA expression of Th1-associated chemokines CXCL9-11 was also found in the EM lesions. Taken together, these findings suggest that the immune response in EM lesions is proinflammatory Th1-like.

Dissemination of *B. burgdorferi* s.l. occurs via direct tissue invasion and through a blood-borne route (12). Studies on human NB have shown that induction of MMPs

may allow *B. burgdorferi s.l.* to invade neural tissue. Production of CXCL11 and CXCL13 contributes to the cell recruitment of T cells and B cells, respectively, into the CSF. The CSF pleocytosis consist of mainly B cells with smaller fractions of T cells and monocytes (12). Intrathecal production of *B. burgdorferi s.l.*-specific antibodies and complement proteins suggest that the immune response in CSF is localised (12,125,175). Previous studies from our group indicate that Th1/Th17 immune response predominate in human NB (175–179), and is eventually followed by an anti-inflammatory response (176,180).

Furthermore, a good clinical outcome of *B. burgdorferi s.l.* infection is associated with strong early Th1-like immune responses in studies that compared immune response between LB patients with good clinical outcome and patients with persistent symptoms six months post-treatment (180–183). Furthermore, the strong early Th1 response was followed by upregulation of Th2 response in patients with good clinical outcome while patients with persistent symptoms had a persistent Th1 response (176,180). Increased number of TNF-secreting blood-derived DCs in asymptomatic *B. burgdorferi s.l.*-infected individuals compared to NB patients, and elevated levels of IL-12p70 from PBMCs of asymptomatics compared to controls, further indicate that a strong proinflammatory Th1 response against the bacteria seem to be associated with a good clinical outcome (182). Taken together, the findings of a Th1 predominating immune response against *B. burgdorferi s.l.* and the association of a strong early Th1 response with good clinical outcome of LB provided the basis for the hypothesis of this thesis.

Aims and hypothesis

Hypothesis

A strong inflammatory Th1-like immune response is required in the early stage of infection in order to achieve both optimal eradication of the *Borrelia burgdorferi sensu lato* bacteria and clinical outcome. The inflammatory response must be down-regulated by an anti-inflammatory response in order to avoid excessive immune responses that will end in tissue injury. The proper down-regulation will also protect against development of a chronic Th1-like inflammatory response, featuring activated cytotoxic cells, which may lead to Lyme borreliosis with persistent symptoms post-treatment.

Overall aim

The overall aim was to investigate the immunological mechanisms for optimal resolution of human *Borrelia burgdorferi sensu lato* infection and to define the aberrant mechanisms leading to development of persistent symptoms.

Specific aims

To investigate

- the risk of developing LB after a bite by a *B. burgdorferi s.l.*-infected tick
- the occurrence of asymptomatic *B. burgdorferi s.l.*-infection after a bite by a *B. burgdorferi s.l.*-infected tick
- whether differences in the early local cytokine expression is associated with clinical outcome of EM
- the presence of possible early immune biomarkers in blood from newly tick-bitten persons during the early events of inflammation caused by *B. burgdorferi s.l.* and association with clinical outcome
- differences in activation of the T-cell subsets Th1, Th2, Th17, cytotoxic T-cells and Treg subsets in an experimental model between immune-deviated and non-deviated *B. burgdorferi s.s.*-infected mice in relation to clinical signs of infection.

Material and Methods

Tick-Borne Diseases (TBD) Sting study

The aims of the TBD Sting study are, among others, to investigate the risk of developing LB after bite by a *B. burgdorferi s.l.*-infected tick; the occurrence of asymptomatic *B. burgdorferi s.l.* infections; whether spirochaete numbers and elapsed time before tick removal correlate with development of LB; the prevalence of *B. burgdorferi s.l.* in ticks that have bitten humans; and to investigate the possible correlation between the quantity of spirochaetes in the tick and development of LB.

The inclusion criteria for those participating in the study were that they should be aged 18 years or older, newly tick-bitten, they had brought the tick to the first visit to the primary health care (PHC) centre, and had signed an informed consent. The exclusion criterion was being on antibiotic treatment during the time of inclusion. Individuals who suffered from similar symptoms to those associated with LB or who were receiving treatment for diseases that would require immunomodulating medications were not excluded but were stratified into specific subgroups.

The study collection was initiated in November 2006 and is to date still on-going. Study subjects are recruited by advertisements on local television, in newspapers, in local grocery stores, libraries, pharmacies, etc. Between June 2007 and January 2008, the population served by nine PHC centres in the County of Östergötland in the south-east of Sweden and the Department of Infectious Diseases at the University Hospital in Linköping were asked to participate in the study if they had recently been bitten by a tick. Since the first year, the study has expanded, and at the time of this thesis includes approximately 60 PHC centres distributed over five southern counties in Sweden (the counties of Jönköping, Kalmar, Skåne, Västra Götaland and Östergötland), two counties in the middle of Sweden (the counties of Dalarna and Dalsland) and one county in the northern part of Sweden (the county of Västerbotten). The study area also includes the inhabitants in the whole of County Blekinge (southern Sweden), and in addition one PHC centre in the southern County of Aust-Agder in Norway and the inhabitants of all archipelago municipalities in the Åland Islands, Finland.

At the time of inclusion, the recruited newly tick-bitten study subjects brought their tick/s to their PHC centre, where they signed an informed consent for study participation, answered a standardized questionnaire with relevant questions, and provided blood samples. The participants were asked to return to the PHC centre for a follow-up visit three months later for new blood sampling, collection of new ticks that

had bitten the subjects (from 2008 and onwards), and to answer a second health questionnaire. All collected material and questionnaires were sent to the University Hospital in Linköping for processing and storage. Study subjects were told to contact health care if they experienced any illness or symptoms possibly associated with the tick bite during the study period. Medical records from such visits were retrieved and scrutinised by three physicians in the TBD Sting study group (PF, DN, JS), all with long experience of diagnosing LB, in order to give a final judgment concerning proper clinical diagnosis of LB.

Subjects (papers I-II, IV)

In total 584 newly tick-bitten individuals or LB patients were included in this thesis (table 4). Eighteen of the study subjects in paper I were also included in paper IV.

Table 4. The clinical outcome of the study subjects included in papers I-II and IV.

Clinical outcome	Paper I Risk of developing LB (n=341)	Paper II Prospective study of EM (n=88)	Paper IV Early biomarkers for LB outcome (n=155)
EM	1 ^b	88	15
NB	-	-	1
Lymphocytoma	-	-	1
LB	-	-	1
Asymptomatic	4	-	28
Controls	-	-	-
Bite by infected tick^a	60	-	54
Bite by uninfected tick^a	276	-	55
Total	341	88	155

a Study subjects did not develop LB or seroconvert during the study time.

b The study subject was bitten by an uninfected tick, thus the developed EM was caused by a tick that was not collected.

LB, Lyme borreliosis; EM, erythema migrans; NB, neuroborreliosis

Papers I and IV

All study subjects included in these two papers were recruited from the on-going TBD Sting study that was initiated in 2007. All newly tick-bitten individuals who were included in the TBD Sting study during 2007 and who completed their participation, by attending the three month follow-up visit were included as study subjects in paper I. Fifty-three of the 394 newly tick-bitten individuals were excluded, leaving 341 study subjects who completed the study.

The study subjects in paper IV were also recruited from the TBD Sting study, participating during 2007-2009 (table 5). However, we actively selected study subjects fulfilling the criteria of being bitten by a *B. burgdorferi* s.l.-infected tick and who, during the study period, developed clinically diagnosed LB (LB patients) or seroconverted but did not develop LB during the period (asymptomatics). In addition to subjects who developed LB or seroconverted we also recruited "controls", i.e. study subjects who did not develop LB or seroconvert after a bite by *B. burgdorferi* s.l.-infected or uninfected ticks, ending up with the first set of 78 subjects divided into four groups (first set of subjects, table 5). The controls were used as controls for the possible influence the tick itself and the actual tick bite might have on the host's immune system. One of the 78 study subjects had to be excluded after analysis, leaving us with a set of four study groups (first set of samples shown in table 5). When more subjects were recruited to the TBD Sting study, another 78 subjects were included (second set of subjects shown in table 5) in paper IV in order to repeat the analyses and confirm the previous results.

Table 5. The study subjects in paper IV.

Group	1	2	3	4
Definition	LB + <i>B. burgdorferi</i> s.l.-infected tick	Seroconversion + <i>B. burgdorferi</i> s.l.-infected tick	<i>B. burgdorferi</i> s.l.-infected tick - LB or seroconversion	<i>B. burgdorferi</i> s.l.-uninfected tick - LB or seroconversion
First set of study subjects				
Number	11	10	27	29
Sex (female/male)	9/2	5/5	21/6	18/11
Age, mean (range)	62 (45-74)	65 (51-79)	59 (24-79)	62 (20-87)
Second set of study subjects				
Number	7	18	27	26
Sex (female/male)	4/3	9/9	18/9	18/8
Age, mean (range)	64 (48-74)	66 (55-81)	62 (30-86)	61 (26-81)

LB, Lyme borreliosis; *B. burgdorferi* s.l., *Borrelia burgdorferi* sensu lato

Paper II

In total, 109 patients with newly discovered EM who were receiving care at Åland Central hospital, Finland were included in paper II. The inclusion criteria were a minimum age of 18 years old and the clinical finding of a circular, expansive skin rash, >5 cm in diameter. A previous tick-bite and EM-associated symptoms such as fever, myalgia, arthralgia and fatigue supported diagnosis. None of the patients had received antibiotic treatment for the current EM before the inclusion. The patients were followed-up after 3, 6, 12 and 24 months. Questionnaires were answered and blood samples collected at each time point, including the time of inclusion. The patients were divided into two groups, according to their health status at the six months follow-up. The groups comprised of those with and without self-reported symptoms associated with EM, or symptoms that had arisen associated with the EM or afterwards. Twenty-one of the 109 patients were excluded from the study, leaving 88 EM patients that completed the study (see below).

Mice

Paper III

Forty-nine 10-week-old female C3H/HeN mice (Taconic M&B, Denmark) arrived and were housed at the Animal Department, Faculty of Health Sciences, (Linköping, Sweden). The mice were acclimatised during the following five weeks and were 15 weeks old at the onset of the experiment (day -15 post-infection with *B. burgdorferi* s.s. (p.i.)). The mice were housed in groups of seven in steel-wire cages in a room with light cycles of 12 hours dark/12 hours light. Pellets and tap water were given *ad libitum*. Before the start of the experiment, all cages were randomised into three groups; with three cages with *B. burgdorferi* s.s.-infected mice (Bb), three cages with immune-deviated *B. burgdorferi* s.s.-infected mice (BbId) and one cage of untreated uninfected mice (controls) (table 6).

The mice strain was chosen because it is known to be susceptible to *B. burgdorferi* s.l. infection in the form of *B. burgdorferi* s.l.-induced arthritis (184). In addition, the mice strain of choice also needed to be susceptible to immune-deviation by mercury treatment in order to shift the immune response to a Th2-like response prior to the bacterial infection. The general health of all the mice was supervised by the hospital/university veterinarian to ensure that no needless pain or suffering were occurring, in which case the experiment would have been terminated.

Table 6. The mice included in paper III.

Clinical outcome	Paper III
	T cell subsets during <i>B. burgdorferi s.s.</i>
	infection (n=49)
Arthritis	42
Controls	7
Total	49

B. burgdorferi s.s., *Borrelia burgdorferi sensu stricto*

Excluded study subjects and mice

In total, 76 individuals were excluded from the papers. In paper I, 46 of the 394 newly tick-bitten individuals were excluded because they did not complete the study by attending the three month follow-up visit despite receiving a reminder. Another seven individuals were excluded after completion; two due to suffering from similar symptoms to those associated with LB at the time of inclusion, two individuals due to being on antibiotic treatment at the time of inclusion, two whose blood samples were not analysed by both ELISA serology methods and one individual whose blood samples were damaged during transport. In paper II, 18 of the 109 EM patients were excluded due to missing data from the six month follow-up, and three patients due to presence of symptoms >3 weeks before inclusion in the study. In paper III, no mouse was excluded. In paper IV, one newly tick-bitten individual from Group 1 from the first set of 78 study subjects was excluded after completion of the study due to reassessment of the results from the *B. burgdorferi s.l.* DNA screening of the subject's tick showing that the tick was uninfected with *B. burgdorferi s.l.* and not infected, as was thought from the first analysis.

LB diagnosis (papers I, II and IV)

The clinical diagnosis of EM was made based on the occurrence of an expanding (>5 cm diameter) rash at the site of the tick bite. The NB patient attended health care three weeks after the study inclusion due to head ache and neck pain. The symptoms persisted one week later and the patient was referred to the Department of Infectious diseases at the university hospital in Linköping, Sweden, where the patient received the NB diagnose. The diagnosis of NB was based on the presence of symptoms associated with NB, mononuclear pleocytosis in CSF and intrathecal production of *B. burgdorferi s.l.*-specific antibodies. The Borrelial lymphocytoma patients had been bitten by a tick during the summer 2009 and attended health care in November the same year due to experience of general malaise and diffuse arthralgia. Patient did not receive a diagnosis until the second visit to health care in January 2010 when the

patient was diagnosed by the finding of an expanding red-blue swelling on the same ear lobe as the preceding tick bite. Infiltrates were present in the swelling and the serology was positive for *B. burgdorferi* s.l.-specific IgM and IgG antibodies.

In papers I and IV, the EM had to have occurred approximately one week after the time of inclusion and at the latest at the three month follow-up for it to be a new LB case during the study time. Three of the co-authors (PF, DN, JS), physicians with long experience of diagnosing LB, scrutinised the medical records in order to give a final judgment concerning proper clinical diagnosis of LB.

In paper II, the EM had to have occurred within three weeks prior to the time of inclusion of EM patients. The limit for debut symptoms was >3 weeks prior to inclusion. The six month evaluation was set because symptoms consistent with non-infectious complications or treatment failure with dissemination of the infection should have occurred by this time point. All patients were treated with the Åland Islands' standard treatments of amoxicillin (or doxycycline in case of penicillin allergy) for 14 days.

Questionnaires (papers I, II and IV)

Two sets of different questionnaires were used in the studies in this thesis. The first set of questionnaires was used in the TBD Sting study (papers I and IV) and asked questions related to tick bites. The second set of questionnaires were used in paper II and asked questions about the EM patients' health status.

In papers I and IV, the first questionnaire, answered at the time of inclusion, contained questions concerning the time of the tick bite, estimated time of tick-exposure and geographical area, previous treatments for LB and current medications. The second questionnaire that was answered, at the three months follow-up, included questions about new tick bites since the time of inclusion, the subject's general health condition during the study period, experiences of symptoms possibly associated with LB, and whether or not the study subjects had attended health care due to the symptoms they had experienced. The listed symptoms possibly associated with LB were headache, fatigue, fever, neck pain, loss of appetite, nausea, weight loss, vertigo, cognitive difficulties, radiating pain, myalgia, arthralgia and numbness.

In paper II, the questionnaire at time of inclusion asked for information about participant's age, sex, general health, other diseases that may have influenced the immune system, immunosuppressive treatments, antibiotic treatments within two months prior to the inclusion, symptoms at baseline, EM location and duration, number and size of the EM-lesions and if a preceding tick-bite had been noticed. The

questionnaires at the three, six, 12 and 24 months follow-ups asked questions about the patient's health status during the study period.

Clinical signs of infection (paper III)

Weight and joint swelling

All mice in paper III were continuously weighed and the average thicknesses of the tibiotarsal joints of both hind legs were measured with a pair of electronic digital callipers. The weight and joint parameters were measured seven days prior to the *B. burgdorferi* s.s. inoculation, every fifth day during days 0-19 p.i., and every third day during days 22-40 p.i. The joint thickness was measured at the thickest portion of the extended tibiotarsal joint. The joint thickness data is presented as joint swelling, defined as the increased in joint diameter compared to the diameter from the measurement taken before the start of the experiment.

Analysis of histopathology

In paper III, the thickest rear tibiotarsal joint from each mouse was fixed in 4% formaldehyde (Histolab Products AB, Göteborg, Sweden), decalcified, and embedded in paraffin. The tissue sections were stained with haematoxylin and eosin in order to detect cell infiltration and tissue destruction at the infection site in the tibiotarsal joints. The tissue sections were analysed by giving a total score for the tissue damage and cell infiltration in the tibiotarsal joints, but also scoring specifically for the degree of neutrophil infiltration, mononuclear cell infiltration, synovial thickness and cartilage hyperplasia. The scores were determined according to the following score scale: 0 - normal; 0.5 - minimal; 1 - slight; 2 - moderate and 3 - severe. All slides were blinded before one of the co-authors (PH) analysed them.

Bacterial strains and culture

In paper III, *B. burgdorferi* s.s. N40 were grown in Barbour Stoenner Kelly (BSKII) medium supplemented with 6% normal rabbit serum (Department of Microbiology, Umeå University, Sweden) at 35° C prior to infection. The BSKII medium was specifically developed as a culture medium for *B. burgdorferi* s.l. (185) (Barbour 1984). Mice were anaesthetised with Isofluran (Forene®, Abbott Scandinavia AB, Solna, Sweden) and injected subcutaneously at the tail root with 10⁵ *B. burgdorferi* s.s. N40 in 100 µL BSKII. Ear biopsies and blood (negative control) were cultured for eight days in BSKII medium supplemented with 6% normal rabbit serum, 50µg/mL sulfamethoxazol (Sigma-Aldrich) and 400µg/mL phosphomycin (Sigma-Aldrich) at 35°C. The cultures were counted in a blinded fashion in Bürker chambers by phase contrast microscopy.

Immune-deviation treatment

The treatment consisted of non-toxic doses of inorganic mercury (approximately 190 µg/Hg/kg body weight/day) for 14 days with start nine days prior *B. burgdorferi s.s.* inoculation. The mercury treatment was prepared by dissolving 10 mg/L HgCl₂ (Fluka Chemie, Switzerland) in tap water that was given *ad libitum*. Fresh solutions were given once a week.

Detection of *Borrelia burgdorferi sensu lato*

In paper I, the detection and quantification of *B. burgdorferi s.l.* were performed with a real-time PCR assay based on the Light Upon eXtension™ technique (Invitrogen Corp., USA), as previously described (186). Briefly, the *B. burgdorferi s.l.* Total DNA was extracted from the tick samples with a DNeasy® Blood and Tissue kit (Qiagen, Germany), according to a Qiagen supplementary protocol for detection of *B. burgdorferi s.l.* DNA (186). In paper IV, the extraction method had changed and the ticks were therefore homogenised with TissueLyser (Qiagen) and total nucleic extraction was performed with a MagAttract® RNA Tissue Mini M48 kit (Qiagen, unpublished data). The cDNA synthesis was performed with an Illustra™ Ready-to-Go RT-PCR Beads kit (GE Healthcare, UK) according to the manufacturer's instructions. *B. burgdorferi s.l.* DNA was detected and quantified with the group-specific 16S rRNA primers B16S_FL and B16S_R, targeting all *B. burgdorferi s.l.* species so far detected in Europe (186). The detection limit for the LUX real-time PCR assay was less than 10 gene copies, the equivalent of the number of copies that exist in one *B. burgdorferi s.l.* cell (187). *B. burgdorferi s.l.* positive samples were further species-determined by two nested conventional PCR assays with primers targeting genetically diverse regions within 5S-23S and 16S-23S intergenic spacers. MacroGen Inc. (South Korea) performed the nucleotide sequencing of the PCR products obtained from the nested PCR assays.

