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Cytokine responses in human Lyme borreliosis

**The role of T helper 1-like immunity and aspects of
gender and co-exposure in relation to disease course**

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To my family; past, present and future

ABSTRACT

Lyme borreliosis was first described some 30 years ago in the USA. Today, it is the most common vector borne disease in Europe and the USA. The disease can have multiple stages and symptoms can manifest from various parts of the body; joints, skin heart and nervous system. In Europe, neuroborreliosis is the most frequent late stage diagnosis. Although Lyme borreliosis is treatable with antibiotics and the causative spirochete has not been shown to be resistant to drugs, some patients do not recover completely. They have persistent symptoms and are diagnosed with chronic or persistent Lyme borreliosis. The mechanism behind the lingering symptoms is unclear but might be due to tissue damage caused by the immune system. The aim of this thesis was to study the immunological differences between patients with different outcome of Lyme borreliosis, i.e. chronic, subacute and asymptomatic, and various factors that might influence the course of the disease.

The *Borrelia*-specific IFN- γ and IL-4 secretion was detected in blood and cerebrospinal fluid from patients with chronic and subacute neuroborreliosis during the course of the disease. Blood samples were also obtained from patients with erythema migrans (EM) and acrodermatitis chronica atrophicans. An early increase of IFN- γ with a later switch to an IL-4 response was observed in patients with a subacute disease course whereas the IFN- γ secretion continued to be elevated in chronic patients.

The *Borrelia*-specific Th1-response was further investigated in chronic, subacute and asymptomatic individuals by studying the expression of the Th1-marker IL-12R β 2, on a protein and mRNA level. The cytokine secretion and Foxp3, a marker for regulatory T-cells, were also analyzed. Chronic patients had a lower IL-12R β 2 expression on CD8+ T-cells and a lower number of *Borrelia*-specific IFN- γ secreting cells compared to asymptomatic individuals. Chronic patients also displayed a higher expression of *Borrelia*-specific Foxp3 than healthy controls.

The conclusions for these two studies were that a strong Th1-response early in the infection with a later switch to a Th2-response is beneficiary whereas a slow or weak Th1-response corresponds to a prolonged disease course.

The influence of a previous infection with another pathogen, seen to suppress the immune response in animals, and the possible gender difference in immune response was also investigated. Patients with EM were screened for antibodies to *Anaplasma phagocytophilum* (*Ap*) as a sign of a previous exposure to these tick-borne bacteria. Blood lymphocytes from *Ap* seronegative, *Ap* seropositive and healthy controls were stimulated with *Borrelia* antigen and the secretion of IL-4, IL-5, IL-12, IL-13 and IFN- γ was detected by ELISPOT. *Ap* seropositive patients had a lower number of cells responding with IL-12 secretion compared to the other groups which might indicate an inhibited Th1-response.

Reinfections with Lyme borreliosis was in a previous study, done by Bennet et al, found to be more frequent in postmenopausal women than in men. To investigate if there was an immunological explanation to the gender discrepancy, blood lymphocytes from individuals reinfected with Lyme borreliosis and individuals infected only once were stimulated with various antigens. The cytokine secretion was detected by ELISPOT, ELISA and Immulite. There were no differences between reinfected and single infected individuals. However, women, regardless of times infected, displayed a Th2-derived and anti-inflammatory spontaneous immune response compared to men.

A previous infection with the bacteria *Ap* might possibly have a long term effect on the immune system and might be of disadvantage when mounting a Th1-response to a *Borrelia* infection. Also, the Th2-derived response displayed by postmenopausal women could indicate why more women than men get reinfected with *Borrelia burgdorferi*.

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ABBREVIATIONS

ACA	acrodermatitis chronicum atrophicans	NK	natural killer
APC	antigen presenting cell	OF	outer surface protein enriched fraction
cDNA	complimentary DNA	OND	other neurological diseases
CSF	cerebrospinal fluid	Osp	outer surface protein
DC	dendritic cell	PBL	peripheral blood lymphocytes
dNTPs	deoxyribonucleotides	PCR	polymerase chain reaction
ELISA	enzyme linked immuno assay	PHA	phytohemagglutinin
ELISPOT	enzyme linked immuno-spot	PPD	purified protein derivative of tuberculin
EM	erythema migrans	R	receptor
Foxp3	forkhead box 3	RA	rheumatoid arthritis
HGA	human granulocytic anaplasmosis	RT	reverse transcription
HGE	human granulocytic ehrlichiosis	Tc	cytotoxic T-cell
HIV	human immunodeficiency virus	T _{CM}	central memory T-cell
IFA	immunofluorescence antibody	TCR	T-cell receptor
IFN	interferon	T _{EM}	effector memory T-cell
Ig	immunoglobulin	TGF	transforming growth factor
IL	interleukin	Th	T-helper
LFA	leukocyte function-associated antigen	TLR	Toll-like receptor
LPS	lipopolysaccharide	TNF	tumor necrosis factor
MHC	major histocompatibility complex	Treg	regulatory T-cell
mRNA	messenger RNA	VlsE	variable major protein-like sequence, expressed

ORIGINAL PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals (I-IV).

- I. **Widhe M, Jarefors S, Ekerfelt C, Vrethem M, Bergström S, Forsberg P and Ernerudh J. (2004).** *Borrelia*-specific interferon-gamma and interleukin-4 secretion in cerebrospinal fluid and blood during Lyme borreliosis in humans: association with clinical outcome. *J Infect Dis* 189(10): 1881-91.
- II. **Jarefors S, Karlsson M, Eliasson I, Forsberg P, Ernerudh J and Ekerfelt C. (2006).** Reduced number of IL-12 secreting cells in patients with Lyme borreliosis previously exposed to *Anaplasma phagocytophilum*. *Clin Exp Immun* 143(2): 322-8.
- III. **Jarefors S, Bennet L, You E, Forsberg P, Ekerfelt C, Berglund J and Ernerudh J. (2006).** Lyme borreliosis reinfection: might it be explained by gender difference in immune response? *Accepted for publication in Immunology*
- IV. **Jarefors S, Janefjord CK, Forsberg P, Jenmalm MC and Ekerfelt C.** Importance of induction and secretion of interferon-gamma for optimal resolution of human Lyme borreliosis – differences between different outcomes of the infection. *Submitted*

INTRODUCTION

Lyme borreliosis

In 1975 a geographic cluster of children with arthritis in the town of Old Lyme, Connecticut, USA caught the attention of the scientific community (Steere et al. 1977). This led to the discovery of what was later called Lyme borreliosis. Lyme borreliosis is now known to be the most common vector borne disease in Europe and the USA. The causative bacteria are transmitted from the reservoir, usually small rodents, to humans via the *Ixodes* tick.

Pathogen

Lyme borreliosis is caused by the spirochete *Borrelia burgdorferi sensu lato* (Benach et al. 1983, Burgdorfer et al. 1982, Johnson et al. 1984, Steere et al. 1983) which can be subdivided into at least 10 species of which *Borrelia burgdorferi sensu stricto*, *Borrelia garinii* and *Borrelia afzelii* are pathogenic to humans (Wang et al. 1999b). A fourth new human pathogenic species, A14S, has been isolated from the skin of two patients (Wang et al. 1999a) and has been suggested the name *Borrelia spielmani* (Richter et al. 2004). In Europe, all four human pathogenic subspecies are found (Ornstein et al. 2002, Wang et al. 1999a), in contrast to the USA where only *B. burgdorferi s. s.* has been identified (Wang et al. 1999b). *B. burgdorferi s. l.* is a gram-negative bacterium, 10-30 μm long, with an inner membrane surrounding the protoplasmic cylinder and an outer membrane surrounding the periplasmic space (Burgdorfer et al. 1982) (Figure 1).

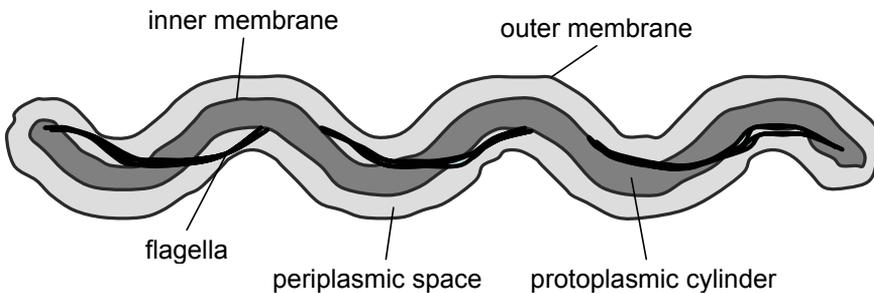


Figure 1. Schematic illustration of *Borrelia burgdorferi sensu lato*

The composition of the outer membrane is high in its abundance of lipoproteins (Brandt et al. 1990), including the outer surface proteins (Osps) A-F (Lam et al. 1994), and the membrane lacks lipopolysaccharide (LPS) (Takayama et al. 1987). To each end of the inner membrane flagella are attached and twisted around the cylinder (Burgdorfer et al. 1982). The flagella consist of flagellar outer sheath protein, FlaA which is unique for spirochetes,

and a core protein, FlaB, also called flagellin (Ge et al. 1998). The spirochete can rotate its flagella and protoplasmic cylinder in opposite directions thereby causing movement. The flagella constitute an important virulence factor, a flagella-less mutant of *B. burgdorferi s. l.* showed a 95% reduction of invasion (Sadziene et al. 1991).

The genome of *B. burgdorferi s. l.* consists of a small, linear chromosome (Baril et al. 1989) and several plasmids containing either linear or circular DNA (Fraser et al. 1997). The plasmids encode many of the important virulence factors such as Osps. The genes for OspA and B are located in the same operon suggesting that they have similar function (Howe et al. 1986). The expression of Osps is dependent on temperature and pH. Therefore different Osps are expressed on the spirochete when in ticks or in humans. OspA and B are down regulated when *B. burgdorferi s. l.* is transmitted to humans and at the same time OspC is up regulated (Obonyo et al. 1999). In the tick, OspA and B are essential for the spirochetes ability to bind to the midgut tissue but the proteins were not necessary for infection, dissemination or pathogenesis in mice (Yang et al. 2004). In contrast, OspC is important for infection shown by a OspC-deficient *B. burgdorferi s. l.* inability to infect mice (Grimm et al. 2004).

The heterogeneity of the different proteins varies. OspC has a 54-68% amino acid sequence identity between the subspecies of *B. burgdorferi s. l.* whereas flagellin is almost homogeneous (Wilske 2003). This diversity makes it difficult to manufacture reliable diagnostic tests and to develop vaccines. There are also proteins with high heterogeneity but with conserved immunogenic epitopes such as the C6 peptide of the variable major protein-like sequence expressed (VlsE) (Liang et al. 1999).

Clinical manifestations

Lyme borreliosis is a multi faceted disease with symptoms from e.g. skin, joints, heart and nervous system. There are three possible stages of illness; early localized disease, disseminated disease and persistent/chronic disease.

Early localized disease

The typical first symptom is the circular skin lesion, erythema migrans (EM), which is seen in over 70% of borreliosis patients (Berglund et al. 1995). Patients may also display symptoms such as fever, headache, neck stiffness, arthralgia, myalgia or fatigue (Smith et al. 2002). EM generally appears at the site of the tick bite after five to 48 days, median 12 days (Oschmann et al. 1998). The lesion should have a diameter of at least 5 cm and there can be a central clearing (Stanek et al. 1996). If the lesion is smaller it might be a reaction to the tick bite. EM caused by *B. afzelii* are more often annular (round or oval, sharply demarked with central clearing) whereas *B. garinii* is the

cause of non-annual (no central clearing) EM (Bennet et al. 2006, Carlsson et al. 2003).

The diagnosis of EM is made clinically. Serological testing with currently used methods is yet of no or little value since only 30-40% of patients with EM display antibodies to *B. burgdorferi s. l.* at this early stage of the disease (Berglund et al. 1995, Lomholt et al. 2000, Nowakowski et al. 2003).

A rare early manifestation is lymphocytoma. It is a painless, bluish-red tumor-like nodule on the earlobe or the nipple which can arise close to a previous or concurrent EM (Stanek et al. 2003). Lymphocytoma is more frequently seen in children than adults (Stanek et al. 1996).

Disseminated disease

From the skin, the spirochete can migrate to various organ systems, thus causing several different symptoms. It should be noted, however, that disseminated disease can present without a previous skin manifestation. The three genospecies of *B. burgdorferi s. l.* can be found in various tissues but they each seem to have specific preference (Balmelli et al. 1995). Manifestations from the joints are usually caused by *B. burgdorferi s. s.* whereas *B. garinii* seems to be more neurotropic and causes symptoms from the central and peripheral nervous system (Balmelli et al. 1995, Ekerfelt et al. 1998, van Dam et al. 1993). *B. afzelii*, on the other hand, stays in the skin and can give rise to the chronic manifestation of acrodermatitis chronicum atrophicans (ACA) (Balmelli et al. 1995).

In Europe the most common form of disseminated borreliosis is neuroborreliosis (Berglund et al. 1995). The clinical signs appear several weeks after the tick bite and include meningitis, facial palsy, radiculitis, headache, fatigue, neck stiffness or paraesthesia (Halperin 2003, Oschmann et al. 1998, Stanek et al. 1996). A lumbar puncture typically shows lymphocyte pleocytosis ($\geq 5 \times 10^6$ mononuclear cells/l) and intrathecal production of specific antibodies, immunoglobulin (Ig) M or IgG (Oschmann et al. 1998). A disturbance in the blood-brain-barrier, seen as an elevated albumin cerebrospinal fluid (CSF)/serum ratio, might also occur (Tumani et al. 1995). Antibodies in serum might be absent in the initial stage of the disease but should be detected in the convalescent phase, i.e. six to eight weeks after onset (Stanek et al. 1996). However, cases have been reported where patients remain seronegative though other laboratory findings, such as positive polymerase chain reaction (PCR) or T-cell reactivity, confirm an existing *B. burgdorferi s. l.* infection (Dattwyler et al. 1988, Dejmekova et al. 2002, Lawrence et al. 1995).

Arthritis is more often seen in the USA than in Europe, 33% of American patients with Lyme borreliosis displayed arthritic symptoms (CDC 2004) whereas the manifestation was found in 7% of Swedish patients (Berglund et al. 1995). Lyme arthritis affects one or several joints, primarily large joints

such as the knee. Recurrent attacks of pain and swelling lasting for a week with remission periods of four weeks are characteristic. Laboratory tests for rheumatoid factor and antinuclear antibodies are usually negative (Steere et al. 1977) but high levels of *B. burgdorferi s. l.* specific antibodies are found in serum and synovial fluid (Stanek et al. 1996).

Chronic disease

As mentioned earlier, *B. afzelii* can persist in the skin and cause a chronic form of Lyme borreliosis called ACA. The disease progression is often slow and is characterized by a bluish-red lesion and thinning skin with prominent veins (Stanek et al. 2003). There is often an association of peripheral neuropathy (Kindstrand et al. 1997). Serological IgG findings are almost always positive in this group of patients. ACA is more often seen in patients over 40 years of age and women are overrepresented (Stanek et al. 2003).

Despite treatment there are patients with Lyme arthritis and neuroborreliosis that continue to have symptoms lasting longer than six months. They are diagnosed as having chronic or persistent Lyme borreliosis. Approximately 10% of patients with Lyme arthritis have persistent symptoms for months or years after completing antibiotic treatment (Steere 2001).

Neuroborreliosis patients usually suffers from musculoskeletal pain, subjective alteration of cognition and fatigue (Treib et al. 1998, Vrethem et al. 2002). The frequency of treatment failure varies between studies. Berglund et al showed that 25% of neuroborreliosis patients reported sequelae five years after completing treatment (Berglund et al. 2002). Vrethem et al found that 50% of patients previously treated for neuroborreliosis had persistent symptoms after 32 months, which was significantly higher than in a control group (Vrethem et al. 2002). Comparable numbers was reported by Asch and colleagues, where 53% of patients with different manifestations of Lyme borreliosis showed an incomplete recovery (Asch et al. 1994). However, Seltzer et al found similar frequency of symptoms in an age matched control group compared to patients previously treated for Lyme borreliosis (Seltzer et al. 2000).

