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IMMUNE EVASION BY *BORRELIA BURGDORFERI*

– WITH SPECIAL REFERENCE TO CD38-MEDIATED CHEMOTAXIS OF NEUTROPHILS AND DENDRITIC CELLS

by

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ABSTRACT

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Immune Evasion by Borrelia burgdorferi – With Special Reference to CD38-mediated Chemotaxis of Neutrophils and Dendritic Cells

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Lyme borreliosis is a tick-transmitted infection caused by the spirochete bacterium *Borrelia burgdorferi* sensu lato. The tick injects bacteria into host skin, where a first line defence, mainly the complement system, neutrophils, dendritic cells and macrophages are ready to attack foreign intruders. However, in the case of Lyme borreliosis, the original immune response in the skin is untypically mild among bacterial infections. A further untypical feature is the ability of *B. burgdorferi* to disseminate to distant organs, where, in some patients, symptoms appear after years after the original infection. This study aimed at uncovering some of the immune evasion mechanisms utilized by *B. burgdorferi* against the complement system, neutrophils and dendritic cells.

B. burgdorferi was shown to inhibit chemotaxis of human neutrophils towards n-formyl-methyl-leucyl-phenylalanine (fMLP). Outer surface protein B (OspB) of *B. burgdorferi* was shown to promote resistance to the attack of the complement system and neutrophil phagocytosis at low complement concentrations.

B. burgdorferi was shown to inhibit migration of dendritic cells in vitro towards CCL19 and CCL21 and also in an in vivo model. This effect was shown to be due to the absence of CD38 on the borrelia-stimulated dendritic cell surface. A defect in p38 mitogen-activated-protein-kinase (p38) signaling was linked to defective CD38 expression. A defect in CD38 expression on *B. burgdorferi*-stimulated neutrophils was also observed.

In this study, a number of novel immune evasion strategies utilized by *B. burgdorferi* were chracterized. However, further studies are needed as other immune evasion mechanisms await to be uncovered.

Keywords: B. burgdorferi, complement, neutrophils, dendritic cells, immune evasion

TIIVISTELMÄ

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Borrelia ja immuuniväistö – erityistarkastelussa neutrofiilien ja dendriittisolujen CD38-välitteinen kemotaksis

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Lymen borrelioosi on punkkien (puutiaisten) levittämä tauti, jonka aiheuttaa *Borrelia burgdorferi* sensu lato bakteeri. Suomessa noin 3000-5000 ihmistä sairastuu tähän tautiin vuosittain. Infektiokohdan ihossa luonnollisen puolustusjärjestelmän komponentit, mm. komplementtijärjestelmä, neutrofiiliset valkosolut, dendriittisolut sekä makrofagit, kohtaavat ensimmäisenä ihoon tunkeutuneet taudinaiheuttajat. Tärkeitä luonnollisen puolustusjärjestelmän puolustuskeinoja ovat solujen hakeutuminen (kemotaksis) infektiopaikalle, komplementti-järjestelmän aktivoituminen ja taudinaiheuttajien tappaminen solusyönnin (fagosytoosi) ja komplementin avulla. Dendriittisolut fagosytoivat taudinaiheuttajia ja kulkeutuvat läheisiin imusolmukkeisiin (migraatio), jossa ne esittelevät taudinaiheuttajat lymfosyyteille ja stimuloivat hankinnaisen immuunivasteen käynnistymisen. Lymen borrelioosissa ensivaiheen puolustusreaktiot ovat poikkeuksellisen heikkoja, mistä kertoo taudin ensioireen, erythema migrans–ihottuman, vaimeus verrattuna muiden bakteerien aiheuttamiin ihotulehduksiin. Tauti voi rajoittua ihoon ja rauhoittua jopa ilman hoitoa, mutta toistaiseksi tuntemattomasta syystä osalla potilaista bakteeri väistää immuunipuolustuksen hyökkäyksen ja leviää useisiin eri elimiin aiheuttaen mitä moninaisimpia oireita viikkoja tai kuukausia ensi-infektion jälkeen. Tämä tutkimus tähtäsi borrelian käyttämien immuuniväistökeinojen selvittämiseen komplementtijärjestelmän, neutrofiilien ja dendriittisolujen osalta.

Tutkimuksessa havaittiin, että *B. burgdorferi* pystyy estämään neutrofiilisten valkosolujen kemotaksiksen n-formyl-methyl-leucyl-phenylalanine (fMLP) nimistä molekyyliä kohtaan. fMLP on yksi voimakkaimmista neutrofiilien kemotaksista aiheuttavista aineista. Lisäksi *B. burgdorferin* pintaproteiini OspB:n todettiin estävän neutrofiilien fagosytoosia sekä komplementtijärjestelmän aktivaatiota ja tappavaa vaikutusta matalassa komplementti-pitoisuudessa. Ihmiselimistössä matala komplementtipitoisuus on mm. nivelissä ja keskus-hermostossa.

Tutkimuksessa havaittiin myös, että *B. burgdorferi* ei stimuloi CD38 molekyylin ilmaantumista dendriittisolujen pinnalle. Tämä johtaa puutteelliseen migraation CCL19 ja CCL21 kemokiineja kohtaan in vitro. Dendriittisolujen migraatiota tutkittiin myös hiirikokeessa, jossa Suomessa esiintyvä borrelialaji *B. garinii* sai aikaan puolet pienemmän dendriittisolujen migraation imusolmukkeisiin kuin *Escherichia coli* bakteeri. Puutteellisen CD38-ekspression osoitettiin johtuvan alentuneesta signalointiaktiivisuudesta p38 mitogen-activated-protein-kinase (p38) –signalointireitissä. Puutteellinen CD38-ekspressio todettiin myös neutrofiileissä *B. burgdorferi*- ja *B. garinii*-stimulaation jälkeen.

Tutkimuksessa onnistuttiin selvittämään useita *B. burgdorferi*-bakteerin käyttämiä immuuniväistökeinoja. Jatkotutkimuksia vielä selvittämättömien immuuniväistökeinojen löytämiseksi tarvitaan yhä, koska taudin patogeneesin ymmärtäminen on välttämätöntä hoitojen oikean kohdentamisen kannalta.

Avainsanat: *B. burgdorferi*, komplementti, neutrofiilit, dendriittisolut, immuuniväistö

CONTENTS

ABBREVIATIONS

ACA	acrodermatitis chronica atrophicans
ADPR	adenosine diphosphate ribose
APC	antigen presenting cell
Ba	*Borrelia afzelii*
Bb	*Borrelia burgdorferi* sensu lato
Bb s.s.	*Borrelia burgdorferi* sensu stricto
Bg	*Borrelia garinii*
cADPR	cyclic adenosine diphosphate ribose
CD	cluster of differentiation
CCL	CC chemokine ligand
CFSE	carboxyfluorescein diacetate succinimidyl ester
CCR	CC chemokine receptor
DC	dendritic cell/cells
EM	erythema migrans
ERK	extracellular signal-regulated kinase
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
fMLP	formyl-methionyl-leucyl-phenylalanine
FPR	formyl-peptide receptor
GM-CSF	granulocyte-macrophage colony stimulating factor
HLA	human leukocyte antigen
Ig	immunoglobulin
IL	interleukin
JNK	c-jun N-terminal kinase
LB	Lyme borreliosis
LC	Langerhans cell
LPS	lipopolysaccharide
LTB4	leukotriene B4
MAC	membrane attack complex
MAPK	mitogen activated protein kinase
MBL	mannose binding lectin
MHC	major histocombatibility complex
NAADP	nicotinic acid adenine dinucleotide phosphate
NAD+	nicotinamide adenine dinucleotide
NADP+	nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NHS	normal human serum

Osp	outer surface protein
PBS	phosphate buffered saline
PBMC	peripheral blood derived mononuclear cell
PCR	polymerase chain reaction
PE	phycoerythrin
PMN	polymorphonuclear leukocyte
RT-PCR	reverse-transcriptase polymerase chain reaction
STAT1	signal transducer and activator of transcription 1
TLR	toll-like receptor
TNF	tumor necrosis factor

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following articles. Additional data on neutrophil CD38 expression has been added.

I Hartiala P., Hytönen J., Pelkonen J., Kimppa K., West A., Penttinen MA., Suhonen J., Lahesmaa R., and Viljanen MK.: Transcriptional Response of Human Dendritic Cells to *Borrelia garinii* - Defective CD38 and CCR7 Expression Detected, *Journal of Leukocyte Biology* 2007 82(1):33-43

II Hartiala P., Hytönen J., Suhonen J., Leppäranta O., Tuominen-Gustafsson H., Viljanen M.K.: *Borrelia burgdorferi* OspB inhibits human neutrophil functions, *Microbes and Infection* 2008 10(1):60-68

III Hartiala P., Hytönen J., Yrjänäinen H., Honkinen M., Terho P., Söderström M., Penttinen M., Viljanen MK.: Borrelia does not induce p38 MAPK dependent expression of CD38 on dendritic cells which leads to defective migration in vitro and in vivo, Submitted 11/2008

1 INTRODUCTION

Lyme borreliosis (LB) is a multisystemic infection caused by the spirochete *Borrelia burgdorferi* and it is the most common tick-borne disease in Europe and the United States. The disease was discovered in 1977 when Allen Steere and colleagues reported an outbreak of arthritis mainly in children in the town of Old Lyme in Connecticut, USA. Some patients had a history of an erythematous skin lesion earlier that spring or summer and some recalled a preceding tick-bite at the site of the skin lesion. The finding that penicillin had a beneficial effect on the symptoms suggested that the causative agent was a bacterium. The causative agent, *B. burgdorferi*, was later isolated and cultivated from the skin, blood and cerebrospinal fluid of LB patients.

LB has been reported in North America, Europe and Asia. In Finland, an estimated 3000-5000 cases occur each year and in 2007 1332 cases of LB were microbiologically confirmed according to the National Infectious Disease Register of the National Public Health Institute of Finland. The diagnosis of LB is challenging due to the wide variety of symptoms and requires clinical expertise and, in many cases, help from microbiological laboratory tests. Recent research has helped in developing better diagnostic tools for more accurate diagnosis, has clarified basic pathogenetic mechanisms and revealed some immune evasion strategies utilized by *B. burgdorferi*. However, research on the dissemination of borrelia and the pathogenesis of late and treatment-refractory disease is needed.

This study focused on the immune evasion strategies of *B. burgdorferi* against important innate immune players, namely the complement system, neutrophils and dendritic cells.

2 REVIEW OF THE LITERATURE

2.1 Borrelia burgdorferi

2.1.1 Classification

Borrelia burgdorferi sensu lato belongs to the order *Spirochaetales* along with leptospirae and treponemae. The genus *Borrelia burgdorferi* sensu lato currently includes three major pathogenic species, *B. burgdorferi* sensu stricto*, B. garinii and B. afzelii*, which cause LB. It also includes at least eight closely related species that rarely cause human infections. In Finland, *B. garinii* and *B. afzelii* are the prevalent species (Junttila et al. 1994). Other species not belonging to the *Borrelia burgdorferi* sensu lato complex cause tick- and louse-borne relapsing fever (e.g. *B. recurrentis, B. duttonii*). *Borrelia burgdorferi* sensu lato is abbreviated in this thesis as Bb or *B. burgdorferi*, *Borrelia burgdorferi* sensu stricto as Bb s.s., *Borrelia garinii* as Bg and *Borrelia afzelii* as Ba.

2.1.2 Structure and growth requirements in vitro

Bb is a gram-negative helicoid bacterium, which is 15-25 µm long and 0,2-0,5 µm wide and has 7-11 periplasmic flagellae (Barbour and Hayes 1986), which mediate the motility and shape of borreliae. Analogously to other gram-negative bacteria Bb has an outer membrane surrounding the periplasmic space and an inner cytoplasmic membrane surrounding the cytoplasm (Barbour and Hayes 1986). Exceptionally, no lipopolysaccharide (LPS) is present in the outer membrane of Bb.

Bb can be visualized without staining by dark-field or phase-contrast microscopy (Preac-Mursic and Wilske 1993). **Figure 1** is a phase-contrast microscopy image of Bb bacteria and a dendritic cell phagocytosing the bacteria. The organism can be detected by light microscopy after Wright, Giemsa or silver staining and by fluorescence microscopy using immunocytochemistry.

Bb can be grown in vitro in microaerophilic conditions at 33 °C in a liquid medium called Barbour-Stoenner-Kelly (BSK II) (Barbour 1984). Under unfavourable conditions, such as low pH or under the influence of antibiotics, the spirochete develops membraneous blebs (Preac-Mursic et al. 1986).

2.1.3 Genome

Bb has a linear chromosome of approximately 1Mb in size (Fraser et al. 1997) and it usually contains at least four linear plasmids and several circular plasmids. Most of the genome of Bb s.s. strain B31 was published in 1997 (Fraser et al. 1997) and the complete genome was published in 2000 (Casjens et al. 2000). The genome contains a large number of genes encoding lipoproteins including outer surface proteins (Osps) from A to F (Fraser et al. 1997). On the other hand, the genome of Bb encodes very few proteins with biosynthetic activity and, thus, the spirochete is dependent on the host for nutritional requirements.

2.1.4 Outer surface proteins

Outer surface protein A (OspA) and OspB are two major lipoproteins on the outer surface membrane of Bb. In the genome of Bb s.s. strain B31, the genes encoding OspA and OspB are located on linear plasmid 54 (lp54). OspB and OspA share a common promoter and thus are coordinately transcribed (Howe et al. 1986; Bergstrom et al. 1989). Bb is transmitted to humans by ticks and studies have shown that Bb regulates the expression of many surface proteins during the transmission phase. OspA and OspB are expressed by Bb in the tick midgut. Soon after entry into a vertebrate host, they are down-regulated and expression of OspC is increased (Schwan et al. 1995; Schwan and Piesman 2000). OspA and OspB antibodies are detectable in some patients in the early phase of the disease, at the late stage during arthritis (Kalish et al. 1995; Chen et al. 1999), and also in treatment-refractory Lyme arthritis (Lengl-Janssen et al. 1994; Chen et al. 1999) suggesting that OspA and OspB are also expressed at some stage during persistent infection. Moreover, OspA antigens (Coyle et al. 1993) and antibodies (Schutzer et al. 1997) have been identified in the cerebrospinal fluid of patients with neuroborreliosis. In a study by Batsford et al., approximately 80 % of sera of patients with arthritis or achrodermatitis chronica atrophicans (ACA) and 23 % of sera of patients with erythema migrans (EM) recognized OspA or OspB antigens (Batsford et al. 1998)

2.2 Lyme borreliosis

2.2.1 Epidemiology

Bb spirochetes live in nature in enzootic cycles involving ticks and a wide range of animals including mice and voles (Xu et al. 2003). The main vectors are *Ixodes ricinus* ticks in Europe, *I. persulcatus* in Asia, and *I. scapularis* and *I. pacificus* in North America. Ticks have larval, nymphal and adult stages and they require a blood meal at each stage. Larvae and nymphs feed mostly during spring and summer while adults feed mostly in the fall. Bb is usually transmitted to humans by nymphs and adult ticks. Studies on laboratory animals have shown that transmission of the disease usually requires attachment of the tick for as long as 48-72 hours and if the tick has been attached for less than 24 hours, risk of infection can be considered low (Piesman et al. 1987; Piesman 1993; des Vignes et al. 2001).

LB is common in Northern America and in Central and Eastern Europe. In Finland, ticks are common in the Åland archipelago, the south-western archipelago and in Southern and Central Finland. Approximately 10 % - 30 % of ticks in Finland are infected (Junttila et al. 1999). It has been estimated that 3000 - 5000 cases of primary infection occur each year in Finland. According to the National Infectious Disease Register of the National Public Health Institute of Finland, there were 1332 cases confirmed by laboratory testing in Finland in the year 2007, most of these being late disease manifestations as laboratory testing in the early phase of the infection is not usually performed.

2.2.2 Pathogenesis

To maintain its enzootic cycle, Bb must adapt to different host environments. Inside the tick, Bb expresses outer surface protein A (OspA), which keeps it attached to the tick receptor for OspA (TROSPA) in the tick gut (Pal et al. 2004). During the blood meal of the tick, Bb changes the expression of a number of genes including down-regulation of the gene encoding OspA. Simultaneously, the expression of OspC is up-regulated, which is required for infection of mammals (reviewed in Steere et al. 2004). OspC has been shown to bind the tick salivary protein Salp15, which has many immunosuppressive functions (reviewed in Hovius et al. 2008).

After days to weeks of transmission to mammalian skin, Bb may disseminate to various organs. To facilitate its dissemination, Bb adheres to integrins, proteoglycans or glycoproteins on host cells and extracellular matrix (reviewed in Steere et al. 2004). Bb can bind plasminogen and plasminogen activator urokinase to better penetrate through endothelial cell layers. A 47-kDa borrelial protein, BBK32, has been shown to bind fibronectin; a 66 kDa outer surface protein of Bb binds to the fibrinogen receptor and the vitronectin receptor; and a 26 kDa protein binds to glycosaminoglycans on endothelial and neuronal cells. In addition, decorin binding proteins A and B bind to a collagen associated proteoglycan, decorin (reviewed in Steere et al. 2004).