In paper II, the PCR method that was used was developed previously by Comstedt et al. (188). Briefly, DNA from one half of the biopsies was extracted with the Puregene DNA isolation protocol (Gentra systems, USA) and stored at -20° C. DNA extracts were analysed with a quantitative PCR assay with probe and primers specific for the 16S rRNA gene. Serially diluted *B. burgdorferi* 31 and *B. hermsii* HS1 DNA were used as standards. *B. burgdorferi s.l.* Subtypes were identified by direct sequencing amplicons generated from the /rrs/(16S)-/rrl/(23S) intergenic spacer or 16S gene PCRs. When necessary, nested modification of these assays was used to increase amplification success. Further details are found in paper II.

In paper III, the quantification of the spirochaetal load in urinary bladders was performed by extracting total DNA from bladders with the DNeasy® Blood and Tissue kit (Qiagen) using the above mentioned Qiagen supplementary protocol for detection

of *B. burgdorferi* s.l. DNA as described in paper III. The bladder DNA was detected and quantified with 16S rDNA primers and a *B. burgdorferi* s.s. B31 probe on a Bio-Rad iCycler. Further details are found in paper III and have been described previously (189). The cytokine mRNA expression was detected and quantified by extraction of total RNA from inguinal lymph nodes with an Rneasy Lipid Tissue mini kit (Qiagen) and homogenisation of lymph nodes with TissueLyser (Qiagen) according to the manufacturer's instructions. cDNA was produced with a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, the Netherlands), according to the manufacturer's instructions. The cDNA was analysed with TaqMan Gene Expression assays (Applied Biosystems) for detection of IL-2, IL-4, IL-7, IL-10, IL-12p35, IL-12p40, IL-15, IL-17A, IL-22, IL-23p19, IFN- γ , Granulocyte-macrophage colony-stimulating factor (GM-CSF), Foxp3, Epstein-Barr virus induced gene 3 (EBI-3) and the endogenous control Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The data was analysed with the relative quantification ($\Delta\Delta C_T$) method with control mice as a calibrator and GAPDH as a reference gene.

Analysis of cytokine production

Immunohistochemistry (paper II)

Immunohistochemistry (IHC) is a method for detecting specific molecules in tissues or cell compartments. The method uses antibodies conjugated with enzyme or labelled with fluorochrome. The antibodies bind to a monolayer of cells or a frozen section of the tissue of interest and are visualised using a fluorescence microscope (a conventional microscope for enzyme-conjugated antibodies). A confocal microscope is the preferred instrument for visualising fluorochrome-labelled antibodies due to the microscope's ability to filter out unfocused fluorescent light.

In paper II, the cryostat-sectioned biopsies were fixed in a cold acetone bath immediately before use and then air-dried. A saponin containing phosphate buffered saline buffer was used for permeabilisation, dilution and as a washing buffer throughout the assay. Two separate blocking steps were performed before addition of the primary antibodies. First, a streptavidin/biotin blocking kit was used to block the endogenous biotin in the tissue and secondly, blocking with 5% normal sera mixture (goat, rat and mouse serum) was carried out. The primary unlabeled-antibodies specific for IL-4, IL-10, IL12p70 and IFN- γ , as well as their corresponding isotype antibodies, were added to the slides, which were incubated in humidified chamber for 1h at room temperature. The slides were washed and then finally incubated with the secondary Cy3- or FITC-conjugated antibodies in a humidified chamber for another 1h at room temperature (RT). A drop of SlowFade Antifade (Molecular probes) mounting solution was added to each well before the cover glasses were mounted. The stained slides were stored at +4° C for a maximum of 48h due to fluorochrome fading before analysis. The slides were analysed blinded according to a protocol by two of the

authors (JS and LF) using a Nikon Eclipse E600 instrument and Nikon EZ-C1 v.3.30 software (Nikon Instruments Europe, the Netherlands). The fluorochromes were excited at wavelengths 546nm (Cy3) and 488nm (FITC). The antibodies were all titrated for maximal fluorescence in sections of inflamed tonsil (190). The staining method was validated by the continuous use of isotype antibodies and PBS negative controls. The cytokine expression in the tissues was estimated on a scale from 0 to +++, where +, ++ and +++ corresponded to 1-10, 11-20 and >20 cytokine expressing (positive) cells/section. These estimations were converted to numeral values (0=0, +=1, ++=2 and +++=3) in order to perform a semi-quantitative comparable estimation of the cytokine expression in the skin biopsies.

Enzyme-linked immunosorbent assays (papers I, II and IV)

The enzyme-linked immunosorbent assay (ELISA) is used for quantification of antigen or antibody concentrations in solutions. The principle of ELISA is that the unknown quantity of antigen/antibody in the sample solution is captured and bound to the antibody/antigen coated wells in polystyrene microwell plates. Detection antibodies conjugated with an enzyme bind to the captured antigen/antibody, and after the addition of a substrate, an enzymatic reaction occurs that will produce a coloured product in the wells. An alternative to detection antibodies is the use of tracer antibodies that are conjugated with biotin. The biotin binds to added streptavidin-peroxidase protein complexes, and after addition of the substrate, an enzymatic reaction occurs producing a coloured product. The enzymatic reaction is stopped by adding an acidic stop solution. The colour intensity is analysed in a spectrophotometer, which measures the optical density (OD) of the sample. The OD-value correlates to the amount of antigen/antibody present in the analysed sample.

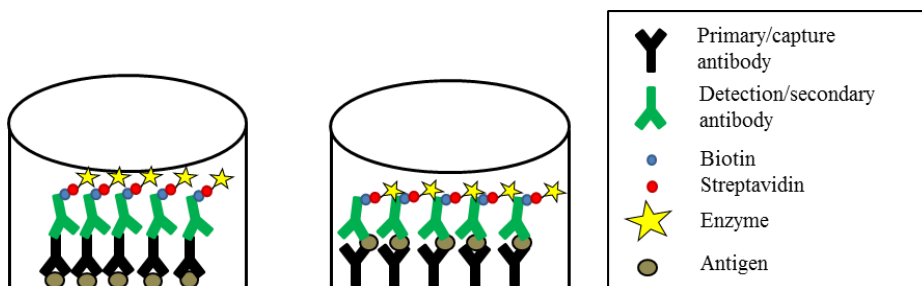


Figure 6. Enzyme-linked immunosorbent assays (ELISA). Indirect ELISA assay (left) and sandwich ELISA assay (right).

Indirect ELISA, papers I and IV

In the commercial IDEIA™ *Borrelia burgdorferi* IgG (Oxoid) and C6 *B. burgdorferi* (Lyme) IgM/IgG (Immunetics®) assays the polystyrene microwells were pre-coated with purified native *Borrelia afzelii* strain DK1 flagellum and a synthetic C6 peptide

derived from the cell membrane protein VlsE protein, respectively. Diluted serum samples from newly tick-bitten individuals were analysed in pairs, *i.e.* sera from the time of inclusion and from the three month follow-up. Unbound serum proteins were removed by washing before addition of the human IgG-specific detection antibody conjugated with the enzyme horseradish peroxidase (HRP) and the detection IgM/IgG conjugate complex conjugated with HRP, respectively. Following the washes, the substrate, 3,3'-5,5'-tetramethylbenzidine (TMB), was added. The enzymatic reaction was stopped by addition of stop solution containing sulphuric acid. The colour intensity was measured in a spectrophotometer at 450nm and reference measures were also made at 600 nm (Oxoid) and 655 nm (Immunetics). The OD -value (Oxoid) and the calculated Lyme Index (Immunetics) for each individual's first and second serum samples were compared in order to detect the seroconversion that had taken place during the study period, *i.e.* first sample being seronegative and the second being seropositive or a twofold increase of the OD-value in the second sample compared to the seropositive first sample.

Sandwich ELISA, paper IV

EDTA-plasma collected from newly tick-bitten individuals a few days after the tick bite were screened for calprotectin (commercial kit, Hycult® biotech) and CCL18 (191). The microwell plates for calprotectin were pre-coated with antibodies specifically directed against human calprotectin, while Costar 3690 microwell plates were coated with carbonate buffer containing monoclonal anti-human CCL18 antibodies (MAB394, clone 64507, R&D Systems, Minneapolis, MN, USA). Unbound coating antibodies were removed by washing with 5% Tween-PBS washing buffer before addition of blocking solution (PBS supplemented with 2% low-fat milk). Excessive blocking solution was removed by washing. Calprotectin standards and recombinant CCL18 standards (R&D Systems), and diluted EDTA-plasma were added to the microwell plates. Biotinylated tracer antibodies (CCL18, BAF394, R&D Systems) specific for respective analyte were added to the wells after removal of unbound proteins by washing. Unbound tracer antibodies were removed by washing before addition of streptavidin-HRP. Following another washing step, the substrate TMB was added. The enzymatic reaction was stopped by addition of stop solutions containing oxalic acid (calprotectin) or 1.8M sulphuric acid (CCL18). The colour intensity was measured with a spectrophotometer at 450nm with a reference measurement for CCL18 at 540 nm. The concentrations of calprotectin and CCL18 in each sample were calculated from the sample's OD-values and the standard curve for the respective analyte.

Multiplex bead assays (paper II-IV)

The fluidics of xMAP Luminex technology was developed from the principles of flow cytometry. The xMAP technology is based on the use of 5.6 µm polystyrene microspheres. These microsphere beads are dyed with different intensities of red and

infrared fluorophores. The combination of these two dyes results in 100 unique bead sets with unique spectral signals, which make it possible to detect several different analytes at the same time in the same sample. The beads can be coated with different kinds of capture molecules (*e.g.* antibodies, receptors, oligonucleotides and peptides) specific for a certain analyte. During the assay procedure, pre-dyed and pre-coated beads are incubated with the sample solution containing the analytes. Analytes present in the sample will bind to the capture molecules on the beads. Detection antibodies conjugated with biotin are added in the following step and bind to the captured analytes. In the last step, addition of streptavidin conjugated with phycoerythrin (PE) results in binding to the detection antibodies that can be detected due to the fluorescent PE molecule. The fluid flow in the Luminex system will align the microspheres into single file before they pass through the detection chamber where a red laser excites both internal dyes in order to identify the microsphere bead, and a green laser excites the PE bound on the detection antibodies. The fluorescence signals, mean fluorescence intensity (MFI), from the PE molecules is used for quantifying the amount of the specific analyte in the analysed sample. Standard curves with known concentrations of the analytes need to be used in order to be able to quantify the amounts of analytes present.

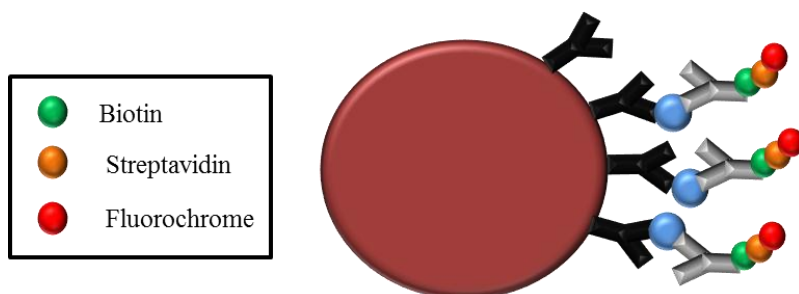


Figure 7. Basic principles of multiplex bead assay technique. Beads, dyed with a mixture of red and infrared, are coated with capture antibodies against the target analyte. Biotinylated detection antibodies conjugated with reporter fluorochrome are added. The beads are excited by a red laser (identify analyte) and the fluorochromes are excited by a green laser (quantification).

In paper II serum samples from the time of inclusion and EDTA-plasma samples from the three and six month follow-ups were analysed according to the manufacturer's instructions for the presence of the cytokines IL-4, IL-10, IL-12p70 and IFN- γ , using the Milliplex MAP High Sensitivity Human Cytokine kit (HSCYTO-60SK, Millipore Corporation, USA). The analysis was performed with a Luminex 100™ System and the data acquisition and analysis were performed with the StarStation software v.3.0 (Applied Cytometry, UK). Values below the detection limit were assigned half the value of the detection limit.

In paper III sera were collected from *B. burgdorferi* s.s.-infected mice, immune-deviated *B. burgdorferi* s.s.-infected mice, and untreated uninfected controls on days 15, 28 and 43 p.i. Blood was also collected from the two groups of infected mice prior to the bacteria inoculation at day 0 p.i. Sera diluted 1:2 were analysed according to the manufacturer's instructions for the presence of the Th1-associated cytokines/chemokines IL-12p70, IFN- γ , CCL3, CCL4, CCL5, CXCL9 and CXCL10 using a Milliplex MAP Mouse Cytokine/chemokine kit (MPXMCYTO-70K, Millipore Corporation, USA). Undiluted sera were analysed according to the manufacturer's instructions for the presence of the Th17-associated cytokines/chemokines IL-17A, IL-22, IL-23 and CCL20 using a Milliplex MAP Mouse Th17 Magnetic bead kit (MTH17MAG-47K, Millipore Corporation, USA). The Th1 immune markers were analysed on a Luminex 100™ System and the data acquisition and analysis were performed with the StarStation software 3.2 (Applied Cytometry, UK). The Th17 markers were analysed on a Luminex 200™ System and the data acquisition and analysis were performed with the software xPONENT 3.1 (Luminex Corporation, USA). Values below the detection limit were assigned half the value of the detection limit.

In paper IV, sera collected from newly tick-bitten individuals a few days after the tick bite were analysed according to the manufacturer's instructions for the presence of the matrix metalloproteinase MMP-3, MMP-8 and MMP-9 with Human Fluorokine MAP kits (LMP513, LMP908 and LMP911, R&D Systems, USA). Sera were diluted 1:10 for analysis of MMP-3 and MMP-8, and diluted 1:100 for analysis of MMP-9. EDTA-plasma were analysed according to the manufacturer's instructions for the presence of the cytokines/chemokines IL-1 β , CXCL8/IL-8, IL-12p70, TNF using a Milliplex MAP High Sensitivity Human Cytokine kit (HSCYTO-60SK, Millipore Corporation, USA), IL-6, IL-17A, CCL22 with a Milliplex MAP Human Cytokine/Chemokine kit (MPXHCYTO-60K, Millipore Corporation, USA), IL-27, CCL20, CXCL1, CXCL10, CXCL11 with a LEGENDplex Custom Human 5-plex Cytokine kit (SPRBL42005, BioLegend, USA) and CXCL9 with a Human MIG Singlplex (LHC1091, Invitrogen, USA). All analytes were analysed on a Luminex 100™ System and the data acquisition and analysis were performed with the software StarStation v.3.0 for the first set of samples and with StarStation 3.2 for the second set of samples (Applied Cytometry, Sheffield, UK). Values below the lowest value of the standard curve were assigned half the values of the lowest standard point.

Allergy propensity

In paper II, the patients' allergic propensity was examined by measuring the total and allergen-specific IgE antibodies in plasma (ImmunoCAP™, Phadia AB, Sweden), according to the manufacturer's instructions. In addition, specific IgE antibodies directed against common food (egg white, cow milk, fish, wheat, peanut and soybean) and inhaled allergens (timothy grass, birch, mugwort, animal dander (cat, horse and

dog), house dust mite *Dermatophagoides pteronyssinus* and spores of the mould *Cladosporium herbarum*) were measured with the Phadiatop Combi[®] assay (Phadia AB). These tests indicated if individuals were sensitised to a specific allergen.

Collection and storage of samples (paper I-IV)

Blood samples

In papers I and IV, blood samples were obtained by venipuncture. The blood was collected in tubes without additive for sera samples, and tubes with EDTA as additive for plasma samples. The collection tubes were sent to the University Hospital (Linköping, Sweden) where they were centrifuged at 1500 g for 10 min at RT. Sera and plasma aliquots were stored at -70° C. In paper II, sera and plasma were collected at Åland Central Hospital, Finland.

In paper III, blood was obtained from the retro-orbital venous plexus at days 0, 15, 28 and 43 p.i. The blood was allowed to clot over-night at 4° C and the sera were stored at -70° C. In addition, one drop of blood (negative control) was added to BSKII culture medium supplemented with 6% normal rabbit serum, 50 µg/mL sulfamethoxazol (Sigma-Aldrich) and 400 µg/mL phosphomycin (Sigma-Aldrich) for eight days of culturing at 35° C.

Ear biopsies

Biopsies were transferred into BSKII culture medium supplemented with 6% normal rabbit serum, 50 µg/mL sulfamethoxazol (Sigma-Aldrich) and 400 µg/mL phosphomycin (Sigma-Aldrich). The biopsies were cultured for eight days at 35° C.

Lymph nodes (paper III)

Inguinal lymph nodes were transferred immediately after sacrifice into RNAlater solution for storage at -70° C until analysis.

Skin biopsy (paper II)

The patients in paper II were given local anaesthesia before a 4 mm skin punch biopsy was taken from the outer red edge of the EM zone and a control biopsy was taken from healthy skin at the opposite body site. Each biopsy was split into two pieces before snap freezing in a mixture of isopentane and liquid CO₂, followed by storage at -70° C. At a later time point, one piece of each biopsy was serially cryostat-sectioned into 6 µm-thick sections (Leitz CM3050 cryostat, Leica Microsystems, Sweden). Two to six sections were placed in each well of a 0.1% poly-L-lysine pre-coated three-well slide. The slides were air-dried before storage at -70° C.

Tibiotarsal joints (paper III)

Immediately after sacrifice the joints were put into a 4% formaldehyde solution and stored at room temperature until required for further histopathology preparations.

Urinary bladders (paper III)

Immediately after collection the urinary bladders were put on ice before they could be transferred to storage at -70° C until spirochaetal load analysis.

Statistics

The statistical analyses in this thesis were mostly of the non-parametric type because most of the data were assumed not to be normally distributed.

A student T-test was used for comparison of age between the two tick-bitten groups because the study population was considered to be normally distributed (paper I). In paper III, a one-way ANOVA was used for comparison of both joint swelling and weight between groups. Bonferroni correction was performed after the ANOVA analysis to correct for multiple comparisons. Logistic regression was used for comparison of clinical parameters between two groups (paper II).

