Immunomodulation and new therapeutic strategies in Lyme borreliosis

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Dedicado a Carlos y a mi familia

List of publications

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- **Diterich, I.** and T. Hartung. 2001. 2001. *Borrelia burgdorferi* s.l., the infectious agent of Lyme borreliosis. *Contrib Microbiol* 8:72-89.

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Abbreviations

Bb	*Borrelia burgdorferi*
BLP	bacterial lipoprotein
CHO	chinese hamster ovary
CpG	synthetical bacterial DNA, oligonucleotides with CG-rich motives
CRASP	complement regulator-acquiring surface protein
ELISA	enzyme-linked immunosorbent assay
GAP-DH	glyceraldehyde-3-phosphate dehydrogenase
G-CSF	granulocyte colony-stimulating factor
hLFA-1	human lymphocyte-function-associated antigen-1
i.v.	intravenous
IFNγ	interferon gamma
IL	interleukin
IRAK	interleukin-1 receptor-associated kinase
LA	Lyme arthritis
LAM	arabinose-capped lipoarabinomannan
LB	Lyme borreliosis
LPS	lipopolysaccharid
LTA	lipoteichoic acid
MAP	mitogen-activated protein
MALP	macrophage-activating lipopeptide
MHC	major histocompatibility complex
mu	murine
MyD88	myeloid differentiation protein
NF-κB	nuclear factor kappa B
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PMN	polymorphonuclear neutrophil
r	recombinant
RT-PCR	reverse transcription polymerase chain reaction
S. aureus	*Staphylococcus aureus*
s.c.	subcutaneous
s.l.	sensu lato

s.s.	sensu stricto
SEM	standard error of the mean
Th	T helper
TLR	Toll-like receptor
TNF	tumor necrosis factor
vlsE	variable major protein-like sequence expressed
vs	versus
WBC	white blood cell

Table of Contents

1 Introduction

1.1 Lyme borreliosis

Lyme borreliosis (LB), which was first described in the mid-1970's, represents the most frequent vector-borne disease in many European countries (1) and the USA (2). In endemic regions of Southern Germany an incidence of LB between 50 – 600 per 100.000 inhabitants was found (3, 4). According to recent studies up to 52% of highly exposed individuals in endemic areas of Baden Württemberg are infected with *Borrelia burgdorferi (B. burgdorferi)* and up to 24% of ticks are infected with this pathogen (5). *B. burgdorferi*, the causative agent of LB, is a corkscrew-shaped bacterium which belongs to the family of *Spirochaetaceae*. Infection which occurs via a tick bite either leads to a subclinical stage or results in a range of clinical symptoms divided into three stages: the early localized, the disseminated and the late, chronic stage. Different organs can be affected, including the heart, joints, skin and central nervous system. Diagnosis of LB still represents a major problem since the commercially available and in-house tests do not offer the desirable standardized performance. If infection with *B. burgdorferi* is not adequately treated, i.e. as early as possible and with the recommended antibiotics, it may lead to a chronic multisystemic disorder, which is difficult to cure.

The most puzzling feature of LB is that *B. burgdorferi* is often not eradicated even in the presence of an active immune response. Several hypotheses have been put forward to explain persistence of Borrelia in the human host during months and years in spite of active immune cells. In the thesis presented here the question how Borrelia persist in the human host was addressed, providing some evidence that the pathogen modulates the host's immune response by inducing anti-inflammatory responses and rendering monocytes and macrophages anergic.

1.2 Toll-like receptors

Recently the toll family of highly conserved transmembrane receptor proteins has been identified. The members of this family share a highly homologous cytoplasmic domain, similar to the IL-1-receptor, a very short transmembrane domain, and an extracellular portion consisting of a various number of leucine-rich repeats. They are expressed on immune cells and on tissue cells and represent a critical link between

immune stimulants produced by microorganisms and the initiation of host defense. Activation of these receptors results in the release of antimicrobial peptides, inflammatory cytokines and costimulatory molecules that initiate adaptive immunity. Up to now at least 10 different members have been identified, which vary in their ligand specificity, expression patterns and presumably in signal transduction pathways, consequently activating different genes. Toll-like receptor (TLR) 4, which was the first TLR found in humans, was identified as the major LPS recognition receptor. TLR2 is characterized by associating with TLR1 and TLR6 and having the broadest spectrum of ligands, including lipoteichoic acid from Gram positive bacteria, peptidoglycan, zymosan and lipoproteins from Treponema, Mycoplasma and also *B. burgdorferi*. However, the role of TLR2 in recognition of other membrane components of Borrelia is not conclusively clear and will be addressed in this thesis.

1.3 Tolerance and cross-tolerance

In its simplest terms “endotoxin tolerance” refers to a hyporesponsive state following a second or additional dose of endotoxin in contrast to the responses observed after an initial exposure to endotoxin. Since its first descriptions in the 1960s extensive research has been undertaken to understand the molecular and cellular background of this phenomenon. The "lipopolysaccharide-tolerant" phenotype of macrophages and monocytes is characterized by reduced TNFα-, IL-1β- and IL-6-release, enhanced cyclooxygenase-2 activation, inhibition of mitogen-activated protein kinase activation, and impaired NFκB-translocation upon re-stimulation with LPS. Similarly as human monocytes and macrophages can become tolerant *in vitro*, monocytic cells from patients with systemic inflammatory response syndrome and sepsis have many characteristics of endotoxin tolerance. It is postulated that the clinical significance of this desensitization is a natural regulatory mechanism aimed to control an otherwise autodestructive sytemic inflammation. Recently, increasing evidence is coming up showing that other stimuli than LPS including lipoteichoic acid, *Staphylococcus aureus*, macrophage-activating lipopeptide (MALP) -2, bacterial DNA (CpG), and arabinose-capped lipoarabinomannan (LAM) render macrophages anergic to each other as well as to LPS. Based on these findings the term “cross-tolerance” or “hetero-tolerance” has been coined to describe tolerance induction between different stimuli. In the present study it was investigated whether *Borrelia burgdorferi* also has the capacity to desensitize macrophages. Since Borrelia-induced

hyporesponsiveness could represent a mechanism enabling the survival of this pathogen in the host despite the presence of immune cells.

1.4 Granulocyte colony-stimulating factor (G-CSF)

In line with its name the granulocyte colony-stimulating factor (G-CSF) is a hematopoietic growth factor which recruites granulocytes from the bone marrow. However, since its discovery two decades ago, much more functions of this pleiotropic protein have been described. In addition to controlling production and maturation of neutrophilic granulocytes in the bone marrow it also primes mature granulocytes resulting in an increased oxidative burst or phagocytosis. Furthermore, it exerts pronounced anti-apoptotic effect on these cells. While G-CSF acts on neutrophils as a pro-inflammatory cytokine by augmenting their bactericidal functions, it influences monocytes with its anti-inflammatory properties, reducing their release of pro-inflammatory cytokines such as TNFα, IL-12 and IL-1β. The lymphocytes seem not to be influenced directly by G-CSF, but lacking monocyte factors attenuate IFNγ-formation.

Due to its unique anti-infectious and hematopoietic pharmacological properties on the one hand, and the fact that it is very safe on the other hand, G-CSF has become one of the most prominent endogenous proteins produced biotechnologically in broad clinical use. In this work G-CSF was employed in combination with antibiotics as an immunosupportive treatment to test a novel therapy for late stage LB.

1.5 Aims of the study

Lyme borreliosis (LB) is the most common tick borne disease in European countries and the United states. An incidence of 50 – 600 per 100.000 inhabitants has been reported and up to 24% *Borrelia burgdorferi*-infected ticks were found in highly endemic areas. Since the first description of LB in the mid 1970s, it is still unclear how Borrelia persist facing the bodies phagocytic and other immune clearance mechanisms. They sometimes even persist in case of antibiotic therapy. Thus, understanding the immunopathology of Borrelia infection represents still a major challenge.

A vaccine, which was only partially effective since it exclusively protected against infection with *Borrelia burgdorferi* s.s., but not against the two other strains pathogenic for humans in Europe, has been available for about three years. However, since its withdrawal in spring 2002 no vaccine exists to protect population from this infection. The lack of a vaccine on the one hand and the sometimes unsatisfactory treatment on the other hand, illustrate the need to find new concepts in treatment of this disease. Since a better understanding of immunopathology of LB is important for novel therapeutic interventions, immune avoidance of Borrelia was examined in the first part of the present thesis: In particular the following issues were addressed:

- Characterization of the immune response of blood leukocytes from late stage LB patients in comparison to healthy donors
- Comparison of Borrelia- and LPS-induced cytokine release in blood from healthy donors
- Characterization of Borrelia-induced immunosuppression as a possible immune evasion mechanism
- Characterization of Borrelia-induced anergy of blood leukocytes in an *in vitro* model of immunomodulation and comparison with endotoxin tolerance

Based on the results of the first part, the aim of the second part was to propose a new concept in LB therapy. The procedure was as follows:

- Establishing the mouse model of Lyme borreliosis
- Testing the effect of immunosupportive treatment with the hematopoietic growth factor G-CSF (Filgrastim) on ankle swelling and bacterial burden in two different Borrelia-infected mouse strains

These studies represented the basis for a clinical trial, in which the combination therapy of antibiotics *plus* Filgrastim in a late stage LB patient was tested.

2 *Borrelia burgdorferi* s.l., the infectious agent of Lyme borreliosis

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2.1 Introduction

Classical symptoms and manifestations of Lyme borreliosis (LB) were first described at the beginning of the 20th century in Sweden (6) and Germany (7), and an association to a tick-borne non-pyogenic bacterium responsive to penicillin was postulated (8). In the mid-1970's, in Lyme, Connecticut, USA, the rheumatologist Allen Steere observed a geographic clustering of children with juvenile rheumatoid arthritis, which was often preceded by a distinctive skin rash, the Erythema migrans (EM), and linked to antecedent tick bites. The multisystemic nature of the illness was recognized, with adoption of the term Lyme disease (or Lyme borreliosis) (9).
In 1983, W. Burgdorfer detected spirochetes in the midgut of the tick *I. ricinus* and identified this ectoparasite in this way as a vector of the newly described LB (10). A few years later, Barbour succeeded in culturing the spirochetes in a modified Kelly's medium (BSK).

2.2 Transmission vectors

Different authors report that transmission of LB bacteria to humans occurs by birds and insects such as mosquitoes, flies and fleas, however, the most important vectors for these bacteria are the ticks. Humans do not represent the natural hosts for these ectoparasites which normally feed on mice, deer, other mammals and birds. The presence of *Borrelia burgdorferi* sensu lato (*B. burgdorferi*), the causative agent of LB, has been shown in many different tick species, but not all of them transmit the bacteria effectively. Four species have been identified as the main vectors for LB:

Ixodes ricinus (I. ricinus) and *I. persulcatus* in Eurasia, and *I. scapularis* and *I. pacificus* in the United States.

Ticks have a three-stage life cycle (Figure 2.1), which extends over a period of two to six years and begins in spring when the larvae hatch from a batch of about 2000 eggs deposited by a female tick in the ground in fall. After their first feed (preferentially on small rodents) they molt into nymphs and are inactive during the winter. In the following early spring, the nymphs feed, usually on small mammals and humans, and molt into adults in fall. These adult ticks attach to larger animals, especially deer, where they feed and mate. Finally, female ticks drop and lay their eggs on the ground, thus the cycle starts again in the next spring with the hatch of the larvae.

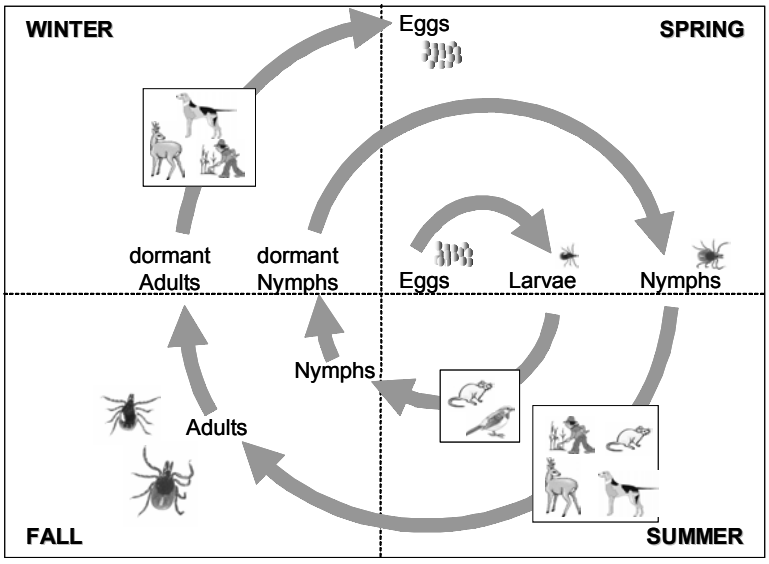

Figure 2.1. Life cycle of Lyme borreliosis ticks

During each of the three stages, ticks feed only once on an animal host and this is when infection occurs. After a tick feeds on an infected host, the bacteria remain confined to the midgut of the tick until the next feed. When the tick, in its next stage, attaches to another host and blood enters the tick gut, the bacteria migrate to the tick's salivary glands and are injected with its saliva into the host.

2.3 Pathogen: *Borrelia burgdorferi* s.l.

Borrelia burgdorferi, the causative agent of LB, is a Gram-negative, corkscrew-shaped, microaerophilic bacterium which belongs to the family of *Spirochaetaceae*. Like all spirochetes, *B. burgdorferi* has a protoplasmic cylinder that is surrounded first

by a cell membrane, then by 7 to 11 periplasmic flagellae, and finally by an outer membrane, that is only loosely associated with the underlying structures. The outer membrane of *B. burgdorferi* is composed of 51% lipids, 46% proteins and 3% carbohydrates. The outer membrane of *B. burgdorferi* is made up of at least 30 different immunogenic proteins, including major outer surface proteins and prominent antigens such as OspA (30 kDa), OspB (34 kDa), and OspC (23 kDa), OspD (28kDa), OspE (19.2 kDa), OspF (26.1 kDa), OspG (22 kDa) and a 93 kDa-protein. Quantitative and qualitative differences in the protein profile have been described between isolates of *B. burgdorferi* genospecies from Europe and the United States.

The sequencing of the complete genome of *B. burgdorferi* s.s., including its various plasmids was achieved by C.M. Fraser et al. (11), demonstrating that *B. burgdorferi* has a linear chromosome (910 725 base-pairs) and 11 plasmids. Unexpectedly, the genome did not contain high numbers of putative virulence genes, however, it was full of multi-copy plasmid-encoded genes for the proteins of the outer membrane. The function of these extrachromasomal genes is still unclear, but it is hypothesized that they determine the antigenic identity of these organisms and are responsible for adaptive antigenic variation.

At present, *B. burgdorferi sensu lato* can be divided taxonomically into at least ten different species, which are often restricted to different “continents”: *B. burgdorferi sensu stricto* is present in Europe and in USA, but absent from Russia and Asia. The genospecies *B. garinii, B. afzelii, B. valaisiana* and *B. lusitaniae* are found in Eurasia whereas *B. japonica*, *B. tanukii* and *B. turdae* are restricted to Japan and finally *B. andersonii* and *B. bissettii* are only present in the USA. Moreover, a number of genomic groups, not yet named, increase this diversity.

Only three of the 10 different Borrelia genospecies, are undoubtedly pathogenic for humans: *Borrelia burgdorferi sensu stricto*, *B. garinii*, and *B. afzelii*. The other genospecies have until now not been isolated from human cases of LB and are only known from isolates obtained from ticks or wild animals. Indirect serologic methods as well as PCR results suggest that *B. valaisiana* could be associated with pathologic symptoms. In Europe and Asia, *B. valaisiana* has been isolated from different tick species and recently this genospecies has been reported to be most common in ticks in Ireland. The least information is available for *B. bissettii*, a species mostly encountered in California. No strain belonging to this species has been isolated from a human patient in USA, although rare cases of human disease due to this species

have been reported in Europe (12). Reports on *B. lusitaniae* are still rare, and only a few strains have been isolated from ticks in Portugal, Central Europe, and Tunisia.

2.4 Lyme borreliosis

2.4.1 Incidence and seroprevalence of Lyme borreliosis

At present LB is the most common arthropod-borne infectious disease in temperate climate zones around the world. Clinically confirmed cases of LB have been reported all over Eurasia (13), (14), and the USA (15). According to the Center for Disease Control and Prevention (CDC), LB accounts for more than 95% of all reported vector-borne illness in the United States and the overall incidence rate of reported cases is about 5 per 100,000 population and year. It is evident that the prevalence of Lyme borreliosis varies considerably in different European countries with an overall increasing prevalence from west to east. The overall incidence rate of reported cases in Germany is approximately 25 per 100.000 habitants (16), and in some high endemic areas there is a seroprevalence of 17% (17).

Table 2.1. Estimated annual incidence of Lyme borreliosis in selected European countries[a]

Country	Incidence per 100,000	Annual number of cases
UK*	0.3	200
Ireland	0.6	30
France	16.0	7200
Germany	25.0	20000
Switzerland	30.4	2000
Czech Republic	39.0	3500
Bulgaria	55.0	3500
Sweden (south)	69.0	7120
Slovenia	120.0	2000
Austria	130.0	14000

[a]based on Report of WHO workshop on Lyme borreliosis Diagnosis and Surveillance, Warsaw, Poland, 20-22 June, 1995, WHO/CDS/VPH/95. (1996) 141-1.

It should be taken into account, that the epidemiological data are mostly based on heterogeneous studies using either direct methods such as prospective clinical studies or indirect methods such as the measurement of seroprevalence, the assessment of the abundance of ticks in general or the prevalence of *B. burgdorferi*-infected ticks. Additionally, the data are misrepresented by the difficulty of serodiagnostic criteria, the under-reporting of EM by the patient and the incoherence

of seropositivity and disease outcome. Probably the best method to obtain “correct” epidemiological data is to combine different methods, i.e. to assess the prevalence of *B. burgdorferi*-infected ticks and to correlate these data to seroprevalence studies in the same region.

The transmission occurs through salivation during the feeding process on an animal host, however, the transmission risk depends on the duration of tick feeding. Apparently the time of transmission varies between the United States, where it usually takes place after the tick has been feeding for more than 36 hours (18), and Europe, where it has been reported that transmission can already occur after 24 hours (19). In general, it is assumed that no infection is transmitted during the first 12 hours after a tick bite.

2.4.2 Borrelia: an emerging pathogen?

Since surveillance of LB was initiated in the mid-1980’s, the annual average incidence of reported cases has continually increased. This appears even more surprising as knowledge, attitude and behavior of both clinicians and the public towards the disease has improved vastly in the last few years. There are different possible explanations for this increase: First, the development of better serological methods in diagnosis, the optimization of clinical case definitions, and further the improved information about the distribution of genospecies, ticks and reservoir hosts, might have led to less false negative and to more accurate diagnosis. In the United States and Europe the incidence of new LB cases per population and their geographical distribution are continually monitored on a national level. Public health authorities must rely on laboratory and physician reports to evaluate trends, identify areas of high and low risk and develop new strategies in disease control. However, there is still considerable underreporting and surveillance methods still vary among different countries.

Second, some authors believe that human demographics, including reforestation and suburban migration lead to increased human exposure to ticks and thus to higher risk of developing LB. According to their opinion, habitats with large numbers of infected ticks are increasingly frequented as a consequence of the changed outdoor recreational activities and occupations in the last few years. Thus conflicting views exist in this respect.

In addition to the overall trend of increasing LB incidence in already established endemic areas, there is also a geographic spread of Bb to new areas. This could be due to the growing mobility of host population including pets and other animals. Different measures have been explored to eliminate deer and rodents, known to be the main hosts for ticks, from high endemic areas. However the results of these experiments were never satisfactory as many other animals are reservoir hosts and the methods were not practical for large-scale use. Measures to control the tick population have also been tried, which also did not lead to convincing results. At present, the most effective preventative measures to stop the increasing incidence of LB is to inform the public about the disease and increase the awareness of LB.

2.4.3 Diagnosis

At present diagnosis of LB still represents a major problem since the commercially available and in-house tests still vary considerably in their specificity and sensitivity and therefore do not meet the desirable standardized performance. False positive tests, resulting for instance from cross-reactivity, lead to misdiagnosis and inappropriate treatment. False negative results, originating from the lack of sensitivity, have more serious effects for the patients since the disease might develop into a chronic stage, which is more difficult to treat. Although clinical manifestations of the illness are variable and rarely exclusive for *B. burgdorferi* infection, diagnosis must be made in the light of careful evaluation of the patient's clinical history, physical findings, laboratory evidence and exposure risk evaluation. On the other hand, infection with *B. burgdorferi* should not be excluded if awareness or recollection of a tick bite are not present as this is not always the case.

2.4.3.1 Microbiological detection method

The isolation of the causative agent in culture is a direct method to detect the live pathogen. The disadvantage is that it is expensive, time consuming and difficult, because of the need for a special bacteriologic medium and laborious observation of cultures. *B. burgdorferi* can be cultured from 80% or more of biopsy specimens taken from early Erythema migrans lesions, but only from about 10% of cerebrospinal fluid (CSF) samples.

2.4.3.2 Serology

The most commonly used laboratory test is currently the detection of antibodies against B.b. in patient's serum or CSF by staining methods. The limitations of this test are the delay in the development of an antibody response, cross-reactivities with other organisms, difficulty in distinguishing past from present infections, and lack of sensitivity and standardization.

A two-step serological approach has been proposed to increase specificity of diagnosis. It is recommended to examine first with a sensitive first test, either an enzyme-linked immunosorbent assay (ELISA) or an indirect immunofluorescence assay (IFA), followed secondly by testing with the more specific Western immunoblot, to corroborate unclear or positive results obtained with the first test. In this second test, IgM or IgG antibodies against individual Bb antigens, which have been separated by gel electrophoresis, can be detected. The Western immunoblot is a suitable procedure to distinguish differentiated immune responses, but the interpretation of the number and intensity of bands must still be standardized.

Except for early and late neuroborreliosis, where antibody production should better be tested in cerebrospinal fluid, serum is the convenient clinical specimen for the detection of *B.b*.-specific antibodies. Patients with early, disseminated or late-stage disease usually have strong serological reactivity and demonstrate specific antibody binding patterns to *B. burgdorferi* antigens. Thus, antibiotic treatment in early, localized disease may blunt or abrogate the antibody response and lead to false-negative results. Further, it is important to consider that antibodies often persist for months or years following successfully treated or untreated infection. This reveals that seroreactivity alone cannot be used as a marker of active disease.

2.4.3.3 Molecular biological detection method by polymerase chain reaction

Diagnosis by polymerase chain reaction (PCR), a method which amplifies genomic DNA of *B. burgdorferi*, is receiving increasing attention, but so far it has not been standardized for routine diagnosis of LB, as the results are still not sufficiently reliable to be used on their own. Skin, blood, urine, cerebrospinal and synovial fluid are the clinical specimens that can be utilized for the detection of Borrelia DNA. Different target sequences (e.g. rRNA genes, intergenic spacers, fla and OspA genes) are currently used. The advantage of PCR is represented by its high sensitivity and the possibility to sequence the PCR amplificate to determine Borrelia subspecies.

Interestingly, the PCR result in the synovial fluid rapidly turns negative after successful antibiotic therapy (20). On the other hand, PCR cannot distinguish between live and dead organisms and it is very susceptible to false-positive results.