2.2.3 Immune response to *B. burgdorferi*

Once Bb is in the skin, the first host factors encountering the bacteria are components of the complement system and cells of the innate immune system. Depending on Bb genospecies, complement-mediated lysis of the spirochetes may be the first defence mechanism of the host (Breitner- Ruddock et al. 1997). The usual first clinical symptom, the EM lesion, consists of lymphocytes, dendritic cells (DCs), macrophages and a small number of plasma cells (Mullegger et al. 2000). However, only very few neutrophils are present, which is an untypical finding in bacterial infections (Steere et al. 1983). Inflammatory cells in EM produce proinflammatory cytokines, most prominently TNFα and INFγ. An optimal Th1 response in the early phase of the disease has been associated with good outcome (Sjöwall et al. 2005).

The infection can be limited to the skin and subside even without treatment, but, for yet unknown reasons, in some patients the bacterium invades into the blood circulation and disseminates into several organs. Symptoms can appear even years after the primary infection. It seems that in the very early phase of the disease cell-mediated Th1 responses are important in defence against the bacteria while antibody-mediated Th2 responses become important in the later phase of LB (Oksi et al. 1996; Sjöwall et al. 2005). A schematic representation of the early immune response towards Bb is shown in **Figure 2.**

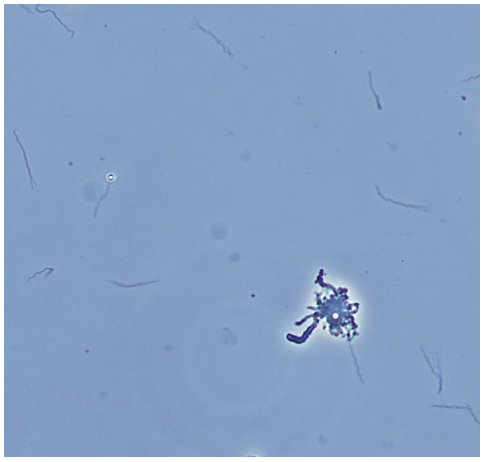

Figure 1. Dendritic cell phagocytosing *B. garinii* as seen with phase-contrast microscopy.(Picture: P. Hartiala)

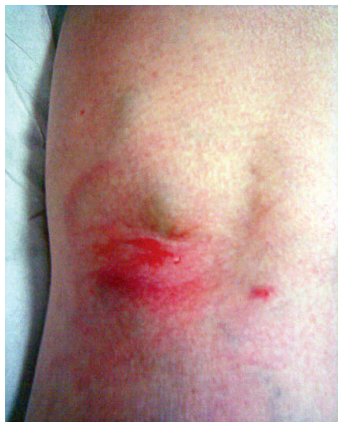

Figure 3. Erythema migrans rash on the dorsal skin of the knee joint. The red area in the center was caused by bleeding of the skin due to tick removal. (Picture: P.Hartiala)

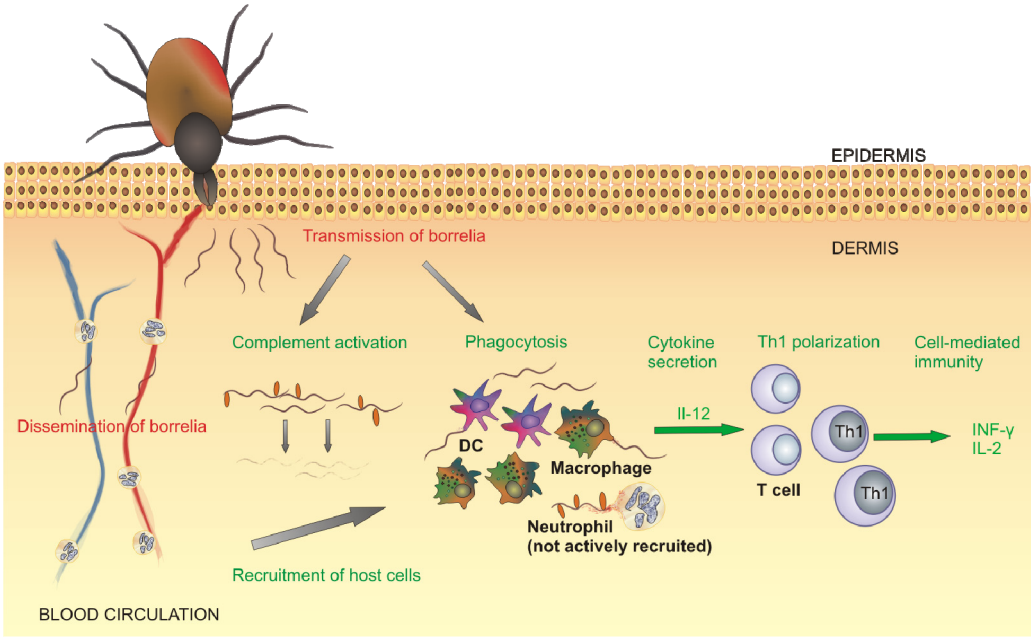

Figure 2. Early events in the immune response against *B. burgdorferi*. Transmission and dissemination of *B. burgdorferi* (written in red) are events that should be inhibited by the immune system. Complement activation, recruitment of host immune cells, phagocytosis, cytokine secretion inducing Th1 polarizarion and cell-mediated immunity (written in green) are events that should take place in an optimal immune response against *B. burgdorferi*. They are also targets of immune evasion by *B. burgdorferi*.

2.2.4 Clinical manifestations

The development of LB can be divided into early local infection, early disseminated infection and late chronic infection (Steere et al. 1984). However, the three stages overlap and the stating is not clear in all LB cases. The first dermatologic symptom of Bb infection, EM, is present usually 3-32 days after the infection **(Figure 3)**. The skin lesion can be accompanied by flu-like symptoms, such as malaise, fatigue, headache, arthralgias, myalgias and fever (Steere et al. 1983). The early neurological manifestations include cranial and peripheral neuropathies, a typical example being facial nerve palsy, lymphocytic meningitis, and meningoradiculitis.

Typical disseminated LB skin manifestations include multiple EM lesions, borrelial lymphocytoma and ACA, which is a manifestation of late stage LB. Lyme arthritis is usually a manifestation of late LB but it can sometimes occur earlier during the disease. Artritis is typically characterized by recurrent attacks of joint swelling either as a monoarthritis or oligoarthritis. The late neurological manifestations of LB include meningitis, painful radiculitis and in some cases just numbness or tingling (reviewed in Hytönen et al. 2008)

There are a large number of disseminated and late stage LB manifestations that include, in addition to the above mentioned most common manifestations, carditis, that may present as conduction disturbances, endomyocarditis, or pericarditis (Steere 1989), and various ocular findings including conjunctivitis, keratitis and uveitis (Lesser 1995). Some cases of hepatitis (Kazakoff et al. 1993), splenomegaly (Cimmino et al. 1989), orchitis, and microscopic hematuria (Steere 1989) have been reported in association with LB. Bb infection has a strong tendency to become chronic (Berger et al. 1983; Steere et al. 1983; Steere et al. 1983).

Tissue tropism among Bb genospecies is an established concept, suggesting that arthritis is in most cases caused by Bb s.s. strains, whereas Bg is responsible for the various neurological symptoms of the patients, and cutaneous manifestations are often associated with Ba infections.

2.2.5 Diagnosis and treatment

In the early phase of LB, diagnosis is usually based on typical clinical findings, most importantly the EM rash, and laboratory testing is not recommended. If an EM rash is suspected, the patient should be treated with amoxycillin 500-1000 mg three times a day for 14 to 21 days. Doxycycline, cefuroxime axetil or macrolides can also be used.

At the late stage of the disease, serological testing is recommended to support clinical diagnosis. Production of IgM antibodies starts at two weeks after infection peaking at two months. Gradually, the production of IgG antibodies begins and that of IgM declines. A two-step approach is generally used in LB serology. The first step is a sensitive ELISA (Enzyme-Linked ImmunoSorbent Assay) screening assay, which is followed by an immunoblot assay in positive or borderline positive cases (reviewed in Hytönen et al. 2008). Bb can be cultured in liquid BSK II medium. As a result of the

long generation time of Bb, cultures need to be incubated for up to 2-3 months (reviewed in Hytönen et al. 2008). One diagnostic method is the detection of Bb DNA from biopsy samples or body fluids (e.g. synovial fluid, cerebrospinal fluid, blood) using polymerase-chain reaction (PCR) (reviewed in Stanek and Strle 2003). In general, the sensitivities of Bb culture and PCR are low leaving serology the method of choice in laboratory diagnosis of LB.

The treatment of late infection varies according to the affected organ system. Most commonly, intravenous ceftriaxone 2 g per day for 14 to 21 days is recommended. In some cases oral doxycycline can be used (reviewed in Hytönen et al. 2008).

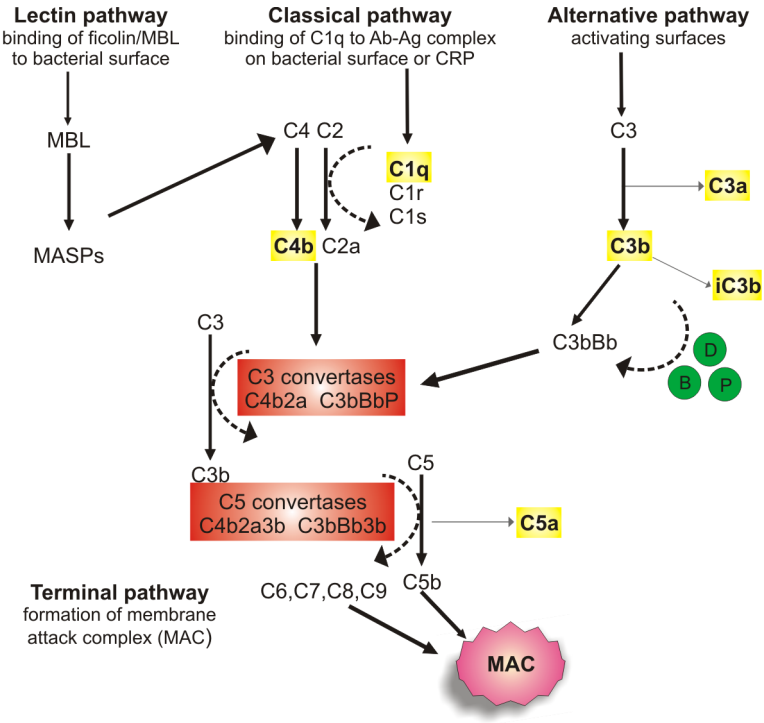

Figure 4. A simplified presentation of the different complement activation pathways. MBL = mannose binding lectin, MASP = mannan-binding lectin-associated serine protease, B = factor B, D = factor D, P = properdin. B, D and P are associated with activation of the alternative pathway. Components acting as opsonins or anaphylotoxins are highlighted with yellow. Figure adapted from Oksjoki et al. 2007.

2.3 Complement system

The complement system belongs to the first line of defence. The following section focuses on the functions of the complement system in defence against microbes. The system consists of a great number of proteins and its activation follows a cascade analogous to e.g. the blood coagulation system. The main functions of the complement system in defence against bacteria are lysis of bacteria through formation of the

membrane attack complex (MAC) and potentiation of phagocytosis by opsonization of bacteria, the major opsonins being C1q, C3b, C3bi and C4b. C3a and C5a act as chemoattractants for immune cells and mediate various inflammatory effects by acting as anaphylatoxins.

The complement system can be activated through three different pathways, the classical pathway, the alternative pathway and the mannan binding lectin (MBL) pathway. Activation of the classical pathway occurs primarily when the C1q component binds to antigen-bound IgG or IgM antibodies or C-reactive protein (reviewed in Oksjoki et al. 2007)). The alternative pathway involves constant low-grade spontaneous activation of C3 in plasma. If the formed C3b is bound to complement-activating surface, e.g. bacteria, parasites, fungi, it may bind factor B, which is then cleaved by factor D, allowing the formation of the alternative pathway C3-convertase, C3bBb (reviewed in Oksjoki et al. 2007). The activation of the MBL pathway occurs when MBL binds to mannose groups or other carbohydrates on microbes. The pathway is analogous to the classical pathway. After cleavage of C5, the three pathways merge to a common terminal pathway, which results in the formation of the MAC. **Figure 4.** shows a simplified presentation of the different complement activation pathways.

Because complement is spontaneously and constantly activated, the presence of several inhibitory molecules is crucial in limiting the activation so that it will not be harmful to the host. Soluble inhibitors of complement include the C1-inhibitor (C1INH), C4b-binding protein (C4bp), factor H, factor I, properdin, S protein, and clusterin. Surface-bound inhibitors include decay accelerating factor (DAF), membrane cofactor protein (MCP, also known as CD46), protectin (CD59), complement receptor 1 (CR1), and the newly described CRIg (Helmy et al. 2006; Oksjoki et al. 2007).

Microbes have developed a variety of mechanisms to inhibit complement attacks. The strategies include 1) blocking effective complement attachment by cell wall or capsule structures, 2) directing the attack away from the microbial cell wall, 3) antigenic variation to evade MAC-mediated lysis and opsonophagocytosis, 4) binding complement regulatory molecules to the cell surface and thus inhibiting further complement activation (Rautemaa and Meri 1999; Kraiczy and Wurzner 2006; Lambris et al. 2008).

2.4 Neutrophils

Neutrophils are cells of the innate immune system and constitute the main cellular defence system against bacteria. They are able to sense chemotactic agents, migrate towards a chemotactic gradient and phagocytose and kill micro-organisms by exploiting oxidative and non-oxidative mechanisms. They differentiate from hematopoietic stem cells in the bone marrow, and in humans around 100 billion neutrophils enter and leave the blood circulation every day (reviewed in Urban et al. 2006). Neutrophils mature in the bone marrow for two weeks after which they migrate to the blood circulation where their half-life is approximately 6-7 hours in humans (Dancey et al. 1976). Neutrophils have a large segmented nucleus and the cytoplasm

contains various granules that have been classified into four subsets based on density and composition: azurophilic (primary) granules, specific (secondary) granules, gelatinase (tertiary) granules, and secretory vesicles (Faurschou and Borregaard 2003). Exocytosis of granules and secretory vesicles plays a pivotal role in most neutrophil functions from early activation to the destruction of phagocytosed microbes.

2.4.1 Neutrophil chemotaxis

To accumulate into the infected skin area, neutrophils need to be able to migrate to the site of infection. Neutrophils respond to a large number of chemoattractants with directional cell movement. These chemoattractants include the bacterial chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP), activated complement component 5 (C5a), leukotriene B_4 platelet-activating factor and a superfamily of chemokines. Chemoattractants activate G-protein-coupled seven-trans-membrane receptors expressed on various cell types.

Formylmethionine peptides are derived from the NH2-terminal regions of newly synthesized prokaryotic protein (Adams and Capecchi 1966) while eukaryotic cells initiate protein synthesis mostly with nonacetylated methionine (Yoshida et al. 1970). The chemoattractant fMLP has three receptors, the high-affinity formyl peptide receptor (FPR) and the low affinity variants FPR-like 1 (FPRL1) and FPRL2. Neutrophils express FPR and FPRL1 while FPRL2 is only expressed by monocytes and dendritic cells (Rabiet et al. 2007).

C5a is a protein fragment released from complement component C5. In humans, the polypeptide contains 74 amino acids. The two receptors for C5a on neutrophils are complement component 5a receptor 1 (C5aR1) and C5aL (Ohno et al. 2000).

Microbes have developed many different strategies for inhibiting neutrophil chemotaxis. *Porphyromonas gingivalis*, that causes adult parodontitis, produces a protease which can cleave the C5a receptor on neutrophils (Jagels et al. 1996), whereas group A streptococci produce a C5a peptidase which rapidly cleaves and inactivates the chemoattractant C5a (Hill et al. 1988; Chen and Cleary 1990). *Staphylococcus aureus* can also inhibit the chemotaxis of neutrophils towards C5a and fMLP by secreting a chemotaxis inhibitory protein that affects C5a and FPR on neutrophils (Veldkamp et al. 2000; de Haas et al. 2004).

2.4.2 CD38 in neutrophil chemotaxis

CD38 is expressed on many lymphoid and myeloid cells. The general properties of CD38 are described in detail in section 2.5.2. In vivo studies have shown that $CD38^{-/-}$ mice are susceptible to bacterial infection as a result of a defect in the ability of $CD38^{-/-}$ neutrophils to directionally migrate to the site of infection (Partida-Sanchez et al. 2001). In their article, Partida-Sanchez et al. showed that $CD38^{-/-}$ neutrophils can be activated to migrate by bacterial chemoattractants but they are unable to follow the chemotactic gradient (Partida-Sanchez et al. 2001). Murine CD38 is expressed at relatively low levels on bone marrow myeloid precursors, whereas on macrophages or neutrophils isolated at sites of infection and inflammation CD38 expression is

increased. CD38 catalyses the formation of cyclic adenosine diphosphate ribose (cADPR), which controls calcium influx and chemotaxis of murine neutrophils towards fMLP.

