The interplay between innate and adaptive immunity in Lyme Borreliosis

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Colophon:
The research presented in this thesis was performed at the department of Experimental Internal Medicine in the Radboud University Medical Center, Nijmegen, the Netherlands, part of the Radboud Institute of Molecular Life Sciences.

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Chapter 1

General introduction
Lyme Borreliosis

In 1976, Lyme Borreliosis was first described in the town of Old Lyme in the United States. The disease was recognized as a tick-transmitted disease due to the geographical clustering of children suffering from arthritis [1, 2]. Since then, Lyme Borreliosis has become the most common human vector borne disease in Western Europe and the United States. In 1981, the causal bacterium was identified as a spirochete of the *Borrelia* genus and is transmitted by bites from ticks of the genus *Ixodes* [3-5]. *B. burgdorferi* is localized at the site of the tick bite where it first encounters host immune cells [6, 7]. Three genospecies of *B. burgdorferi* sensu lato are well-known causes of Lyme Borreliosis in humans: *B. burgdorferi* sensu stricto, *B. garinii* and *B. afzelii* [4]. While *B. burgdorferi* s. s. is prevalent in the northern part of the United States, *B. garinii* and *B. afzelii* infections mainly occur in Eurasia. [4, 10]. This thesis will focus on these most studied pathogenic genospecies.

Since the 1990s, the number of reported Lyme Borreliosis cases has increased in Europe due to changes in vector abundance, distribution of the ticks, environmental and behavioural changes. In Europe, Lyme Borreliosis is transmitted by *Ixodes ricinus* and *I. persulatus*, while *I. scapularis* and *I. pacificus* are responsible for the spread of the disease throughout the United States. These ticks can also carry other microorganisms such as *Phagocytophilium*, *Babesia* spp. and tick-borne encephalitis virus [4, 5]. Early localized *B. burgdorferi* s.l. infection is often identified and diagnosed by the pathognomonic sign erythema migrans (EM), a bull’s-eye rash around the tick bite [3, 5, 8]. EM occurs in approximately 70-80% of Lyme Borreliosis patients [9]. From the skin, the pathogen can disseminate throughout the human body, using the blood and lymphatic systems [10-13], causing further long-term complications, such as Lyme arthritis, neuroborreliosis or skin complications in the human host.

Lyme Borreliosis is difficult to diagnose in the absence of clear pathognomonic signs, such as a typical EM, or the presence of a tick bite. Serological testing, often used for the diagnosis of infectious diseases, has its limitations for Lyme Borreliosis. Early in infection, antibodies may still be undetectable, and antibiotic therapy interferes in the development of serological responses [14, 15]. Once formed, IgG antibodies can be detectable for years in previous patients, even after the infection has passed and has been cleared from the human body [16, 17]. Incorrect diagnosis enhances the risk of further development of the disease in patients, when the infection may be effectively targeted with antibiotics, such as doxycycline [4, 18].
In a small percentage of patients, symptoms persist, even after antibiotic treatment. The exact mechanism responsible for this persisting phenotype are still investigated [1].

The spirochete and its vector

Structure

*B. burgdorferi* is one of the largest bacteria known to man. Its genome consists of a linear chromosome, approximately 910 kb in size, and 21 plasmids; 9 circular and 12 linear, ranging from 5-56 kb [19, 20]. Different regulatory systems control the gene expression *B. burgdorferi* requires during the various life cycle phases. *B. burgdorferi* is a micro-aerobic bacterium with a dierm consisting of an inner and outer membrane with a thin layer of peptidoglycans in between. The outer membrane consists of a fluid bilayer that is devoid of liposaccharides. However, the membrane does contain outer surface lipoproteins (Osp)s, which play a role in *B. burgdorferi* virulence, and several membrane-spanning proteins. The inner membrane is another lipid bilayer rich in integral membrane proteins, most of which are transporters. A peptidoglycan layer separates the inner and outer membrane, as well as flagellar fibrils in its periplasmic space [21]. Because the outer membrane does not contain any lipopolysaccharides, but the bacterium has a double membrane structure with a thin peptidoglycan layer, it is not a typical a gram-positive or -negative bacterium.

As a spirochete, each bacterium has 7 to 11 bundled periplasmic flagella. What makes *B. burgdorferi* unique is that the bacterium can survive without iron, using manganese for growth [22]. As an extreme auxotroph, lacking genes encoding enzymes for the citric acid cycle and oxidative phosphorylation, *B. burgdorferi* derives energy from fermentation of sugars through glycolysis [19, 23]. The spirochete is also capable of producing the second messenger cyclic-dimeric-GMP (c-di-GMP). This second messenger upregulates the expression of multiple genes such as OspE/BbCRASP, surface lipoproteins known to be involved in inhibition of complement-mediated lysis by binding to factor H [24]. *B. burgdorferi* is sensitive to proteases released by the host defence system. Moreover, the bacterium expresses a variety of outer membrane proteins that modulate the immune system, OspBs are a well-known example. In humans, OspC is for example inversely correlated with OspA expression [25]. It is expressed during initial *B. burgdorferi* infection. By binding to plasminogen and promoting cytokine production to
manipulate the vasculature, OspC promotes *B. burgdorferi* dissemination and complicates immune cell recognition by the host [26, 27].

**Figure 1: *B. burgdorferi* life cycle**

The life cycle of the *B. burgdorferi* spirochetes starts with the eggs of a female tick *Ixodes* that hatch into spirochete-free larvae. These larva feeds on its first host, which is infected with *B. burgdorferi*. When the larva is considerably engorged it drops to the ground and molts to a nymph. The infected nymph feeds on a second host, while it takes its blood meal it releases saliva infected with *B. burgdorferi* spirochetes into the second host, before dropping to the ground again. The tick moults into an infected adult and infects a third host.

**Life cycle**

*B. burgdorferi* life cycle starts when the egg of the tick hatch into spirochete-free larvae (Figure 1). These larvae feed on their first hosts such as mice, birds or
squirrels. One of these hosts may be infected with *B. burgdorferi* spirochetes. After feeding, larva drop to the ground and moults to a nymph. During the time it takes for the tick to find its second host, the *B. burgdorferi* spirochete migrates from the gut to the salivary ducts, securing its spread to another host. The infected nymph feeds on the second host and infects them with *B. burgdorferi* in the process. Once the nymph is fully engorged and *B. burgdorferi* bacteria have infected a new host, the nymph drops to the ground and moults to an adult [21, 28].

In 2007-2013, the average risk of incurring Lyme Borreliosis following a tick bite in the Netherlands was 2.6%. This risk was higher if the tick was considerably engorged, increasing the infection rate to 5.5% [29]. Ticks are often attached to the hosts for several days to acquire their blood meals. In order to survive, they have a broad range of immune evasion strategies to avoid rejection by the host [30, 31].

**Tick saliva**

One of these immune invasion strategies is the use of tick saliva. Tick saliva suppresses a broad range of immune responses (Figure 2), generating an immune privileged site that protects the tick against the host immune responses during its blood meal and facilitates *B. burgdorferi* dissemination throughout the host [32]. *Ixodes* saliva targets various immune cell functions such as dendritic cell (DC) maturation by prostaglandin E2 (PGE2), T- and B-cell proliferation, keratinocyte activation, impairment of natural killer cells, IL-8 secretion through evasin-E3 important for neutrophil attraction, IFN-γ production by Natural Killer (NK) cells, binding of histamine, blocking of chemokine activity, inhibition of antimicrobial peptides (AMPs) complement activation and suppression of cytokine production [31, 33-44]. Tick saliva may also prime DCs to induce Th2-immune responses *in vitro* and *in vivo*. PGE2 was shown to be responsible for DC priming towards a Th2 cell phenotype *in vitro*. LCs can be stimulated by PGE2, and express prostanoid synthases which are another source of PGE2. PGE2 may also induce a tolerogenic phenotype in LCs [45].

Another protein, Salp15 also interferes with the CD4 receptor on T cells and DC-SIGN signalling in DCs [46-48]. In murine macrophages, sialostatin L2 has been shown to inhibit caspase-1 activation, reducing IL-1β and IL-18 production [49]. These mechanisms aid in constructing a tick–host–pathogen interface with a less hostile environment favouring tick attachment and pathogen spread to the host, thus promoting *B. burgdorferi* survival and dissemination from of the infection site.
Figure 2: The effect of tick saliva on host cell function
Representation of the immune suppression induced by different components of tick saliva. The Complement cascade is inhibited by OMCI, Salp20, Salp15, Isac and Irac. Natural killer cell, fibroblast and keratinocyte function is affected by saliva gland extract (SGE). Evasins block chemokine activity and saliva in general affects neutrophil adhesion and phagocytosis. Dendritic cell secretion of IL-6, TNF-α and IL-12p70 is inhibited by Salp15, whereas macrophage IFN-γ and NO production is suppressed by Iris, caspase-1 by sialostatin L2, and mast cell function is limited by histamine binding.

Internalization and recognition by the host

Cell interaction
*B. burgdorferi* is transmitted through the skin where it will first come into contact with tissue resident cells such as keratinocytes, fibroblasts, skin dendritic cell and macrophages [7]. This process is followed by the arrival of peripheral blood immune cells at the infection site through immune cell recruitment strategies and *B. burgdorferi* migration from the initial infection site to specific target sites [10, 11].

Upon detection, immune cells can recognize and attach to *B. burgdorferi* using different receptors, leading to activation of various signalling pathways and
induction of receptor specific gene transcription [50-53]. This results in the production of cytokines, chemokines or AMPs, in order to protect the host against the spirochete invasion. However, during Lyme Borreliosis some of these pathways are affected by either tick saliva (Figure 2) or via specific proteins expressed by the bacterium that mislead the immune cells, eventually resulting in a suppressed or more tolerant immune response, which without antibiotic treatment may fail to clear the bacteria [6, 54, 55].

Internalization
Early studies in murine and human primary phagocytes have shown how *B. burgdorferi* internalization may occur through coiling, rather than conventional phagocytosis [56, 57]. *B. burgdorferi* endocytosis is mediated through the surface molecule CD14. By CD14 binding of integrin αMβ2 it traffics *B. burgdorferi* to lipid rafts, organizing the endocytosis of the bacterium. Integrin αMβ2 is vital in *B. burgdorferi* attachment to the cell [58]. In contrast, integrin α3β1 was not involved in mediating *B. burgdorferi* attachment, but instead facilitates *B. burgdorferi* induced toll-like receptor (TLR) 2/1 ligand signalling [59]. Of interest, TLR2-mediated lipoprotein signalling is also enhanced through interaction with CD14 [60]. CD14 is an important co-receptor of both TLR2 and TLR4 [61, 62].

Phosphatidylinositol 3-kinase (PI3K) signalling was also required for *B. burgdorferi* phagocytosis by murine macrophages [63]. PI3K’s exact role remains to be determined, however, it may be related to its function in the autophagy pathway, which is activated during *B. burgdorferi* infection [64]. Moreover, scavenger receptor macrophage receptor with collagenous structure (MARCO) and CD36 enable *B. burgdorferi* internalization, possibly via cooperation with TLR2 [65, 66]. Both MARCO and PI3K are upregulated and activated by *B. burgdorferi* induced MyD88. Of note, PI3K is also associated with TRIF, a signalling molecule induced by various TLRs [63, 65].

Pathogen-associated molecular pattern and their pattern recognition receptors
During the process of internalization, pathogen-associated molecular patterns (PAMPs) are recognized through binding to pathogen recognition receptors (PRRs). There are several types of PRRs which are mostly expressed on innate immune cells to detect specific pathogenic structures expressed by a broad range of pathogens such as TLRs, nucleotide-binding oligomerization domain-containing (NOD)-like receptors (NLRs), C-type lectin receptors (CLRs) and RIG-I like receptors (RLRs).
**Toll-like receptors**

TLRs are important in pathogen recognition by innate immune cells, but they are also expressed by endothelial cells and mucosal epithelial cells. TLR location within the cell is vital to their function. TLR3, TLR7, TLR8 and TLR9 are located within the cell, whereas TLR1, TLR2, TLR4, TLR5 and TLR6 are expressed on the cellular surface [50]. Recently a study demonstrated TLR10 is expressed both intra- and extracellularly. However, TLR10 probably exerts its main biological function extracellularly and in contrast to other TLRs, it mediates anti-inflammatory effects by competing with other TLRs for the available ligands [67]. Mourits et al. (2020) also showed *B. burgdorferi* probably activates TLR10 expression (Table 1), since inhibition of TLR10 enhanced pro-inflammatory cytokine production [67].

*B. burgdorferi* is often recognized by immune cells through TLR2 [51]. TLR2 mediates signalling from endosomal vesicles containing *B. burgdorferi* spirochetes [68, 69]. TLR2 also cooperates with other (endosomal) TLRs to generate a targeted inflammatory response. It has been demonstrated to interact with TLR1 to form TLR1/2 heterodimers, while TLR2/TLR6 heterodimers were not or only slightly involved in cytokine production [53, 70].

TLR2 recruitment to the phagosome has been demonstrated extensively. TLR2 traffics to the vacuole from the plasma membrane and an unknown location from which it traffics together with TLR8. Cervantes et al. (2011) clearly demonstrated the co-localization of (degraded) spirochetes with TLR2 and TLR8 in a phagosomal vacuole, partially embedded in the monocyte’s membrane, and in the cytoplasm. Once TLR2 reaches the phagosomal vacuole it becomes available for signalling. Endosomal TLR8 is probably activated in the phagosomal vacuole through exposure to *B. burgdorferi* mRNA of the degraded spirochetes, and responsible for the production of IFN-β. TLR8 may assist TLR2 in the production of NF-κB–dependent cytokines [53].

TLR7 and TLR9 may be activated by *B. burgdorferi* [52, 53, 71, 72]. Petzke et al. (2009) showed the dependency of IFN-α production by pDCs by stimulation of TLR7 and TLR9 with *B. burgdorferi*, possibly through stimulation of TLR9 by *B. burgdorferi* DNA [72]. However, IRF7, the only IRF significantly upregulated in PBMCs exposed to *B. burgdorferi*, is dependent upon the TLR8 pathway. Recent studies have shown the impact of endosomal TLR activation can interfere with the ability of a host cell to respond to the activation of other TLRs [69, 73]. These observations suggest TLR2 may be cooperating with TLR8 to induce (pro-)inflammatory genes.
Though TLR3 has not been shown to play a role in recognition of *B. burgdorferi* spirochetes, TRIF, the adaptor molecule of TLR3, is activated in response to *B. burgdorferi*. Host cells damaged by tick mouthparts may generate dsRNAs that activate TLR3 and TRIF, and potentially impact the TLR2-*B. burgdorferi* induced inflammatory process [32, 63].

TLR4 is another TLR known to utilize TRIF in its cascade, however, *B. burgdorferi* does not express the main ligand for TLR4, lipopolysaccharides, on its surface. Cassiani et al. (2006) detected no changes in surface and mRNA expression of TLR4 or TLR6 on primary human monocytes and microglia upon stimulation with *B. burgdorferi*. In contrast, a robust increase in TLR1 and TLR2 expression was observed [51, 74].

Bacterial motility is achieved from flagella, TLR5 can identify pathogens through their expression of flagellin, a major component in flagella. TLR5 binding to flagellin induces degradation of Iκβ, activating the NF-κB pathway. *B. burgdorferi* express flagellin but thus far it has no significant effect on *B. burgdorferi* induced cytokine production [52], probably due to the location of the flagella between the inner and outer membrane of the spirochete.

### Table 1: Pathogen recognition Receptors involved in *B. burgdorferi* recognition

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- **Green**: *B. burgdorferi* recognition
- **Yellow**: Parts of the signalling cascade involved *B. burgdorferi* recognition
- **Orange**: Unknown
- **Red**: Not involved in *B. burgdorferi* recognition

**NOD-Like receptors**

NOD1 and NOD2 are involved in bacterial sensing through the recognition of peptidoglycans [75, 76]. While NOD1 recognizes pathogens through mesodiaminopimelic, NOD2 binds to muramyl dipeptides [75-77]. A human PBMC
and murine study showed that NOD2 is involved in the immune response towards *B. burgdorferi*. However, without NOD2 activation, TLR2 was still effective in producing an immune response. In contrast to NOD2, NOD1 was not involved in *B. burgdorferi* species recognition [78].