2.4.3.4 Species-specific diagnosis

As has been shown in 4.3. up to now no laboratory LB test is definitive nor valid as a "gold standard". Consequently, the main objective is to develop new approaches in LB diagnosis, such as species-specific testing. Increasing data indicate that particular clinical manifestations are associated with different species of *B. burgdorferi* (see chapter 4.5.). Therefore, detection of the given genospecies in the patients' specimens might allow a more systematic and accurate treatment of clinical manifestations. A useful method to detect *B. burgdorferi* genospecies in clinical samples could be "real-time Polymerase Chain Reaction (PCR)", offering the advantage, that different species can be tested simultaneously in one single run by melting point analysis.

Another important point to address is the considerable polymorphism of antigen composition among the individual genospecies. Taking this into account, an interesting approach would be the use of different Borrelia antigens in each endemic region to detect antibodies in patient serum from the corresponding area.

2.4.4 Clinical manifestations

Infection with *Borrelia burgdorferi* can be subclinical, or result in a range of clinical symptoms, depending on the length of time after the infection and the organs affected. Clinical presentations can generally be divided into three stages (Figure 2.2): the early, localized, the early, disseminated and the late, chronic stage. If left untreated, the illness progresses from an acute to a chronic stage. The intervals between these stages can vary considerably in their duration. Furthermore, some patients present with late manifestations without having experienced, or noticed, early stage symptoms.

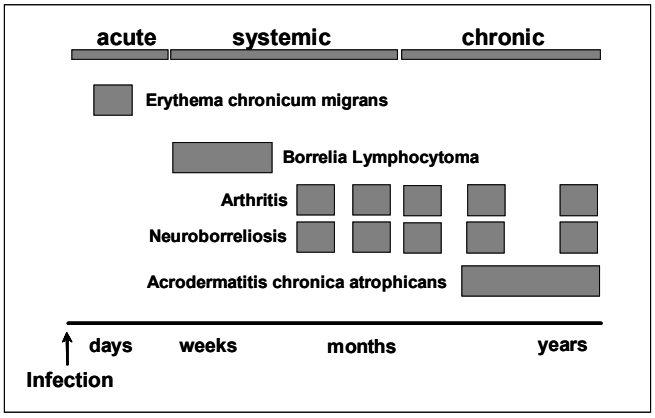

Figure 2.2. Clinical course of Lyme borreliosis

2.4.4.1 Early, localized stage

A typical early symptom of LB, is a slowly expanding red rash (EM), often with central clearing at the site of the tick bite. The rash only emerges in about 50% of all cases, it usually appears within a week to a month after the bite and then slowly expands over several days. The cause for this characteristic skin manifestation is the infiltration of plasma and immune cells, which are recruited to attack the pathogen. The EM, which resolves spontaneously within three to four weeks, is sometimes overlooked by the affected person. General flu-like symptoms such as headache, myalgia, arthralgia, fever and stiff neck can also appear at this stage.

2.4.4.2 Early, disseminated stage

The generalized stage, which starts after about 8 to 10 weeks after the infection, is marked by a bacteremia, this means a systemic spread of the pathogen in the body. The spirochetes disseminate via the bloodstream and lymphatic system to multiple sites, including the heart, the liver, and the nervous system. Manifestations of this stage may include multiple secondary skin lesions (lymph adenoma), mild hepatitis, carditis, arthritis with effusion (joint swelling) and a spectrum of neurologic abnormalities.

2.4.4.3 Late, chronic stage

If left untreated, *B. burgdorferi* infection may progress to the late disseminated stage weeks to months or even years after infection. The most common presentation at this stage is chronic Lyme arthritis, which is characterized by intermittent swelling and pain of one or a few, usually large, weight-bearing joints such as the knees.

Another presentation is acrodermatitis chronica atrophicans (ACA), an unusual skin affection characterized by a lymphocytic infiltration, which is mixed with plasma cells, in the dermis and often also in the subcutis. The long-standing red discoloration, usually on extensor surface of extremities, sometimes with dough-like swelling, ultimately becomes atrophic. It is characterized by a violaceous plaque with epidermal atrophy, hyperkeratosis, and destruction of the epidermal appendages.
Finally, late stage neurological disorders include chronic Lyme meningoencephalitis, chronic axonal polyneuropathy, or encephalopathy, the latter usually manifested by cognitive disorders, sleep disturbance, fatigue, and personality changes. LB is not fatal, however the damages which occur during an infection are often irreversible and can be disabling.
There is increasing evidence that each Borrelia genospecies correlates with different disease manifestations of LB, i.e. *B. afzelii* is often associated with symptoms of ACA (21), which is relatively common in central Europe and in Scandinavia, but very rare in the United States. In western Europe neurological symptoms seem to be the most common manifestation and they are most frequently associated with *B. garinii* (22). Finally, infection with *B. burgdorferi sensu stricto* appears to lead more frequently to arthritis, which is the most frequent manifestation in the United States (23). The pathognomic symptom Erythema migrans occurs after infection with all three species showing that there also exists overlap between the species in relation to infection associated symptoms.

2.5 Immunpathogenesis and persistence

Although a Borrelia infection leads to activation of monocytes and granulocytes, and further induces a prominent antibody and T helper (Th) cell cytokine response in humans, no protective immunity is conferred, indicating that Borrelia-induced activation of the immune system alone is not sufficient to eradicate the pathogen and to protect against ongoing infection. Several hypotheses have been suggested to explain the persistence of Borrelia in the human host (Figure 2.3): Localization of the spirochetes in immunepriviledged sites, such as intracellular compartments (24), as well as in the extracellular matrix (25), has been shown. Others suspect a high variation of surface antigens in *B. burgdorferi* (26), similar to *Borrelia hermsii* which causes relapsing fever (27). Furthermore, a shift in the T helper cell response is discussed as the cause of the treatment resistant form of LB (28). A self-propagating

induction of autoimmunity following infection with Borrelia spec. represents an alternative explanation for the development of chronic disease. This view is corroborated by the finding of homology between the Borrelia outer surface protein A (OspA) and the human LFA-1 antigen (29). Another hypothesis is that the host's immune response is modulated by the pathogen (30, in press): based on the ability of microorganisms to shift or suppress the host's immune response in a direction favorable to the survival of the pathogen has been shown for viral, bacterial and parasitic infections, and has hence led to the concept of microbial modulins. Indeed there is some evidence that patients with persistent LB show an impaired immune response not only to Borrelia but also to other bacterial stimuli (13).

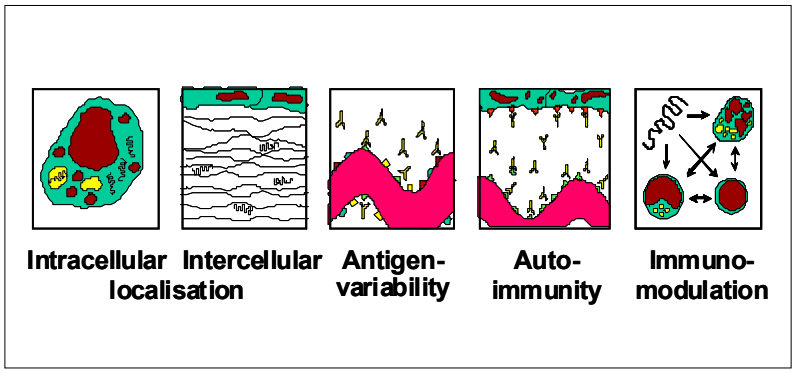

Figure 2.3. Hypotheses on the cause of persistent Lyme borreliosis

2.5.1 T-cell response

The first indications for the involvement of a cellular immune response in LB evolved from animal models, showing that arthritis-susceptible mouse-strains predominantly produced T helper 1 (Th1) cytokines, whereas in arthritis-resistant mice mainly T helper 2 (Th2) cytokines were released (31). Later, further evidence that the cellular immune response against *B. burgdorferi* is predominantly characterized by a T helper cell type 1 pattern was found by Gross et al. demonstrating that in synovial fluid specimens from patients with arthritis the Th1-like cytokine pattern dominated (32). In accordance with these results, others could show in a mouse model, that the Th1-response during a Borrelia infection and the severity of arthritis could be reduced by treatment with anti-IL-12 (33).

In summary, the preferential Th1-like situation associated with LB reflects that T-cells seem to have a modulating capacity, leading to an imbalance between Th1/Th2

cytokine response. Accordingly, pathology of LB could partially be a consequence of changes in T-cell-subsets.

2.5.2 Phagocytosis

Electron microscopy studies revealed that professional phagocytes incorporate the long and thin Borrelia through active uptake processes, either by conventional phagocytosis (34) or by one of the two preferential processes: „coiling phagocytosis“ (35), which is used by monocytes, and “tube phagocytosis” seen with granulocytes (36). Coiling phagocytosis seems to be a mixture of macropinocytosis and conventional phagocytosis. Briefly, spirochetes attach to the phagocyte and are then wrapped in single folds of the plasma membrane, called pseudopod coils, which enroll the bacteria in multiple turns until finally engulfing them completely. “Tube phagocytosis”, which takes approximately 20 minutes, starts with a head-on attachment of Borrelia to the neutrophils, which induces the latter to form a thin, tube-like protrusion surrounding increasingly the often intensively moving spirochete and finally covering them completely. Finally, the bacteria are drawn into the cell and the tube is retracted.

Lysosomal (34) and non-lysosomal degradation of Borrelia in the phagocytes (37) have been reported. Moreover NO and oxygen radicals seem to be involved in the killing of Borrelia in the macrophages (38). Suhonen et al. observed that neutrophil functions such as oxidative burst, calcium mobilization and phagocytosis are induced by Borrelia in a complement-dependent manner (39). At present, not very much is known about the phagocytosis-promoting receptors for Borrelia. There is some evidence that the integrin CR3 (39) and the Fc-receptor (40) seem to be receptors involved in the interactions and adherence of the spirochete and the phagocytes. Others observe that non-Fc-mediated phagocytosis takes place when the spirochetes are not opsonized (41). The identification of Borrelia receptors and the regulatory mechanisms involved in phagocytosis still have to be elucidated, which will probably lead to a better understanding of the pathology of LB in humans.

2.5.3 Inflammation versus anti-inflammation

LB is characterized by a puzzling discrepancy: Borrelia persist quietly during long periods at lesional sites without obviously activating the immune system, but on the other hand, they are capable of inducing a strong local inflammatory reaction during

short lasting clinical manifestations leading to tissue damage, which can be detected clinically and histologically. Different authors could show that isolated lipoproteins from the outer membrane of *B. burgdorferi*, strongly activate monocytes in vitro, resulting in a strong pro-inflammatory response, characterized by the release of TNFα, IL-1β and IL-6 (42), (43), (44). In line with these observations is the finding that treatment with the anti-inflammatory IL-11 reduced arthritis in murine LB (45). However, the induction of a strong pro-inflammatory response conflicts with the persistence of the spirochetes in the tissues during months and years in the presence of active immune cells. Importantly, increasing evidence is coming up showing that Borrelia also induce anti-inflammatory features such as the release of anti-inflammatory cytokines i.e. IL-10 in PBMC (peripheral blood mononuclear cells) from humans and rhesus monkeys (46). Additionally, the cytokine pattern induced by Borrelia lysate in human whole blood differed significantly from that induced by bacterial endotoxins, showing a reduced release of TNFα and IFNγ versus an enhanced secretion of IL-10 and G-CSF (30). The anti-inflammatory influence of Borrelia is also reflected by the reduced release capacity of pro-inflammatory cytokines in ex-vivo stimulated blood from patients with persistent LB, in comparison to the blood from healthy volunteers (30). Further a decrease in expression of MHC markers on Langerhans cells in skin of ACA patients has been observed. In summary, the data about the pro- and anti-inflammatory effects of Borrelia on immune cells are still ambiguous. However, anti-inflammatory changes elicited by Borrelia might favor the persistence of the spirochete, and in this way contribute to its pathogenicity.

2.6 Therapy

2.6.1 Prophylaxis

The use of antibiotics following a tick bite is not generally recommended for the following reasons: The results of different studies, carried out to assess the value of the prophylactic use of antibiotics immediately after tick removal, were very contradictory. Further, only a minority of ticks except in few endemic areas are infected and finally infection can be prevented by prompt removal of the tick. The immediate, correct (no squeezing, no oil, no glue) removal of the tick appears to be the most effective prophylactic measure.

2.6.2 Antibiotic treatment

Important strategies to avoid the difficulties and costs of complicated and late-stage LB are the early, correct diagnosis and proper antibiotic treatment. Treatment is recommended for patients showing symptoms with adequate supporting laboratory evidence for diagnosis to prevent possible progression of the disease. A range of antibiotics are available (tetracyclines, penicillin, cephalosporins, etc.) and their selection and use vary in different countries. However, different studies have shown quinolones, first–generation cephalosporins, rifampicin, and aminoglycosides are not sufficiently effective in their activity against *B. burgdorferi*. Furthermore, the combination, prolongation (>1month), or repetition of antimicrobial therapy in different studies led to contradictory data. Within 24h of the start of antibiotics, patients may transiently have intensified signs and symptoms consistent with a Jarisch-Herxheimer reaction. Prognosis is good for most persons treated early and correctly, however about 10% of LB patients do not respond sufficiently even to repeated antibiotic treatment. Different hypotheses are currently discussed to explain the reason for these treatment resistant cases (20), (26).

2.6.3 Vaccine

The development of safe and effective vaccines is of great importance due to the difficulties presented by both the diagnosis and treatment of LB. Interest has focused on several highly immunogenic outer surface proteins (OspA, B, C) and the most intensively studied of these is OspA. A vaccine for use in humans is now available in the United States (LYMErix™, Smithkline Beecham Pharmaceuticals). It is made from lipidated rOspA of *B. burgdorferi* sensu stricto. Evidence from several studies in animals indicates that rOspA vaccine may exert its principal protective effect by eliciting antibodies that kill LB spirochetes within the tick gut, when the ingested blood meets the bacteria.

LYMErix™ is administered by intramuscular injection. Three doses are required for optimal protection: the first dose should be followed by the second dose after one and the third dose after 12 months. The safety and immunogenicity of alternate dosing schedules are currently being evaluated and approval of this vaccine was guarded as there are still doubts about the long-term usage of the product (47).

The OspA vaccine is designed for USA where *B. burgdorferi* s.s. (OspA serotype 1) appears to be the only human pathogen of LB. Unfortunately, antibodies generated

against this OspA serotype are not cross-protective, since this outer surface protein varies considerably between the different Borrelia species. Furthermore, it is only expressed in the tick, but not in humans, as a shift from OspA to OspC occurs following transmission into the host's blood.

Since European *B. burgdorferi* s.l. appears to be much more heterogeneous, it will probably be necessary to produce a more complex mixture of immunogenic proteins to achieve full protection in Europe. The necessity for vaccination is likely to vary considerably in different areas. Therefore, the geographic risk as well as a person's activities and behaviors relating to tick exposure should be taken into account before the use of the vaccine is recommended.

2.7 Conclusion, perspectives

2.7.1 Co-infection and co-transmission

The tick vector of *B. burgdorferi* s.l., *Ixodes ricinus*, also transmits separately or simultaneously to LB-bacteria other zoonotic organisms, including *Babesia*, *Ehrlichia* and encephalitis viruses (48), (49). Co-infections of these pathogens in humans have been documented. The impact of these co-infections on the clinical course of LB are still uncertain, but actually it has been suggested, that they often interact with LB diagnosis and epidemiology and lead to a confusing mixture of manifestations in patients. Sometimes the manifestations caused by different pathogens overlap and one of the infections is overlooked, resulting in failure to provide appropriate treatment. Cross-reactions are very probable and often lead to false positive serodiagnosis. Another important observation is that some of the zoonotic pathogens (e.g. *Ehrlichia* and *Babesia*) transmitted by *I. ricinus* are known to be immunosuppressive, therefore they may affect the severity and duration of infection of co-transmitted pathogens.

2.7.2 Immunomodulation versus host-predisposition

Recent findings indicate that LB development is on the one hand affected by Borrelia-derived components, which may be responsible for the infectivity of the spirochetes, and on the other hand by host-derived factors, actually influencing the disease pathology (50).

Borrelia-derived components include the immunogenicity of the outer surface proteins, the competence to disseminate in the host and the resistance to the hosts complement system (51). Experiments with low and high passaged Borrelia showed, that with increasing passage number outer surface protein expression varies and infectivity decreases considerably.

Host-derived factors seem to be important determinants for the pathology of LB. The control of a Borrelia invasion is characterized by the release of cytokines and of oxygen radicals and the upregulation of adhesion molecules. Depending on the magnitude of the inflammatory response its result can either be the resolution of infection or, if the immune response is excessive, damages such as arthritis, or if the response is too weak, ineffective clearance of the spirochetes.

Important insight into host factors which are responsible for the development of LB arose from studies in the murine model of LB. As, after a subcutanous inoculation of spirochetes, some inbred mouse strains only develop moderate and others severe arthritis, mice were grouped according to their susceptibility towards a Borrelia infection. C57Bl/6 and Balb/c mice do not develop disease, but interestingly they have the same spirochete burden as C3H/HeN mice which are susceptible to Borrelia infection and develop strong arthritis. The genetic resistance of Balb/c mice seems to be associated with the expansion of a Th2 (IL-4 producing) subset of lymphocytes. In contrast C3H/HeN mice show the expansion of a Th1 lymphocyte subset with concomitant IFNγ production (52). The immunomodulating role of IL-10 in LB was revealed in experiments with C57Bl/6J IL-10 knockout mice, which developed a more severe arthritis than the resistant wildtype C57Bl/6J. Unexpectedly, this increase in arthritis was associated with a significant decrease in spirochete burden in the knockout mice in comparison to the wildtype strain. The authors hypothesized that the reduced arthritis in the resistant C57Bl/6J mice is related to enhanced levels of Borrelia induced IL-10, which modulates the inflammatory reaction inhibiting cytokine release and at the same time reducing the killing and elimination of the pathogen (53).

Thus, the host response to *B. burgdorferi* is likely to play a role in the pathogenesis of LB. Such predisposing host factors for the development of a persistent infection despite treatment with antibiotics may be genetic and immune factors leading to a greater susceptibility. In 1990, Steere et al. already postulated that in individuals with the major histocompatibility complex class II alleles HLA DR2 and HLA DR4, failure

of antibiotic therapy is more likely to appear (54), and a few years later the IgG-reactivity against OspA was identified as another “risk factor” for a lack of response to therapy (55).

In addition, there is also some evidence that the activation of potentially autoreactive T-cells may be responsible for chronic, treatment resistant LB, as in some patients spirochetes could not be detected, although manifestations of Lyme arthritis were obvious. A homology search to the immunodominant epitope of OspA revealed the human leukocyte function-associated antigen-1 (hLFA-1) as a candidate autoantigen, which might be able to induce cross reactivity, providing a model of molecular mimicry in the pathogenesis of LB. In line with the sequence homology the authors also detected a specific T-cell response to hLFA-1 which was exclusively found in patients with treatment resistant arthritis.

Information gained from the published genome sequence of *B. burgdorferi* combined with further experimental results, will provide new insights into the pathogenesis of LB, which could help to clarify how this pathogen persists in its natural reservoir and hosts, and further how it infects humans, interacts with the host defense or avoids it. These new insights into the pathogenesis will lead us to novel diagnostic, preventative and therapeutic methods.

2.8 New therapy concepts

The OspA vaccine protects humans from *B. burgdorferi sensu stricto* infection, but not from *B. garinii*, and *B. afzelii* infection, which are the most frequent ones in Europe. Further, the vaccine is not useful, if the infection is already established and treatment is needed to cure the illness. Therefore, the aim should be to identify new targets existing in all strains pathogenic for human, which could also be used to treat ongoing infections and thus achieve full protection.

chronic LB. As we mentioned in (chapter) 6.3. OspA is exclusively protective against *B. burgdorferi sensu stricto* infections because of the strong antigenic variation of this outer surface protein between the different species. Recently, new targets have already been tested for the development of a novel vaccine, including vaccination with plasmid DNA containing the ospA gene, which lead to protection against *B. burgdorferi* infection in the mouse model. Further pG, a novel lipoprotein which is preferentially expressed in the host, was identified. Infection of mice with Borrelia resulted in the induction of specific antibodies against it. A drawback of pG is its

heterogeneity among the different Borrelia species. Promising data showing efficient protective immunogenicity of the *B. burgdorferi* adhesin decorin-binding protein A (DbpA) in the murine model were questioned by others demonstrating, that the protective antibody response was only induced by needle inoculation of cultivated spirochetes, but not by infestation with infected ticks. Therefore, the suitability of this protein for immunoprophylaxis in LB was retracted. Finally, another vaccine, based on OspC, which is one of the most variable of the immunogenic outer surface proteins, but which is expressed by spirochetes during active infections of humans, is under development. Mouse experiments showed, that immune sera to recombinant OspC led to resolution of chronic arthritis, and further to clearance of disseminated spirochetes in infected mice (56). Thus, new strategies in the development of a vaccine, characterized by conferring full protection and suitable for therapeutic use are underway, but at present their effectiveness is limited to experiments in mice.

New antibiotics which are selective for Borrelia have not been presented yet. Currently, the incidence of treatment resistant LB obviously does not prompt such developments. However, the growing awareness of Borrelia-induced complications and treatment deficits might further the development of such agents.

Since there is increasing evidence indicating that pathology of LB is associated with modulation of the host's immune response adjuvant immunotherapy should be taken into account as an additional therapy to vaccination and antibiosis. The reconstitution of the patients immune competence e.g. with immunosuppressive or activating cytokines represents an attractive target for supportive treatment to antibiosis in chronic LB.

3 Modulation of cytokine release in ex vivo stimulated blood from borreliosis patients

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3.1 Abstract

In LPS-stimulated blood from 71 late stage borreliosis patients, the ex vivo cytokine release capacity of TNFα and IFNγ was reduced to 28 ± 5% and to 31 ± 5% ($p \leq 0.001$), respectively, compared to that of 24 healthy controls. White blood cell counts were normal in both groups. In order to investigate direct interactions between the pathogen and the immune cells, blood from healthy controls was exposed in vitro to live or heat-killed Borrelia, or to Borrelia lysate. Compared to the pattern induced by bacterial endotoxins, a reduced release of TNFα and IFNγ versus an enhanced secretion of IL-10 and G-CSF was found. In blood from 10 borreliosis patients stimulated with Borrelia lysate, TNFα formation was decreased to 31 ± 14% and IFNγ to 8 ± 3% ($p \leq 0.001$) compared to the cytokine response of blood from healthy controls (n=24). We propose to consider anti-inflammatory changes elicited by Borrelia of the blood cytokine response capacity as a condition that might favour the persistence of the spirochete.

3.2 Introduction

Lyme borreliosis is a multisystemic disease caused by the spirochete *Borrelia burgdorferi (B. burgdorferi* s.l.) which is transmitted to humans by the bite of Ixodes ticks (57). In general, acute infections with *B. burgdorferi* are successfully treated with antibiotics. However, if left untreated, persistent infection may result which may eventually develop into chronic Lyme borreliosis, manifesting in neurological and/or

articular symptoms such as Lyme arthritis. It is still unclear, how Borrelia infection can persist in an immunocompetent host. Several hypothesis are discussed:

(i) Localization of the spirochetes in immunepriviledged sites such as intracellular compartments (24), as well as in the extracellular matrix (25) as a rationale why the pathogen escapes the immune system.
(ii) A high variation of surface antigens in *Borrelia burgdorferi* (26), similar to *Borrelia hermsii* which causes relapsing fever (27). This surface antigen modulation could explain how Borrelia evade the immune response.
(iii) A shift in the T helper cell response as the cause of the treatment resistant form of Lyme borreliosis (28).
(iv) A self-propagating induction of autoimmunity following infection with Borrelia to become a chronic disease, recently supported by the finding that the Borrelia outer surface protein A (OspA) is homologue to the human LFA-1 antigen (29).
(v) A feasible further hypothesis is that the host's immune response is modulated by the pathogen such that the bacteria shift or suppress the host's immune response in a way that enables survival of the pathogen.