Subsequently, Partida-Sanchez et al. showed that a cADPR antagonist and a CD38 substrate analogue inhibited the chemotaxis of human phagocytic cells to FPRL1 receptor ligands (Partida-Sanchez et al. 2004). cADPR modulates intracellular free calcium levels by inducing influx of extracellular calcium without intracellular calcium release, and cADPR is needed for FPRL1, CXCR4, CCR1 and CCR5 mediated chemotaxis in human cells (Partida-Sanchez et al. 2004). It has been suggested that cADPR regulates the activation of L-type calcium channels and store operated Icrac channels. fMLP and IL-8 down-regulate CD38 on the human neutrophil surface while CD38 in supernatant is increased and this is dependent on p38 MAP kinase (Fujita et al. 2005).

2.4.3 Neutrophil phagocytosis and oxidative burst

After chemotaxis to the inflamed area, neutrophils start to phagocytose bacteria. Neutrophils recognize pathogens by cell-surface receptors, either directly or through opsonisation of the microbe with complement components or antibodies. The most important phagocytosis receptors on neutrophils include complement receptors 1 (CR1) and CR3 and Fc-receptors I-III. CR1 binds the complement components C3b and C4b and CR3 binds iC3b and bacterial lipopolysaccharide (LPS) (Brown 1991). These receptors highlight the importance of effective complement opsonisation in phagocytosis. Fc receptors, on the other hand, participate in antibody-mediated phagocytosis by binding the Fc fragment of immunoglobulin G.

After phagocytosis, ingested bacteria are usually trapped inside a phagosome, which fuses with a lysosome to form a phagolysosome. Microbes are then killed by oxygen-independent and oxygen-dependent mechanisms. The oxygen-independent mechanisms utilize antimicrobial proteins and peptides, which are stored in neutrophil granules. These antimicrobial agents include lactoferrin, lipocalin, lysozyme, gelatinase and metalloproteinase released from secondary and tertiary granules and α-defensins, seprocidins, cathepsin G, proteinase 3 and neutrophil elastase released from primary granules.

The oxygen-dependent mechanisms involve the generation of reactive oxygen species (ROS) and are called the oxidative burst. Once the neutrophil is activated, cytosolic and transmembrane NADPH-oxidase complexes catalyse the production of microbicidal superoxide anions (O_2-). Myeloperoxidase then catalyses the reaction of superoxide with H_2O_2 to form hypochlorous acid (HOCI). HOCI is the most bactericidal oxidant of neutophils.

Pathogens have developed many strategies to prevent neutrophil phagocytosis and killing. Phagocytosis can be prevented by the use of physical barriers such as polysaccharide capsules that prevent recognition of the microbe. Uropathogenic *Escherichia coli* utilizes this approach through its capsular antigens O75 and K5, which mediate resistance to phagocytosis. Another approach is to interfere with

opsonisation. An example of this is the secretion of a complement inhibitor SCIN by *S. aureus*, which inhibits complement-mediated phagocytosis. A third approach is to interfere with the organization of the actin cytoskeleton. *Yersinia pestis, Y. pseudotuberculosis and Y. enterocolitica* inject effector proteins (YopE, YopH, YopT and YopO) into the cytoplasm of the phagocytosing cell and these proteins inhibit the functions of the actin cytoskeleton. (reviewed in Urban et al. 2006).

If a microbe is phagocytosed and it can not take advantage of the phagocytosis inhibiting strategies mentioned above, it can still try to inhibit killing inside the neutrophil. The first strategy is to inhibit the fusion of the phagosome and lysosome and is exploited e.g. by *Streptococcus pyogenes* through its M and M-like proteins that prevent the phagosomal fusion of primary granules. *S. pyogenes* can also survive inside the phagolysosome, with the help of M- and H-proteins. Another strategy aiding survival inside the phagolysosome is interference with the NADPH-oxidase complex by *Helicobacter pylori*. It does induce an oxidative burst but it can direct the burst to extracellular spaces leaving the microbe unharmed inside the cell. A third strategy is to escape from the phagolysosome and is exploited once again by *S. pyogenes* with the help of large capsule formation. (reviewed in Urban et al. 2006)

2.5 Dendritic cells

Dendritic cells (DCs) are a heterogeneous family of cells of hematopoietic origin. They are in close contact with mucosal surfaces and are among the first cells to meet invading pathogens in the body. DCs can differentiate via two distinct pathways resulting into plasmacytoid DCs and myeloid DCs. The DC subtypes can be characterized in many ways. One way is to divide them regarding their origin and migratory pattern to plasmacytoid DCs, $CD8\alpha^+$ DCs, Langerhans cells (LCs) and conventional myeloid DCs (Randolph et al. 2007). Human skin DCs can be divided into LCs ($CD1a^+$, $CD14^-$, $langerin^+$), and to two dermal subpopulations ($CD1a^-$ $CD14^+$ and $CD1a^+$ $CD14^-$) (Klechevsky et al. 2008). Klechevsky et al. recently analyzed the functions of the different human skin DC subpopulations. $CD14^+$ DCs primed $CD4^+$ T cells into cells that induce naive B cells to switch isotype (IgM to IgG or IgA) and become plasma cells secreting large amounts of antibodies. In contrast, LCs preferentially induced the differentiation of $CD4^+$ T cells secreting Th2 cell cytokines and were efficient at priming naive $CD8^+$ T cells into potent cytotoxic effectors. The third DC population, $CD14^-CD1a^+$ DC, which resides in the dermis, could activate $CD8^+$ T cells better than $CD14^+$ DCs but less efficiently than LCs. (Klechevsky et al. 2008). In mice at least six DC subtypes have been described (Blanco et al. 2008). DCs reside in peripheral tissues in a steady state. After suitable stimuli, such as bacteria, LPS and a variety of cytokines, DCs undergo a maturation process and migrate to lymph nodes, where they present foreign antigens to T cells (Banchereau et al. 2000). DCs also influence the type of T cell response and participate in the activation and recruitment of immature DCs, NK cells, macrophages, granulocytes and B cells through chemokine and cytokine production (Banchereau et al. 2000).

DCs can be activated through a variety of pattern-recognition receptors which include Toll-like receptors (TLR) and C-type lectin receptors (Blanco et al. 2008). DCs express TLR1–

4, TLR6, and TLR8 in vitro (Iwasaki and Medzhitov 2004). Upon maturation, DCs change the expression of several molecules. E-cadherin (Schwarzenberger and Udey 1996) and CCR6 (Dieu et al. 1998) are down-regulated and the expression of major histocompatibility complex (MHC) class I, MHC class II, chemokine receptor 7 (CCR7), CD80, CD83, CD86, CD40 are up-regulated (Banchereau et al. 2000). DC maturation is accompanied by morphological changes including loss of adhesive structures, cytoskeleton reorganization and increase in cellular motility (Winzler et al. 1997).

Through cytokine secretion DCs, like other antigen-presenting cells, can polarize differentiation of naïve T cells into Th1, Th2, T-regulatory (Treg) or Th17 subtypes. Th1 cells are important for cell-mediated immunity and in defence against intracellular pathogens, while Th2 cells are associated with humoral responses and are important in defence against extracellular pathogens (reviewed in Zhu and Paul 2008). Th17 cells have been characterized recently and are associated with the development of autoimmunity as well as defence against extracellular pathogens, while Treg cells are important in mediating immune tolerance and regulation of immune responses (Zhu and Paul 2008). The presence of IL-12 produced by DCs promotes Th1 polarization of adaptive immune responses and that of IL-4 Th2 polarization. Th17 polarization is driven by IL-23, when it is secreted without simultaneous IL-12 production (Roses et al. 2008) and transforming growth factor β (TGF-β), while Treg polarization is induced by IL-2 and TGF-β (Zhu and Paul 2008). Th1 cells produce proinflammatory cytokines (INF-γ and IL-2) and Th2 cells anti-inflammatory cytokines (IL-4, IL-10, IL-13) and IL-5 (Blanco et al. 2008; Zhu and Paul 2008). Th17 cells produce IL-17 and Treg cells produce IL-10, among other cytokines (Zhu and Paul 2008). **Figure 5** shows the cells participating in and the events occurring during Th polarization.

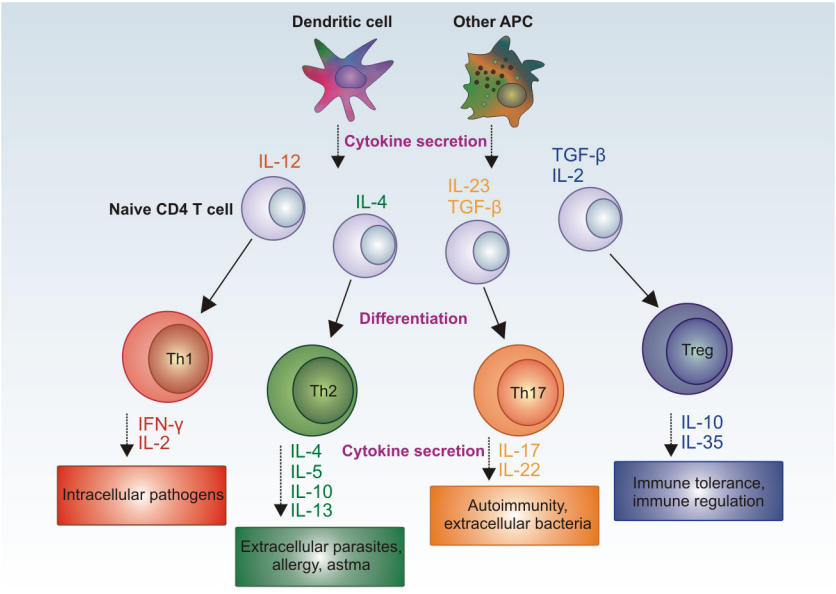

Figure 5. Schematic presentation of events leading to Th1, Th2, Th17 and Treg polarization of naïve CD4+ T cells. The major functions of subpopulations are also listed.

Microbes have been shown to inhibit DC functions in many ways. *Salmonella* causes apoptosis of immature DCs, *Leishmania mexicana* inhibits DC maturation and Herpes simplex virus inhibits DC migration (Rescigno and Borrow 2001).

2.5.1 Dendritic cell migration to lymph nodes

DC migration to lymph vessels and their positioning in lymph nodes is prominently controlled by CCR7 (Forster et al. 1999; MartIn-Fontecha et al. 2003), although various other factors affecting DC migration have also been discovered (Randolph et al. 2005). CCL19 and CCL21, ligands of CCR7, are expressed by lymphatic endothelium and by stromal cells and endothelial cells in lymph nodes and by DCs themselves (MartIn-Fontecha et al. 2003). However, the expression of CCR7 alone is not sufficient to enable DC migration to lymph nodes. DNAX activating protein of 12 kD, cysteinyl leukotrienes, prostaglandin E2 and CD47 also have roles in DC migration (Randolph et al. 2005; Hagnerud et al. 2006). Although CCR7 has been credited with being the dominant mediator of DC migration, there is contradictory information of its importance in DC migration. A recent study by Velan et al. showed that DCs pulsed with *Y. pestis* showed decreased migration towards CCL19 in an in vitro assay and in an in vivo assay but still showed up-regulated CCR7 expression (Velan et al. 2006). In a study concerning the role of CD47 in DC migration, $CD47^{-/-}$ mature DCs showed normal CCR7 expression but impaired migration to CCL19 in an in vitro assay (Hagnerud et al. 2006).

2.5.2 CD38 and its role in dendritic cell migration

Recently, the ectoenzyme CD38 has been ascribed an important role in DC chemotaxis and migration (Partida-Sanchez et al. 2004; Salmi and Jalkanen 2005; Frasca et al. 2006). CD38 is an ectoenzyme expressed on many lymphoid cells. It has been attributed many roles in the immune system (Lund et al. 1998). It is involved in the regulation of calcium release and the entry of extracellular calcium into cells due to catalysis of formation of calcium-mobilizing metabolites (Lee 2000). The extracellular domain of CD38 contains an enzymatic site that can catalyse the formation of cyclic ADPribose (cADPR) and ADPribose (ADPR) from nicotine adenine dinucleotide (NAD^+) and the formation of nicotinic acid adenine dinucleotide phosphate (NAADP) from NADP (Howard et al. 1993). cADPR is transported into cells where it binds to FK506 binding protein (FKBP) on the Ryanodine receptor and stimulates Ca^{2+} release from the endoplastic reticulum (reviewed inMorita et al. 2008). It can also sustain adhesion and rolling of lymphocytes through interaction with its counter-receptor CD31 on endothelial cells (Deaglio et al. 1996). CD38 is down-regulated during the differentiation of immature monocyte derived DCs and again expressed upon maturation (Fedele et al. 2004). It was considered to be part of the common cellular response to infection in the study by Jenner and Young, and it is up-regulated in DCs by various microbes, including LPS-expressing bacteria as well as viruses, yeasts and LPS-lacking bacteria (Jenner and Young 2005). Recent studies have revealed novel functions for CD38 in DCs. In murine models, CD38 is involved in chemotaxis and transendothelial migration of both polymorphonuclear leukocytes and DCs, and this function requires its enzymatic activities (Partida-Sanchez et al. 2001; Partida-Sanchez

et al. 2004). CD38-deficient DCs are inefficiently recruited from the skin to local lymph nodes after antigenic stimulation (Partida-Sanchez et al. 2004). This results in poor priming of T cells and impaired induction of humoral immune responses. CD38 is needed for the chemotaxis of immature and mature DCs towards CCL2, CCL19, CCL21 and CXCL12 (Partida-Sanchez et al. 2004). A recent study showed that monoclonal antibodies and other reagents interfering with CD38-mediated signals lead to powerful inhibition of human DC migration in vitro (Frasca et al. 2006).

Studies with CD38-knockout mice have revealed other interesting roles for CD38. A recent study by Hin et al. showed that CD38-knockout mice have marked defects in maternal nurturing and social behaviour with higher locomotor activity and the plasma level of oxytocin, an important hormone regulating e.g. social behaviour, was strongly decreased in these mice. This can be explained by the fact that secretion of oxytocin is dependent on Ca^{2+} release from intracellular stores which requires catalysis of cADPR formation by CD38. (Jin et al. 2007).Another recent and interesting study by Barbosa et al. showed that CD38-knockout mice are protected against high-fat diet-induced obesity as a result of a higher metabolic rate compared to control mice. Analysis of the mechanism revealed that CD38, via regulation of NAD levels, can control sirtuin enzyme (SIRT) activity and activation of peroxisome proliferator-activated receptor γ coactivator (PGC1α) which, in turn, regulates energy metabolism and obesity. SIRT-PGC1α is a well established cascade, involved in the regulation of mitochondrial biogenesis and energy homeostasis. (Barbosa et al. 2007). These recent studies highlight the diverse roles of CD38 regulated Ca^{2+} metabolism ranging from immune regulation to energy metabolism and behaviour.

2.5.3 Approaches to studying the migration of skin dendritic cells in vivo (Langerhans cells and dermal dendritic cells)

The following methods were studied in detail when setting up the in vivo experiment later described in this thesis. Because there was no recent review available on the topic, the different methods are described in this section. The effect of bacteria on DC migration in vivo has previously been studied by several techniques including adoptive transfer, fluorescein isothiocyanate (FITC) painting and direct labelling of bacteria. Ato et al. (Ato et al. 2002) studied the migration of naïve DCs or DCs isolated from *Leishmania donovani* infected C57BL/6 mice by adoptive transfer. DCs from infected and naïve animals were isolated and labelled with Hoechst 33342 and transferred to recipient animals. The mice were killed after 24 hours and their spleens were isolated. Spleen sections were photographed under normal light and UV light and the number of DCs was counted.

Khader et al. (Khader et al. 2006) used both adoptive transfer and direct labelling of bacteria to study the migration of DCs in *Mycobacterium tuberculosis* infected IL-12p35- and IL-12p40-knockout mice. Using the direct labelling method, irratiated *M. tuberculosis* or LPS in a carboxyfluorescein diacetate succinimidyl ester (CFSE) solution was delivered intratracheally to recipient mice. After 18 hours, draining lymph nodes were harvested and single-cell suspensions were analyzed for CD11c and CFSE double positive cells using flow cytometry.

Velan et al. (Velan et al. 2006) also used a direct labelling technique when studying the effect of *Y. pestis* on DC migration. An orange cell tracker, CMTMR, was administered intratracheally and bacteria were administered via the same route five hours later. After 18 hours, draining lymph nodes were isolated and the amount of CMTMR and CD11c double positive cells was counted using flow cytometry.

Rotta et al. (Rotta et al. 2003) used various methods when studying the effect of *Salmonella* Typhimurium and various other bacteria on DC migration in vivo. Their primary approach was to inject green fluorescent protein (GFP) tagged salmonella intradermally and search draining lymph nodes for GFP and CD11c double positive cells. Unfortunately, no GFP positive cells could be found in drainig lymph nodes, probably because DCs were able to kill salmonella efficiently and metabolize the GFP. The second approach they used was to inject FITC-coated latex beads simultaneously with bacteria and search draining lymph nodes for FITC-positive DCs. This method was originally described by Randolph et al. (Randolph et al. 1999). In an attempt to study the effect of salmonella on LC migration, skin was painted with FITC and bacteria were injected intracutaneously either at the same site or contralaterally. However, it is now thought that FITC painting does not solely stain epidermal cells, as dermal cells are also stained (Randolph et al. 2005).