*B. burgdorferi* spirochetes are probably recognized through TLR2, taken up by the cell and degraded in lysosomes. *B. burgdorferi*-derived peptidoglycans are released during this process and can bind to activated NOD2. Activated NOD2, bound by peptidoglycans, recruits receptor-interacting serine/threonine kinase (RICK), resulting in the activation of NF-kB and cytokine transcription of IL-1β, IL-6, IL-10 and TNF-α. [78]. NOD2 may be responsible for enhancing TLR2 responses and enhance pro-inflammatory cytokine production[79].

NOD2 activation is known to lead to autophagy. Autophagy involves the degradation of damaged organelles and long-lived proteins to sustain cell survival. To degrade the proteins, small membrane structures surround the sequestering proteins. Formation of this autophagosome is initiated by autophagy related gene (ATG) 16L1 and type III PI3K [80, 81]. Autophagosome maturation occurs by lysosome fusion resulting in protein degradation. 3MA, a PI3K inhibitor, and wortmannin, which inhibits lysosomal fusion, increased mRNA expression and production of IL-6 and IL-1β, but not TNF-α, in response to *B. burgdorferi* exposure compared to the medium control [64]. Of interest, both of these inhibitors are involved in the autophagy related, mTOR pathway [82, 83].

**C-Type lectin receptors**

CLRls, such as Dectin-1, Dectin-2, Mincle, DC-SIGN, mannose receptor (MR), CLEC9A and mannose-binding lectin, are PRRs involved in the recognition of polysaccharides of micro-organisms. There are two main CLR signalling pathways: Syk (Dectin-1, -2 or Mincle) and Raf-1 (Dectin-1 and DC-SIGN). Upon injection of live *B. burgdorferi* into knee-joints of Dectin-2 deficient mice, a trend of reduced inflammation was observed. However, when studied in human PBMCs, Dectin-1 and -2 silencing or absence, through a non-functional SNP, had no significant effect on TNF-α and IL-1β production. In contrast, spleen tyrosine kinase (Syk) increased IL-6 and IL-1β production in response to *B. burgdorferi* exposure. This study demonstrates the importance of Syk induction, independent from Dectin-1 or -2 signalling. In contrast, Raf-1 inhibition had no effect on cytokine levels [84]. Of interest, murine studies have demonstrated a role for Syk, through interaction with integrin β1, MARCO, and Src, in *B. burgdorferi* phagocytosis [85, 86].
It is yet unknown if sugar-like structures of *B. burgdorferi*: ACGal and MGalD, can be recognized by Dectin-1 or -2 [84]. Furthermore, it has been proposed that MR might be involved in *B. burgdorferi* recognition [78]. A study by Cinco *et al.* (2001) showed importance of MR in attachment of monocytes and macrophages to *B. burgdorferi*, which suggests MR may facilitate phagocytosis of *B. burgdorferi* spirochetes and influence inflammatory cytokine production [87].

**RIG-I-like receptors and AIM2**

RLRs are a family of DExD/H box RNA helicases that function as cytoplasmic sensors of PAMPs within viral such as RIG-1, MDA5 or LGP2 [88]. There is also the DNA sensing AIM2 inflammasome complex. RLRs and AIM2 function during *B. burgdorferi* infection has not been yet fully elucidated [89], and more studies are warranted.

**Antigen presentation**

Antigen presentation is essential for the induction of specific T- and B-cells responses and the initiation of a proper and organised adaptive immune response. Monocytes, dendritic cells (DCs), macrophages and B cells are immune cells who are capable of antigen presentation to naïve or memory T cells. To fulfil this function, these immune cells have a complex diverse cellular signalling pathway called the antigen presentation pathway, who’s cascades result in the expression of a stable major histocompatibility complex (MHC) I and MHC class II (MHCII) loaded with antigens. MHCI and MHCII are the central mediators in establishing communication between the innate and adaptive immune system, as well as the activation and [90]. Though CD4+ T cells are stimulated by MHCII generally with exogenous antigens and CD8+ T cells via MHCI presenting mostly endogenous peptides [91], this balance may sometimes be shifted so that the antigens end up in the other MHC class [92]. The MHCII antigen presentation pathway starts in the endoplasmic reticulum (ER), where MHCII dimer subunits and CD74, also called the invariant chain, proteins are produced (Figure 3).

Without a properly functioning antigen presentation pathway, the immune system cannot recognize and distinguish host peptides from pathogenic ones. Therefore, no memory function can be established, and invading pathogens survive and thrive. Thus, antigen presentation a key pathway for pathogens to target and disrupt,
increasing pathogen persistence. For certain pathogens, this pathway has been studied broadly [93], but for other infections such as Lyme Borreliosis it has never been thoroughly explored. This topic will be further investigated in Chapter 2.

Figure 3: The major histocompatibility complex class II (MHCII) antigen presentation pathway. Class II transactivator (CIITA) activates MHC(-related) genes. Following transcription of MHC (-related) genes RNA in the nucleus, translation occurs in the endoplasmic reticulum were MHCII subunits $\alpha$ and $\beta$ are produced. In the endoplasmic reticulum (ER), MHCII dimer subunits and CD74, also called the invariant chain, proteins are produced. In the ER, CD74 trimer binds MHCII glycoproteins of three MHCII alpha-beta dimers, forming a nonameric complex. CD74 is crucial for MHCII ER egress. CD74 facilitates MHCII transport from the ER to the Golgi and from the Golgi to the late endosomal compartments. In these compartments CD74 is partially degraded by resident proteases, including: asparagine endopeptidase (AEP), Cathepsin S and L, leaving only the CLIP molecule in the P1 pocket. CLIP has different affinities for the variety of MHCII proteins. By its influence on the processing of exo- and endogenous antigens, CD74 shapes the repertoire of presented peptides in antigen presenting cells. For those with a high affinity for CLIP, HLA-DM induces CLIP dissociation, making the groove accessible for peptides. CLIP binding to MHCII results in a peptide selection in specific compartments under influence of HLA-DM. CLIP is then broken down by SPPL2a. HLA-DM affects the Trp residue at the edge of the peptide binding groove, the P1 and P2 pockets. Allowing peptide exchange to take place until a high affinity peptide binds and occupies the pockets, flipping the Trp residue inwards and preventing further HLA-DM interaction with HLA-DR. Though the bond with
CLIP is generally not very potent and HLA-DM association is usually not necessary for its release from CLIP, HLA-DM stabilizes empty MHCII proteins significantly. HLA-DM selectively promotes peptide binding to MHCII by inducing dissociation of low affinity peptides. After forming an unstable bond with MHCII, only peptides that can compete with this bond can cause HLA-DM release from MHCII and bind the molecule for antigen presentation. Peptide exchange is enhanced by the low pH environment of the endosomal peptide loading compartment. In contrast, peptide loading is reduced at the cell surface by the neutral pH environment. Interestingly covalent linkage of the peptide to MHCII results in immediate release of HLA-DM. Therefore, HLA-DM only binds to MHCII with an empty P1 pocket and does not destabilize MHCII-peptide complexes. Peptides can only stably bind MHCII when they effectively compete with HLA-DM for access to the P2 and P1 sites. MHCII often aggregate when their binding groove is not occupied with.

Initial *B. burgdorferi* s.l. infection: Lyme Borreliosis in the skin

Human skin constitutes a complex mucosal barrier protecting the host against invading pathogens. The epidermis is the first layer of this immune barrier and consists of keratinocytes and Langerhans cells (LCs). The second layer of resident skin cells is comprised of dermal fibroblasts, various dendritic cell subsets, mast cells and macrophages [44, 94]. These cells possess specific PRRs that recognize PAMPs, which leads to the activation of different molecular signalling pathways, depending on the receptor induced. These signalling cascades result in changes in gene transcription and the production of inflammatory molecules, including chemokines, cytokines and AMPs [95].

The early stages of Lyme Borreliosis are marked by the tick bite and injection of tick saliva and spirochetes into the dermal layers of the human skin [6, 7]. *B. burgdorferi* transmission from tick salivary glands to host tissues occurs 24-48h after the onset of the blood meal [96, 97]. Up to 48h after tick detachment, and 6-8 days after tick placement, *B. burgdorferi* spirochetes were observed in murine skin [98]. In the dermis, pathogens will first encounter host immune cells such as LCs, migrating down from the epidermis, a range of other DC subsets and macrophages, as well as keratinocytes and fibroblasts, which also contribute to skin immunity [7].

In order to detect antigens, LCs express both a range of TLRs and CLRs. LCs can process antigens and present them to T cells using MHCII. They also have the ability to cross present via MHC-I. In response to pathogens, LCs may produce IL-8, TNFα and CXCL10, but only low levels of IL-1β, IL-6 or IL-10 [99, 100]. Disruption of E-cadherin connections between LCs and keratinocytes in the skin can stimulate
immature LC migration. This can lead to the induction of tolerance in the host, partially through cytokine stimulation of Th2 responses. In mice, LCs appear to suppress the development of Th1 responses. Previous studies have shown how LCs can modulate T cell priming by other antigen-presenting cells [101-103]. *I. scapularis* ticks feeding on mice is enough to result in systemic immune deviation of T-helper (Th) responses toward the anti-inflammatory Th2 type, as assessed by mitogen stimulation of splenic T cells harvested 7 to 10 days after tick placement [30, 104]. Vesley *et al.* (2009) shows how LCs suppress Th1-cell development. LCs were required for the tick-mediated diminution of Th1 responses in regional lymph nodes, independently from IL-4 production, but not in the spleens of mice infected with specific-pathogen-free ticks demonstrate the presence of *B. burgdorferi* can overcome Th1-inhibitory effects of tick feeding on the host. No differences in pathogen burden were observed at the tick feeding site in LC-deficient and WT mice. However, during this study significant differences were observed between the male and female mice, demonstrating that gender has an effect on the functional roles of DC subsets in mice [30].

LCs are present in both EM and acrodermatitis chronica atrophicans (ACA), a chronic inflammation of the epidermis and dermis of the skin that eventually results in atrophy of the skin [105]. Higher LC numbers were observed in ACA than in normal skin. Also, MHCII expression of LC was down-regulated in EM and ACA compared to normal skin sections[6, 106]. LCs and melanocytes may be invaded and damaged by *B. burgdorferi*. During invasion, LCs demonstrated signs of degeneration in the form of intracellular oedema and vacuole formation, as well as mitosis of LCs at the periphery of EM tissue [6]. Inflammatory cells in EM produce increased levels of TNF-α and IFN-γ [106].

Keratinocytes are the major cell type present in the epidermis of human skin, whereas fibroblasts are the prominent cell type of the dermis. Both are directly involved in the skin injury caused by the tick bite [107]. In keratinocytes, triacylated *B. burgdorferi* OspC induces an inflammatory response by activating TLR1/2 in cooperation with CD14 [32, 60, 61]. Activation of these signalling pathways leads to the production of TNF-α and AMPs [44, 108]. *B. burgdorferi* alone induces much lower cytokine responses in primary human keratinocytes in combination with other TLR agonists. In contrast, tick saliva inhibited keratinocyte immune responses against these pathogenic ligands [32]. Both fibroblasts and keratinocytes produced AMPs in response to *B. burgdorferi*, including human β-defensin-2 and cathelicidin.
Tick saliva successfully inhibited IL-8 and defensin production by fibroblasts and keratinocytes [107].

In addition to fibroblasts, *B. burgdorferi* encounters dermal DC subsets and tissue resident macrophages [100, 109]. To simulate the tick bite and *B. burgdorferi* transmission into the skin, *B. burgdorferi* spirochetes were injected into healthy human skin [7]. Migration of LCs and dermal DCs from the injected skin was studied using a biopsy from the infected site and culturing it in media, the cells from three biopsies were pooled. Compared to a mock injection, a significant increase in LC and dermal DC migration was observed, but this was prevented by blocking TLR2. TLR2 blockage had no effect on DC cytokine production, suggesting induction of other PRRs by *B. burgdorferi* is enough to induce cytokine production in DCs. OspC is probably one of the factors involved in TLR2 stimulation, as an OspC mutated *B. burgdorferi* strain decreased DC migration slightly but not significantly [7].

*B. burgdorferi* infected skin tissue showed increased secretion of IL-1β, IL-6, IL-8 and IL-10 at 48h. Migrating DCs from the tissue expressed higher levels of CD83 in 6/10 donors and, while no differences were observed in HLA-DR, CCR7, CD38 and CD86 expression or IL-12 and TNF-α production. Indicating that migrating DCs have a partially matured but not fully activated phenotype [7]. Similar observations, with regards to CD38 and CCR7 expression, were shown by Hartiala *et al.* (2007 and 2010) in monocyte derived DCs (MDDCs) [106, 110]. The variation in DC maturation markers may be caused by the different DC subsets studied.

Mason *et al.* (2016) demonstrated that DC migration peaked at a concentration of $10^5$ spirochetes, increased migration occurred between 24-48h of incubation [7]. DC migration may occur through different mechanisms then CD38 and CCR7 expression, including prostaglandin E2 and MMPs [26, 106], as well as support from neighbouring keratinocytes and fibroblasts [111]. During the study, Mason *et al.* (2016) observed low numbers of LCs migrating out of the tissue 2-6%. Low levels of LC migration may be caused by intradermal inoculation or due to inhibition of LC migration [7]. In contrast to previous studies, no change in MHC class II expression was observed in LCs, while Silberer *et al.* (2000) observed a downregulation of MHC class II expression on LCs [6]. However, in the study by Mason *et al.* (2016) the segregation between the LCs and dermal DC subsets was slight and therefore the LC subset may be contaminated with dermal DCs. Last, the study did not take the existence of the Langerin+ cDC2 subset into account which is both positive for Langerin and CD1a, contaminating the studied LC population [7, 109].
Fibroblasts are important during the initial cutaneous *B. burgdorferi* infection and the induction of anti-inflammatory response. Human fibroblasts were assessed for changes in gene expression upon contact with the three different *B. burgdorferi* sensu lato strains: *B. burgdorferi* s.s., *B. afzelii* and *B. garinii*. CCL2, CXCL1, CXCL2, CXCL6, CXCL10, IL-6, IL-8, as well as the genes encoding for SOD2, NF-κB and interferon-related proteins were upregulated. The upregulation of IL-8, CXCL1, IL-6 and SOD2 was also confirmed at mRNA level. The differences at protein levels were only assessed for IL-8. Not only can dermal fibroblasts sense pathogens, but like keratinocytes, they also play a key role in the anti-*B. burgdorferi* response and may help to steer the immune response towards Th2 auto-immunity [112].

**The support of the blood system**

**Polymorphonuclear leukocytes**

Shortly after *B. burgdorferi* is recognized, there is an influx of immune cells from the peripheral blood into the site of infection. Polymorphonuclear leukocytes (PMNs) such as neutrophils, basophils, eosinophils and mast cells, are the first cells to enter the infected tissue and have been shown to be activated through direct interaction with OspA [113]. Freshly isolated human PMNs, mostly eradicated spirochetes through extracellular processes such as ROS production. Whereas differentiated monocyte-derived macrophages ingest the bacteria and killed spirochetes without the necessity of opsonization. PMN clearance of opsonized *B. burgdorferi* also occurred more rapidly [114]. Spirochetes ingested by macrophages are quickly localized in endosomes and lysosomes [115]. In a SCID and C3H/HeN murine study with peritoneal macrophages, OspC was shown to protect the spirochete against phagocytosis [116].

**Natural Killer cells, monocytes, dendritic cells and macrophages**

Moore *et al.* (2007) observed that *B. burgdorferi* induces IFN-γ production in natural killer (NK) cells. They determined the effect of monocyte depletion on the production of cytokines and revealed that monocytes were the primary source of inflammatory cytokines, while DCs were responsible for IFN-γ production. Thus, phagocytosis of live spirochetes initiates differential cell activation programs in monocytes and DCs [117].