Examples of this latter type are known for viral (58), bacterial (59) and parasitic infections (60), and has hence led to the concept of microbial cytokine-inducing or suppressing molecules named modulins (61, 62). The effects of Borrelia infection on the acquired immune response have been investigated extensively: The strain- and disease stage-specific production of antibodies (63), as well as the T-cell responses (64) have been analyzed in great detail. Although infection with Borrelia induces a prominent antibody response in the human host, no protective immunity is conferred, indicating that Borrelia-induced antibody production alone is not sufficient to eradicate the pathogen. Similarly, the Th-1-type cytokine response alone is not able to protect against ongoing infection (65).

In contrast to these variations in the specific immune response, only few data exist on the consequences of the innate immune response during the course of an infection with Borrelia. Recent findings indicated that host-derived factors, like an aberrant or exuberant immune response, may actually be responsible for the onset of the disease, while Borrelia-derived components, such as outer surface proteins, may influence infectivity and persistence of the spirochete in the host (50, 66). Only

recently, it was observed that the anti-inflammatory cytokine IL-10 was induced in peripheral blood mononuclear cells (PBMC) by Borrelia antigen (46).

Since we were interested in investigating the influence of an ongoing Borrelia infection on the effector cells of the innate immune system, we chose the ex vivo stimulated cytokine release from human whole blood as a convenient and simple surrogate approach to characterize changes in immune function due to the disease (67), (68), (69), (70), (71), (72). In the first part of a pilot study we compared the LPS-elicited cytokine release capacity of whole blood taken from late stage borreliosis patients with that of blood from healthy volunteers. Since we observed that also in blood from healthy donors a modulation of the cytokine response to Borrelia lysate occurred in comparison to LPS, we investigated in a second part the response of blood from borreliosis patients to Borrelia lysate. From the attenuated release of pro-inflammatory cytokines under such conditions we conclude that also the status of the innate immune system might represent a critical determinant in the course of an infection with Borrelia.

3.3 Material and Methods

3.3.1 Patients and healthy controls

The mean age of the 24 control subjects, 7 women and 17 men, was 29 years (range 22 to 42 years). The mean age of the 71 patients with Lyme borreliosis enrolled in this study, 33 women and 38 men, was 54 (range 15 to 84 years). Inclusion criteria for the patients were clinical symptoms indicative of late stage Lyme borreliosis (arthritis, neurological complications and Acrodermatitis chronica atrophicans), as judged by an experienced physician (D.H.). Of these 71 patients, 14 had not been treated with antibiotics against Borrelia before, the other 57 patients had been treated once (32 patients), or at least twice (25 patients) with antibiotics. In all patients, symptoms of active Lyme borreliosis as summarized in table 3.1 were present at the time of the investigation. Infection with Borrelia sp. was confirmed by positive serology (positive serum IgM titer $\geq$ 1:32 and/or IgG titer $\geq$ 1:256) and positive Western blot, with a minimum of two highly Borrelia-specific (22kDa, 31/34 kDa, 94 kDa) bands. 10 of the patients (34 to 67 years of age, mean 54) were randomly selected, and their blood tested for cytokine release induced by Borrelia lysate in comparison to that of the 24 healthy controls. With regard to ex vivo endotoxin

stimulation, this patient subgroup did not behave statistically different from the entire patient group.

The controls were recruited from laboratory personal after informed consent. All controls had no history of tick bites or borreliosis and tested negative in the Enzygnost Borreliosis ELISA (Dade Behring, Marburg, Germany).

Table 3.1. Clinical and serological characteristics of borreliosis patients (LB) and healthy control subjects

	n	sero-positive	°Rh factor	age mean	age range	tickbite recall	*EM recall	§ACA	#NC	arthritis
LB-patient	71	71	-	46.9	15-84	35	18	5	30	36
control	24	-	-	35.4	22-42	-	-	-	-	-

°Rh rheumatoid factor, age is given in years, *EM erythema migrans, §ACA acrodermatitis chronica atrophicans, #NC neurological complications (such as meningitis and/or neuropathy)

3.3.2 Cultivation of *Borrelia burgdorferi*

All reagents used throughout the study were ultra-pure and pyrogen-free. *Borrelia burgdorferi sensu stricto* (N40), *Borrelia afzelii* (VS461) and *Borrelia garinii* (PSth) were cultivated at 33°C in BSK-H medium (Sigma, Deisenhofen, Germany), supplemented with 10% normal rabbit serum. Addition of amphotericin B (5.5 µg/ml), phosphomycin (1060 µg/ml) and rifampicin (30 µg/ml) (all Sigma) inhibited fungal and microbial growth, respectively. All Borrelia strains were kindly provided by T. Kamradt (Berlin, Germany). The strains were passaged fewer than 8 times after isolation from mice. For heat-inactivation, 10 ml of a Borrelia culture grown into log-phase ($\geq 10^8$/ml) was incubated for 5 min at 95°C and viability of remaining Borrelia was checked visually under the microscope.

3.3.3 Preparation of Borrelia lysate

A Borrelia culture (300 ml) grown until late log-phase was washed twice (20 min, 14°C, 10,000 x g) with pyrogen-free saline solution supplemented with 1 mM $MgCl_2$. The cell pellet was resuspended in 7.5 ml saline with 1 mM $MgCl_2$ and aliquots of 2.5 ml were lysed by sonification (Branson Sonifier model 250/450 with a 3 mm microtip, Schwäbisch Gmünd, Germany). Sonification was carried out on ice at a power setting of 5 at 50% interval for 2 min and lysate checked for absence of intact cells under the microscope. Protein concentration of the lysate was determined with the BSA protein assay (Pierce, Rockford, USA) following manufacturer's protocol and

protein concentration in the lysate adjusted to a final concentration of 1 mg/ml with pyrogen-free saline. The lysate preparation contained less than 0.03 endotoxin units per 10 µg protein, as assessed by *Limulus* amoebocyte assay (Bio Whittaker, Verviers, Belgium).

3.3.4 Whole blood incubation

Heparinized venous blood was freshly drawn from either healthy donors or patients with Lyme borreliosis and diluted 1:5 in RPMI 1640 medium (Biochrom Berlin, Germany) supplemented with 2.5 IU heparin (Liquemin®, Hoffmann LaRoche; Grenzach-Wyhlen, Germany) and incubated in the presence of different stimuli: live or heat-inactivated Borrelia, Borrelia lysate, or endotoxins (LPS) from *Salmonella abortus equi* (Sigma)*, Escherichia coli* (026-B6, Sigma), *Klebsiella pneumoniae* (RIBI, Hamilton, Montana, USA), *Bordetella pertussis* (List, Quadratech, Epsom, England), *Vibrio cholerae* (Sigma), *Pseudomonas aeruginosa* (Sigma) and *Salmonella enteritidis* (Sigma), or without a stimulus (control). After incubation for 24 h at 37°C in the presence of 5% CO_2, blood was resuspended and subsequently centrifuged at 16,000 x g for 2 min, the cell-free supernatant was frozen and stored at -80°C until cytokines were measured. White blood cell count (WBC) was determined by staining with Türk's solution. Furthermore, blood smears were made for differential leukocyte counts and stained according to Pappenheim.

3.3.5 Cytokine measurement

The concentrations of TNFα, IL-1β, IFNγ, G-CSF and IL-10 in the supernatants were measured by in-house sandwich ELISA using commercially available antibody pairs and recombinant standards. Monoclonal antibody pairs against TNFα, IL-1β and IFNγ were purchased from Endogen (Eching, Germany) and recombinant TNFα (Bender, Vienna, Austria), IL-1β (Endogen) and IFNγ (Thomae, Bieberach, Germany) were used as standards. Anti-G-CSF antibodies from R&D (Wiesbaden, Germany) and recombinant G-CSF from Amgen (Thousand Oaks, USA) were used. For the measurement of IL-10, monoclonal antibodies from R&D and standard from Pharmingen (Hamburg, Germany) were used.

Assays were carried out in flat bottom, ultrasorbant 96-well plates (Greiner, Frickenhausen, Germany). The secondary biotinylated antibodies were detected with

horse-radish-peroxidase-conjugated streptavidin (Dianova, Hamburg, Germany) and tetramethylbenzidine solution (Sigma) used as substrate.

3.3.6 Statistics

Data are shown either as mean ± SEM or as box-and-whiskers plots. Cytokine release was calculated per ml blood, i.e. corrected for the dilution factor of 5 since 20% blood was used. Statistical analyses were performed by two-tailed, non-parametric Mann-Whitney U-test. For the comparison of parametric data, the two-tailed, paired Tukey-test was used. All tests are options of GraphPad Prism 3.0 (San Diego, USA). P-values $\leq$ 0.05, 0.01 and 0.001 were considered significant and are depicted as *, ** and ***, respectively.

3.4 Results

3.4.1 Comparison of ex vivo endotoxin inducible cytokine release in whole blood from borreliosis patients and healthy controls

The stimulated whole blood cytokine release capacity was taken as a surrogate marker to test the hypothesis that persistent Borrelia infection is associated with a modulation of the immune status. The ex vivo cytokine release capacity for TNFα, IFNγ, G-CSF and IL-10 was measured in stimulated blood from 24 healthy donors and compared to that of 71 patients which fulfilled the inclusion criteria for late stage borreliosis. The data in Figure 3.1 illustrate that the release of the cytokines TNFα (- 72 ± 5.2%, $p \leq 0.001$), IFNγ (- 69 ± 5%, $p \leq 0.001$) and G-CSF (-26 ± 16%, $p \leq 0.01$) is attenuated in blood from borreliosis patients stimulated with endotoxin compared to healthy controls. In contrast, the release of the anti-inflammatory cytokine IL-10 did not differ in the two groups. This finding suggests an association between the modulation of cytokine release capacity of blood and Borrelia infection.

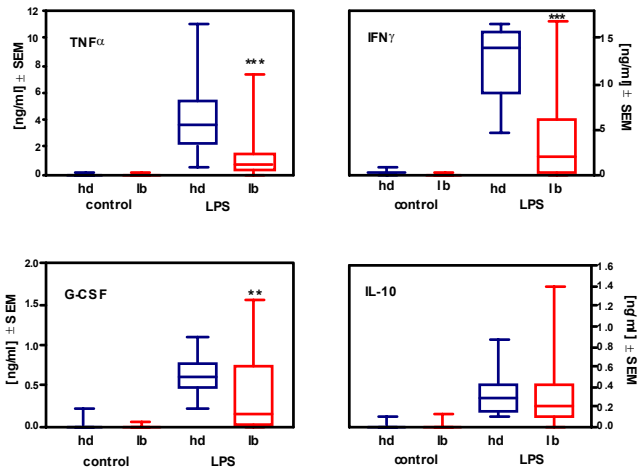

Figure 3.1. Ex vivo endotoxin -inducible cytokine release capacity of blood from healthy donors or borreliosis patients
Whole blood from healthy donors (hd, n=24) or borreliosis patients (lb, n=71), diluted 1:5 was incubated in the presence of 100 ng/ml endotoxin from S. abortus equi for 24 h at 37°C. Cytokines in the cell-free supernatant were measured by ELISA. Data are depicted as Box-and-Whiskers plots (Box shows median and the upper 75% and lower 25%, whiskers show the 95th percentile). P-values of * 0.01 and 0.001 were considered significant and are marked with ** or ***, respectively

Since the study was carried out with 71 outdoor borreliosis patients of a general practitioner, leukocyte counts could not be immediately measured by flow cytometry. Therefore, we repeated our experiments with a subset of 14 patients and 10 healthy controls, counted 200 leukocytes per smear, and then normalized the cytokine release to the number of monocytes present in each sample. Also under this normalization, the expression levels of cytokines per number of mononuclear cells were similar to the results previously obtained with whole blood. Simultaneously, a possible influence of different patient and donor age and regional settings were tested in this subpart of the study: Patient- and control group (n=14 and n=10 respectively) were age-matched (mean: 46.9 ± 4.9 and 35.4 ± 4.4 respectively, n.s.) and the incubations were carried out in parallel, i.e. at the same temporal, demographic and geographic setting. As is shown in table 3.2, the results of this second study corresponded to the ones obtained in the first study.

Table 3.2. Cytokine release from borreliosis patients and healthy control blood[a]

Stimulus	TNFα (%)	TNFα/ $1x10^6$ mono (%)	IFNγ (%)	G-CSF (%)	G-CSF/ $1x10^6$ mono (%)	IL-10 (%)	IL-10/ $1x10^6$ mono (%)
100 pg/ml LPS	- 34.5 ± 21	- 42.9 ± 28	- 57 ± 30	+ 96.2 ± 104	+ 33.3 ± 42	+ 85.2 ± 100	+ 14.3 ± 25
100 ng/ml SEB	- 20.4 ± 39	- 21.3 ± 43	- 5.5 ± 35	- 17.5 ± 75	- 46.2 ± 38	+ 106 ± 113	+ 111 ± 84

[a] Blood was taken from 14 borreliosis patients and 10 healthy controls. Blood cytokine release is shown as % of healthy control group induced by 100 pg/ml LPS (*S. abortus equi*) or 100 ng/ml SEB.

In whole blood from borreliosis patients and from healthy controls stimulated ex vivo with 100 pg/ml LPS, the release of the pro-inflammatory cytokines TNFα and IFNγ of the former group was uniformly lower than of the controls. The release capacity of the anti-inflammatory cytokine IL-10 was slightly higher in the blood from borreliosis

patients than in the control group, however, statistically not significantly different. In this small patient group, we also tested the cytokine release induced by further stimuli, i.e. 100 ng/ml SEB (Staphylococcal enterotoxin B), 10 µg/ml Borrelia lysate and a higher concentration of LPS (100 ng/ml). The results were uniform in so far as the release of TNFα and IFNγ in whole blood from borreliosis patients was always lower in comparison to the release in blood from healthy controls, while the release of IL-10 was insignificantly elevated in the patients blood, compared to the control group (data not shown). We furthermore tested if any measurable amounts of the cytokines TNFα, IFNγ, G-CSF and IL-10 could be detected in plasma, as was described for TNFα by (42), but we found no significant difference in cytokine plasma levels in the two groups: we detected 55 $\pm$ 35 pg TNFα/ml, 19 $\pm$ 8 pg IFNγ/ml, 134 $\pm$ 74 pg G-CSF/ml and 260 $\pm$ 157 pg IL-10/ml in patients plasma and 3.2 $\pm$ 1.5 pg TNFα/ml, 11 $\pm$ 3 pg IFNγ/ml , 38 $\pm$ 36 pg G-CSF/ml and 246 $\pm$ 222 pg IL-10/ml in plasma from healthy donors.

3.4.2 Cytokine release induced by heat-killed or sonified Borrelia in whole blood from healthy donors

The hypothesis that the presence of Borrelia or components of the bacterium directly induce modulations of the blood cytokine response was tested in vitro in blood from healthy donors: Both heat-inactivated Borrelia, incubated at a ratio of ten Borrelia to one leukocyte, and a corresponding concentration of Borrelia lysate (10 µg protein/ml) induced a significant release of TNFα (1,6 $\pm$ 0,4 ng/ml and 1,6 $\pm$ 0,5 ng/ml respectively, n=4). The extent of cytokine release induced by either 10 µg protein/ml Borrelia lysate (corresponding to approximately 2 x 10^7 sonified Borrelia) or by the same number of heat-inactivated Borrelia was comparable for all cytokines measured, showing that Borrelia lysate from *Borrelia burgdorferi* s.s. and heat-inactivated Borrelia are approximately equipotent stimuli. The different preparations of lysate from the three Borrelia species were equipotent with regard to the capacity to induce cytokine release in whole blood (e.g. 1.1 $\pm$ 0.3, 0.8 $\pm$ 0.2, 1.2 $\pm$ 0.3 ng TNFα/ml induced by 10 µg/ml lysate from *B. burgdorferi*, *B. garinii* and *B. afzelii*, respectively). This observation and the similarities of the cytokine pattern (data not shown) suggests that a highly conserved principle is responsible for cytokine induction by Borrelia species.

For the following experiments Borrelia lysate was used, because it can be quantified by its protein content. The cytokine release induced by stimulating blood with Borrelia lysate from *B. burgdorferi s.s.* showed a concentration dependence at lysate protein concentrations ranging from 0.1 to 100 µg/ml (Figure 3.2). The highest cytokine release for all cytokines tested was seen at the highest concentration tested, i.e. 100 µg Borrelia lysate/ml, which was not toxic for the cells as no reduction in cytokine release was seen. Similar results were obtained using lysates from *B. garinii* and *B. afzelii* (data not shown).

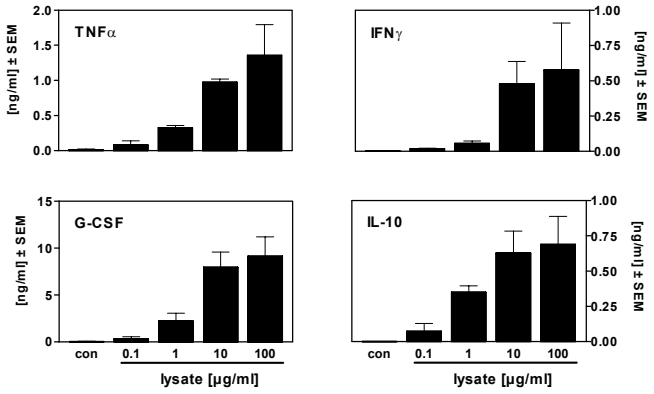

Figure 3.2. Concentration dependence of blood cytokine release from healthy donors stimulated with Borrelia lysate
Human whole blood diluted 1:5 was incubated without a stimulus (con) or in the presence of Borrelia lysate (0.1-100 µg/ml) for 24 h at 37°C. Cytokines in the cell-free supernatant were measured by ELISA. Data are depicted as means ± SEM from 4 healthy donors.

3.4.3 Comparison of cytokine release induced by Borrelia lysate and by endotoxin in blood from healthy donors

Endotoxin derived from Gram-negative bacteria, i.e. lipopolysaccharide (LPS), induces the release of a multitude of cytokines in blood in a concentration-dependent fashion. The potency of endotoxins from different bacterial species varies considerably: The data in Table 3.3 demonstrate that for endotoxins from some *E. coli* or *Salmonella species* pg/ml concentrations suffice to induce cytokine release in blood, while ng or µg per ml concentrations are required for other LPS species e.g. *Pseudomonas aeruginosa* or *Bordetella pertussis*. The Borrelia lysate concentrations which were needed to induce comparable amounts of cytokine release correspond approximately to those required for *Bordetella pertussis* endotoxin, i.e. these two stimuli have comparable low stimulatory activity. Either Borrelia lysates contain highly active material or large amounts of less active components.

Table 3.3. Threshold of IL-1β-induction in human whole blood[a]

	Borrelia lysate			LPS						
	B. burgdorferi s.s.	*B. garinii*	*B. afzelii*	*S. enteritidis*	*Kl pneumoniae*	*S. abortus. equi*	*E. coli 026-B6*	*B. pertussis*	*P. aeruginosa*	*Vibrio Cholerae*
IL-1β-treshold (pg/ml)	1000	1000	1000	10	10	10	1000	1000	10000	10000

[a]Human whole blood diluted 1:5 in RPMI was incubated in the presence of Borrelia lysate (0.1 ng - 100 µg/ml) or various endotoxins (1 pg - 10 µg/ml) in 10fold serial dilutions for 24 h at 37°C. IL-1β in the cell-free supernatant was measured by ELISA. Data represent the lowest concentration of the given stimulus in pg/ml where blood from all 4 healthy donors released significant amounts of IL-1β.

To compare the pattern of cytokine release induced by LPS and Borrelia lysate the concentrations of endotoxin from four different LPS preparations were adjusted to induce the same levels of TNFα-release as seen with 10µg/ml Borrelia lysate. The release of the cytokines TNFα, IFNγ, G-CSF and IL-10 induced by endotoxins from *Salmonella abortus equi* (200 pg/ml), *Escherichia coli* (10 ng/ml), *Klebsiella pneumoniae* (100 pg/ml) and *Salmonella enteriditis* (50 pg/ml) was uniform in blood from healthy volunteers (Figure 3.3) suggesting that different endotoxins share a leukocyte activation principle. However, a pronounced difference was seen between the four LPS preparations and the Borrelia lysate: at concentrations which induced the same TNFα-release as 10 µg/ml Borrelia lysate, endotoxins induced much more IFNγ compared to Borrelia lysate. Instead, Borrelia lysate induced a 5 to 10 fold higher release of the anti-inflammatory cytokines IL-10 and G-CSF than the LPS preparations. The lysates from other Borrelia species, i.e. *B. afzelii* and *B. garinii*, induced the same cytokine pattern as *B. burgdorferi* (data not shown). These findings show that LPS predominantly induces the release of the pro-inflammatory cytokine IFNγ, while Borrelia lysate is a stronger inducer of the anti-inflammatory cytokines IL-10 and G-CSF. Such an inverse cytokine induction pattern demonstrates that the immunostimulatory components of *Borrelia burgdorferi* differ from endotoxins.

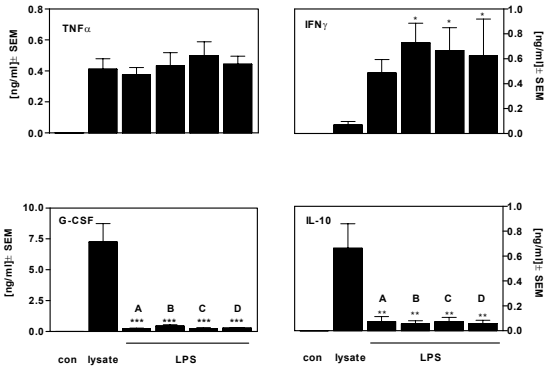

Figure 3.3. In vitro cytokine release capacity of blood from healthy donors stimulated with Borrelia lysate or LPS
Human whole blood diluted 1:5 was incubated without a stimulus (con) or with either Borrelia lysate (10 µg/ml), A; endotoxin from *S. abortus equi* (200 pg/ml), B; from *E. coli* (10 ng/ml), C; from *Kl. pneumoniae* (100 pg/ml) or D; from *S. enteritidis* (50 pg/ml) for 24 h at 37°C. Cytokines in the cell-free supernatant were measured by ELISA. Data are depicted as means * SEM from 4 healthy donors. P-values of 0.05, * 0.01 and * 0.001 versus lysate were considered significant and are depicted as *, ** and *** respectively.

3.4.4 Comparison of ex vivo cytokine release from borreliosis patients to healthy controls in response to Borrelia lysate

It was now of interest whether a similar immunomodulation by Borrelia might also be detected in the patients' blood. Therefore, the ex vivo cytokine response to Borrelia lysate of a group of ten borreliosis patients was compared with that of 24 healthy controls. In blood from patients with Lyme borreliosis, the cytokine release capacity for TNFα (-61 ± 14.3%, p≤ 0.001), IFNγ (-92.0 ± 3.2%, p≤ 0.001) and G-CSF (-84 ± 7.0%, p≤ 0.001) in response to Borrelia lysate was significantly reduced compared to healthy controls (Figure 3.4). However, again no difference between controls and patients was seen with regard to the release of the anti-inflammatory cytokine IL-10 in stimulated blood. These data indicate that ex vivo stimulated blood from borreliosis patients responds differently to LPS as well as to Borrelia lysate in comparison to healthy volunteers.