Diagnostics

There is a variety of laboratory tests available to aid and confirm a clinical diagnosis of Lyme borreliosis. Unfortunately, no golden standard has been agreed upon. To this day, the only reliable way to verify a *B. burgdorferi s. l.* infection is to culture the bacteria. It has been done from skin biopsies, blood and CSF. The recovery rate from skin with EM is good, 50-86% (Berger et al. 1992, Nowakowski et al. 2001), however from body fluids the recovery rate is much lower; blood 25-50% (Nowakowski et al. 2001, Wormser et al. 1998, Wormser et al. 2005) and CSF 10% (Karlsson et al. 1990). Cultivation is also time-consuming due to the slow growing rate of the spirochete, further

making the method unsuitable for use in clinical laboratories. An alternative to culture is PCR where the spirochetes' DNA is detected. This method has about the same sensitivity as that of culture (Wilske 2003).

In patients with symptoms of disseminated disease detection of antibodies in serum or CSF is the most reliable approach to validate a clinical diagnosis. However, serological tests can give false positive results, especially for IgM, due to cross-reactions (Smith et al. 2005). The method of enzyme linked immunosorbant assay (ELISA) uses whole cell preparation of *B. burgdorferi s. l.* or specific proteins as antigen (Kaiser 1998). This is a convenient method but the sensitivity and specificity varies between commercial kits (Ekerfelt et al. 2004) thereby making it complicated to compare results from different laboratories. To further evaluate samples and to rule out cross-reaction with other microorganisms, Western blot can be applied. This method allows detection of antibodies to specific antigens (Hauser et al. 1998). However, ELISA and Western blot can not differentiate between an ongoing and a previous infection since antibodies can be detected in patients for many years after the infection has cleared (Kalish et al. 2001, Lomholt et al. 2000). Different immunogenic proteins have been tried as antigen in ELISA to find a test that discriminates between past and ongoing infection. The antibody response to C6 peptide has been shown to decline in patients successfully treated for Lyme borreliosis (Philipp et al. 2001, Philipp et al. 2003) but there are also studies showing conflicting results (Fleming et al. 2004, Peltomaa et al. 2003).

Treatment and prevention

B. burgdorferi s. l. has not been shown to be resistant to antibiotics (Hunfeld et al. 2005) and Lyme borreliosis is therefore considered to be a treatable disease. The recommendations vary between countries, both in type of antibiotic and length of treatment. In Sweden, EM is treated with phenoxymethyl penicillin for ten days and neuroborreliosis with doxycycline for 14 days. Arthritis and ACA are also treated with doxycycline but the duration of treatment is 20 days (Läkemedelsverket 1998). The efficacy is high in EM patients, >90% (Bennet et al. 2003, Nowakowski et al. 2003, Smith et al. 2002) whereas the complete recovery of patients with neuroborreliosis is slightly lower, 80% according to Karkkonen et al (Karkkonen et al. 2001). There is no evidence of an ongoing *B. burgdorferi s. l.* infection in patients with chronic Lyme borreliosis (Klempner 2002) which might explain why long term antibiotic therapy does not improve the clinical picture of these patients (Kaplan et al. 2003, Klempner et al. 2001, Krupp et al. 2003). However, the inflammatory skin lesion in patients with ACA do improve after adequate treatment although symptoms of peripheral nerve deficit may persist (Kindstrand et al. 2002). This condition is, on the other hand, associated with a persistent infection and *B. burgdorferi s. l.* has been

isolated from ACA skin biopsies 10 years after clinical onset (Asbrink et al. 1985).

Several studies have been performed with the intent to find the optimal therapy for Lyme borreliosis. Bennet et al compared phenoxymethyl penicillin and doxycycline treatment in patients with EM. Penicillin was shown to be more effective than doxycycline but this might have been due to over representation of penicillin treatment in the study group (Bennet et al. 2003). Treatment with doxycycline for 10 days has been shown to be as effective as a 20-days course (Wormser et al. 2003). No association has been made between the type of antibiotic treatment used and the clinical outcome in form of chronic manifestations (Berglund et al. 2002).

A vaccine for Lyme borreliosis, LYMERix, was approved in the USA in 1998. It consisted of purified OspA which generated antibody production in humans and when the tick feed it ingested the antibodies. Since OspA is expressed on the surface of *B. burgdorferi s. l.* when the spirochete is in the tick, the antibodies opsonized and killed the bacteria in the tick thereby preventing transmission (Fikrig et al. 1992). Although the efficacy of the vaccine was high, 92% after a booster dose (Sigal et al. 1998), it was removed from the market in the spring 2002. A public concern that the vaccine might cause arthritis due to an autoimmune cross-reactivity, led to decline in sales. However, there has been no proven association between the vaccine and arthritis (Guerau-de-Arellano et al. 2005). Willett et al recently reported of a second-generation OspA vaccine where the auto reactive epitope has been removed (Willett et al. 2004). Other surface proteins are also being investigated as possible vaccine components. Brown et al used a mixed vaccine of decorin binding protein, fibronectin-binding protein and OspC and showed that this was more effective than if one single protein was used (Brown et al. 2005). Nonetheless, at this time there is no vaccine for Lyme borreliosis available.

Human granulocytic anaplasmosis (HGA)

Veterinary medicine has been faced with the problem of the tick-borne fever since the 1930's but in humans the disease, human granulocytic anaplasmosis (HGA), was not recognized until 1994, in the USA, (Bakken et al. 1994, Chen et al. 1994) and 1997 in Europe (Petrovec et al. 1997). As for *B. burgdorferi s. l.*, the vector is *Ixodes* ticks and the main reservoir is believed to be small rodents.

Pathogen

The causative agent of HGA was first thought to be of the genus *Ehrlichia* and the disease was therefore named human granulocytic ehrlichiosis (HGE). After further genetic studies the bacteria was classified as belonging to the

genus *Anaplasma* (Dumler et al. 2001) and given the name *Anaplasma phagocytophilum* (Editor 2002). Carlyon and Fikrig suggested that the disease also should be renamed (Carlyon et al. 2003), hence HGE will throughout this thesis be called HGA.

A. phagocytophilum is a gram-negative, obligate intracellular bacteria which invades granulocytes, mainly neutrophils, and propagate in membrane-bound vacuoles (Webster et al. 1998). These vacuoles can be seen in blood smears by means of Giemsa staining and are referred to as morula (Carlyon et al. 2003). *A. phagocytophilum* uses P-selectin glycoprotein ligand-1 on leukocytes as a receptor (Herron et al. 2000) and once inside the morula the bacteria blocks lysosome fusion thereby escaping the fatal enzymes (Gokce et al. 1999). Neutrophils also use toxic oxygen intermediates to destroy phagocytosed microorganisms. By inhibiting the enzyme involved in the production of the oxygen intermediates, *A. phagocytophilum* is able to avoid this killing mechanism as well (Banerjee et al. 2000, Mott et al. 2000). The bacteria also delays apoptosis in otherwise short-lived neutrophils (Yoshiie et al. 2000). Akkoyunlu and colleagues showed that *A. phagocytophilum* induced interleukin-8 (IL-8) secretion, a neutrophil attractant chemokine, which would recruit naive neutrophils to the infection site, thereby facilitating bacterial dissemination (Akkoyunlu et al. 2001).

Clinical manifestations

The most common symptoms of HGA are headache and fever accompanied by more diffuse manifestations such as myalgia, chills, malaise and arthralgia (Brouqui et al. 2004). Laboratory findings of lymphopenia and/or thrombocytopenia and elevated liver enzymes may also be seen (Bakken et al. 1996, Bjöersdorff et al. 1999a, Brouqui et al. 2004). Serological tests will in over 95% of the patients show specific antibodies in a titer ≥ 80 (Aguero-Rosenfeld 2002). Compared to the USA, cases of acute HGA are rare in Europe but epidemiological studies show a high seroprevalence, up to 28% (Strle 2004).

HGA is in most cases a mild illness (Brouqui et al. 2004) but if the patient is immunocompromised or taking immunosuppressive medication there is a five times greater risk for the need of hospitalization (Bakken et al. 2002). Interestingly, animals infected with *A. phagocytophilum* are prone to secondary infections (Larsen et al. 1994) indicating that the infection itself might cause an immunosuppression.

Diagnostics

In the USA morula are often seen in blood from patients in the acute stage of disease (Bakken et al. 2000, Bakken et al. 2001). However, in Europe this visual diagnostic test is of little use since morula are very seldom detected. PCR, on the other hand, have been used successfully to identify the presence

of *A. phagocytophilum* in blood (Bjöersdorff et al. 1999a) although the method has not been standardized and may therefore give discrepant results (Brouqui et al. 2004).

The diagnosis of HGA is usually aided by serological testing, combined with clinical data. The most commonly used serological method is immunofluorescence antibody (IFA) test which utilizes infected cells as antigen (Bjöersdorff et al. 1999b). Patients might remain seropositive for up to 42 months (Bakken et al. 2002, Lotric-Furlan et al. 2001), indicating that a positive serology is not a complete proof of an ongoing infection. Therefore, the criterion of an ongoing infection is a four-fold or greater change in antibody titer between the acute and convalescent sample, taken at least four weeks apart (Bakken et al. 2000). ELISA and Western blot are also used to determine antibodies to *A. phagocytophilum* (Ijdo et al. 1997, Ijdo et al. 1999, Tajima et al. 2000) but no commercial products have reached the market.

Treatment

Due to the often mild course of HGA it is suggested that the majority of infected patients never need to consult a physician and therefore never receive treatment. The disease is in these cases self-resolving (Strle 2004). In more severe cases the recommended treatment is doxycycline for seven to 14 days (Bakken et al. 1996). *A. phagocytophilum* have been shown to be resistant to several antibiotics, i.e. ampicillin, ceftriaxone, and azithromycin, which can be used in the treatment for Lyme borreliosis (Klein et al. 1997).

Immunology

Our body is under constant attack by bacteria, viruses and other microorganisms. To protect ourselves, an elaborate system of cells and proteins has evolved. These constitute the immune system which is divided into two parts; innate and adaptive. The innate immune system can be found in both plants and animals whereas the more specific adaptive system is unique to vertebrates (Ausubel 2005).

Innate immunity

The first line of defense against pathogens is the innate immunity. It can be divided into four types of barriers; anatomic (skin and mucous membranes), physiological (temperature, low pH and chemical mediators), phagocytic (macrophages, dendritic cells and neutrophils) and inflammatory (serum proteins with antibacterial activity) (Goldsby et al. 2000). The innate immunity is said to be non-specific in that it does not focus on a particular pathogen but works in a more general defense manner. It does however possess a certain specificity since it can discriminate between self and non-self.

If pathogens invade the tissue they will most likely encounter the phagocytic cell types macrophages and dendritic cells (DCs) (Figure 2a). These cells have various receptors that will recognize and bind to structures that are only found on pathogens. Macrophages express CD14 which binds LPS, a molecule found in the cell wall of gram-negative bacteria, and mannose receptors that can bind certain sugar moieties on the surface of bacteria and viruses. DCs have a type of receptor, CD1, which is specialized for lipid molecules (De Libero et al. 2005). Another important group of receptors is the toll-like receptors (TLRs). The toll protein was first identified in the fruitfly, *Drosophila*, where it showed anti-fungi properties (Lemaitre et al. 1996). A homologous protein was later discovered in humans (Medzhitov et al. 1997) and now 10 human TLRs are identified (Chuang et al. 2001), each bind particular structures found only on pathogens. TLRs are expressed on leukocytes but in different patterns; TLR1 is omnipresent whereas TLR2, TLR4 and TLR5 are restricted to macrophages, DC and polymorphonuclear cells and TLR3 is only found on DC (Muzio et al. 2000).

The binding of the pathogen to a receptor initiates a sequence of signals activating the cell. This causes the cell to increase its expression of co-stimulatory molecules, CD80 and CD86 which are collectively called B7 (Janeway et al. 2005), and secretion of pro-inflammatory cytokines and chemokines, e. g. IL-1 β , IL-6, IL-8, IL-12 and tumor necrosis factor (TNF)- α (Puccetti et al. 2002) (Figure 2b). The cytokines affect the blood flow and increases the adhesion molecules on endothelial cells in the vessels which facilitates for leukocytes in the blood to migrate into the tissue. Furthermore, cytokines such as IL-12 activates natural killer (NK) cells to become more aggressive in destroying virus or bacterial infected cells. The co-stimulatory molecules are important for the activation of the adaptive immune response (Janeway et al. 2005).

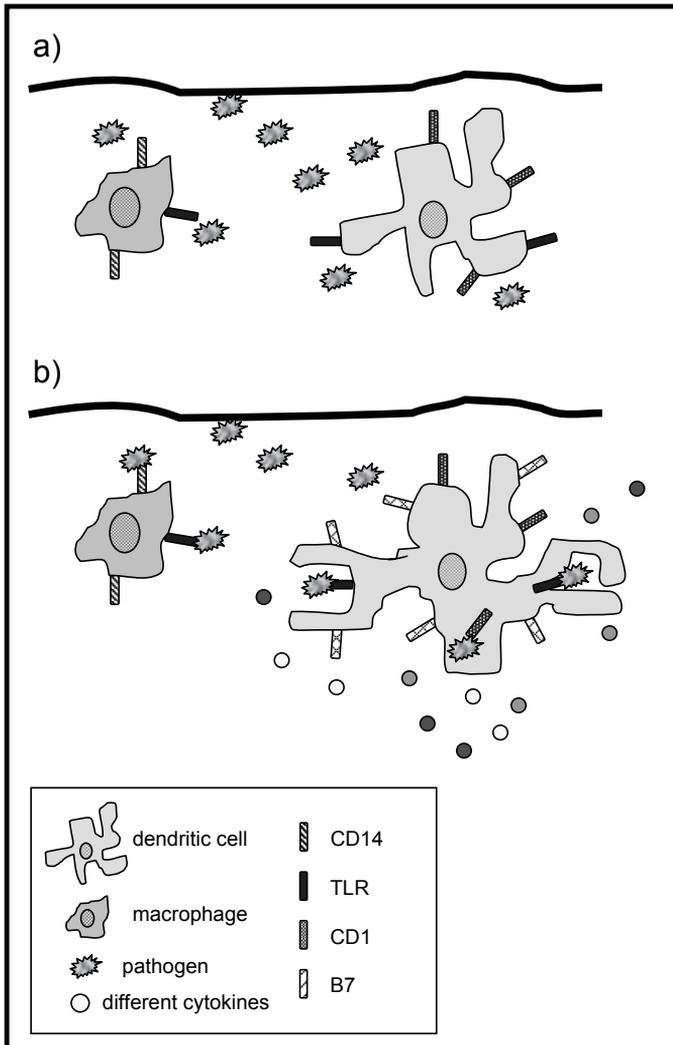


Figure 2. Schematic illustration of innate immunity: a) The invading pathogen encounter antigen presenting cells (APCs). b) APCs bind the pathogens to receptors and phagocytose the microorganisms. The APCs then become activated and up-regulate the expression of co-stimulatory molecules, i.e. B7, and secrete pro-inflammatory cytokines.

DCs, macrophages and B-cells are called antigen presenting cells (APCs) since they can display the pathogens on their cells' surface and also express co-stimulatory molecules. B-cells capture pathogens and toxins by way of their B-cell receptors whereas DCs and macrophages ingest and break down the pathogen before presenting the peptides. Depending on where in the APCs the pathogen is digested it is presented by major histocompatibility complex (MHC) molecules class I or II. Intracellular pathogens such as viruses are processed in the cytosol and the fragmented peptides are bound to MHC I

whereas extracellular microorganisms are processed in vesicles and fuses with MHC II (Janeway et al. 2005).

Adaptive immunity

After becoming activated, DCs migrate to lymphoid organs, i.e. the spleen and the lymph nodes, where they encounter T-cells (Banchereau et al. 1998). DCs bind naive T-cells with low affinity through different receptors (Figure 3), for example leukocyte function-associated antigen (LFA)-1 on the T-cells will bind ICAM-1 on DCs, and then the DC can present the antigen peptide to the T-cell. The T-cell receptor (TCR) is specific for foreign antigens but will probably bind to several different peptide sequences and not to only one specific (Mason 1998). If the T-cell recognizes the antigen the bond will become stronger, if not the cells will let go and the T-cell will try its luck with the next DC.