2.6 The innate immune system and dendritic cells in Lyme borreliosis

2.6.1 Complement and *B. burgdorferi*

Bb genospecies differ in their ability to activate and tolerate complement (Brade et al. 1992; Breitner- Ruddock et al. 1997; van Dam et al. 1997). Bb s.s. strains vary in their serum sensitivity, whereas Ba strains are serum-resistant and most Bg strains serum-sensitive (Breitner- Ruddock et al. 1997; van Dam et al. 1997). Complement resistance is mediated by borrelial surface proteins that bind host complement regulatory factors factor H. B313, a mutant derivative of *B. burgdorferi* s.s. B31 lacking lp54 among other plasmids, is susceptible to complement whereas the parental strain B31 is complement resistant (Sadziene et al. 1995). This can most likely be explained by the absence of complement regulator-acquiring surface protein 1 (CRASP-1), an important mediator of complement resistance in Bb, from the B313 surface (Brooks et al. 2005). Borrelial OspE and OspE paralogs have been shown to mediate complement resistance by binding factor H (Hellwage et al. 2001; Alitalo et al. 2002). However, the role of OspA and OspB in the complement resistance of Bb is not known.

2.6.2 Neutrophils and *B. burgdorferi*

Bb is phagocytozed by (Benach et al. 1984; Peterson et al. 1984; Szczepanski and Fleit 1988) and induces the oxidative burst in neutrophils (Peterson et al. 1984; Szczepanski and Fleit 1988; Georgilis et al. 1991). Neutrophils ingest Bb by coiling phagocytosis (Rittig et al. 1992) and tube phagocytosis (Suhonen et al. 1998), and phagocytosis of Bb is complement-dependent (Suhonen et al. 2000). After internalization, Bb can be killed by a variety of agents released by neutophils (Lusitani et al. 2002). OspA has

been shown to prime and activate human neutrophils (Morrison et al. 1997) and induce neutrophil chemotaxis (Benach et al. 1988).

CR3 is an adhesion molecule expressed on neutrophils. It is involved in neutrophil-borrelia interactions and its main ligand is the complement component iC3b (Todd 1996). The CD11c chain of CR3 participates in the oxidative burst and calcium mobilization induced by Bb (Suhonen et al. 2000). OspA and OspC up-regulate CR3 on neutrophils (Wooten et al. 1998; Cinco et al. 2000), and OspA and OspB have been shown to bind to CR3 in a iC3b-independent manner (Garcia et al. 2005). The roles of OspA and OspB in Bb induced phagocytosis and oxidative burst of neutrophils is not currently known.

Saliva of *I. dammini* ticks inhibits the function of peritoneal-derived rat neutrophils, as measured by anaphylatoxin-induced aggregation, fMLP-induced granule enzyme secretion, zymosan-induced superoxide secretion, and phagocytosis of *B. burgdorferi* spirochetes (Ribeiro et al. 1990). The saliva also reduces neutrophil adhesion via downregulation of β2-integrins and decreases the efficiency of neutrophils in the uptake and killing of spirochetes but does not affect neutrophil orientation or chemotaxis (Montgomery et al. 2004). However, little is known about the direct chemotaxis inhibitory properties of Bb.

2.6.3 Dendritic cells and *B. burgdorferi*

DCs phagocytose Bg strain Å218 and Bb s.s. strain B31, process borrelia-specific antigens and activate borrelia-specific T cells (Filgueira et al. 1996; Suhonen et al. 2003). These strains also induce DC maturation (Suhonen et al. 2003). DCs also secrete IL-8 after borrelial encounter in a manner similar to LPS leaving the reasons for the sparse neutrophil infiltrate in EM unclarified (Suhonen et al. 2003). LCs, which are cells of the DC lineage, are present in EM and ACA, the late skin manifestation of LB (Hulinska et al. 1994; Silberer et al. 2000). Although the number of LCs has been found to be higher in ACA than in normal skin, in both EM and ACA the MHC II expression of LCs has been found to be down-regulated compared to normal skin (Silberer et al. 2000).

Sjöwall et al. have characterized the cytokine response of DCs differentiated from various LB patient groups to Bg (Sjöwall et al. 2005). Their results show that the overall number of DCs secreting IL-4, IL-10, IFN-γ and IL-12p70 is low. In contrast, the number of TNF-α secreting cells is high and it is specifically high in the seropositive asymptomatic patient group. Sjöwall et al. also looked at the cytokine secretion of whole blood cells after Bg stimulation to look mainly at the macrophage response. IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNF-α were detectable in the supernatants after Bg stimulation, and the level of IL12p70 was elevated in asymptomatic individuals compared to seronegative controls.

However, little is known about the effect of Bb on other functions of DCs in addition to the above mentioned. No studies have been carried out on the effect of Bb on DC gene expression or cytokine secretion.

3 AIMS OF THE STUDY

The present study was carried out to reveal immune evasion strategies utilized by *B. burgdorferi*. The study focused on crucial innate immune players, namely the complement system, neutrophils and dendritic cells. Three broader aims concerning the pathogenesis of Lyme borreliosis were delineated:

1) to discover why the local defence reaction at *B. burgdorferi* infection site in the skin is mild and untypical of a bacterial infection,

2) to discover the means *B. burgdorferi* uses in dissemination from the skin to distant organs,

3) to clarify early events that could favour the development of the chronic phase of Lyme borreliosis.

The aims were studied with special regard to the function of the complement system, neutrophils and dendritic cells. On a molecular level, Bb OspA and OspB and dendritic cell CD38 were of special interest.

4 MATERIALS AND METHODS

4.1 Bacteria

4.1.1 *Borrelia burgdorferi* sensu lato

Low-passage Bb s.s. strain B31 (kindly provided by Sven Begström, University of Umeå), Bb strain Å218 (a tick-isolate) and high-passage strains of Ba (isolate 1082/93, isolated from a skin biopsy) and strain B313 were used in the experiments. B313 is a B31 derived mutant lacking several plasmids including linear plasmid 54, which contains among others genes encoding OspA, OspB and CRASP-1 surface molecules (Sadziene et al. 1992; Sadziene et al. 1995). The strain was kindly provided by Thomas Kamradt (Deutches Rheumaforschungszentrum, Berlin, Germany). Borreliae were cultivated in Barbour-Stoenner-Kelly II medium (BSK II) at 34°C and passaged weekly. Prior to the experiments, borreliae were counted in a Neubauer counting chamber, centrifuged at 1400 x g for 3 minutes and resuspended in phosphate-buffered saline (PBS) at a concentration of 6 x 10^7 /ml.

4.1.2 Generation of outer surface protein complemented *B. burgdorferi*

A DNA fragment containing *ospA* and *ospB* genes from *B. burgdorferi* s.s. B31 was PCR-amplified using the primers OspAB-forward (5'-AATA GCA TGC TAA CTT TTC TTT TTC CTG AAA G-3') and OspAB-reverse (5'-AATA GGA TCC GAG ACT TTT TCC AGA AGT AAC-3'). The fragment was then digested with restriction enzymes *Sph*I and *Bam*HI and ligated into the pBSV2 shuttle vector (a generous gift from Patricia Rosa, National Institutes of Health, Hamilton, MT, USA) at the corresponding sites. The ligation mixture was used to transform *E. coli* DH5α, and kanamycin-resistant clones were selected on Luria-Bertani plates. One correct clone (pBSV2-*ospAB*) was subjected to large scale plasmid preparation.

B313 bacteria were grown to a final concentration of 2×10^7 to 10^8/ml. After centrifugation, the bacteria were washed twice with 15 ml ice-cold electroporation solution (EPS; 0.27 M sucrose, 15 % (v/v) glycerol, sterile filtered). The cell pellet was suspended in EPS to a final concentration of 10^9–10^{10} cells/ml. Fifty micrograms of the plasmid was electroporated (2.5 kV, 25 µF, 200 Ω) into 50 µl of B313. The bacteria were transferred to 20 ml BSK II without antibiotics and incubated for 18–24 h. Kanamycin was added at a concentration of 200 µg/ml, and the suspension was aliquoted to 96-well plates. After incubation for 2–4 weeks, bacteria in wells that had turned yellow as a result of bacterial growth were subjected to further analyses (see below). The resulting strain was named $B313_{OspAB}$. A control strain with pBSV2 without an insert was created similarly and named $B313_{pBSV2}$.

To generate a B313 strain expressing OspA but not OspB, the plasmid pBSV2-o*spAB* was digested with *Ahd*I and *Bst*API to remove a 214 bp fragment from the 5' end of *ospB*. The resulting linear plasmid was blunt-ended and self-ligated to yield the

circular plasmid pBSV2-*ospA*. This plasmid was propagated in *E. coli* DH5α and electroporated into B313 as described above. The resulting strain was named $B313_{OspA}$.

4.1.3 Characterization of surface protein complemented mutant bacteria

$B313_{OspAB}$ and $B313_{OspA}$ strains were first subjected to PCR analysis to verify the presence of the plasmids and then to Western blot analysis to confirm the expression of OspA and OspB proteins. In Western assays, monoclonal anti-OspA (H5332) and anti-OspB (H6831) antibodies (gifts from Sven Bergstöm, University of Umeå, Sweden) and HRP-conjugated anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used to probe whole cell lysates of the bacteria (5×10^6 bacteria) separated on 10 % SDS-PAGE gels and transferred to nitrocellulose membranes. Anti-Flagellin B (anti-FlaB) antibody (H9724) (a gift from S. Bergstöm) was used as a control.

Flow cytometric analysis was used to detect OspA and OspB molecules on the surface of Osp complemented borrelia. Briefly, bacteria were washed and resuspended as described above and stained with monoclonal anti-OspA (H5332) or anti-OspB (H6831) antibodies for 15 min at room temperature. Alexa fluor 488 donkey anti-mouse IgG (Invitrogen, Carlsbad, CA, USA) was used as a secondary antibody. Fluorescence was measured using the FL1-H channel of a flow cytometer (FACScalibur, Becton Dickinson, Franklin Lakes, NJ, USA). The flow cytometric data were analyzed using CellQuest software (Becton Dickinson).

4.1.4 *Escherichia coli*

A clinical uropathogenic *E. coli* isolate was used for DC stimulations and in vivo studies. The bacteria were grown in Luria-Bertani medium at 37 °C overnight, diluted in fresh medium and counted in a Neubauer counting chamber. Bacteria were then centrifuged at 8000 x g for 3 min and resuspended in RPMI medium (Gibco Invitrogen, Carlsbad, California, USA) or PBS.

4.2 Cell isolation and differentiation

4.2.1 Neutrophils

Human neutrophils were isolated from the heparinized venous blood of healthy volunteers. Dextran T-500 (Pharmacia Biotech, Uppsala, Sweden) sedimentation and Percoll (Pharmacia Biotech) gradient centrifugation were used. 20-50 ml of venous blood (10 ml per tube) was incubated with 6 % Dextran T-500 for approximately 30 min. After sedimentation, the supernatant was layered over 56 % Percoll and centrifuged at 450 x g for 15 min. The remaining erythrocytes were lysed with 0.87 % ammonium chloride. The cells were centrifuged at 150 x g for 10 min and washed twice with PBS. After counting the cells in a Neubauer counting chamber they were suspended in Hank's balanced salt solution (HBSS) supplemented with 0.25 % bovine serum albumin (BSA, Wilfrid Smith, Edgvare, UK) (HBSS/BSA) at a concentration of 10^7/ml for phagocytosis and chemiluminescence assays and in RPMI supplemented

with 1 mg/ml BSA (RPMI/BSA) at a concentration of $2.5x10^6$/ml for chemotaxis assays.

4.2.2 In vitro generation of dendritic cells

PBMC were isolated from buffy coats of healthy donors (Finnish Red Cross Blood Transfusion Service, Turku, Finland) by Ficoll-paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. $CD14^+$ positive monocytes were isolated by magnetic-activated cell separation (MACS) with MACS CD14 micro beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. 10^6 cells per well were cultured on 24-well plates (Costar, Cambridge, MA, USA) in Iscove's modified Dulbecco's medium (IMDM; Gibco BRL, Grand Island, NY, USA) with phenol red, supplemented with 10% heat inactivated fetal calf serum (FCS, HyClone Logan, Utah, USA), 1 mmol/l of HEPES, 0.1 mmol/l of 2-mercaptoethanol and 100 mg/ml of gentamycin (Biological Industries, Kibbutz beit Haemek, Israel). Recombinant human IL-4 (1000 IU/ml; R&D systems, Minneapolis, MN, USA) and granulocyte-macrophage colony-stimulating-factor (GM-CSF, 375 IU/ml; R&D systems) were added to the culture on days 1, 3 and 5. Prior to addition of stimuli, DCs were repeatedly shown to be $CD1a^+$, $CD14^{-/low}$ by flow cytometry

Alternatively, peripheral blood mononuclear cells were isolated by Ficoll-paque density gradient centrifugation. Mononuclear cells were further subjected to a second density gradient centrifugation using 1,064 g/ml Percoll according to a published protocol (Lehner and Holter 2002). Monocytes were further adhered for 1 h in RPMI without serum and nonadherent cells were removed. Adhered monocytes were cultured in RPMI supplemented with Glutamax, 10% heat inactivated FCS, 1 mmol/l of HEPES, and 100 mg/ml of gentamycin (Biological Industries,). Recombinant human IL-4 (1000 IU/ml) and GM-CSF (375 IU/ml) were added to the culture on days 1, 3 and 5. After 6-7 days of culture, cells were shown to be $CD14^{low/-}$, $CD1a^{high}$ and DC-$SIGN^{high}$.

Bg, Bb s.s. and Ba used at various bacteria:cell ratios, *E. coli* (bacteria:cell ratio 9:1) or LPS from *E. coli* serotype O127:B8 (final concentration 1 µg/ml; Sigma, St. Louis, MO, USA) were added on day 7. Unstimulated DCs were used as control cells.

4.3 Antibodies and other reagents

The antibodies used in flow cytometric studies are listed in Table I, and antibodies used in Western blotting experiments are listed in Table II. When indicated, inhibitors of p38 mitogen activated protein kinase p38 (SB202190, final concentration 10 µM), extracellular signal-regulated kinase ERK (PD98059, final concentration 50 µM) and c-jun N-terminal kinase JNK (SP600125, final concentration 10 µM) (all from Calbiochem, San Diego, CA, USA) were added 15 min prior to stimulants, and cells were incubated at 37 °C before addition of stimulants. Inhibitor for NF-kB (Celastrol, Calbiochem) was added 15 min prior to stimulants at a final concentration of 270 nM or 1000 nM (adjusted according to information on the manufacturer's data sheet) and

inhibitor for STAT1 (fludarabine, Fludara, Schering, Berlin, Germany) was added 15 min prior to stimulants at a final concentration of 50 μM (Frank et al. 1999).

4.4 Flow cytometry and Western blotting

4.4.1 Flow cytometric analysis of dendritic cell and neutrophil surface molecules

DCs were generated as described above. Bg ($10x10^6$ bacteria per 1 $x10^6$ cells) and *E. coli* LPS (final concentration 1 μg/ml) were added on day 7 of culture. Ba or Bb s.s. were used at the above mentioned bacteria to cell ratio when indicated. For dose dependency studies DCs were stimulated with $1x10^6$, $10x10^6$ or $100x10^6$ Bg. Cells were stained with a phycoerythrin (PE)-conjugated monoclonal antibody for CD38, CCR7 or CD83 (BDPharmingen, San Diego, CA, USA) before stimulation and after 7, 24 and 48 h of stimulation. Isotype-matched antibodies were used as negative controls. Cells were analyzed using the FACScalibur flow cytometer with CellQuest software or using Cyflogic software.

Neutrophils were incubated with different concentrations of bacteria for 30 min at 37 °C in RPMI containing 1 mg/ml BSA (RPMI/BSA). Neutrophils were washed once to discard the remaining bacteria and stained with a PE-conjugated monoclonal antibody for FPR (BDPharmingen).

In another setting, neutrophils were incubated with 1:5 bacteria for different time points at 37 °C in RPMI/BSA. Neutrophils ($3x10^5$) were washed once to discard remaining bacteria and incubated with different concentrations of fMLP at 37 °C for 30 min. Neutrophils were stained with a PE-conjugated monoclonal antibody for CD38 (BDPharmingen). Cells were analyzed using flow cytometry and CellQuest and Cyflogic software.

4.4.2 Use of flow cytometry in other assays

Flow cytometric techniques were also used in the DC migration studies (4.5.4), in the in vivo migration studies (4.7), in the bacterial studies when studying the activation of complement (4.6.2) and when studying the expression of borrelial outer surface proteins (4.1.3).