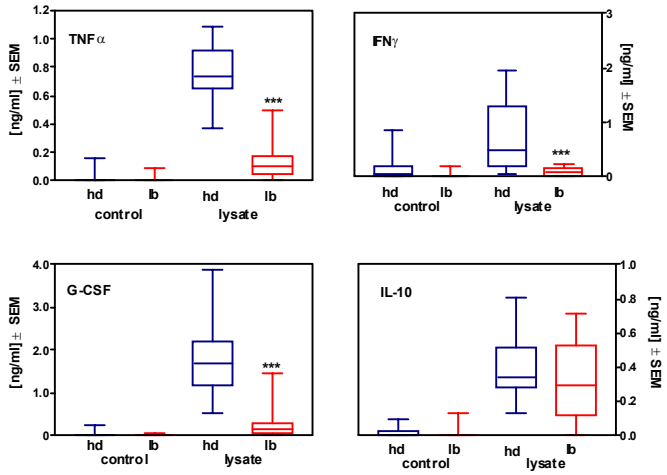

Figure 3.4. Ex vivo cytokine release capacity of blood from healthy donors or borreliosis patients stimulated with Borrelia lysate
Whole blood, either from healthy donors (hd, n=24) or patients with Lyme borreliosis (lb, n=10), diluted 1:5 was incubated with 10 µg protein/ml Borrelia lysate for 24 h at 37°C. Cytokines in the cell-free supernatant were measured by ELISA. Data are shown as Box-and-Whiskers plots. P-values of * 0.001 were considered significant and are marked with ***.

3.5 Discussion

Our study suggests at persistent infection with Borrelia spirochetes is associated with an attenuation of the release capacity of some cytokines, in particular the pro-inflammatory cytokines TNFα and IFNγ, in blood. Such an attenuation was not only seen with Borrelia specific antigen, but also after stimulation with the endotoxin of other Gram-negative bacteria (LPS), thus indicating a more general underlying mechanism.

The cytokine release capacity of patient blood and healthy controls can be used for comparative studies and alterations have been detected in blood from patients with multiple sclerosis (67), malignant melanoma (68), rheumatoid arthritis (69), multiple myeloma (70), HIV (73) and children infected with enterohemorrhagic *E. coli* (74). Especially because leukocyte counts are not affected in Borrelia infection (75, 76) it seemed appropriate to take a similar approach to characterize possible changes of the immune response in patients with persistent Borrelia infection.

There is no clear consensus on the accurate diagnosis of chronic infection and it is not yet possible to separate the symptoms of the persistent infection from possible sequalae of a successful eradication of the pathogen. Therefore we defined the following inclusion criteria both for the patient group and for the control group: The clinical diagnosis of persistent Lyme borreliosis set by an experienced practitioner in an endemic area plus well established serology were used. Furthermore, patients with further non-borrelia-associated diseases were excluded. The healthy control group was tested seronegative for Borrelia antibodies and had no history of tick bites or borreliosis.

Using these criteria, a significant attenuation of the pro-inflammatory cytokine response of blood was found in the patient compared to the healthy control group. Due to the exploratory character of the main study, a number of confounding factors have to be considered: Obviously, the age of patient and control group differed and cytokine release was not controlled for differences in leukocyte counts. Therefore, a control study was performed using healthy patients of the same general practitioner as parallel control group. This control experiment resulted in the same tendency towards attenuated pro-inflammatory cytokine response, although the lower number of blood donors did not allow to reach statistical significance. Thus, an influence of an imbalance in patient and control group selection can not be finally excluded.

The immunostimulatory properties of Borrelia antigen have been investigated extensively in the past, with live or heat-killed pathogen or purified antigen preparations (77), (78). For our experimental approach, we needed a standardized Borrelia stimulus. Heat-inactivated and sonified Borrelia induced similar cytokine release patterns and displayed a similar concentration dependence. We also compared the immunostimulatory properties of 3 different Borrelia strains (*B. burgdorferi* s.s*., B. garinii, B. afzelii*), however, no significant differences were found with regard to concentration dependence or pattern of cytokines induced. With a relatively high concentration (at least 10 ng protein/ml) of the lysate, a measurable cytokine release was induced that was quantitatively comparable to endotoxin from *Pseudomonas aeruginosa*. Since e.g. in murine Borrelia infections, accumulations of up to 10^5 spirochetes were found in different organs of infected mice (79) it is feasible that high concentrations of antigens are present at the site of infection that induce local cytokine formation.

Further, Borrelia lysate- and LPS-induced cytokine release is qualitatively different as to the pattern of predominant cytokines released. Although Borrelia belong to the group of Gram-negative bacteria (57), they lack the typical endotoxin LPS (80, 81). Instead, Borrelia express lipoproteins, e.g. outer surface proteins (Osp), which have the ability to induce the release of TNFα (42), IL-1β (43) and IL-6 (44) when incubated with isolated leukocytes, which pointed towards a probably characteristic pro-inflammatory nature of Borrelia antigen (82). However, when we compared Borrelia lysate and LPS at a concentration which induced an equipotent TNFα-release, we found that Borrelia lysate induced greater amounts of the anti-inflammatory cytokines G-CSF and IL-10 than LPS. Our results which indicate that Borrelia induce an anti-inflammatory response are corroborated by recent findings showing IL-10 induction in monocytes by Borrelia antigen (46).

We are well aware of the fact that OspA and further Borrelia-derived components are able to evoke a cytokine response from isolated peripheral blood monocytes (83), (84). We repeated and confirmed these experiments also with our Borrelia lysate and observed complete inhibition of TNFα-release after neutralization of CD14 in analogy to previous work with other bacterial stimuli (85). However, under identical conditions, the cytokine response of whole blood to Borrelia was not attenuated (data not shown). This phenomenon is currently under further investigation. It implies that the

cytokine pattern released from whole blood induced by Borrelia lysate is unrelated to these known CD14-mediated initiating mechanisms.

On the basis of published data and the study presented here, we propose to consider the attenuated release capacity of white blood cells for pro-inflammatory cytokines such as TNFα and IFNγ as a mechanism that weakens the immune response of borreliosis patients towards circulating spirochetes. This might be due to a direct recognition of Borrelia components by immunocompetent cells or represent a consequence of an enhanced local production of the anti-inflammatory factor IL-10 as published by others (84). In any case, the reconstitution of the patients immune competence represents an attractive target for supportive treatment to antibiosis in chronic Lyme borreliosis.

4 *Borrelia burgdorferi* induced immune anergy as a model of persistence via immunosuppression

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submitted to *Infection and Immunity*

4.1 Abstract

If left untreated, infection with *Borrelia burgdorferi* s.l. may lead to chronic Lyme borreliosis. It is still unknown how this pathogen manages to persist in the host in the presence of competent immune cells. We recently reported that Borrelia suppress the host's immune response, so perhaps preventing their elimination (30). Here, we further characterize Borrelia-induced immunomodulation in order to develop a model of this anergy. We observed that different Borrelia preparations we tested, i.e. live, heat-inactivated and sonicated Borrelia, could desensitize human blood monocytes, as shown by attenuated cytokine release on re-stimulation with either stimulus. Next, we investigated whether these Borrelia-specific stimuli render monocytes tolerant towards another toll-like receptor (TLR)2-agonist such as lipoteichoic acid from Gram-positive bacteria or towards the TLR4-agonist lipopolysaccharide. Cross–tolerance was induced towards all tested stimuli. Furthermore, using primary bone marrow cells from TLR2-deficient mice and from mice with a non-functional TLR4 (C3H/HeJ), we demonstrated that the TLR2 was required for tolerance induction by Borrelia and identified IL-10 by neutralizing antibodies as the key mediator involved. PBMC tolerized by Borrelia exhibited a reduced TLR2-mRNA level, which might contribute to tolerance. In summary, we characterized tolerance induced by *Borrelia burgdorferi*, i.e. a model of desensitization which might mirror the

immunosuppression recently attributed to persistence of Borrelia in immunocompetent hosts.

4.2 Introduction

Borrelia burgdorferi s.l. is the causative agent of Lyme borreliosis (LB), the most common vector-borne disease in the USA (2) and in many European countries (1). If infection with this pathogen is not treated adequately with antibiotics, it may lead to a chronic multisystemic disorder which is difficult to cure. The mechanism how Borrelia survive in their natural reservoir, and in human tissues remains unclear. Although they are recognized by the host's immune defense and occasionally induce strong inflammatory reactions, Borrelia are often not eradicated. Thus, how Borrelia persist in immunologically competent hosts is a key question in understanding the pathogenesis of LB and remains an area of debate. It has been postulated that Borrelia interact with the complement system, inactivating the complement regulatory proteins FHL-1/reconectin and Factor H (86, 87). Others have proven by electron microscopy, that Borrelia can hide themselves in immunopriviledged sites like the collagen fibers of the connective tissue (88) or in human synovial cells (24). The antigenic variation of the *Borrelia burgdorferi* outer membrane is also discussed as a possible strategy to evade the immune response (26, 89, 90). Data from Gross et al. suggest alternatively, that Borrelia induce an autoimmune process, as they could identify a homology between the Borrelia outer surface protein A (OspA) and the human lymphocyte-function-associated antigen-1 alpha (hLFA-1) and also found cross reactive T-cells (29).

We investigated whether Borrelia modulate the host's immune system in order to persist. Blood cells from patients suffering from persistent Lyme borreliosis released significantly lower levels of pro-inflammatory cytokines (i.e. TNFα and IFNγ) in response to either a Borrelia-specific stimulus or to lipopolysaccharide (LPS) in comparison to the cells from healthy volunteers (30). Based on their findings in patient specimens, others have also described Borrelia as an immunomodulator, supporting the hypothesis of Borrelia-induced immundysregulation (91), (92). In line, Borrelia seem to influence the balance between the pro- and anti-inflammatory immune response. We and others found that Borrelia not only induce pro-inflammatory cytokines, but also lead to a strong formation of IL-10, which is an important down-regulator of the pro-inflammatory immune response (30, 46, 84). In

addition, the modulatory and regulatory capacity of IL-10 with regard to Borrelia-induced cytokine release has been found in different *in vitro* models (93, 94). For Borrelia lipoproteins the toll-like receptor 2 (TLR2) has been identified as the major signal transducing receptor (95-99). However, recently the existence of TLR2-independent, non-lipoprotein Borrelia components was postulated (100). In order to address these conflicting results we tested the role of the TLR2 and TLR4 in the recognition of different Borrelia-specific stimuli.

The phenomenon of endotoxin (LPS) tolerance has been investigated extensively *in vitro* and *in vivo* (for review see (101)). It describes a status of macrophage hyporesponsiveness after exposure to low LPS doses to a subsequent high or lethal LPS dose (102, 103). Recently, similar desensitization experiments were reported which demonstrated, that stimuli other than LPS, i.e. highly purified lipoteichoic acid (LTA) (104) or macrophage-activating lipopeptide-2 (MALP-2) from mycoplasma (105), can also render macrophages tolerant to subsequent re-stimulation. Furthermore, it was shown in these same studies, that tolerance can also be induced by two heterologous stimuli, independent of the receptor involved in their recognition and signaling. In this case the appropriate term is cross-tolerance or hetero-tolerance. To our knowledge it has not yet been investigated whether *Borrelia burgdorferi* also has the capacity to desensitize macrophages. However, Borrelia-induced hyporesponsiveness could represent a mechanism enabling the survival of this pathogen in the host despite the presence of immune cells.

We tested this hypothesis in desensitization experiments with Borrelia-specific stimuli and with other well characterized TLR2- and TLR4-agonists. Additionally, the involvement of the TLR2 and the TLR4, as well as of endogenous IL-10 formation in tolerance and cross-tolerance induction was addressed.

4.3 Material and Methods

4.3.1 Borrelia cultivation and preparation of Borrelia-specific stimuli

Borrelia burgdorferi sensu stricto (s.s.) (strain N40, kindly provided by T. Kamradt, Berlin, Germany) was cultivated as described previously (30). Borrelia cultures passaged fewer than 8 times after isolation from mice were grown to log-phase ($\geq 10^8$/ml) and differentially prepared for stimulation experiments.

For experiments with live Borrelia, *B. burgdorferi* numbers were determined by microscopy using a Thoma-counting-chamber, with a modified depth of 0.02 mm. The culture was adjusted to 5 x 10^5 Borrelia/ml. Due to the low replication rate of Borrelia and to their high requirements with regard to the culture conditions an increase in bacterial numbers during the 24 h of subsequent incubation is improbable.

Before heat-inactivation or sonication, Borrelia cultures were washed twice. Briefly, cultures were centrifuged at 10000 g for 30 min, the supernatant was removed and the pellet was resuspended with pyrogen-free saline solution. Subsequently, the bacterial numbers were determined as described above. Finally, washed Borrelia were either incubated for 30 min at 56°C for heat-inactivation, or they were sonicated yielding Borrelia lysate as described elsewhere (30). Protein concentration of the lysate was adjusted to a final concentration of 1 mg/ml. Remaining viable spirochetes were excluded visually under the microscope after one week incubation under standard cultivation conditions.

The amount of endotoxin units (EU) contained in 10 µg protein of sonicated Borrelia and of 1 x 10^7 heat-inactivated Borrelia was below the detection limit (0.1 EU and 0.05 EU respectively), assessed by *Limulus* amoebocyte lysate assay (LAL) (QCL-1000, Charles River Endosafe, Charleston, SC, USA). The spike recovery (0.5 EU), was 107% and 103%, respectively. Live Borrelia were only used for the first set of experiments, because they represent a highly variable stimulus which is difficult to standardize as they change their surface protein expression depending on cultivation conditions (106). All the other experiments were carried out with the same batch of heat-inactivated or sonicated Borrelia.

4.3.2 Isolation of human peripheral blood mononuclear cells

Peripheral blood mononuclear cells were isolated with cell preparation tubes (Vacutainer CPT, sodium citrate, Becton Dickinson Biosciences, Heidelberg, Germany) according to the manufacturer's instructions. Briefly, blood from healthy donors was centrifuged in the tubes for 20 minutes at 1650 g. PBMC, separated from erythrocytes and neutrophils by the gel phase were transferred into 50 ml polypropylene tubes (bio-one, Greiner, Frickenhausen, Germany) and washed twice with RPMI 1640 (Bio Whittaker, Apen, Germany) supplemented with 2.5 IU/ml heparin (Liquemin®, Hoffmann LaRoche, Grenzach-Whylen, Germany). The overall

cell count of PBMC (i.e. monocytes and lymphocytes) was determined with a Pentra60 (ABX Diagnostics, Montpellier, France). PBMC were adjusted to a final concentration of 1 x 10^7 PBMC/ml with RPMI 1640 supplemented with heparin. 100 µl of cells were pipetted per well of a 96-well cell-culture plate (Greiner) and 10% autologous plasma was added to each well.

4.3.3 Mice

Female C3H/HeJ-mice, characterized by a non functional TLR4 due to a natural point mutation in the TLR4-gene (107) and the corresponding wildtype mouse strain (C3H/HeN) were purchased from Charles Rivers Laboratories (Sulzfeld, Germany). TLR2 deficient mice generated by homologous recombination were a generous gift from Tularik, (South San Francisco, CA, USA) and the corresponding wildtype mice (129Sv/B57BL/6) were bred in the animal facilities of the University of Konstanz at 24°C, 55% humidity, 12 h day-night rhythm on a diet of Altromin C 1310 (Altromin, Lage, Germany). Mice were used for the experiments at 8 to 10 weeks of age.

4.3.4 Isolation of primary bone marrow cells from mice

Mice were killed by terminal pentobarbital anaesthesia (Narcoren, Merial, Hallbergmoos, Germany). Femurs were lavaged with 10 ml ice-cold sterile PBS (Life Technologies, Karlsruhe, Germany). The lavages were transferred to siliconized glass tubes (Vacutainer, Bioscience, Heidelberg, Germany) for isolation of bone marrow cells. Bone derived debris were removed by re-suspending the lavage and transferring it into a new glass tube after 1 minute sedimentation. After centrifugation, primary murine bone marrow cells were re-suspended in RPMI 1640 containing 10% FCS (Biochrom, Berlin, Germany) and 100 IU/ml penicillin/streptomycin (APP-laboratories, Linz, Austria). Cell counts were determined with a Pentra60 (see above) and plated to 96-well culture plate (Greiner) at a density of 5 x 10^5 cells per well.

4.3.5 In vitro desensitization and re-stimulation experiments

Immediately after isolation, cells (PBMC or primary murine bone marrow cells (BMC)) were desensitized with different concentrations of the following stimuli: live, heat-inactivated or sonicated Borrelia (lysate), LTA from *S. aureus* (isolated and prepared in house as described previously (108)), endotoxin (LPS from *Salmonella abortus equi*) (Sigma-Aldrich, Seelze, Germany), or left untreated for the controls. The

volume was adjusted to 220 µl with medium (RPMI supplemented with 100 IU/ml penicillin/streptomycin, containing 10% FCS for the BMC and 2.5 IU/ml heparin for the PBMC). After incubation for 24 h at 37°C in the presence of 5% CO_2, the supernatants were transferred into 96-well round-bottom plates (Greiner) and stored at -70°C until cytokines were measured. The remaining adherent, tolerized, monocytes in the plate were washed twice with RPMI 1640, and subsequently re-stimulated with 1 ng/ml LPS, 10 µg/ml LTA, 10 µg/ml Borrelia lysate or 1 x 10^6 heat-inactivated *Borrelia burgdorferi* s.s. (Bb)/ml. 10% autologous plasma was added to the PBMC. The final volume of the incubation was adjusted to 220 µl RPMI 1640 (supplemented as described above). After 24 h incubation at 37°C and 5% CO_2 the supernatants were stored at –70°C until cytokine measurement. To study the involvement of mediators in tolerance induction, neutralizing antibodies (anti-IL-10 (Endogen, Eching, Germany), pan-specific anti-TGFβ (R&D Systems, Wiesbaden, Germany), and polyclonal anti-mu-G-CSF-sheep IgG raised in our laboratory (109)) were added to the first 24 h incubation period.

4.3.6 MTT-assay

MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide, Sigma) stock solution (5 mg/ml in PBS) was diluted 1:5 in RPMI 1640. 200 µl of this working solution was added to the adherent cells in a 96-well culture-plate for 2 h at 37°C and 5% CO_2. Cells with mitochondrial activity convert dissolved MTT to insoluble purple formazan. After the incubation the supernatant was removed and the cells were lysed for 10 minutes with 95% isopropanol, 5% formic acid. Absorbance of converted dye was measured at 555 nm against 690 nm as reference wavelength.

4.3.7 Cytokine measurement in culture supernatant by ELISA

The concentrations of human and murine TNFα in the supernatants were measured by in-house sandwich ELISA using commercially available antibody pairs and recombinant standards. Monoclonal antibody pairs against human TNFα were purchased from Endogen (Perbio Science, Bonn, Germany). Recombinant human TNFα was a gift from Dr. S. Poole (National Institute for Biological Standards and Controls, London, GB) and was used as standard. For the measurement of murine TNFα, polyclonal antibodies from R&D Systems (Wiesbaden, Germany) and standard from Pharmingen (BD Bioscience, Heidelberg, Germany) were used.

Assays were carried out in flat-bottom, ultrasorbant 96-well plates (Nunc, Wiesbaden, Germany). Binding of secondary biotinylated antibodies was detected with horseradish-peroxidase-conjugated streptavidin (Biosource, Camarillo, CA, USA) and 3',3', 5', 5'-tetramethylbenzidine solution (Sigma) used as substrate.

4.3.8 RNA-extraction and TLR2-mRNA-quantification

5 x 10^6 PBMC/ml were pipetted into 24 well-culture plates in 1 ml medium. After 24 h incubation at 37°C and 5% CO_2 with 10 µg/ml lysate or without a stimulus the supernatant was removed and cells were either lysed for RNA extraction or they were washed and re-stimulated with lysate (10 µg/ml) or with LPS (1 ng/ml) for another 3 h. After removal of the supernatant, RNA was prepared from the adherent cells with QIAmp RNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, including DNA digestion with the RNase-free DNase-Set (Qiagen).

6 µl RNA was reverse transcribed in a sample volume of 20 µl containing 2.5 µM Oligo dT16 (Gibco BRL, custom primer), $MgCl_2$ (5 mM), dNTP (1 mM each), RNase inhibitor (1 U/µl), murine leukemia virus reverse transcriptase (2.5 U/µl) in PCR buffer (all PE Applied Biosystems, Weiterstadt, Germany). Samples were incubated at 21°C for 10 min, 42°C for 15 min, 94°C for 5 min and 5°C for 5 min in a GeneAmp PCR System 2400 (PE Applied Biosystems).

For relative quantification real-time PCR was performed using a LightCycler rapid thermal cycler system (Roche Diagnostics GmbH, Mannheim, Germany). The cDNA for TLR2 and GAP-DH was amplified using LightCycler-FastStart DNA Master SYBR-Green (Roche Diagnostics, Germany) according to the manufacturer's protocol. The sequence for the primers were ggc cag caa att acc tgt gtg (forward), agg cgg aca tcc tga acc t (reverse) and gaa ggt gaa ggt cgg agt c (forward), gaa gat ggt gat ggg att tc (reverse) for TLR2 and GAP-DH, respectively. The $MgCl_2$ concentration was adjusted to 2 mM for TLR2 and 4 mM for GAP-DH. The thermal cycling was performed according to the manufacturer's protocol (50 cycles) with an annealing temperature of 58°C and 65°C and an elongation time of 3 sec and 11 sec for TLR2 and GAP-DH, respectively. The amplification was followed by a melting program which started at 65°C for 15 sec and then increased to 95°C at 0.1°C/sec. The specific melting temperatures for TLR2 and GAP-DH products were 86°C and 86.5°C, respectively. The TLR2 results were normalized with GAP-DH.

4.3.9 Statistics

Data are shown either as mean ± SEM of 4 different blood donors or of primary cells from 4-7 individual mice. To take into account the varying viability of the cells on day 2, TNFα-concentrations were divided by the mitochondrial activity assessed in the MTT-assay. Repeated measure analysis of variance followed by Dunnett's multiple comparison test were performed using GraphPad Prism 3.00 (GraphPad Software, San Diego, CA, USA). ≤ 0.05, 0.01 and 0.001 were considered significant and are depicted as *, ** and ***, respectively.

4.4 Results

4.4.1 Borrelia-induced tolerance in human PBMC

We tested whether Borrelia are able to induce a state of hyporesponsiveness in human monocytes *in vitro*. On the one hand, we used the same Borrelia preparations, i.e. heat-inactivated, sonicated and live Borrelia, for pre-stimulation and for re-stimulation. On the other hand, we tested all combinations of the different Borrelia preparations in the two sequential incubation periods. When human PBMC were pre-treated with either sonicated, heat-inactivated or live Borrelia for 24 h, they showed a significantly reduced release of TNFα in response to a secondary stimulation with the same or either of the other Borrelia preparations (Figure 4.1). In contrast, cells which had not been stimulated during the first incubation period were fully responsive to the second stimulus, as demonstrated by the non-pretreated control cells. Thus, all Borrelia-specific stimuli tested could desensitize macrophages to either the same or to another Borrelia-specific stimulus, which is termed Borrelia-tolerance.