Fisher's exact test was used for comparison of nominal data and the frequency of undetectable cytokine variables between two groups (papers I and II). Pearson's chi-square test was used for comparison of nominal data between the four groups in paper IV. Spearman rank correlation test was used for correlation analysis of the serology assays (paper I) and the cytokine expression (paper II).

In paper II, a Mann-Whitney U test was used for comparison between two unrelated groups. A manual Bonferroni correction was performed to correct for multiple comparisons. A Mann-Whitney U test was also used for comparison of unpaired data within groups (paper III). A Wilcoxon signed rank test was used for comparison of paired data within two groups (paper II). A Kruskal-Wallis H test was used for comparison between unrelated groups (papers III and IV). A Mann-Whitney U test was used as a post hoc test when the Kruskal-Wallis H test indicated significant differences between the groups. $P\text{-value} \leq 0.05$ (papers I-IV) or $p\text{-value} \leq 0.0125$ (*i.e.* $p \leq 0.05/4$) after manual Bonferroni correction (paper II) were considered significant. $P\text{-values} \leq 0.1$ were considered to indicate a trend (paper III).

All statistical analyses were performed using SPSS 15 for Windows (SPSS Inc., Chicago, IL, USA, paper I), PASW Statistics 18 for Windows (papers II and III) and IBM® SPSS® Statistics 20 for Windows (IBM Corp., Armonk, NY, USA, papers III and IV). The randomization of study subjects in paper IV was conducted by using

Research Randomizer (Urbaniak, G.C., & Plous, S. (2011). Research Randomizer version 3.0. Retrieved on April 2010 and June 2012, from <http://www.randomizer.org/>.

Results and Discussion

Risk of *Borrelia burgdorferi sensu lato* infections (papers I and IV)

Prevalence of *B. burgdorferi s.l.* in the collected ticks (paper I and IV)

DNA screening of the collected ticks revealed that 64 (19%) of the tick-bitten study subjects in paper I had been bitten by a tick that was infected with detectable levels of *B. burgdorferi s.l.* (186). In paper IV, 489 (26%) of the ticks collected during the three years of study were infected with detectable levels of *B. burgdorferi s.l.* The frequencies of infected ticks in paper I and IV are in line with other prospective studies on *B. burgdorferi s.l.* infection in general populations which report infection rates of 10-32.8% in ticks collected from tick-bitten individuals (46,47,192,193). However, all the studies, except for Huegli et al. (46), were differently designed compared to our TBD Sting study because the tick-bitten individuals were enrolled into the studies when they contacted general practitioners instead of being invited to visit their local health care after the occurrence of a tick bite (our study and Huegli et al.). The different study designs could affect the composition and bias of the study groups due to differences in anxiety and concerns about the risk of developing disease in the persons who actively attended health care compared to those who were urged to participate.

Table 7. Occurrence of asymptomatic and clinical LB in the TBD Sting study

Tick season	2007		2007-2009	
Outcome	Bitten by infected tick n, (%)	Bitten by uninfected tick n, (%)	Bitten by infected tick n, (%)	Bitten by uninfected tick n, (%)
Asymptomatic infection	4 (6)	6 (2.1)	24 (4.9)	37 (2.6)
Lyme borreliosis (LB)	0	1 (0.4) ^a	18 (3.7) ^b	16 (1.2) ^c
Absence of seroconversion or LB	60 (94)	270 (97.5)	447 (91.4)	1348 (96.2)
Total	64 (100)	277 (100)	489 (100)	1401 (100)

One (a), five (b) and four tick-bitten subjects (c) who developed LB also seroconverted during the study time.

Seroconversion after a bite by a *B. burgdorferi* s.l.-infected tick (papers I and IV)

In paper I, 136 (40%) paired blood samples from the 341 newly tick-bitten individuals who were recruited to the TBD Sting study had detectible levels of anti-C6 antibodies, whereas 39 (11%) paired samples had detectible levels of anti-flagellum antibodies. Of the 136 individuals who were seropositive for *B. burgdorferi* s.l.-specific antibodies at the time of enrolment, 56 reported a past history of symptomatic LB. Four of the 64 (6%) individuals bitten by *B. burgdorferi* s.l.-infected ticks had seroconverted during the study period in at least one of the ELISA tests (table 7). The seroconversion was verified by the development of new *B. burgdorferi* s.l.-specific antibodies in the RecomeLine test. Seven of the 277 (2.5%) subjects bitten by *B. burgdorferi* s.l.-uninfected ticks had also seroconverted, verified by the RecomeLine test, during the study period (table 7). In paper IV, the study population contained two additional years of study participants compared to one year in paper I, leaving a total of three years of study data in paper IV. Twenty-four (4.9%) study subjects bitten by infected and 37 (2.6%) bitten by uninfected ticks seroconverted during the three study years (table 7). The frequency among the subjects bitten by infected ticks who developed clinical LB over the three years of the study was 3.7% (n=18), and among subjects bitten by uninfected ticks it was 1.2% (n=16) (table 7). A latter prospective study from Switzerland (46) also reported rates of seroconversion after tick bite with lower frequency of seroconversion in tick-bitten individuals bitten by *B. burgdorferi* s.l.-infected ticks (1.6% compared to 6% in the TBD Sting study). Interestingly, the frequency of seroconversion in individuals bitten by uninfected ticks in the study conducted by Huegli et al. (46) was similar to our reported frequency (2.4% and 2.5%, respectively).

In paper I, 52% (n=33) of the study subjects bitten by *B. burgdorferi* s.l.-infected ticks and 50% (n=139) of the subjects bitten by uninfected ticks were bitten by at least one more tick during the study period. The high frequency of additional tick bites during the study period is probably one of the most likely explanations for the number of seroconversions that occurred in the group bitten by uninfected ticks. Another likely explanation might be that the ticks were infected with *B. burgdorferi* s.l. subtypes that were not detected by the PCR assay.

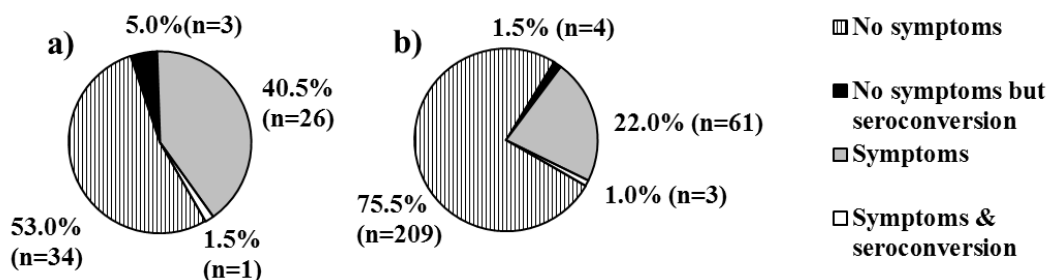


Figure 8. The frequency of self-reported symptoms in study subjects bitten by *B. burgdorferi s.l.*-infected (a, n=64) and -uninfected (b, n=277) ticks. Ninety-one of the 341 study subjects, of which 27 were bitten by *B. burgdorferi s.l.*-infected ticks, reported experiencing symptoms possibly associated with Lyme borreliosis during the three month study period.

Self-reported symptoms and development of clinical Lyme borreliosis (paper I)

Self-experienced symptoms possibly associated with LB were reported by 27 (42%) subjects bitten by *B. burgdorferi s.l.*-infected ticks and 64 (23%) of the subjects bitten by uninfected ticks (figure 8). Although both groups of tick-bitten subjects reported symptoms that may be associated with a current *B. burgdorferi s.l.* infection, the frequency of subjects reporting symptoms was higher in the group bitten by infected ticks; 42% compared with 23% of subjects bitten by uninfected ticks ($p=0.003$). However, the total number of each reported symptom was similar between the two groups (table 8). Furthermore, it has been shown in a Slovenian prospective clinical trial (56) that these, mainly non-specific, symptoms also occur quite frequently in normal non-*B. burgdorferi s.l.*-infected clinical subjects. One of the four subjects who seroconverted after a bite by a *B. burgdorferi s.l.*-infected tick reported experiencing fatigue and myalgia/arthritis during the study period but did not attend health care for the reported symptoms. In contrast, two of the subjects who seroconverted after a bite by an uninfected tick sought health care for their symptoms (vertigo and myalgia/arthritis, respectively). The former subject was diagnosed with an EM.

Table 8. Self-reported symptoms in 341 tick-bitten study subjects

Frequency of different symptoms reported by tick-bitten study subjects^a			
	All tick-bitten subjects (n=341)	Subjects bitten by a <i>B. burgdorferi</i> s.l.-infected tick (n=64)	Subjects bitten by a <i>B. burgdorferi</i> s.l.-uninfected tick (n=277)
No. of subjects reporting symptoms	n=91	n=27	n=64
Reported symptoms (%)			
Fatigue	18	17	19
Myalgia/arthritis	17	16	17
Headache	14	15	14
Neck pain	11	13	10
Numbness	10	9	10
Vertigo	9	10	7
Concentration difficulties	5	6	5
Nausea	5	6	5
Radiating pain	4	3	5
Fever	3	0	4
Loss of appetite	2	4	1
Loss of weight	2	1	3

^a several individuals reported more than one symptom. Erythema migrans was not included in the list of symptoms in the questionnaire.

***Borrelia burgdorferi sensu lato* infections (papers I and IV)**

The relative risk of acquiring a *B. burgdorferi* s.l. infection (seroconversion or development of LB) after a tick bite in paper I was 2.473 (95% CI 0.746-8.195), and increased during the three years of the TBD Sting study to 2.609 (95% CI 1.739-3.913). A latter report from a prospective study conducted in a highly endemic area of Switzerland reported a relative risk of 1.46 of developing *B. burgdorferi* s.l. infection after a tick bite (46). Although the relative risk was lower in the study from Switzerland, they reported a similar frequency of infection in subjects bitten by infected ticks (8.2%) compared to our TBD Sting study (6-8.6%). However, Huegli et al. (46) reported a higher frequency of infection in subjects bitten by uninfected ticks (5.6%) than our study (2.5-3.1%). Previous studies have shown frequencies of infection after a tick bite of 5.3% (192) and 26.7% (193), however one thing to bear in mind is that these two studies had a different design to our study, which could affect the reported infection frequency. Another interesting result was that we found the occurrence of asymptomatic *B. burgdorferi* s.l. infections, i.e. the absence of development of clinical LB, in 5-6% of the individuals bitten by infected ticks, which is a much higher frequency than the reported 1.6% by Huegli et al. (46).

Asymptomatic infections are not a new phenomenon since they have been described in previous studies, *e.g.* studies screening for the presence of anti-*B. burgdorferi s.l.* antibodies in blood donors (53) and in a vaccinal trial (51). However, paper I, and of course the TBD Sting study in its entirety, seem to be the first larger prospective studies, covering nine different counties of Sweden, of tick-bitten individuals to report that asymptomatic infections after bites by *B. burgdorferi s.l.*-infected ticks seem to be quite frequent among individuals who become infected.

Taken together, we found that the risk of developing LB after a tick bite by a documented *B. burgdorferi s.l.*-infected tick is small. In addition, asymptomatic *B. burgdorferi s.l.* infection, *i.e.* documented by seroconversion in the absence of symptoms possibly associated with LB, appears to be frequent among individuals who become infected.

The early immune response towards *Borrelia burgdorferi sensu lato*

Previous studies have shown an association between good clinical outcome of LB and a strong Th1-like proinflammatory immune response towards the *B. burgdorferi s.l.* bacteria (170,180–183,194,195). However, these studies were designed to investigate the immune response during disseminated LB, which occurs weeks to months after the tick bite. To our knowledge, none have previously investigated the association between clinical outcome and the very early immune response towards *B. burgdorferi s.l.* Therefore, in paper II, we turned our attention towards EM, which is one of the first and the most common manifestation of LB in Europe (196). However, EM lesions occur days to weeks (49) after the tick bite so the early local immune response in EM patients is still at least 1-2 weeks old. Therefore, further investigation of even earlier episodes of the immune response would be of great interest. This was done in paper IV, for the first time to our knowledge, investigating the presence of possible immune biomarkers for the clinical outcome of LB in blood collected from newly tick-bitten individuals a few days after the tick bite.

Erythema migrans patients (paper II)

The 88 included EM patients (55 women, 33 men, with a mean age 57 years) had a median of six days' duration of EM at the time of inclusion, except for one patient who had an EM duration of 215 days (commented on in paper I). Three of the EM patients had multiple EM at the time of inclusion but all three were asymptomatic at the six months follow-up. Out of the 88 EM patients, 58 patients (66%) had observed a preceding tick bite. All patients were treated with antibiotics after skin biopsies and blood samples were collected at the time of inclusion and all except for one completed their treatments. The one asymptomatic patient who disrupted the treatment did so due to a suspected allergic reaction after seven days.

Seven (8%) of the 88 EM patients had experience of persisting symptoms at the six month follow-up, hereafter referred to as symptomatic EM patients. The persisting symptoms were arthralgia in the elbow (2/7), knee (2/7), ankle (1/7), wrist (1/7), or fatigue (1/7), back pain (1/7) and hypoesthesia in the skin close to the previous site of the EM (1/7). Five of the seven patients with persisting symptoms still experienced persisting symptoms at the 12 month follow-up, with three of them still having symptoms at the 24 month follow-up. However, none of the patients were retreated with antibiotics and none showed signs of disseminated infection, such as arthritis, NB or ACA. Taken together, the overall clinical outcome after antibiotic treatment of the EM lesions was good, which is in line with observations from other studies (56,197,198). There were no differences between the asymptomatic and symptomatic patients concerning clinical characteristics, *B. burgdorferi* s.l. serology or allergic trait (table 9).

Table 9. Clinical characteristic of the included EM patients in paper II.

Variable	Patients with symptoms at six months (A)	Patients with no symptoms at six months (B)	Statistical comparison (A vs B)	In total (A+B)
N	7	81		88
Sex F/M	5/2	50/31	NS	55/33
Age (years)				
range	30-68	23-88		23-88
median	55	58	NS	57
Single/Multiple EM	7/0	78/3	NS	85/3
Reported tick bite, Y/N	3/4	55/25	NS	58/29
EM size (cm)				
range	5-30	3-37		3-37
median	15	10	NS	10
Borrelia antibodies				
Pos/neg analysis				
C6 serology				
3 mos	4/7	39/81	NS	43/88
6mos	4/7	36/78	NS	40/85
IgM serology				
3 mos	7/7	48/81	NS	55/88
6mos	6/7	43/78	NS	49/85
IgG serology				
3 mos	5/7	51/81	NS	56/88
6mos	7/7	46/78	NS	53/85
Allergy Y/N	1/6	25/56	NS	26/62
EM duration^a				
(days)				
range	2-215	0-63		0-215
median	4	6	NS	6

^a Duration of EM prior to antibiotic treatment. EM, erythema migrans

Newly tick-bitten subjects (paper IV)

In paper IV, study subjects were consecutively divided into two sets, and the analyses of the blood samples were analysed on two different occasions for the two sets of samples. The first set of study subjects consisted of 77 subjects divided into four groups with respect to outcome and whether the tick was *B. burgdorferi s.l.*-infected, while the second set of study subjects consisted of 78 subjects (table 10). Nine of the 11 study subjects from the group diagnosed with LB in the first set of subjects were diagnosed with EM, one subject with NB, and the last one had received the diagnosis Borrelial lymphocytoma. In the second set of study subjects with clinical diagnosis (Group 1) six out of seven subjects had EM, and the last one had been diagnosed with LB. All LB cases developed during the study period in the TBD Sting study except for the Borrelial lymphocytoma which was diagnosed approximately six months after the study period. However, the lymphocytoma had developed during a six month period at the same site as the tick bite on the subject's earlobe and therefore was probably associated with the tick bite at this site.

Table 10. Study subjects in paper IV.

Group	1	2	3	4
Definition	LB + <i>B. burgdorferi</i> <i>s.l.</i> -infected tick	Seroconversion + <i>B. burgdorferi</i> <i>s.l.</i> -infected tick	<i>B. burgdorferi</i> <i>s.l.</i> -infected tick - LB or seroconversion	<i>B. burgdorferi</i> <i>s.l.</i> -uninfected tick – LB or seroconversion
First set of subjects				
Number	11	10	27	29
Sex (female/male)	9/2	5/5	21/6	18/11
Age, mean (range)	62 (45-74)	65 (51-79)	59 (24-79)	62 (20-87)
Second set of subjects				
Number	7	18	27	26
Sex (female/male)	4/3	9/9	18/9	18/8
Age, mean (range)	64 (48-74)	66 (55-81)	62 (30-86)	61 (26-81)

LB, Lyme borreliosis; *B. burgdorferi s.l.*, *Borrelia burgdorferi sensu lato*.

B. burgdorferi s.l. subtypes in EM and in ticks (papers II and IV)

Seventy-five (81.5%) of the 92 collected EM biopsies in paper II were screened positive for *B. burgdorferi s.l.* DNA, of which 48 were successfully sequenced. Thirty-six (48%) of the EM biopsies contained DNA from *B. afzelii*, 11 (15%) from *B. garinii* and 1 (1%) from *B. burgdorferi s.s.* In addition, 20 of 87 (23%) of the healthy skin control biopsies were initially screened positive for *B. burgdorferi s.l.* but could not be

sequenced, hence they were found to be false positive for *B. burgdorferi s.l.* DNA. The frequency of *B. afzelii* and *B. garinii* in the skin biopsies did not differ between symptomatic and asymptomatic EM patients.

Out of the 100 newly tick-bitten study subjects, bitten by *B. burgdorferi s.l.*-infected ticks, in paper IV, 41 were bitten by ticks infected with *B. afzelii* (n= 41%), 25 were bitten by *B. garinii*-infected ticks (n=25%), four were bitten by ticks infected with *B. burgdorferi s.s.* (n=4%), two were bitten by ticks infected with *B. spielmanii* (n=2%), four by ticks infected with *B. valaisiana* (n=4%) and two were bitten by ticks infected with *B. miyamotoi*-like (n=2%) (table 11). *B. afzelii* and *B. garinii* were the most frequent subtypes detected in the ticks that had bitten the study subjects, which is in line with previous reports of both subtypes predominating in Europe (21,32). Interestingly, two other studies, with a similar design to our TBD Sting study, have investigated the risk of developing LB after tick bite in the Netherlands (47) and in Switzerland (46), and both studies reported that *B. afzelii* was the most frequent subtype detected in the collected ticks, followed by *B. garinii* (47) or by *B. valaisiana* (46). Sixteen (16%) of the ticks in our study were screened positive for *B. burgdorferi s.l.* DNA but were untypable. In addition, two tick-bitten individuals had been bitten by *B. burgdorferi s.l.* co-infected ticks and another four individuals had been bitten by several ticks of which each were infected with different *B. burgdorferi s.l.* subtypes (table 11). The frequency of *B. afzelii* and *B. garinii* in the ticks did not differ between symptomatic and asymptomatic tick-bitten study subjects.