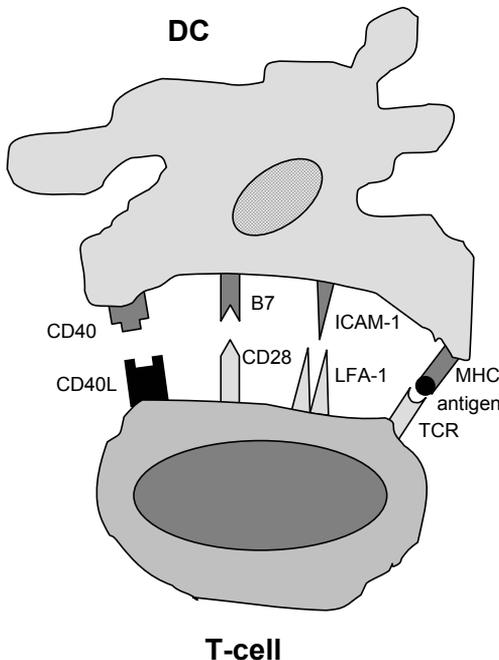


Figure 3. The various receptors and ligands involved in activation of a T-cell by a dendritic cell (DC).

Once a T-cell has been presented with an antigen it will start to mature (Janeway et al. 2005). This process demands co-stimulatory signals from the DC, through the ligation of the DC receptor B7 and CD28 or CD2 on the T-cell (Green et al. 2000), and IL-2 secreted by the T-cell. The T-cell will also express CD40 ligand, which binds to CD40 on the DC, stimulating both cells. The maturation takes several days after which the T-cell migrates via the blood to the infected site (Janeway et al. 2005).

T-cells

There are several types of T-cells; the most abundant are CD4+ T-helper (Th) cells and CD8+ cytotoxic T (Tc) cells. Tc-cells recognize antigens presented by MHC I, i.e. peptides derived from the cytosol whereas the TCR on Th-cells bind to MHC II, which presents peptides derived from extracellular proteins.

CD4+ Th-cells can be subdivided into several types (Mosmann et al. 1996) but the major types are Th1 and Th2. They originate from the same precursor and, depending e.g. on the cytokine milieu at antigen presentation, they mature into different subsets. Th1-cells develop if IL-12 or interferon (IFN)- γ are present (O'Garra 1998). These cells are important for the cell-mediated immunity since the IFN- γ secreted by Th1-cells stimulates macrophage and neutrophil activation and the synthesis of opsonizing antibodies. If the precursor Th-cell is exposed to IL-4 during its maturation it will develop into a Th2-cell. Typical cytokines secreted by Th2-cells are IL-4, IL-13 (McKenzie 2000), IL-5 (Lalani et al. 1999) and IL-9 (Zhou et al. 2001) which will activate eosinophils and mast-cells and increase the antibody production by B-cells. Th2-type immunity is called humoral (Kelso 1998) or phagocyte-independent defense.

It has been suggested by Maldonado and colleagues that Th1 development is the default response whereas Th2 needs to be specially induced. They demonstrated that TCR and IFN- γ receptor (R) co-localized when the Th-cell was activated, which lead to the development of a Th1-cell. The IL-4R did not display this co-localization unless IL-4 was present (Maldonado et al. 2004).

Apart from the different cytokine patterns secreted by Th1- and Th2-cells they can be distinguished by the presence of the IL-12R. The functional high-affinity IL-12R is a heterodimer, consisting of two chains, IL-12R β 1 and IL-12R β 2, the latter being the primary signal transduction component (Presky et al. 1996). IL-12R β 1 is consecutively expressed on activated T- and NK-cells (Desai et al. 1992), whereas IL-12R β 2 is only found on cytotoxic T-cells, Th1- and NK-cells (Rogge et al. 1997, Rogge et al. 1999).

As the name implies Tc-cells kill other cells that are displaying a foreign peptide on the surface. Tc-cells contain granules with cytotoxic proteins, such as perforin and granzymes. When released they will induce apoptosis in the target cell (Barry et al. 2002). The release of cytokines by Tc-cells also aid in the elimination of infections. IFN- γ has an inhibitory effect on viral replication and TNF- α and lymphotoxin- α activate macrophages (Janeway et al. 2005). The Tc-cells are more dangerous than Th-cells and therefore their activation is under strict control. To activate a Tc-cell the APC first has to bind a Th-cell and receive a stimulatory signal which will then enable the APC to activate a Tc-cell (Bevan 2004).

B-cells

B-cells are, as mentioned above, regarded as APCs. To become fully mature and be able to produce antibodies, they however need to be activated by a Th-cell, of a Th1- or Th2-type. The B-cell receptor is, like the T-cell receptor, more or less specific for one antigen. When the cell comes into contact with an antigen, the antigen is internalized, degraded and presented on the surface by a MHC II molecule. The already activated Th-cell, which recognizes the same antigen, will bind to the B-cell and stimulate it to mature into an antibody producing plasma cell (Janeway et al. 2005).

Immunological memory

After an invading pathogen has been cleared an immunological memory can be created. If a reinfection with the same pathogen occurs a response will be mounted much more quickly since mature antigen specific cells are already present. The process of activation of naive cells is omitted (Antia et al. 2005).

Memory cells are of both T- and B-cell type. As with effector T-cells, different subsets of memory cells can be found; Th1-, Th2- and Tc-cells (Sallusto et al. 2004). How these cells are maintained is not quite understood. One hypothesis was that the memory cells consisted of a non-dividing population but after animal studies showing that memory cells did undergo division this hypothesis was rejected (Tough et al. 1994). Furthermore, the presence of antigen does not seem to be required (Lau et al. 1994). Other cells might stimulate the memory cells or there can be a cross-reactive stimulation by self-antigen or unrelated pathogens (Antia et al. 2005).

There are two different groups of memory T-cells; effector (T_{EM}) and central (T_{CM}) memory T-cells (Sallusto et al. 2004). T_{EM} , mostly CD8⁺ cells, are responsible for the protective memory and migrate to the inflamed tissue where they can have immediate effect. T_{CM} , on the other hand, consist mostly of CD4⁺ cells and are found in the lymph nodes. These cells have no direct effect on the infection but can differentiate into T_{EM} in response to antigen stimulation. Some of the T_{CM} are pre-programmed to become Th1- or Th2-cells and others are induced depending on the cytokine milieu at the site of induction (Sallusto et al. 2004). T_{EM} also show Th1- or Th2-phenotype but can switch due to influence of cytokines (Messi et al. 2003).

Cytokines

Cytokines are small proteins (Janeway et al. 2005) which generally act paracrine and/or autocrine. They can be involved in activation, inhibition, growth and they determine the type of immune response to be mounted against a pathogen (Borish et al. 2003). Some cytokines are produced and secreted by many different cell types, e.g. IL-6 and TNF- α , and others are more restricted to specific cells, e.g. IL-2 and IL-4 (Kelso 1998). Cytokines are usually pleiotropic. A good example of this is IL-10 which affects most hemopoietic cell types (Moore et al. 2001). Depending on the route of antigen presentation, IL-10 has different effects on Tc-cells. In the presence of APC IL-10 acts suppressive on Tc-cell but if the Tc-cell is activated via its TCR, IL-10 has a growth-promoting effect (Groux et al. 1998).

IL-12 and IL-23 are two different cytokines but with much in common. They are heterodimeric cytokines that share the p40 subunit. IL-12 is also comprised of p35 and IL-23 of p19. Since the p40 subunit binds to IL-12R β 1, this chain is present in both the IL-12R and IL-23R complex. Furthermore, both cytokines are mainly produced by DCs and macrophages and affects the same types of cells, T-cell, NK-cells and APCs. However, they also have different attributes. IL-23 secretion seems to be less dependent on IFN- γ than IL-12 production. Naive T-cells respond well to IL-12 but poorly to IL-23 whereas memory T-cells show the opposite response pattern (Langrish et al. 2004). The IL-12 subunits can form different combinations that have opposite effects. IL-12 p70 (p40 and p35 heterodimer) has an activating effect on macrophages and Th1-cells whereas the p40 subunit homodimer, p80, may function as an antagonist to p70 by binding to and blocking the receptor (Holscher 2004).

The cytokines investigated in the papers of this thesis are described by origin and principal effects in Table 1.

Table 1. Summary of cytokines investigated in papers I-IV

Cytokine	Producer cell	Action	Paper	Reference
IFN- γ	Th1-cells NK-cells Macrophages Tc-cells	- activates macrophages - suppresses Th2 differentiation	I, II, III, IV	(Borish et al. 2003, Shtrichman et al. 2001)
TNF- α	macrophages Tc-cells NK-cells neutrophils mast cells	- induces inflammation - activates endothelial cells	III	(Borish et al. 2003, Ma 2001)
IL-4	Th2-cells mast cells	- activates B-cells - induces Th2 differentiation - suppresses Th1 differentiation - induces isotype switch from IgM to IgE	I, II, III, IV	(Borish et al. 2003)
IL-5	Th2-cells mast cells	- promotes eosinophil growth	II, IV	(Borish et al. 2003)
IL-6	DC macrophages T-cells B-cells	- promotes T- and B-cell growth - induces acute phase proteins production - induces IL-4 production thereby stimulating polarization of Th2-cells - inhibits differentiation of Th1-cells by blocking IFN- γ R signaling	III	(Borish et al. 2003, Diehl et al. 2000, Rincon et al. 1997)
IL-10	DC macrophages B-cells Treg T-cells	- inhibits macrophages - enhances B-cell proliferation and survival - inhibits Th1- and Th2-cells - activates Tc-cells	III, IV	(Borish et al. 2003, Ding et al. 1993)
IL-12	DC macrophages B-cells neutrophils	- activates NK-cells - induces Th1 differentiation - activates Tc-cells	II, IV	(Borish et al. 2003, Manetti et al. 1994)
IL-13	Th2-cells	- promotes B-cell growth - inhibits Th1-cells - induces isotype switch from IgM to IgE	II, IV	(Borish et al. 2003)

Abbreviations: IFN, interferon; Th-cell, T-helper cell; Tc-cell, cytotoxic T cell; TNF, tumor necrosis factor; IL, interleukin; DC, dendritic cell; NK, natural killer.

Regulation

The immune system is regulated on various levels and in different ways. Th1- and Th2-type immune responses balance each other in that IL-4 inhibits the production of IFN- γ and IL-12 and IFN- γ inhibits the production of IL-4 (Paludan 1998). Certain cytokines can also have a down-regulatory effect on immune cells. IL-10 inhibits the production of many cytokines and chemokines (Moore et al. 2001) and has been shown to be important in regulating the inflammatory response as mice deficient of IL-10 develop chronic enterocolitis (Kuhn et al. 1993). Macrophages respond to IL-10 by down-regulating the expression of B7 which affects the antigen presentation and T-cell activation (Ding et al. 1993).

In the last years the research field of T-cell involved in suppression of the immune system has been given a renaissance. The term suppressor T-cells has been changed to regulatory T-cells (Treg). These subsets of T-cells display the CD4 molecule and the alpha part of the IL-2R (CD25) but can differ in their cytokine pattern. Inducible Treg, that is they derive from conventional CD4⁺ Th-cells which are exposed to specific stimulatory conditions (Belkaid et al. 2005), such as Treg1 secrete IL-10 whereas Th3 induces suppression via production of transforming growth factor (TGF)- β (McGuirk et al. 2002). A third subset called natural occurring CD4⁺CD25⁺ Treg (hereafter referred to as Treg) develop into suppressor cells in the thymus and secrete little or no IL-10 or TGF- β . These cells constitute 5-10% of peripheral CD4⁺ T-cells and use cell-cell contact to suppress *in vitro* but might act through cytokines *in vivo*. Treg do not inhibit primary T-cell response but since they proliferate upon specific antigen stimulation this expansion act to suppress continuous immune responses (Thompson et al. 2004). There are T-cells lacking suppressor function which express CD25 making this marker not absolute for localization of Treg. The forkhead transcription factor Foxp3 has been found in high levels in Treg but not in Tc- or B-cells and can therefore be used as a marker for Treg (Hori et al. 2003).

As the Swedish saying goes “lagom är bäst” (approximate translation: just right is best), the cells involved in the immune response have to be balanced to obtain the best result. If Th1 dominates an inflammation will occur, if Th2 is over expressed there is a risk for allergy and if the Treg population is enlarged inhibition of a vital immune response might be the outcome (McGuirk et al. 2002). The last scenario has been seen by Stoop et al in patients with chronic hepatitis B. This group of patients displayed an increase in the Treg population compared with healthy controls and individuals with a resolved hepatitis B infection (Stoop et al. 2005). Another study showed that Treg suppresses the T-cell response to *Helicobacter pylori* in patients infected with the bacteria (Lundgren et al. 2003).

Circulating cytokines can be harmful if they are present at high concentrations and during long periods of time, e.g. systemic exposure to TNF- α leads to septic shock (Ma 2001). The regulation of cytokines is therefore important and achieved in various ways. Cells secrete soluble cytokine receptors which can bind and block the activity of the cytokine. On the other hand, the soluble receptors might also act as agonists by protecting the cytokine from degradation and prolonging its half-life (Kelso 1998). These receptors can impose a problem in methods that measure soluble cytokines since they prevent the detection of cytokines and therefore the result of the analysis is incorrect.

Factors influencing the immune response

The immune system evolves and changes during our life time (Mund 2003). The response is also affected by numerous factors, for example gender, drugs, chronic diseases such as atopy and diabetes.

Sex hormones

Women are overrepresented in diseases such as multiple sclerosis, rheumatoid arthritis and Sjogren's syndrome. This is believed to be coupled to sex hormones. Estrogen and testosterone have different effect on the immune response; estrogen seems to be immunostimulatory whereas testosterone might function as a suppressor (Da Silva 1999, D'Agostino et al. 1999). Cytokine levels have been shown to correlate with hormones. Verthelyi et al demonstrated that estrogen correlated with IL-4 secretion in premenopausal women and dehydroepiandrosterone sulfate, a precursor of sex hormones, correlated with the production of IFN- γ in both men and women (Verthelyi et al. 2000). The antibody-mediated immune response after vaccination is higher in women than men (Struve et al. 1992).

During menopause the hormone levels decrease in women (Cioffi et al. 2002, Pietschmann et al. 2003). These changes affect the immune response, though the data are conflicting. Spontaneous TNF- α secretion was shown to be lower in postmenopausal women than in women before menopause (Cioffi et al. 2002, Verthelyi et al. 2000). However, in another study TNF- α was seen to be elevated in women who had had their ovaries removed by surgery. The TNF- α secretion decreased if the women were given estrogen replacement therapy (Pacifici et al. 1991). Verthelyi et al also showed that spontaneous IFN- γ was lowered in postmenopausal women (Verthelyi et al. 2000) whereas Pietschmann et al demonstrated that mitogen stimulated cells from postmenopausal women secreted higher levels of IFN- γ than premenopausal women (Pietschmann et al. 2003).

The proportion of CD4+ and CD8+ cells also changes in women during this time of life. The percentage of CD4+ T-cells in blood and bronchoalveolar lavage fluid increases in women over 43 years of age, compared to women

≤40 years, whereas CD8+ T-cells decreases in bronchoalveolar lavage fluid at the same time. These changes are not seen for men (Mund et al. 2001).

Co-infections

Some infections have profound effect in the immune system and its function. Human immunodeficiency virus (HIV) is probably the most well known immunosuppressive pathogen. HIV infects and destroys CD4+ T-cells thereby reduce the immune systems ability to respond to other infections. The measles virus also alters the immune response by binding to CD46 leading to inhibition of IL-12 production. This suppression can persist for months after the acute infection (Atabani et al. 2001) and might be due to persistent viral antigen in lymphoid tissue (Ciurea et al. 1999).