It was shown that 272 genes were upregulated in MDDCs by *B. garinii* or LPS in direct and indirect comparison. Of these genes, only 26 were upregulated by *B. garinii*, while LPS enhanced expression of 246 genes, demonstrating the significant
differences in immune response between this well-known bacterial outer membrane component and *B. burgdorferi*. Both microarray and quantitative RT-PCR analysis was performed to assess gene expression. CD38, CXCL10, CCL5, Klf4, CCR7 and STAT6 expression was upregulated to a greater extent by *B. garinii*. Genes encoding NAB2, MMP9, MMP12, and MMP19 were significantly upregulated by *B. garinii* compared to LPS. Microarray results showed that SAMHD1, responsible for inhibiting reverse transcriptase, and CD31 (PECAM1), which is the counter-receptor of CD38, were also down-regulated at 6 and 8 h in *B. garinii*-stimulated cells [106].

CD38 regulates of calcium release and entry of extracellular calcium [118]. Generally, CD38 is downregulated during the differentiation of immature MDSC and expressed upon DC maturation [119]. CD38 also affects CD83 expression, a cell marker which has been implicated in T cell co-stimulation, enhancing T cell priming and chemotaxis of DCs. CD38-deficient DC are inefficiently recruited from the skin to local lymph nodes after antigenic stimulation, resulting in poor priming of T cells and impaired induction of humoral immune responses [120], as is witnessed during *B. burgdorferi* infection [106, 110]. In a follow-up study, similar effects were observed when these DCs are stimulated with *B. garinii*. No difference was observed in CD38 levels in *B. garinii* exposed DCs [106]. *B. garinii* failed also to induce p38 MAPK, STAT1 and NF-κB activation, leading to a defective upregulation of CD38 and impaired CCR7-mediated migration toward the chemotactic attractants CCL19 and CCL21 [110]. *B. burgdorferi* may specifically target the migration potential of DCs and may explain the increased number of LCs observed in the ACA lesions, as well as the reduced migration observed by Mason et al. (2016) [7].

*B. burgdorferi* induces MMP production in host cells using a TLR-independent manner by binding to integrin α3, β1, which is also involved in *B. burgdorferi* internalization. Human monocytes produce MMP1 and MMP9 in response to *B. garinii* [121], while MMP9 is up-regulated in EM skin lesions of acute Lyme Borreliosis patients [122]. Hartiala et al. (2007) demonstrated upregulation of MMP9, -12 and -19 by *B. garinii*. MMP inhibitory factors, TIMP-1 and TIMP-2, production was decreased significantly. Moreover, increased IL-10 production was observed [106].

It is believed that for a good clinical outcome during infection, the infected host requires an effective T helper cell (Th) 1 type response that is adequately balanced
by a Th2 response [123]. *B. burgdorferi* suppresses host Th1 responses, promoting a Th2 cytokine response that reduces the protection against tick-transmitted *B. burgdorferi* in mice [124]. An early and effective Th1 response through IL-12p70 secretion has been shown to lead to a good disease outcome [125]. Symptomatic Lyme Borreliosis patient’s posttreatment showed decreased expression of Th1-associated cytokine IFN-γ in the EM biopsies and correlated with IL-12p70, which induces and maintains IFN-γ secretion [123]. Therefore, *B. burgdorferi* may polarize T cell responses away from a Th1 phenotype and toward a Th2 dominant response, by reducing the number of IFN-γ producing T cells [110, 126].

*B. burgdorferi* can also induce IFN-β production in human monocytes and macrophages independent from MyD88 or TRIF adaptor molecules and NOD2 activation, but instead it is initiated through IRF3 [53, 127]. In mice, *B. burgdorferi* induced IFN type I production and stimulating B cell accumulation in the lymph node. However, unlike IFN type I production, the B cell accumulation is independent of MyD88 and TRIF signalling. Because IFNAR1 knockdown on B cells did not affect B cell accumulation while deletion on all cells did, suggesting that other cells regulate B cell accumulation through IFN type I stimulation. Additionally, type I IFNs promoted inflammatory cell death of *B. burgdorferi* infected monocytes [53]. Moreover, in mice, type I IFNs induced Lyme arthritis [127].

*B. burgdorferi* induces the expression of TLR2 and CD14 in peripheral blood monocytes and murine microglia, the macrophages of the central nervous system which are not present in the peripheral blood [128]. However, the modulation of several MHCII-related genes differed greatly between the two cell types. At 48 hours, there was significant downmodulation of numerous HLA-associated genes in monocytes, whereas their expression was not significantly altered (except for one) in microglial cells. HLA-DR and HLA-DQ, two MHC molecules expressed on the cell surface, were downregulated specifically in monocytes and upregulated in microglia. Additionally, these monocytes produced increased levels of IL-10 and TNF-α compared to microglia. These cytokines may increase the expression of CXCL13, SOCS3 and downregulate MHCII transactivator. CXCL13 is a B cell attracting chemokine. Stimulation of microglia with *B. burgdorferi* also increased transcription of CXCL12, but not in monocytes. In contrast, CXCL13 expression was enhanced in monocytes, while decreased in microglia. Microglia stimulation with *B. burgdorferi* induced the expression of CD11c, a DC marker, generating a subpopulation of the microglial cells. This expression may be correlated to CXCL12 expression, since it is known to direct DCs as well as neural/glial progenitor cells to CNS sites of injury.
Also, increased secretion of IL-6 by microglial cells was observed, possibly responsible for stimulating tissue inflammation[51]. *B. burgdorferi* might modulate TLR signalling and MHC pathway and function to its own advantage [6, 129]. TLR1/2 signalling could also support the induction of a state of tolerance in specific host cells in order to suppress the inflammatory reaction that would otherwise eliminate *B. burgdorferi* [51, 130].

**Objectives and outline of this thesis**

Over the last few years, the knowledge of Lyme Borreliosis, the *B. burgdorferi* spirochete, and the role of its vector in the early stages of infection has increased significantly. Current research demonstrates how early on in *B. burgdorferi* infection there is a failed induction of a balanced immune response possibly leading to disseminated infection at a later timepoint. This thesis shows how the interaction of *B. burgdorferi* spirochetes with different immune cell subsets leads to a tolerant immune response due to a diverse range of immune evasion and immunosuppression mechanisms: from inhibition of antigen presentation, very low and delayed IFNγ production, absence of IL-12 and STAT4 activation, lack of T cell stimulation and finally inhibition of ROS.

During early infection as well as the late stages of the disease, there are still gaps in our understanding of the dysregulated immune responses and the development of the disease. Most studies that investigated tissue or organ specific immune responses towards *Borrelia* have only been performed in mice and these results are not directly translatable to humans. Furthermore, many studies assess only one or a few cell subsets and therefore lack the complete overview of what is occurring during *B. burgdorferi* infection. Therefore, experimental models that closely resemble the *in vivo* situation during initial *B. burgdorferi* infection need to be designed and used to determine the crucial changes that occur during the interaction and communication of *B. burgdorferi* with antigen presenting cells and the bridge between the innate and adaptive immunity networks. When the mechanisms involved in the host response to *B. burgdorferi* are fully known, these limitations in innate and adaptive immune responses can be targeted, treated and utilized for the design of a better diagnostic method.

To understand the early stages of Lyme Borreliosis and investigate possible diagnostic targets, the interaction between *B. burgdorferi* and the immune system
was studied. This thesis focusses on communication between and within different immune cell subsets after *B. burgdorferi* exposure. First, peripheral mononuclear blood cells (PBMCs) were stimulated with *B. burgdorferi* spirochetes to assess their responses, both as a population as a whole and individual cell types. When PBMCs were stimulated with *B. burgdorferi*, a significant change in gene expression was observed. One of our major findings was the downregulation in antigen presentation genes in Chapter 2. Once *B. burgdorferi*-induced inhibition of antigen presentation proteins was also observed, the mechanism of this inhibition was explored further. A pathway involving RIP1 significantly inhibited antigen presentation, possible through TNFα signalling.

The importance of antigen presentation in the immune response and the genetic differences in these proteins per cell type are further explored in Chapter 3. Moreover, the importance of these genes during various infections is investigated.

The consequences of antigen presentation inhibition were also observed in the absence of IFNγ production by T cells, and specific STAT and SOCS signatures in various innate and adaptive immune cell types. SOCS1 and SOCS3 activation may also be responsible for the RIP1 mediated inhibition of antigen presentation. As a result of *B. burgdorferi* stimulation, both CD4 and CD8 T cells do not produce any IFNγ, probably due to an absence of STAT1/4 activation and cytokines such as IL-12, which can normally induce its expression. The method of inhibition was further explored in this Chapter 4, were the expression of the MHCII (-related) genetic regulator called CIITA, influenced by STAT1 and STAT3 expression, was investigated in Chapter 4.

Next, the role of IL-12 and IL-7, as well as the STAT4 pathway, was further evaluated in EM patients and Dutch foresters, who are exposed to *B. burgdorferi* infected ticks daily in Chapter 5.

During our studies in Chapter 6, natural killer (T) cells seemed to be the most capable cells of instigating a Th1 IFNγ response. Therefore, their role in IFNγ production was explored. Alone, NK cells were very capable of producing IFNγ. Therefore, their role in skin cell immunity was studied further by incubating them with *B. burgdorferi* and keratinocytes to observe the changes that occurred in NK cells in response to *B. burgdorferi* spirochetes in the skin.
In an additional study in Chapter 7, the role of reactive oxygen species was evaluated in PBMCs and monocytes incubated with *B. burgdorferi* infection. NADPH dependent ROS was significantly inhibited by *B. burgdorferi*.

Novel treatment strategies for restoration of specific immune responses were explored in Chapter 8 Combining IL-12 with various interleukins enhanced both IFNγ responses and antigen presentation protein expression to different levels. Last, the consequences of *B. burgdorferi* infection and its strategic immune evasion in initial and disseminated infection are discussed in the final Chapter 9, in the context of Lyme arthritis.

All findings in this thesis are summarized and discussed in Chapter 10. Moreover, future perspectives are provided, such as further investigation into whether *B. burgdorferi*-induced inhibition of antigen presentation molecules, STAT and SOCS signatures are also the reason why T cell dependent B cell activation does not occur effectively in the initiation stage of the disease and how these *in vitro* models may be translated to patients and be involved in the development of disseminated disease.
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Chapter 1


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Chapter 2

*B. burgdorferi* sensu lato-induced inhibition of antigen presentation is mediated by RIP1 signalling resulting in impaired functional T cell responses towards *Candida albicans*


Ticks and Tick Borne Diseases (2021)
Abstract

Antigen presentation is a crucial innate immune cell function that instructs adaptive immune cells. Loss of this pathway severely impairs the development of adaptive immune responses. To investigate whether *B. burgdorferi* sensu lato. spirochetes modulate the induction of an effective immune response, primary human PBMCs were isolated from healthy volunteers and stimulated with *B. burgdorferi* s.l. Through cell entry, TNF receptor I, and RIP1 signalling cascades, *B. burgdorferi* s.l. strongly downregulated genes and proteins involved in antigen presentation, specifically HLA-DM, MHC class II and CD74. Antigen presentation proteins were distinctively inhibited in monocyte subsets, monocyte-derived macrophages, and dendritic cells. When compared to a range of other pathogens, *B. burgdorferi* s.l.-induced suppression of antigen presentation appears to be specific. Inhibition of antigen presentation interfered with T-cell recognition of *B. burgdorferi* s.l., and memory T-cell responses against *Candida albicans*. Re-stimulation of PBMCs with the commensal microbe *C. albicans* following *B. burgdorferi* s.l. exposure resulted in significantly reduced IFN-γ, IL-17 and IL-22 production. These findings may explain why patients with Lyme borreliosis develop delayed adaptive immune responses. Unravelling the mechanism of *B. burgdorferi* s.l.-induced inhibition of antigen presentation, via cell entry, TNF receptor I, and RIP1 signalling cascades, explains the difficulty to diagnose the disease based on serology and to obtain an effective vaccine against Lyme borreliosis.
Introduction

Lyme borreliosis is the most common human vector-borne disease in Western Europe and the United States. The disease is caused by spirochetes of the Borrelia genus transmitted by ticks of the genus Ixodes. Since the 1990s, the number of reported Lyme borreliosis cases has significantly increased in Europe due to increased infection rates of the vector, changes in vector abundance, distribution of the ticks and environmental and behavioural changes (Hubalek, 2009; Jahfari et al., 2016; Lindgren et al., 2012; Smit and Postma, 2015; Smith and Takkinen, 2006). The major causal microorganisms of Lyme borreliosis are the three genospecies of Borrelia burgdorferi sensu lato: *B. burgdorferi* sensu stricto, *B. garinii* and *B. afzelii* (Smit and Postma, 2015; Strle et al., 1999). Their initial and localized infection of the skin is often identified by the pathognomonic sign erythema migrans (EM), a bull's-eye rash around the tick bite, that is observed in around 60–80% of the patients (Burgdorfer et al., 1982; Cervantes et al., 2011a; Jahfari et al., 2016; Smit and Postma, 2015; Stanek et al., 2012; Strle et al., 1999). From the skin, the pathogen disseminates throughout the human body, using blood and lymphatic systems, causing what is called the disseminated stage (Jahfari et al., 2016; Smith and Takkinen, 2006). Depending on the *B. burgdorferi* s.l. genospecies, the disease specifically targets certain organs leading to either Lyme arthritis, carditis, neuroborreliosis and acrodermatitis chronica atrophicans (ACA) (Smit and Postma, 2015; Steere, 1989; Steere et al., 2004; van Dam et al., 1993). Currently, Lyme borreliosis is diagnosed based on the presence of a tick bite with an EM, and a broad range of early symptoms (Association, 2019; Control, 2019). Due to the lack of a proper adaptive immune response, serology often results in incorrect diagnosis of Lyme patients (Association, 2019; Control, 2019; Smit and Postma, 2015), increasing the risk of further advancement of Lyme borreliosis in patients, while the infection could be effectively treated with antibiotics such as doxycycline (Little et al., 2010; Smit and Postma, 2015).

The course of *B. burgdorferi* s.s. infection was studied in two independent mouse models (Hastey et al., 2012; Tunev et al., 2011). In both models, a disorganized lymph node structure was observed, along with a robust increase in naïve B cell numbers through clonal expansion. Concomitantly, there was an absence of T cell-dependent B cell activation, lack of T cell recruitment to the lymph nodes and no development of T cell zones. Although, T-helper (Th) 2 cytokines, IL-4 and IL-21
levels increased compared to uninfected mice, low IFN-γ and IL-2 production were observed in infected animals. This development of a T cell independent B cell response may explain the low specificity and sensitivity of testing patient’s serology against *B. burgdorferi* s.s. (Hastey et al., 2012; Leeflang et al., 2016; Tunev et al., 2011). The delayed antibody response observed in patients may be caused by the absence of an organized and effective communication between antigen presenting cells and T cells, resulting in a delayed and unspecific B cell response (Hastey et al., 2012; Tunev et al., 2011). Under normal circumstances, following pathogen detection, innate immune cells present specific antigens to adaptive immune cells through the major histocompatibility complexes (MHC), further stimulating their activation through upregulation of co-stimulatory molecules by secreted cytokines. This suggests that the antigen presentation complexes may be compromised in Lyme borreliosis.

After their gene transcription is induced via MHCII trans activator (CIITA) binding to the MHCII promoters, MHCII protein complexes are produced in the ER. Here, a CD74 trimer, also called the invariant chain, immediately binds to the novel MHCII glycoprotein. This invariant chain is important for MHCII ER egress (Bikoff et al., 1993; Elliott et al., 1994). Moreover, CD74 facilitates MHCII transport to and from the Golgi and to the late endosomal compartments (Neefjes et al., 2011). In the endosomal compartments, CD74 is partially degraded by resident proteases, leaving only a small CD74 fragment in the MHCII binding groove. For MHCII complexes with a high affinity for this fragment, HLA-DM induces its dissociation, preparing the peptide binding groove for antigens (Blum et al., 2013; Busch et al., 2005). MHCII are (highly) unstable in the absence of one of its chaperones, CD74 and HLA-DM, respectively (Busch et al., 2005).