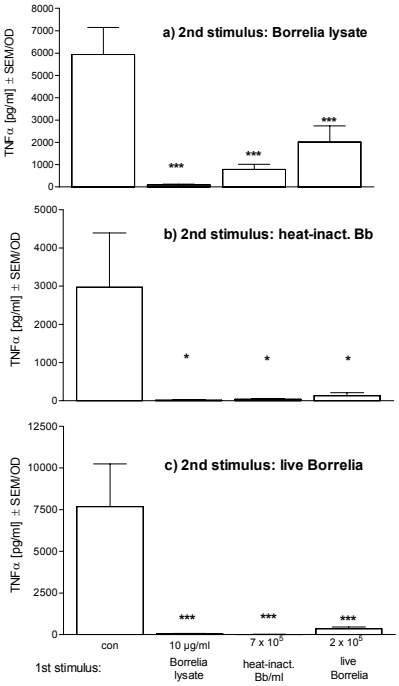

Figure 4.1. Borrelia-induced tolerance in human PBMC

1 x 10^6 human PBMC from 4 healthy donors were incubated with culture medium (con), 10 µg/ml Borrelia lysate, 7 x 10^5 heat-inactivated or 2 x 10^5 live Borrelia during 24 h, then washed and incubated for another 24 h in the presence of a) 10 µg/ml Borrelia lysate, b) 7 x 10^5 heat-inactivated or c) 2 x 10^5 live Borrelia. The concentrations of TNFα were measured by ELISA. Data are expressed as the mean ± SEM divided by the OD assessed by the MTT-assay. The statistical significance was compared with the non-tolerized (con) cells. *, $p < 0.05$, ***, $p < 0.001$ based on ANOVA, followed by Dunnett's multiple comparison test.

4.4.2 Comparison of TNFα-inducing potency of different bacterial stimuli

We next tested TNFα-inducing capacity of different bacterial stimuli during 24 h incubation in the *in vitro* model. Human PBMC treated with LPS, LTA or with two Borrelia-specific stimuli (heat-inactivated Borrelia and Borrelia lysate) induced dose-dependent TNFα-release as measured by ELISA. 1 ng/ml LPS, 10 µg/ml LTA, 1 x 10^6 heat-inactivated Bb/ml and 10 µg/ml Borrelia lysate induced comparable amounts of TNFα in the range of 200-500 pg/ml. Therefore, these concentrations were used in the subsequent re-stimulation experiments.

4.4.3 Borrelia-induced cross-tolerance to LTA and LPS

Next, we investigated whether Borrelia-specific stimuli - which according to literature (95, 96, 98, 99) act via TLR2 - render macrophages tolerant towards another TLR2-agonist such as lipoteichoic acid (LTA) from Gram-positive *Staphylococcus aureus* or towards a TLR4-agonist, i.e. the lipopolysaccharide from *Salmonella abortus equi*. PBMC were first treated overnight with either heat-inactivated Borrelia or Borrelia lysate. Then, the cells were re-stimulated either with 10 µg/ml LTA or with 1 ng/ml LPS. Figure 4.2a and 4.2b demonstrate, that pre-stimulation with Borrelia-specific stimuli led to reduced responsiveness of the PBMC to re-stimulation with LTA as well as with LPS. Tolerance induction was concentration dependent. The stronger the

stimulus during the pre-incubation period, the lower the TNFα-response to the second stimulus. Thus, Borrelia induced cross–tolerance towards two different heterologous stimuli, one also signaling via TLR2, the other TLR4-mediated.

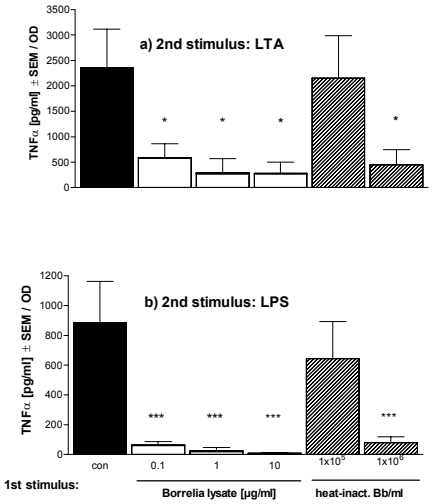

Figure 4.2. Induction of Borrelia cross-tolerance to LTA or LPS in human PBMC

Human PBMC (1 x 10^6/well) from 4 different donors were incubated with culture medium (con, black bars), 0.1, 1, 10 µg/ml Borrelia lysate (white bars),1 x 10^5 and 1 x 10^6 heat-inactivated Borrelia (hatched bars) during 24 h, then washed and incubated for another 24 h in the presence of 10 µg/ml LTA (a) or 10 ng/ml LPS (b). The concentrations of TNFα were measured by ELISA. Data are expressed as the mean ± SEM divided by the OD assessed by the MTT-test. The statistical significance was compared with the non-tolerized (con) cells, *, $p < 0.05$, ***, $p < 0.001$ based on ANOVA, followed by Dunnett's multiple comparison test.

4.4.4 LPS and LTA-induced cross-tolerance to Borrelia-specific stimuli

Stimulation of macrophages with LPS renders the cells tolerant to a subsequent LPS-stimulation. This kind of tolerance has been known since the 1960-1970s (110-112). Recently, we reported tolerance-induction by LTA, including cross-tolerance inducible by LTA towards LPS and vice versa, indicating that cross-tolerance between TLR2 and TLR4-agonists is possible (104). Therefore, our results prompted us to test whether cells rendered tolerant by LPS or LTA were also hyporesponsive to re-stimulation with Borrelia lysate.

In the experiments shown in Figure 4.3, LPS or LTA were used for pre-stimulation. For subsequent re-stimulation, Borrelia lysate was added to the cells. Similarly to the previous experiments, cytokine release in response to the second stimulus was decreased in a dose-dependent fashion depending on the concentration of the first stimulus. The same results were obtained when heat-inactivated Borrelia were used for re-stimulation instead of Borrelia lysate (data not shown).

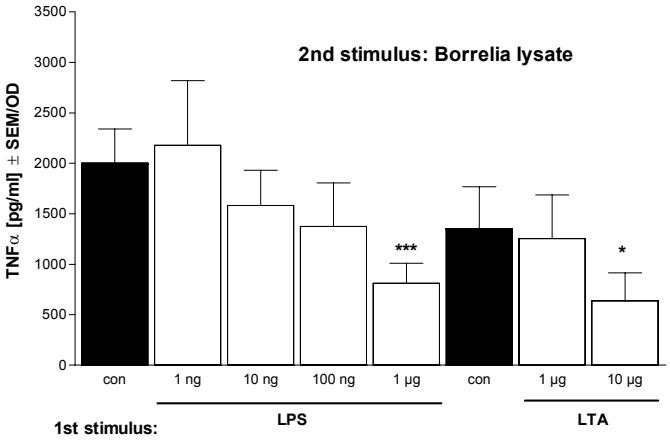

Figure 4.3. Effect of pre-incubation with LPS or LTA on re-stimulation with Borrelia lysate

Human PBMC (1 x 10^6 / well) were incubated with culture medium (con, black bars), 1, 10, 100 and 1000 ng/ml LPS (white bars), or with 1 and 10 µg/ml LTA (white bars) during 24 h, then washed and incubated for another 24 h in the presence of 10 µg/ml Borrelia lysate. The concentrations of TNFα were measured by ELISA. Data are expressed as the mean ± SEM divided by the OD assessed by the MTT-assay. The statistical significance was compared with the non-tolerized (con) cells, *, $p < 0.05$, ***, $p < 0.001$, based on ANOVA, followed by Dunnett's multiple comparison test.

4.4.5 IL-10 is involved in tolerance induction by Borrelia and LPS

Next we were interested in the mechanism of Borrelia-induced tolerance. We first checked whether soluble mediators are involved. It has been shown, that endogenous anti-inflammatory factors such as IL-10 and TGFβ mediate the phenomenon of LPS tolerance in human monocytes *in vitro* (113). In order to examine this mechanism, neutralizing antibodies were incubated simultaneously with the Borrelia lysate during the pre-incubation period. In Figure 4.4 pre-treatment of PBMC with Borrelia and specific neutralizing antibodies against the anti-inflammatory cytokines IL-10, TGFβ and G-CSF showed that neither antibodies against TGFβ nor G-CSF alone, nor the combination of both could block Borrelia-induced tolerance. The neutralizing activity of the antibodies was assured in control experiments blocking the immunosuppressive effect of recombinant cytokines on LPS inducible cytokine release (data not shown). Anti-IL-10 antibodies alone partially prevented tolerance induction by Borrelia. Addition of anti-TGFβ or anti-G-CSF antibodies or both did not augment the inhibitory effect of anti-IL-10 antibodies. These findings suggest that IL-10, not G-CSF or TGFβ, is involved in tolerance induction by Borrelia in human PBMC, but other mediators are additionally needed to completely prevent the process.

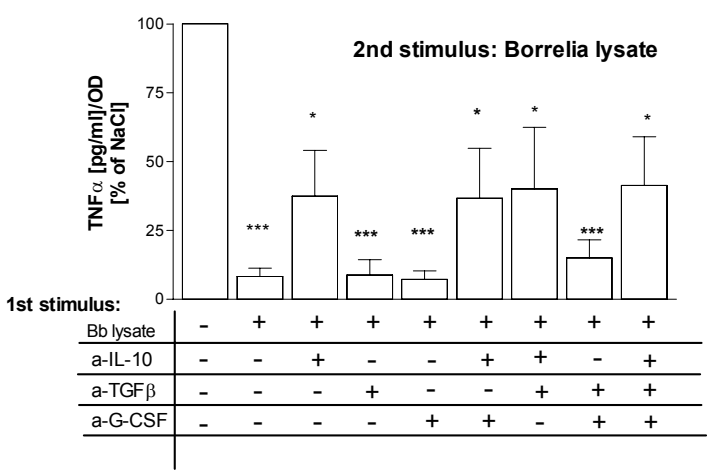

Figure 4.4. Neutralization of IL-10, TGFβ and G-CSF during pre-incubation prevents establishment of desensitization by Borrelia lysate

PBMC were cultured for 24 h in the presence of 10 µg/ml Borrelia lysate and neutralizing anti-TGFβ (1 µg/ml) and anti-IL-10 (10 µg/ml) monoclonal antibodies, 1% anti-mu-G-CSF-sheep IgG or combinations of the neutralizing antibodies and serum. Controls were cultured without Borrelia lysate. After washing, cells were re-stimulated with 10 µg/ml Borrelia lysate for further 24 h at 37°C. Cytokines in supernatants were determined by ELISA. The statistical significance was compared with the non-tolerized (con) cells, *, $p < 0.05$, ***, $p < 0.001$ based on ANOVA, followed by Dunnett's multiple comparison test.

4.4.6 TLR2-downregulation by Borrelia-induced tolerance

Recently published experiments from Wang et al. suggest that the TLR2 is downregulated in synthetic bacterial lipopeptide-tolerance (114). We therefore measured the TLR2-mRNA expression of human PBMC in Borrelia-induced tolerance. 24 h stimulation of PBMC in the presence of Borrelia lysate (10 µg/ml) or LPS (1 ng/ml) led to a significantly reduced TLR2-mRNA expression, which remained attenuated after re-stimulating these tolerized cells (Figure 4.5). These data suggest that downregulation of the TLR2 might contribute to suppressed TNFα-formation upon re-stimulation in Borrelia-induced tolerance.

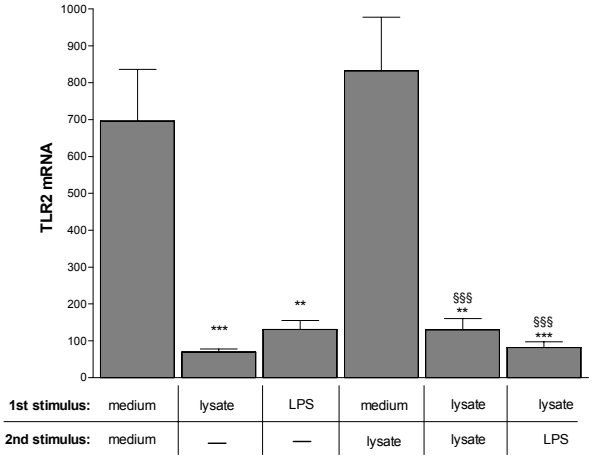

Figure 4. 5. TLR2-mRNA expression in human PBMC

5 x 10^6 PBMC from 4 different donors were incubated with culture medium, 1ng/ml LPS or 10 µg/ml Borrelia lysate during 24 h and either lysed or incubated for another 3 h in the presence of medium, 10 µg/ml Borrelia lysate or 1 ng/ml LPS. The cDNA for TLR2 and GAP-DH was amplified and relative quantification was performed using a LightCycler. The TLR2 results were normalized to GAP-DH. **, $p < 0.01$ and ***, $p < 0.001$ based on ANOVA, followed by Bonferroni's multiple comparison test. * vs. medium - medium, § vs. medium - lysate.

4.4.7 TLR2 but not TLR4 is required for tolerance- and cross-tolerance-induction by Borrelia

To further investigate the role of TLR2 and TLR4 in Borrelia-induced tolerance, we carried out desensitization experiments using primary murine bone marrow cells from TLR2-knock-out mice and C3H/HeJ-mice (with a non-functional TLR4) and corresponding wildtype cells. As expected there was no measurable TNFα- or IL-10-release in TLR2$^{-/-}$ bone marrow macrophages after stimulation with either heat-inactivated Borrelia or Borrelia lysate on the first day after 8 h or 24 h (data not shown). Hereby, we confirmed data from others (95, 96, 98, 99), showing that Borrelia-specific stimuli employ TLR2. However, cells from TLR2$^{-/-}$mice responded to LPS to the same extent as cells from wildtype mice, reflecting the normal responsivity to a TLR4-agonist (data not shown). Cells from TLR2- plus TLR4-defective mice obtained by crossing both strains, did neither release TNFα upon stimulation with Borrelia, nor with LPS or LTA (data not shown). We controlled the responsiveness of the cells to exclude that they were desensitized by activating them with other stimuli such as CpG-oligonucleotides, a TLR9-mediated stimulus modeling bacterial DNA, and by the phorbolesther PMA, a receptor-independent stimulus (data not shown).

Pre-incubation of cells from TLR2$^{-/-}$mice with a Borrelia-specific stimulus had no significant effect on their responsiveness to LPS (Figure 4.6b). They behaved similar to saline-pretreated cells. This result indicates that the cells had not been desensitized by heat-inactivated Borrelia and demonstrates that TLR2 is required for Borrelia-induced tolerance. Furthermore, as expected, no TNFα-release could be measured upon re-stimulation of the TLR2-deficient cells with either lysate (Figure 4.6d) or heat-inactivated Borrelia (data not shown) independent of the pre-incubation stimulus. In contrast, re-stimulating wildtype and TLR2$^{-/-}$-cells after LPS pre-treatment with LPS again, resulted as expected in strongly reduced TNFα-release, showing that the cells had been rendered tolerant (Figure 4.6a). Thus, the TLR2 was not required for LPS-induced tolerance. In line with our PBMC-results (Figure 4.1, Figure 4.3) this experiment shows that pre-stimulation of primary murine wildtype cells with LPS or Borrelia lysate tolerized the cells to Borrelia lysate.

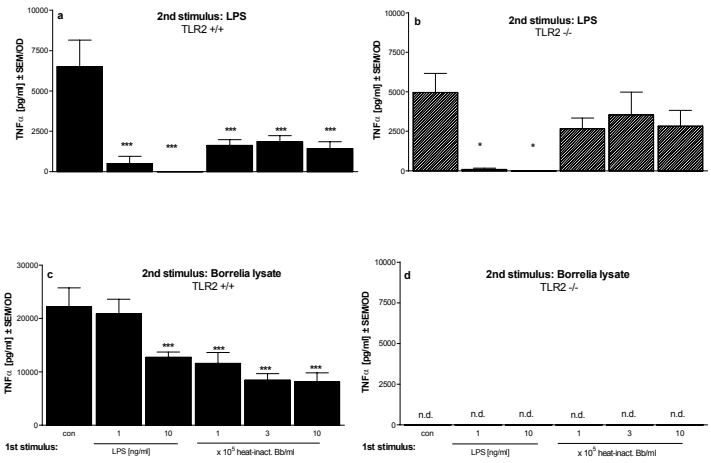

Figure 4.6. Induction of tolerance and cross-tolerance in primary bone marrow cells from TLR2$^{+/+}$ and TLR2$^{-/-}$ mice

Bone marrow cells (5 x 10^5/well) from TLR2$^{+/+}$-mice (black bars, a, c) and TLR2$^{-/-}$-mice (hatched bars, b, d) were incubated with culture medium (con), 1 and 10 ng/ml LPS, or with 1 x 10^5, 3 x 10^5, 1 x 10^6 heat-inactivated Borrelia during 24 h, then washed and incubated for another 24 h in the presence of a) and b) 1 ng/ml LPS or c) and d) 10 µg/ml Borrelia lysate. The concentrations of TNFα were measured by ELISA. Data are expressed as the mean ± SEM divided by the viability assessed by MTT-assay. The statistical significance was compared with the non tolerized cells. *, $p < 0.05$, ***, $p < 0.001$. n.d. not detectable.

The same experiments were also conducted with primary murine bone marrow cells from C3H/HeJ mice which lack a functional TLR4 and their corresponding wildtype cells. Cells from both mouse strains responded similarly to Borrelia-specific stimuli during the pre-incubation period (data not shown), confirming that TLR4 is not required for signaling of the Borrelia-specific stimuli. Consequently, cells from C3H/HeJ mice behaved like wildtype-cells, showing a reduced TNFα-release to re-stimulation with heat-inactivated Borrelia after pre-treatment with the same Borrelia-specific stimulus (Figure 4.7a). Furthermore, in line with our expectations, neither tolerance to Borrelia (Figure 4.7b) nor to LPS (data not shown) could be induced by LPS-pre-treatment in cells without a functional TLR4.

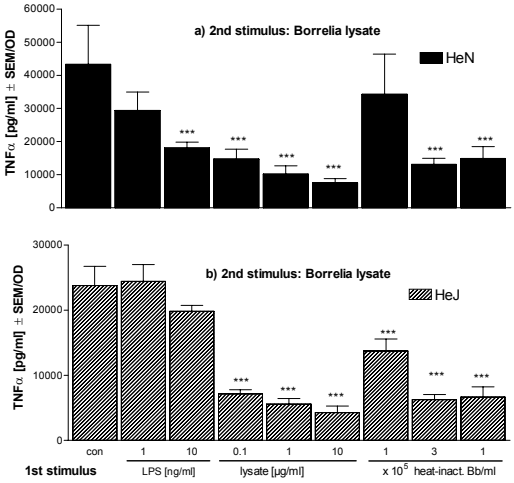

Figure 4.7. Induction of tolerance and cross-tolerance in primary bone marrow cells from C3H/HeN and C3H/HeJ mice

Bone marrow cells (5 x 10^5/well) from C3H/HeN-mice (black bars) and C3H/HeJ mice (hatched bars) were incubated with culture medium (con), 1 and 10 ng/ml LPS, or with 1 x 10^5, 3 x 10^5, 1 x 10^6 heat-inactivated Borrelia during 24 h, then washed and incubated for another 24 h in the presence of 10 µg/ml Borrelia lysate. The concentrations of TNFα were measured by ELISA. Data are expressed as the mean ± SEM divided by the viability assessed by MTT-assay. The statistical significance was compared with the non tolerized cells. ***, $p < 0.001$ based on ANOVA, followed by Dunnett's multiple comparison test.

Hence, in the absence of TLR2, no cytokine release could be induced by Borrelia and no state of hyporesponsiveness for re-stimulation with LPS was achieved. In line, no desensitization to Borrelia stimuli could be induced by LPS in the absence of functional TLR4.

4.5 Discussion

Understanding the immunopathology of LB is still a major challenge. Although inducing strong immune activation, e.g. in phases of arthritis, the causative agent of LB persists and leads to a chronic pathology in the immunocompetent host. Noteworthy, the inflammatory episodes in LB are typically self-limiting and the site of manifestation often changes e.g. between different joints. These phenomena suggest counter-regulatory anti-inflammatory mechanisms and the long phases of latency indicate phases of immune evasion.

We recently proposed a possible mechanism for survival of Borrelia in the immunologically competent host: Since our *ex vivo* experiments with whole blood from patients with LB revealed a significantly reduced capacity to release TNFα and IFNγ in comparison to blood cells from healthy controls, we postulated that Borrelia modulate the host's immune system in order to evade immune clearance (30). There are few reports about Borrelia acting as an immunomodulator (91, 92, 115). We found further evidence for Borrelia-induced immunomodulation by demonstrating, that Borrelia induce a stronger anti-inflammatory cytokine-response than endotoxins from various other Gram-negative bacteria (30). These data are in line with findings from other authors, indicating that *Borrelia burgdorferi* is a potent inducer of the anti-inflammatory cytokine IL-10 (46, 84, 116). In addition, the modulatory and regulatory capacity of IL-10 with regard to Borrelia-induced cytokine release has been found in different *in vitro* models (93, 94).

In the present study, we examined whether tolerance could represent a possible model for Borrelia-induced immunomodulation. First, we checked whether Borrelia can render cells hyporesponsive and second we investigated some of the underlying mechanisms involved in this immunomodulation. Borrelia could indeed render human PBMC tolerant, i.e. unable to react to a second stimulation with Borrelia, as shown by a reduced capacity of TNFα-release (Figure 4.1). All the tested settings led to a significantly reduced TNFα-release as a result of pre-incubation with Borrelia, compared to saline controls. As no major differences between live and killed Borrelia

with respect to their ability to render the monocyte hyporesponsive could be observed, we selected the more standardized stimuli (heat-inactivated Borrelia and Borrelia lysate) for the subsequent experiments. Based on these experiments we demonstrated, that Borrelia are able to modulate the monocytic immune response. Next, assuming that Borrelia can induce a more general desensitization in the cell, we investigated whether a monocyte rendered tolerant by Borrelia was also hyporesponsive to a different stimulus. Differences in the potency of TNFα-induction among the bacterial stimuli (Borrelia, LPS and LTA) were excluded by choosing equipotent concentrations, i.e. concentrations which induced comparable amounts of TNFα-release during 24 h of incubation.

According to literature, Borrelia signal via the TLR2 (95, 96, 98, 99). This could be confirmed in our experiments with primary murine bone marrow cells from mice lacking either a functional TLR2 or TLR4 or both. The findings in Figure 4.2 a, b demonstrate that Borrelia desensitize monocytes in a more general manner, because they also rendered PBMC hyporesponsive to subsequent stimulation with heterologous stimuli such as the TLR2 agonist LTA or the TLR4 agonist LPS. The extent of desensitization was concentration dependent. Using LPS or LTA to tolerize the cells similarly led to hyporesponsiveness to Borrelia (Figure 4.3). We did not observe any difference in cross-tolerance induction by the tested stimuli on the level of TNFα-release. These findings confirm our data regarding cross-tolerance between LTA and LPS, showing that the degree of hyporesponsiveness induced by the TLR2 or the TLR4 agonist, does not vary (104). Opposing results have been published by others, which postulate that LPS pre-treatment is less effective in other cross-tolerance models. Sato et al., for example describe poor tolerance induction by LPS towards MALP-re-stimulation (105).