Table 11. *B. burgdorferi* s.l. subtypes detected in EM lesions and in ticks (papers II, IV).

Group/ Outcome	EM patients (II) n (%)	LB (Group 1, IV) n (%)	Asymptomatics (Group 2, IV) n (%)	Bitten by infected tick (Group 3, IV) n (%)
<i>B. afzelii</i>	36 (48.0)	11 (61.1)	9 (32.1)	21 (38.9)
<i>B. garinii</i>	11 (15.0)	3 (16.7)	7 (25)	15 (27.8)
<i>B. burgdorferi</i> s.s.	1 (1.0)	-	3 (10.7)	1 (1.8)
<i>B. spielmanii</i>	-	-	1 (3.6)	1 (1.8)
<i>B. valaisiana</i>	-	-	3 (10.7)	1 (1.8)
<i>B. miyamotoi</i> - like	-	-	-	2 (3.7)
Coinfections ^a	-	-	1 ^b (3.6)	5 ^c (9.3)
Untypable	27 (36.0)	4 (22.2)	4 (14.3)	8 (14.8)
Total	75 (100)	18 (100)	28 (100)	54 (100)

^a Co-infections in one tick or tick bites from several infected ticks at the same time.

^b One subject bitten by tick co-infected with two *B. afzelii* strains.

^c One subject bitten by tick co-infected with two *B. burgdorferi* s.s. strains, and subjects bitten by several infected-ticks; two ticks with *B. afzelii* strains; three ticks with *B. afzelii* strains; one tick with *B. afzelii* and one with untypable subtype; one tick with *B. afzelii*, one with *B. garinii* and the third tick with *B. valaisiana* subtype.

EM, erythema migrans; LB, Lyme borreliosis; *B.*, *Borrelia*; s.s., *sensu stricto*

Cytokine expression in EM (paper II)

Seven symptomatic and 18 asymptomatic EM patients were included in the IHC analysis of the local cytokine expression in skin biopsies. Ten of the asymptomatic patients were chosen in order to match age and sex with the then ten symptomatic patients (before the exclusion of three of them) while the other eight asymptomatics were unmatched.

Symptomatic EM patients had decreased expression of IFN- γ and a strong tendency for decreased expression of IL-12p70 in the EM lesions compared with asymptomatic patients ($p=0.003$ and $p=0.013$, respectively. To adjust for multiple comparisons in this study, $p \leq 0.0125$ was considered a significant manual Bonferroni correction. Figure 9a-b). The expression of IFN- γ and IL-12p70 correlated in both biopsies from EM

lesions and control skin ($p \leq 0.01$ for both, $\rho = 0.687$ and $\rho = 0.771$, respectively), which validates the finding since IL-12p70 induce and maintains secretion of IFN- γ . The expression of IL-4 and IL-10 did not differ between the asymptomatic and the symptomatic patients (figure 9c-d). Both IL-4 and IL-10 were generally found in small amounts in the biopsies. None of the four cytokine expressions in the control skin biopsies differed between the two patient groups. Furthermore, no differences were found between the cytokine expressions in the EM biopsies compared with the corresponding control biopsies (figure 9a-h), nor were there any differences in the systemic expression levels of the cytokines between symptomatic and asymptomatic patients. No correlation was found between cytokine expression in the skin and the systemic cytokine expression, which indicates that the inflammatory response towards the *B. burgdorferi s.l.* infection is local, not systemic.

The decreased local expression of the proinflammatory cytokine IFN- γ in EM lesions from symptomatic patients compared with asymptomatic patients supports the hypothesis that a strong Th1-type response is required early in the *B. burgdorferi s.l.* infection to ensure a successful clinical outcome. The expression of the proinflammatory IL-12p70 also tended to be reduced in symptomatic EM patients, a tendency that was strengthened by the correlation between the expressions of the two proinflammatory cytokines. Surprisingly, the local cytokine expression did not differ between biopsies from EM lesions and from unaffected control skin. Speculatively, the lack of difference in cytokine expression between EM lesions and unaffected skin could reflect a temporary general elevation of cytokine levels in the skin due to *B. burgdorferi s.l.* infection. However, other studies on cytokine expression in normal unaffected skin show no or very low cytokine expression compared with infected or diseased skin (172,199). Another speculative explanation for the cytokine expression in the unaffected skin is the possibility of constitutive expression of cytokines in the entire skin as a part of the first line of defence against the outer environment, keeping the immune system in a constant state of alertness. There are a few reports of the Th1-associated cytokines IL-12p70 and IL-18 that have been shown to be constitutively expressed by keratinocytes (200,201). The differences in cytokine expression could not be explained by bacteria strain, or the patient's allergy predisposition. These results are the first, to our knowledge, to find a possible association between the early local cytokine expression in EM lesions of LB patients and the clinical outcome.

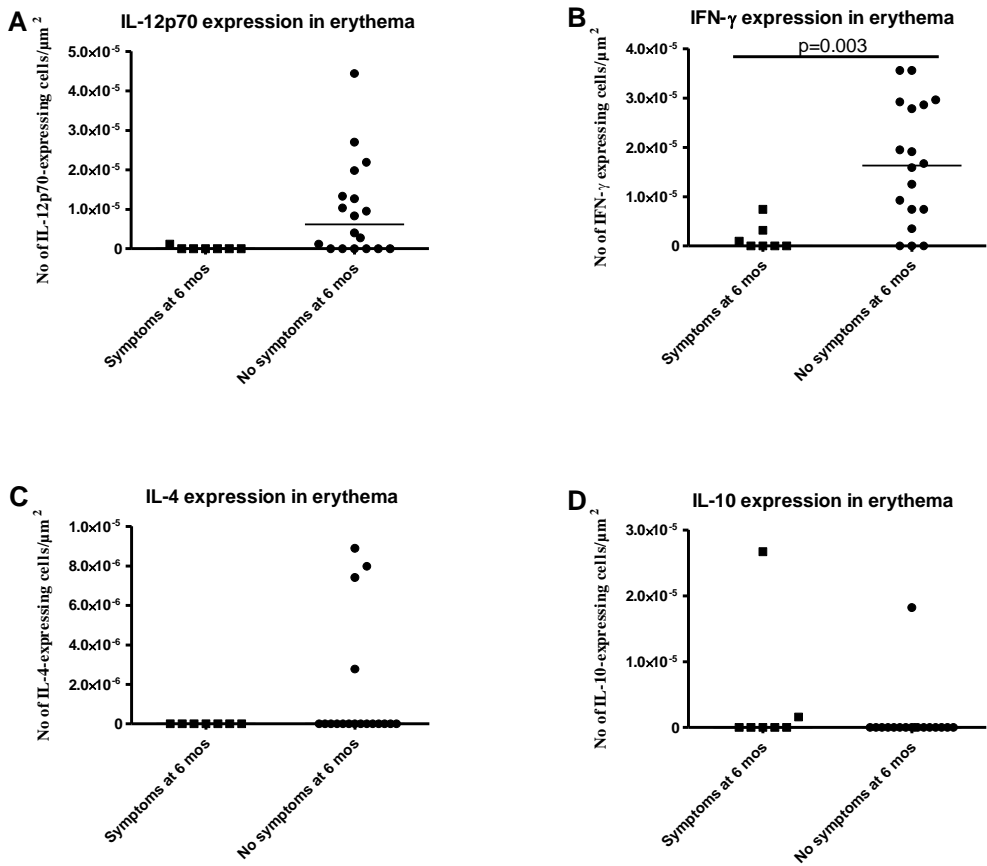


Figure 9 A-D. Immunohistochemical analysis of the number of cytokine-expressing cells in skin biopsies from erythema migrans lesions. Expression of IL-12p70 (A), IFN- γ (B), IL-4 (C) and IL-10 (D) from patients with ($n=7$) and without ($n=18$) persistent symptoms six months post-treatment. Horizontal lines represent the median. The Mann-Whitney U-test followed by a manual Bonferroni correction was used for statistical analysis. $P \leq 0.0125$ was considered significant.

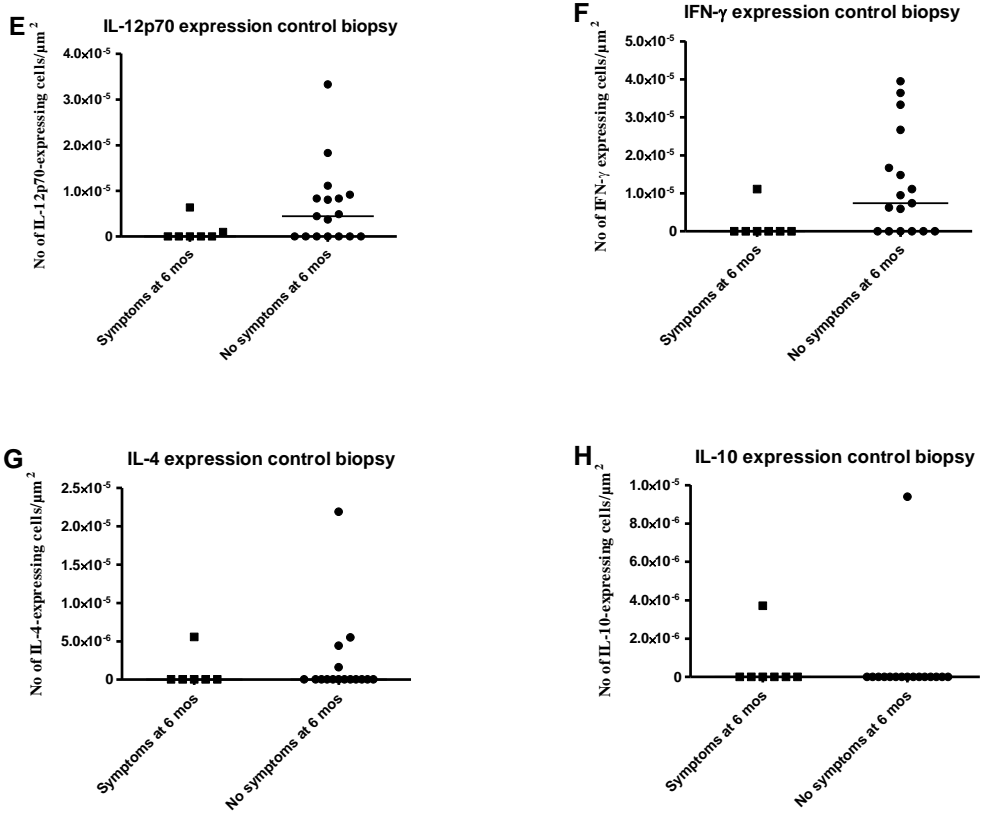


Figure 9 E-H. Immunohistochemical analysis of the number of cytokine-expressing cells in skin from unaffected skin biopsy controls. Expression of IL-12p70 (E), IFN- γ (F), IL-4 (G) and IL-10 (H) from patients with ($n=7$) and without ($n=18$) persistent symptoms six months post-treatment. Horizontal lines mark the median. The Mann-Whitney U-test followed by a manual Bonferroni correction was used for statistical analysis. $P \leq 0.0125$ was considered significant.

Cytokine expression after tick bite (paper IV)

The systemic expression of three of the 18 analysed immune markers differed when compared between the four groups of study subjects in the first set of samples. The neutrophil-associated calprotectin was detected at higher levels in study subjects who developed clinical LB (Group 1) after a bite by a *B. burgdorferi s.l.*-infected tick compared with subjects who seroconverted but did not develop clinical LB (asymptomatics, Group 2) after a bite by an infected tick, and with subjects bitten by a *B. burgdorferi s.l.*-uninfected tick (uninfected, Group 4) (figure 10a). The IFN- γ -induced chemokine CXCL10 was detected at higher levels in study asymptomatic

subjects compared with all other study groups (figure 10b). Higher levels of MMP-3 were detected in blood from subjects bitten by uninfected ticks compared with subjects who neither developed clinical LB nor seroconverted after a bite by an infected tick (Group 3) (figure 10c). However, the levels of MMP-3 did not differ between the three groups of subjects bitten by *B. burgdorferi s.l.*-infected ticks.

Only one of the 18 analysed immune markers differed when compared between the four groups of study subjects in the second set of samples. Higher levels of IL-12p70 were detected in blood from asymptomatic subjects compared with both subjects who developed clinical LB after a bite by a *B. burgdorferi s.l.*-infected tick and subjects bitten by uninfected ticks (figure 10d).

A discriminating immune biomarker could neither be found when comparing the immune marker expressions between tick-bitten individuals bitten by *B. burgdorferi s.l.*-infected ticks and individuals bitten by uninfected ticks, nor when comparing the subjects bitten by infected ticks but who had different clinical outcomes. However, although no biomarker was found, differences between the study groups with different outcomes were found and the findings showed that tick-bitten study subjects who later on would become asymptomatics had higher expression of the Th1-associated chemokines CXCL10 (first set of subjects) and cytokine IL-12p70 (second set of subjects) than tick-bitten individuals who would develop clinical LB. These results further strengthen the hypothesis that an early strong Th1-type immune response is required for a good clinical outcome. Calprotectin was expressed in higher levels in blood from study subjects who would develop LB compared with asymptomatic subjects, which surprised us since calprotectin is abundant in neutrophils, which are cells that would probably favour proinflammatory responses, resulting in a good clinical outcome. Furthermore, IFN- γ is important for enhancing the neutrophils' activity and functions (202) and therefore the decreased levels of CXCL10 in combination with the increased levels of calprotectin could speculatively suggest a decreased influence of IFN- γ on neutrophils, with impaired neutrophil function as a result.

Taken together, the results from both the EM patients and the newly tick-bitten individuals corroborate previous reports of predominating Th1 responses in LB patients (172–174,176,177,182,203), but also corroborate previous reports of an association between good clinical outcome of disseminated LB and strong proinflammatory Th1-like immune responses (180,182,194,204). A previously reported experimental study on mice susceptible to *B. burgdorferi s.l.* (170) showed that the successful outcome of *B. burgdorferi s.l.* infection also depends on a downregulating anti-inflammatory Th2-like immune response following the early strong proinflammatory response, in order to balance and properly shut down the inflammatory response.

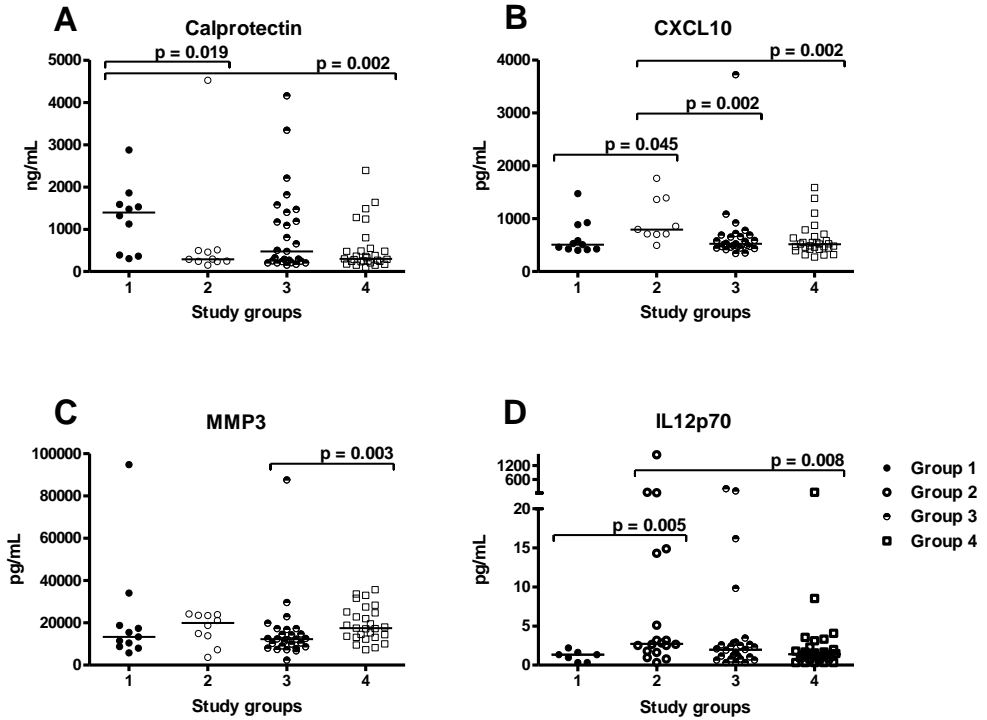


Figure 10. Multiplex bead assay detection of biomarkers in sera from newly tick-bitten study subjects from the first set of subjects (A-C) and the second set of subjects (D). Systemic levels of calprotectin (A), CXCL10 (B), MMP-3 (C) and IL-12p70 (D). The study groups consisted of individuals bitten by a *B. burgdorferi s.l.*-infected tick and who developed clinical LB (Group 1, 1st set n=11, 2nd set n=7), bitten by a *B. burgdorferi s.l.*-infected tick and who seroconverted but did not develop clinical LB (Group 2, 1st set n=10, 2nd set n=18), bitten by a *B. burgdorferi s.l.*-infected tick and who did not seroconvert or develop LB (Group 3, 1st set n=27, 2nd set n=27) and bitten by a *B. burgdorferi s.l.*-uninfected tick and who did not seroconvert or develop LB (Group 4, 1st set n=29, 2nd set n=26). Horizontal lines mark the median. The Kruskal-Wallis test showed significant ($p \leq 0.05$) changes across groups, and was followed by a Mann-Whitney U-test (significant p-values shown in the figure) for comparison between the groups. P value ≤ 0.05 was considered significant.

Differences in T cell markers during the course of experimental *B. burgdorferi* s.s. infection (paper III)

A good clinical outcome after a *B. burgdorferi* s.l. infection is associated with an early strong pro-inflammatory Th1-like immune response that is downregulated by an anti-inflammatory Th2-like response. This has been shown in studies on both human LB patients and on experimentally infected mice (170,180,182,194,195,205). However, Th1 and Th2 immune responses do not act alone during the immune response towards *B. burgdorferi* s.l. since it has been shown that cytotoxic T cells, Th17 cells and Treg are present during the immune response or respond to *B. burgdorferi* s.l. stimulation (176,177,181,203). In order to elucidate the interplay between the different T cell subsets during the course of infection and to investigate if there are differences in the activation of the different subsets, we studied T cell markers in relation to the outcome of *B. burgdorferi* s.s. infection in mice that were immune-deviated and compared it to mice with *B. burgdorferi* s.s. infection without immune deviation.

Clinical signs of infection

Both Bb and the immune-deviated BbId mice showed increased joint swelling compared with untreated uninfected controls. The BbId mice showed increased tibiotarsal joint swelling compared with Bb mice on day 26 p.i. ($p=0.035$) but the total histopathology score did not differ between the two groups. However, the BbId mice showed increased infiltration of neutrophils in the joints compared to Bb mice on day 28 p.i. ($p=0.049$).