An infection with *A. phagocytophilum* can predispose animals and humans to secondary infections (Lepidi et al. 2000). It has been speculated that this predisposition is caused by the leukopenia seen in many HGA patients. However, there might also be other factors involved. Woldehiwet showed that blood cells from *A. phagocytophilum* infected sheep responded poorly to mitogen stimulation possibly due to toxic products released from dead infected granulocytes (Woldehiwet 1987). This suppression might not be restricted to the acute stage of the infection. Larsen et al found that serum from healthy sheep, previously infected with *A. phagocytophilum*, significantly lowered the response to mitogen in cells from healthy non-infected sheep (Larsen et al. 1994). Immunosuppression has, in another study on sheep, been seen to last for eight weeks post-infection (Whist et al. 2003).

Although *A. phagocytophilum* infect neutrophils the bacteria seem to affect other immunological cells as well. When human peripheral blood leukocytes were stimulated with *A. phagocytophilum* or the surface protein p44 expression of IL-1 β , TNF- α and IL-6 messenger RNA (mRNA) was induced in monocytes whereas only IL-1 β mRNA was elevated in neutrophils (Kim et al. 2002). Furthermore, *A. phagocytophilum* might interact with other cells and generate pro-inflammatory cytokines (Rikihisa 2003).

Immunology of Lyme borreliosis

Animal studies have been useful for studying the immune response to *B. burgdorferi s. l.* However, studies of neuroborreliosis can not be performed in mice since the *Borrelia* infection does not involve the nervous system in rodents. Instead, the use of non-human primates has shown to be a good model for neuroborreliosis. Pachner and colleagues found the spirochete to be widely disseminated throughout the central and peripheral nervous system (Pachner et al. 2001). They also found strong inflammatory responses in the tissue investigated but the level of inflammation was not coupled to the spirochete load; the cerebrum had a large load of spirochetes but showed no inflammation.

Innate immune response

The first line of defense, the antigen presenting cells DCs, can be found in CSF from patients with neuroborreliosis (Pashenkov et al. 2001, Pashenkov et al. 2002). These cells react to the lipid portion of Osps (Beermann et al. 2000a, Häupl et al. 1997, Morrison et al. 1997) leading to secretion of the pro-inflammatory cytokines TNF- α , IL-1 β , IL-6 and IL-12 (Radolf et al. 1995). The anti-inflammatory cytokine IL-10 is also secreted in response to *Borrelia* antigens (Giambartolomei et al. 1998). IL-10 has been shown to inhibit IL-6 and IL-12 production but had no effect on TNF- α and IL-1 β because these cytokines were secreted before or at the same time as IL-10 (Murthy et al. 2000).

Osps are recognized by TLR2 heterodimerized with TLR1 or TLR6, found on macrophages and DCs, and this receptor has an important roll in controlling the spirochete load but it is not necessary for the development of an antibody response (Wooten et al. 2002). Lipopeptides are also presented to T-cells by the CD1 receptor (De Libero et al. 2005, Gumperz et al. 2001).

Adaptive immune response, Th1 and Th2

The adaptive and more specific immune response to an infection with *B. burgdorferi s. l.* was shown to be of a Th1-type in mice susceptible to Lyme borreliosis whereas resistant mice displayed a Th2-response (Keane-Myers et al. 1995). However, the investigators did not look at the immune response during the course of infection. This was later done by Kang and colleagues and they found contradictory results. The resistant mice first displayed a Th1-response and then switched over to a Th2-type response. In the susceptible mice the Th1-response was slower and they lacked the switch to Th2. Kang et al therefore postulated that it is the deficiency of a Th1-response early in the infection that is the cause of the more sever symptoms (Kang et al. 1997). However, two studies done by Anguita et al showed that mice lacking Th2-response developed more severe arthritis than wild type mice whereas mice lacking Th1-response showed milder symptoms of arthritis but higher spirochete load than normal mice (Anguita et al. 1996, Anguita et al. 1998). In both studies all the mice recovered within 60 days. Similar results were seen in pregnant mice. The mice had a dominant Th2-type immune response and milder arthritis symptoms (Moro et al. 2001). The Th1-type response seems to be the cause of the symptoms but at the same time is also responsible for the clearance of spirochetes.

Human studies confirm the results seen in animals, where patients with chronic Lyme borreliosis have been found to display a Th1-response (Oksi et al. 1996, Pohl-Koppe et al. 1998). Furthermore, T-cells from synovial fluid show a predominant Th1-response and the ratio of Th1/Th2 cells correlated to the severity of arthritis (Gross et al. 1998b).

Autoimmunity

Chronic Lyme borreliosis, especially arthritis, might be caused by an autoimmune reaction. Treatment-resistant chronic arthritis has been associated with specific classes of major histocompatibility complex, HLA-DR4 and HLA-DR2 (Steere et al. 1990) and T-cell reactivity to specific OspA peptides (Chen et al. 1999). Antibody reactivity to OspA or B might also trigger an autoimmune response (Kalish et al. 1993). A peptide from the LFA-1 has been found to show homology with OspA (Gross et al. 1998a) and T-cells reacting to OspA were also found to respond to LFA-1 (Kalish et al. 2003). The OspA peptide involved in treatment-resistant Lyme arthritis differs between *B. burgdorferi s. s.*, *B. garinii* and *B. afzelii*. Lymphocytes from these patients react to the *B. burgdorferi s. s.* peptide but not to peptides from the other strains (Drouin et al. 2004).

The role of autoimmune reactions in neuroborreliosis is less known. Antibodies to gangliosides, a lipid found in high concentrations in cells in the nervous system, can be found in patients with neuroborreliosis and an animal study showed that *B. burgdorferi s. l.* could induce these antibodies (Garcia-Monco et al. 1995). Furthermore, two OspA epitopes have been identified which share immune cross-reactivity with proteins in human neural tissue (Alaedini et al. 2005). Flagellin is another possible antigen thought to be able to elicit an autoimmune response. The protein was found to have homology with human myelin basic protein (Weigelt et al. 1992).

Evasion strategies of the *Borrelia* spirochete

The *Borrelia* spirochete elicits an immunological response when recognized by the human immune system but the bacteria have found ways of protecting themselves. A tick salivary protein, Salp15, binds to OspC on *B. burgdorferi s. l.* and protects the spirochete from antibody-mediated killing (Ramamoorthi et al. 2005). Other proteins, such as OspE, OspE-related proteins and complement-regulator-acquiring surface proteins, protects the spirochete from the immune system by binding the human complement regulatory protein factor H (Kraiczky et al. 2001) thereby inhibiting the complement cascade. *B. burgdorferi s. l.* possible releases soluble antigens to which antibodies are bound and forms immune complexes (Brunner et al. 2000). This strategy will decrease antibody opsonization of the spirochete. It would also inhibit the detection of antibodies for diagnostic purposes leading to false negative results (Lawrence et al. 1995, Schutzer et al. 1990).

The spirochete might also hide physically by entering the joints and central nervous system. Normally, these sites do not contain circulating immune cells making them, in part, immunological privileged sites. It has also been speculated that the spirochete might reside in intracellular compartments thereby escaping the immune response (Hu et al. 1997) but this has not been demonstrated *in vivo*. If derived of nutrient (Brorson et al. 1997) or exposed

to β -lactam antibiotics the *Borrelia* spirochete has been shown to transform into a non-motile cystic form (Murgia et al. 2002). The cysts can then revert into its original spiral shape when the conditions are restored.

AIM OF THE THESIS

The general aim of this thesis was to find out if immunological differences could be found between patients with different outcome of Lyme borreliosis and to study the role of various factors that might influence the course of the disease.

The specific aim for each paper was:

- I. to investigate the *Borrelia*-specific IFN- γ (Th1) and IL-4 (Th2) response during different stages and clinical outcomes of Lyme borreliosis
- II. to compare the Th1- and Th2-type immune response to *Borrelia* antigen in patients with Lyme borreliosis with or without a previous exposure to *Anaplasma phagocytophilum*
- III. to elucidate if host immune status could explain the increased risk of Lyme borreliosis reinfection in postmenopausal women
- IV. to investigate if there was a constitutive difference in the ability to mount a Th1-type immune response between patients with different outcomes of Lyme borreliosis and if the *Borrelia*-specific regulatory T-cells response was altered in chronic Lyme borreliosis patients

MATERIALS AND METHODS

Subjects

Patients and controls (Table 2) were recruited from the south eastern part of Sweden. The healthy controls were blood donors or staff at the University Hospital in Linköping. Also, a group of patients undergoing elective orthopedic surgery were included as healthy CSF controls. Four healthy controls participated in both paper II and IV and one patient with subacute borreliosis was included in both paper I and IV. Altogether, 260 individuals were included in this thesis.

Table 2. Patients and controls included in paper I-IV

Diagnosis	Paper I	Paper II	Paper III	Paper IV
EM	12	15	38	
Subacute borreliosis	12			14
Chronic borreliosis	32			12
Asymptomatic borreliosis				14
Borreliosis reinfection			24	
HGA, previous exposure		8		
OND	13			
Healthy controls:				
blood donors/staff	23	15		14
CSF controls	19			
Total	111	38	62	54

Abbreviations: EM, erythema migrans; HGA, human granulocytic anaplasmosis; OND, other neurological diseases; CSF, cerebrospinal fluid

In paper III, a control group of healthy individuals was not included since the aim was to compare individuals single or reinfected with *B. burgdorferi* s. l. A control group would show the specificity of the *Borrelia* antigen used in the study. However, a control group would not contribute information regarding the constitutive immune response since the persons included in paper III were healthy at the time of sampling, which was indicated by low levels of C-reactive protein.

The skewed gender distribution in the subacute and in the asymptomatic group in paper IV was not intentional. The asymptomatic individuals were found by screening blood donors. This group consisted of more men than women from the start; 451 men (58.3%) and 322 women (41.7%) which is a significant difference (Chi2, $p < 0.0001$). Therefore, it was not surprising that

more male asymptomatic individuals were found and thus were available for the study. The gender skewness in the subacute group was due to limited number of patients and should not be seen as reflection of subacute patients in general.

Diagnostic criteria

Patients in papers I and IV were diagnosed by the same experienced physicians (co-authors) and the same criteria, summarized in Table 3, were used in all four papers for the different diseases. Patients in papers II and III were diagnosed by their general practitioner.

Clinical outcome

In paper I and IV patients with borreliosis were grouped into chronic or subacute/nonchronic, according to duration of symptoms. Chronic borreliosis was defined as symptoms lasting longer than six months whereas the patients in the subacute/nonchronic group recovered within six months. Patients diagnosed with ACA were placed in a separate group in paper I but were included in the chronic group in paper IV. The reason for this inconsistency in subdivision was that in paper I the immune response was studied in the primary infected compartment, i.e. CSF or the skin, whereas in paper IV the systemic memory response was investigated.

Reinfection

The patients in paper III who were defined as being reinfected with *B. burgdorferi s. l.* had been diagnosed with an EM (≥ 5 cm in diameter) between May 1992 and the end of April 1993 and had then, between May 1993 and May 1998, been diagnosed with a new EM. The diagnoses were made by a physician at both occasions.

Controls

The term healthy control was in regard to Lyme borreliosis (papers I-IV) and HGA (paper II). Other common diseases were not taken into consideration although these persons were not taking immunomodulating medication and did not have an ongoing infection when the sample was collected.

A CSF control group was included in paper I. This group consisted of patients with the diagnosis of for example multiple sclerosis or tick-borne encephalitis, termed other neurological diseases (OND), and patients undergoing elective orthopedic surgery. All patients were negative for *Borrelia*-specific antibodies in serum and CSF. Furthermore, the orthopedic patients had no neurological symptoms and no pleocytosis in CSF.

Table 3. Definitions of diagnoses described in papers I-IV

Diagnosis	Definition
EM	red circular rash, ≥ 5 cm in diameter
Neuroborreliosis	<ul style="list-style-type: none"> - clinical symptoms ^a - intrathecal <i>B. burgdorferi s. l.</i> specific antibody production (IgG and/or IgM) - mononuclear pleocytosis in cerebrospinal fluid ($\geq 5 \times 10^6$ cells/l)
ACA	<ul style="list-style-type: none"> - clinical symptoms ^b - <i>B. burgdorferi s. l.</i> specific antibodies in serum
Asymptomatic borreliosis	<ul style="list-style-type: none"> - no clinical symptoms - <i>B. burgdorferi s. l.</i> specific antibodies in serum - <i>B. burgdorferi s. l.</i> specific T-cell reaction
HGA, acute	<ul style="list-style-type: none"> - clinical symptoms ^c - fourfold or greater change in <i>A. phagocytophilum</i> specific antibody titer or a positive PCR assay or intracytoplasmic morula
HGA, previous exposure	<ul style="list-style-type: none"> - no clinical symptoms - <i>A. phagocytophilum</i> specific antibody titer $\geq 1:80$
OND	<ul style="list-style-type: none"> - neurological symptoms - no history of EM - negative intrathecal <i>B. burgdorferi s. l.</i> specific antibody production - negative serology for <i>B. burgdorferi s. l.</i>
Healthy controls	negative serology for <i>B. burgdorferi s. l.</i> and/or <i>A. phagocytophilum</i> specific antibodies

Abbreviations: EM, erythema migrans; ACA, acrodermatitis chronicum atrophicans; HGA, human granulocytic anaplasmosis; PCR, polymerase chain reaction; OND, other neurological diseases

^a Facial palsy, neck and/or back pain, head ache, muscle pain and/or radiculitis

^b Discolored bluish/red skin

^c Fever, head ache, myalgia and/or malaise

Antigens

The *Borrelia* antigen used in all four papers was prepared at our laboratory in Linköping. *B. garinii* strain Ip90 was generously provided by professor Sven Bergström, Umeå University and the Osps were collected by membrane fractioning as previously described (Magnarelli et al. 1989). The final product, called outer surface protein enriched fraction (OF), was analyzed with Western blot for the presence of OspA and B, which are the main proteins in OF. The concentration was also optimized and set to be used at a final concentration of 10 µg/ml. OF has been shown to discriminate between *B. burgdorferi* s. l. seronegative and seropositive individuals, seen as a predominant IFN-γ response but IL-4 was also secreted (Forsberg et al. 1995). *Borrelia* lipoproteins however stimulated macrophages to secrete IL-1β, IL-6, IL-10 and TNF-α in a non specific manner (Giambartolomei et al. 1998, Häupl et al. 1997).

In Sweden all children born before 1975 were vaccinated against tuberculosis therefore most adults should have an immunological memory to purified protein derivative of tuberculin (PPD). This makes PPD useful as a reference antigen and it was used in papers III and IV. The bacteria causing tuberculosis is intracellular and the immunological response should therefore be of a Th-1 type (elGhazali et al. 1993).

Phytohemagglutinin (PHA) is lectin extracted from the red kidney bean *Phaseolus vulgaris*. It is a mitogen, i.e. it stimulates cell division, and activates NK- and T-cell through CD2 (O'Flynn et al. 1985) in a non-specific manner. PHA was used in all four papers, usually as a positive control, but since CD2 is more highly expressed on Th1-cells PHA can also be considered a Th1-type derived antigen (Rogge et al. 2000).

Peptidoglycan is a complex of polysaccharides and peptides found in the cell wall of both gram-positive and gram-negative bacteria. The layer of peptidoglycan is thicker in gram-positive than negative bacteria. It binds to TLR2 on macrophages and induces a pro-inflammatory response (Hessle et al. 2005). Peptidoglycan was used in paper III.

Methods

The methods used in this thesis were: cell separation (paper I-IV), enzyme linked immunospot (ELISPOT, paper I-IV), ELISA (paper III and IV), Immulite (paper III), flow cytometry and real time reverse transcription (RT) PCR (paper IV). The principals of these methods are described below.

Cell separation (paper I-IV)

Mononuclear cells were separated from heparinized peripheral blood by density gradient centrifugation (Boyum 1968). The blood was diluted 1:3 in buffer salt solution and a polysaccharide solution, Lymphoprep (paper I-III)

or Ficoll-Paque (paper IV), was applied by syringe beneath the blood. Lymphoprep and Ficoll-Paque have the same density as mononuclear cells (1.077 g/ml) therefore, when centrifuged, these cells will be collected in the interface between the Lymphoprep/Ficoll-Paque and the buffer, which also includes plasma, whereas other cells will go straight through and will be found at the bottom of the tube (Figure 4).