4.4.3 Western blotting of dendritic cell signalling proteins

For Western blotting, $2x10^6$ unstimulated DCs or DCs stimulated for 30 min (p38 blotting) or 24 h (STAT1 blotting) with LPS (final concentration of 1 μg/ml) or Bg (9 x 10^6 bacteria) were lysed in Buffer C (420 mM NaCl, 25 % [volume/volume] glycerol, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 20 mM HEPES [pH 7.9], 0.5 mM dithiothreitol) and 0.5 mM phenylmethylsulfonyl fluoride (PMSF; pH 7.4) and centrifuged at 13 000 x g at 4 °C for 15 min. The protein content of the supernatant (whole cell extract) was measured by protein assay (Bio-Rad, Hercules, CA, USA). SDS NuPage sample buffer (Invitrogen, Carlsbad, CA, USA) was added to the supernatant and samples were incubated at 95 °C for 8 min. Proteins were separated on 10 % SDS-PAGE gels and gels were transferred to nitrocellulose membranes. Antibodies specific for p38, phosphorylated p38, STAT1 and phosphorylated STAT1 (Cell Signalling, Danvers, MA, USA) and HRP-conjugated goat

anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology) were used to probe the membranes. Ponceau S (Sigma) staining confirmed that there were similar amounts of protein in the samples after protein transfer.

4.5 In vitro cell function assays

4.5.1 Neutrophil chemotaxis assay

Chemotaxis of human neutrophils towards chemoattractants C5a and fMLP was determined using a 48-well microchemotaxis chamber (Neuroprobe, Bethesda, MD, USA) in which a 3 µm pore-sized nitrocellulose filter (Neuroprobe) separates the upper and lower wells. Neutrophils ($0.125x10^6$) were incubated with 0.75 $x10^6$ bacteria at 37 °C for 30 min. The cells were washed once and resuspended in RPMI/BSA at $2.5x10^6$ cells/ml. 25 µl aliquots of 0.05 % partially purified C5a, $1x10^{-9}$ M fMLP (Sigma-Aldrich, St Louis, MO, USA) or RPMI/BSA were added to the lower wells and neutrophils, added to the upper wells, were allowed to migrate at 37 °C for 30 min.

In another setting, borreliae were added to the chemoattractant solutions prior to the experiment. 25 µl aliquots of chemoattractant solution ($1x10^{-9}$ M fMLP, 0.05 % C5a) or control medium (RPMI/BSA) containing $0.5x10^6$ bacteria were added to the bottom wells. 50 µl of neutrophil suspension ($0.125x10^6$ neutrophils per well) was added to the upper wells and neutrophils were allowed to migrate in a humified atmosphere at 37 °C for 30 min.

After incubation, the membrane was fixed with methanol and stained with Mayer's hematoxylin solution (Sigma-Aldrich). The dehydrated membranes were mounted on microscope slides and the migration depth of neutrophils (µm) was quantified by measuring the distance from the upper surface of the membrane to the leading front of two cells from five fields of the sample area using 40x magnification. **Figure 6 (A)** shows the protocol for the first setting.

4.5.2 Neutrophil phagocytosis assay

Normal human serum (NHS) was used as the source of complement. Serum was prepared by allowing venous blood from 6 healthy volunteers to clot separately at room temperature for 30 min. After centrifuging at 1200 x g for 10 min the serum was removed from the samples, pooled, divided into aliquots and stored at –70 °C. Tested by two different enzyme immunoassays (an in-house EIA (Viljanen and Punnonen 1989) and a commercial EIA (IDEIA™ *Borrelia burgdorferi* IgG and IgM; Oxoid, Cambridgeshire, UK) the serum pool was found negative for IgG and IgM antibodies against Bb. Heat-inactivated serum (NHSin) was prepared by incubating the serum at 56 °C for 30 min.

Bacteria were washed once in PBS and resuspended in 500 µl of PBS and stained with CFSE (Molecular Probes, Eugene, Oregon, USA) for 10 min at room temperature in the dark in a final volume of 1 ml (final concentration of CFSE 5 µM). Bacteria were washed twice in PBS containing 5 % FCS, counted and resuspended in HBSS/BSA at a concentration of $1.5x10^8$/ml. $0.5x10^6$ neutrophils, $3x10^6$ bacteria and different

concentrations of NHS (14 %, 0.7 % or 14 % NHSin) were incubated for 5, 20 or 60 min at 37 °C, and the phagocytosis was stopped by adding 500 µl ice-cold PBS into the tubes. The samples were washed once, fixed with PBS containing 1 % formaldehyde, and analyzed using flow cytometry.

4.5.3 Measurement of neutrophil oxidative burst

Oxidative burst of neutrophils was measured using a chemiluminometric assay. Bacteria were counted and suspended as described above. Neutrophils ($1.2x10^6$ cells) were incubated with luminol (Sigma) in polystyrene measuring cuvettes at 37 °C for 30 min. The total volume in the cuvettes was 500 µl. Bacteria ($6x10^6$ bacteria, Bacteria-to-neutrophil-ratio 5:1) and complement were added at the concentrations mentioned above (14 % NHS, 0.7 % NHS, 14 % NHSin). The cuvettes were placed in a luminometer (Luminometer 1250; Wallac, Turku, Finland) and measuring was performed with MultiUse software (BioOrbit, Turku, Finland). Chemiluminescence was measured at 150 second intervals for 60 min. Duplicate cuvettes for each sample were used.

4.5.4 Dendritic cell migration assay

Chemotaxis of human DCs towards CCL19 and CCL21 (R&D systems) was determined using a Transwell migration assay. 10^5 LPS-stimulated and 10^5 Bg-stimulated DCs were allowed to migrate towards 600 µl of 0,1 % CCL19 and 0,1 % CCL21 in a 24-well Costar Transwell chamber (8 µm pore size; Corning, Lowell, MA, USA) in a humified atmosphere at 37 °C for 90 min. Cells that had migrated to the lower chamber were collected, washed, fixed and counted by flow cytometry using TruCount beads (Becton Dickinson). **Figure 6 (B)** shows the protocol for the assay.

4.6 Complement assays

4.6.1 Serum sensitivity assay

The serum sensitivity of B31, B313, $B313_{OspAB}$ and $B313_{OspA}$ was studied by incubating $2x10^7$ bacteria at 37 °C for 2 h with different concentrations of NHS in a total volume of 350 µl (Suhonen et al. 2002). After incubation, 10 µl aliquots were pipetted on glass slides and the bacteria were examined using phase contrast microscopy to categorize the serum-resistant and serum-sensitive strains. Bacteria were considered killed when loss of motility and/or excessive blebbing were observed.

4.6.2 iC3b deposition assay

B31, B313, $B313_{OspAB}$ and $B313_{OspA}$ were incubated with different NHS concentrations as described above at 37 °C for 30 min. The bacteria were stained with an anti-iC3b antibody (A209 Quidel Corporation, San Diego, California, USA) and, after washing with PBS supplemented with 2 % BSA and 5 mM MgCl (PBS-Mg-BSA), bacteria were stained with an isotype-specific, PE-conjugated secondary antibody (goat anti-mouse IgG_{2b}, Southern Biotechnology Associated, Inc., Birningham, AL). The bound iC3b was detected using flow cytometry.

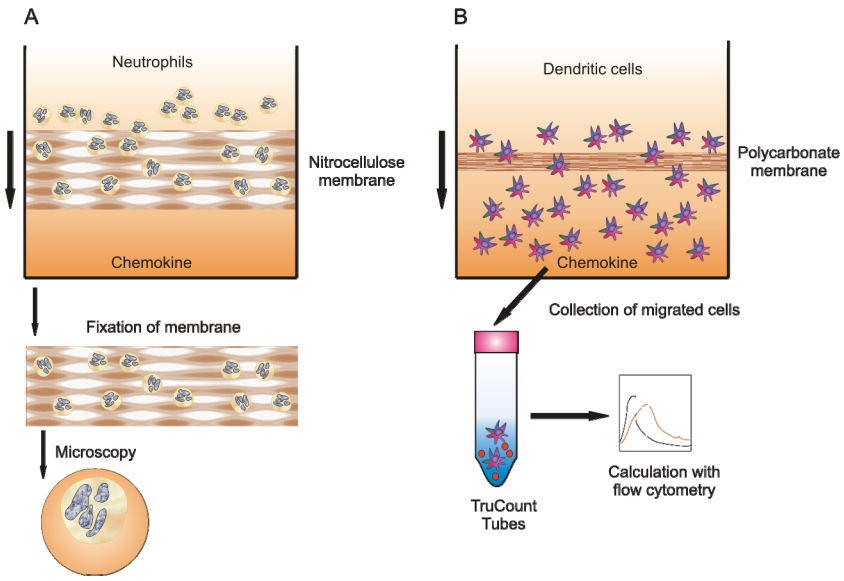

Figure 6. Protocols for in vitro migration experiments. **(A)** Neutrophils are placed in the upper chamber of a microchemotaxis chamber. A 200 µm nitrocellulose membrane separates the upper chamber from the lower chamber containing the chemokine solution. Cells are allowed to migrate into the nitrocellulose membrane through 3 µm pores for 30 minutes, after which the membrane is fixed and the migration depth of neutrophils (µm) is quantified by measuring the distance from the upper surface of the membrane to the leading front of two cells from five fields of the sample area. **(B)** Dendritic cells are placed in the upper chamber of a Transwell chamber. A thin polycarbonate membrane separates the upper chamber from the lower chamber containing the chemokine solution. Cells are allowed to migrate through the membrane through 8 µm pores for 90 minutes. Cells that have migrated to the lower chamber are collected and counted using TruCount tubes and flow cytometry.

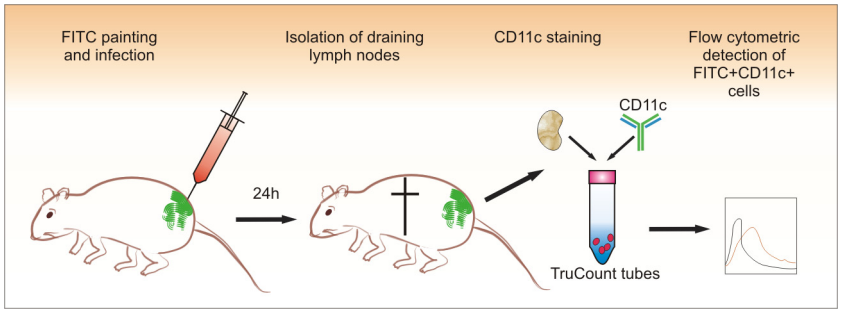

Figure 7. Protocol for in vivo migration assay. Mice are painted with FITC on their lower back followed by intradermal injection of Bg or *E. coli*. 24 hours later mice are killed and draining inguinal lymph nodes are collected. A single-suspension is prepared and cells are stained with a CD11c antibody. The number of FITC+/CD11c+ cells is calculated using TruCount beads and flow cytometry

4.7 Methods for studying dendritic cell migration in vivo

4.7.1 FITC painting assay

A method utilizing FITC painting and simultaneous injection of bacteria (Bg and *E.* coli) was used (Rotta et al. 2003; Bonasio et al. 2006). Draining lymph nodes were searched for FITC and CD11c double positive cells. Live *E.* coli was used as a control bacterium because the doses could be made comparable using two live bacteria. The CD11c antibody used showed nonspecific binding to a population of small cells, and therefore the results were analyzed using a multiple-gating strategy to include only DCs in the final analysis.

Balb/c mice were obtained from the Turku University Animal Center (Turku, Finland) and were 4-6 weeks of age at the time of experiments. The dorsal skin of mice was shaved and 200 µl of a 0.5 % solution of FITC in acetone and olive oil (4:1 volume/volume) was applied on the skin (Rotta et al. 2003; Bonasio et al. 2006). Control mice were also shaved and olive oil was applied on the skin. Immediately after FITC painting, 10^6 Bg or *E. coli* (in 100 µl PBS) or 100 µl PBS was injected intradermally. Mice were killed after 24 hours and their organs were collected for further analysis. Inguinal lymph nodes were shredded and treated with 1 mg/ml collagenase D (Roche Diagnostics, Mannheim, Germany) at 37°C for 45min. Cells were passed through a 70-µm cell-strainer (BD Falcon, Franklin Lakes, NJ, USA) to generate a single-cell suspension. After washing twice with PBS, cells were stained with an allophycocyanin (APC)-conjugated monoclonal antibody for CD11c or matching isotype control (BDPharmingen). Two thirds of the cells were stained with anti-CD11c and one third with the isotype control. Before analysis, samples were transferred to TruCount tubes (Becton Dickinson) to enable counting of the absolute number of double-positive (FITC+/CD11c+) cells. The counting was carried out using flow cytometry. The County Administrative Board granted permission for the animal studies (Permission numbers 1359/03 and 1656/06). A schematic protocol for the in vivo migration study can be seen in **Figure 7.**

4.7.2 Immunohistochemistry

Lymph nodes were embedded in Tissue-Tek optimal cutting temperature compound (Sakura, Finetek, Torrance, CA, USA) and snap-frozen in liquid nitrogen. Sections of 6 µm in thickness were cut and fixed with acetone. The sections were stained with an anti-CD11c antibody (BDPharmingen) and a biotinylated anti-hamster IgG cocktail was used as a secondary antibody, and Anti-Hamster Ig HRP detection kit (BDPharmingen) was used for visualization according to the manufacturer's instructions.

4.8 Array techniques

4.8.1 RNA preparation and cDNA microarray hybridization

DC RNA was extracted before stimulation at 0 h and after 2, 4, 6 or 8 h of Bg or LPS stimulation using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). cDNA was synthesized and labelled with fluorescent dyes. The reference sample was labelled with

FluoroLink™ Cy3-dUTP (Amersham Pharmacia Biotech, Uppsala, Sweden) and the samples of interest with Cy5-dUTP (Amersham Pharmacia Biotech). The reference sample and the sample of interest were mixed in one tube before hybridization.

Hybridization was carried out using direct and indirect comparison approaches. In direct comparison, LPS-stimulated DC RNA was used as the reference sample and Bg-stimulated DC RNA was used as the sample of interest in the same array. In the indirect comparison method, unstimulated DC RNA was used as a reference sample and LPS or Bg-stimulated DC RNA was used as samples of interest.

HUM-16K cDNA microarrays (Finnish DNA Microarray Centre, Turku, Finland), representing approximately 10 500 genes, were hybridized with cDNA originating from 20 μg of total RNA. The hybridization was performed as previously described (Nikula et al. 2005), and three biological and two technical replicates at each time point were hybridized independently. **Figure 8** shows a simplified protocol for microarray hybridization.

4.8.2 Microarray data analysis

Hybridized HUM 16K cDNA microarrays were scanned using the ScanArray® Express optical scanner (Perkin Elmer, Wellesley, MA, USA) to determine the fluorescent intensities of Cy3 and Cy5 dyes at each spot. Microarrays were scanned first at a 633 nm and then at a 543 nm wavelength to acquire separate images for Cy3 and Cy5 dyes. The images were combined and the spots were identified using ScanArray® Express microarray analysis software (Perkin Elmer). Spots were specified using the histogram method. Microarray data were analyzed using Kensington software (InforSense Knowledge Discovery Environment, London, UK). Gene expression levels were determined from the background-decreased log-transformed intensity ratio values. Systematic variation in measured intensity values was eliminated using lowess-normalization. A gene was considered differently expressed if at least a twofold difference between the sample of interest and the reference sample was seen in all three replicates and the difference was also statistically significant at a risk level of $p<0.05$. Statistical significance was computed using the two-sided t-test. Hierarchical clustering was used to visualize gene expression profiles (reviewed in Pelkonen 2006). To classify genes and to divide them into functional groups, different databases (GenBank, Gene, Kegg and GeneOntology) were searched. The results concerning the expression level of a limited number of genes were verified using quantitative RT-PCR carried out at the Finnish DNA Microarray Centre.

4.8.3 Cytokine antibody array

Cell-free culture media supernatants of unstimulated, Bg-stimulated and LPS-stimulated DCs were collected after 8h stimulation. RayBio®Human Cytokine Antibody Array C Series 1000 (RayBiotech, Inc, Norcross, GA, USA) containing 120 different cytokines was used to detect cytokine levels in culture medium. The arrays were prepared according to the manufacturer's instructions. Membranes were exposed to x-ray film (Biomax XAR, Kodak, New Haven, CT, USA) within 30 min of exposure to the substrate. Biotin-conjugated IgG served as a control, and each membrane

contained six positive control spots. The image was analyzed with MCID Image analysis system M5+ software (InterFocus Imaging Ltd, Linton, UK). Baseline optical density was subtracted from total optical density. The samples were made comparable by subtracting the optical density level of the membrane's negative control from all studied spots and by dividing the optical density of the studied spots by the optical density level of the membrane's positive controls.