The BbId mice had increased spirochaetal load in their ear biopsies from day 28 p.i. and 43 p.i. compared with day 15 p.i. ($p=0.040$ and $p=0.010$, respectively. Figure 11). No such changes were found in ear biopsies from Bb mice. Trend for increased spirochaetal load in BbId mice compared with Bb mice was found on day 28 p.i. and had become a significant difference on day 43 p.i. ($p=0.008$).

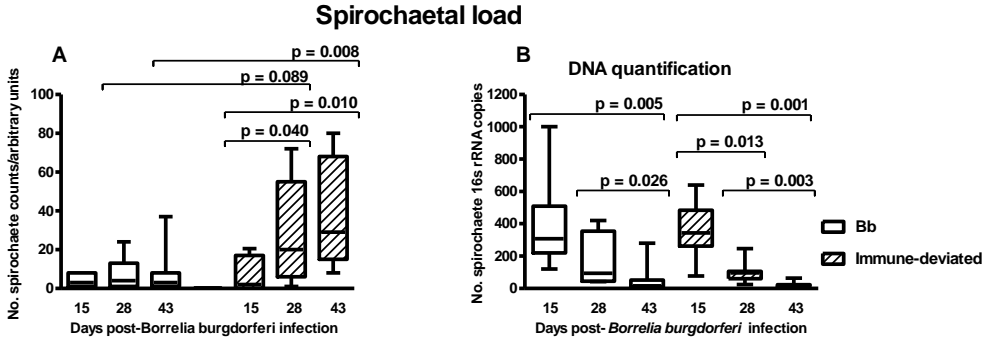


Figure 11. Spirochaetal load in ear biopsies and urinary bladders from experimental *B. burgdorferi* s.s.-infected C3H/HeN mice. Measurement of spirochaetal load in ear biopsies (A) and *B. burgdorferi* s.s. DNA quantification in urinary bladders (B) in two experimental groups (n=21 in each group). The boxes represent the median with 25th and 75th percentiles and with min-max whiskers. A Mann-Whitney U-test and Kruskal-Wallis test followed by a post hoc Mann-Whitney U test were used for the comparisons between and within the groups, respectively.

The increased joint swelling and delayed eradication of *B. burgdorferi* s.s. bacteria in BbId mice compared to Bb mice corroborate our previous report on this experimental model (195). Interestingly, a discrepancy was found between the spirochaetal load in the ear biopsies and the urinary bladders. The discrepancy was probably due to the difference in tissue expression of the *B. burgdorferi* s.l.-binding extracellular protein decorin (206), which is highly expressed in skin but not in bladders (207,208).

Local mRNA expression of T cell subset markers

The local mRNA expression of the 14 different T cell subset markers in inguinal lymph nodes did not change significantly in BbId mice during the course of infection, although a trend for decreased expression of EBI-3 mRNA (subunit of IL-27 and IL-35) was found ($p=0.064$). Bb mice showed decreased mRNA expression of IL-4 ($p=0.018$), IL-10 ($p=0.018$) and EBI-3 ($p=0.009$) over the course of infection (figure 12). The decrease of the Th2-associated IL-4 mRNA in inguinal lymph nodes in Bb mice but not in BbId mice suggests a Th2-deviated response in the BbId mice, which is also in line with our previous study (195). The decrease of both Th1-antagonistic IL-4 and the mainly anti-inflammatory IL-10 in the Bb mice may suggest a decrease in the anti-inflammatory response during the resolution of the disease. EBI-3 is one of the subunits of both the Th1-associated IL-27 and the Treg-associated IL-35 cytokines, thus the decrease of EBI-3 in Bb mice during the course of infection may be interpreted as a decreased Th1 or Treg response. Irrespective of the exact nature of the

response, a decrease in the T cell subsets indicates that the inflammatory response has been terminated in Bb mice.

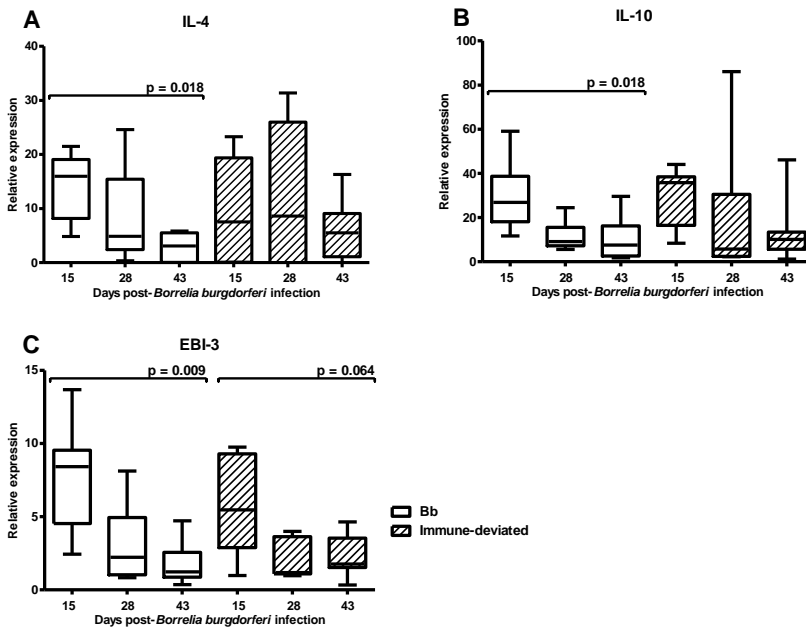


Figure 12. mRNA expression of markers for Th1 (EBI-3), Th2 (IL-4 and IL-10), Treg (EBI-3) subsets in two experimental *B. burgdorferi* s.s.-infected ($n = 21$ in each group) groups of C3H/HeN mice. The relative expression was calculated with the $\Delta\Delta C_T$ method with control mice ($n = 7$) as a calibrator and GAPDH as a reference gene. The boxes represent the median with 25th and 75th percentiles and with min-max whiskers. A Kruskal-Wallis test followed by a post hoc Mann-Whitney U test was used for the comparisons within the groups.

No significant differences between BbId and Bb mice mRNA expression were found at any time point for any of the 14 markers. This may be because the life of cytokines is short and dynamic and therefore we may not have chosen the most optimal time frame for measurement. However, a strong trend for higher levels of IL-12p40 (subunit of the homodimer IL-12p80, the heterodimers IL-12p70 and IL-23) mRNA expression was found in Bb mice on day 43 p.i. compared with BbId mice ($p=0.057$, figure 13a). BbId mice had at the same time point strong trends of higher levels of both Foxp3 (Treg) and GM-CSF (cytotoxicity) mRNA expression compared with Bb mice ($p=0.053$ and $p=0.057$, respectively, Figure 13b-c). The trend for higher expression of IL-12p40 in Bb mice at day 43 p.i. may suggest a resolution of the inflammation due to the IL-12p40 homodimer's function as an IL-12 receptor antagonist, but could likewise suggest an increase of the Th1 and/or Th17 response in

the Bb mice due to the role of IL-12p40 as a subunit in the Th1- and Th17-associated cytokines IL-12p70 and IL-23. The growth factor GM-CSF has been reported to increase both the number and activity of cytotoxic effector cells (209,210) and the trend for higher expression of GM-CSF in BbId mice compared to Bb mice at day 43 p.i. may therefore suggest that the inflammation is still on-going in the BbId mice. Lastly, the Treg-associated transcription factor Foxp3 (211,212) also had a trend for higher expression in BbId mice compared to Bb mice at the late phase of infection and this may suggest that Tregs are also present during this time point of infection, compatible with an on-going inflammation. The finding of Tregs being present in the late phase of infection is in line with one of our previous reports that patients with persistent symptoms of LB had higher *B. burgdorferi s.l.*-induced expression of Foxp3 in blood mononuclear cells than healthy controls (181).

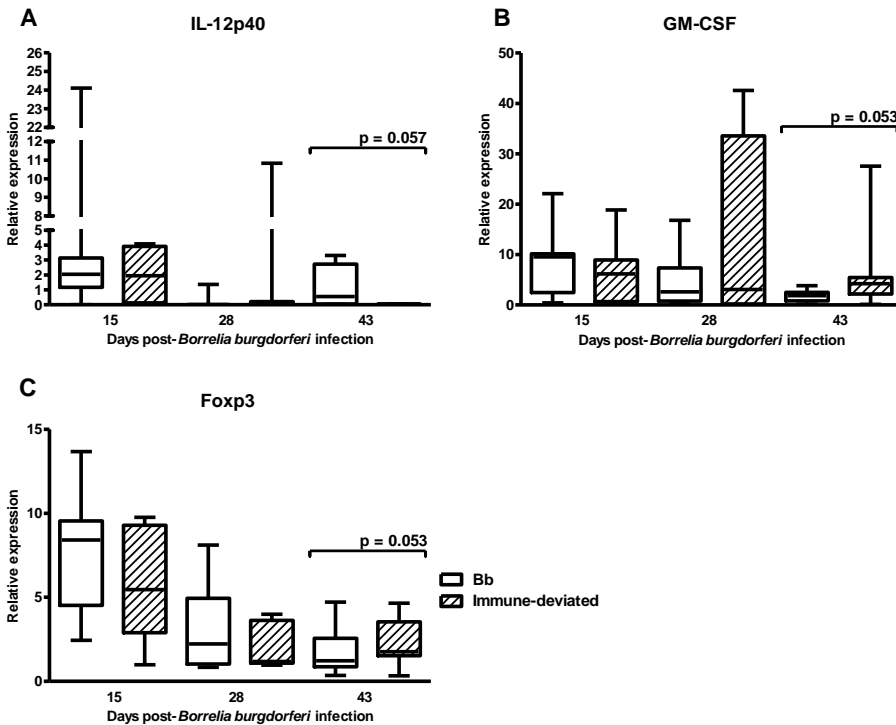


Figure 13. mRNA expression of markers for Th1 (IL-12p40), Th2 (IL-12p40), Treg (Foxp3) subsets and cytotoxicity (GM-CSF) in two experimental *B. burgdorferi s.s.*-infected ($n = 21$ in each group) groups of C3H/HeN mice. The relative expression was calculated with the $\Delta\Delta C_T$ method with control mice ($n = 7$) as a calibrator and GAPDH as a reference gene. The boxes represent the median with 25th and 75th percentiles and with min-max whiskers. A Mann-Whitney U-test was used for the comparisons between the groups.

Systemic levels of T cell subset markers

Three of the eleven immune markers analysed in sera differed between Bb, BbId and healthy control mice. The BbId group showed decreased levels of the Th1-associated chemokine CXCL9 compared with Bb mice on day 15 p.i. ($P=0.007$, figure 14b), suggesting a decreased Th1 response, which is compatible with a Th2 deviation. Bb mice showed an increase in both Th1-associated chemokine CXCL9 and CXCL10 at all time points compared with day 0 p.i. ($p\leq 0.015$ for all, figure 15c), suggesting an on-going Th1-type response in the Bb mice. BbId mice, on the other hand, showed an initial decrease in both chemokines on day 15 p.i. compared with day 0 p.i. ($p\leq 0.008$ for all, figure 15d), which instead suggests a weakened or absent Th1 response. Both Bb and BbId mice showed increased levels of CXCL9 and CXCL10 compared with controls at several time points (figure 14b-c). The third immune marker that differed, the Th17-associated IL-22, showed increased levels in BbId mice compared with controls on day 43 p.i (figure 14a).

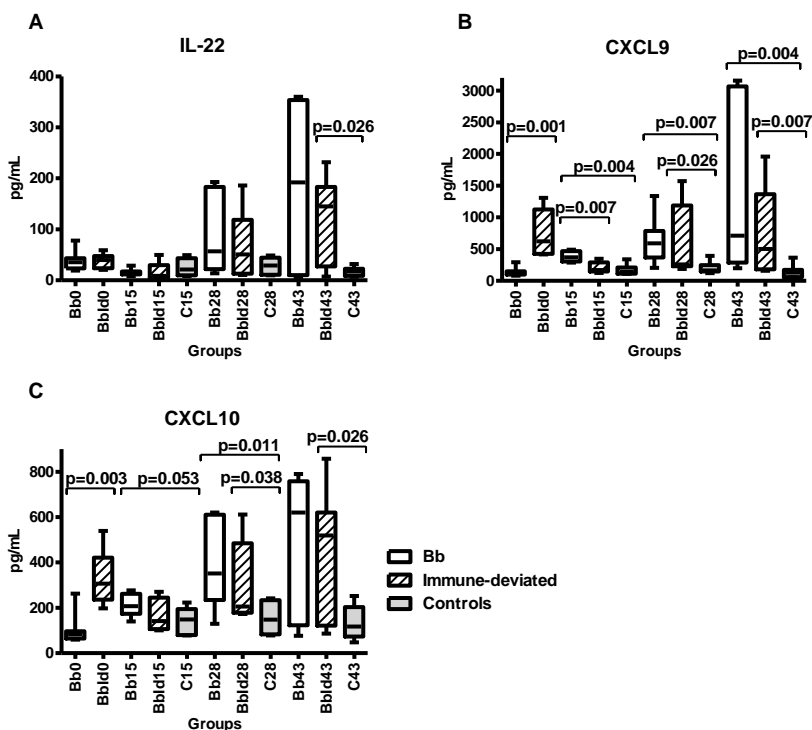


Figure 14. Systemic protein levels of markers for Th1 (CXCL9 and CXCL10) and Th17 (IL-22) subsets in sera from two experimental *B. burgdorferi* s.s.-infected (n = 21 in each group) groups and one untreated control group (n = 7) of C3H/HeN mice. The boxes represent the median with 25th and 75th percentiles and with min-max whiskers. A Mann-Whitney U-test was used for the comparisons between the groups.

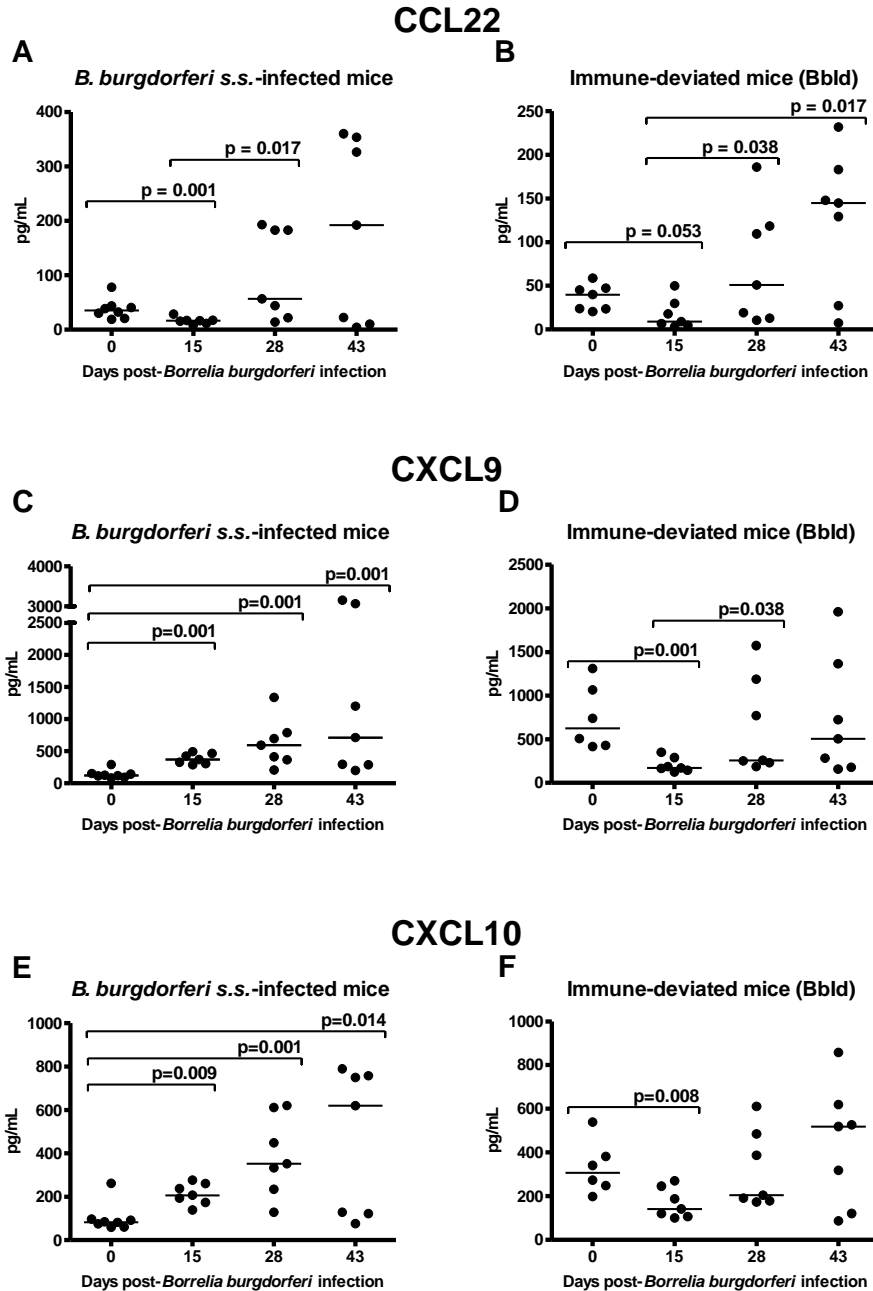


Figure 15. Systemic changes of protein levels of markers for Th1 (CXCL9 and CXCL10) and Th17 (IL-22) subsets in sera during the course of infection in two experimental *B. burgdorferi* s.s.-infected ($n = 21$ in each group) groups of C3H/HeN mice. The boxes represent the median with 25th and 75th percentiles and with min-max whiskers. A Kruskal-Wallis test followed by a post hoc Mann-Whitney U test was used for the comparisons within the groups.

Taken together, the findings in this study support the hypothesis that a strong initial Th1-like response, followed by an anti-inflammatory response, is needed for optimal resolution of *B. burgdorferi s.l.* infection. Although the differences in mRNA expression between the two groups of mice only reached the level of trends, these findings were also in line with the hypothesis. Even though the findings are in line with our hypothesis, the anti-inflammatory response seems to be more complex than just a Th1-antagonistic Th2 response.