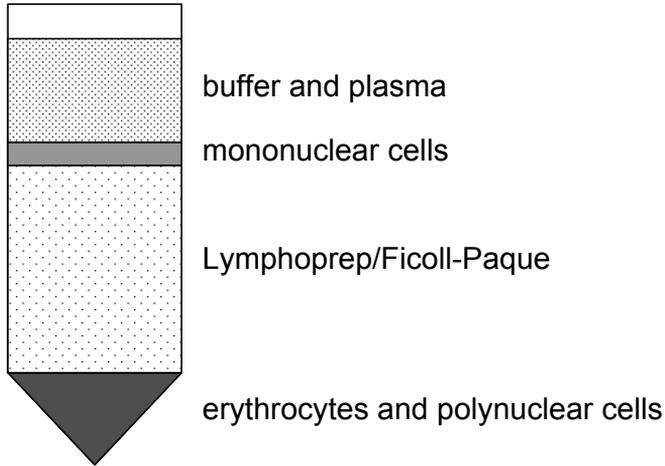


Figure 4. Separation of mononuclear cells by density gradient centrifugation.

The mononuclear cells were removed and washed with buffer at 4°C (papers I-III) or at room temperature (paper IV).

The reason for using two different approaches for the cell separation was that in paper IV this step was performed by another laboratory where the Ficoll-Paque protocol is utilized. However, the two approaches of separating cells gave the same yield of mononuclear cells.

ELISPOT (paper I-IV)

The ELISPOT method used in this thesis was first described by Czerkinsky et al (Czerkinsky et al. 1988) and thereafter modified according to Forsberg et al (Forsberg et al. 1995). It is a sensitive technique where the cytokine secretion can be detected on a single cell level. Capture antibodies are coated onto a nitro-cellulose surface (Figure 5a) and unspecific binding sites are blocked by use of cell culture medium. A suspension of cells is then added at a density which makes the cells form a monolayer. The cells can then be stimulated with different antigens or mitogens. To assess the spontaneous secretion of cytokine, samples of non-stimulated cells should always be included in the assay as well as a negative control consisting of medium only, i.e. no cells. The cytokine is captured by the capture antibodies immediately after secretion

(Figure 5b) and to get a clear “foot print” of each secreting cell it is important that the culture is kept still during the incubation period.

The cells are removed by washing and then, to visualize the cytokine from each cell, a secondary biotinylated antibody is used which binds to a different epitope of the cytokine compared to the capture antibody (Figure 5c). Streptavidin, conjugated with an enzyme, is then added and will bind to the biotin (Figure 5d). The biotin is used for enhancement since several biotin molecules can be attached to each antibody thereby increasing the number of streptavidin-enzyme complex. The last step is to add a mixture of two substrates which, catalyzed by the enzyme, will react and form an insoluble complex (Figure 5e). This complex falls to the bottom and will make the cell’s cytokine “foot print”, the spot, visible (Figure 5f).

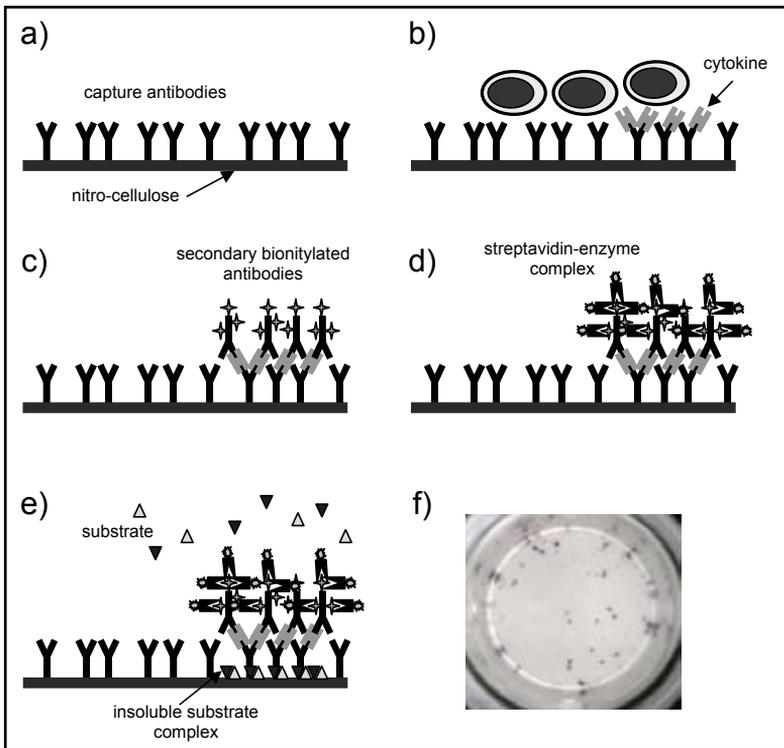


Figure 5. Enzyme linked immunospot (ELISPOT): a) Nitro-cellulose coated with capture antibodies. b) Cells secrete cytokine that is capture by the capture antibodies. c) Secondary biotinylated antibodies are added which will bind to the cytokine. d) Streptavidin-enzyme complexes bind to the biotin. e) The enzyme catalyzes the formation of an insoluble complex. f) Photograph of spots.

Spots can be counted manually in a microscope or automatically with the aid of computer software. Since different persons make different assessment of what constitutes a spot (Janetzki et al. 2004), it is important that one person

evaluates and counts the spots in a certain study. However, data from our laboratory show that there can also be a strong counting correlation between two persons ($\rho=0.95$ for IFN- γ and $\rho=0.88$ for IL-4, calculated with Spearman's rank correlation test). For the different papers in this thesis different persons counted the spots but the spots in each study was evaluated by only one person. If a computer is used for the counting one needs to recognize that spots formed by different cytokines do not have the same appearance and therefore can not be analyzed with the same settings.

When cells are mixed with a protein antigen it has to be presented to the T-cells by an APC. To obtain the optimal antigen presentation a concentration of 16% monocytes are needed (Schmittl et al. 2001) which is the normal value of the cell suspension after density gradient centrifugation.

The advantage of ELISPOT is foremost its sensitivity. Schmittl et al coated beads with IFN- γ and found that they could detect almost 100% of the beads using ELISPOT (Schmittl et al. 1997). For detection of cytokines which are secreted at low concentrations, e.g. IL-4, ELISPOT is a superior technique over ELISA and real time PCR (Ekerfelt et al. 2002). One problem seen in other methods is that cytokines can be consumed or bound to soluble receptors thereby making the results unreliable. With ELISPOT the cytokine is captured by the capture antibodies immediately after secretion thus overcoming this obstacle. The disadvantages with the method are that it is time consuming and more importantly has a high inter assay variation (31% for IFN- γ and 38% for IL-4) (Ekerfelt 1999). The intra assay variation can also pose as a problem with between 7%, at high counts (mean 490 spots), and 25%, at low counts (mean 33 spots), for IFN- γ and 25% for IL-4 (Widhe 2003). Some might argue that the use of median, not mean, values of the triplicate wells would lessen the intra assay variation. However, the median and mean values of the samples included in this thesis correlate well (IFN- γ $\rho=0.97$, IL-4 $\rho=0.99$ and IL-10 $\rho=1.0$, calculated with Pearson's correlation test).

ELISA (paper III and IV)

ELISA is based on the same principle as ELISPOT but ELISA measures molecules in solution, i.e. the concentration. As with ELISPOT, capture antibodies are bound to a solid surface. Unspecific binding sites are blocked, e.g. by milk proteins, and the samples are added in duplicates to diminish the intra assay variation. The sample can be plasma, serum, CSF, cell supernatants, saliva or any other solution. To quantify the measured molecule, a set of samples with known concentrations are used to calculate a standard curve. Medium only is added to assess the background and a control sample, that is continually used, is included to evaluate the inter assay variation.

A secondary biotin conjugated antibody followed by enzyme-streptavidin and enzyme substrate is then used. The enzyme reaction is stopped by sulfuric

acid and the color change in the solution can then be measured optically. The known values of the samples/points of the standard curve are entered into the computer and the unknown concentrations of the other samples are calculated on the basis of this curve.

ELISA is a convenient method; quick, easy and relatively inexpensive. However, it requires rather large volumes of sample and the inter assay variation can be high (paper IV, IFN- γ 8%, IL-5 29% and IL-10 11%). Also, only one cytokine, receptor or hormone can be detected in each assay which makes it time-consuming if several substances are analyzed.

Immulate (paper III)

Immulate, like ELISA, is used for measuring soluble substances. It utilizes chemiluminescence to visualize the detected molecule. A bead coated with antibodies directed towards the substance of interest is mixed with the sample. Then, enzyme-labeled secondary antibodies are added and last chemiluminescent reagent. The reaction between the enzyme and the chemiluminescent reagent results in light production, which can be measured. A standard curve is run once and saved in the machine. When samples are analyzed two control samples, one high and one low, are included and on the basis of these points the standard curve is adjusted. Several other control samples with known concentrations are also analyzed in each run. For the run to be approved these controls have to fall within specific ranges.

Compared to ELISA, Immulate is less time-consuming and since the method is automated, which reduces laboratory errors, the inter-assay variation is lower. However, Immulate is more expensive, mainly due to the cost of the machine. Immulate and ELISA do not differ in terms of sensitivity.

Flow cytometry (paper IV)

To detect membrane bound structures the method of flow cytometry is most useful. By attaching fluorescent dyes to antibodies, which will then bind to the cell, the structure of interest can be detected and analyzed in a flow cytometer. The labeled cells are lined up in a single-cell stream and passed through a laser beam. The light scatter is detected; the forward scatter determines cell size and side scatter determines granularity (Figure 6). The laser also causes the fluorochromes to emit light which is separated into different colors by filters and registered by a computer. Thus, from analyzing cells in a flow cytometer information about each cell's size, granularity and surface markers can be collected. If many fluorescent dyes with different emission spectra are used various membrane structures can be investigated on each cell at the same time. The data is then analyzed with the aid of computer software.

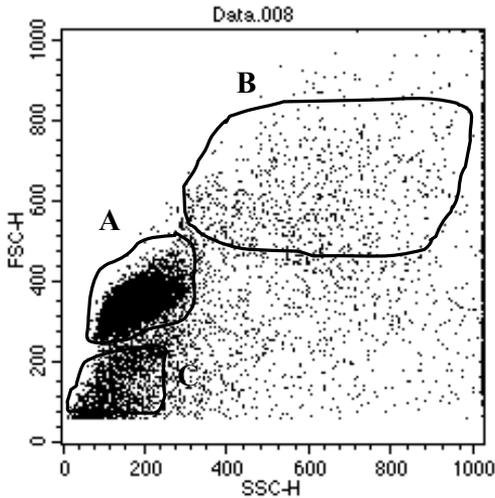


Figure 6. Analysis of peripheral blood lymphocytes using forward (FSC) and side scatter (SSC).

- A. Lymphocytes
- B. Monocytes
- C. Cell debris

The flow cytometer is a delicate instrument that needs to be adjusted and serviced on occasion. This can result in variations of detection. It is therefore vital for the user to make appropriate adjustments to compensate for these differences, if serial measurements are made at different time points. By using standardized beads and modifying the settings at every analyzing occasion the variation of the instrument can be corrected for.

In paper IV, the presence of IL-12R β 2 and the phenotype of the cells were analyzed with flow cytometry. The IL-12R β 2 is expressed in low numbers and therefore a signal reinforcement step was needed in form of biotin-streptavidin. Antibodies directly conjugated with a fluorochrome was tried but yielded a weaker signal than when biotin-streptavidin was used. The method was also validated by driving lymphocytes towards Th1 or Th2. Th1-cells were generated by incubating cells with IL-12 and anti-IL-4, and Th2-cells were incubated with IL-4 and anti-IL-12. As a control cells were also incubated without cytokine stimulation. The different cells types were then labeled as described in paper IV. For the Th1-cells 14-27%, depending on phenotype, expressed IL-12R β 2 whereas the receptor could not be detected on the Th2-cells (Figure 7). The conclusion of this experiment was that the method used could detect IL-12R β 2 and did discriminate between Th1- and Th2-type.

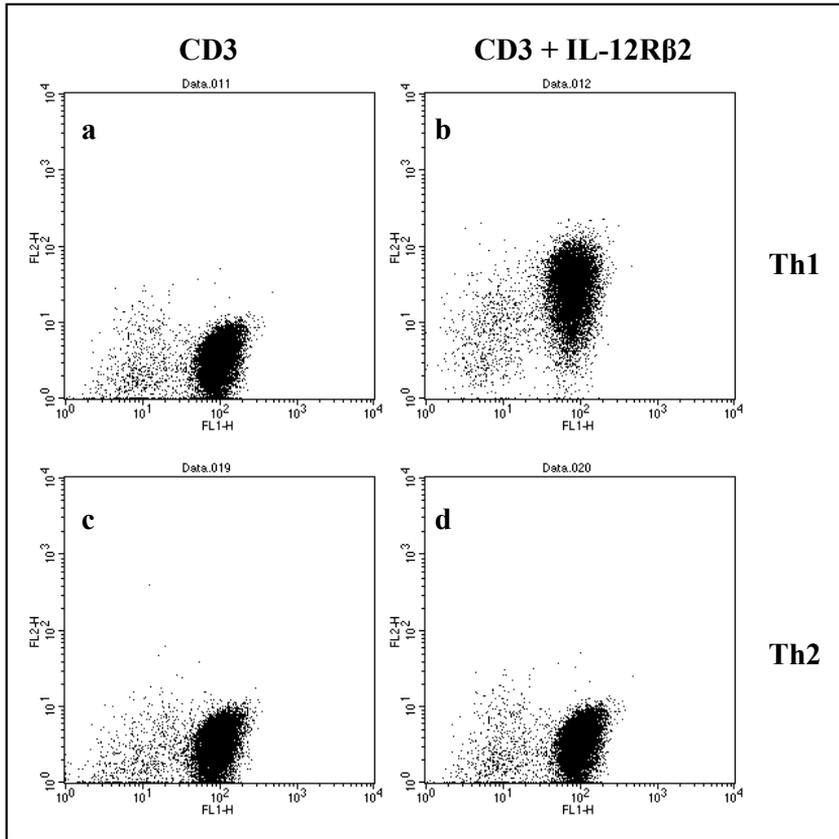


Figure 7. Analysis by flow cytometry of peripheral blood lymphocytes deviated to a Th1- or Th2-phenotype. a) Th1-cells labeled with anti-CD3 antibodies. b) Th1-cells labeled with anti-CD3 and anti-IL-12R β 2 antibodies. c) Th2-cells labeled with anti-CD3 antibodies. d) Th2-cells labeled with anti-CD3 and anti-IL-12R β 2 antibodies.

No standardized controls were used in paper IV. Each patient was analyzed once and non-stimulated cells were used to set the detection limit. Therefore, the difference between the stimulated and the non-stimulated cells were not dependent of the sensitivity of the instrument and possible oscillations during the study did not affect the final value of each analysis.

Real time RT PCR (paper IV)

ELISPOT, ELISA and flow cytometry all detect proteins secreted or expressed by the cell. Since these methods might not always be sensitive enough to detect small amounts of proteins, it can also be valuable to study the cell's reaction to stimuli before the actually protein is produced. This can be done on a genetic level by measuring the amount of mRNA. When the

eukaryotic cell produces a protein its DNA is first transcribed into RNA which, after splicing to remove the non-coding intron regions, is called mRNA. mRNA is then translated into the final protein.

There are a few different approaches that can be utilized to measure mRNA but the method of choice that is becoming more and more common is real time RT PCR. Total RNA is extracted from lysed cells and converted to complimentary DNA (cDNA) by the enzyme reverse transcriptase. The second part of the technique is the real time PCR. The cDNA is mixed with primers, Taq polymerase and deoxyribonucleotides (dNTPs). These are used in the PCR reaction where primers anneal to the cDNA and dNTPs are added on by Taq polymerase, creating mRNA. Each PCR cycle doubles the amount of mRNA. A probe specific for the protein of interest is also added. The probe has a fluorescent dye on one end and a quencher dye on the other (Figure 8a).