Interestingly, Langerhans cells (LCs) isolated from *B. burgdorferi* s.s. rash EMs and *B. garinii*-induced ACA lesions showed significant downregulation of MHCII expression when compared to healthy individuals (Hartiala et al., 2007; Silberer et al., 2000). These findings evince that the antigen presentation machinery may be inhibited by *B. burgdorferi* s.s. spirochetes. In another study, wild-type mice were compared to mice that either lacked CD4 T cell co-stimulatory molecules (CD40 L, ICOS and SAP) or to mice which were treated with anti-CD4. These mouse groups were then stimulated with *B. burgdorferi* s.s. or a medium control. In the absence
of co-stimulatory molecules similar B cell numbers were observed between the groups, suggesting T helper cell presence and MHC stimulation were not important for the accumulation of B cells in the lymph node observed in the *B. burgdorferi* s.s.-infected mice (Hastey et al., 2012; Tunev et al., 2011). However, the authors did not study the functionality of MHCI and MHCII and their antigen presentation capacity, which may have been affected by the spirochete presence. Furthermore, the authors did not study the effect of lack of co-stimulation on T cell activation, which may also play a role in the development of this T cell independent B cell proliferation.

In this study, we showed that PBMC gene expression changed significantly upon *B. burgdorferi* s.l. exposure. One of the signalling cascades specifically downregulated by *B. burgdorferi* s.l. was the antigen presentation pathway. Downregulation of gene transcription was followed by significantly reduced antigen presentation protein expression. The mechanism behind this inhibition was shown to be induced via TNF receptor I and RIP1 signalling. Antigen presentation suppression appears to be specific to *B. burgdorferi* s.l., since protein expression was significantly different from CD14⁺ monocytes exposed to other pathogens tested. The identification of the mechanism of *B. burgdorferi* s.l.-induced inhibition of antigen presentation is of great relevance, providing us with novel clues on the development of the early immune response against *B. burgdorferi* s.l. spirochetes and possible biomarkers to improve future diagnosis Lyme borreliosis patients. In addition, it may be of great importance for the development of a vaccine against Lyme borreliosis.

**Materials and methods**

**PBMC and monocyte isolation**

PBMCs were isolated from buffy coats obtained from the Sanquin Blood Bank in Nijmegen or blood donated by anonymous healthy volunteers, after written informed consent. These healthy volunteers were healthy blood donors, not currently infected with *B. burgdorferi* s.l. species, may have been previously exposed. Ethical approval was obtained from the committee on research involving human subjects (CMO) Arnhem-Nijmegen (NL32357.091.10). Blood was diluted 1:1 in PBS. Using a Ficoll-Paque density gradient centrifugation, the PBMC interphase was collected. PBMCs were washed twice in ice-cold PBS. After the last wash,
PBMCs were resuspended in RPMI 1640 Dutch Modified supplemented with glutamate, gentamycin and pyruvate. The donors used across the various assays differ, they are not from the same pool of donors. PBMC viability was assessed after 72 h and 7 days of culture using a flow cytometry cell viability stain (Fig. S7A).

A monocyte-enriched population was isolated by Percoll density gradient centrifugation following Ficoll isolation. The interphase was collected and washed three times in ice-cold PBS, followed by resuspension in RPMI 1640, Dutch Modified; an addition of sodium bicarbonate and 20 mM HEPES, supplemented with glutamate, gentamycin and pyruvate.

**B. burgdorferi** s.l. spirochetes

*B. burgdorferi* s.s., American Type Culture Collection (ATCC) 35210 type strain B31, *B. afzelii* ATCC 51567 and *B. garinii* ATCC 51383 were cultured at 24 °C in Barbour–Stoenner–Kelly (BSK)-H medium (Sigma-Aldrich), supplemented with 6% rabbit serum until spirochete growth commenced. Spirochetes were grown at 34 °C to late-logarithmic phase and checked for motility by dark-field microscopy. Spirochetes were quantified using a Petroff–Hauser counting chamber. Organisms were harvested following centrifugation at 7000 x g for 15 min, washed three times with sterile PBS (pH 7.4), diluted in culture medium to the required concentration and stored at -80 °C in sterile PBS until use. When thawed for cell stimulation, *B. burgdorferi* s.l. mix (BMix) was prepared by mixing equal amounts of *B. burgdorferi* s.s., *B. afzelii*, and *B. garinii*. In most experiments a final concentration of 106 spirochetes/mL, equal to a multiplicity of infection (MOI) of 0.2, was used. Unless otherwise stated, this mix of *B. burgdorferi* s.l. species was used to stimulate the various cell populations. The decision was made to use a mix of these species because similar trends were observed for all strains (Fig. 2E) and this way the results could be extrapolated to the three major *B. burgdorferi* s.l. strains.

*S. aureus* strain ATCC 29213 was grown overnight in culture medium, washed twice with cold PBS, and heat-killed for 30 min at 100 °C. Aliquots were stored at –80 °C throughout the study. *E. coli* ATCC 25922 was grown overnight in culture medium, washed three times with PBS, and heat-killed for 60 min at 80 °C. Aliquots were stored at –80 °C throughout the study. Cultures of H37Rv *M. tuberculosis* were grown to mid-log phase in Middlebrook 7H9 liquid medium (Difco, Becton-
Burkholderia burgdorferi inhibition of antigen presentation

Dickinson) supplemented with oleic acid/albunin/dextrose/catalase (OADC) (BBL, Becton-Dickinson), washed in sterile saline and heat-killed for 30 min at 110 °C. Heat-killed, at 65 °C for 60 min, *Candida albicans* blastoconidia (strain ATCC MYA-3573, UC 820) in a concentration of 106 CFU/mL were used throughout this study. The Japanese *B. miyamotoi* strain HT31 was cultured according to the previously published protocol (Koetsveld et al., 2017). N-acetylmuramyl-ananyl-d-isoglutamine (MDP) was purchased from Sigma (A-9519) and used at a final concentration of 5 μg/mL. Pam3Cys, a TLR1/2 ligand, was purchased at EMC microcollections (L-2000) and used in a final concentration of 10 μg/mL.

*Coxiella burnetii* strains were cultured on Buffalo green monkey cells at Wageningen Bioveterinary Research (former Central Veterinary Institute), the Netherlands, as described previously (Roest et al., 2013). Two strains were used, the frequently used Nine Mile (RSA 693) strain (Roest et al., 2012). The Nine Mile strain is a laboratory strain isolated from a tick and is generally used for research purposes (Seshadri et al., 2003).

**Monocyte-derived macrophages**
Following Percoll isolation, monocytes were left to adhere to a 96 well flat-bottom plate for 1–2 h, after which non-adherent cells were washed away using PBS. Monocytes received fresh RPMI 1640 Dutch Modified supplemented with glutamate, gentamycin, pyruvate and 10% human pool serum. Monocytes were cultured in this medium for 3 days, before receiving fresh medium. At day 5 of culture the phenotype of MDMs were determined by flow cytometry using CD11b and CD68 and stimulated with *B. burgdorferi* s.l. mix for 24 or 48 h.

**Monocyte-derived dendritic cell culture**
Percoll monocytes were cultured in a 75 cm2 flask (Corning, VWR) for 1 h in fresh RPMI 1640 Dutch Modified supplemented with glutamate, gentamycin, pyruvate and 1% human pool serum. Non-adherent cells were washed away using PBS, after which remaining monocytes were cultured at 5 × 10^5 cells/mL in Dutch modified RPMI 1640 (Company) supplemented with 10% heat-inactivated FBS (Gibco, Fischer Scientific), 50 ng/mL GM-CSF (Miltenyi Biotech) and 20 ng/mL IL-4 (R&D) for 7 days at 37 °C, before differentiation was complete. Non-adherent cells were used for analysis. The phenotype of immature monocyte-derived dendritic cells (MDDCs)
was determined using MMR, CD54, DC-SIGN, CD83, CD86, CD14 (dim), HLA-DR (neg) and CD80 seeded on a round-bottom 96 well plate and stimulated for 24 or 48 h. MDDC viability was assessed after 24 h of culture with or without stimuli using an Annexin PI stain (Fig. S7B).

**Cell stimulations and inhibitors of cell signalling**

5 × 10⁵ PBMCs, monocytes and MDDCs were seeded in a round bottom 96 well plate, while MDMs were cultured in a flat bottom plate at the same concentration. To study the various monocyte subsets, 7.5 × 10⁵ monocytes were cultured in polystyrene tubes (Falcon, VWR) and stimulated with 10⁶ B. burgdorferi s.l. species and a medium control for 16 h to avoid adherence and differentiation of the cells. Cells were treated with different stimulants and inhibitors: IFN-γ-1β (Immunkine, Boehringer Ingelheim), Ponatinib (AP24534; Selleck chemicals), Anti-hTLR2-IgA2, Clone: B4H2 (maba2-htrl2, SAS-Inivogen), human TNFRI antibody (MAB225; R&D), 7-Cl-O-Nec1; metabolically stable RIP1 inhibitor (ab221984 Abcam), Caspase 8 inhibitor Z-IETD-FMK (FMK007; R&D), Humira (Abbvie) and Enbrel (Pfizer), as well as vehicle controls: DMSO (Invitrogen, Life Technologies), Mouse IgG1 (MAB002; R&D) and Human IgA2 Isotype Control (maba2-ctrl, Sas-Inivogen).

The different pathogenic stimuli used include live B. miyamotoi 10⁶/well, MOI of 0.2, heat-killed Candida albicans 10⁶ CFU/well, MOI of 0.2, heat-killed Coxiella 10⁶ CFU/well, MOI of 0.2, heat-killed Escherichia coli 10⁶ CFU/well, MOI of 0.2, 100 ng/well IFN-γ-1β, heat-killed Mycobacterium tuberculosis 10⁶ CFU/well, MOI of 0.2, and heat-killed Staphylococcus aureus 10⁶ CFU/well, MOI of 0.2. Both live and dead whole B. burgdorferi s.l. mix were used for comparison to the live and dead pathogenic stimuli used. After addition of the stimuli to the cells the content of the wells were resuspended.

**Microarray**

Previously published transcriptome data (GEO: GSE42606) was used and the raw data was re-analysed for our study (Smeekens et al., 2013). PBMCs were isolated from 48 healthy volunteers and stimulated with RPMI or B. burgdorferi s.s. (1 × 10⁶ spirochetes/mL) for 4 h and 24 h at 37 °C and 5% CO₂. Total RNA was extracted using 800 μL of TRIzol reagent (Invitrogen). Global gene expression was profiled with an Illumina Human HT-12 Expression BeadChip version 4, according to the
manufacturer’s instructions. Using functions of the Bioconductor package limma (Ritchie et al., 2015), the raw binary output files (IDAT, BGX) were imported in the R software environment (R-3.5.1), background-corrected followed by quantile normalization (neqc) (Shi et al., 2010), removal of unspecific probes (Barbosa-Morais et al., 2010), where after differentially expressed probes were identified by using linear models and an intensity-based moderated t-statistic (Ritchie et al., 2015; Sartor et al., 2006). P-values were corrected for multiple testing using the Benjamini–Hochberg procedure (Benjamini and Hochberg, 1995). Probes that satisfied the criterion of FDR < 0.01 were considered to be significantly regulated.

Changes in gene expression upon *B. burgdorferi* s.l. treatment were related to biologically meaningful changes by using conservative significant thresholds (FDR < 0.01) in Ingenuity Pathway Analysis (Qiagen) or Enrichr (Chen et al., 2013; Kuleshov et al., 2016).

**RNA sequencing**

PBMCs were isolated from 8 healthy volunteers and stimulated with RPMI or *B. burgdorferi* s.s. (1 × 106 spirochetes/mL) for 24 h at 37 °C and 5% CO2. Total RNA was extracted using 800 μL of TRIzol reagent (Invitrogen). Single-ended sequencing was performed at the UMCG. Global gene expression was profiled using Illumina Hi-seq (2000) according to manufacturer’s instructions. Sequencing reads were mapped to the human genome using STAR (version 2.3.0) (Dobin et al., 2013). The aligner was provided with a file containing junctions from Ensembl GRCh37.71. Htseq-count of the Python package HTSeq (version 0.5.4p3) was used to quantify the read counts per gene based on annotation version GRCh37.71, using the default union-counting mode (The HTSeq package, [http://www.huber.embl.de/users/anders/HTSeq/doc/overview.html](http://www.huber.embl.de/users/anders/HTSeq/doc/overview.html)). Data were analysed using DESeq2 ([http://bioconductor.org/packages/release/bioc/html/DESeq2.html](http://bioconductor.org/packages/release/bioc/html/DESeq2.html)) (Howie et al., 2011). The standard p-value correction was replaced with a Benjamini-Hochberg correction based on just the provided set of genes (Benjamini and Hochberg, 1995). The threshold for statistical significance was set at (FDR P < 0.05 and Fold Change >2).

**Quantitative polymerase chain reaction**

Following the removal of supernatant, cells were resuspended in TRlzol
(ThermoFisher) and RNA was isolated according to the manufacturer’s instructions. RNA purity was checked and RNA reverse transcription into cDNA was performed using the Iscript cDNA synthesis kit (Biorad). Quantitative PCR was analysis was performed using Power Sybr Green PCR Master Mix (Applied Biosystems) and a 7300 Real-time PCR system (Applied Biosystems). Primer sequences were used for the detection and quantification of transcriptional levels of various antigen presentation proteins: CD74 FW: GCTGGACAAACTGACAGTCAC; CD74 RV: CAGGTGCATCACATGGTCCT; HLA-DMα FW: CCTGCACACAGTGTACTGC; HLA-DMα RV: CACCCGAGTGTCTGGGAA; HLA-DMβ FW: ACCTGTGCTGATGATGATGCT; HLA-DMβ RV: CGCAAGGGCCATCTTATTCT; HLA-DRα FW: AGTCCCTGTGTGATGATGATGCT; HLA-DRβ1 FW: CGGGGTGTGGAGAGCTTC; HLA-DRβ1 RV: AACCACCTGACTTGAATGCTG; HLA-DOα FW: CCTACGGACCGCCCTTCTA; HLA-DOα RV: GGCCTCGCTTTTCTTCAGG; HLA-DQα FW: AGATGAGCAGTCTCTACGTGGA; HLA-DQα RV: ACGGGAGACTTGGGAAACACT; B2M (Housekeeping gene) FW: TGACCGTGATCTTTCTGGTG; B2M (Housekeeping gene) RV: ATTTGAGGTGGTGGGAAACTG.

**Flow cytometry**

PBMCs were collected from the incubator and immediately washed and stained. Following PBS supplemented with 1% BSA buffer wash (PBA), PBMCs were stained for 15 min at RT with CD45 APC (Biolegend), CD14 AlexaFluor 700 (Biolegend), CD19 PE-Cy7 (Biolegend), CD74 FITC (BD Bioscience), HLA-DM PE (Biolegend), HLA-DR BV421 (BD Bioscience). Cells were washed in PBA and permeabilized using a fixation and permeabilization buffer set (eBioscience, 00-5523-00), by incubation for 45 min at 4 °C. Following a wash with the permeabilization buffer kit, half of the cells were analysed for extracellular staining, while remaining cells were stained intracellularly with CD74 FITC (Becton Dickinson), HLA-DM PE (Biolegend), HLA-DR BV421 (Becton Dickinson) for 15 min at RT. The gating strategy is demonstrated in Fig. S8 and the isotype controls are displayed in Fig. S9.

Monocyte subsets were stained for 15 min at RT with CD45 APC (Biolegend), CD56 PE-Cy5.5 (Beckman Coulter), CD14 AlexaFluor 700 (Biolegend), CD16 APC-Cy7 (Biolegend), CD74 FITC (Becton Dickinson), HLA-DM PE (Biolegend). Intracellular staining was performed after permeabilization for CD74 FITC and HLA-DM PE.
MDMs were stained for 15 min at RT with CD45 APC (Biolegend), CD14 AlexaFluor 700 (Biolegend), CD74 FITC (Becton Dickinson), HLA-DM PE (Biolegend) and HLA-DR BV421 (BD). MDDCs were stained for 15 min at RT with CD14 AlexaFluor700 (Biolegend, ITK-Diagnostics), DC-SIGN APC (Biolegend, ITK-Diagnostics), CD80 BV421 (Becton Dickinson), CD83 PE-CF594 (Becton Dickinson), CD86 PE-Cy7 (Becton Dickinson), CD74 FITC (Becton Dickinson), HLA-DM PE (Becton Dickinson), HLA-DR PerCP/Cy5 (Biolegend, ITK-Diagnostics). Intracellular staining was also performed on both MDMs and MDDCs after permeabilization for CD74, HLA-DM and HLA-DR. Cellular expression was measured with a CytoFleX (Beckman Coulter). Results were analysed on Kaluza version 1.5a (Beckman Coulter).