Conclusions

- 25% of the ticks that had bitten individuals included in this thesis were infected by *B. burgdorferi s.l.* However, few of the ticks transmitted the bacteria to the human host and caused an infection since only 3.7% developed LB and another 4.9% of the individuals bitten by infected ticks developed asymptomatic infection. The relative risk of developing *B. burgdorferi s.l.* infection is $RR=2.6$ for individuals bitten by *B. burgdorferi s.l.*-infected ticks compared to individuals bitten by uninfected ticks. Approximately one third of all tick-bitten individuals (27%) experienced symptoms possibly associated with LB despite the low risk of disease and infection. Individuals bitten by infected ticks were more likely to report experiencing symptoms than individuals bitten by uninfected ticks. The number of reported symptoms did not differ between the two groups, which is not surprising since these non-specific symptoms are also quite common in the general population. Thus, the risk of developing LB after a bite by a *B. burgdorferi s.l.*-infected tick is low.
- The majority of the tick-bitten individuals who developed *B. burgdorferi s.l.* infection, irrespective of the infectious status of the tick, had an asymptomatic infection, *i.e.* they did not develop LB. These results suggest that an asymptomatic infection is the most common outcome after a tick bite transmitting the infection, even if the tick was infected with *B. burgdorferi s.l.*
- Symptomatic EM patients, *i.e.* those with persistent symptoms at the six-month follow-up, showed decreased expression of Th1 cytokines in EM lesions compared with asymptomatic EM patients. Furthermore tick-bitten individuals who developed asymptomatic infection showed an increase of early Th1-associated biomarkers in blood, only a few days after the tick bite, compared to individuals who developed clinical LB. Taken together, these results suggest that a good clinical outcome of LB is associated with a strong Th1 response early in the infection, supporting the hypothesis.
- An early immune biomarker for the clinical outcome of a *B. burgdorferi s.l.* infection after tick bite could not be found when screening the blood samples from tick-bitten individuals. Although three immune markers differed between individuals who developed LB and individuals with asymptomatic infection, none of the markers clearly discriminated between the groups.
- Th2-immune-deviated *B. burgdorferi s.s.*-infected mice could not eradicate the spirochaete as efficiently as *B. burgdorferi s.s.*-infected mice. The immune-deviated

mice also had more pronounced clinical signs of infection than infected mice. The non-deviated infected mice showed a decrease of mRNA expression associated with Th2, anti-inflammatory and Treg/Th1 responses during the course of infection, which suggests a termination of the inflammatory response that was not seen in the immune-deviated mice. The trends for increased expression of pro-inflammatory GM-CSF and Treg marker Foxp3 in immune-deviated mice suggested ongoing inflammation. Non-deviated mice showed increases of CXCL9 and CXCL10 at all time points compared with day 0 p.i. ($p \leq 0.014$), whereas immune-deviated mice showed an initial decrease in both chemokines at day 15 p.i. compared with day 0 p.i. ($p \leq 0.008$). Thus, although the findings are not fully clear-cut, the clinical signs of infection together with the findings of mRNA expression in inguinal lymph nodes and systemic chemokine expression support the hypothesis of a need for a strong Th1 response followed by anti-inflammatory response for optimal resolution.

In conclusion, the risk of developing LB after a tick bite is low, and no infection or asymptomatic infections are the most common outcome after a tick bite. The findings on the early immune response in humans and the immune response towards *B. burgdorferi* s.s. infection in mice support the hypothesis that a strong pro-inflammatory Th1 response is needed for an optimal clinical outcome and eradication of bacteria.

Future perspectives

In view of the results in this thesis, it would be interesting to continue recruiting newly tick-bitten individuals to the TBD Sting study and continue to investigate the risk of developing both LB and asymptomatic infection after tick bite, and the prevalence of different subtypes of *B. burgdorferi s.l.* in the ticks, and correlate the clinical outcome with the prevalence of *B. burgdorferi s.l.*. In addition, epidemiological and microbiological aspects should also be investigated. The TBD Sting study is one of the few studies designed to investigate newly tick-bitten individuals and the ticks that bite them, instead of investigating LB patients who were bitten by a tick weeks to months prior to participation in a study. Therefore the study is a unique opportunity to study the early events in the immune response against the *B. burgdorferi s.l.* bacteria.

Another investigation I would like to see continued is the study of early immune biomarkers conducted on blood samples collected from the study subjects in the TBD Sting study. A continuation would consist of larger groups of individuals that are analysed and a larger spectrum of immune markers. In this thesis we did not find a discriminatory early immune marker for the clinical outcome after a tick bite. However, it may be that there is no single marker for the clinical outcome; perhaps a combination of several early immune markers together may serve as a prognostic marker.

Another investigation of interest would be to study the inherent differences in the immune system of different individuals. This could be conducted by investigating the expression levels of immunological genes from LB patients with and without persistent symptoms post-treatment, but also from individuals with asymptomatic infection, and healthy individuals. The gene expression could be screened with a microarray assay, which can analyse a huge amount of genes simultaneously. This investigation could hopefully, like the study on early biomarkers, pinpoint the immunological markers that differ between individuals with different clinical outcomes of LB.

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References

1. Burgdorfer W. Discovery of the Lyme disease spirochete: a historical review. *Zentralbl Bakteriol Mikrobiol Hyg A*. 1986 Dec;263(1-2):7–10.
2. Afzelius, A. Verhandlungen der dermatologischen gesellschaft zu Stockholm, 28 Oktober 1909. 1910;101:404.
3. Steere AC, Malawista SE, Snyderman DR, Shope RE, Andiman WA, Ross MR, et al. Lyme arthritis: an epidemic of oligoarticular arthritis in children and adults in three connecticut communities. *Arthritis Rheum*. 1977 Feb;20(1):7–17.
4. Steere AC, Broderick TF, Malawista SE. Erythema chronicum migrans and Lyme arthritis: epidemiologic evidence for a tick vector. *Am. J. Epidemiol*. 1978 Oct;108(4):312–21.
5. Burgdorfer W, Barbour AG, Hayes SF, Benach JL, Grunwaldt E, Davis JP. Lyme disease—a tick-borne spirochetosis? *Science*. 1982 Jun 18;216(4552):1317–9.
6. Hanincová K, Taragelová V, Koci J, Schäfer SM, Hails R, Ullmann AJ, et al. Association of *Borrelia garinii* and *B. valaisiana* with songbirds in Slovakia. *Appl. Environ. Microbiol*. 2003 May;69(5):2825–30.
7. Kurtenbach K, Peacey M, Rijpkema SG, Hoodless AN, Nuttall PA, Randolph SE. Differential transmission of the genospecies of *Borrelia burgdorferi* sensu lato by game birds and small rodents in England. *Appl. Environ. Microbiol*. 1998 Apr;64(4):1169–74.
8. Humair PF, Gern L. Relationship between *Borrelia burgdorferi* sensu lato species, red squirrels (*Sciurus vulgaris*) and *Ixodes ricinus* in enzootic areas in Switzerland. *Acta Trop*. 1998 Jun;69(3):213–27.
9. Gray JS, Kahl O, Lane RS, Stanek G. Lyme Borreliosis: Biology, Epidemiology and Control. First. CABI; 2002.
10. Kurtenbach K, Sewell HS, Ogden NH, Randolph SE, Nuttall PA. Serum complement sensitivity as a key factor in Lyme disease ecology. *Infect. Immun*. 1998 Mar;66(3):1248–51.
11. Anderson JF. Epizootiology of Lyme borreliosis. *Scand J Infect Dis Suppl*. 1991;77:23–34.
12. Samuels DS, Radolf JD, editors. *Borrelia: Molecular Biology, Host Interaction and Pathogenesis*. 1st ed. Caister Academic Press; 2010.
13. Richter D, Debski A, Hubalek Z, Matuschka F-R. Absence of Lyme disease spirochetes in larval *Ixodes ricinus* ticks. *Vector Borne Zoonotic Dis*. 2012 Jan;12(1):21–7.

14. Crippa M, Rais O, Gern L. Investigations on the mode and dynamics of transmission and infectivity of *Borrelia burgdorferi* sensu stricto and *Borrelia afzelii* in *Ixodes ricinus* ticks. *Vector Borne Zoonotic Dis.* 2002;2(1):3–9.
15. Piesman J, Mather TN, Sinsky RJ, Spielman A. Duration of tick attachment and *Borrelia burgdorferi* transmission. *J. Clin. Microbiol.* 1987 Mar;25(3):557–8.
16. Des Vignes F, Piesman J, Heffernan R, Schulze TL, Stafford KC 3rd, Fish D. Effect of tick removal on transmission of *Borrelia burgdorferi* and *Ehrlichia phagocytophila* by *Ixodes scapularis* nymphs. *J. Infect. Dis.* 2001 Mar 1;183(5):773–8.
17. Kahl O, Janetzki-Mittmann C, Gray JS, Jonas R, Stein J, De Boer R. Risk of infection with *Borrelia burgdorferi* sensu lato for a host in relation to the duration of nymphal *Ixodes ricinus* feeding and the method of tick removal. *Zentralbl. Bakteriologie.* 1998 Jan;287(1-2):41–52.
18. Barbour AG, Hayes SF. Biology of *Borrelia* species. *Microbiol. Rev.* 1986 Dec;50(4):381–400.
19. Takayama K, Rothenberg RJ, Barbour AG. Absence of lipopolysaccharide in the Lyme disease spirochete, *Borrelia burgdorferi*. *Infect. Immun.* 1987 Sep;55(9):2311–3.
20. Brandt ME, Riley BS, Radolf JD, Norgard MV. Immunogenic integral membrane proteins of *Borrelia burgdorferi* are lipoproteins. *Infect. Immun.* 1990 Apr;58(4):983–91.
21. Strle F, Stanek G. Clinical manifestations and diagnosis of Lyme borreliosis. *Curr. Probl. Dermatol.* 2009;37:51–110.
22. Richter D, Schlee DB, Allgöwer R, Matuschka F-R. Relationships of a novel Lyme disease spirochete, *Borrelia spielmani* sp. nov., with its hosts in Central Europe. *Appl. Environ. Microbiol.* 2004 Nov;70(11):6414–9.
23. Fingerle V, Schulte-Spechtel UC, Ruzic-Sabljic E, Leonhard S, Hofmann H, Weber K, et al. Epidemiological aspects and molecular characterization of *Borrelia burgdorferi* s.l. from southern Germany with special respect to the new species *Borrelia spielmanii* sp. nov. *Int. J. Med. Microbiol.* 2008 Apr;298(3-4):279–90.
24. Maraspin V, Ruzic-Sabljic E, Strle F. Lyme borreliosis and *Borrelia spielmanii*. *Emerging Infect. Dis.* 2006 Jul;12(7):1177.
25. Rijpkema SGT, Tazelaar DJ, Molkenboer MJCH, Noordhoek GT, Plantinga G, Schouls LM, et al. Detection of *Borrelia afzelii*, *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* and group VS116 by PCR in skin biopsies of patients with erythema migrans and acrodermatitis chronica atrophicans. *Clin. Microbiol. Infect.* 1997 Feb;3(1):109–16.
26. Saito K, Ito T, Asashima N, Ohno M, Nagai R, Fujita H, et al. Case report: *Borrelia valaisiana* infection in a Japanese man associated with traveling to foreign countries. *Am. J. Trop. Med. Hyg.* 2007 Dec;77(6):1124–7.
27. Hulínská D, Votýpka J, Vanousová D, Hercogová J, Hulínský V, Drevová H, et al. Identification of *Anaplasma phagocytophilum* and *Borrelia burgdorferi* sensu lato in patients with erythema migrans. *Folia Microbiol. (Praha).* 2009;54(3):246–56.

28. Maraspin V, Cimperman J, Lotric-Furlan S, Ruzić-Sabljić E, Jurca T, Picken RN, et al. Solitary borrelial lymphocytoma in adult patients. *Wien. Klin. Wochenschr.* 2002 Jul 31;114(13-14):515–23.
29. Rudenko N, Golovchenko M, Růžek D, Piskunova N, Mallátová N, Grubhoffer L. Molecular detection of *Borrelia bissettii* DNA in serum samples from patients in the Czech Republic with suspected borreliosis. *FEMS Microbiol. Lett.* 2009 Mar;292(2):274–81.
30. Rudenko N, Golovchenko M, Mokráček A, Piskunová N, Ruzek D, Mallatová N, et al. Detection of *Borrelia bissettii* in cardiac valve tissue of a patient with endocarditis and aortic valve stenosis in the Czech Republic. *J. Clin. Microbiol.* 2008 Oct;46(10):3540–3.
31. Collares-Pereira M, Couceiro S, Franca I, Kurtenbach K, Schäfer SM, Vitorino L, et al. First isolation of *Borrelia lusitaniae* from a human patient. *J. Clin. Microbiol.* 2004 Mar;42(3):1316–8.
32. Hubálek Z, Halouzka J. Distribution of *Borrelia burgdorferi* sensu lato genomic groups in Europe, a review. *Eur. J. Epidemiol.* 1997 Dec;13(8):951–7.
33. Rauter C, Hartung T. Prevalence of *Borrelia burgdorferi* sensu lato genospecies in *Ixodes ricinus* ticks in Europe: a metaanalysis. *Appl. Environ. Microbiol.* 2005 Nov;71(11):7203–16.
34. Van Dam AP, Kuiper H, Vos K, Widjojokusumo A, De Jongh BM, Spanjaard L, et al. Different genospecies of *Borrelia burgdorferi* are associated with distinct clinical manifestations of Lyme borreliosis. *Clin. Infect. Dis.* 1993 Oct;17(4):708–17.
35. Balmelli T, Piffaretti JC. Association between different clinical manifestations of Lyme disease and different species of *Borrelia burgdorferi* sensu lato. *Res. Microbiol.* 1995 May;146(4):329–40.
36. Ornstein K, Berglund J, Nilsson I, Norrby R, Bergström S. Characterization of Lyme borreliosis isolates from patients with erythema migrans and neuroborreliosis in southern Sweden. *J. Clin. Microbiol.* 2001 Apr;39(4):1294–8.
37. Hanincová K, Schäfer SM, Etti S, Sewell HS, Taragelová V, Ziak D, et al. Association of *Borrelia afzelii* with rodents in Europe. *Parasitology.* 2003 Jan;126(Pt 1):11–20.
38. Humair PF, Postic D, Wallich R, Gern L. An avian reservoir (*Turdus merula*) of the Lyme borreliosis spirochetes. *Zentralbl. Bakterirol.* 1998 May;287(4):521–38.
39. Wormser GP, Dattwyler RJ, Shapiro ED, Halperin JJ, Steere AC, Klempner MS, et al. The clinical assessment, treatment, and prevention of lyme disease, human granulocytic anaplasmosis, and babesiosis: clinical practice guidelines by the Infectious Diseases Society of America. *Clin. Infect. Dis.* 2006 Nov 1;43(9):1089–134.
40. Hubálek Z. Epidemiology of lyme borreliosis. *Curr. Probl. Dermatol.* 2009;37:31–50.
41. Bennet L, Halling A, Berglund J. Increased incidence of Lyme borreliosis in southern Sweden following mild winters and during warm, humid summers. *Eur. J. Clin. Microbiol. Infect. Dis.* 2006 Jul;25(7):426–32.

42. Institutet för hälsa och välfärd [Internet]. Available from: http://www.thl.fi/sv_SE/web/sv/startsidea. Retrieved 26 November 2012.
43. Berglund J, Eitrem R, Ornstein K, Lindberg A, Ringér A, Elmrud H, et al. An epidemiologic study of Lyme disease in southern Sweden. *N. Engl. J. Med.* 1995 Nov 16;333(20):1319–27.
44. Bennet L, Berglund J. Reinfection with Lyme borreliosis: a retrospective follow-up study in southern Sweden. *Scand. J. Infect. Dis.* 2002;34(3):183–6.
45. Jarefors S, Bennet L, You E, Forsberg P, Ekerfelt C, Berglund J, et al. Lyme borreliosis reinfection: might it be explained by a gender difference in immune response? *Immunology.* 2006 Jun;118(2):224–32.
46. Huegli D, Moret J, Rais O, Moosmann Y, Erard P, Malinverni R, et al. Prospective study on the incidence of infection by *Borrelia burgdorferi* sensu lato after a tick bite in a highly endemic area of Switzerland. *Ticks Tick Borne Dis.* 2011 Sep;2(3):129–36.
47. Tijssse-Klasen E, Jacobs JJ, Swart A, Fonville M, Reimerink JH, Brandenburg AH, et al. Small risk of developing symptomatic tick-borne diseases following a tick bite in The Netherlands. *Parasit Vectors.* 2011;4:17.
48. Dandache P, Nadelman RB. Erythema migrans. *Infect. Dis. Clin. North Am.* 2008 Jun;22(2):235–260, vi.
49. Stanek G, O'Connell S, Cimmino M, Aberer E, Kristoferitsch W, Granström M, et al. European Union Concerted Action on Risk Assessment in Lyme Borreliosis: clinical case definitions for Lyme borreliosis. *Wien. Klin. Wochenschr.* 1996 Dec 13;108(23):741–7.
50. Läkemedelsverket [Internet]. Report No.: 4:2009. Available from: <http://www.lakemedelsverket.se/>. Retrieved 26 November 2012.
51. Steere AC, Sikand VK, Schoen RT, Nowakowski J. Asymptomatic infection with *Borrelia burgdorferi*. *Clin. Infect. Dis.* 2003 Aug 15;37(4):528–32.
52. Fahrner H, Van der Linden SM, Sauvain MJ, Gern L, Zhioua E, Aeschlimann A. The prevalence and incidence of clinical and asymptomatic Lyme borreliosis in a population at risk. *J. Infect. Dis.* 1991 Feb;163(2):305–10.
53. Ekerfelt C, Masreliez C, Senvik M, Ernerudh J, Roberg M, Forsberg P. Antibodies and T-cell reactivity to *Borrelia burgdorferi* in an asymptomatic population: a study of healthy blood donors in an inland town district in the south-east of Sweden. *Scand. J. Infect. Dis.* 2001;33(11):806–8.
54. Wormser GP, Nadelman RB, Nowakowski J, Schwartz I. Asymptomatic *Borrelia burgdorferi* infection. *Med. Hypotheses.* 2001 Oct;57(4):435–8.
55. Smith RP, Schoen RT, Rahn DW, Sikand VK, Nowakowski J, Parenti DL, et al. Clinical characteristics and treatment outcome of early Lyme disease in patients with microbiologically confirmed erythema migrans. *Ann. Intern. Med.* 2002 Mar 19;136(6):421–8.