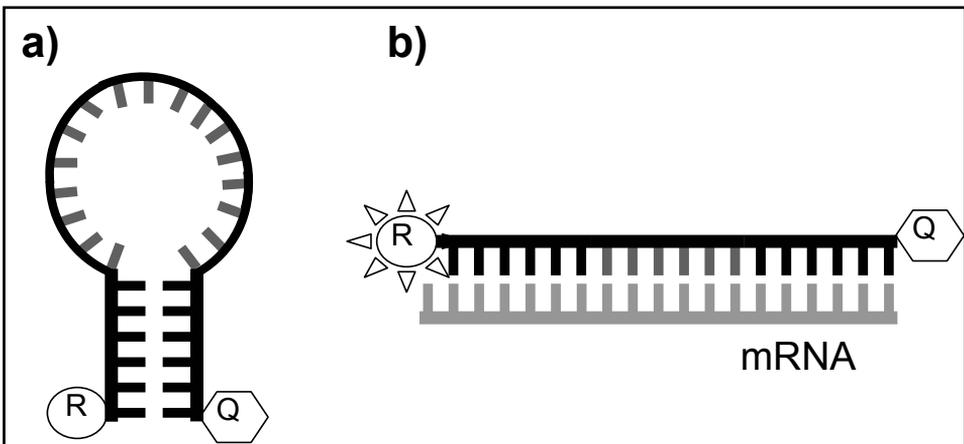


Figure 8. Schematic drawing of the principal of real time polymerase chain reaction (PCR): a) The probe has a fluorescent dye (R) attached to one end is and a quencher (Q) at the other end. b) When the probe binds to mRNA, the product of the PCR reaction, Q can no longer block the light from R.

The quencher absorbs the light emitted by the fluorescent dye when these molecules are close together but when the probe anneals to the mRNA the quencher is no longer able to block the light (Figure 8b). The signal that is detected is proportional to the number of mRNA copies and since the mRNA is doubled with every cycle the fluorescent signal should also be doubled if it is an optimal reaction.

A standard curve is used to calculate the value of the samples. The values of the points in this curve are set arbitrary and therefore the values of the samples are semi-quantitative. Since the amount of RNA/cDNA might vary between samples a reference gene is measured and all other markers are divided by the value of the reference gene. In paper IV we used the 18s gene

located on ribosomal RNA which, according to Bustin and colleagues, is the most stable reference target (Bustin et al. 2005).

To check for contaminations a negative control, water, is always included in the assay and to assess the inter assay variation an internal control is used. The inter assay variation was quit high in paper IV; 18s 30%, Foxp3 16% and IL-12R β 2 24%. To diminish the intra assay variation samples are run in duplicates and are only approved if the variation is less than 15%.

The primers used in the reaction should be designed to anneal over an exon-exon junction, which do not exist in genomic DNA, and therefore if the sample is contaminated by genomic DNA this is not amplified. The primers used to quantify Foxp3, IL-12R β 2 and 18s did not amplify genomic DNA.

Statistics

Nonparametric tests were used to analyze cytokine secretion and expression of receptors since it was not known if these types of data are normally distributed within the population. Also, the sample sizes in each paper were small ($n < 100$) which further makes the use of nonparametric test preferable. These tests give each sample a rank value, regardless of its exact/measured value. Therefore, outliers will not have an effect on the final p-value.

Where more than two groups were compared, Kruskal-Wallis test was used and Mann-Whitney U-test (paper I, IV) or Dunn's test (paper II) was used as post hoc. By applying Dunn's test a compensation for multiple comparisons were made. This was not done in papers I and IV. In these studies, however, the different parameters analyzed were part of a pattern and the significances found supported each other making corrections for mass significances less necessary. In paper III, only Mann-Whitney U-test was used since there were just two groups and to compensate for multiple comparisons the limit for a significant p-value was set to < 0.01 . The paired analysis in paper I was made by use of Wilcoxon signed rank test.

Most parameters analyzed in this thesis had been investigated previously in the same type of material but Treg and the expression of Foxp3, measured in paper IV, had not been studied before in patients with Lyme borreliosis. Thus, a lower level of significance was set for Foxp3 and, although more than two groups were involved, no Kruskal-Wallis test was used but only Mann-Whitney U-test.

Fisher's exact test is used when analyzing 2x2 tables. It should be used if the sample size in any cell is less than five but can also be used on larger materials. This test was utilized in paper I to compare the intervals with regard to patients IFN- γ or IL-4 predominance, in paper III to calculate the frequency of diseases and in paper IV to evaluate the frequency of atopy.

Student's t-test check for significant differences of the mean value between two groups and the observations must be normally distributed. This test was

used in paper II to compare cell count and in paper III to assess the age distribution.

In paper IV correlations between cytokine secretion and receptor expression were analyzed. These data were not normally distributed, thus the nonparametric Spearman's ranked correlation test was used.

Statistical power calculations were not performed. In each paper sample size of the material collected was limited by the source of available patients.

All statistical calculations were done with SPSS for Windows, version 10.0 (paper I) or 11.5 (paper II-IV), except for Dunn's test which was calculated using GraphPad Prism version 4.03. A p-value of <0.05 was used in all four papers, with the exception in paper III as mentioned above.

Ethics

All patients and controls included in the papers of this thesis gave their informed consent to participate. The studies were approved by The Ethics Committee of Linköping University (paper I, II and IV) or by The Ethical Committee at the University of Lund (paper III).

RESULTS AND DISCUSSION

Immune balance, Th1 vs. Th2

In paper I the Th1/Th2 balance was investigated, in blood and CSF, during the course of Lyme borreliosis. Patients with neuroborreliosis displayed a stronger *Borrelia*-specific response in CSF compared to that seen in blood, for both IFN- γ and IL-4. This was not an unexpected result since the symptoms originate from the central nervous system this is where the most intense immunological response is likely to be located. Ekerfelt et al demonstrated the same findings in a previous study (Ekerfelt et al. 1997).

When subacute and chronic neuroborreliosis patients were compared no significant difference was seen in the intervals. However, chronic patients had a continued IFN- γ response in CSF, compared to controls, during the course of the disease which was not the case for the subacute patients (Figure 9a). For IL-4 the opposite condition was found; IL-4 increased over time in subacute patients but did not change in chronic patients (Figure 9b).

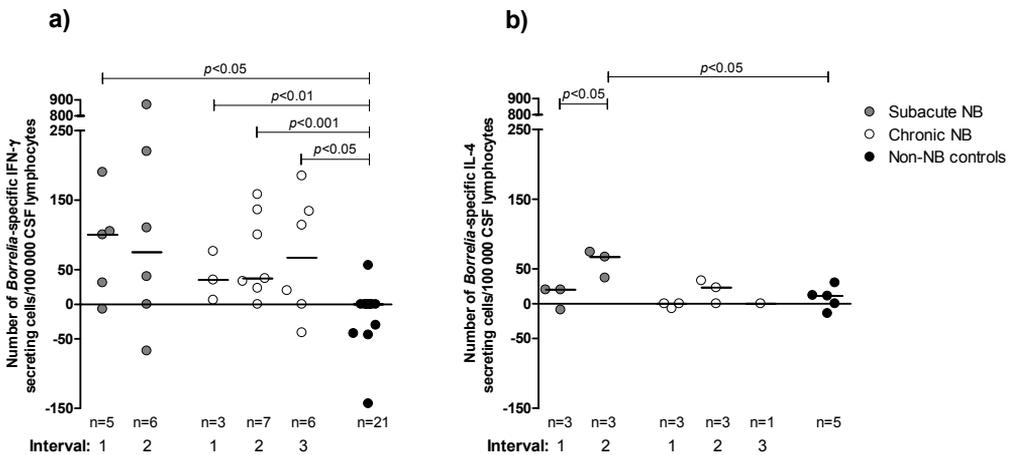


Figure 9. Number of *Borrelia*-specific IFN- γ (a) and IL-4 (b) secreting cells/100 000 lymphocytes in the cerebrospinal fluid (CSF) from patients with neuroborreliosis (NB), in association with clinical outcome. P-values show statistical significant differences from comparison with Mann-Whitney U-test. Each point represents one individual and the lines mark the median values.

These findings were further supported by the results from patients with ACA, the chronic Lyme borreliosis skin manifestation. They displayed a higher *Borrelia*-specific IFN- γ response, in blood, than the control group but no difference was seen in the IL-4 secretion. Patients with the benign skin manifestation EM had the same cytokine pattern as the subacute

neuroborreliosis patients; an initial IFN- γ response with a later switch to IL-4 secretion.

The conclusion drawn from these results is that an initial Th1-type response with a later switch to a Th2-type response is compatible with a subacute prognosis of Lyme borreliosis. On the other hand, a slowly increasing Th1-response and lack of Th2 might correspond to a chronic or prolonged disease course. This is in line with the animal study done by Kang et al (Kang et al. 1997). Also, in a later study we have found that children with neuroborreliosis display a Th1-response in CSF compared to controls. Furthermore the children displayed a higher Th2-response compared to adults with the same diagnosis (Widhe et al. 2005). A chronic disease course is seldom seen in children (Berglund et al. 2002) therefore these findings further support the results in paper I.

A strong Th1-response will probably both aid in the eradication of the spirochete and also induce a Th2-switch. Since all neuroborreliosis patients in our study received antibiotic therapy and there is no evidence that *B. burgdorferi s. l.* is resistant to antibiotics (Hammers-Stiernstedt 1998), the assumption can be made that the infection is cleared in an adequate manner. Therefore, the Th1-response might not be needed for the clearance of the spirochete in the patients but is of more importance for the immunological switch.

Two different types of DC have been described; DC1 and DC2. DC1 induce naive Th-cells to differentiate into Th1-cells and DC2 induce Th2-type cells (Rissoan et al. 1999). A negative feedback loop controls these DCs. IL-4 can kill DC2 whereas IFN- γ can protect DC2 from apoptosis. Thus, the Th2-response can down-regulate itself and the Th1-immunity can up-regulate a Th2-type response. Furthermore, IFN- γ stimulated DCs induced NKT-cells that secreted IL-4 (Minami et al. 2005). If a slow Th1-response is initiated at the beginning of the infection, before therapy is started, it might not set the regulatory wheels in motion which is required for a down regulation. The consequence could be an ongoing Th1-response which in time will damage the tissue and cause the symptoms seen in chronic patients. Th1-responses have been shown to induce injury in different types of diseases, such as gastric ulcers (Mohammadi et al. 1996), celiac disease (Wapenaar et al. 2004) and mycobacterial infection in mice (Zganiacz et al. 2004).

The reason for the slow Th1-response seen in some individuals infected with *B. burgdorferi s. l.* is unknown. Genetic background, such as HLA haplotype, has been coupled to chronic Lyme arthritis (Steere et al. 1990) but not to chronic neuroborreliosis. The primary cytokine milieu is vital for shaping the immune response thus there might be an inaccuracy in this early stage which is later reflected in the adaptive response. Although the development of chronic borreliosis does not necessarily mean that this particular patient has a general defect in the immune system. Patients with the

sever type of leprosy do not mount a Th1-response to the causative pathogen of leprosy but they respond appropriately to other microbial agents (Kim et al. 2001).

Studies have shown that chronic patients do not benefit from antibiotic treatment, further supporting the assumption that these patients do not have an ongoing infection (Klempner 2002). However, certain antibiotics might be able to modulate the immune response. Benzylpenicillin binds to IFN- γ and inhibits the cytokines activity (Brooks et al. 2001). Benzylpenicillin can also conjugate to IL-4 and IL-13 but does not inhibit the effect of these cytokines (Brooks et al. 2003). The effect seen in the odd chronic patients that do improve during antibiotic therapy might be due to down regulation of the immune response, not the eradication of an infection.

Memory response

In paper III and IV the memory response to *Borrelia* antigen was further investigated. The asymptomatic individuals (paper IV) and the individuals with previous EM (paper III) showed a Th1-response to *Borrelia* stimulation, in comparison to controls (Figure 10), which is in contrast to the Th2-like memory response seen in EM-patients in paper I. However, the material in paper I was small and this might have contributed to an incorrect result.

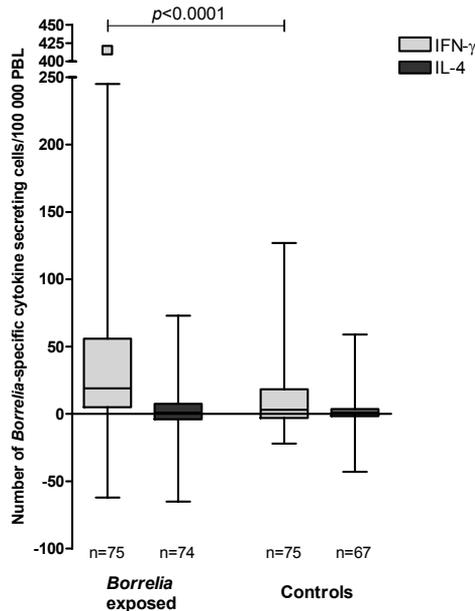


Figure 10. Number of *Borrelia*-specific IFN- γ and IL-4 secreting cells/100 000 peripheral blood lymphocytes (PBL) from individuals with a previous *Borrelia* exposure (patients with erythema migrans [paper III] and seropositive asymptomatic individuals [paper IV]) and controls with no signs of *Borrelia* exposure. P-values show statistical significant differences from comparison with Mann-Whitney U-test. The median (line), interquartile range (box) and maximum-minimum (whiskers) are marked.

Furthermore, when the memory response was analyzed in all patients with neuroborreliosis (the material from paper I and IV combined) the subacute patients showed a *Borrelia*-specific IFN- γ response compared to controls ($p < 0.01$) but no increase in IL-4 (Figure 11a and b). Whereas the chronic neuroborreliosis patients displayed a memory response of both Th1- ($p < 0.05$) and Th2-type ($p < 0.05$), compared to controls (Figure 11a and b).

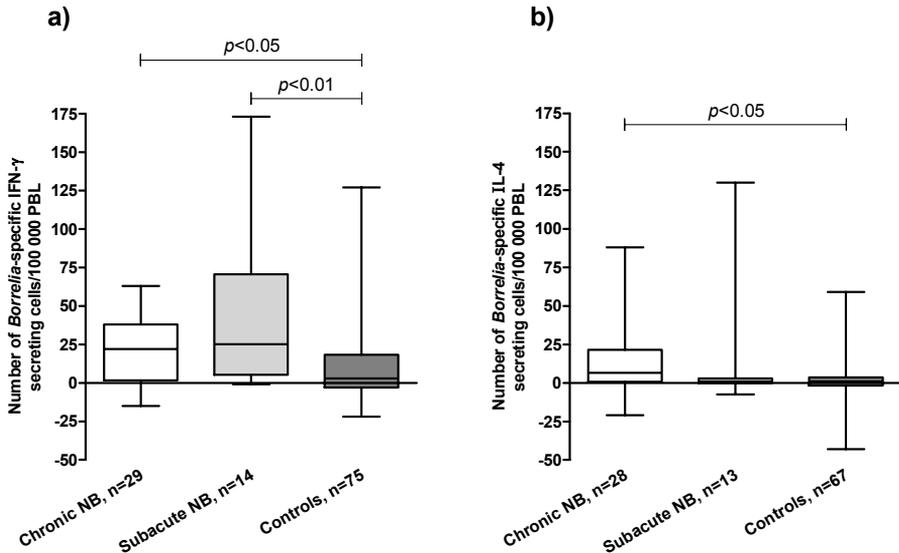


Figure 11. Number of *Borrelia*-specific IFN- γ (a) and IL-4 (b) secreting cells/100 000 peripheral blood lymphocytes (PBL) from patients with chronic or subacute neuroborreliosis (NB) and controls with no signs of *Borrelia* exposure. P-values show statistical significant differences from comparison with Dunn's test. The median (line), interquartile range (box) and maximum-minimum (whiskers) are marked.