Tolerance experiments presented here suggest that the underlying mechanisms which lead to Borrelia-tolerance seem to be very similar to those in tolerance induced by cell wall components from other bacteria since no difference could be observed between the tested combinations of tolerance and cross-tolerance. Furthermore, cross-tolerance data indicate, that signal pathways shared by LPS and TLR2 agonists seem to be impaired. Similar heterologous tolerance phenomena have recently also been described for macrophage-activating lipopeptides (MALP) from mycoplasma (105), bacterial DNA (CpG) (117), *Staphylococcus aureus* (118), LTA

(104), arabinose-capped lipoarabinomannan (119) and LPS. There is accumulating evidence that tolerance induced by different TLR2- and TLR4-agonists shares common intracellular signal transduction pathways (114), (105). However, recently Jacinto et al. suggested unique TLR2 signaling components downstream of TLR2 and upstream of MyD88/IRAK in LTA tolerance by demonstrating that IRAK expression and IRAK activity differs in LPS and LTA tolerance (120). Controversial data are reported regarding the involvement of the TLR in LPS and non-LPS tolerance and cross-tolerance. Our findings with primary murine bone marrow cells show that in the absence of the Borrelia-recognition receptor, TLR2, no tolerance could be induced (Figure 4.6b). Similarly the TLR4 was required for LPS-induced tolerance (Figure 4.7b) indicating that these two receptors are essential in Borrelia- and LPS-induced tolerance, respectively.

Differing results regarding the regulation of the TLR in tolerance have been published (105, 119, 121). Wang et al. showed downregulation of TLR2 in tolerance induced by synthetic bacterial lipopeptide (BLP), but not LPS (114). Our results regarding the TLR2-mRNA are partially in line with these observations, since the TLR2 was significantly downregulated in cells tolerized by Borrelia lysate upon re-stimulation with the same stimulus. As a major difference to Wang et al., we also observed a downregulation of the TLR2 by LPS. The opposing results could be ascribed to the fact that cross-tolerance we observe is complete and likewise induced by the two stimuli, unlike Wang et al. who report an incomplete LPS-induced cross-tolerance to BLP. Our data suggest that downregulation of the TLR2 seems to occur in tolerance, representing a probable explanation for the observed state of unresponsiveness to a second stimulation.

We also addressed the involvement of soluble mediators. Data from Randow et al. demonstrated that LPS-induced tolerance is mediated by endogenous cytokines such as IL-10 and TGFβ (113). Our results with regard to tolerance induced by Borrelia in the presence of IL-10-, TGFβ- and G-CSF-neutralizing antibodies (Figure 4.4) indicate that IL-10 is indeed involved in induction of Borrelia-tolerance. However, tolerance induction could not be completely prevented by blocking the mediator, suggesting that other mediators also play a role in this process. TGFβ, which also contributed to LPS-tolerance in human PBMC (113), was apparently not required for Borrelia-tolerance, nor was G-CSF. Further studies will be necessary to definitely

settle this point, since our data oppose data from others, showing that peritoneal macrophages from wildtype and IL-10$^{-/-}$ mice could be similarly tolerized by LPS and MALP, suggesting that IL-10 was not involved (105). Own results from coculture experiments with TLR2- and TLR4-deficient primary murine cells and the corresponding wildtype cells stimulated with LTA and LPS also argue against soluble factors responsible for suppression of TNFα upon secondary stimulation (104).

Our experiments comparing LPS and Borrelia-induced tolerance cannot provide final evidence regarding differences and similarities of these two phenomena, since our observations were restricted to TNFα-release, neglecting other mediators induced by bacteria. So far, except for TLR-involvement there was little difference between both phenomena. Taken together, in this study we characterized Borrelia-induced desensitization in human monocytes which is cross-reactive to LPS and to LTA and IL-10- and TLR2-dependent. Based on this ability of Borrelia to render cells hyporesponsive we propose that this might mirror a mechanism how this human pathogen avoids elimination and persists in the host leading to chronic disease.

5 Experimental Therapy of Lyme borreliosis with Granulocyte Colony-Stimulating Factor (Filgrastim)

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submitted to *Wien Klin Wochenschrift*

5.1 Abstract

Therapy of late stage Lyme borreliosis (LB) is difficult due to the limited success of antibiotic treatment in some cases. Here, we report an anecdotal observation of a late stage LB patient, who was successfully treated by a combination therapy of the cephalosporin antibiotic Ceftriaxone *plus* Filgrastim. As the patient became free of any symptoms with this new regimen, we were interested to investigate the effects of Filgrastim treatment of LB in more detail. Based on this case and on our recent report, that Borrelia modulate the host's immune response impairing their elimination (30), we raised the hypothesis that Filgrastim might restore the pathogen-induced dysfunction of immune competence. In order to study the therapeutic effects of Filgrastim in Borrelia infection we carried out experiments in two different mouse models. We first assessed the ankle swelling of Borrelia-infected immunocompetent disease-susceptible C3H/HeN mice treated with Filgrastim compared to placebo-treated controls and found that treatment had no beneficial effect on the pathology, i.e. joint swelling. In the next experiment the bacterial load was determined by quantitative real-time PCR. Less spirochetes were found in the bladder and in the ankle of Filgrastim-treated C3H/HeN mice compared to the placebo-treated. In severe combined immunodeficiency (SCID) mice infection again resulted in severe arthritis in both groups, however, the numbers of Borrelia were less in all organs tested of the Filgrastim-treated group. Taken together, the positive effects of Filgrastim treatment in the patient case as well as in mice prompted us to propose a clinical study on immunosupportive therapy of LB.

5.2 Introduction

While antibiotic treatment of early stage LB has a high success rate (122), treatment often fails in chronic stages (123-128), which are characterized by a broad variety of symptoms that often only develop after many symptom-free years. When a patient with symptoms and serology indicative of Lyme arthritis, who had failed in a first antibiotic therapy with cephalosporin infusion (2 grams of Ceftriaxone daily for two weeks) presented, we combined the repetition of this treatment with Filgrastim application, i.e. with a recombinant hematopoietic growth factor in clinical use with some prospects in strengthening host defense against infection. The therapeutic benefit of this experimental approach prompted us to study the value of Filgrastim as an adjuvant immunotherapy in preclinical settings. We reported recently that late stage Lyme borreliosis patients were characterized by a reduced release of pro-inflammatory cytokines in ex vivo stimulated whole blood in comparison to healthy controls (30). This finding suggests that immunosuppression and persistence of Borrelia might be causally linked. Since in particular Borrelia induced the predominantly anti-inflammatory, immunosuppressive IL-10 in blood from healthy donors (30), we raised the hypothesis that downregulation of immune response might represent a virulence mechanism of Borrelia, lending support to explain the clinical experience of reported antibiosis failure despite no documented resistance of Borrelia. A typical scenario of transient amelioration of symptoms under antibiosis might be explained by the fact that Borrelia numbers are reduced but not entirely eradicated because the lack of endogenous immune defense allows persistence and repopulation with recurrence of the symptoms.

Filgrastim (granulocyte colony-stimulating factor, G-CSF) is a recombinant, hematopoetic growth factor that primarily recruits and primes neutrophilic granulocytes, but also induces mono- and lymphocytosis in human volunteers (72). It has immunomodulatory properties, acting both as a pro-inflammatory (129-131) and as an anti-inflammatory cytokine (for review see (132, 133) (134)) possessing anti-infectious properties (135, 136). In addition, this endogenous growth factor has been shown to ameliorate the course of infection in more than 40 studies in non-neutropenic animals (for review see (137, 138)).

There is accumulating evidence that neutrophilic granulocytes contribute to host defense against Borrelia (39, 139-141). An obvious rationale in the case of LB might either be attributed to an enhanced defense capacity of granulocytes by Filgrastim, or

the recruitment of new, non-anergic leukocytes from the bone marrow. We therefore investigated this issue in the mouse model of Lyme borreliosis (142) which represents a model of the common form of LB in humans, and is accepted to study the development and regulation of subacute LB. Joint swelling was monitored and bacterial burden was quantified by real-time PCR during Filgrastim treatment of Borrelia infection in two disease-susceptible mouse strains, one being immunocompetent (C3H/HeN), the other one being genetically immunodeficient (SCID).

5.3 Materials and Methods

5.3.1 Case report

A 51 year old patient with a history of frequent exposures to tick bites presented with polyarthritis in the fingers and feet. Arthritic destruction of synovial clefts mainly in the metacarpophalangial and in the proximal interphalangial joints of fingers and feet could be demonstrated by X-ray. Low, but clearly positive, serum titers of Borrelia IgG by ELISA and immunoblot (p100 +++) and a negative IgM-ELISA (both Max-Pettenkofer-Institute, Munich, Germany) corroborated diagnosis of late stage Borrelia infection. A standard two week i.v. treatment with 2 g/day Ceftriaxone (Rocephin®, Hoffmann LaRoche, Grenzach-Whylen, Germany) led to transient improvement of symptoms, i.e. subjective decline of arthritis, that lasted for eight weeks. Then, the inflammatory symptoms returned and became progressively worse, indicating that the treatment had probably failed. We hypothesized that persistence of Borrelia might be due to a disabled immunocompetence of the patient. Therefore, we tested whether a complete eradication of the pathogen could be achieved by combining immunosupportive treatment with antibiosis. The experimental treatment regimen, applied with the informed consent of the patient, was as follows: First week 2 g Ceftriaxone (Rocephin®) i.v. daily, second week 480 µg s.c. Filgrastim (Neupogen®, Amgen, Thousand Oaks, USA) every second day, and third week 2 g Ceftriaxone daily *plus* 300 µg Filgrastim every second day (Figure 5.1). Neutrophil counts were determined by a Coulter STKS counter (Coulter, Krefeld, Germany).

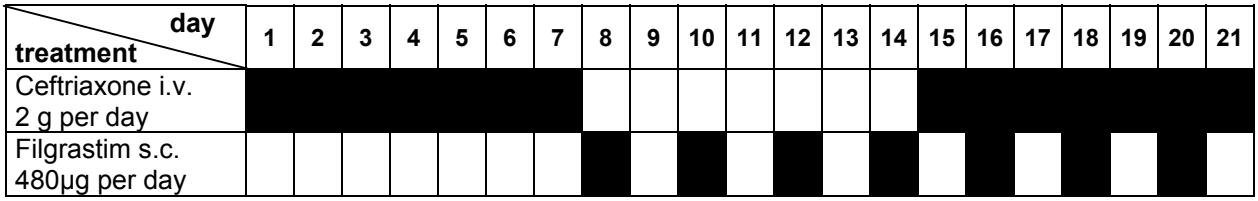

Figure 5.1: Treatment schedule of the combination therapy with Ceftriaxone and Filgrastim
The patient received intravenous infusion of 2 g/d Ceftriaxone within one hour and / or 480 µg Filgrastim s.c. on the days indicated by black boxes.

5.3.2 Whole blood incubation

20% citrate blood in RPMI 1640 medium (Biochrom, Berlin, Germany) *plus* 2.5 IU heparin (Liquemin®, Hoffmann LaRoche) was stimulated in open polypropylene reaction tubes (Eppendorf, Hamburg, Germany) at 37°C and 5% CO_2 for 24 h with either 0.1 µM phorbol myristate acetate (PMA), 10 µg/ml *Salmonella abortus equi* endotoxin (LPS), or 1 µg/ml *Staphyloccocus enterotoxin* B (SEB). All stimuli were from Sigma (Deisenhofen, Germany). Cell-free supernatants obtained after resuspension and centrifugation at 3000 g for 2 min were stored at -80°C until cytokine measurement.

5.3.3 Experiments with C3H/HeN mice

Female C3H/HeN mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and housed in micro-isolator cages. They were provided with food and water ad libitum. All animals received humane care in accordance with the National Institutes of Health guidelines and the legal requirements in Germany. Mice were randomized by weight into groups. At 4 weeks of age (range of weight: 10 - 16.5 g) mice were needle inoculated intradermally into dorsal skin at the base of the tail with 2 x 10^6 *B. garinii* A218 P3, late log-phase (a kind gift from H. Martilla, National Public Health Institute, Department in Turku, Finland) in 100µl Barbour-Stoenner-Kelley medium (BSK, Sigma-Aldrich, Seelze, Germany). Control animals were mock injected with BSK. Tibiotarsal joints were measured twice a week with a metric caliper (Mitutoyo, Tokyo, Japan), and weight was assessed. Measurements were taken in the anterior-to-posterior position, with the ankle extended, through the thickest diameter of the ankle. Filgrastim was injected subcutaneously in the dorsal skin. In the first experiment (arthritis development), Filgrastim treatment was as follows: (250 µg/kg) on days -3, -2, -1, 0 (= day of infection), 1, 2 (Sunday) and from then on on Mondays, Wednesdays and Fridays until day 38. Treatment schedule for the second experiment (bacterial burden) was: 40 µg/kg on day -3, -2, -1, 0 (= day of infection),

1, 2 and, starting at day 3 with 250 µg/kg, every second day until day 12. Untreated controls received saline-injections on the respective days. At the end of the observation period, mice were killed by terminal pentobarbital anesthesia (Narcoren®, Merial, Hallbergmoos, Germany).

5.3.4 Experiments with SCID mice

C.B-17.SCID mice were bred under specific pathogen-free conditions at the Max-Planck-Institute for Immunobiology, Freiburg, Germany. Virulent, low-passage tick isolate *B. burgdorferi* ZS7 was grown in BSK-medium and harvested as described (143). 6 - 8 week old female mice were randomized by weight into 3 groups: group A: uninfected control mice (n = 3), group B: infected mice (n = 7) and group C: infected mice *plus* Filgrastim treatment (n = 7). Filgrastim (250 µg/kg) was injected subcutaneously into the dorsal skin, on days -3, -2, -1, 0 (= day of infection), 1, 2 (Sunday) and from then on on Mondays, Wednesdays and Fridays until day 27. Untreated controls received saline-injections on the respective days. Mice were needle inoculated intradermally (s.c.) with 1 x 10^5 *B. burgdorferi* ZS7. Control animals were mock-injected with BSK. Mice were monitored for the development of clinical arthritis under blinded conditions. The development of arthritis in the right and the left tibiotarsal joints was scored as described (144) using swelling and reddening as criteria: ++, severe; +, prominent; (+), moderate; +/-, mild; (+/-), mainly reddening without significant swelling; and -, no clinical signs of arthritis. At the end of the observation period (day 27 p.i.), mice were killed by terminal pentobarbital anesthesia (Narcoren®).

5.3.5 Murine leukocyte counts

Blood was obtained by cardiac puncture under terminal pentobarbital anesthesia. White blood cell counts were determined microscopically in a Neubaur chamber after erythrocyte lysis with Türk's solution (Merck, Darmstadt, Germany). Leukocyte differential counts of 100 cells each were done on May-Grünwald/Giemsa-stained smears.

5.3.6 Determination of cytokine production by peritoneal lavage cells

Mice were killed by terminal pentobarbital anaesthesia. Peritoneal cavities were lavaged with 10 ml ice-cold PBS (Life Technologies, Karlsruhe, Germany). The lavage was transferred to siliconized glass tubes (Vacutainer, Becton Dickinson,

Heidelberg, Germany) for isolation of peritoneal cells. After centrifugation, cells were resuspended in medium (RPMI 1640, Bio Whittaker, Apen, Germany) containing 10% FCS (Biochrom) and 100 IU/ml penicillin/streptomycin (Biochrom) and plated to 96-well culture plate (bio-one, Greiner, Frickenhausen, Germany) at a density of 1 x 10^5 cells per well. Cells were stimulated immediately with pyrogen-free saline (Braun, Melsungen, Germany), 0.01 µg/ml – 10 µg/ml LPS from *Salmonella abortus equi* (Sigma) or 0.1 – 10 µg/ml Borrelia lysate (30). After incubation for 24 h at 37°C in the presence of 5% CO_2, the supernatants were transferred into 96-well round-bottom plates (Greiner) and stored at -80°C until cytokines were measured.

5.3.7 Quantitative real-time PCR of Borrelia DNA in murine tissue

Ear tissues, contralateral rear ankle joints, lymph nodes, meninges, entire hearts, brains and bladders were harvested from experimental mice at the end of the observation period. Brain and heart samples were homogenized with a polytron (PT1200; Kinematik, Lucerne, Switzerland) in order to test a representative aliquot from these organs. DNA was prepared from the tissues with Qiagen DNeasy™ tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA content was determined by measuring the absorbance at 260 nm against 280 nm and adjusted to 10 µg DNA/ml. Quantification of Borrelia DNA was performed by real-time PCR as described previously (145).

5.3.8 Cytokine ELISA

Monoclonal antibody pairs against human IFNγ were purchased from Endogen (Biomar, USA / Endogen). Recombinant human IFNγ (a generous gift from Thomae Biberach, Germany) was used as standard. For the measurement of murine (mu) TNFα, polyclonal antibodies from R&D Systems (Wiesbaden, Germany) and standard from Pharmingen (BD Bioscience, Heidelberg, Germany) were used. For the detection of mu IL-10 the OptEia kit from PharMingen was used. Assays were carried out in flat bottom, ultrasorbant 96-well plates (Nunc, Wiesbaden, Germany). Binding of secondary biotinylated antibodies was detected with horseradish-peroxidase-conjugated streptavidin (Biosource, Camarillo, CA, USA) and 3',3',5',5'-tetramethylbenzidine solution (Sigma) used as substrate.

5.3.9 Statistics

Data in the figures and in the tables are given as means ± SEM. Analysis of bacterial burden and leukocyte numbers were done with the unpaired t-test or the two-sided Welch's correction test for two groups. For experiments with more than two groups one-way ANOVA was performed, followed by Bonferroni's multiple comparison test for selected groups or Dunnett's test for comparison with the control group. All tests were performed using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, USA). $P \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$ were considered significant and are depicted as *, ** and *** respectively.

5.4 Results

5.4.1 Patient case report

The combination therapy of Ceftriaxone *plus* Filgrastim was well tolerated. Only after the first injection of Filgrastim the patient reported acute but moderate pain in the previously affected joints i.e. the shoulder, fingers and knees. Circulating neutrophil counts increased from 1400 to 17000 cells/µl within 24 h after the first Filgrastim injection. Monocyte numbers increased about two-fold, while there was little effect on lymphocytes (Figure 5.2a). The plateau of neutrophil counts at about 17000 cells/µl blood was maintained until one day after the end of treatment. The subjective symptoms disappeared during the following six weeks after the treatment. The patient reported that he was able to resume previously abandoned sporting activities including mountain climbing and downhill skiing. Moreover, fine mechanical skills needed for piano playing were restored. After three months, the Borrelia IgG titer was negative. The intensity of the immunoblot at this time point was significantly reduced (from +++ to +) and two years later it was negative. Eight years after treatment the patient is still free of arthritic symptoms.

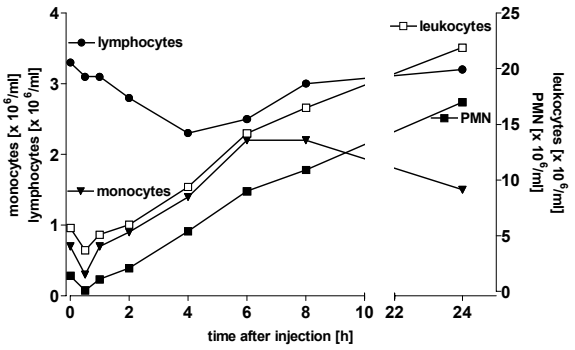

Figure 5.2a. Leukocyte counts of a Lyme borreliosis patient after first injection of 480 µg Filgrastim s.c.
On day 8 of the therapy the patient was injected with 480 µg Filgrastim s.c. at 8 a.m. (= time point 0). Blood was withdrawn at the time points indicated and the differential white blood cell count was determined (PMN: polymorphonuclear neutrophil).

In order to monitor the immunological response of our patient, we followed the attenuation of IFNγ-release capacity of whole blood taken at various time points after Filgrastim injection, which is a well-known effect of this treatment (146). The release of the lymphocyte-derived pro-inflammatory cytokine IFNγ was stimulated ex vivo by different stimuli, i.e. PMA (0,1 µM), LPS (10 µg/ml) or SEB (1 µg/ml). As expected, the inducible release of IFNγ in blood of the Filgrastim-treated patient was abrogated from 2 h after Filgrastim injection onwards (Figure 5.2b), indicating that Filgrastim treatment was effective.

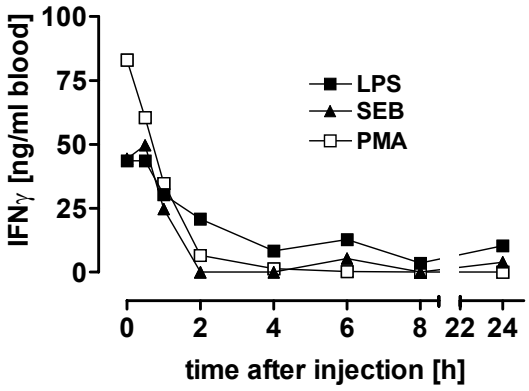

Figure 5.2b. Ex vivo IFNγ-release capacity of blood from a Lyme borreliosis patient after first injection of 480 µg Filgrastim s.c.
The patient was injected with 480 µg Filgrastim s.c. at 8 a.m., i.e. time point 0. Blood was withdrawn at the time points indicated and stimulated with 10 µg/ml LPS, 1 µg/ml SEB or 0.1 µM PMA for 24 h at 37°C. Cytokines in the supernatant were determined by ELISA.

5.4.2 Course of *B. garinii*-infection in C3H/HeN mice under Filgrastim treatment

This anecdotal therapeutic success of Filgrastim in the patient prompted us to carry out further experimental studies. In order to address a possible effect of Filgrastim more systematically, we carried out experimental treatments in different mouse strains.

Arthritis-susceptible C3H/HeN mice were needle inoculated with Borrelia and arthritis development was monitored by measuring joint swelling for 38 days. This is shown

for infection with and without Filgrastim treatment in figure 5.3 in comparison to uninfected control mice. There was no significant difference in ankle swelling between the Filgrastim-treated and placebo-treated group. From day 21 p.i. ankle swelling was similar in all infected mice. It peaked by day 28 and then resolved continually. Thus, in spite of a slight delay in measurable arthritis manifestations, Filgrastim treatment did not attenuate the extent of transient arthritis observed in infected mice.

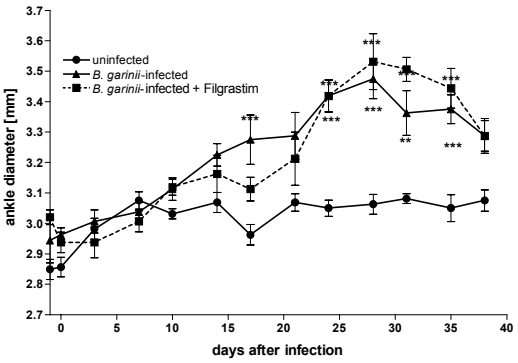

Figure 5.3. Effect of Filgrastim treatment on ankle swelling following infection of mice with *B. garinii*
C3H/HeN mice (n = 8) were inoculated s.c. in the base of the tail. Measurements were taken in the anterior-to-posterior position, with the ankle extended, through the thickest diameter of the ankle. Results are depicted as mean diameter (mm) ± SEM, **, $p \leq 0.01$ and ***. $p \leq 0.001$ vs. control.

5.4.3 Determination of dissemination kinetics of *B. garinii*-infection in C3H/HeN mice

Kinetics of Borrelia dissemination into various tissues of inbred mouse strains have been studied by several groups (50, 79, 147-149). It is well known that the course of infection varies depending on the mouse strain and on the Borrelia strain used for infection. We therefore characterized our model with regard to the spirochetal load over the time, to determine when peak spirochete numbers are reached. C3H/HeN mice were infected with *B. garinii* or mock-injected with BSK-medium. On day 1, 3, 22 and 43 post infection, bacterial load was assessed by quantitative real-time PCR in four different organs from four infected mice and their respective controls. In order to exclude the presence of inhibitors, which might interfere with the detection of Borrelia DNA by PCR, spike experiments were carried out with all organs. No interference could be seen, since spike recovery was around 100% for all the tissues. The bacterial load of the tested organs differed strongly (Figure 5.4). Bladder and ankle contained high numbers of spirochetes, whereas in the meninx and the lymph node less spirochetes were detected. *B. garinii* spread quickly from the infection site (base of the tail) to other tissues. The highest numbers of spirochetes were detected at day 10 p.i. in all organs tested correlating with the evolving arthritis and declined

rapidly over time. In an additional long-term experiment, in spite of strong ankle swelling around day 30 p.i., no Borrelia DNA at all could be detected by PCR on day 135 p.i. (data not shown), indicating that infection had completely been cleared by the immune cells of the disease-susceptible inbred C3H/HeN mice.