56. Cerar D, Cerar T, Ruzić-Sabljic E, Wormser GP, Strle F. Subjective symptoms after treatment of early Lyme disease. *Am. J. Med.* 2010 Jan;123(1):79–86.
57. Kowalski TJ, Tata S, Berth W, Mathiason MA, Agger WA. Antibiotic treatment duration and long-term outcomes of patients with early lyme disease from a lyme disease-hyperendemic area. *Clin. Infect. Dis.* 2010 Feb 15;50(4):512–20.
58. Bennet L, Danell S, Berglund J. Clinical outcome of erythema migrans after treatment with phenoxymethyl penicillin. *Scand. J. Infect. Dis.* 2003;35(2):129–31.
59. Asch ES, Bujak DI, Weiss M, Peterson MG, Weinstein A. Lyme disease: an infectious and postinfectious syndrome. *J. Rheumatol.* 1994 Mar;21(3):454–61.
60. Klempner MS, Hu LT, Evans J, Schmid CH, Johnson GM, Trevino RP, et al. Two controlled trials of antibiotic treatment in patients with persistent symptoms and a history of Lyme disease. *N. Engl. J. Med.* 2001 Jul 12;345(2):85–92.
61. Henningsson AJ, Malmvall B-E, Ernerudh J, Matussek A, Forsberg P. Neuroborreliosis--an epidemiological, clinical and healthcare cost study from an endemic area in the south-east of Sweden. *Clin. Microbiol. Infect.* 2010 Aug;16(8):1245–51.
62. Eikeland R, Ljøstad U, Mygland A, Herlofson K, Løhaugen GC. European neuroborreliosis: neuropsychological findings 30 months post-treatment. *Eur. J. Neurol.* 2012 Mar;19(3):480–7.
63. Vrethem M, Hellblom L, Widlund M, Ahl M, Danielsson O, Ernerudh J, et al. Chronic symptoms are common in patients with neuroborreliosis -- a questionnaire follow-up study. *Acta Neurol. Scand.* 2002 Oct;106(4):205–8.
64. Ljøstad U, Mygland Å. The phenomenon of “chronic Lyme”; an observational study. *Eur. J. Neurol.* 2012 Aug;19(8):1128–35.
65. Djukic M, Schmidt-Samoa C, Nau R, Von Steinbüchel N, Eiffert H, Schmidt H. The diagnostic spectrum in patients with suspected chronic Lyme neuroborreliosis--the experience from one year of a university hospital's Lyme neuroborreliosis outpatients clinic. *Eur. J. Neurol.* 2011 Apr;18(4):547–55.
66. Kalish RA, Kaplan RF, Taylor E, Jones-Woodward L, Workman K, Steere AC. Evaluation of study patients with Lyme disease, 10-20-year follow-up. *J. Infect. Dis.* 2001 Feb 1;183(3):453–60.
67. Auwaerter PG. Point: antibiotic therapy is not the answer for patients with persisting symptoms attributable to lyme disease. *Clin. Infect. Dis.* 2007 Jul 15;45(2):143–8.
68. Marques A. Chronic Lyme disease: a review. *Infect. Dis. Clin. North Am.* 2008 Jun;22(2):341–360, vii–viii.
69. Murray PR, Rosenthal KS, Pfaller MA. Medical microbiology [Elektronisk resurs] / Patrick R. Murray, Ken S. Rosenthal, Michael A. Pfaller. Philadelphia : Mosby/Elsevier, cop. 2009; 2009.

70. Brouqui P, Bacellar F, Baranton G, Birtles RJ, Bjoërsdorff A, Blanco JR, et al. Guidelines for the diagnosis of tick-borne bacterial diseases in Europe. *Clin. Microbiol. Infect.* 2004 Dec;10(12):1108–32.
71. Swanson SJ, Neitzel D, Reed KD, Belongia EA. Coinfections acquired from ixodes ticks. *Clin. Microbiol. Rev.* 2006 Oct;19(4):708–27.
72. Bogovic P, Lotric-Furlan S, Strle F. What tick-borne encephalitis may look like: clinical signs and symptoms. *Travel Med Infect Dis.* 2010 Jul;8(4):246–50.
73. Aguero-Rosenfeld ME, Wang G, Schwartz I, Wormser GP. Diagnosis of lyme borreliosis. *Clin. Microbiol. Rev.* 2005 Jul;18(3):484–509.
74. Turvey SE, Broide DH. Innate immunity. *J. Allergy Clin. Immunol.* 2010 Feb;125(2 Suppl 2):S24–32.
75. Abbas, Abdul K, Lichtman, Andrew H, Pillai, Shiv. *Cellular and Molecular Immunology*. 7th ed. Saunders; 2011.
76. Borish L. IL-10: evolving concepts. *J. Allergy Clin. Immunol.* 1998 Mar;101(3):293–7.
77. Saraiva M, O’Garra A. The regulation of IL-10 production by immune cells. *Nat. Rev. Immunol.* 2010 Mar;10(3):170–81.
78. Moore KW, De Waal Malefyt R, Coffman RL, O’Garra A. Interleukin-10 and the interleukin-10 receptor. *Annu. Rev. Immunol.* 2001;19:683–765.
79. Bonilla FA, Oettgen HC. Adaptive immunity. *J. Allergy Clin. Immunol.* 2010 Feb;125(2 Suppl 2):S33–40.
80. Chaplin DD. Overview of the immune response. *J. Allergy Clin. Immunol.* 2010 Feb;125(2 Suppl 2):S3–23.
81. Murphy K. *Janeway’s Immunobiology*. 8th ed. Garland Science; 2011.
82. Jankovic D, Kullberg MC, Feng CG, Goldszmid RS, Collazo CM, Wilson M, et al. Conventional T-bet(+)Foxp3(-) Th1 cells are the major source of host-protective regulatory IL-10 during intracellular protozoan infection. *J. Exp. Med.* 2007 Feb 19;204(2):273–83.
83. Anderson CF, Oukka M, Kuchroo VJ, Sacks D. CD4(+)CD25(-)Foxp3(-) Th1 cells are the source of IL-10-mediated immune suppression in chronic cutaneous leishmaniasis. *J. Exp. Med.* 2007 Feb 19;204(2):285–97.
84. McGeachy MJ, Bak-Jensen KS, Chen Y, Tato CM, Blumenschein W, McClanahan T, et al. TGF-beta and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology. *Nat. Immunol.* 2007 Dec;8(12):1390–7.
85. Spits H, Cupedo T. Innate lymphoid cells: emerging insights in development, lineage relationships, and function. *Annu. Rev. Immunol.* 2012;30:647–75.

86. Zhu J, Paul WE. CD4 T cells: fates, functions, and faults. *Blood*. 2008 Sep 1;112(5):1557–69.
87. Zygmunt B, Veldhoen M. T helper cell differentiation more than just cytokines. *Adv. Immunol.* 2011;109:159–96.
88. Commins SP, Borish L, Steinke JW. Immunologic messenger molecules: cytokines, interferons, and chemokines. *J. Allergy Clin. Immunol.* 2010 Feb;125(2 Suppl 2):S53–72.
89. Maddur MS, Miossec P, Kaveri SV, Bayry J. Th17 cells: biology, pathogenesis of autoimmune and inflammatory diseases, and therapeutic strategies. *Am. J. Pathol.* 2012 Jul;181(1):8–18.
90. Vignali DAA, Collison LW, Workman CJ. How regulatory T cells work. *Nat. Rev. Immunol.* 2008 Jul;8(7):523–32.
91. Zhang N, Bevan MJ. CD8(+) T cells: foot soldiers of the immune system. *Immunity*. 2011 Aug 26;35(2):161–8.
92. Beetz S, Wesch D, Marischen L, Welte S, Oberg H-H, Kabelitz D. Innate immune functions of human gammadelta T cells. *Immunobiology*. 2008;213(3-4):173–82.
93. Akdis M, Burgler S, Cramer R, Eiwegger T, Fujita H, Gomez E, et al. Interleukins, from 1 to 37, and interferon- γ : receptors, functions, and roles in diseases. *J. Allergy Clin. Immunol.* 2011 Mar;127(3):701–721.e1–70.
94. Brombacher F, Kastelein RA, Alber G. Novel IL-12 family members shed light on the orchestration of Th1 responses. *Trends Immunol.* 2003 Apr;24(4):207–12.
95. Bonecchi R, Galliera E, Borroni EM, Corsi MM, Locati M, Mantovani A. Chemokines and chemokine receptors: an overview. *Front. Biosci.* 2009;14:540–51.
96. Borish LC, Steinke JW. 2. Cytokines and chemokines. *J. Allergy Clin. Immunol.* 2003 Feb;111(2 Suppl):S460–475.
97. Moser B, Loetscher P. Lymphocyte traffic control by chemokines. *Nat. Immunol.* 2001 Feb;2(2):123–8.
98. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol.* 2004 Dec;25(12):677–86.
99. Parks WC, Wilson CL, López-Boado YS. Matrix metalloproteinases as modulators of inflammation and innate immunity. *Nat. Rev. Immunol.* 2004 Aug;4(8):617–29.
100. Goyette J, Geczy CL. Inflammation-associated S100 proteins: new mechanisms that regulate function. *Amino Acids*. 2011 Oct;41(4):821–42.
101. Stríz I, Trebichavský I. Calprotectin - a pleiotropic molecule in acute and chronic inflammation. *Physiol Res*. 2004;53(3):245–53.

102. Sbardella D, Fasciglione GF, Gioia M, Ciaccio C, Tundo GR, Marini S, et al. Human matrix metalloproteinases: an ubiquitarian class of enzymes involved in several pathological processes. *Mol. Aspects Med.* 2012 Apr;33(2):119–208.
103. Yang XF, Pal U, Alani SM, Fikrig E, Norgard MV. Essential role for OspA/B in the life cycle of the Lyme disease spirochete. *J. Exp. Med.* 2004 Mar 1;199(5):641–8.
104. Pal U, Li X, Wang T, Montgomery RR, Ramamoorthi N, Desilva AM, et al. TROSPA, an Ixodes scapularis receptor for Borrelia burgdorferi. *Cell.* 2004 Nov 12;119(4):457–68.
105. Tokarz R, Anderton JM, Katona LI, Benach JL. Combined effects of blood and temperature shift on Borrelia burgdorferi gene expression as determined by whole genome DNA array. *Infect. Immun.* 2004 Sep;72(9):5419–32.
106. Grimm D, Tilly K, Byram R, Stewart PE, Krum JG, Bueschel DM, et al. Outer-surface protein C of the Lyme disease spirochete: a protein induced in ticks for infection of mammals. *Proc. Natl. Acad. Sci. U.S.A.* 2004 Mar 2;101(9):3142–7.
107. Kotsyfakis M, Sá-Nunes A, Francischetti IMB, Mather TN, Andersen JF, Ribeiro JMC. Antiinflammatory and immunosuppressive activity of sialostatin L, a salivary cystatin from the tick Ixodes scapularis. *J. Biol. Chem.* 2006 Sep 8;281(36):26298–307.
108. Guo X, Booth CJ, Paley MA, Wang X, DePonte K, Fikrig E, et al. Inhibition of neutrophil function by two tick salivary proteins. *Infect. Immun.* 2009 Jun;77(6):2320–9.
109. Tyson K, Elkins C, Patterson H, Fikrig E, De Silva A. Biochemical and functional characterization of Salp20, an Ixodes scapularis tick salivary protein that inhibits the complement pathway. *Insect Mol. Biol.* 2007 Aug;16(4):469–79.
110. Valenzuela JG, Charlab R, Mather TN, Ribeiro JM. Purification, cloning, and expression of a novel salivary anticomplement protein from the tick, Ixodes scapularis. *J. Biol. Chem.* 2000 Jun 23;275(25):18717–23.
111. Lieskovska J, Kopecky J. Effect of tick saliva on signalling pathways activated by TLR-2 ligand and Borrelia afzelii in dendritic cells. *Parasite Immunol.* 2012 Sep;34(8-9):421–9.
112. Slámová M, Skallová A, Páleníková J, Kopecký J. Effect of tick saliva on immune interactions between Borrelia afzelii and murine dendritic cells. *Parasite Immunol.* 2011 Dec;33(12):654–60.
113. Kern A, Collin E, Barthel C, Michel C, Jaulhac B, Boulanger N. Tick saliva represses innate immunity and cutaneous inflammation in a murine model of lyme disease. *Vector Borne Zoonotic Dis.* 2011 Oct;11(10):1343–50.
114. Gillespie RD, Dolan MC, Piesman J, Titus RG. Identification of an IL-2 binding protein in the saliva of the Lyme disease vector tick, Ixodes scapularis. *J. Immunol.* 2001 Apr 1;166(7):4319–26.

115. Sá-Nunes A, Báfica A, Lucas DA, Conrads TP, Veenstra TD, Andersen JF, et al. Prostaglandin E2 is a major inhibitor of dendritic cell maturation and function in *Ixodes scapularis* saliva. *J. Immunol.* 2007 Aug 1;179(3):1497–505.
116. Liveris D, Wang G, Girao G, Byrne DW, Nowakowski J, McKenna D, et al. Quantitative detection of *Borrelia burgdorferi* in 2-millimeter skin samples of erythema migrans lesions: correlation of results with clinical and laboratory findings. *J. Clin. Microbiol.* 2002 Apr;40(4):1249–53.
117. Coburn J, Fischer JR, Leong JM. Solving a sticky problem: new genetic approaches to host cell adhesion by the Lyme disease spirochete. *Mol. Microbiol.* 2005 Sep;57(5):1182–95.
118. Gebbia JA, Coleman JL, Benach JL. *Borrelia* spirochetes upregulate release and activation of matrix metalloproteinase gelatinase B (MMP-9) and collagenase 1 (MMP-1) in human cells. *Infect. Immun.* 2001 Jan;69(1):456–62.
119. Zhao Z, Chang H, Trevino RP, Whren K, Bhawan J, Klempner MS. Selective up-regulation of matrix metalloproteinase-9 expression in human erythema migrans skin lesions of acute lyme disease. *J. Infect. Dis.* 2003 Oct 15;188(8):1098–104.
120. Kirchner A, Koedel U, Fingerle V, Paul R, Wilske B, Pfister HW. Upregulation of matrix metalloproteinase-9 in the cerebrospinal fluid of patients with acute Lyme neuroborreliosis. *J. Neurol. Neurosurg. Psychiatr.* 2000 Mar;68(3):368–71.
121. Ramamoorthi N, Narasimhan S, Pal U, Bao F, Yang XF, Fish D, et al. The Lyme disease agent exploits a tick protein to infect the mammalian host. *Nature.* 2005 Jul 28;436(7050):573–7.
122. Schuijt TJ, Hovius JWR, Van Burgel ND, Ramamoorthi N, Fikrig E, Van Dam AP. The tick salivary protein Salp15 inhibits the killing of serum-sensitive *Borrelia burgdorferi* sensu lato isolates. *Infect. Immun.* 2008 Jul;76(7):2888–94.
123. Hovius JWR, Ramamoorthi N, Van't Veer C, De Groot KA, Nijhof AM, Jongejan F, et al. Identification of Salp15 homologues in *Ixodes ricinus* ticks. *Vector Borne Zoonotic Dis.* 2007;7(3):296–303.
124. Hovius JW, Schuijt TJ, De Groot KA, Roelofs JJTH, Oei GA, Marquart JA, et al. Preferential protection of *Borrelia burgdorferi* sensu stricto by a Salp15 homologue in *Ixodes ricinus* saliva. *J. Infect. Dis.* 2008 Oct 15;198(8):1189–97.
125. Ekdahl KN, Henningsson AJ, Sandholm K, Forsberg P, Ernerudh J, Ekerfelt C. Immunity in borreliosis with special emphasis on the role of complement. *Adv. Exp. Med. Biol.* 2007;598:198–213.
126. Kraiczy P, Skerka C, Kirschfink M, Brade V, Zipfel PF. Immune evasion of *Borrelia burgdorferi* by acquisition of human complement regulators FHL-1/reconectin and Factor H. *Eur. J. Immunol.* 2001 Jun;31(6):1674–84.
127. Kraiczy P, Skerka C, Brade V, Zipfel PF. Further characterization of complement regulator-acquiring surface proteins of *Borrelia burgdorferi*. *Infect. Immun.* 2001 Dec;69(12):7800–9.

128. Kraiczy P, Skerka C, Kirschfink M, Zipfel PF, Brade V. Mechanism of complement resistance of pathogenic *Borrelia burgdorferi* isolates. *Int. Immunopharmacol.* 2001 Mar;1(3):393–401.
129. Hellwage J, Meri T, Heikkilä T, Alitalo A, Panelius J, Lahdenne P, et al. The complement regulator factor H binds to the surface protein OspE of *Borrelia burgdorferi*. *J. Biol. Chem.* 2001 Mar 16;276(11):8427–35.
130. Kraiczy P, Hellwage J, Skerka C, Becker H, Kirschfink M, Simon MM, et al. Complement resistance of *Borrelia burgdorferi* correlates with the expression of BbCRASP-1, a novel linear plasmid-encoded surface protein that interacts with human factor H and FHL-1 and is unrelated to Erp proteins. *J. Biol. Chem.* 2004 Jan 23;279(4):2421–9.
131. Kurtenbach K, De Michelis S, Etti S, Schäfer SM, Sewell H-S, Brade V, et al. Host association of *Borrelia burgdorferi* sensu lato--the key role of host complement. *Trends Microbiol.* 2002 Feb;10(2):74–9.
132. Van Dam AP, Oei A, Jaspars R, Fijen C, Wilske B, Spanjaard L, et al. Complement-mediated serum sensitivity among spirochetes that cause Lyme disease. *Infect. Immun.* 1997 Apr;65(4):1228–36.
133. Anguita J, Thomas V, Samanta S, Persinski R, Hernanz C, Barthold SW, et al. *Borrelia burgdorferi*-induced inflammation facilitates spirochete adaptation and variable major protein-like sequence locus recombination. *J. Immunol.* 2001 Sep 15;167(6):3383–90.
134. McDowell JV, Sung S-Y, Hu LT, Marconi RT. Evidence that the variable regions of the central domain of VlsE are antigenic during infection with lyme disease spirochetes. *Infect. Immun.* 2002 Aug;70(8):4196–203.
135. Wilske B, Barbour AG, Bergström S, Burman N, Restrepo BI, Rosa PA, et al. Antigenic variation and strain heterogeneity in *Borrelia* spp. *Res. Microbiol.* 1992 Aug;143(6):583–96.
136. Gilmore RD Jr, Mbow ML, Stevenson B. Analysis of *Borrelia burgdorferi* gene expression during life cycle phases of the tick vector *Ixodes scapularis*. *Microbes Infect.* 2001 Aug;3(10):799–808.
137. Ohnishi J, Schneider B, Messer WB, Piesman J, De Silva AM. Genetic variation at the vlsE locus of *Borrelia burgdorferi* within ticks and mice over the course of a single transmission cycle. *J. Bacteriol.* 2003 Aug;185(15):4432–41.
138. Indest KJ, Howell JK, Jacobs MB, Scholl-Meeker D, Norris SJ, Philipp MT. Analysis of *Borrelia burgdorferi* vlsE gene expression and recombination in the tick vector. *Infect. Immun.* 2001 Nov;69(11):7083–90.
139. Posey JE, Gherardini FC. Lack of a role for iron in the Lyme disease pathogen. *Science.* 2000 Jun 2;288(5471):1651–3.