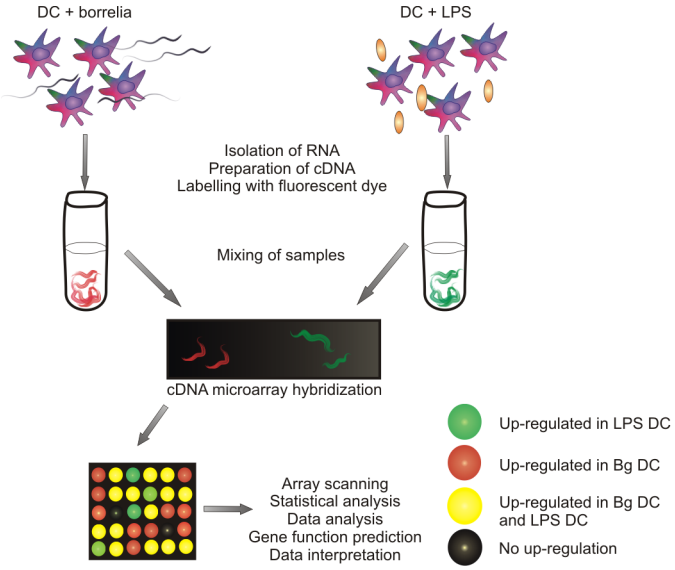

Figure 8. Protocol for microarray experiments. Dendritic cells (DC) are stimulated with *B. burgdorferi* or LPS for 24 h and DC RNA is isolated. cDNA is prepared and labeled with different fluorescent dyes. The cDNA microarray is hybridized and scanned after which a statistical analysis is carried out to identify differentially expressed genes (green, red or yellow). After obtaining the group of differentially expressed genes, their functions need to be predicted and the significance of each finding has to be established.

Table I The anti-human antibodies used in the flow cytometric studies

antibody	source
anti-CD1a-phycoerythrin (PE)	BDPharMingen
anti-CD14-PE	BDPharMingen
anti-CD80-PE	BDPharMingen
anti-CD83-PE	BDPharMingen
anti-CD86-PE	BDPharMingen
anti-CD38-PE	BDPharMingen
anti-HLA-DR-PE	BDPharMingen
anti-CCR7-PE	BDPharMingen
anti-DC-SIGN-PE	BDPharMingen

Table II The antibodies used in Western blotting

antibody	source
mouse anti-human CD38	BDPharMingen
rabbit anti-human phospho p38	Cell Signalling
rabbit anti-human p38	Cell Signalling
rabbit anti-human phospho STAT1	Cell Signalling
rabbit anti-human STAT1	Cell Signalling
goat anti-mouse IgG-HRP	Santa Cruz Biotech
goat anti-rabbit IgG-HRP	Santa Cruz Biotech

5 RESULTS

5.1 Interaction between *B. burgdorferi,* human neutrophils and the complement system

5.1.1 Interactions between *B. burgdorferi* OspA and OspB and the complement system

Several borrelial surface proteins have previously been associated with complement resistance of Bb. According to previous studies, B31 is complement sensitive and B313 complement resistant. The role of OspA and OspB in complement sensitivity and deposition of iC3b as a sign of complement activation was investigated using B31, B313 and the generated recombinant strains $B313_{OspAB}$ and $B313_{OspA}$. B31, as expected, was serum-resistant at all studied NHS concentrations, whereas all other strains were serum-sensitive in 28% and 14% NHS. $B313_{OspAB}$ tolerated 0.7% NHS slightly better than B313 and $B313_{OspA}$.

In 14% NHS, nearly equal amounts of iC3b were deposited on $B313_{OspAB}$, B313, and $B313_{OspA}$ surfaces. In 0.7% NHS $B313_{OspAB}$ had slightly smaller amounts than B313 and $B313_{OspA}$. The results suggest that OspB is able to inhibit complement activation at low complement concentrations.

5.1.2 Interactions between *B. burgdorferi* OspA and OspB and human neutrophils

B. burgdorferi s.s B31, B313 and OspA and OspA + OspB-complemented mutant strains were used to study the role of these surface proteins in the chemotaxis, phagocytosis and oxidative burst of neutrophils. Neutrophils preincubated with any of the strains showed significantly reduced fMLP-mediated chemotaxis (untreated neutrophils 113.3 ±3.21 µm vs. borrelia-treated neutrophils 75.0-87.5 ± 2.1-9.9 µm varying between strains). However, Bb strains caused no change in the random migration and C5a-mediated chemotaxis of neutrophils. In a further experiment the effect of borreliae, that were added simultaneously with fMLP or C5a in the chemotaxis well, (as opposed to pre-incubation with neutrophils) on neutrophil chemotaxis and the chemotaxis induced by borreliae alone were evaluated. No significant inhibition of C5a-mediated or fMLP-mediated chemotaxis was seen when different Bb strains were added simultaneously with fMLP or C5a into the bottom wells. $B313_{OspA}$ was the only strain to induce neutrophil chemotaxis alone, whereas B31, B313, and $B313_{OspAB}$ alone did not stimulate neutrophil chemotaxis.

To study the role of OspA and OspB in phagocytosis by neutrophils, the percentages of neutrophils that had ingested CFSE-stained borreliae at different time points were counted. The maximum oxidative burst induced by these strains was measured with a luminometer. In 0.7% NHS the phagocytosis of $B313_{OspAB}$ was significantly lower than that of B313 and that of $B313_{OspA.}$ A corresponding trend was seen in 14% NHS, but the difference was not statistically significant. The phagocytosis of $B313_{OspAB}$ was similar to that of the wild-type strain B31 in the presence of 0.7% NHS. The oxidative

burst induced by $B313_{OspAB}$ in 0.7% NHS was significantly lower than that induced by $B313_{OspA}$.

The results of the phagocytosis and oxidative burst assays suggest that the presence of OspB is unfavourable to phagocytosis and oxidative burst especially at low complement concentrations. In contrast, the presence of OspA enhances oxidative burst.

5.1.3 Effect of *B. burgdorferi* and LPS on CD38 and formyl peptide receptor expression on human neutrophils

The results of this experiment are not presented in the separate articles and are thus given in more detail. As described above, neutrophils incubated with Bb showed a chemotactic defect towards fMLP. To clarify the mechanism behind defective chemotaxis, formyl peptide receptor (FPR) expression on neutrophils after incubation with Bb s.s. and Bg was investigated. However, FPR expression was unchanged in Bg- and Bb s.s.-stimulated neutrophils compared to control neutrophils.

Previously, a link between defective CD38 expression and defective chemotaxis of neutrophils has been shown (Fujita et al. 2005). When neutrophils are incubated with fMLP, CD38 expression decreases as chemotaxis occurs (Fujita et al. 2005). An experiment was set up to study this phenomenon. Neutrophils were first incubated with LPS, Bg, Bb s.s. or control medium for 60 min. After washing, cells were further incubated with fMLP for 30 minutes and CD38 expression was analyzed using flow cytometry. The results show that after incubation with fMLP, CD38 expression decreased as described previously in the literature. After incubation with LPS, CD38 expression remained unchanged and when these cells were incubated further with fMLP, CD38 expression decreased as described. However, when neutrophils were incubated with Bb s.s. or Bg before fMLP, CD38 expression already decreased at such a magnitude, that a further decrease after incubation with fMLP could not be seen **(Figure 9).**

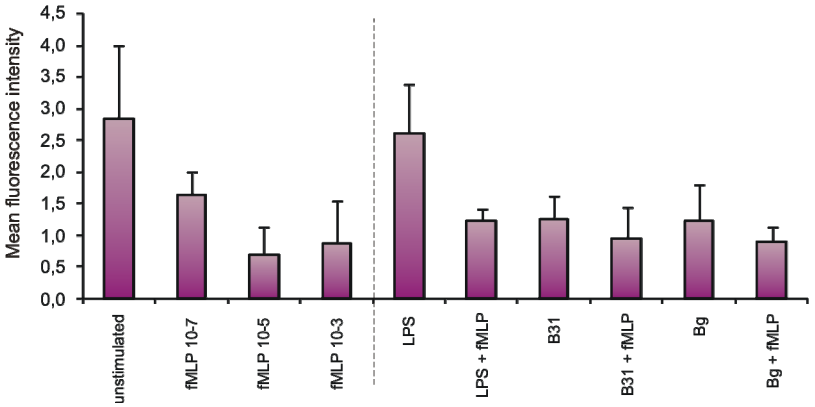

Figure 9. Effect of different concentrations of fMLP and LPS, *B. burgdorferi ss.* strain 31 (B31) or *B. garinii* (Bg) on neutrophil CD38 expression. Data are shown as mean fluorescence intensity ± standard deviation (geo mean fluorescence intensity subtracted by geo mean fluorescence intensity of negative control) of three experiments done with neutrophils from different donors.

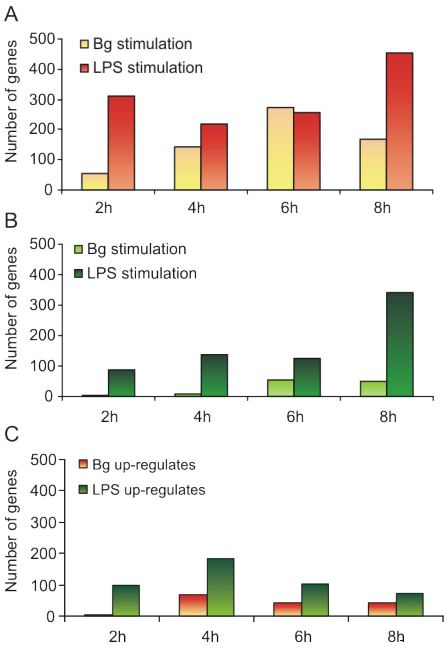

Figure 10. Differentially regulated genes at different time points. **(A)** Number of up-regulated genes in DCs after *B. garinii* (Bg) and LPS stimulation as observed by indirect comparison (unstimulated cells = reference sample, Bg stimulated or LPS stimulated cells = sample of interest). **(B)** Number of down-regulated genes in DCs after Bg and LPS stimulation. **(C)** Differentially regulated genes at different time points- direct comparison. Numbers of Bg and LPS specific up-regulated genes as observed by the direct comparison method (LPS stimulated cells = reference sample, Bg stimulated cells = sample of interest).

5.2 Interaction between *B. burgdorferi* and human dendritic cells

5.2.1 Transcriptional response of human dendritic cells to *B. garinii*

To clarify interactions between DCs and Bb, a cDNA microarray experiment was carried out. The transcriptional response of DCs after Bg and LPS stimulation was studied at 2, 4, 6 and 8 hours of stimulation. The number of genes regulated by LPS was greater at all time points studied **(Figure 10)**. A population of similarly regulated genes was found, while the majority of genes were differentially regulated by Bg and LPS. Genes that were similarly up-regulated by Bg and LPS included an endocytosis-associated gene (RAB5A), a cell junction protein-coding gene (CLDN1), genes encoding inflammatory cytokines (TNF-α, IL-1α , IL-1β and IL-6), and TNF-α -related genes [TNF-α-induced proteins 3 (apoptosis inhibitor) and 6, TNFR superfamily member 5, and TRAF1]. Genes encoding IL-7R, neutrophil chemoattractants [CXCL1 (GROα), CXCL2 (GROβ)], DC differentiation and maturation markers (human ADAM19, CD83, SLAMF1), macrophage stimulants and other chemokines [CSF-1, CCL3 (MIP-1α), CCL20 (MIP-3α)], PG receptor EP4, and adhesion molecules (CD58, tenascin C, ninjurin 1) were also similarly up-regulated. Genes encoding apoptosis inhibitors [BIRC 2, BIRC 3, BCL2A1, PBEF1 (inhibits neutrophil apoptosis), CFLAR] and various genes related to metabolism, signal transduction, transcription, and transport were up-regulated. It seems that despite of the silent nature of the EM a great number of inflammatory genes are regulated.

Bg specific up-regulated genes include genes encoding three matrix metalloproteinases (MMP9, MMP12, MMP19). Genes encoding the chemokine receptor CXCR4 and chemokines CXCL7 (LDGF-PBP) and CCL2 (MCP-1) were up-regulated as well as

genes encoding protein kinase C,nu (many functions, e.g. in B/T-cell receptor mediated signalling) and TRAF3. Cell cycle/apoptosis related genes such as BTG3 (antiproliferative, may interact with CCR4), CYCS (mitochondrial electron transport, involved in apoptosis initiation) and genes encoding SGCD (cytoskeleton, forms a link between f-actin and extracellular matrix), CDC42EP3 (mediates actin cytoskeleton reorganization at the plasma membrane), NFKBIA (inhibits NFKB complex) and NAB2 (transcriptional repressor) were up-regulated. Bg specific down-regulated genes included those encoding the adhesion molecule CD31 (PECAM1, counter-receptor of CD38,) and SAMHD1 (dendritic cell-derived IFNG-induced protein). **Figure 11** shows a hierarchical cluster of differentially regulated immunity- and inflammation-associated genes (indirect comparison method).

The gene encoding CD38 was found not to be up-regulated by Bg, whereas LPS highly increased its expression. This finding was confirmed further by RT-PCR and flow cytometry. Using RT-PCR, the gene encoding CCR7, a dominant mediator of DC migration, was also studied and it was found to be eleven-times more up-regulated in LPS-stimulated DCs compared to Bg DCs.

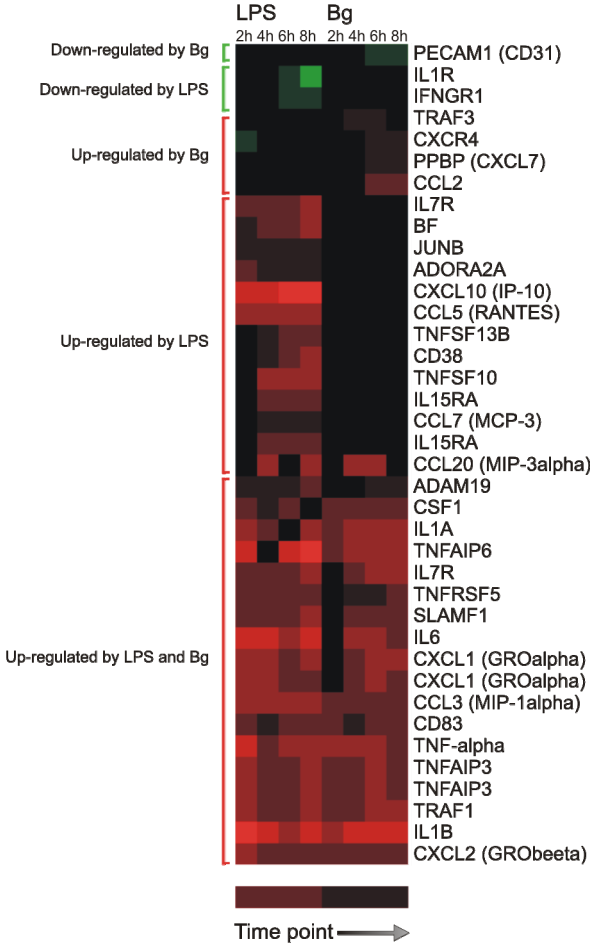

Figure 11. Representative simplified hierarchical cluster of immunity and inflammation related genes up-regulated by Bg, LPS, or both. The genes were grouped according to function on the basis of public databases and published studies. Red indicates up-regulation and green down-regulation. PECAM1 (platelet-endothelial cell adhesion molecule 1), TRAF3 (Tnf-receptor associated factor 3), PPBP (Pro-platelet basic protein), BF (B-factor, properdin), JUNB (jun B proto-oncogene), ADORA2A (adenosine A2a receptor), TNFSF13B (tumor necrosis factor (ligand) superfamily, member 13b), ADAM19 (a disintegrin and metalloproteinase domain 19 (meltrin beta)), CSF1 (colony stimulating factor 1 (macrophage)), TNFAIP6 (tumor necrosis factor, alpha-induced protein 6), SLAMF1 (signaling lymphocytic activation molecule family member 1), TRAF1 (TNF receptor-associated factor 1), IP-10 (IFN-inducible protein 10), GROα (growth-related oncogene α).

5.2.2 Cytokine secretion of human dendritic cells in response to *B. garinii*

To compare transcriptional and protein level results, the levels of different cytokines produced and secreted by unstimulated DCs, LPS-stimulated DCs and Bg-stimulated DCs were measured using the RayBio® Human Cytokine Antibody Array at 8 hours of stimulation. In general, LPS and Bg seemed to induce similar cytokine secretion profiles in DCs, and significant differences were seen only in a small number of cytokines. DCs were found to constitutively secrete Eotaxin-2, IL-4, IL-8, MIP-1α, MCP-1, MCP-4, PARC, TARC, EGF-R, TIMP1, TIMP2 and uPAR. Both Bg and LPS induced the secretion of eotaxin-2, IL-10, TNFα, GRO, IL-8 and MIP-1α, whereas the secretion of TIMP-1 and TIMP-2 was decreased. Bg specifically increased the secretion of MCP-1, whereas LPS did not specifically induce the secretion of any cytokine. However, eotaxin-2, PARC, uPAR and TIMP-1 secretion decreased in LPS-stimulated cells less than in Bg-stimulated cells.

5.2.3 Effect of *B. garinii* and LPS on CD38, CCR7 and CD83 expression of dendritic cells

The mRNA level experiments showed that CD38 and CCR7, two factors important in DC migration, were deficiently up-regulated in Bg-stimulated DCs. Flow cytometric assays were performed to confirm the expression at protein level. The results showed that there was a clear difference in CD38 expression between LPS-stimulated and Bg-stimulated DCs. However, the difference in CCR7 and CD83 expression was not as great as the difference in CD38 expression.