In Fig. S2A we demonstrate that the differences in markers between PBMCs stimulated in medium with and without 10 % human pool serum were not serum dependent. Moreover, Fc receptor antibodies were used to show that Fc receptor stimulation had no effect on the differences in antigen presentation observed between *B. burgdorferi* s.l.-stimulated and unstimulated CD14+ monocytes at 24 h (Fig. S2B).

**Statistical analysis**
qPCR data was normalized to a housekeeping gene, followed by direct comparison to the delta Ct value of the medium control, the delta delta Ct value was used for the final analysis. Flow cytometry results were normalized to the medium control for each donor at every time point. Statistics for measurements of cytokine levels, protein and qPCR data were performed using GraphPad Prism version 5.03 for Windows (GraphPad Software). Data represent mean ± SEM of n different donors. Unless otherwise stated, means were compared using the nonparametric Wilcoxon matched pairs signed ranks test, with two-tailed significance level set as P > 0.05. Statistical significance was accepted at p<0.05 and indicated as follows: n.s. not significant, * p<0.05 and **p< 0.01. Additional statistical details are stated in the appropriate figure legends.

**Results**

*B. burgdorferi* s.l. induces transcriptional inhibition of antigen presentation and associated proteins in human PBMCs
The specific immune cell pathways induced by *B. burgdorferi* s.s. spirochetes were
studied by stimulating human PBMCs with live *B. burgdorferi* s.s. for 24 h. The heatmap shows the top 500 significantly modulated genes by *B. burgdorferi* s.s. compared to the medium control (Fig. 1A). Four different gene clusters were observed when studying the relationships between the various genes. Overrepresentation analysis of each of these clusters revealed that in cluster 1 and 3, the genes involved in cytokine and receptor signalling, were significantly enriched. Cluster 2 showed significant downregulation of genes involved in antigen presentation, whereas cluster 4 was enriched in genes belonging to metabolic pathways, such as modifications in HIF-1 signalling. An Ingenuity Pathway analysis was performed showing the most significantly affected pathways by *B. burgdorferi* s.s were related to cellular adhesion and phagocytosis, T and B cell differentiation, signalling and activation, and communication between innate and adaptive immune cells. Focusing on the antigen presentation pathway (Supplementary Materials; Fig. S1), HLA-DM subunits α and β, CD74, HLA-DRα, HLA-DRβ1, HLA-DRβ3, HLA-DRβ4, HLA-DPα1, HLA-DPβ1, HLA-DOα, and HLA-DQα1, showed a robust downregulation (Fig. 1A). The micro-array results were validated using RNA-seq performed in cells isolated from 8 healthy volunteers, stimulated with live *B. burgdorferi* s.s. or a medium control for 24 h (Fig. 1B). In addition, qPCR confirmation using PBMCs isolated from healthy donors was performed for CD74, HLA-DMα, HLA-DMβ, HLA-DRα and HLA-DRβ1 (Fig. 1C). Gene expression levels of CD74, HLA-DM and HLA-DR were significantly downregulated after *B. burgdorferi* s.s. exposure.
B. burgdorferi inhibition of antigen presentation

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<tr>
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Figure 1: *B. burgdorferi* s.s. specifically inhibited the antigen presentation pathway in human PBMCs. (A) Heat-map of Illumina micro-array gene expression data of top 500 significantly affected genes, showing the adjusted p-values, 48 healthy volunteers were stimulated for 24 h with a medium control and live *B. burgdorferi* s.s. $10^6$/mL. The top 500 most significantly regulated genes were subjected to hierarchical, unsupervised bi-clustering analysis of samples (columns) and gene expression (rows). Gene expression values were centered across samples and subjected to hierarchical bi-clustering analysis using the Euclidean distance and the complete linkage clustering method. This resulted in the identification of 5 main gene clusters with specific gene expression patterns. For each cluster genes were related to biological functions using the EnrichR tool. The black box shows the cluster of genes related to the antigen presentation pathway. (B) Gene expression data of the antigen presentation pathway obtained by RNA-seq of 8 healthy volunteers stimulated for 24 h with a medium control and live *B. burgdorferi* s.s. $10^6$/mL before RNA was isolated and prepared for sequencing. (C) PBMCs from 3-5 healthy volunteers, from three independent experiments, were stimulated with live *B. burgdorferi* s.s. $10^6$/mL for 24 h and gene expression of CD74, HLA-DMα, HLA-DMβ, HLA-DRα, HLA-DRβ1 and the B2M housekeeping gene was determined using quantitative PCR analysis. qPCR data was normalized to the housekeeping gene, followed by direct comparison to the delta Ct value of the medium control, the delta delta Ct value was used to determine the fold-change.

**Antigen presentation is inhibited in primary human CD14+ monocytes, dendritic cells and macrophages**

To examine whether *B. burgdorferi* s.l. exposure also results in reduced protein expression of antigen presentation (-related) proteins, PBMCs were isolated and stimulated with different *B. burgdorferi* s.l species (Fig. 2). CD74, HLA-DM and HLA-DR expression was determined at a protein level, across different cell types: PBMCs, monocyte subsets, monocyte-derived macrophages (MDMs), and monocyte-derived dendritic cells (MDDCs), following stimulation with a mix of the three *B. burgdorferi* s. l. genospecies for 24 h and 48 h. Extra- and intra-cellular expression were determined by flow cytometry. Within the PBMC fraction, CD14+ monocytes demonstrated a significant decrease in intracellular expression of CD74 at both time points (Fig. 2A). Following the reduction in CD74 intracellular expression, a significant decrease in extracellular expression was observed at 48 h (Fig. 2A). In contrast, CD19+ B cells showed an increasing trend in extra- and intra-cellular CD74 expression at 24h (Fig. 2B).

In separate experiments, monocytes were differentiated into MDMs and MDDCs.
MDMs and MDDCs were stimulated for 24 h and 48 h with *B. burgdorferi* s.l. to observe changes in CD74 expression. MDMs showed a significant decrease in extracellular CD74 expression at 24 h. At 48 h *B. burgdorferi* s.l. inhibited intracellular CD74 expression in MDMs (Fig. 2C). MDDCs expressed higher basal expression levels of antigen presentation proteins (Fig. S2C) in comparison to other APCs studied. MDDC stimulation with *B. burgdorferi* s.l. significantly decreased extracellular CD74 expression and showed a decreasing trend in intracellular expression at both time points (Fig. 2D). Overall, CD74 was enhanced in B cells and downregulated in MDDCs, MDMs and CD14+ monocytes following exposure to *B. burgdorferi* s.l.

**Inhibition of antigen presentation proteins by *B. burgdorferi* s.l. species is dose- and time-dependent**

To determine if there were differences between the induction of *B. burgdorferi* s.l.-mediated inhibition of antigen presentation proteins across the various *B. burgdorferi* s.l. strains, CD14+ monocytes were stimulated with the three genospecies. As positive control, CD14+ monocytes were exposed to IFN-γ, a well-known inducer of MHCII gene expression, via CIITA pIII and pIV (Fig. 2E). IFN-γ significantly upregulated CD74 expression in CD14+ monocytes. No significant differences were observed between the various *B. burgdorferi* s.l. species (Fig. 2E). Stimulations with live and dead *B. burgdorferi* s.l. species, consisting of the three main genospecies of *B. burgdorferi* s.l.: *B. burgdorferi* s.s., *B. garinii* and *B. afzelii*, showed no significant difference in the extent to which they downregulated CD74 protein expression (Fig. 2E).
Figure 2: *B. burgdorferi* s.l.-induced inhibition of antigen presentation proteins in human innate immune cells. Protein expression analysis performed by flow cytometry, PBMCs from six healthy volunteers were stimulated with a medium control, live *B. burgdorferi* s.s. 10^6/mL, *B. afzelii* 10^6/mL, *B. garinii* 10^6/mL, a live mix of these three *B. burgdorferi* sensu lato species: BMix 10^6/mL or a range of dosages (10^3/mL – 10^7/mL) and 1 μg/mL IFN-γ for 24 h, 48 h or 24 h - 7 days. Data shown is mean ± SEM. The dotted line represents the expression level of the medium control, to which the stimulated expression levels are normalized for each time point. To compare the differences in intracellular and extracellular CD74 expression between the different antigen presentation cell subsets: (A) CD14+ monocytes (n=9, three independent experiments), (B) CD19+ B cells (n=6, two independent experiments), (C) monocyte derived dendritic cells (MDDCs) (n=8, three independent experiments) and (D) monocyte derived macrophages (MDMs) (n=8, three independent experiments), after stimulation with BMix 10^6/mL for 24 h and 48 h. (E) To study the different *B. burgdorferi* s.l. species, live and dead, compared to the medium control and positive control in CD14+ monocytes, furthermore, differences between intra- and extracellular expression of CD74 were compared (n=3, one independent experiments). (F) Test of different BMix dosages from 10^3/mL – 10^7/mL on the intracellular expression of CD74 in CD14+ monocytes because the first and most significant effect was observed here (n=9, three independent experiments). Induced inhibition of intra- and extracellular expression of (G) CD74, (H) HLA-DR and (I) HLA-DM was studied over the course of 1-7 days in CD14+ monocytes of the PBMC population stimulated with BMix 10^6/mL in RPMI+++ with 10% human pool serum (n=9, three independent experiments). Protein expression analysis performed by flow cytometry. (J) Percoll monocytes from six healthy volunteers were stimulated with a medium control, live BMix 10^6/mL for 16 h in polystyrene tubes. In the percoll monocyte population, CD45+ cells were selected followed by a selection of the classical (CD14++, CD16+), intermediate (CD14++, CD16+) and non-classical (CD14+, CD16+) monocytes, the effect of BMix stimulation on intra- and extracellular expression of CD74 was determined in these subsets (n=6, two independent experiments). Data were normalized by dividing the geometric mean fluorescent intensity (MFI) of each condition to the MFI of the medium control per timepoint and demonstrated as the fold-change. A non-parametric Wilcoxon matched-pairs signed rank test was used to determine significance. Significance indications shown above the bars demonstrate whether there is a significant change compared to the medium control.

CD14+ monocytes were stimulated with different dosages of *B. burgdorferi* s.l. species for 24 h to determine the optimal and minimal dosage to induce antigen presentation inhibition. When 5 × 10^5 cells were exposed to a thousand bacteria in a volume of 100 μl (BMix 104/mL), a significant reduction in CD74 expression was observed (Fig. 2F). For HLA-DM a similar trend was observed, reaching
at a ten-fold higher dose (Fig. 2F). At 24 h, intracellular HLA-DR expression was not significantly affected by the range of *B. burgdorferi* s.l. species dosages due to its long half-life of a few days to a week (Landsverk et al., 2011; Lazarski et al., 2005; Muller et al., 1993) (Fig. 2F), but the trend in HLA-DR expression showed a dose-dependent reduction between 104-106/mL bacteria.

To study the variation of the expression of antigen presentation proteins over time, 5 × 10^5 PBMCs were exposed to 106/mL *B. burgdorferi* s.l. species for one to seven days (Fig. 2G). This concentration was chosen based on the previous dose-dependent results for the antigen presentation proteins (Fig. 2E and F). Protein expression was determined every 24 h by flow cytometry. A time dependent decrease was observed for CD74, HLA-DM and -DR expression (Fig. 2G, H and I). Intracellular CD74 levels decreased significantly after 24 h of stimulation, followed by a reduction in extracellular expression 48 h after stimulation (Fig. 2G). HLA-DM and HLA-DR expression decreased in a similar manner, HLA-DM showed a higher fold of decrease in both extracellular and intracellular protein expression (Fig. 2H and I). Intracellular HLA-DR expression was significantly inhibited after 48 h (Fig. 2H). Followed by a decrease in extracellular HLA-DR expression at 72 h. At 24 h, HLA-DR expression exhibited a high inter-individual variation. While most donors showed a reduction in HLA-DR expression, other donors demonstrated an increased expression in comparison to the medium control. Extracellular HLA-DR decreased at 72 h, within 24 h after the intracellular drop (Fig. 2H). *B. burgdorferi* s.l. significantly reduced the expression of various proteins of the antigen presentation pathway in CD14+ monocytes over time.

**CD74 is specifically inhibited in classical (CD14+/CD16-) and intermediate (CD14+/CD16-) monocyte subsets**

As demonstrated previously, *B. burgdorferi* s.l. exposure to primary human monocytes results in significant inhibition of CD74, HLA-DM and HLA-DR expression. To evaluate whether this inhibition was specific for one of the monocyte subsets, a suspension of monocytes was stimulated with *B. burgdorferi* s.l. species 106/mL or medium, as a negative control, for 16 h, before their intra- and extracellular levels of CD74 were assessed by flow cytometry. Classical and intermediate monocyte subsets demonstrated a similar degree of inhibition of intracellular CD74 expression (Fig. 2J). In contrast, non-classical monocytes showed
an increase in intracellular CD74 expression and *B. burgdorferi* s.l. had no effect on extracellular CD74 expression (Fig. 2J). No inhibition of extracellular CD74 expression was observed in the classical monocyte subset after 16 h of *B. burgdorferi* s.l. infection (Fig. 2J). Extracellular expression of CD74 was significantly inhibited in intermediate monocytes (Fig. 2J). During the incubation and stimulation of PBMCs with *B. burgdorferi* s.l., enhanced CD14+ expression was observed (Fig. S2D) in intermediate and classical monocytes. In summary, *B. burgdorferi* s.l. stimulation enhanced CD14 expression on monocytes and significantly inhibits intracellular CD74 expression in classical and intermediate monocyte subsets. Intracellular CD74 expression in non-classical monocytes is enhanced while in intermediate monocytes *B. burgdorferi* s.l. significantly inhibited extracellular CD74 expression after 16 h of stimulation.

**Figure 3: MDDCs significantly mature in response to BMix stimulation.** Protein expression analysis performed by flow cytometry, monocytes from eight healthy volunteers and three independent experiments were differentiated in RPMI + 10% FBS, GM-CSF and IL-4 enriched medium for 7 days. Following differentiation, MDDCs were stimulated with a medium control, a live mix of *B. burgdorferi* sensu stricto, *B. garinii* and *B. afzelii* species: BMix 10^6/mL for 24 and 48 h in a 96 well round bottom plate. Data shown is mean ± SEM. The
dotted line represents the expression level of the medium control, to which the stimulated expression levels are normalized for each time point. MDDC population was first analysed by selecting CD45+ cells, followed by a selection of DC-SIGN+ CD14dim and HLA-DR+ cells as MDDCs, the effect of BMix 10⁶/mL stimulation of intra- (grey) and/or extracellular (black) expression of (A)CD80, (B)CD83, (C) CD86 and (D) HLA-DR was determined in these MDDCs. Data were normalized by dividing the geometric mean fluorescent intensity (MFI) of each condition to the MFI of the medium control per time point and demonstrated as the fold-change. A non-parametric Wilcoxon matched-pairs signed rank test was used to determine significance. Significance indications shown above the bars demonstrate whether there is a significant change compared to the medium control.

_B. burgdorferi _s.l. significantly enhanced CD80, CD83 and CD86 expression on monocyte-derived dendritic cells

In the cultured MDDCs, maturation markers were assessed after _B. burgdorferi _s.l. stimulation to determine whether _B. burgdorferi _s.l. targets innate APC activation in general or if the bacteria only specifically interfere with the antigen presentation pathway in these cells. MDDC activation markers CD80, CD83 and CD86 were significantly enhanced after 24 and 48 h of _B. burgdorferi _s.l. stimulation (Fig. 3A–C). Extracellular HLA-DR expression was significantly increased at 24 h, at 48 h it showed more variation (Fig. 3D). At an intracellular level, HLA-DR expression was not significantly enhanced at either timepoint (Fig. 3D). Overall, _B. burgdorferi _s.l. stimulation increased DC maturation markers.