140. Lazarus JJ, Kay MA, McCarter AL, Wooten RM. Viable *Borrelia burgdorferi* enhances interleukin-10 production and suppresses activation of murine macrophages. *Infect. Immun.* 2008 Mar;76(3):1153–62.
141. Bockenstedt LK, Barthold S, Deponate K, Marcantonio N, Kantor FS. *Borrelia burgdorferi* infection and immunity in mice deficient in the fifth component of complement. *Infect. Immun.* 1993 May;61(5):2104–7.
142. Lawrenz MB, Wooten RM, Zachary JF, Drouin SM, Weis JJ, Wetsel RA, et al. Effect of complement component C3 deficiency on experimental Lyme borreliosis in mice. *Infect. Immun.* 2003 Aug;71(8):4432–40.
143. Woodman ME, Cooley AE, Miller JC, Lazarus JJ, Tucker K, Bykowski T, et al. *Borrelia burgdorferi* binding of host complement regulator factor H is not required for efficient mammalian infection. *Infect. Immun.* 2007 Jun;75(6):3131–9.
144. Malawista SE, De Boisfleury Chevance A. Clocking the Lyme spirochete. *PLoS ONE.* 2008;3(2):e1633.
145. Cassimeris L, McNeill H, Zigmond SH. Chemoattractant-stimulated polymorphonuclear leukocytes contain two populations of actin filaments that differ in their spatial distributions and relative stabilities. *J. Cell Biol.* 1990 Apr;110(4):1067–75.
146. Gruler H, De Boisfleury Chevance A. Chemokinesis and necrotaxis of human granulocytes: the important cellular organelles. *Z. Naturforsch., C, J. Biosci.* 1987 Oct;42(9-10):1126–34.
147. Montgomery RR, Lusitani D, De Boisfleury Chevance A, Malawista SE. Human phagocytic cells in the early innate immune response to *Borrelia burgdorferi*. *J. Infect. Dis.* 2002 Jun 15;185(12):1773–9.
148. Lusitani D, Malawista SE, Montgomery RR. *Borrelia burgdorferi* are susceptible to killing by a variety of human polymorphonuclear leukocyte components. *J. Infect. Dis.* 2002 Mar 15;185(6):797–804.
149. Montgomery RR, Malawista SE. Entry of *Borrelia burgdorferi* into macrophages is end-on and leads to degradation in lysosomes. *Infect. Immun.* 1996 Jul;64(7):2867–72.
150. Montgomery RR, Nathanson MH, Malawista SE. The fate of *Borrelia burgdorferi*, the agent for Lyme disease, in mouse macrophages. Destruction, survival, recovery. *J. Immunol.* 1993 Feb 1;150(3):909–15.
151. Oosting M, Ter Hofstede H, Sturm P, Adema GJ, Kullberg B-J, Van der Meer JWM, et al. TLR1/TLR2 heterodimers play an important role in the recognition of *Borrelia* spirochetes. *PLoS ONE.* 2011;6(10):e25998.
152. Cinco M, Cini B, Murgia R, Presani G, Prodan M, Perticarari S. Evidence of involvement of the mannose receptor in adhesion of *Borrelia burgdorferi* to monocyte/macrophages. *Infect. Immun.* 2001 Apr;69(4):2743–7.
153. Medzhitov R. Toll-like receptors and innate immunity. *Nat. Rev. Immunol.* 2001 Nov;1(2):135–45.

154. Strle K, Drouin EE, Shen S, Khoury JE, McHugh G, Ruzic-Sabljic E, et al. *Borrelia burgdorferi* stimulates macrophages to secrete higher levels of cytokines and chemokines than *Borrelia afzelii* or *Borrelia garinii*. *J. Infect. Dis.* 2009 Dec 15;200(12):1936–43.
155. Defosse DL, Johnson RC. In vitro and in vivo induction of tumor necrosis factor alpha by *Borrelia burgdorferi*. *Infect. Immun.* 1992 Mar;60(3):1109–13.
156. Ma Y, Weis JJ. *Borrelia burgdorferi* outer surface lipoproteins OspA and OspB possess B-cell mitogenic and cytokine-stimulatory properties. *Infect. Immun.* 1993 Sep;61(9):3843–53.
157. Miller LC, Isa S, Vannier E, Georgilis K, Steere AC, Dinarello CA. Live *Borrelia burgdorferi* preferentially activate interleukin-1 beta gene expression and protein synthesis over the interleukin-1 receptor antagonist. *J. Clin. Invest.* 1992 Sep;90(3):906–12.
158. Radolf JD, Norgard MV, Brandt ME, Isaacs RD, Thompson PA, Beutler B. Lipoproteins of *Borrelia burgdorferi* and *Treponema pallidum* activate cachectin/tumor necrosis factor synthesis. Analysis using a CAT reporter construct. *J. Immunol.* 1991 Sep 15;147(6):1968–74.
159. Talkington J, Nickell SP. *Borrelia burgdorferi* spirochetes induce mast cell activation and cytokine release. *Infect. Immun.* 1999 Mar;67(3):1107–15.
160. Giambartolomei GH, Dennis VA, Lasater BL, Philipp MT. Induction of pro- and anti-inflammatory cytokines by *Borrelia burgdorferi* lipoproteins in monocytes is mediated by CD14. *Infect. Immun.* 1999 Jan;67(1):140–7.
161. Gebbia JA, Coleman JL, Benach JL. Selective induction of matrix metalloproteinases by *Borrelia burgdorferi* via toll-like receptor 2 in monocytes. *J. Infect. Dis.* 2004 Jan 1;189(1):113–9.
162. Tai KF, Ma Y, Weis JJ. Normal human B lymphocytes and mononuclear cells respond to the mitogenic and cytokine-stimulatory activities of *Borrelia burgdorferi* and its lipoprotein OspA. *Infect. Immun.* 1994 Feb;62(2):520–8.
163. Widhe M, Ekerfelt C, Forsberg P, Bergström S, Ernerudh J. IgG subclasses in Lyme borreliosis: a study of specific IgG subclass distribution in an interferon-gamma-predominated disease. *Scand. J. Immunol.* 1998 Jun;47(6):575–81.
164. Olsson I, Hammarström L, Smith CI, Hovmark A, Asbrink E. IgG subclasses of specific antibodies in *Ixodes ricinus*-borne borreliosis. *Clin. Exp. Immunol.* 1987 Sep;69(3):618–23.
165. Collins C, Wolfe J, Roessner K, Shi C, Sigal LH, Budd RC. Lyme arthritis synovial gammadelta T cells instruct dendritic cells via fas ligand. *J. Immunol.* 2005 Nov 1;175(9):5656–65.
166. Roessner K, Wolfe J, Shi C, Sigal LH, Huber S, Budd RC. High expression of Fas ligand by synovial fluid-derived gamma delta T cells in Lyme arthritis. *J. Immunol.* 2003 Mar 1;170(5):2702–10.

167. Vincent MS, Roessner K, Sellati T, Huston CD, Sigal LH, Behar SM, et al. Lyme arthritis synovial gamma delta T cells respond to *Borrelia burgdorferi* lipoproteins and lipidated hexapeptides. *J. Immunol.* 1998 Nov 15;161(10):5762–71.
168. Kinjo Y, Tupin E, Wu D, Fujio M, Garcia-Navarro R, Benhnia MR-E-I, et al. Natural killer T cells recognize diacylglycerol antigens from pathogenic bacteria. *Nat. Immunol.* 2006 Sep;7(9):978–86.
169. Kumar H, Belperron A, Barthold SW, Bockenstedt LK. Cutting edge: CD1d deficiency impairs murine host defense against the spirochete, *Borrelia burgdorferi*. *J. Immunol.* 2000 Nov 1;165(9):4797–801.
170. Kang I, Barthold SW, Persing DH, Bockenstedt LK. T-helper-cell cytokines in the early evolution of murine Lyme arthritis. *Infect. Immun.* 1997 Aug;65(8):3107–11.
171. Böer A, Bresch M, Dayrit J, Falk TM. Erythema migrans: a reassessment of diagnostic criteria for early cutaneous manifestations of borreliosis with particular emphasis on clonality investigations. *Br. J. Dermatol.* 2007 Jun;156(6):1263–71.
172. Salazar JC, Pope CD, Sellati TJ, Feder HM Jr, Kiely TG, Dardick KR, et al. Coevolution of markers of innate and adaptive immunity in skin and peripheral blood of patients with erythema migrans. *J. Immunol.* 2003 Sep 1;171(5):2660–70.
173. Müllegger RR, McHugh G, Ruthazer R, Binder B, Kerl H, Steere AC. Differential expression of cytokine mRNA in skin specimens from patients with erythema migrans or acrodermatitis chronica atrophicans. *J. Invest. Dermatol.* 2000 Dec;115(6):1115–23.
174. Jones KL, Muellegger RR, Means TK, Lee M, Glickstein LJ, Damle N, et al. Higher mRNA levels of chemokines and cytokines associated with macrophage activation in erythema migrans skin lesions in patients from the United States than in patients from Austria with Lyme borreliosis. *Clin. Infect. Dis.* 2008 Jan 1;46(1):85–92.
175. Ekerfelt C, Ernerudh J, Bunikis J, Vrethem M, Aagesen J, Roberg M, et al. Compartmentalization of antigen specific cytokine responses to the central nervous system in CNS borreliosis: secretion of IFN-gamma predominates over IL-4 secretion in response to outer surface proteins of Lyme disease *Borrelia* spirochetes. *J. Neuroimmunol.* 1997 Nov;79(2):155–62.
176. Henningsson AJ, Tjernberg I, Malmvall B-E, Forsberg P, Ernerudh J. Indications of Th1 and Th17 responses in cerebrospinal fluid from patients with Lyme neuroborreliosis: a large retrospective study. *J Neuroinflammation.* 2011;8:36.
177. Nordberg M, Forsberg P, Johansson A, Nyman D, Jansson C, Ernerudh J, et al. Cytotoxic mechanisms may play a role in the local immune response in the central nervous system in neuroborreliosis. *J. Neuroimmunol.* 2011 Mar;232(1-2):186–93.
178. Grusell M, Widhe M, Ekerfelt C. Increased expression of the Th1-inducing cytokines interleukin-12 and interleukin-18 in cerebrospinal fluid but not in sera from patients with Lyme neuroborreliosis. *J. Neuroimmunol.* 2002 Oct;131(1-2):173–8.

179. Ekerfelt C, Ernerudh J, Forsberg P, Bergström S. Augmented intrathecal secretion of interferon-gamma in response to *Borrelia garinii* in neuroborreliosis. *J. Neuroimmunol.* 1998 Aug 14;89(1-2):177–81.
180. Widhe M, Jarefors S, Ekerfelt C, Vrethem M, Bergstrom S, Forsberg P, et al. *Borrelia*-specific interferon-gamma and interleukin-4 secretion in cerebrospinal fluid and blood during Lyme borreliosis in humans: association with clinical outcome. *J. Infect. Dis.* 2004 May 15;189(10):1881–91.
181. Jarefors S, Janefjord CK, Forsberg P, Jenmalm MC, Ekerfelt C. Decreased up-regulation of the interleukin-12Rbeta2-chain and interferon-gamma secretion and increased number of forkhead box P3-expressing cells in patients with a history of chronic Lyme borreliosis compared with asymptomatic *Borrelia*-exposed individuals. *Clin. Exp. Immunol.* 2007 Jan;147(1):18–27.
182. Sjöwall J, Carlsson A, Vaarala O, Bergström S, Ernerudh J, Forsberg P, et al. Innate immune responses in Lyme borreliosis: enhanced tumour necrosis factor-alpha and interleukin-12 in asymptomatic individuals in response to live spirochetes. *Clin. Exp. Immunol.* 2005 Jul;141(1):89–98.
183. Widhe M, Grusell M, Ekerfelt C, Vrethem M, Forsberg P, Ernerudh J. Cytokines in Lyme borreliosis: lack of early tumour necrosis factor-alpha and transforming growth factor-beta1 responses are associated with chronic neuroborreliosis. *Immunology.* 2002 Sep;107(1):46–55.
184. Barthold SW, Beck DS, Hansen GM, Terwilliger GA, Moody KD. Lyme borreliosis in selected strains and ages of laboratory mice. *J. Infect. Dis.* 1990 Jul;162(1):133–8.
185. Barbour AG. Isolation and cultivation of Lyme disease spirochetes. *Yale J Biol Med.* 1984 Aug;57(4):521–5.
186. Wilhelmsson P, Fryland L, Börjesson S, Nordgren J, Bergström S, Ernerudh J, et al. Prevalence and diversity of *Borrelia* species in ticks that have bitten humans in Sweden. *J. Clin. Microbiol.* 2010 Nov;48(11):4169–76.
187. Ornstein K, Barbour AG. A reverse transcriptase-polymerase chain reaction assay of *Borrelia burgdorferi* 16S rRNA for highly sensitive quantification of pathogen load in a vector. *Vector Borne Zoonotic Dis.* 2006;6(1):103–12.
188. Comstedt P, Bergström S, Olsen B, Garpmo U, Marjavaara L, Mejlön H, et al. Migratory passerine birds as reservoirs of Lyme borreliosis in Europe. *Emerging Infect. Dis.* 2006 Jul;12(7):1087–95.
189. Tsao JJ, Wootton JT, Bunikis J, Luna MG, Fish D, Barbour AG. An ecological approach to preventing human infection: vaccinating wild mouse reservoirs intervenes in the Lyme disease cycle. *Proc. Natl. Acad. Sci. U.S.A.* 2004 Dec 28;101(52):18159–64.
190. Andersson J, Abrams J, Björk L, Funä K, Litton M, Agren K, et al. Concomitant in vivo production of 19 different cytokines in human tonsils. *Immunology.* 1994 Sep;83(1):16–24.

191. Sandberg M, Frykman A, Ernerudh J, Berg G, Matthiesen L, Ekerfelt C, et al. Cord blood cytokines and chemokines and development of allergic disease. *Pediatr Allergy Immunol.* 2009 Sep;20(6):519–27.
192. Nahimana I, Gern L, Blanc DS, Praz G, Francioli P, Péter O. Risk of *Borrelia burgdorferi* infection in western Switzerland following a tick bite. *Eur. J. Clin. Microbiol. Infect. Dis.* 2004 Aug;23(8):603–8.
193. Maiwald M, Oehme R, March O, Petney TN, Kimmig P, Naser K, et al. Transmission risk of *Borrelia burgdorferi sensu lato* from *Ixodes ricinus* ticks to humans in southwest Germany. *Epidemiol. Infect.* 1998 Aug;121(1):103–8.
194. Oksi J, Savolainen J, Pène J, Bousquet J, Laippala P, Viljanen MK. Decreased interleukin-4 and increased gamma interferon production by peripheral blood mononuclear cells of patients with Lyme borreliosis. *Infect. Immun.* 1996 Sep;64(9):3620–3.
195. Ekerfelt C, Andersson M, Olausson A, Bergström S, Hultman P. Mercury exposure as a model for deviation of cytokine responses in experimental Lyme arthritis: HgCl₂ treatment decreases T helper cell type 1-like responses and arthritis severity but delays eradication of *Borrelia burgdorferi* in C3H/HeN mice. *Clin. Exp. Immunol.* 2007 Oct;150(1):189–97.
196. Stanek G, Wormser GP, Gray J, Strle F. Lyme borreliosis. *Lancet.* 2012 Feb 4;379(9814):461–73.
197. Luger SW, Paparone P, Wormser GP, Nadelman RB, Grunwaldt E, Gomez G, et al. Comparison of cefuroxime axetil and doxycycline in treatment of patients with early Lyme disease associated with erythema migrans. *Antimicrob. Agents Chemother.* 1995 Mar;39(3):661–7.
198. Lipsker D, Antoni-Bach N, Hansmann Y, Jaulhac B. Long-term prognosis of patients treated for erythema migrans in France. *Br. J. Dermatol.* 2002 May;146(5):872–6.
199. Caproni M, Torchia D, Cardinali C, Volpi W, Del Bianco E, D'Agata A, et al. Infiltrating cells, related cytokines and chemokine receptors in lesional skin of patients with dermatomyositis. *Br. J. Dermatol.* 2004 Oct;151(4):784–91.
200. Yawalkar N, Limat A, Brand CU, Braathen LR. Constitutive expression of both subunits of interleukin-12 in human keratinocytes. *J. Invest. Dermatol.* 1996 Jan;106(1):80–3.
201. Companjen AR, Van der Velden VH, Vooys A, Debets R, Benner R, Prens EP. Human keratinocytes are major producers of IL-18: predominant expression of the unprocessed form. *Eur. Cytokine Netw.* 2000 Sep;11(3):383–90.
202. Ellis TN, Beaman BL. Interferon-gamma activation of polymorphonuclear neutrophil function. *Immunology.* 2004 May;112(1):2–12.
203. Ekerfelt C, Jarefors S, Tynngård N, Hedlund M, Sander B, Bergström S, et al. Phenotypes indicating cytolytic properties of *Borrelia*-specific interferon-gamma

- secreting cells in chronic Lyme neuroborreliosis. *J. Neuroimmunol.* 2003 Dec;145(1-2):115–26.
204. Jarefors S, Karlsson M, Forsberg P, Eliasson I, Ernerudh J, Ekerfelt C. Reduced number of interleukin-12 secreting cells in patients with Lyme borreliosis previously exposed to *Anaplasma phagocytophilum*. *Clin. Exp. Immunol.* 2006 Feb;143(2):322–8.
205. Wang WZ, Fredrikson S, Sun JB, Link H. Lyme neuroborreliosis: evidence for persistent up-regulation of *Borrelia burgdorferi*-reactive cells secreting interferon-gamma. *Scand. J. Immunol.* 1995 Dec;42(6):694–700.
206. Guo BP, Norris SJ, Rosenberg LC, Höök M. Adherence of *Borrelia burgdorferi* to the proteoglycan decorin. *Infect. Immun.* 1995 Sep;63(9):3467–72.
207. Järveläinen HT, Kinsella MG, Wight TN, Sandell LJ. Differential expression of small chondroitin/dermatan sulfate proteoglycans, PG-I/biglycan and PG-II/decorin, by vascular smooth muscle and endothelial cells in culture. *J. Biol. Chem.* 1991 Dec 5;266(34):23274–81.
208. Liang FT, Brown EL, Wang T, Iozzo RV, Fikrig E. Protective niche for *Borrelia burgdorferi* to evade humoral immunity. *Am. J. Pathol.* 2004 Sep;165(3):977–85.
209. Shi Y, Liu CH, Roberts AI, Das J, Xu G, Ren G, et al. Granulocyte-macrophage colony-stimulating factor (GM-CSF) and T-cell responses: what we do and don't know. *Cell Res.* 2006 Feb;16(2):126–33.
210. Wada H, Noguchi Y, Marino MW, Dunn AR, Old LJ. T cell functions in granulocyte/macrophage colony-stimulating factor deficient mice. *Proc. Natl. Acad. Sci. U.S.A.* 1997 Nov 11;94(23):12557–61.
211. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat. Immunol.* 2003 Apr;4(4):330–6.
212. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science.* 2003 Feb 14;299(5609):1057–61.