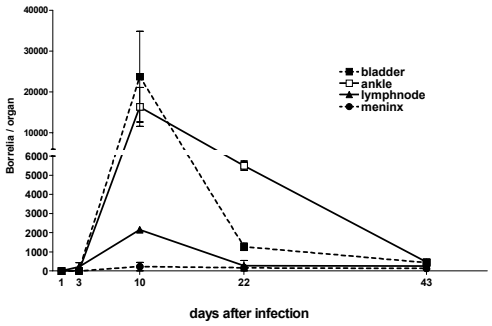

Figure 5.4. Kinetics of spirochete distribution in tissues from mice
C3H/HeN mice were infected with 2 x 10^6 *B. garinii*. At the time points indicated 4 mice per group were sacrificed and DNA was isolated from selected tissues. The numbers of spirochetes were determined by real-time PCR. The mean numbers of spirochetes ± SEM per organ are given.

In order to characterize the immune response of mice during the course of *B. garinii*-infection, we determined leukocyte numbers and performed stimulation experiments with isolated macrophages *in vitro*. Neither overall leukocyte numbers, nor monocyte, lymphocyte, or neutrophil counts differed between infected and uninfected mice during the observation period of 43 days (Figure 5.5). In addition, mice's weights were not influenced during the whole course of the experiment (data not shown). These results confirm that Borrelia-infection in mice does not represent a severe infection, which would interfere strongly with the host's immune system. When peritoneal lavage macrophages from infected or uninfected control mice were stimulated with either LPS or Borrelia lysate for 24 h, there was no significant difference in release of pro-inflammatory TNFα and anti-inflammatory IL-10 between the two groups at any time point tested (i.e. day 1, 3, 10, 23, 43). The TNFα levels induced by 10 µg/ml Borrelia lysate ranged from 100 to 200 pg/ml, and IL-10 levels between 200 pg/ml and 300 pg/ml were measured. No measurable TNFα levels could be detected in plasma of infected and uninfected mice.

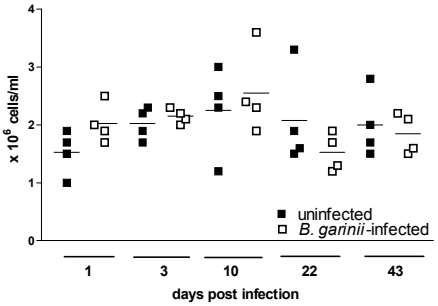

Figure 5.5. Leukocyte counts from uninfected and *B. garinii*-infected C3H/HeN mice
At the indicated days post infection 4 uninfected (dark symbols) and 4 infected (light symbols) mice were sacrificed and leukocyte counts in the blood were determined in a Neubaur chamber. The line corresponds to the mean of the 4 values.

5.4.4 Effect of Filgrastim treatment on *B. garinii* numbers in tissues of C3H/HeN mice

It has been shown, that symptomatic manifestations in both mice and humans are strongly associated with the presence of bacteria, however, the levels of tissue spirochetes and the severity of pathology do not correlate strictly with each other (50, 150). Therefore, we determined the effects of Filgrastim on spirochete burden in the host's tissues, instead of concentrating only on a possibly resolving arthritis.

Based on our results from the dissemination kinetics, day 12 p.i. was chosen to terminate a further experiment, since by this time point joint inflammation had started to evolve and spirochetes had reached maximum numbers in the organs. The distribution of *B. garinii* in seven tested organs (ankle, bladder, lymph node, meninx, ear, brain, heart) was not influenced uniformly by Filgrastim (Table 5.1). There was a decrease in Borrelia numbers in the bladder and in the ankle of Filgrastim-treated mice, however the reduction was not significant. The lymph node was similarly infected with *B. garinii* in both groups, but it has to be taken into account that spirochete levels were around the detection limit in this organ. Surprisingly, no Borrelia at all could be detected in meninx, ear, brain, and heart.

Table 5.1. Borrelia numbers in selected tissues from C3H/HeN mice during Filgrastim treatment [a]

	ankle	bladder	lymph node	meninx	ear	brain	heart
C3H/HeN	5960 ± 1220	2280 ± 950	340 ± 180	n.d.	n.d.	n.d.	n.d.
C3H/HeN + Filgrastim	3170 ± 1030*	560 ± 200*	460 ± 180*	n.d.	n.d.	n.d.	n.d.

[a] Filgrastim-treated (treatment schedule see material and methods) and untreated mice were infected with 2 x 10^6 *B. garinii* (A218) and sacrificed at 12 days p.i.. DNA was extracted from the tissues and amplified by real-time PCR as described in materials and methods. Borrelia numbers / organ were quantified with a standard curve. Means ± SEM from 8 mice are given, (* not significant vs. C3H/HeN, n.d. not detectable).

To control the effectiveness of Filgrastim treatment, leukocyte counts were determined. Neutrophil numbers were increased in Filgrastim-treated compared to untreated mice (3.5 x 10^6 / ml ± 0.7 vs. 1.9 x 10^6 / ml ± 0.3, p = 0.08). However, the effect of recruiting neutrophils from the bone marrow was less pronounced than expected (109).

5.4.5 Effect of Filgrastim treatment on *B. burgdorferi* infection in SCID mice

Murine *Borrelia burgdorferi* infection is often studied in severe combined immunodeficient (SCID) mice which, despite lacking B- and T-cells, develop severe

arthritis (148, 151). We were interested to investigate whether Filgrastim possesses beneficial effects on arthritis development and spirochete clearance in these mice. Therefore we repeated the experiments in C.B-17 SCID mice infected with *B. burgdorferi* s.s. ZS7 at the Max-Planck-Institute in Freiburg. The same treatment protocol as with immunocompetent mice was applied. Development of clinical arthritis was again assessed by scoring the redness and degree of swelling of the joint during the infection. Filgrastim-treated and untreated *B. burgdorferi*-infected SCID mice developed similarly strong ankle swelling compared to the uninfected control mice (Figure 5.6).

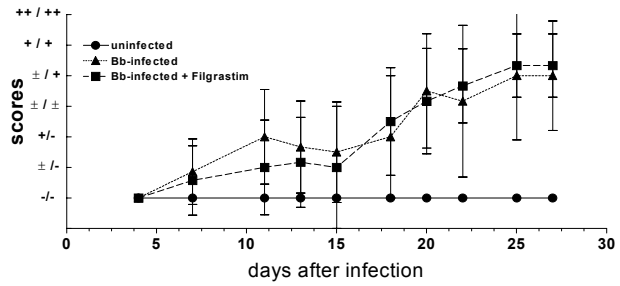

Figure 5.6. Effect of Filgrastim treatment on ankle swelling of CB-17 SCID mice following infection with *B. burgdorferi* s.s. ZS7
CB-17 SCID mice (n = 4 control group, n = 7, infected groups) were inoculated s.c. in the base of the tail. The development of arthritis in the right and the left tibiotarsal joints was scored as described using swelling and reddening as criteria: ++, severe; +, prominent; (+), moderate; +/-, mild; (+/-), mainly reddening without significant swelling, and -, no clinical signs of arthritis. Results are depicted as the mean diameter (mm) ± SEM.

As in the experiment with C3H/HeN mice, Filgrastim treatment caused a slight delay in the development of ankle swelling, however, from day 18 p.i. the extent of swelling was the same in the two infected groups. Thus, Filgrastim treatment did not seem to have an attenuating effect on Borrelia-induced ankle swelling in immunodeficient SCID mice. By the end of the experiment (day 27 p.i.), spirochetal burden in 5 different organs was assessed by quantitative real-time PCR. Borrelia DNA could be detected in all tested organs from both, Filgrastim treated and untreated *B. burgdorferi*-infected mice. There was an 18-fold reduction in the numbers of Borrelia in the pericardium and a 9-fold reduction in the ankles. Bladder, lymph node and meninx from Filgrastim-treated mice contained 2-times less Borrelia than the respective organs from untreated mice (Table 5.2). Thus, in general, Filgrastim-treated SCID mice harbored fewer spirochetes in the investigated tissues than untreated mice, indicating that Filgrastim treatment might improve elimination of Borrelia from the tissues of immunodeficient mice.

Table 5.2. Borrelia numbers in tissues from SCID mice during Filgrastim treatment [a]

	ankle	bladder	lymph node	meninx	pericardium
SCID	7670 ± 4360	4640 ± 2040	2970 ± 1290	880 ± 260	3670 ± 3350
SCID + Filgrastim	880 ± 170*	1950 ± 280*	1240 ± 510*	480 ± 230*	200 ± 80*

[a] Filgrastim-treated (treatment schedule described in material and methods) and untreated C.B.17-SCID mice were infected with 1 x 10^5 *B. burgdorferi* s.s. (ZS7) and sacrificed at day 27 p.i.. Means ± SEM from 4 mice are given, * not significant vs. SCID (for further details see Table 5.1 legend).

5.5 Discussion

While early stages of LB respond to therapy with antibiotics, therapy of late stage LB is still unsatisfactory. About 10% of all cases of late stage LB are therapy-resistant, i.e. they cannot be cured with standard therapies (124, 152-154).

Immunosupportive therapy represents a novel promising approach in the treatment of chronic LB. We chose Filgrastim for a combination therapy with antibiotics, due to its unique anti-infectious and hematopoietic pharmacological properties, and the fact that it is well tolerated on the other hand, as shown in many clinical trials (138). The treatment regimen for the patient was set on the basis of previous experiences from various studies treating healthy volunteers with Filgrastim (72, 146, 155, 156). The major reservation was the intention to avoid a Herxheimer reaction, i.e. a stage of hypertension, fever and rigors, subsequently followed by a stage of hypotension after initiation of antibiosis in LB patients. Therefore, we started Filgrastim treatment after one week of antibiotic therapy when the bacterial burden should have declined and a Herxheimer reaction was less likely. During this week antibiotic treatment was interrupted to avoid possible interferences. Finally, the treatment course was completed with one week of combined therapy. The increasing experience of the safety of Filgrastim treatment in infectious diseases and the experience from this first case reduces these precautions retrospectively. Therefore, in future experimental trials, interruption of antibiosis might not be necessary, though we still feel that initiation of antibiosis should precede Filgrastim therapy. The injection of Filgrastim every other day resulted from our experience with healthy volunteers, where both neutrophilia and immunomodulation by Filgrastim lasted for 48 hours after single injection of Filgrastim (146). Since the effects of Filgrastim on leukocytes other than neutrophils were most prominent after 5 days of daily treatment, we would nowadays consider this the most promising regimen to be combined with antibiosis (72). Noteworthy a later study in healthy volunteers indicated that G-CSF treatment thrice a week maintains its hematopoietic but not its anti-inflammatory effects (156). The

case reported here represents a relatively mild case of chronic Lyme arthritis. It is not clear whether positive effects of Filgrastim would also translate to more severe cases, especially to patients with neuroborreliosis, where bacteria are difficult to reach for leukocytes in the immunopriviledged central nervous system.

Our recent observation that blood from patients with chronic LB shows a suppressed pro-inflammatory cytokine release when stimulated ex vivo (30) made it attractive to speculate that the immunomodulatory effects of Filgrastim contributed the therapeutic efficacy. Properties of Filgrastim which most likely enhanced the beneficial effects of the treatment include amplified hematopoesis, which leads to an enforcement of the innate immune system, allowing a greater number of non-anergic, naive activated neutrophils to reach and kill bacteria even at remote sites of infection. The enhanced anti-bacterial capacity might be especially efficient under the chosen conditions: antibiotic pre-therapy would lead to lysis of bacteria thus releasing membrane components that allow Filgrastim-activated neutrophils to be attracted by chemotactic signals to eradicate the bacteria at poorly perfused, e.g. intraarticular locations. Additional effects of Filgrastim on neutrophils which might also have contributed to the therapeutic efficacy of the combination treatment include increased microbicidal activity (157), enhanced oxidative burst (130, 158), and improved adherence and phagocytic capacity (129, 159). It is well-known that repetitive treatment with antibiotics increases the probability of Borrelia eradication and success of the second course of treatment might be explained by this simple fact. However, it is very intriguing that the patient reported warmth and pain for two days after the first two injections of Filgrastim only in those joints which were affected by arthritic pain and immobility over years before. This incident could be tentatively explained by a successful granulocytic local attack on residual bacteria in the joints.

To investigate in more detail the effects of Filgrastim in Borrelia infection we employed the mouse model of immunocompetent C3H/HeN and immunodeficient SCID mice. Filgrastim could not attenuate edema formation, as monitored by ankle swelling, neither in immunocompetent inbred mice, nor in SCID mice**.** However, increasing evidence indicates that the degree of arthritis (assessed as ankle swelling) does not necessarily correlate with histopathology. Anti-IL-12 treatment for example did not affect the degree of ankle swelling, but arthritis documented by histopathology was improved significantly (33). Wooten et al. found that ankle swelling provides only information on the degree of edema within the ankle tissue, but neglects joint

pathology (100). This notion is supported by recent findings indicating that ankle swelling and arthritic lesion are two traits which are regulated independently by genetic loci mapping to distinct locations (160). Histological analyses of the joints could not be performed retrospectively. Thus we cannot rule out that, despite lacking an effect on joint swelling, Filgrastim might have attenuated the inflammation in the joint, i.e. sheath thickness and inflammatory cell infiltration.

Different authors observed that arthritis severity does not always correlate with spirochete numbers in the organs from infected mice. A comparison between different inbred mouse strains showed that, in spite of equal levels of spirochetes in the ankles, the degree of arthritis pathology differed strongly (50) (161). Anti-IL-12 treatment reduced arthritis severity, but led to increased spirochetal load in the ear (33) and IL-10$^{-/-}$ mice harbored less Borrelia in their ear, ankles and hearts, but displayed a more severe arthritis compared to their wildtype littermates (53). Consequently we next determined whether Filgrastim treatment altered the number of spirochetes in infected mice. The bacterial burden was assessed by quantitative real-time PCR (145).

Dissemination kinetics of Borrelia in mice have been studied extensively by many different authors (50, 79, 147-149). However, it is well known, that on the one hand Borrelia strains spread differently depending on the immunocompetence of the host, and on the other hand mouse strains are differently susceptible depending on the Borrelia strain used for infection (162-165). Our results on dissemination kinetics of *B. garinii* in C3H/HeN mice are in line with data from others (50, 147-149), finding peak spirochetal numbers by day 10 of infection.

The numbers of spirochetes in C3H/HeN mice were only moderately influenced by immunosupportive Filgrastim treatment. Some organs of the Filgrastim-treated mice appeared to contain less spirochetes at the time points tested (bladder, ankle), while others (brain, ear, heart, and meninx) did not show any Borrelia. It is not clear whether the chosen time point (day 12 p.i.) is representative for the whole course of the infection and treatment. Noteworthy, the treatment dosage and administration protocol was set following the case report. Possibly a lower dosage given daily would have had stronger effects on arthritis development and / or bacterial load. The fact that the effects of Filgrastim treatment on neutrophil numbers were less pronounced than in a previous study, in which a corresponding dosis of murine G-CSF was given on two consecutive days leading to a 5-fold increase indicates that the administration

protocol was suboptimal. Importantly a high species cross-reactivity has been shown by Moore et al. (166) making it unlikely that in our experiment the poor effectiveness was due to the use of human Filgrastim in mice.

Additional experiments demonstrated that Borrelia-induced immune dysfunction and longterm persistence cannot be investigated in immunocompetent mice: firstly, Borrelia numbers declined over time and were no longer found at day 135, indicating a long-lasting but, in contrast to humans, self-limiting infection; secondly, unlike chronically infected patients, when testing the influence of Borrelia-infection on macrophages of immunocompetent mice, no alteration of ex vivo inducible cytokine production in infected mice was found, as was characteristic for LB patients (30), suggesting that mice are not immunosuppressed by Borrelia. These results are in line with data from Montgomery et al., who showed, that Borrelia neither induce a global downregulation of phagocytic functions in mice (167) nor reduce inflammatory activity in infected organs of these animals (168). It is attractive to speculate but difficult to prove that the lack of immunomodulation is linked to the self-limiting character of the infection in mice in contrast to humans.

Since the model of Borrelia infection in immunocompetent mice appeared to be unsuitable to study persistence of Borrelia, we studied the effects of Filgrastim in SCID mice, lacking B- and T-cells, which develop an array of symptoms such as chronic progressive arthritis typical of LB in man (144) and which do not show resolution of disease over time (66, 148). By treating SCID mice with Filgrastim a reduction of spirochete levels in ankle, bladder, pericardium, lymphnode and meninx was observed. Our observation that Filgrastim given without antibiotics to immunodeficient mice, which do no possess an acquired immune system, resulted in some reduction of bacterial burden, suggests that Filgrastim mainly acted on the innate immune system, enhancing monocyte and neutrophil recruitment. This is of special importance because neutrophils have been shown to play a critical role in spirochetal clearance (140).

In conclusion, the concept of Borrelia-induced immune dysfunction as an explanation of persistence and frequent antibiotic failure cannot be studied in the mouse model. However, considering the unsatisfactory current antibiotic treatment, the positive experience of an experimental therapy with Ceftriaxone *plus* Filgrastim in a LB patient, and the tendency of enhanced elimination of Borrelia from mice with

Filgrastim alone, are quite promising and encouraged an experimental clinical trial which is currently underway.

6 Discussion

Understanding the pathogenesis of a bacterial infection is of major importance when ameliorating diagnostics and searching for preventive and therapeutic strategies. This study provides insights into the pathogenesis of Lyme borreliosis, which could help to clarify how the causative agent, *B. burgdorferi*, interacts with the host defense in order to persist.

6.1 Borrelia-induced immunogenicity versus host predisposition

When investigating Borrelia-induced pathology it has to be taken into account that the pathogen as well as the host contribute to the outcome of LB, thus when studying host-pathogen interactions both aspects have to be considered.

6.1.1 Borrelia-induced immunogenicity

Several reports have been published demonstrating, that Borrelia-derived components, such as the outer surface proteins (150, 169), the Erp-proteins (170), and the variable major protein-like sequence expressed (VlsE) (171), contribute to the immunogenicity of the pathogen and therefore affected LB development. Experiments with low and high passaged Borrelia demonstrate, that with increasing passage number outer surface protein expression varies and infectivity decreases considerably (172-174). Further, complement regulator-acquiring surface protein (CRASP) 1 and CRASP-2 Borrelia have been identified by Kraiczy et al. demonstrating that these two outer suface proteins bind human complement regulators and control complement activation on their surface, preventing the formation of toxic activation products and conferring resistance to Borrelia (87). Another property of Borrelia which contributes to the development of LB is its ability to invade various host tissues. Several candidate bacterial ligands haven been identified, including decorins, fibronectin, glycosaminolgycanes and β_3-chain integrins (for review see (175)), which might facilitate binding to the mammalian cells. Recent studies underline the interplay between host and pathogen signals in both directions showing that pro-inflammatory responses of the mammalian host trigger recombination in the variable protein-like sequence expressed (vlsE) locus of the spirochete resulting in the appearance of antigenically altered vlsE gene products (176).

6.1.2 Host predisposition

More recently, there is accumulating evidence that host-derived factors play a critical role in disease pathology (50). The control of Borrelia invasion in the human body is characterized by the release of cytokines (78, 177, 178) and of oxygen radicals (38, 179) as well as the upregulation of adhesion molecules (180, 181). Depending on the magnitude of the inflammatory response its result can either be the resolution of infection or, if the immune response is excessive, damages such as arthritis. On the other hand ineffective clearance of the spirochetes will occur if the response is too weak. Thus, the strength of the immune response is decisive for the outcome of the disease. Using genetically and immunologically manipulated mice the importance of the host's immune system has been investigated and led to the identification of several host genes, controlling spirochete growth and influencing the severity of arthritis, possibly by regulating the balance of pro- and anti-inflammatory responses (reviewed in (182)). Steere et al. demonstrated that particular class II major histocompatibility complex alleles represent important determinants for the host's immune response to *B. burgdorferi* and consequently for the pathology of LB. According to their findings, the presence of HLA-DR4 and HLA-DR2 correlates with the failure to respond to antibiotic therapy and consequently with chronic Lyme arthritis (54). A genetic predisposition for chronic arthritis in humans has additionally been associated with the appearance of Borrelia-specific T-cells that cross react with self antigens (29). Recently the role of the TLR in Lyme borreliosis has been addressed. Identifying Lyme disease vaccine low responders characterized by a lower cell-surface expression of Toll-like receptor (TLR) 1, but normal expression of TLR2, as compared to normal cells, Alexopoulou et al. demonstrated that defects in the TLR1/2 signaling pathway may account for human hyporesponsiveness to OspA vaccination (183).

In the present study, LB pathogenesis has been elucidated from two aspects: first, the immune responsiveness of Borrelia-infected patients was characterized and second, the effect of Borrelia on the immune response of healthy blood cells was examined.

6.2 Hypotheses of Borrelia persistence

Although Borrelia-infection leads to activation of monocytes and granulocytes, and further induces a prominent antibody and T helper (Th) cell cytokine response in

humans, no protective immunity is conferred, indicating that Borrelia-induced activation of the immune system alone is not sufficient to eradicate the pathogen and to protect against ongoing infection. Several hypotheses have been put forward to explain the persistence of Borrelia in the human host:

It has been postulated that Borrelia interact with the complement system, inactivating the complement regulatory proteins FHL-1/reconectin and Factor H (86, Kraiczy, 2002 #912). Others have proven that Borrelia can hide themselves in immunoprivileged sites such as the collagen fibers of the connective tissue (88) and in human synovial cells (24) as well as in the extracellular matrix (25). The antigenic variation of the *B. burgdorferi* outer membrane is also discussed as a possible strategy to evade the immune response (26, Zhang, 1997 #938, Wilske, 1996 #939). Data from Gross et al. suggest alternatively, that Borrelia induce an auto-immune process, as they could identify a homology between the Borrelia outer surface protein A (OspA) and the human lymphocyte-function-associated antigen-1 alpha (hLFA-1) and also found cross reactive T-cells (29).

6.2.1 Immunomodulation as a possible immune evasion strategy

In the present study a further hypothesis was raised postulating that the host's immune response is modulated by the pathogen such that the bacteria shift or suppress it in a way that enables survival of the pathogen. Examples of this latter type are known for viral (58), bacterial (59) and parasitic infections (60), and has hence led to the concept of microbial cytokine-inducing or suppressing molecules named modulins (61, 62). Our hypothesis of Borrelia-induced immunomodulation as a possible immune evasion mechanism is based on the findings that ex vivo stimulated whole blood from patients with chronic LB revealed a significantly reduced capacity to release TNFα and IFNγ in comparison to blood cells from healthy controls (see chapter 3). There are few other reports about Borrelia acting as an immunomodulator**:** Mullegger et al. found a lack of IFNγ expression in the LB-specific chronic skin lesions, i.e. acrodermatitis chronica atrophicans (ACA), and postulated reduced effectiveness of spirochetal killing (91). Furthermore, the anti-inflammatory influence of Borrelia is reflected in a decreased expression of MHC II markers on Langerhans cells in skin of ACA patients (92). An unusual subset of T-cells, characterized by secreting Th1 (IFNγ) and Th2 (IL-10) cytokines concomitantly, were identified in the joints of LB patients in which Borrelia were present (115). Sigal et al.

designated Borrelia “a potent immunomodulator due to its observed potential for immundysregulation” (82).