_B. burgdorferi _s.l.-induced inhibition of antigen presentation is highly specific compared to other pathogens

To determine the specificity of _B. burgdorferi _s.l.-induced antigen presentation inhibition, the expression of MHCII(-related) proteins was evaluated in CD14+ monocytes after stimulation for 72 h with diverse range of human pathogens, known to bind the same or various other pattern recognition receptors (PRR), and activators of different immune pathways. Of high interest, the inhibition of antigen presentation by B. miyamotoi at 72 h was significantly lower compared to _B. afzelii, B. garinii and B. burgdorferi _s. s.-induced downregulation (Fig. 4). _B. burgdorferi _s.l. was the only pathogen significantly inhibiting extracellular and intracellular CD74 expression (Fig. 4). Other pathogens had significantly higher extracellular CD74 expression levels compared to _B. burgdorferi _s.l. species (Fig. 4). A similar trend was observed for HLA-DR (Fig. S3). After 72 h of incubation, the decrease in extracellular
MHCII(-related) protein expression was specific for *B. burgdorferi* s.l. infection. In contrast to extracellular CD74 expression and HLA-DR expression, at 72 h, *B. burgdorferi* s.l.-induced inhibition of intracellular CD74 expression was not significantly different from *E. coli* and *Mycobacterium tuberculosis* (Fig. 4).

**Figure 4:** *B. burgdorferi* s.l.-induced inhibition of extracellular CD74 expression is significantly different from other pathogens after 72 h of stimulation. Protein expression analysis performed by flow cytometry, PBMCs from six healthy volunteers and two independent experiments were stimulated with a medium control, a dead and live mix of *B. burgdorferi* sensu stricto, *B. garinii* and *B. afzelii* species both at $10^6$/mL, live *B. miyamotoi* $10^6$/mL, heat-killed *Candida albicans* $10^6$/mL, heat-killed *Coxiella* $10^6$/mL, heat-killed *Escherichia coli* $10^6$/mL, 1 µg/mL IFN-γ, heat-killed *Mycobacterium tuberculosis* $10^6$/mL and heat-killed *Staphylococcus*. Data shown is mean ± SEM. The dotted line represents the expression level of the medium control, to which the stimulated expression levels are normalized for each time point. To study the effect of various pathogens on the intra- and extracellular expression of antigen presentation proteins CD74 and HLA-DR (Supplemental Figure S2) in CD14+ monocytes, a diverse range of pathogens were compared to the mix of *B. burgdorferi* s.l. species. Data were normalized by dividing the geometric mean fluorescent intensity (MFI) of each condition to the MFI of the medium control per time point and demonstrated as the fold-change. A non-parametric Wilcoxon matched-pairs signed rank test was used to determine significance. Significance indications shown above the bars demonstrate whether there is a significant change compared to live BMix.
**B. burgdorferi** s.l. uptake and RIP1/TNFRI signaling are crucial for inhibition of antigen presentation in CD14+ monocytes

To investigate the mechanism of **B. burgdorferi** s.l.-induced downregulation of antigen presentation, **B. burgdorferi** s.l. cell entry and activation of different receptors and signaling pathways were inhibited. TLR2 antibodies and cytochalasin B, known to prevent pathogen cell entry through actin interaction and polymerization inhibition, TNFRI antibodies, RIP1 inhibitor 7-CL-O-Nec, and RIP2 inhibitor ponatinib, were used to study their role in **B. burgdorferi** s.l.-induced inhibition of the antigen presentation pathway. Anti-TLR2 had no significant impact on the inhibition of antigen presentation following **B. burgdorferi** s.l. stimulation (Fig. 5A). Cytochalasin B successfully prevented **B. burgdorferi** s.l.-induced suppression of antigen presentation (Fig. 5B). Of high interest, TNFRI antibodies and 7-CL-O-Nec significantly prevented **B. burgdorferi** s.l.-induced downregulation of antigen presentation (Fig. 5C and D), while caspase 8 inhibitors had no significant effect on the **B. burgdorferi** s.l.-induced downregulation (Fig. S4A). RIP1 inhibitors also showed an increasing trend in IFN-γ response following **B. burgdorferi** s.l. stimulation (Fig. S4C). CD14+ monocytes stimulated with TNFα, showed a decrease in intracellular CD74 expression (Fig. S4B). Pre-incubation with ponatinib did not prevent **B. burgdorferi** s.l.-induced inhibition of CD74, but it showed a significant decrease in inhibition of intracellular CD74 expression compared to untreated CD14+ monocytes stimulated with **B. burgdorferi** s.l. spirochetes. The phagocytosis of **B. burgdorferi** s.l. followed by TNF production, TNFRI binding and RIP1 activation is crucial for the induction of **B. burgdorferi** s.l.-induced CD74 and HLA-DR downregulation.

**B. burgdorferi** s.l.-induced downregulation of class II trans activator inhibits transcription of antigen presentation genes

To determine whether **B. burgdorferi** s.l.-induced antigen presentation inhibition may be induced by the MHCII(-related) transcriptional regulator called CIITA, upstream regulators were assessed in the RNAseq analysis. Using RNAseq data of 8 healthy volunteers, whose PBMCs were stimulated with live **B. burgdorferi** and a medium control, a significant inhibition of CIITA gene expression was observed (Fig. S5). CIITA is an important regulator of MHCII antigen presentation genes. RNAseq analysis demonstrated how the observed **B. burgdorferi** s.l.-induced inhibition of the antigen presentation pathway is regulated via CIITA suppression (Fig. S5).
Figure 5: Cell entry and RIP1/TNFα signalling pathways are crucial in *B. burgdorferi* s.l.-induced inhibition of intracellular CD74 expression. Protein expression analysis performed by flow cytometry, PBMCs from healthy volunteers were pre-incubated with αTLR2 (n=6, two independent experiments), TNFRI IgG1 antibody (n=6, two independent experiments), 7-Cl-O-Nec (RIP1 inhibitor; n=6, two independent experiments), Ponatinib (aRIPK2, n=8, three independent experiments), IgA2 (n=8, three independent experiments) and IgG1 Isotype Control (n=6, two independent experiments) for an hour, and with Cytochalasin (n=8, three independent experiments) and a DMSO vehicle control for 30 min before stimulating the PBMCs with a medium control, a live mix of *B. burgdorferi* sensu stricto, *B. garinii* and *B. afzelii* species: BMix 10⁶/mL, Pam3Cys 10 µg/mL and MDP 5 µg/mL for 24 h (Figure 5A, B and E) or 72 h (Figure 5C and D). Data shown is mean ± SEM. The dotted line represents the expression level of the medium control, to which the stimulated expression
levels are normalized for each time point. To study the involvement of specific PRR pathways on the intracellular expression of CD74 in CD14+ monocytes, PBMCs were pre-incubated with specific PRR inhibitors for 1 h and compared to medium control cells or isotype controls after 24 h or 72 h of stimulation with live BMix and a medium control. Data were normalized by dividing the geometric mean fluorescent intensity (MFI) of each condition to the MFI of the medium control per time point and demonstrated as the fold-change. A non-parametric Wilcoxon matched-pairs signed rank test was used to determine significance. Significance indications shown above the bars demonstrate whether there is a significant change compared to the medium control.

**B. burgdorferi** s.l. inhibits T-cell responses against *C. albicans*

To determine whether downregulation of antigen presentation by *B. burgdorferi* s.l. has an impact on the adaptive immune response after exposure to a non-related pathogen, PBMCs were stimulated with *B. burgdorferi* s.l. species 106/mL for 24 h, followed by stimulation with 106 heat-killed Candida albicans/mL for 48 h and 5 days. After these 48 h and 5 days, protein expression of CD74, HLA-DM and HLA-DR was assessed using flow cytometry, both extra- and intracellularly, as well as the production of IFN-γ (48 h), IL-17 (5 days) and IL-22 (5 days) by ELISA. Before stimulation with *C. albicans*, changes in antigen presentation protein expression following *B. burgdorferi* s.l. exposure were confirmed by flow cytometry, shown by the 24 h timepoint (Fig. 6A–C). When compared to the RPMI control for that timepoint, antigen presentation through CD74, HLA-DM and HLA-DR was decreased one- to two-fold of the unstimulated levels after 72 h and 5 days upon exposure to *B. burgdorferi* s.l. (Fig. 6A–C). *C. albicans* could not fully restore CD74, HLA-DM and HLA-DR levels to those of the medium control. At 72 h, 48 h of exposure to *C. albicans* did significantly increase extracellular CD74, HLA-DM and HLA-DR expression compared to 72 h of exposure to *B. burgdorferi*.

To further explore the functional consequence of the inhibition of antigen presentation on the adaptive immune response, T helper -cell response towards a well-known human pathogen *C. albicans* was studied. IL-17, IL-22 and IFN-γ production, known from previous studies to be induced especially in memory T cells (van de Veerdonk et al., 2009), were significantly inhibited if cells were pre-treated with *B. burgdorferi* s.l. species before *C. albicans* stimulation (Fig. 6D–F). Human PBMCs pre-exposed to *B. burgdorferi* s.l. species inhibited the Th1 and Th17 responses against *C. albicans*. 
Figure 6: BMix exposure has a permanent effect on antigen presentation in CD14+ monocytes, shown by severe inhibition of the adaptive immune responses to Candida stimulation following BMix stimulation. Intra- and extracellular protein expression of (A) CD74, (B) HLA-DM and (C) HLA-DR analysis in CD14+ monocytes was performed by flow cytometry. PBMCs from six healthy volunteers and two independent experiments were stimulated with a medium control, 24h of live BMix 10^6/mL, 72 h live BMix 10^6/mL, 24 h RPMI followed by 48 h stimulation with Candida albicans, 24 h BMix 10^6/mL followed by 48 h stimulation with Candida albicans, 24 h RPMI followed by 5 days of stimulation with
Candida albicans, 24 h BMix 10^6/mL followed by 5 days of stimulation with Candida albicans. BMix consists of a mix of B. burgdorferi sensu stricto, B. garinii and B. afzelii species. Data were normalized by dividing the geometric mean fluorescent intensity (MFI) of each condition to the MFI of the medium control at 24h and demonstrated as the fold-change. To study the functional impact of BMix-induced inhibition of antigen presentation, cytokine levels of (D) IFN-γ at 72 h, (E) IL-17 at day 6 and (F) IL-22 at day 6 were determined by ELISA. PBMCs from six healthy volunteers and two independent experiments were stimulated with a medium control, (D) 72 h live BMix 10^6/mL, 24h RPMI followed by 48 h heat-killed Candida albicans 10^6/mL, 24 h BMix 10^6/mL followed by 48 h stimulation with Candida albicans and (E and F) 24h RPMI followed by 5 days of stimulation with Candida albicans. Data shown is mean ± SEM. The dotted line represents the expression level of the medium control, to which the stimulated expression levels are normalized for each time point. A non-parametric Wilcoxon matched-pairs signed rank test was used to determine significance. Statistical significance was accepted at p<0.05 and indicated as follows: n.s. not significant, * p<0.05 and **p<0.01. Significance indications shown above the bars demonstrate whether there is a significant change compared to the medium control.

Discussion

This study unravels the molecular mechanism of B. burgdorferi s.l.-induced inhibition of antigen presentation in primary human PBMCs, and how this may interfere with the development of a functional adaptive immune response against B. burgdorferi s.l. infection. A small number of B. burgdorferi s.l. spirochetes, dead or alive, were sufficient to significantly downregulate the antigen presentation pathway (Fig. 2E and F). A cumulative inhibition was observed in several antigen presentation molecules which follow different kinetics (Fig. 2G, H and I). CD74 and HLA-DM expression decrease first, followed by HLA-DR. Stable HLA-DR complexes require both HLA-DM and CD74 expression. Studies have shown how CD74 absence in transfected cell lines decreased the stability of both HLA-DM and HLA-DR, though CD74 does not directly interfere with their expression (Mellins and Stern, 2014; Serradell et al., 1999; van Lith et al., 2010). However, HLA-DR transcription decreased concomitantly with HLA-DM and CD74 expression (Fig. 1), suggesting the delayed reduction in protein expression is correlated to the half-life and recycling kinetics of the different antigen presentation proteins. MHCII loaded with peptides has a half-life from a few days to a week, depending on the organ and cell type (Landsverk et al., 2011; Lazarski et al., 2005; Muller et al., 1993).
inhibition of proteasome activity showed how a decrease in degradation can contribute to maintaining the original expression levels of MHCII-related proteins (Fig. S6). Interestingly, intracellular expression of all MHCII(-related) proteins decreased first (Fig. 2G, H and I), followed by extracellular expression within 24 h, indicating the inhibition transpires at gene expression level or by post-translational modification. Therefore, it seems the production of novel antigen presentation proteins is inhibited, while regular degradation and recycling is not modulated or is enhanced by *B. burgdorferi* s.l. exposure.

*B. burgdorferi* s.l. was able to inhibit the expression of antigen presentation proteins regardless whether it was dead or alive and this inhibition was similar between the different *B. burgdorferi* s.l. species (Fig. 2E), suggesting the involvement of a generally expressed *B. burgdorferi* s.l. ligand or mechanism. Of note, killed *B. burgdorferi* s.l. consist of whole cell, non-heat killed bacteria (Fig. 2E and 4). These bacteria died due to the thawing and resting in medium, before the stimulation, without the addition of high nutrient serum, called BSK-H medium supplemented with rabbit serum, in which they are normally cultured. Without this nutrient rich environment, the spirochetes don’t survive and die within an hour of resting. This method was preferred since previous studies have shown how heat killing affects cytokine production and changes in gene expression significantly (Cruz et al., 2008; Petzke et al., 2009).

Unlike other pathogens, *B. burgdorferi* s.l. specifically inhibited extracellular CD74 and HLA-DR expression within 72 h (Fig. 4 and S3). Similar to *B. burgdorferi*, *S. aureus* expresses both TLR2 and NOD2 ligands, however, it induced no significant changes in MHCII-related proteins (Fig. 4 and S3). Positive control *C. albicans* and IFN-γ enhanced HLA-DR and CD74 expression after 72 h. In contrast, the intracellular bacterium, Coxiella burnetti, induced no significant changes in CD74 and HLA-DR expression, as was previously observed during MHCII expression studies in bovine macrophages (Sobotta et al., 2016). Some of these other pathogens did show a decrease in intracellular MHCII(-related) protein expression, such as the commensal bacterium TLR4 binding E. coli and Mycobacterium tuberculosis. However, *B. burgdorferi* s.l. was the only pathogen that significantly suppressed extracellular CD74 and HLA-DR expression. *M. tuberculosis* was already known for its suppression of antigen presentation proteins. It slightly affected HLA-
DR expression on monocytes and murine bone marrow-derived macrophages (Gercken et al., 1994; Noss et al., 2000). However, these studies used a very high MOI (Gercken et al., 1994) or high levels of bacterial lysate (Wang et al., 2005). In contrast, in the current study we used a lower concentration of *B. burgdorferi* s.l. spirochetes and showed a significant downregulation of MHCII(-related) protein expression. *B. burgdorferi* s.l. spirochetes decreased the overall expression and function of antigen presentation proteins in innate immune cells more significantly than *B. miyamotoi, Coxiella burnetti, S. aureus, E. coli* and *M. Tuberculosis*.

*B. burgdorferi* s.l. exposure significantly inhibited antigen presentation in three major innate immune cell types (Fig. 2A–D). While, in the PBMC population, B cells showed an increasing trend in antigen presentation molecules, CD14⁺ monocytes exhibited significant downregulation (Fig. 2A, B and J). In MDDCs, the decrease in antigen presentation molecules we observed was less severe than those observed in macrophages and monocytes. Intriguingly, monocytes become more CD14⁺ during *B. burgdorferi* s.l. exposure in comparison to the medium control (Fig. S2D), and it is this CD14⁺ population of monocytes that shows severe inhibition of the antigen presentation machinery. Monocytes are well-known for their plasticity and antigen presenting functions (Jakubzick et al., 2017; Lee et al., 2017). Moreover, their plasticity differs per subset, classical monocytes have been shown to exhibit the largest changes in MHCII expression upon cytokine stimulation (Lee et al., 2017). Classical monocytes may also enhance CD14 expression once they are exposed to *B. burgdorferi* s.l. spirochetes, since CD14 is an important co-receptor for *B. burgdorferi* s.l. uptake via TLR2 (Behera et al., 2006; Hirschfeld et al., 1999), possibly resulting in further augmentation of TNFα production. In contrast, non-classical CD16⁺ CD14dim monocytes increased intracellular CD74 and showed similar levels of extracellular expression compared to the medium control (Fig. 2J). This may be the result of different regulatory mechanisms for the antigen presentation pathway or non-classical monocyte-resistance to *B. burgdorferi* s.l.-induced inhibition due to the lack of certain receptors, proteins or signaling pathways, such as RIP1 and TNFRI which are normally present in CD14⁺ monocytes and essential for *B. burgdorferi* s.l. interference.