The memory response is believed to be a reflection of the initial response (Sallusto et al. 2004). If this is true, then asymptomatic individuals, patients with EM and patients with subacute neuroborreliosis responded with a Th1-type and chronic neuroborreliosis patients responded with both a Th1- and Th2-type. There is no information of the actual initial cytokine response in asymptomatic individuals, since the time of infection is not known, this group has to be left behind in this discussion. For the results in paper I it seems as chronic, subacute and EM patients displayed an IFN- γ secretion in the early stage of the disease. The chronic patients did however not show a Th2-response at this point. Then why is this seen in the memory response? The initial immune response was investigated in the target organ, CSF, but the memory response was seen systemically in blood. There might be a discrepancy in the immunological response in the different sites.

Further analysis of Th1-immunity

Working from the hypothesis that an insufficient initial Th1-response might lead to a chronic course of Lyme borreliosis and also that the memory response mirrors the early immune reaction, the *Borrelia*-specific Th1-

immunity was further investigated in paper IV. Blood lymphocytes from *Borrelia* seropositive asymptomatic individuals, patients with chronic and subacute Lyme borreliosis and healthy controls were stimulated with *Borrelia* antigen, the mitogen PHA and a reference antigen, PPD. The expression of the Th1-cell marker IL-12R β 2 was analyzed on a protein and mRNA level. The cytokine secretion was also evaluated by ELISPOT and ELISA.

There was no significant difference between the subacute and the chronic Lyme borreliosis patients for any of the variables investigated. However, the chronic patients displayed lower expression of *Borrelia*-induced IL-12R β 2 on CD8 $^{+}$ cells (Figure 12) and a lower number of *Borrelia*-specific IFN- γ

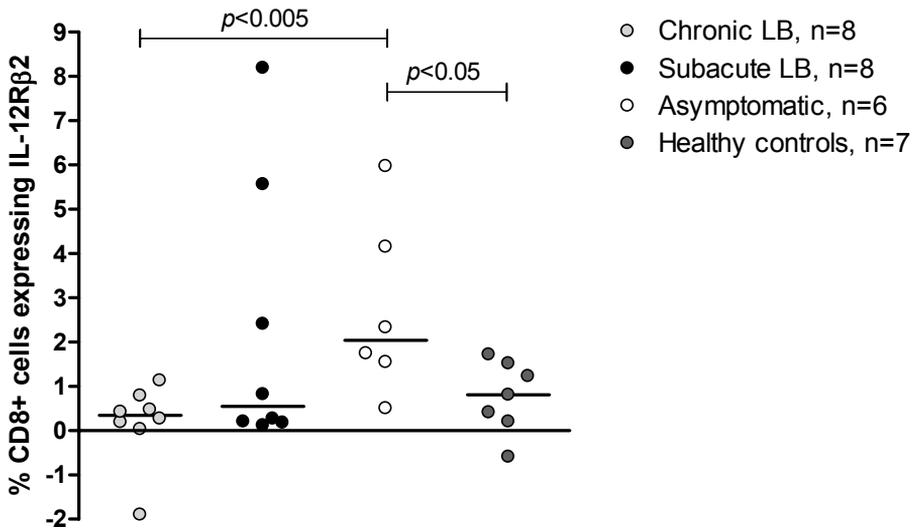


Figure 12. Percentage of CD8 $^{+}$ cells expressing IL-12R β 2 in response to *Borrelia* stimulation detected by flow cytometry. P-values show statistical significant differences from comparison with Mann-Whitney U-test. Each point represents one individual and the lines mark the median values. LB, Lyme borreliosis.

secreting cells (Figure 13) compared with asymptomatic individuals. On the mRNA level the difference in IL-12R β 2 expression was not seen. mRNA was detected for the whole lymphocyte population and not divided into specific phenotypes which might explain the discrepancy in the results. There was no difference between the four diagnostic groups in the number of cells, for any of the phenotypes investigated.

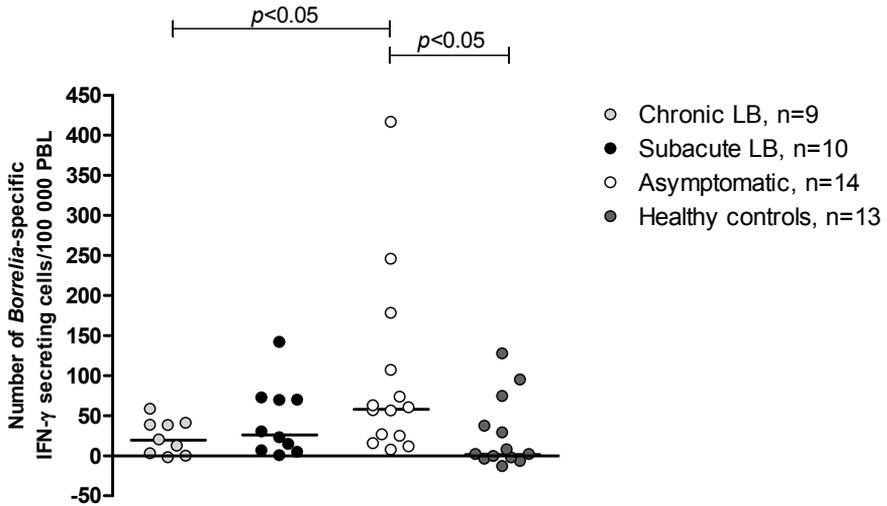


Figure 13. Number of *Borrelia*-specific IFN- γ secreting cells/100 000 peripheral blood lymphocytes (PBL). P-values show statistical significant differences from comparison with Mann-Whitney U-test. Each point represents one individual and the lines mark the median values. LB, Lyme borreliosis.

Beermann et al showed that DCs loaded with *Borrelia* antigen induce maturation of CD8+ Tc-cells (Beermann et al. 2000b) and CD8+ Tc-cells have later been found to be the main producers of *Borrelia*-specific IFN- γ (Ekerfelt et al. 2003). The finding in paper IV that CD8+ cells from chronic patients have a lower expression of IL-12R β 2 is therefore interesting. IL-12 is important in inducing naïve T-cells to mature into Th1-cells and for the maximum IFN- γ production (Manetti et al. 1994). The β 2-chain increases the affinity of cytokine binding and is the signaling component of the receptor (Rogge et al. 1997). The chronic patients did respond to *Borrelia* stimulation with an IL-12 response but since the CD8+ cells showed a low expression of IL-12R β 2 there might not be a response to the IL-12 stimulation. Thus, these cells might not have an optimal IFN- γ secretion which was confirmed by the low numbers of *Borrelia*-specific IFN- γ secreting cells.

The results found after *Borrelia* stimulation was not seen when cells were stimulated with PPD. However, due to limited number of cells PPD stimulation was not performed on samples from all patients. The expression of IL-12R β 2 was not analyzed on PPD stimulated cells.

The findings in paper IV further supports the results from paper I that a Th1-response might be of importance for the outcome of Lyme borreliosis. Individuals who develop chronic Lyme borreliosis possibly have an aberrant Th1-type response to the *Borrelia* infection. Still, what causes this immune response in some individuals and not in others is yet unknown.

Regulatory T-cells

A weak or slow pro-inflammatory immune response might be caused by an over compensating suppression. In paper IV the expression of the Treg marker Foxp3 was investigated and compared between groups of Lyme borreliosis patients with different clinical outcome. Chronic patients showed a higher expression of *Borrelia*-specific Foxp3 than healthy controls. However, this significance was not strong ($p=0.05$) and there was no difference between chronic, subacute and asymptomatic individuals in the *Borrelia*-specific Foxp3 expression. Also, the result was not supported by other findings such as difference in the secretion of the Treg cytokine IL-10. The detection of cytokine production from Treg should be done with regard to phenotype, i.e. CD4+CD25+ cells should be selected and analyzed. Treg secrete IL-10 and TGF- β but they are not the only cells producing these cytokines. Therefore, if the correlation between Foxp3 and cytokine secretion is to be correct consideration should be made to the origin of the cytokine.

The expression of Foxp3 is not, according to Hori et al, up-regulated by stimulation; Foxp3 expression is stable in CD4+CD25+ cells. In paper IV lymphocytes were stimulated with *Borrelia* antigen and the mitogen PHA. If the Foxp3 expression is stable then these stimulations would not have an effect on the transcription factor. Nevertheless, the Foxp3 expression was significantly higher in cells stimulated with PHA than *Borrelia* antigen ($p<0.0001$, Figure 14). Since PHA is a mitogen one possible explanation

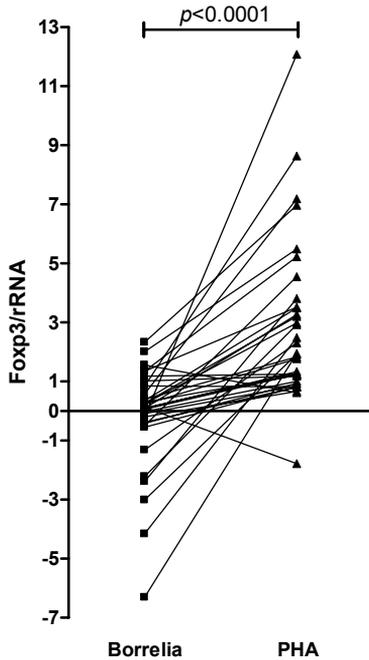


Figure 14. Expression of *Borrelia*-specific and PHA induced Fosp3 mRNA/rRNA in peripheral blood lymphocytes from patients with Lyme borreliosis and asymptomatic individuals (n=35), quantified using real-time polymerase chain reaction. The p-value show statistical significant difference from comparison with Wilcoxon signed rank test.

could be that there are more Treg in this culture. However, Treg proliferate poorly *in vitro* (McGuirk et al. 2002) and furthermore the expression of Fosp3 in paper IV was normalized by the division of rRNA. Stimulation of the TCR on CD4+CD25- cells have been found to induce both Fosp3 and CD25 expression and these cells also display Treg suppressor functions (Walker et al. 2003). This induction of Treg cells might indicate that Treg can be generated from memory cells.

Although, the results regarding Treg in paper IV are weak, it is an interesting angle of the pathogenesis of Lyme borreliosis and should be further investigated. A study done on mice showed that CD4+CD25+ T-cells were able to control the inflammation in the joints following a *B. burgdorferi* s. l. challenge thereby preventing Lyme arthritis (Nardelli et al. 2005). However, the Treg had to be derived from previously *Borrelia* infected mice to have a suppressive effect. This shows that antigen recognition might be required for Treg suppression and also that there can be Treg with an immunological memory.

Gender and its influence on the immune response

A study done by Bennet et al found that postmenopausal women had an increased risk of being reinfected by *B. burgdorferi s. l.* (Bennet et al. 2002). In paper III reinfected and single infected individuals were investigated with respect to immune responses to various antigens and to confounding health factors such as autoimmune diseases. Since women were overrepresented in the reinfected group (Table 4) comparisons were also made, using the same variables, with regard to gender.

Table 4. Patients included in paper III, divided into gender and number of *B. burgdorferi s. l.* infections

	Women		Men	
	n	age mean (range)	n	age mean (range)
Reinfected	21	68 (51-81)	3	58 (43-84)
Single infected	20	66 (53-85)	18	62 (43-78)
Total	41	67 (51-85)	21	62 (43-84)

No differences were found between reinfected and single infected individuals. Nor were there a difference between reinfected women and single infected women. Since the reinfected men were few, no comparison was made between this group and the single infected male group. The individuals in paper III were all tick bitten to the same extent. The reinfected group might just have had the misfortune of gotten bitten by *Borrelia* infested ticks. But why should more women than men be unlucky in this sense? When comparing the cytokine secretion in men with that seen in women, regardless of times infected with *B. burgdorferi s. l.*, women displayed a higher spontaneous secretion of all cytokines measured (IL-4, IL-6, IL-10, IFN- γ and TNF- α). They also seemed to have a more Th2-type immune response (Figure 15a) and a bias for an anti-inflammatory response (Figure 15b). The adaptive and memory response, though under the influence of the innate immunity, is also controlled by the cytokine milieu at the site of antigen presentation. The Th2-like environment displayed by the women might have impact on the type of adaptive immune response mounted against a pathogen. Biedermann et al have shown that if IL-4 is present when a T-cell is activated, the T-cell will develop into a Th2-cell (Biedermann et al. 2001).

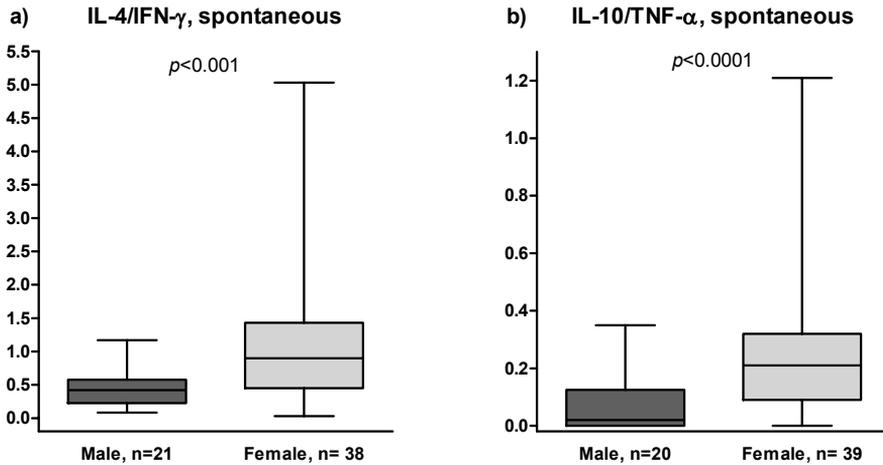


Figure 15. Ratio of spontaneous cytokine secretion from peripheral blood lymphocytes (PBL). a) Number of cells/100 000 PBL detected by ELISPOT. b) Amount (pg/ml) secreted from PBL detected by ELISA (IL-10) or Immulite (TNF- α). P-values show statistical significant difference from comparisons with Mann-Whitney U-test. The median (line), interquartile range (box) and maximum-minimum (whiskers) are marked.

In paper III, the innate immune response was also investigated by analyzing IL-6, IL-10 and TNF- α . Since these cytokines are primarily produced by APCs, which do not display antigen specific memory, it might be more correct to consider the accumulative secretion rather than the specific response. Men and women did not differ in the amount of innate cytokine secretion after stimulation with peptidoglycan or *Borrelia* antigen (Figure 16). The two groups seem to have the same initial response to these antigens.

Neither was there any difference between men and women for the antigen specific or mitogen induced memory responses (IFN- γ and IL-4), with one exception. Men displayed a higher number of PHA-induced IFN- γ secreting cells ($p < 0.01$) compared to women. PHA is regarded as giving a Th1-type derived response therefore the men might be considered to have a stronger Th1-immune response than women. This would be beneficial when infected by *B. burgdorferi s. l.*

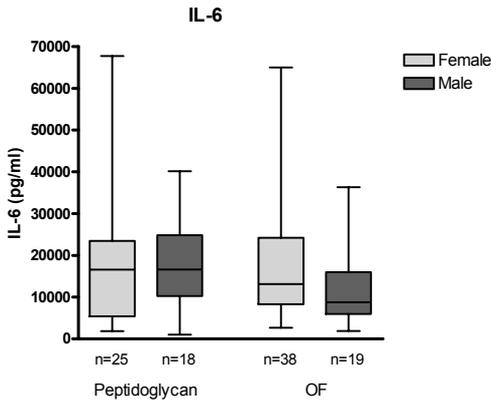
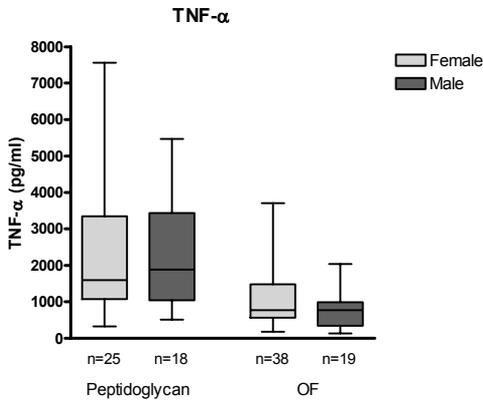
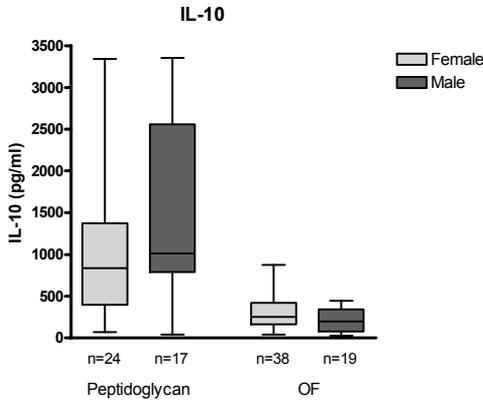


Figure 16. Amount (pg/ml) of IL-6, IL-10 and TNF- α secreted from peripheral blood lymphocytes after stimulation with peptidoglycan or *Borrelia* antigen (OF).