To investigate possible inter-individual variation in DC surface molecule expression, CD38, CCR7 and CD83 expressions after LPS and Bg stimulation of PBMC differentiated DCs from 19 individuals were compared. LPS induced a strong expression of CD38, CCR7 and CD83 on all DCs tested, whereas Bg-induced CD38 expression remained low on all cells (percentage of CD38 positive cells, Bg-stimulated DCs 1,06 ± 1,44 vs LPS-stimulated DCs 23,8 ± 19,7, $P<0.01$). CCR7 and CD83 expressions were also significantly lower, but not absent, on Bg-stimulated DCs than on LPS-stimulated DCs.

5.2.4 Characterization of defective CD38 expression on *B. garinii*-stimulated dendritic cells

It has been previously shown that the transcriptional responses in DCs induced by *E. coli* can almost completely be mimicked by LPS (Huang et al. 2001). The results of our study show that the responses induced by *E .coli* and LPS derived from *E. coli* were also similar regarding CD38, CCR7 and CD83 protein level expression. Next, the dose dependency of surface molecule expressions was determined. The expression of CD38 remained negative regardless of Bg dose, and was thus dose independent. CCR7 expression remained at the same level regardless of Bg dose. In contrast, CD83 expression was highest when the cells were stimulated with 100 $x10^6$ Bg, thus showing dose-dependency.

To investigate whether the inability to induce CD38 expression on DCs is a common property among borrelia genospecies, CD38 expression on Bb s.s.- and Ba-stimulated DCs was analyzed and compared to Bg- and LPS-induced expression. As with Bg, Bb s.s. and Ba did not induce expression of CD38 on DCs.

To investigate whether the lack of CD38 expression on Bg-stimulated DCs is due to an active process by the bacteria, the following strategy was applied:

First, DCs were stimulated with Bg and LPS simultaneously to see whether Bg could inhibit LPS induced CD38 expression. The mean±standard deviation of 9 experiments showed that CD38 expression was similar after LPS and LPS+Bg stimulation (percentage of positive cells 25.7±18.9% for LPS-stimulated DCs vs 25.7±21.1% for LPS+Bg-stimulated DCs).

Second, DCs were stimulated with heat-inactivated Bg to see whether inhibition of CD38 up-regulation is a property of only live Bg. However, heat-inactivated Bg induced similar responses in CD38, CCR7 and CD83 expressions as live Bg suggesting that live borrelia or borrelial proteins in their original conformation are not a prerequisite for defective CD38 expression.

Third, DCs were stimulated with Bb s.s. strain B313. Again, CD38 expression was similar after Bg and B313 stimulation suggesting that the borrelial surface proteins encoded on the plasmids missing from B313 were not responsible for defective expression of CD38 on the DC surface. This also suggests that the lipoproteins missing from B313 do not block the action of surface molecules that would be able to induce CD38 expression.

5.2.5 Signalling pathways regulating CD38 expression

To understand the mechanisms that result in defective CD38 molecule expression in Bg DCs, the role of different signalling pathways in the up-regulation of CD38 was investigated. The specific p38 inhibitor, SB 202190, efficiently inhibited CD38 up-regulation in LPS-stimulated DCs (mean percentage of positive cells decreased from 30.6 ± 14.7% to 6.4 ± 6.5%). The Erk inhibitor, PD98059, also had a small but statistically significant inhibitory effect on CD38 up-regulation while the inhibition caused by the JKN inhibitor, SP600125, was not statistically significant. The effect of the p38 inhibitor on CCR7 and CD83 up-regulation was also investigated. CD83 up-regulation was also p38-dependent, whereas CCR7 up-regulation was MAP p38-independent. The inhibitors caused no change in CD38 expression on Bg-stimulated DCs, which was already low. The effects of the inhibitors on CCR7 and CD83 expressions were similar on Bg-stimulated and LPS-stimulated DCs.

Because the p38 inhibitor inhibited CD38 up-regulation in LPS-stimulated DCs, it was predicted that stimulation of DC with Bg might not activate p38 and, therefore, CD38 expression would remain low. To investigate this, Western blotting of p38 was performed. After 30 minutes of stimulation, p38 was effectively phosphorylated in LPS-stimulated DCs while very little phosphorylation occurred in Bg-stimulated DCs.

The amount of total p38 was similar in LPS-stimulated and Bg-stimulated DCs at this time point. These results further suggest that signalling through p38 is defective in Bg-stimulated DCs.

Next, the role of transcription factors that act downstream of p38 was investigated. First, previous microarray data were reviewed for different p38 MAPK-related transcription factors. There were no differences between LPS-stimulated and Bg-stimulated DCs in the mRNA levels of NF-kB, ATF-2 (activating transcription factor 2) or AP-1 (activator protein 1). However, the amount of STAT1 mRNA was up-regulated in LPS-stimulated DCs at 6 and 8 hours of stimulation, while no corresponding up-regulation was seen in Bg-stimulated DCs at any time point studied. Thus, the amount of STAT1 protein in LPS-stimulated and Bg-stimulated DCs was compared by Western blotting. LPS induced an increase in STAT1 protein and phosphorylated STAT1 in DCs, while in Bg-stimulated DCs the amount of these proteins remained low and at a similar level as in unstimulated control cells.

Although in the microarray data NF-kB was not differently expressed in LPS-stimulated and Bg-stimulated DCs, CD38 up-regulation in DCs has previously been shown to be NF-kB- dependent (Fedele et al. 2004). Therefore, the role of both STAT1 and NF-kB in DC surface molecule expression was studied by treating DCs with a STAT1 inhibitor fludarabine or a NF-kB inhibitor Celastrol before addition of Bg or LPS. Both 270 nM and 1000 nM concentrations of Celastrol could effectively inhibit LPS-induced CD38 up-regulation. A clear inhibition in CD83 expression was also seen. However, CCR7 expression was only slightly decreased with Celastrol. In contrast, fludarabine did not inhibit CD38, CCR7 or CD83 expression on LPS-stimulated or Bg-stimulated DCs.

5.2.6 DC migration in vitro and in vivo

In order to investigate the functional relevance of defective CD38 expression on Bg-stimulated DCs, in vitro and in vivo migration studies were carried out. Bg-stimulated DCs showed deficient migration towards CCL19 and CCL21 in an in vitro Transwell migration assay when compared to LPS-stimulated DCs. Because of the inhibitory effect of the p38 inhibitor on CD38 but not CCR7 expression, the effect of the p38 inhibitor on LPS-induced DC migration was studied, and the results showed that the p38 inhibitor efficiently inhibited migration of LPS-stimulated DCs towards CCL19 and CCL21 but had no effect on random migration. The average fold-decrease of four individual experiments was 7 for CCL19 and 15 for CCL21.

An in vivo model for DC migration exploiting FITC painting of the mouse skin was used. The results unambiguously showed that there was twice as much painting-site derived DCs in regional lymph nodes after *E. coli* infection as after Bg infection. Finally, to confirm and visualize the difference between the DCs of *E. coli*-infected and Bg-infected mice, immunohistochemical staining of lymph nodes was performed. The results support the findings of the flow cytometric analysis showing a clearly increased number of DCs in lymph nodes after *E. coli* infection but not after Bg infection.

6 DISCUSSION

6.1 Borrelia, neutrophils and complement

Serum resistance of Bb s.s. strain B31 is mainly associated with OspE and CRASP-1 (Hellwage et al. 2001; Brooks et al. 2005) and mediated by the binding of complement regulator factor H. OspA and OspC do not bind factor H (Hellwage et al. 2001). However, the role of OspB in serum resistance has not been studied. The present results show that $B313_{OspAB}$ tolerated 0.7 % NHS slightly better than B313 and $B313_{OspA}$. The amount of iC3b deposited on $B313_{OspA}$ was similar to that deposited on B313. These findings indicate that OspB may have a slight inhibitory effect on complement activation at low serum concentrations.

The results also show that Bb inhibits the fMLP-mediated but not C5a-mediated chemotaxis of human neutrophils and the inhibition is Osp-independent. The inhibition of chemotaxis could be due to a soluble factor produced by Bb interfering with FPR, since this is a mechanism already documented to be used by certain microbes and because the inhibitory effect could only be seen when borreliae were incubated with neutrophils. However, the expression of FPR was normal in Bb-stimulated neutrophils. fMLP and IL-8 have been shown to down-regulate the level of CD38 on the surface of human neutrophils. The decrease in CD38 expression induced by fMLP and IL-8 is dependent on p38 MAP kinase, but is independent of ERK (Fujita et al. 2005). fMLP- and IL-8-induced chemotaxis has been shown to decrease dramatically by pre-treatment of neutrophils with a p38 inhibitor, but not to be influenced by an ERK kinase inhibitor (Fujita et al. 2005). The results of the neutrophil CD38 surface expression studies of this thesis suggest that Bb s.s. and Bg down-regulate CD38 expression, and when these cells should migrate towards fMLP, not enough CD38 is left on the cell surface, which leads to defective chemotaxis towards fMLP. However, according to unpublished observations of our group, CD38 expression on neutrophils is very variable, at least in healthy controls. It would be feasible to study this phenomenon further with neutrophils isolated from LB patients.

The complementation of the OspA gene in Bb had no effect on the phagocytosis of Bb by neutrophils. On the other hand, the complementation of OspB significantly reduced the amount of phagocytosis in 0.7% NHS and to a small extent in 14% NHS. As specified above, B31 was the only strain able to survive in 14% NHS, suggesting that in the phagocytosis assay all other strains but B31 might have been harmed or even killed before phagocytosis had occurred in 14% NHS. The amount of iC3b on bacterial surfaces were also high in 14% NHS, suggesting that the possible inhibiting molecules of the bacterium are covered under deposited complement components. In 0.7% NHS, the amount of deposited iC3b is small and the molecules on the bacterial surface could interact with neutrophil surface molecules more easily. An interaction of OspB directly with the CR3 receptor could be exploited to cause a phagocytosis-opposing effect.

These results show that although OspA and OspB are located in the same operon and are coordinately transcribed and similarly regulated during the life cycle of Bb, their

interactions with neutrophils may have different functions. It has previously been speculated that because of the great plasticity of the Bb genome, OspB persistence is a result of positive selection pressure and OspB must therefore be of great value to the bacterium. However, no specific role for OspB has previously been documented (Templeton 2004). The results of the thesis suggest that OspB has an immune evasive function.

6.2 Borrelia and dendritic cells

The study on Bb and DC interactions started by an extensive microarray experiment, and several interesting and meaningful genes could be identified from the long list of differentially regulated genes.

The transcriptional response induced by LPS was greater than that of Bg at all time points studied. Most differentially expressed genes in indirect comparison were specific for either LPS or Bg, and the number of jointly regulated genes was limited. The jointly regulated genes included endocytosis associated genes, genes encoding basic inflammatory mediators, chemokines, adhesion molecules and many apoptosis inhibitors. The results of the jointly regulated genes suggest that the core responses of DCs at the transcriptional level are similar after Bg and LPS stimulation. The similar core responses also indicate that LPS and Bg doses were comparable. These results are in line with previous findings concerning the transcriptional response of DCs and other inflammatory cells to different microbes and their components (Huang et al. 2001; Chaussabel et al. 2003; Jenner and Young 2005). The meta-analysis by Jenner et al. clustered 32 different gene expression studies of host-pathogen interactions and defined a common host response occurring in all the cell types studied (e.g. DCs, macrophages, PBMCs) regardless of the nature of the stimulus. The genes that are part of the common host response and were similarly regulated in our study include genes coding for e.g. CXCL1, CXCL2, CSF-1, CCL3, CCL20, TNF-α, IL-1α, IL-1β, IL-6, BIRC 2, BIRC 3, BCL2A1, PBEF1, CFLAR. The genes encoding DC maturation markers were also similarly regulated. This indicates that the core responses in different functional groups induced by Bg are similar to those induced by other microbes.

Matrix metalloproteinases (MMPs) are a family of zinc proteases degrading extracellular matrix components. They also have other substrates, such as some growth factors, cytokines and chemokines, including monocyte chemoattractant proteins (MCPs) 1-4 (McQuibban et al. 2002; Overall et al. 2002). Several studies have shown that human monocyte derived DC produce MMP9 and MMP2 (Uchi et al. 1998; Kouwenhoven et al. 2002; Osman et al. 2002), but neither of these MMPs were considered to be part of the common cellular response to infection or the common DC response to infection in the above mentioned review article clustering various microarray studies of cell-microbe interactions (Jenner and Young 2005). Bg has been shown to induce the production of MMP1 and MMP9 in human monocytes (Gebbia et al. 2001), and MMP9 is up-regulated in EM skin lesions of patients with acute Lyme borreliosis (Zhao et al. 2003). In our study, transcription of genes encoding MMPs 9

and 12 was specifically up-regulated by Bg at two time points and the gene encoding MMP19 at one time point. The gene encoding MMP9 was included in the genes specifically up-regulated by Bg in both comparison methods at 8 h of stimulation. This is in line with previous findings and supports the important function of MMP9 in Lyme borreliosis pathogenesis.

The gene expression data and protein level data from a limited amount of individuals showed that there was a clear difference in CD38 expression between LPS-stimulated and Bg-stimulated DCs. After these experiments, difficulties with DC differentiation occurred and the monocyte isolation technique was changed (see 4.2.2. alternative protocol). The resulting DCs were phenotypically similar regardless of the isolation method.

With the new methodology, variability in DC surface molecule expression on DCs differentiated from 19 individuals was investigated. In terms of Bg-induced CD38 expression, very small individual variation was seen and the difference of CD38 expression on Bg-stimulated DCs compared to CD38 expression on LPS-stimulated DC was statistically significant. CCR7 as well as CD83 expression was lower, but not absent, in Bg-stimulated DCs compared to LPS-stimulated DCs. Thus, the previous results concerning DC transcriptional response to LPS and Bg correlated well with the observed CD38 protein levels, while the defect in CCR7 expression at protein level was smaller than what could be predicted from the transcriptional data. Compared to LPS-stimulated DCs, CCR7 expression was smaller but not absent on Bg-stimulated DCs. The functional consequences of the slightly decreased level of CCR7 on Bg-stimulated DCs are hard to predict.

The inability to induce CD38 expression was not limited exclusively to Bg, as defective CD38 expression on DCs stimulated with Bb s.s. or Ba was also observed. Studies on whether Bg actively induced defective CD38 up-regulation in DCs showed that 1) Bg can not inhibit the CD38 expression induced by LPS, 2) live Bg are not required for defective CD38 expression and 3) borrelial surface proteins encoded on the missing plasmids of B313 are not responsible for defective expression of CD38 on the DC surface.

DCs express Toll-like receptors (TLR) TLR1-4, TLR6 and TLR8 in vitro (Iwasaki and Medzhitov 2004). Lipoproteins from Bb activate inflammatory cells through TLR2 and TLR1 (Hirschfeld et al. 1999; Alexopoulou et al. 2002), whereas LPS activates the cells through TLR4 (Poltorak et al. 1998). TLR2 signalling leads to NF-κB activation, which is believed to require the adapters myeloid differentiation primary-response protein 88 (MyD88) and MyD88 adapter-like (Mal) protein (O'Neill 2006). TLR4-mediated NF-κB signalling is believed to require the adapters MyD88, Mal, Toll-IL1-like receptor (TIR) related adapter protein inducing interferon (TRIF), and TRIF related adapter molecule (TRAM) (O'Neill 2006). In our microarray results, the adapter protein MyD88 was up-regulated by LPS by both comparison methods at least at two time points but not at all by Bg. This finding supports the existence of alternative signalling pathways for Bg.

CD38 and CCR7 belong to the host core response genes that are up-regulated in DCs after microbial encounter (Jenner and Young 2005). However, recent evidence suggests that this transcriptional response is not always translated to the protein level. Lehner et al. studied DC surface marker expression after stimulation of cells with various TLR targeting ligands. They found that CD38 is expressed on the cell surface after stimulation with LPS, a TLR4 agonist, and poly(I:C), a TLR3 agonist. In contrast, R848 resiquimod, a TLR8 agonist, and peptidoglycan, a TLR2 agonist, failed to up-regulate CD38 on human DCs (Lehner et al. 2007). A similar finding regarding CD38 expression after TLR4 and TLR2 stimulation was observed by Remoli et al. (Remoli et al. 2007). However, the defect in CD38 expression after TLR2 or TLR8 stimulation observed by Lehner et al. did not lead to functional defects concerning DC migration towards CCL19 (Lehner et al. 2007), while other evidence suggests a clear correlation between CD38 expression and the migratory capacity of human DCs (Frasca et al. 2006). In relation to previously published data, defective CD38 up-regulation might be a more general feature regarding stimulation via certain TLRs, mainly TLR2 and TLR8, but studies with additional live pathogens targeting different TLRs are needed to clarify this issue.