A previous study showed how *B. burgdorferi* s.s. induced TLR2 and CD14 expression in both murine monocytes and microglia. In contrast, the induction of several
MHCII(-related) genes differed greatly between the two cell types. At 48 h, there was significant downmodulation of numerous HLA-associated genes in monocytes, namely HLA-DR and -DQ, while their expression was not significantly altered in microglial cells (Rasley et al., 2002). Additionally, these monocytes produced increased levels of IL-10, which is well-known for its suppression of MHCII-related proteins (Chadban et al., 1998; Mittal and Roche, 2015), and TNFα, whose possible involvement in antigen presentation was shown in this study (Figs. 5, 7 and S4), compared to microglia (Rasley et al., 2002). In murine bone marrow macrophages stimulated with *B. burgdorferi* s.l., inhibition of CD74 was also observed in gene expression analysis (Carreras-Gonzalez et al., 2018). Upstream regulators of the antigen presentation proteins in our pathway analysis demonstrated a possible cause for the interference in the antigen presentation pathway. IL-10, MMP-9 and OSCAR, proteins normally suppressed by the ‘master molecule’ CIITA, were upregulated following *B. burgdorferi* s.l. stimulation. Interestingly, human monocytes have been shown to exhibit increased levels of MMP-9 in response to *B. garinii* (Gebbia et al., 2001). Also, MMP-9 is upregulated in EM skin legions of Lyme borreliosis patients (Zhao et al., 2003), suggesting an absence of CIITA activation. In this study, MHCII(-related) gene expression is shown to be reduced through suppression of CIITA activation (Fig. S5). CIITA downregulation may occur via TNFα production and increased expression of SOCS-3 (Cassiani-Ingoni et al., 2006; Ehlting et al., 2007). There is a strong indication that the transcriptional regulator CIITA is downregulated during *B. burgdorferi* s.l. infections, as observed in Fig. S5, and thus contributing to the decreased expression of antigen-presentation proteins.

Intriguingly, the MHCII pathway is distinctively regulated in various immune cell subsets through the differential expression of CIITA isoforms. While B cells express more pIII and pIV CIITA isoforms (Muhlethaler-Mottet et al., 1997), DCs, macrophages and monocytes are mostly regulated by pI (Muhlethaler-Mottet et al., 1997) and, if activated by IFN-γ, also the pIII isoform (LeibundGut-Landmann et al., 2004; Reith et al., 2005; Waldburger et al., 2001). Activated T cells are also known to express MHCII under regulation of CIITA isoform pIII (Kitamura et al., 2012). High expression levels of pIII have been observed in immature monocyte derived- and murine bone marrow-DCs (Landmann et al., 2001; Muhlethaler-Mottet et al., 1997). Compared to unstimulated DCs, *B. burgdorferi* s.l. stimulation
matured MDDCs significantly, increasing CD80, CD83, CD86 and HLA-DR expression (Fig. 3). Of note, dendritic cell maturation is known to affect their MHCII (-related) protein expression, since DC maturation is known to increase MHCII surface expression through enhanced transport of the MHCII endosomes to the cell membrane and increased half-life (Pierre et al., 1997). Of interest, activity of CIITA pl and pIII is known to be markedly decreased in DCs after DC maturation (Landmann et al., 2001), probably because of this stabilization of MHCII expression. Reduction in CIITA pl and pIII activity may be due to histone deacetylation (Landmann et al., 2001) over the entire regulatory region of MHC2TA, the gene encoding CIITA. In this study, we observed an increase in HLA-DR expression in MDDCs following *B. burgdorferi* s.l. infection at 24 h, while this increase was no longer significantly different at 48 h. Previous studies have also observed an increase in extracellular CD74 expression in maturing DCs (Huang et al., 2008; Nguyen-Pham et al., 2011; Pierre and Mellman, 1998). In contrast, MDDCs exposed to *B. burgdorferi* s.l. spirochetes significantly downregulated extracellular CD74. This suggests that normal stabilization of increased CD74 and HLA-DR expression on the cell surface in matured DCs does not occur *B. burgdorferi* s.l. stimulated DCs and is even significantly decreased at certain timepoints, possibly through differential CIITA regulation in these antigen presentation cells.

*B. burgdorferi* s.l. spirochete cell entry into these antigen presenting cells is crucial for the induction of antigen presentation inhibition (Fig. 5B). This suggests *B. burgdorferi* s.l. phagocytosis (Linder et al., 2001; Rittig et al., 1992) is important in order to inhibit CIITA activity and MHCII(-related) gene transcription. Antibodies against TLR2 did not interfere with *B. burgdorferi* s.l.-induced inhibition of antigen presentation (Fig. 5A), though it plays an important role in *B. burgdorferi* s.l. recognition and TLR2 has also been proposed to co-localize with TLR8 in the phagosomal vacuole (Cervantes et al., 2011b). In contrast, inhibition of TNFRI and RIP1 signaling significantly inhibited the suppression of antigen presentation proteins in CD14+ monocytes (Fig. 5C and D). Interestingly, TNF receptors were upregulated in the micro-array of *B. burgdorferi* s.l. stimulated PBMCs, suggesting increased TNF signaling and production in response to exposure to the pathogen (Fig. 1A). Activation of the TNF receptor might then lead to RIP1 induction, which is a crucial step in the suppression of *B. burgdorferi* s.l.-induced antigen presentation in CD14+ monocytes. Previous studies have also shown how TNFα is produced early on in infection (Sandholm et al., 2014) and that there is an association between
antibiotic-refractory Lyme patients and higher levels of TNFα in the synovial fluid (Shin et al., 2007). These studies demonstrate that the TNF signaling pathway may be relevant in the initiation of a balanced immune response. Furthermore, enhanced TNFα levels have been shown to be induced by TLR2-activated, MHCII:CD74 fragment-bearing B cells, and may also be tied to chronic inflammation and clonal B cell activation (Newell et al., 2010). This, in combination with the increased antigen presentation protein expression on *B. burgdorferi* s.l.-stimulated B cells (Fig. 2B), might explain the clonal B cell expansion observed in mice (Hastey et al., 2012; Tunev et al., 2011). Moreover, another study characterized the TNFR expression levels on the different monocyte subsets and observed that intermediates expressed more TNFRI than classical and nonclassical monocytes (Hijdra et al., 2012). On the other hand, nonclassical monocytes expressed higher levels of TNFRII than intermediates (Hijdra et al., 2012). Of high interest, TNFRI is the one of the two receptors which uses RIP1 as a signaling molecule. This might explain why *B. burgdorferi* s.l. stimulation inhibited antigen presentation more successfully in intermediate and classical monocytes than the non-classical subset (Fig. 2J). RIP1 is a protein that belongs to the receptor interacting protein family (Humphries et al., 2015). The RIP family consists of important signaling molecules for innate immune cells and is involved in cell death pathways. While RIP1 and RIP3 form a signaling complex crucial for cell death pathways, no interaction between RIP1 and RIP2 has been described (Humphries et al., 2015). Therefore, the partial restoration of CD74 expression under influence of ponatinib is probably caused by the fact that ponatinib is quite a broad inhibitor and interferes in the signaling of other RIPs, beside RIP2 (Fig. 5E). RIP1 and TNFRI can form two different signaling complexes, one involved in initiation of cell death, through interaction with Caspase-8, while the other is key in NF-kB and MAPK signaling (Humphries et al., 2015). However, Caspase-8 blockage had no impact on CD74 expression levels (Fig. S4A), suggesting *B. burgdorferi* s.l. induced inhibition is implemented through the latter signaling complex, affecting gene expression levels via these transcription factors. Of high interest, when PBMCs were pre-treated with RIP1 inhibitors during *B. burgdorferi* s.l. infection, IFN-γ production showed an increasing trend after only 72 h of stimulation (Fig. S4C). This shows how the induction of TNFα secretion via innate immune recognition of *B. burgdorferi* s.l., leads to inhibition of antigen presentation through TNFRI and RIP1 signaling (Fig. 7) and, as a result, interferes in the Th1 and Th17 responses against *C. albicans*. 
Figure 7: *B. burgdorferi* s.l.-induced inhibition of antigen presentation via TNF/RIP1 signalling. Following *B. burgdorferi* s.l. recognition by the pattern recognition receptors on innate immune cells, AP1 and NF-κB activated TNFα transcription, resulting in translation and secretion of this protein. TNFα binds to the TNF receptor I which results in the activation of RIP1 and consequentially in inhibition of antigen presentation gene transcription and protein production, leading to decreased intra- and extracellular expression of HLA-DR and CD74 after *B. burgdorferi* s.l. exposure. The antigen presentation pathway is shown in red because it is inhibited via *B. burgdorferi* s.l.-induced TNFRI/RIP1 signalling.

Antigen presentation is the crucial line of communication between the innate and adaptive immune system. Without it, the human immune system fails to instigate a robust and coordinated adaptive immune response against invading pathogens. *B. burgdorferi* s.l. severely inhibits this antigen presentation pathway in three major innate immune cell subsets: monocytes, dendritic cells and macrophages, through downregulation of MHCII(-related) gene expression via *B. burgdorferi* s.l. cell entry and TNFRI/RIP1 signaling cascades, enhancing its chances of survival within the human host. This knowledge contributes to our understanding of the
changes that occur during initial *B. burgdorferi* s.l. infection and the mechanism of antigen presentation inhibition may provide us with novel insights on the belated adaptive immune response observed in Lyme patients. Furthermore, it enhances our comprehension of antigen presentation regulation in various immune cell subsets and may be implemented to develop a more specific diagnostic method and target-based strategy for more efficient vaccines against Lyme borreliosis in the future.

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Supplementary Data

Figure S1: *Borrelia* inhibits antigen presentation in human PBMCs. Ingenuity pathway analysis (IPA) of the Illumina micro-array gene expression data of the antigen presentation pathway in 48 healthy volunteers who were stimulated for 24h with a medium control and live *B. burgdorferi* 10^5. A core analysis was performed with a foldchange cut-off of <1.5 and >1.5. Downregulated genes are shown in green while upregulated genes are red, and no change is shown in grey. The pink lines demonstrate the genes measured in the Micro-Array. Data were analysed using IPA (QIAGEN Inc.,
Figure S2: Effect of various conditions on CD74 expression. (A) Difference between intracellular CD74 expression, shown using the geometric mean fluorescent intensity, in CD14+ monocytes of the PBMC population stimulated with RPMI with and without 10% human pool serum, which was used for in stimulation assays lasting longer than 24h. Normalized, data compared to the RPMI control and the raw data are shown. (B) Fc receptor block before staining PBMCs with the antibody cocktail shows the same trend in CD74 expression in CD14+ monocytes at 24h. Data was normalized to the geometric mean intensity of the medium control, shown by the dotted line (n=3). (C) Difference between intra- and extracellular CD74 expression in CD14+ monocytes of the PBMC population, monocyte-derived macrophages (MDMs) and dendritic cells (MDDCs) stimulated with RPMI with 10% human pool serum, shown using the geometric mean fluorescent intensity. (D) Difference in extracellular CD14 expression on CD14+ monocytes of the PBMC population stimulated with RPMI and BMix (10^6/mL) with 10% human pool serum, shown using the geometric mean fluorescent intensity of CD14. Protein expression analysis performed by flow cytometry, PBMCs from healthy volunteers were incubated with a medium control, or BMix 10^6/mL (D). BMix consists of a mix of Borrelia burgdorferi sensu stricto, Borrelia garinii and Borrelia afzelii species.
Figure S3: Borrelia-induced inhibition of extracellular HLA-DR expression is significantly different from other pathogens after 72h of stimulation. Protein expression analysis performed by flow cytometry, PBMCs from six healthy volunteers were stimulated with a medium control, a dead and live mix of *Borrelia burgdorferi sensu stricto*, *Borrelia garinii* and *Borrelia afzelii* species both at $10^6$/mL, live *B. miyamotoi* $10^6$/mL, heat-killed *Candida albicans* $10^5$/mL, heat-killed *Coxiella* $10^5$/mL, heat-killed *Escherichia coli* $10^5$/mL, 1 µg/mL IFN-γ, heat-killed *Mycobacterium tuberculosis* $10^6$/mL and heat-killed *Staphylococcus*. Data shown is mean ± SEM. The dotted line represents the expression level of the medium control, to which the stimulated expression levels are normalized for each time point. To study the effect of various pathogens on the intra- and extracellular expression of antigen presentation proteins HLA-DR in CD14+ monocytes, a diverse range of pathogens were compared to the mix of *B. burgdorferi* s.l. species. Data were normalized by dividing the geometric mean fluorescent intensity (MFI) of each condition to the MFI of the medium control per time point and demonstrated as the fold-change. Significance indications shown above the bars demonstrate whether there is a significant change compared to live BMix. Statistical significance was accepted at p<0.05 and indicated as follows: n.s. not significant and * p<0.05.
Figure S4: TNFα and RIP1 are crucial in Borrelia-induced inhibition of CD74, while Caspase 8 is not involved. (A and B) Protein expression analysis performed by flow cytometry, PBMCs from healthy volunteers were pre-incubated with (A) Caspase 8 Inhibitor Z-IETD-FMK (n=6), a DMSO control (N=3), (B) Humira and Enbrel TNFα antagonists (n=6) for an hour, before stimulating the PBMCs with a medium control, TNFα 10 and 100 ng/mL (n=6), a live mix of Borrelia burgdorferi sensu stricto, Borrelia garinii and Borrelia afzelii species: BMix 10⁶/mL for 72h. Data shown is mean ± SEM. The dotted line represents the expression level of the medium control. Data were normalized by dividing the geometric mean fluorescent intensity (MFI) of each condition to the MFI of the medium control of the
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matched condition per time point demonstrated as the fold-change. (C) IFN-γ production measured by ELISA from PBMCs pre-incubated with 7-CI-O-Nec (RIP1 inhibitor; n=6) for an hour, before stimulating the PBMCs with a medium control and BMix 10⁶/mL for 72h.