The idea of Borrelia-induced immunomodulation was extended by our findings, that Borrelia are stronger inducers of an anti-inflammatory cytokine response than endotoxins from various Gram-negative bacteria (see chapter 3), confering them the capacity to influence the balance between the pro- and the anti-inflammatory immune response. Our data are in line with findings from other authors, indicating that *B. burgdorferi* is a potent inducer of the anti-inflammatory cytokine IL-10 (46, 84, 116). In addition, the modulatory and regulatory capacity of IL-10 with regard to Borrelia-induced cytokine release has been found in different *in vitro* models (93, Lisinski, 2002 #952). Evidence for the *in vivo* relevance of the modulatory capacity of IL-10 was provided by Brown et al. (53), showing that in the absence of IL-10 the spirochetal burden in IL-$10^{-/-}$ mice was significantly lower than in wildtype mice. In contrast, inflammation in the ankle was significantly higher. These results demonstrate the key role of IL-10 in regulating Borrelia-infection in Lyme arthritis.

6.3 Borrelia lipoproteins and the Toll-like receptor 2

For Borrelia lipoproteins the Toll-like receptor 2 (TLR2) has been identified as the major signal transducing receptor. Using TLR2-cotransfected mice macrophages Brightbill et al. demonstrated, that Borrelia lipoprotein induced IL-12 production and activation of iNOS is TLR2-mediated (98). At the same time Hirschfeld et al. described that TLR2-transfection into a glioma cell-line confers responsiveness to lipoproteins and sonicated Borrelia (95). Experiments transfecting CHO cells with TLR2 renders them responsive to OspA, synthetical lipoproteins and live Borrelia as assessed by induction of pro-inflammatory cytokines and NFκB-translocation (96). Aliprantis et al. postulate that TLR2 is involved in Borrelia-induced cell-activation and apoptosis (99). Further evidence for TLR2-involvement in recognition of Borrelia was provided by Lorenz et al., who performed functional studies with 293T cells transfected with the non-functional Arg753Gln polymorphic TLR2 gene in comparison to the wildtype gene (97). The most convincing data that TLR2 is crucial for host defense to Borrelia were obtained in $TLR2^{-/-}$ mice, which harbored up to 100-fold more spirochetes in tissues than their wildtype littermates. However, since the antibody response was not influenced by the absence of TLR2 it was postulated that the acquired immune response to Borrelia does not require this receptor. The most

surprising result was that TLR2$^{-/-}$ mice developed greater ankle swelling than TLR2$^{+/+}$ mice, suggesting TLR2-independent components to be involved in the inflammatory response to Borrelia. Indeed, although being unresponsive to stimulation with lipoproteins, when stimulating macrophages from TLR2$^{-/-}$ mice with high concentrations (50 µg/ml) of sonicated Borrelia, they released TNFα and NO (100). These findings are in line with our hypothesis that the TLR2 mediated response is primarily of an anti-inflammatory nature.

In order to address these conflicting results, we tested the role of TLR2 and TLR4 in the recognition of different Borrelia-specific stimuli. As demonstrated in stimulation-experiments with bone marrow-derived macrophages from TLR2$^{-/-}$ mice, none of the tested Borrelia-specific stimuli induced cytokine release in the absence of TLR2 (chapter 4). In contrast, despite a nonfunctional TLR4, stimulation of macrophages from C3H/HeJ mice with the same Borrelia-specific stimuli resulted in TNFα-release comparable to wildtype cells. Thus, our results substantiate the view, that TLR2 is the major receptor responsible for Borrelia-induced cytokine production. It has to be mentioned that we could confirm data from Wooten et al., inducing weak but clearly measurable TNFα-release when stimulating macrophages from TLR2-deficient mice with Borrelia lysate concentrations above 10 µg/ml. However, since these TLR2-independent components, which have neither been isolated nor characterized to date, appear to be immunogenic only in high concentrations their relevance still has to be defined.

6.4 Borrelia-induced tolerance and cross-tolerance

The phenomenon of endotoxin (LPS) tolerance has been investigated extensively *in vitro* and *in vivo* (for review see (101)). It describes a status of macrophage hyporesponsiveness after exposure to low LPS doses to a subsequent high or lethal LPS dose (102, 103). In this study we investigated whether *B. burgdorferi* also has the capacity to desensitize macrophages, since Borrelia-induced hyporesponsiveness could represent a mechanism enabling the survival of this pathogen in the immunocompetent host (chapter 4). We tested this hypothesis in desensitization experiments with Borrelia-specific stimuli. Indeed, similarly to the effect of LPS pre-treatment in endotoxin tolerance, pre-treatment with Borrelia lysate could render human PBMC tolerant, i.e. unable to react to a second stimulation with Borrelia, as shown by a reduced capacity of TNFα-release. Thus, Borrelia are able to

modulate the monocytic immune response. They even induce a more general desensitization, since they also rendered human PBMC hyporesponsive to subsequent stimulation with heterologous stimuli such as the well characterized TLR2 agonist LTA or the TLR4 agonist LPS. Recently, similar desensitization experiments were reported which demonstrated, that stimuli other than LPS, i.e. highly purified lipoteichoic acid (LTA) (104) or macrophage-activating lipopeptide-2 (MALP-2) from mycoplasma (105), can also render macrophages tolerant to subsequent re-stimulation. Furthermore, it was shown in these same studies, that tolerance can also be induced by two heterologous stimuli, independent of the receptor involved in their recognition and signaling. In this case the appropriate term is cross-tolerance or hetero-tolerance.

6.4.1 Toll-like receptors and signaltransduction pathways in Borrelia-induced tolerance

Several reports have been published, showing that tolerance induced by different TLR2- and TLR4-agonists shares common intracellular signal transduction pathways: Pre-treatment with synthetic bacterial lipoproteins (BLP) inhibits phosphorylation of ERK, JNK and p38 Map-kinases and reduces NFκB-activation and NFκB-DNA binding activity similarly to LPS pre-treatment (114). In MALP-2 induced cross-tolerance to LPS NFκB-activation and c-Jun NH2 terminal kinase is suppressed (105). Down regulation of IL-1 receptor associated kinases (IRAK) protein levels and kinase activity correlates with development of LPS tolerance and with LPS induced cross-tolerance to LTA, but not with LTA tolerance and LTA-induced cross-tolerance, suggesting differences in tolerance mechanisms of these agonists. Unique TLR2 signaling components downstream of TLR2 and upstream of MyD88/IRAK are postulated in LTA tolerance (120).

Our experiments with bone marrow lavage cells from TLR2$^{-/-}$ mice and from mice lacking a functional TLR4 substantiate the view that common signaling molecules are shared by TLR2 and TLR4. In contrast to LPS, pre-exposure to Borrelia induced macrophage hyporesponsiveness in the absence of a functional TLR4. Instead, pre-exposure to Borrelia lysate failed to confer refractoriness to TLR2-deficient cells, whereas LPS-induced TNFα suppression was unaffected in these cells. Thus, Borrelia-specific stimuli required the TLR2 to induce tolerance and respectively LPS required the TLR4. Based on our findings that stimulation with Borrelia lysate, which

signals via TLR2, results in hyporesponsiveness to TLR4-mediated LPS signaling and vice versa, we postulated that.

6.4.2 Regulation of the TLR in Borrelia-induced tolerance

Differing results regarding the regulation of the TLR in tolerance have been published (105, 119, 121). Wang et al. showed downregulation of TLR2 in tolerance induced by synthetic bacterial lipopeptide (BLP), but not LPS (114). Our results regarding the TLR2-mRNA are partially in line with these observations, since the TLR2 was significantly downregulated in cells tolerized by Borrelia lysate upon re-stimulation with the same stimulus (chapter 4). As a major difference to Wang et al., we also observed a downregulation of the TLR2 by LPS. The opposing results could be ascribed to the fact that cross-tolerance we observe is complete and likewise induced by the two stimuli, unlike Wang et al. who report an incomplete LPS-induced cross-tolerance to BLP. Our data suggest that downregulation of the TLR2 seems to occur in tolerance, representing a probable explanation for the observed state of unresponsiveness to a second stimulation.

6.4.3 Role of soluble mediators in Borrelia-induced tolerance

Several reports have been published on the role of leukocyte-derived inflammatory mediators in tolerance induction of macrophages. According to these publications endogenous anti-inflammatory cytokines such as IL-10, TGFβ or IL-1 are considered to be involved in LPS-induced tolerance by suppressing the formation of pro-inflammatory cytokines factors (113, 184, 185)). Others postulate the existence of soluble yet unidentified suppressor molecules of TNFα-expression after pre-exposure of cells to endotoxin (186). Data from Randow et al. demonstrate that LPS-induced tolerance is mediated by endogenous cytokines such as IL-10 and TGFβ (113). Our results with regard to tolerance induced by Borrelia in the presence of IL-10-, TGFβ- and G-CSF-neutralizing antibodies indicate that IL-10 is indeed involved in induction of Borrelia-tolerance. However, tolerance induction could not be completely prevented by blocking the mediator, suggesting that other mediators also play a role in this process. TGFβ, which also contributed to LPS-tolerance in human PBMC (113), was apparently not required for Borrelia-tolerance, nor was G-CSF. Further studies will be necessary to definitely settle this point, since our data oppose data from others, showing that peritoneal macrophages from wildtype and IL-$10^{-/-}$ mice could be similarly tolerized by LPS and MALP, suggesting that IL-10 was not involved

(105). Own results from coculture experiments with TLR2- and TLR4-deficient primary murine cells and the corresponding wildtype cells stimulated with LTA and LPS also argue against soluble factors responsible for suppression of TNFα upon secondary stimulation in this murine model (104).

6.5 Adjuvant immunotherapy in Lyme borreliosis

While LB at early stages can be managed with antibiotics, therapy of late stage LB still is unsatisfactory. A small percentage of all late stage LB cases are therapy resistant, i.e. they cannot be cured with standard therapies (124, 152-154). In this work evidence has been provided that Borrelia modulates the human immune response representing a possible mechanism to persist in the host. Based on this finding that pathology of LB is associated with modulation of leukocyte functions, adjuvant immunotherapy should be taken into account as an additional therapy to vaccination and antibiosis. The reconstitution of the patients immune competence with activating cytokines represents an attractive target for supportive treatment in chronic LB. Possible candidates could be the endogenous products granulocyte macrophage colony-stimulating factor (GM-CSF) and IFNγ, which possess strong pro-inflammatory properties and have been shown to restore the systemic TNFα-response to endotoxin in LPS-desensitized mice (187) or granulocyte colony-stimulating factor (G-CSF, Filgrastim) which acts as well as a pro- as an anti-inflammatory cytokine, recruits and primes neutrophils and induces mono- and lymphocytosis in human volunteers (72). In the study presented here, G-CSF (Filgrastim) was chosen for the combination therapy with antibiotics, due to its unique anti-infectious and hematopoietic pharmacological properties on the one hand, and the fact that it is well tolerated on the other hand, as shown in many clinical trials (138).

6.5.1 Case report

We report the anecdotal observation of a late stage LB patient, who was successfully treated with a combination therapy of a cephalosporin antibiotic *plus* Filgrastim (chapter 5). Properties of Filgrastim which most likely contributed to the beneficial effects of the treatment include amplified hematopoesis, which leads to an enforcement of the innate immune system, allowing a greater number of activated non-anergic, naive neutrophils to reach and kill bacteria even at remote sites of infection. This is of special importance since it has been demonstrated that

neutrophilic granulocytes contribute substantially to host defense against Borrelia (39, 139-141). The enhanced anti-bacterial capacity might be especially efficient under the chosen conditions: antibiotic pre-therapy could lead to lysis of bacteria, thus releasing membrane components that allow Filgrastim-activated neutrophils to be attracted by chemotactic signals to eradicate the bacteria at poorly perfused, e.g. intraarticular locations. Additional effects of Filgrastim on neutrophils which might also have contributed to the therapeutic efficacy of the combination treatment include increased microbicidal activity (157), enhanced oxidative burst (130, 158) and improved adherence and phagocytic capacity (129, 159). Noteworthy, the patient represented a relatively mild case of chronic Lyme arthritis. It is not clear whether positive effects of Filgrastim would also translate to more severe cases, especially to patients with neuroborreliosis, where bacteria are difficult to reach for leukocytes in the immunopriviledged central nervous system. Another aspect which has to be mentioned is that repetitive treatment with antibiotics increases per se the probability of Borrelia eradication and success of the second course of treatment might be explained by this simple fact. However, it is very intriguing that the patient reported warmth and pain after the first two injections of Filgrastim for two days only in those joints which were affected by arthritic pain and immobility over years before. This incident could be tentatively explained by a successful granulocytic local attack on residual bacteria in the joints.

6.5.2 Mouse model

The murine model of LB developed by Barthold and colleagues demonstrated that certain strains of mice develop severe inflammatory arthritis 3 - 4 weeks after intra-dermal injection of Borrelia with histopathological characteristics similar to the subacute arthritis seen in humans (188). Therefore this mouse model is frequently used to study pathology of Borrelia-infection and to find new treatment strategies in LB. To investigate in more detail the effects of Filgrastim in Borrelia-infection we carried out experiments with immunocompetent C3H/HeN and immunodeficient SCID mice. Filgrastim could not attenuate edema formation, as monitored by ankle swelling, neither in immunocompetent inbred mice, nor in SCID mice**.** However, considering recent findings, it might have influenced lesion severity. Increasing evidence indicates that the degree of arthritis (assessed as ankle swelling) does not necessarily correlate with histopathology. Anti-IL-12 treatment for example did not affect the degree of ankle swelling, but arthritis documented by histopathology was

improved significantly (33). Wooten et al. found that ankle swelling provides only information on the degree of edema within the ankle tissue, but neglects joint pathology (100). This notion is supported by recent findings indicating that ankle swelling and arthritic lesion are two traits which are regulated independently by genetic loci mapping to distinct locations (160). Histological analyses of the joints could not be performed retrospectively. Thus we cannot rule out that, despite lacking an effect on joint swelling, Filgrastim might have attenuated the inflammation in the joint, i.e. sheath thickness and inflammatory cell infiltration.

Differing results have been published regarding the correlation between levels of tissue spirochetes and severity of pathology. Some authors demonstrate that development of arthritis in susceptible mouse strains correlates with higher numbers of spirochetes in these hosts (147, 189). In contrast, comparison between different inbred mouse strains showed that, in spite of equal levels of spirochetes in the ankles, the degree of arthritis pathology differed strongly (50, 161). Anti-IL-12 treatment reduced arthritis severity, but led to increased spirochetal load in the ear (33) and IL-$10^{-/-}$ mice harbored less Borrelia in their ear, ankles and hearts, but displayed a more severe arthritis compared to their wildtype littermates (53). In our experiments the numbers of spirochetes assessed by quantitative real-time PCR in C3H/HeN mice were only moderately influenced by immunosupportive Filgrastim treatment. Some organs of the Filgrastim-treated mice appeared to contain less spirochetes at the time points tested (ankle, bladder, lymph node), while in others (brain, ear, heart, and meninx) no Borrelia could be detected. The chosen time-point might not be representative of the whole course of the infection. Furthermore treatment dosage and administration protocol possibly account for the low effects on arthritis development and bacterial load and could be varied in future studies. Additional experiments demonstrated that Borrelia-induced immune dysfunction and longterm persistence could not be investigated in immunocompetent mice: firstly, Borrelia numbers declined over time and were no longer found at day 135, indicating a long-lasting but, in contrast to humans, self-limiting infection; secondly, unlike chronically infected patients, when testing the influence of Borrelia-infection on macrophages of immunocompetent mice, no alteration of ex vivo inducible cytokine production in infected mice was found, as was characteristic for LB patients (30), suggesting that mice are not immunosuppressed by Borrelia. These results are in line with data from Montgomery et al., who showed, that Borrelia neither induce a global

downregulation of phagocytic functions in mice (167) nor reduce inflammatory activity in infected organs of these animals (168). It is attractive to speculate but difficult to prove that the lack of immunomodulation is linked to the self-limiting character of the infection in mice in contrast to humans.

Since the model of Borrelia-infection in immunocompetent mice appeared to be unsuitable to study persistence of Borrelia, we studied the effects of Filgrastim in SCID mice, lacking B- and T-cells, which develop an array of symptoms such as chronic progressive arthritis typical of LB in man (144) and which do not show resolution of disease over time (66, 148). By treating SCID mice with Filgrastim a reduction of spirochete levels in the ankle, bladder, heart and meninx was observed. Our observation that Filgrastim given without antibiotics to immunodeficient mice, which do no possess an acquired immune system, resulted in some reduction of bacterial burden, suggests that Filgrastim mainly acted on the innate immune system, enhancing monocyte and neutrophil recruitment.

In conclusion, a novel approach in the treatment of chronic LB was tested based on the concept that killing of Borrelia with antibiotics should be combined with reactivation of immune cells which have been modulated by the presence of the pathogen. Considering the unsatisfactory current antibiotic treatment, the positive experience of an experimental therapy with Ceftriaxone plus Filgrastim in a LB patient, and the enhanced elimination of Borrelia from SCID-mice, as well as the tendency to beneficial effects in immunocompetent mice with Filgrastim alone are quite promising and encouraged a clinical trial on the immunosupportive Filgrastim therapy of LB which is currently underway.

7 Summary

If infection with *Borrelia burgdorferi* is not treated adequately with antibiotics in an early stage, it may lead to Lyme borreliosis (LB), a chronic multisystemic disorder which is difficult to cure. In some cases the pathogen survives in spite of antibiotic treatments. It is challenging to understand why Borrelia are often not eradicated, although being recognized by the host's immune defense and occasionally inducing a strong inflammatory reaction. Thus, it remains an area of debate how this pathogen persists in human tissues. This question was addressed in the present thesis, examining possible immune evasion mechanisms of Borrelia.

- Blood cells from patients suffering from persistent LB released significantly lower levels of pro-inflammatory cytokines (TNFα and IFNγ) in response to either Borrelia lysate or to lipopolysaccharide (LPS) in comparison to cells from healthy volunteers.
- In blood from healthy volunteers Borrelia lysate led to strong production of anti-inflammatory IL-10 and G-CSF, while inducing only low levels of pro-inflammatory IFNγ, compared to LPS.
- Similar to endotoxin tolerance, different Borrelia preparations desensitized human blood monocytes on re-stimulation with either stimulus.
- Borrelia-specific stimuli induced cross-tolerance towards heterelogous stimuli such as lipopolysaccharid (LPS) and lipoteichoic acid (LTA) in human monocytes.
- Toll-like receptor (TLR) 2 but not TLR4 was required for Borrelia-induced tolerance and cross-tolerance, as shown in experiments with knock-out mice.
- PBMC tolerized by Borrelia lysate exhibited reduced TLR2-mRNA levels. Further, IL-10 was identified as a key mediator involved in tolerance-induction by Borrelia lysate.
- Combination of Filgrastim treatment with Ceftriaxone in a late stage LB-patient who failed standard antibiotic therapy led to successful eradication of the pathogen and complete regression of symptoms.
- The mouse model of Borrelia infection was set up and characterized in order to study the therapeutic effects of Filgrastim *in vivo*.

- Treating immunocompetent and immunodeficient SCID mice with Filgrastim, as an immunosupportive therapy of LB did not attenuate the characteristic ankle swelling induced by Borrelia infection.
- Regular application of Filgrastim led to an enhanced elimination of Borrelia from various organs in SCID mice. In immunocompetent mice this effect was less pronounced.

In summary, we propose that Borrelia modulate the host's immune system in order to evade clearance in the immunologically competent host. Tolerance could represent the mechanism inhibiting host response thereby enabling survival and persistence of the pathogen. Promising results were obtained testing a novel treatment strategy for late stage LB, a combination of Filgrastim as an immunosupportive therapy with antibiotics. The respective clinical trial based on these findings was recently started.

8 Zusammenfassung

Die Infektion mit *Borrelia burgdorferi*, die nicht frühzeitig und angemessenen behandelt wird, führt im Menschen zu einer chronischen Multisystem-Erkrankung, die schwer zu therapieren ist, da Antibiotika teilweise versagen. Trotz intensiver Forschung ist bisher noch nicht befriedigend geklärt, wie die Borrelien in einem funktionierenden Immunsystem persistieren. In der vorliegenden Arbeit wurde dieser Frage der Borrelienpersistenz nachgegangen.

- Im Blut chronisch erkrankter Borreliosepatienten wurde nach Stimulation mit Borrelienlysat oder LPS im Vergleich zu einem gesunden Kontrollkollektiv eine signifikant reduzierte TNFα- und IFNγ-Freisetzung nachgewiesen.
- Im Blut gesunder Spender stellten Borrelien im Vergleich zu LPS einen starken Stimulus für die Freisetzung der anti-inflammatorischen Zytokine G-CSF und IL-10 dar und nur einen schwachen für das pro-inflammatorische Zytokin IFNγ.
- Die wiederholte Stimulation mit verschiedenen Borrelien-spezifischen Stimuli induzierte eine Desensitivierung von humanen Blutmonozyten, vergleichbar mit der „Endotoxin Toleranz“.
- Borrelien-spezifische Stimuli erzeugten mit LTA oder mit LPS eine Kreuzdesensitivierung von humanen Blutmonozyten.
- Experimente mit Makrophagen von knockout-Mäusen ergaben, dass TLR2 - nicht aber TLR4 - benötigt wird, um mit Borrelienlysat Desensitivierung und Kreuzdesensitivierung zu induzieren.
- Bei der Borrelienlysat-induzierten Desensitivierung und Kreuzdesensitivierung von humanen PBMC wurde die Toll-like receptor (TLR) 2-mRNA herunterreguliert und es konnte die Beteiligung von IL-10 als Mediator nachgewiesen werden, nicht aber von TGFβ oder G-CSF.
- Die Kombination des Immunaktivators Filgrastim mit Ceftriaxon bei einem chronisch infizierten Borreliosepatienten und Therapieversager führte zu der erfolgreichen Eradikation des Erregers und dem vollständigen Rückgang der Krankheitssymptome.

- Das Mausmodell wurde zur *in vivo* Untersuchung der Borrelieninfektion etabliert und charakterisiert, um anschließend Therapiestrategien testen zu können.
- Der Einsatz von Filgrastim im Borreliose-Mausmodell als adjuvante Immuntherapie hatte keinen Effekt auf die Entwicklung der Arthritis, weder in der immundefizienten, noch in der immunkompetenten Maus.
- Die regelmäßige Gabe von Filgrastim vor und während der Borrelieninfektion führte in der immundefizienten Maus zu einer deutlichen Reduktion der Erregerlast in allen Organen, aber in nur einem Organ der immunkompetenten Maus.

Aufgrund dieser Ergebnisse stellt die Modulation der Immunantwort eine mögliche Erklärung für die Persistenz der Borrelien im Patienten dar, indem sie einerseits die anti-inflammatorische Zytokinantwort der Immunzellen begünstigen und andererseits einen Zustand der Desensitivierung im Makrophagen hervorrufen können. Aus dieser Kenntnis der pathophysiologischen Defekte und des erfolgreichen Einzelbefundes, zusammen mit den Daten aus der Maus, stellt der Einsatz von Immunaktivatoren eine vielversprechende therapeutische Interventionen in der Borreliose dar. Die auf diesen Befunden basierende experimentelle klinische Studie wurde kürzlich gestartet.

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