The up-regulation of CD38 and CD83 in LPS-stimulated DCs was shown to be p38-dependent, while CCR7 expression was not significantly altered by p38 inhibition. p38 phosphorylation was induced after 30 minutes in LPS-stimulated DCs, whereas very little phoshporylation was observed in Bg-stimulated DCs. CD38 on human airway smooth muscle cells is involved in normal airway function and in IL-13-induced airway hyperresponsiveness, and is also regulated by TNF-α (Guedes et al. 2008). TNF-α induces activation of ERK, p38 and JNK MAP kinases, and p38 and JNK MAP kinases regulate CD38 expression through activation of NF-κB and AP-1 (Tirumurugaan et al. 2007). A recent study by Frasca et al. showed that LPS-induced CD38 up-regulation in human DCs can be inhibited efficiently by p38 inhibition and also to a lesser degree by ERK inhibition (Frasca et al. 2008). Futher, their results showed induction of STAT1 phosphorylation in DCs stimulated by LPS (Frasca et al. 2008). LPS induced CD38 expression on human DC can be partly inhibited by N-Acetyl-L-cysteine, an antioxidant that inhibits NF-κB activity (Fedele et al. 2004)

Downstream of p38, the activation and effect of several transcription factors on surface molecule expression on DCs was investigated. Interestingly, in Bg-stimulated DCs STAT1 and phosphorylated STAT1 were expressed at a level comparable to unstimulated cells, while in LPS-stimulated DCs effective induction of both STAT1 and phosphorylated STAT1 was observed. Defective induction of STAT1 expression and STAT1 phosphorylation could also be seen in *B. burgdorferi* s.s. -stimulated cells. An NF-κB-inhibitor was capable of inhibiting the expression of CD38 and CD83 while CCR7 expression was unaltered. STAT1 inhibition had no clear effect on CD38, CCR7 or CD83 expression. Thus, a clear defect in STAT1 phosphorylation in Bg-stimulated DCs could be seen, but inhibition of STAT1 had no effect on CD38 up-regulation on LPS-stimulated DCs. Fludarabine has previously been used for STAT1 inhibition in only very few studies, and it could be that its effects are not of a similar magnitude in

all cell types. Thus, future studies are needed to determine the activation and role of these transcription factors in Bg-stimulated DCs. The effect of defective STAT1 phosphorylation also on other cellular functions in Bg-stimulated DCs and the effect of Bg on CD38 expression and related functions in other cell types need to be explored. A schematic diagram of the signals leading to CD38 expression and migration of DCs and the steps that are defective in Bb-stimulated DCs is shown in **Figure 12**.

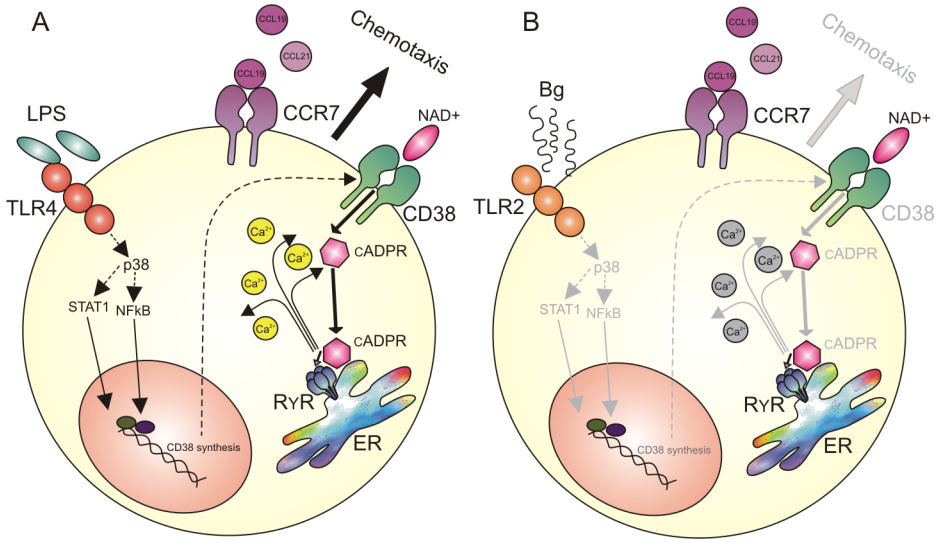

Figure 12. A schematic diagram of the signals leading to CD38 expression and migration of dendritic cells. **(A)** LPS induces the activation of p38 through TLR4 and further the activation of transcription factors STAT1 and NF-kB. This results in up-regulation of CD38 (and CD83). CD38 catalyzes the formation of cADPR from NAD+. cADPR releases Ca^{2+} from endoplasmic reticulum stores through ryanodine receptors (RyR). The calcium release enables the CCR7-dependent migration of DC towards CCL19 and CCL21.
(B) *B. garinii* does not induce the activation of p38 and STAT1. This results in defective up-regulation of CD38, reduced cADPR-dependent release of Ca^{2+} from endoplasmic reticulum stores and defective CCR7-mediated migration towards CCL19 and CCL21. The defective events in *B. garinii*-stimulated DCs are shown in grey.

To investigate the functional consequences of defective CD38 expression, DC migration was studied. Bg-stimulated DCs were shown to have a defect in chemotaxis towards CCR7-ligands CCL19 and CCL21 in vitro. The p38 inhibitor reduced the expression of CD38 and migration towards CCL19 and CCL21, while CCR7 expression was unaltered. These results indicate a strong dependency of DC migration on p38-dependent CD38 expression. CCR7 has long been thought to be the dominant mediator of DC migration. However, a study by Velan et al. (Velan et al. 2006) showed that DCs pulsed with *Y. pestis* showed decreased migration towards CCL19 in an in vitro and in an in vivo assay but still showed up-regulated CCR7 expression. In a study concerning the role of CD47 in DC migration, CD47–/– DC showed normal

CCR7 expression but impaired migration to CCL19 in an in vitro assay (Hagnerud et al. 2006). The results of the chemotaxis experiments support the observations of other factors in addition to CCR7 being crucial in DC migration and further emphasize the role of CD38. One explanation could be that CCR7 is needed because of its receptor function to recognize and sense CCL19 and CCL21, while CD38 is crucial in eliciting an optimal calcium release for chemotaxis to occur.

Finally, an in vivo migration model was used to study DC migration in mice. The results show that *E. coli* induced twice as much DC migration to local draining lymph nodes as an equal amount of Bg. Although the results can not be directly compared with the results obtained from our human DC assays, they highlight the role of CD38 in an optimal immune response and warrant future studies regarding DC migration during LB.

6.3 Why are the defence reactions in erythema migrans attenuated?

In the forthcoming three sections, the results of the thesis are discussed in the light of the aims of the thesis project and compared to recent data on the topic.

As mentioned earlier, a sparse neutrophil infiltrate occurs in the EM skin lesion of LB (Steere et al. 1983). One hypothesis has been that DCs do not effectively attract neutrophils to the site of infection. In the microarray analysis of DCs, neutrophil chemoattractants CXCL1 and CXCL2 were up-regulated by Bg even more than by LPS. However, CXCL1 (GROα) secretion, as measured by the cytokine array, was not increased after Bg or LPS stimulation at 8 h, probably because of the early time point. On the other hand, IL-8 was constitutively secreted, and the secretion was increased after Bg and LPS stimulation. These results point out that the interplay between DCs and neutrophils is successful after Bb stmulation.

However, it seems that Bb is able to interfere directly with neutrophil functions. The chemotaxis assay showed that Bb inhibits the fMLP-mediated but not C5a-mediated chemotaxis of human neutrophils. Defective CD38 expression on Bb-stimulated neutrophils was also observed, although it was not directly linked with defective chemotaxis of neutrophils. Xu et al. recently showed that enhancing the expression of the murine chemokine CXCL1/KC at the inoculation site of Bb in murine skin led to an increase in neutrophil infiltration and this enhanced the host's ability to control the initial infection (Xu et al. 2007). These results combined suggest that the lack of neutrophil migration to the skin could be due to the ability of Bb to prevent neutrophil recruitment. These properties of Bb are an important field of future research.

Another important phenomenon attenuating the defence reactions in EM is the complement resistance of Bb. Serum-resistant Bb strains are able to evade complement-mediated killing by binding to the complement regulators of the alternative complement pathway, i.e., factor H and factor H-like protein-1. The binding to Bb is mediated by Bb proteins CRASP-1 (Brooks et al. 2005), CRASP-2_{Bb} (Hartmann et al. 2006) and OspE and OspE paralogs (Hellwage et al. 2001; Alitalo et

al. 2002; Kraiczy et al. 2003). A recent study by Schjuit et al. showed that the tick salivary protein Salp15 plays a role in the protection of serum-sensitive Bg strains and intermediately resistant Bb s.s. strains against direct killing by the complement system (Schuijt et al. 2008). The data of this thesis concerning OspB and complement resistance show that new and unexpected molecules contribute to complement resistance and that there is still progress to be made in the research of these aspects.

Salp15 is a major immunomodulatory protein in tick saliva. It binds to CD4, thereby inhibiting T cell receptor ligation-induced signals, resulting in impaired IL-2 production and impaired $CD4^+$ T cell activation (reviewed in Hovius et al. 2008). In addition, Salp15 binds to OspC and protects the spirochete from antibody-mediated killing (Ramamoorthi et al. 2005). Salp15 inhibits both Toll-like receptor- and Bb–induced production of pro-inflammatory cytokines IL-12p70, IL-6, and TNF-α by DCs and suppresses DC-induced T cell activation by interacting with DC-SIGN on DCs (Hovius et al. 2008). While feeding on a host, ticks introduce Bb into the host's skin. Local immunosuppression of the host by tick molecules assists Bb in establishing an infection. All these recent findings highlight the importance of both tick- and Bb-carried properties in immunosuppression during the primary infection. However, the potential benefit obtained by the tick from helping the spirochete to infect a mammalian host remains an enigma.

6.4 Why is *B. burgdorferi* able to disseminate?

As reviewed in section 2.2.2, Bb binds various host molecules. Dissemination is aided by binding to integrins, proteoglycans or glycoproteins on host cells and extracellular matrix (reviewed in Steere et al. 2004) as well as to plasminogen and plasminogen activator urokinase. Borrelial proteins have been shown to bind fibronectin, fibrinogen receptor, vitronectin receptor, and glycosaminoglycans on host tissues. Decorin binding proteins A and B have been shown to bind a collagen-associated proteoglycan, decorin (reviewed in Steere et al. 2004).

Neutrophils are present in the joint fluid of Lyme arthritis patients (Steere 1989), suggesting that they contribute to immune defence against Bb during the late stage of LB. According to the results of this thesis, the expression of OspB could be beneficial to Bb because it could better resist phagocytosis and oxidative burst in places where complement is only moderately present, e.g. the joints, skin and central nervous system. This would help the survival and spread of the spirochete. The extensive mechanisms mediating complement resistance discussed above are also important in aiding the dissemination of the bacteria.

Langerhans cells (LCs) are present in EM and their number is increased in ACA, the late skin manifestation of LB, but the MHC II expression of these cells is decreased in both lesions (Silberer et al. 2000). Bb can also be isolated from both these skin lesions (Åsbrink and Hovmark 1985). The decreased MHC II expression suggests that LCs have a decreased capacity to induce immune responses. Our results show that Bb can inhibit the migratory potential of DCs in vitro and in vivo. This would explain the

increased amount of LCs in the ACA lesions. By hampering the migratory potential of DCs, Bb could, in some individuals, delay the antibody response. This would enable efficient dissemination of the bacteria by giving them time for antigenic variation and prolonging the time of their recognition by the host immune system.

According to studies in both human and mice, a strong Th1 response is crucial for eradicating the spirochete in very early infection. Previous work on mice has also demonstrated that *I. scapularis* ticks, the most common *Ixodes* tick in the United States, together with Bb suppress host Th1 immune responses thereby promoting a Th2 cytokine response that it is not protective against tick-transmitted Bb infection in mice. Reconstitution of Th1-associated cytokines or suppression of both Th2 cytokines IL-4 and IL-5 at the time of tick feeding induces resistance to subsequent tick-transmitted infection (reviewed in Zeidner et al. 2008). Sjöwall et al. found that early IL12p70 secretion, and thus effective Th1 response, is associated with a good disease outcome (Sjöwall et al. 2005).

Interestingly, CD38 expression of DCs has been shown to promote Th1 polarization. Inhibition of CD38-mediated signaling results in decreased IL-12 production, and thus interferes with the capacity of DCs to polarize T cell responses toward a Th1 phenotype as measured by the percentage of T lymphocytes producing IFN-γ (Frasca et al. 2006). By failing to induce CD38 expression, Bb could polarize the response towards an unfavourable Th2-dominant phenotype which would then aid the spreading of the spirochete. This evidence indicates that defective CD38 expression on Bb-stimulated DCs causes various functional consequences including impaired migration of DCs, inhibition of Th1 polarization and induction of adaptive immune responses and, finally, insufficient eradication of the spirochetes.

6.5 What immune events promote the development of the chronic manifestations of Lyme borreliosis?

A switch to a Th2 phenotype in the immune response against Bb should occur during the late disease, as persistent or late Th1 responses have been associated with the development of chronic manifestations of LB (Oksi et al. 1996; Widhe et al. 2004). Antibodies have been shown to be responsible for immune protection against LB at the later stage of the disease (Schaible et al. 1990; Barthold and Bockenstedt 1993). Although antibodies to Bb can be detected in most patients with late disease, in some patients, the antibody responses are weak, delayed, or in rare cases, absent (Craft et al. 1986; Wilske et al. 1986; Dattwyler et al. 1988). Increased IFN-γ (Th1 induction) and decreased IL-4 (Th2 suppression) by peripheral blood derived monocytes have been detected in patients suffering from late stages of LB (Oksi et al. 1996) suggesting a persistent Th1 response and insufficient Th2 response in late LB. In blood samples obtained from patients with EM, increased IFN-γ was observed, whereas increased IL-4 was observed after clearance of EM. Patients with ACA have IFN- γ, but not IL-4, detected in blood samples (Widhe et al. 2004). These data suggest that an initial Th1 response, followed by a switch to a Th2 response, is associated with eradication of Bb, whereas a persistent Th1 response may lead to chronic manifestations of LB.

As already mentioned, antibiotic treatment usually cures LB. When untreated, the infection can persist for years or even decades causing a wide variety of symptoms and irreversible damage in the body. The chronic treatment-refractory symptoms of LB have been proposed to be due to persistent infection or infection-induced autoimmunity (Steere and Glickstein 2004). In a study by our research group, Bb-infected mice were treated with ceftriaxone and later with anti-TNF-α. Bb could be isolated by culture from various tissues of mice that received anti-TNF-α treatment but not from the tissues of animals treated with ceftriaxone only (Yrjänäinen et al. 2007). Similar findings have been reported by other groups (reviewed in Hytönen et al. 2008). These observations together with cultivation of Bb from LB patients properly treated with anibiotics support the theory of persistent infection. The theory on autoimmunity relies on the finding that a correlation between OspA-binding HLA-DR phenotypes and increased risk of treatment-refractory arthritis has been documented (reviewed in Steere et al. 2004).

The results of this thesis suggest that by inhibiting the migration of DCs to lymph nodes, Bb would have time to disseminate and find a protective niche where it could hide from the immune system. The ability of OspB to inhibit neutrophil phagocytosis and complement activation and the previous evidence that in some patients antibodies to OspB are present during the treatment-refractory phase of the disease suggest that the expression of OspB during chronic infection could be beneficial for the bacteria. Thus, the results of this study support the theory of persistent infection.

7 CONCLUSIONS

There are around 1000 reported and 3000 – 5000 unreported cases of LB in Finland each year. In a significant number of the patients, the infection is most likely spontaneously cleared by the early immune response and by antibiotic treatment. However, there is still a population of patients that suffers from treatment-refractory symptoms.

This study aimed at gathering answers to three LB pathogenesis-related questions: why are the defence reactions in EM attenuated, why can Bb disseminate and what early immune events may augment the development of the chronic manifestations of LB. These questions were studied concerning the functions of complement, neutrophils and DCs.

Inhibition of fMLP-induced chemotaxis of neutrophils by Bb and OspB-mediated complement resistance may contribute to the attenuated defence reactions in the skin of EM rash. Other factors contributing to this phenomenon include the immunosuppressive effects and complement-resistance mechanisms mediated by tick saliva as well as the surface proteins of Bb.

The defect in DC CD38 expression resulting in inefficient migration of DCs and suppression of Th1 polarization as well as neutrophil and complement inhibitory functions of borrelial OspB and binding to host proteins through various borrelial proteins may promote effective dissemination of the bacteria. Studies on CD38 expression on DCs isolated from different LB patient groups are warranted in the future. Further studies on the mechanisms behind the defective CD38 expression should also be carried out.

By inhibiting the migration of DCs to lymph nodes, Bb would have time to disseminate and find a protective niche from the immune system. Defective DC functions may also result in delayed antibody responses that could aid dissemination. The ability of OspB to inhibit neutrophil phagocytosis and complement activation in the protective niche could favour the development of persistent infection. Other possible reasons for the chronic phase of the disease are insufficient Th2 polarization and autoimmune reactions.

Many immune evasion strategies utilized by Bb have been revealed during recent years. The ability to hamper DC and neutrophil functions can now be added to the list of evasion strategies already including control of complement activation by binding complement regulatory factors, antigenic variation during infection and the ability to induce unfavourable Th1/Th2 responses. The debate between persistent infection vs. autoimmunity as the cause of treatment-refractory symptoms will continue.

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