The first time the immune system comes into contact with *B. burgdorferi s. l.* it can result in the correct type of memory, which is a Th1-type according to previous discussed results in the thesis. This could be protective if a second infection with *B. burgdorferi s. l.* occurs. If, on the other hand, the memory is of the less correct kind, Th2-type, the second infection might not be fought as effectively. However, this time the end result may be a Th1-type memory (Sallusto et al. 2004). Men and women in paper III did not differ in their memory response to *Borrelia* antigen. This could possibly indicate that if challenged by a *B. burgdorferi s. l.* infection again the individuals might respond with the correct type and effectively eradicate the spirochete. Women, with their initial Th2-bias, can have an increased risk of developing the wrong memory response and thereby also an increased risk of reinfections.

A previous infection can render the patient immune to an infection with the same pathogen. This does not seem to be the case for Lyme borreliosis. One explanation for reinfections with *B. burgdorferi s. l.* could be that there are various species and they do not elicit cross-immunity. This has been shown in a mouse model where animals originally infected with *B. burgdorferi s. s.*, *B. garinii* or *B. afzelii* showed resistance to the same species but were infected by a heterologous species (Barthold 1999). However, reinfections occur in the USA (Nowakowski et al. 1997, Nowakowski et al. 2003, Smith et al. 2002) where only *B. burgdorferi s. s.* has been found. Therefore, even different strains of *B. burgdorferi s. s.* might give rise to a reinfection (Golde et al. 1998).

To assess the Th1/Th2 balance a ratio of IFN- γ and IL-4 is calculated. These two cytokines are classical Th1- and Th2-type markers. In paper III, the ratio of IL-10 and TNF- α was used to investigate the pro- and anti-inflammatory response. IL-10 is regarded as an anti-inflammatory cytokine since it inhibits the expression of co-stimulatory molecules on macrophages thereby reducing their ability to present antigen and activate T-cells (Ding et al. 1993). TNF- α has been seen as pro-inflammatory but can also act regulatory. Activated macrophages are more susceptible to inhibition by TNF- α than naive cells which indicates that the regulation has a role in the late stage of an infection (Zakharova et al. 2005). Mice lacking TNF- α develop lethal tissue damage on the lungs if infected by mycobacteria due to a high Th1-response (Zganiacz et al. 2004). The Th1-response was controlled and thereby the tissue damage was limited if the TNF- α -deficiency was reversed. Although TNF- α might have dual properties, in paper III the spontaneous secretion was assessed when TNF- α can be regarded as a proinflammatory mediator.

Previous exposure to *Anaplasma phagocytophilum*

Ticks can be carriers of more than one pathogen at a time which can lead to co-infections (Bjöersdorff et al. 2002). In paper II, patients with EM were also investigated for a co-infection with *A. phagocytophilum*. None of the patients showed any signs of an ongoing HGA but antibodies demonstrating a previous exposure to *A. phagocytophilum* were found in eight patients, termed *Ap* seropositive. When blood cells from *Ap* seropositive, *Ap* seronegative and healthy controls were stimulated with *Borrelia* antigen the *Ap* seropositive group and the control group showed a lack of an IL-12 response (Figure 17a). The IFN- γ secretion did not differ between the *Ap* seropositive and seronegative group, though the *Ap* seronegative group showed a higher number of cytokine secreting cells compared to controls (Figure 17b). There were no differences between the three groups regarding the Th2-cytokines investigated, i.e. IL-4, IL-5 or IL-13.

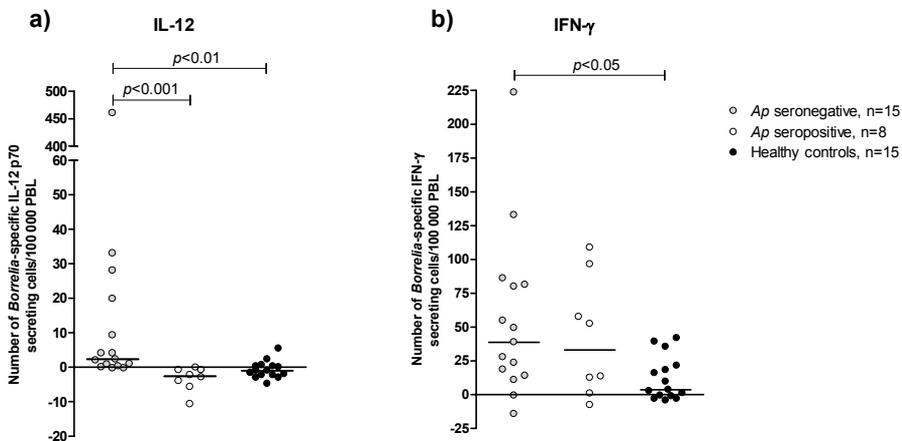


Figure 17. Number of *Borrelia*-specific IL-12 p70 (a) and IFN- γ (b) secreting cells/100 000 peripheral blood lymphocytes (PBL) from erythema migrans patients seropositive or seronegative for *Anaplasma phagocytophilum* (*Ap*) and healthy controls. P-values show statistical significant differences from comparison with Dunn's test. The lines mark the median values.

As mentioned previously, IL-12 has a central roll in inducing Th1-responses. The inability to produce or respond to IL-12 has been connected to the inability to mount a strong Th1-response (Alexander et al. 2005). Although the group of *Ap* seropositive patients did not show a significant increase of the Th1-cytokine IFN- γ in response to *Borrelia* compared to the controls, some of these patients seemed to respond with IFN- γ despite the lack of IL-12. Macrophages, which are the main producers of IL-12, are activated not only by antigens or pathogens, but also by IFN- γ and will then produce more IL-12 (Flesch et al. 1995, Losana et al. 2002, Yun et al. 2002).

Hypothetically, since the cells from *Ap* seropositive patients secrete IFN- γ when stimulated with *Borrelia* antigen but do not secrete IL-12 the inhibition might be due to an inability in macrophages to respond to IFN- γ stimulation. This is supported by the observation that the control group, in line with a lack of memory T-cell response, responded with a generally very low or absent IFN- γ secretion when stimulated with *Borrelia* antigen and a consequently low or absent secretion of IL-12. However, this has to be further investigated.

The *Ap* seropositive patients did not have signs of an ongoing *A. phagocytophilum* infection, yet a previous infection may have a long term effect on the immune response. Larsen et al showed in a study on sheep that serum from previously *A. phagocytophilum* infected animals strongly suppressed the stimulatory effect of mitogens on lymphocytes from healthy animals (Larsen et al. 1994). Microbial circulating proteins can bind to cell receptors and inhibit the cells cytokine production (Coccia et al. 2005, Eisen-Vandervelde et al. 2004, Tang et al. 2004). If this also occurs in HGA has not been established but the findings from the study by Larsen and colleagues is an indication in this direction and should be studied further.

The patients in paper I, III and IV were not screened for antibodies to *A. phagocytophilum*. This should be done on samples taken in the initial stage of disease. If the serology is performed on convalescent samples it is possible that the patient has been infected by *A. phagocytophilum* after the *Borrelia* infection. Unfortunately, early samples were only available from a few of the patients in paper I, III and IV.

Autoimmune or chronic disease such as atopy or rheumatoid arthritis (RA) can influence the immune response and might have an effect on microbial infections. Patients with RA, which is considered a Th1-derived autoimmune disease, who were also atopic (manifested as hay fever) had milder joint symptoms and less IFN- γ secretion than RA-patients without hay fever (Verhoef et al. 1998). The Th2-type response of the hay fever and the Th1-type response of RA possibly controlled each other. Would the atopic Th2-derived response be a disadvantage in Lyme borreliosis? The frequency of atopy was investigated in paper IV and in paper III occurrences of different confounding health factors, such as chronic and autoimmune diseases, was analyzed. In none of the papers a difference was seen between the different groups, which were classified according to outcome in paper IV and number of infections in paper III. Whether this is a true picture or just an error from a small sample size needs to be further explored.

The specificity of the *Borrelia* antigen

The *Borrelia* antigen, OF, was prepared from *B. garinii* strain Ip90. Since neither the *Borrelia* species nor the strain infecting the patients were known in any of the four papers of this thesis, one could question the limitation of the antigen used. Also, reinfections can possibly occur due to lack of immunological memory to different strains. However, OF has previously been shown to discriminate between individuals who have come in contact with the spirochete and those who have not (Forsberg et al. 1995). This was not the case in papers I and IV. Patients with borreliosis in papers I and IV did not show a higher number of blood cells secreting IFN- γ in response to *Borrelia* stimulation than the healthy control group. However, when looking at the memory response in all patients (patients with on ongoing infection was not included, i.e. patients in paper II and patients with ACA in paper I) and controls included in this thesis, a significant difference was seen for IFN- γ ($p < 0.001$) (Figure 18) but not for IL-4 ($p = 0.065$).

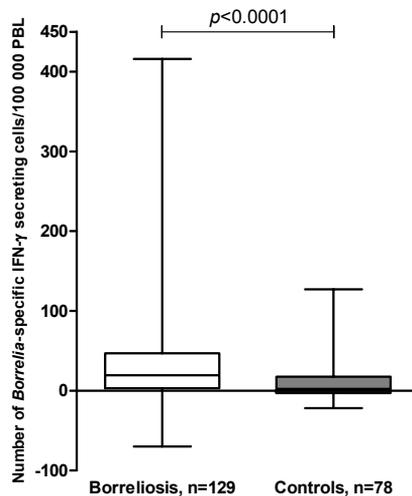


Figure 18. Number of *Borrelia*-specific IFN- γ secreting cells/100 000 peripheral blood lymphocytes (PBL) in patients with a history of Lyme borreliosis but without an ongoing infection and healthy controls. The p-value show statistical significant difference from comparisons with Mann-Whitney U-test. The median (line), interquartile range (box) and maximum-minimum (whiskers) are marked.

Stimulation of blood cells with *Borrelia* antigen elicited an IL-10 response but not a specific one. Healthy controls showed a higher number of *Borrelia*-specific IL-10 secretion than patients exposed to *B. burgdorferi s. l.* (Figure 19a). IL-10 is mainly produced by APCs and was therefore expected to give an nonspecific response to *Borrelia* stimulation (Giambartolomei et al. 1998).

The control group possibly has a stronger response to lipid antigen in general. Unfortunately, observations from stimulation with the reference antigen PPD are too few therefore no conclusions can be made from these data.

IL-12 is also derived from APCs and a specific response was not expected for this cytokine either. However, *B. burgdorferi s. l.* exposed patients did display a higher number of *Borrelia*-specific IL-12 secreting cells than controls (Figure 19b). As discussed previously, IL-12 production seems to be dependent on IFN- γ and the lack of specific IFN- γ response to *Borrelia* antigen, seen in controls (Figure 18), may therefore explain the lack of an IL-12 response.

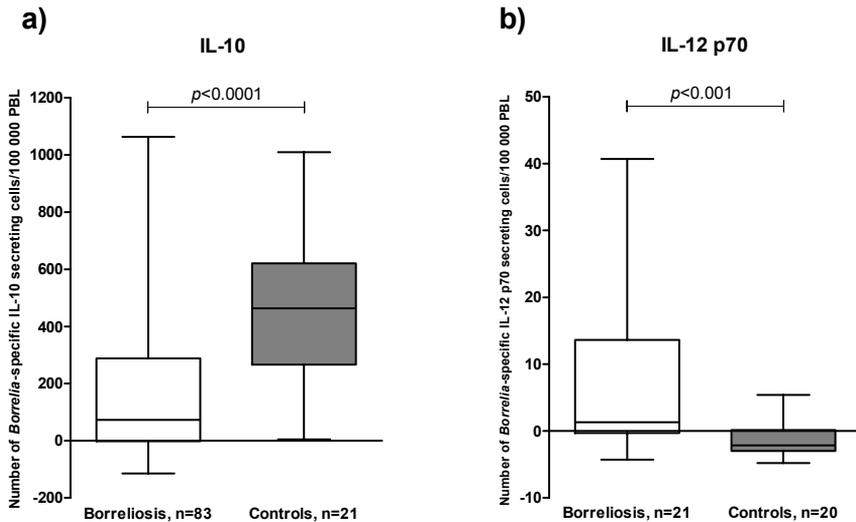


Figure 19. Number of *Borrelia*-specific IL-10 (a) and IL-12 p70 (b) secreting cells/100 000 peripheral blood lymphocytes (PBL) in patients with a history of Lyme borreliosis but without an ongoing infection and healthy controls. P-values show statistical significant difference from comparisons with Mann-Whitney U-test. The median (line), interquartile range (box) and maximum-minimum (whiskers) are marked.

FURTHER STUDIES

It would be interesting to repeat the study described in paper I but with a larger number of patients and also to follow the same patient through the whole clinical course of the disease. The use of a reference antigen, for example a bacterial lipoprotein, could give valuable information on whether the response to the *Borrelia* antigen is exclusively seen for this antigen or is a general response to lipoproteins. The Treg population and its development during time in these patients would also be interesting to investigate. If the Treg cytokine secretion was to be analyzed these cells should be selected for and studied separate.

The immune response in individuals previously exposed to *A. phagocytophilum* needs further investigation. A larger material is essential and a clinical follow up should also be done. Cells could be stimulated with antigen from *A. phagocytophilum* and if a T-cells response is seen this would support the serological findings of a previous infection. The experiment done by Larsen et al (Larsen et al. 1994) where serum from previous exposed sheep was incubated with cells from non-exposed sheep could be applied to human samples. A reference antigen should also be used here to see if the IL-12 suppression is general or specific for Lyme borreliosis.

To investigate the hypothesis that chronic patients have a slow and eventually lingering Th1-response, other markers should be looked at, apart for the classical IFN- γ . IL-23 and IL-18 are interesting candidates.

Since ELISA can only measure one cytokine at a time it would be preferable to use the Luminex method instead. In Luminex antibody-coated fluorescent beads are used. The beads have different intensity and each type of bead is coated with one specific antibody. Therefore, with Luminex many substances can be measured in the same sample at one occasion.

SUMMARY AND CONCLUSION

It seems that an initial Th1-type response with a later switch to a Th2-response is compatible with a subacute prognosis of Lyme borreliosis. On the other hand, a slowly increasing Th1-response and lack of Th2 might correspond to a chronic or prolonged disease course.

The reason behind why some individuals show an inadequate response to *Borrelia* is probably not one single factor but is likely multi factorial. The type of infectious species plays a part due to differences in invasive ability. The immunological status of the individual at the time of infection might also affect the specific response to the *Borrelia* spirochete and thereby also the outcome of the disease. Women, after going through menopause, seem to have an immune response that is not beneficial for combating a *Borrelia* infection. Furthermore, a previous infection by a different pathogen can possibly alter the immune response.

Even though a weak Th1-response appears to be involved in the cause of chronic Lyme borreliosis it would probably be to oversimplify the underlying mechanism. The focus should not be solely on the Th1-Th2 balance since many other factors also have to be considered in the complicated network of immunological responses. The involvement of regulation and the possible contribution of Treg to the pathogenesis of Lyme borreliosis have only just begun to be investigated.

The finding in paper III, that there might be a gender difference in the immune response, illustrates the importance of careful consideration when selecting the patient material for an immunological experimental study. The different groups to be compared should be both age and gender matched to limit the variations in cytokine secretion between men and women. It is never possible to eliminate all confounding factors when working with a human material but the ones within our control should be limited.

The results and findings in this thesis do not solve the mystery behind chronic Lyme borreliosis but might be a piece of the giant jigsaw puzzle that is Lyme borreliosis.

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