![CIITA expression graph]

**Figure S5: CIITA expression is inhibited by *Borrelia burgdorferi*.** Gene expression data of the antigen presentation pathway obtained by RNA-sequencing of 8 healthy volunteers stimulated for 24h with a medium control and live *B. burgdorferi* 10⁶/mL before RNA was isolated and prepared for sequencing. Significance indications shown above the bars demonstrate whether there is a significant change compared to the medium control. Statistical significance was accepted at p<0.05 and indicated as follows: * p<0.05.
Figure S6: Proteasome inhibition restores MHCII (-related) protein expression. (A) CD74, (B) HLA-DM, (C) HLA-DR protein expression analysis performed by flow cytometry, PBMCs from healthy volunteers were pre-incubated with 0.1-2.5 µM of Bortezomib (n=3), for an hour before stimulating the PBMCs with a medium control, a live mix of *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* and *Borrelia afzelii* species: BMix $10^6$/mL for 24h. Data shown is mean ± SEM. The dotted line represents the expression level of the medium control pre-incubated with the same amount of Bortezomib. Data were normalized by dividing the geometric mean fluorescent intensity (MFI) of each condition to the MFI of the medium control per condition and demonstrated as the fold-change.
Figure S7: Monocyte cell death is minimal after 72h and 7 days of PBMC culture in the presence of human serum. (A) Viability of monocytes and PBMCs after 72h or 7 days of culture. (B) Viability of monocyte-derived DCs after 7 days of differentiation and 24h of stimulation. Viability was assessed by flow cytometry using a viability stain and Annexin PI stain, PBMCs from healthy volunteers were stimulated with a medium control and a live mix of *Borrelia burgdorferi sensu stricto, Borrelia garinii and Borrelia afzelii* species at $10^6$/mL. Using the viability stain the percentage of cell death was assessed by gating on the positive, dead cell populations.
Figure S8: Representative plots of determining of protein expression by flow cytometry in PBMCs stimulated with BMix $10^6$/mL for 24h. (A) BMix consists of a mix of *Borrelia burgdorferi sensu stricto, Borrelia garinii and Borrelia afzelii* species. Single cells were used, CD45$^+$ cells were selected, followed by CD14$^+$ monocytes within this population the expression of CD74, HLA-DM and HLA-DR was determined by studying the geometric mean fluorescent intensity. (B) Overlay of the fluorescent markers of CD74, HLA-DM and HLA-DR in the CD14$^+$ monocytes after PBMCs were stimulated for 72h with either RPMI, 1µg/mL IFN-$\gamma$ or BMix $10^6$/mL. BMix consists of a mix of *Borrelia burgdorferi sensu stricto, Borrelia garinii and Borrelia afzelii* species.
Figure S9 Isotype control at 24h: Protein expression analysis performed by flow cytometry, PBMCs from six healthy volunteers were stimulated with a medium control and live mix $10^6$/mL of the three *Borrelia burgdorferi* sensu lato species: *B. burgdorferi*, *B. afzelii* and *B. garinii* for 24h. The monocyte population was gated on using the gating strategy shown in figure S2. Isotype controls (red line) was similar between the two groups and therefore only one isotype control is shown, below the graph the geometric mean used for quantification of the marker’s expression is recorded.
Chapter 3

Genetic regulation of major histocompatibility complex class II expression during infections

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Abstract

Major histocompatibility class II complexes (MHCII) are essential for the recognition of extracellular pathogens and exchange of information between immune cells. Moreover, MHCII include the structures necessary to initiate development of a targeted memory response. Subsequently, MHCII expression is tightly regulated, but many aspects of these regulatory pathways can be manipulated by invading pathogens to avoid recognition and detection, affecting either MHCII expression or functionality. As the main transcription factor of MHCII, Class II transactivator (CIITA) is often the primary target through which signal transducer and activator of transcriptions (STAT) or suppressor of cytokine signalling (SOCS) cascades can interfere, as well as epigenetic changes induced by different infections. In addition, other transcription factors and miRNAs have been shown to be influenced by invading pathogens. In this review, the broad range of MHCII regulatory pathways and how infections affect these pathways are discussed.
Introduction

Major histocompatibility complexes (MHC) have been studied ever since their discovery in mice in 1936 (1). These complexes are important in the presentation of peptides, present or produced inside human cells or that they find in their environment, to other immune cells. Without antigen presentation, immune cells would not be able to recognize self from not-self or develop specific long-term memory responses against invading pathogens. MHC class II (MHCII) and I (MHC) are the two immune complexes responsible for antigen presentation and therefore communication between myeloid and lymphoid immune cells. MHCII is mostly responsible for extracellular peptide presentation, whereas MHC generally presents peptides found or produced within the human immune cell. MHCII and MHCII are part of separate pathways, and their genes are differentially regulated. In this review we will focus on the MHCII machinery.

MHCII and related proteins, including HLA-DM, HLA-DO and CD74, are regulated by a gene called class II transactivator (CIITA). The MHCII pathway activation starts in the nucleus, where transcription of MHCII (-related) proteins is initiated via CIITA binding. From the nucleus, the transcribed mRNA is transported to the endoplasmic reticulum (ER). MHCII dimer subunits and CD74 are produced in the ER. HLA-DR consists of two subunits: HLA-DRα and HLA-DRβ. Following production, trimers of CD74 proteins bind three MHCII glycoproteins, each consists of one MHCII alpha-beta dimers (2). This occurs independently of their polymorphism (3) resulting in the formation of a nonameric complex. CD74 is important in MHCII ER egress (4,5), though the dependency differs between MHCII alleles (6).

Next, these proteins are transported to the Golgi and from there to early endosomes. In the early endosomes, CD74 is partially degraded, leaving only a fragment called CLIP in the MHCII complex (7). CLIP has varying affinities for different MHCII proteins. HLA-DM induces CLIP release, making the groove accessible for peptides (8). CLIP is then broken down by the proteases. After HLA-DM forms an unstable bond with MHCII, only peptides that dissociate HLA-DM from MHCII can bind the molecule for antigen presentation (9).

MHCII genes have a high level of nucleotide diversity and sequence variation in the proximal promoter regions (10,11). The human MHCII encodes three different isotypes: HLA-DR, HLA-DP and HLA-DQ. While HLA-DR is widely known and broadly studied, few data is available on HLA-DP and HLA-DQ. These three isotypes show around 70% similarity to one another, are all highly polymorphic and encoded by
different MHCII genes. Moreover, they also have different subunits. HLA-DRβ has more than 700 known alleles at population level, while of HLA-DRα only has three different alleles (12,13). Also, HLA-DR alleles with low affinity for the CD74 CLIP fragment, in the presence and absence of HLA-DM, are more likely to induce autoimmune responses (14,15).

Figure 1: Genetic regulation of MHCII and related genes. MHCII gene transcription, showing the S, X1, X2 and Y boxes, that bind the enhanceosome complex components in place, when accessible, allowing CIITA to interact and attach to the enhanceosome complex to initiate MHCII (related) gene transcription.

Class II Transactivator

Transcription of MHCII and its related genes (CD74, HLA-DM, -DP, -DQ and -DO) is regulated by CIITA (16–19). CIITA also activates MHC I promoters in vitro, however, its contribution in vivo remains unknown (20). CIITA is a non-DNA-binding nuclear protein. The mature protein contains an N-terminal transcriptional acidic activation domain (AAD), a proline-serine/threonine rich (P/S/T) domain, a GTP binding domain (GBD) and a c-terminal leucine rich region (LRR) (Figure 1) (21–23). A cell type and differentiation specific response is induced by CIITA. The expression pattern induced can be influenced cytokine stimulation, explaining the effects of IFNγ and IL-4 on MHCII expression (24). Furthermore, Fas-ligand (CD95L), collagen A2 and several viral controlling proteins are also regulated by CIITA (25). CIITA overexpression in T cells was observed to suppress FasL, a receptor ligand involved in cytotoxic T cell apoptosis (26), suggesting it may promote immune cell survival.

MHC2TA is CIITA’s coding gene. It is located on chromosome 16p13 (27). CIITA is evenly distributed between the cytoplasm and cell nucleus (28,29). It is also a
member of a large family of structurally related proteins called the nucleotide binding oligomerization domain (NOD)-protein, otherwise known as the CATERPILLER (caspase-recruitment domain (CARD), transcription enhancer, R (purine-) binding, pyrin, lots of LLRs) -protein family. This includes the intracellular receptors NOD1 and NOD2, well-known for their role in innate immunity and pathogen recognition mechanism (22,30,31).

MHCII promoters differ from MHCII promoters in the sense that they lack enhancer A and ISRE, while the sequence alignment in the SXY module is highly conserved (32,33). The promoter of the MHCII gene has DNA-binding proteins wrapped around nucleosomes formed by histone octamers into compact chromatin (22). A genome wide siRNA screen identified 287 factors responsible for the regulation of MHCII expression, of which 140 proteins had unknown functions (34). Some examples of molecules modulating MHCII antigen presentation include chloroquine via inhibition of lysosomal acidification, leupeptin, selective cathepsin S inhibitors through interference with CD74 processing, inhibitors of aspartyl proteases (LY411575 and zLL2k) and AEP affect the presentation of defined epitopes.

Though the promoters of MHCII and related genes are located on numerous chromosomes, CIITA recognizes these genes through the presence of conserved sequence elements called the SYK motif containing the W/S, X, X2 and Y boxes. These sequence elements are bound by several complexes that cooperatively form a highly stable macromolecular nucleoprotein complex referred to as the MHCII enhanceosome (35). Of the MHCII enhanceosome, the DNA-binding protein members regulatory factor X (RFX) 5, RFX-ANK and RFX-AP bind to the X1 box (Figure 1). While another enhanceosome element, the X2BP complex, including cyclic AMP responsive element binding protein (CREB), associates with X2 (36–38). The Y box is recognized by the trimeric nuclear factor Y (NF-Y) complex, including NF-YA, -YB and -YC (39,40). The W/S box is more promiscuous in the proteins it binds. RFX has been demonstrated to associate with the W box. Though, none of these proteins seems to play a functional role (41). The S box functionality is influenced by unidentified factor. However, its presence, integrity and spacing between the S and X box is crucial in CIITA recruitment (42). The enhanceosome complex is used by CIITA to dock to the MHCII (-related) gene promoters to activate gene transcription by recruiting histone modifying enzymes and transcription machinery (31,43).

The motif of CIITA, AAD, is important for the interaction with effector proteins and co-activators that promote gene transcription. The phosphorylation sites in P/S/T
can induce post-translational modifications. GBD and LRR facilitate CIITA association and translocation to the nucleus (44). CIITA promotes transcription through the recruitment of several other regulators such as positive transcription factor b, transcription factor IID (TFIID) and TFIIB, phosphorylation of RNA polymerase, chromatin remodelling factor brahma-related gene 1 (BRG1) and co-activators involved in chromatin remodelling, histone acetylation and methylation. Moreover, specific histone methyltransferases, including co-activator-associated arginine-methyltransferase 1 (CARM1), also known as protein arginine methyltransferase 4 (PRMT4), induce epigenetic changes to the MHCII promoters under influence of CIITA (45). CARM1 has been reported to be required for MHCII expression in B cells and IFNγ induced expression (22).

In contrast, PRMT1 activity has been linked to the suppression of MHCII expression in macrophages through CIITA methylation and degradation (46). PRMT1 specializes in modifying arginine residues of both histones and non-histone factors (47). HLA-A expression was also decreased by PRMT1 activity, while HLA-E remained unaffected in cancer cells (48). Furthermore, PRMT1 represses NF-kB via methylation and preventing RelA binding to target genes, possibly through TRAF6 methylation. In turn, IFNγ stimulation resulted in downregulation of PRMT1 expression, PRMT1 knockout using shRNA resulted in more effective activation of MHCII gene expression using IFNγ (46). Beaulieu et al. (2016) have reported the extreme N terminus of CIITA is responsible for its rapid degradation, this area contains two arginine residues that may be targeted by PRMT1 (49).

ERK1/2 is a regulatory pathway of CIITA. According to Voong et al. (2008), phosphorylation of Serine (Ser)²⁸⁶, Ser²⁸⁸, Ser²⁹³ on CIITA increased CIITA export from the nucleus and downregulated its activity (50). In contrast, Morgan et al. (2015) showed a role for Lys⁶³ ubiquitination in CIITA post translational modification in the cytoplasm also using CIITA transfected COS cells, a monkey cell line derived from kidney (51). This was followed by phosphorylation of CIITA via ERK1/2 signalling possibly induced through the regulatory site Ser²⁸⁰, causing its translocation from the cytoplasm to the nucleus and increasing its activity. These conflicting findings may be attributed to differences in transfection, timing of measurement and activity of other kinases such as ERK5 and cell division protein 2. Lys⁶³ ubiquitination is linked to TRAF6 signalling, allowing additional proteins such as TGFβ activated kinase 1/MAP3K7 binding protein 2 (TAB2) to bind to Lys⁶³ ubiquitinated chains, resulting in phosphorylation and nuclear translocation. IRAK1 is also modulated through the same mechanism. Lys⁶³ could either act as a scaffold for ERK1/2 binding or by driving CIITA’s nuclear translocation it provides a platform
for ERK1/2-CIITA interaction to take place. Morgan et al. (2015) suggested CIITA may be regulated by the same kinase but at multiple locations, having a diverse range of effects on the protein’s activity (51). K63 linked ubiquitination is involved in the crosstalk between CIITA and ERK phosphorylation. This regulatory mechanism of mono-ubiquitination is crucial for CIITA’s activity, mediates cellular movement from the cytoplasm to the nucleus, and K48 polyubiquitin implements its CIITA degradative properties (52).

Long-range interactions between the proximal elements of the MHCII regions and distal X-Y or X-box sequences cause epigenetic changes at the MHCII promoter (43,53,54). One study even reported that RFX and CIITA interact with the SYX module and distal X-Y or X-box like sequences to form a chromatin loop (53), resulting in enhanced histone acetylation (55). CCCTC binding factor for insulator (CTCF) has also been demonstrated to control MHCII gene expression via long distance chromatin interactions (56), which involve CIITA and RFX5 association.

Epigenetic regulation through histone deacetylases (HDACs) and demethylases are also important mediators in gene silencing. HDAC inhibitors, including trichostatin-A, enhance MHCII gene transcription, while HDAC recruitment results in CIITA, NF-
Y and RFX dissociation from the MHCII promoters (57–60). Deacetylation of CIITA, by HDAC2 and SIRT1, differentially control its stability (61,62). While HDAC1 and 2 repress IFNy activation and CIITA function, HDAC4 interacts directly with RFX-ANK, causing MHCII gene repression (60,63,64). Another methyltransferase KMT1C, otherwise known as G9a, is required for epigenetic MHCII silencing processes (65).

The process of ubiquitination via either a degradation-independent path or phosphorylation directly modulates CIITA function (66–69), increasing its binding to MHCII transcription factors. Whereas CIITA phosphorylation modulates CIITA self-association, protein stability and translocation (43). ATPase S6a, a subunit of the 19S proteasome complex, was observed to be vital in the regulation of cytokine induced CIITA expression (70). MLL/COMPASS subunits are also recruited to MHCII promoters following IFNy stimulation, suggesting a role of stabilization of active histone H3-K4 methylation in the induction of MHCII gene transcription (71). Mycobacterium avium repressed IFNy induced histone acetylation in a human monocyte cell line through enhancing the expression of Sin3A, a HDAC1 co-repressor. Also, a 481-nucleotide RNA of trophoblast non-coding RNA was demonstrated to suppress CIITA transcription when transfected into murine B cells (72,73). CIITA repression in various cancer types is dependent on CpG dinucleotide methylation CIITA, as well as histone deacetylase activities (58,59,74,75).
Histone acetyltransferases are also involved in regulating CIITA. Well-known histone acetyltransferases are the lysine acetyltransferases (KATs) p300 (KAT3b), p300/CBP-associated factor (pCAF) and steroid receptor co-activator (SRC)-1, providing a more open chromatin structure for gene transcription (32,76–78).

Cell specific promotor activation

CIITA’s gene is regulated by four different promoters (Figure 2). These four promoters result in unique transcription leading to four different CIITA isoforms that differ in their N-terminus. The CIITA N-terminal moiety contains several transcription activation domains, including components of general transcription machinery, chromatin remodelling factors and other co-activators. In contrast, the C-terminal is implicated in self-association, localization to the nucleus and recruitment of the enhanceosome (23,33,79). CIITA molecules slightly differ in their N-termini due to the distinct exon 1 sequence, promoter type I (pI) for example has an extra CARD domain (31). Promotor type I (pI) to pIII specifically drive MHC2TA transcription in antigen presenting cells (APCs) and pIV is inducible through IFN-γ stimulation in a significant number of cell types (21,80) (Figure 2).

CIITAs four distinct promoters are expressed differentially across various cell-types. The promotors precede alternative first exons, spliced to shared downstream exons, resulting in various 5’ends, e.g. pI has a 101 amino acid N-terminal extension, while pIII only contains 24 amino acids. The pI isoform is thought to be slightly more efficient in activating MHCII promoters (81). The attribution of this increased efficiency has been linked to the N-terminal sequence that has a weak homology to CARDs. A study by Muhlethaler-Mottet et al. (1997) demonstrated various roles for the CIITA promotor isoforms, known at that time. Each isoform, resulting from activation of a distinct promoter, was expressed differentially over a range of cell types. While (monocyte-derived) DCs expressed high levels of pI and pIII, the thymus, spleen and several B cell lines expressed high levels of pII and pIV, and no to little pI (21,82–84). CIITA pIII is also the isoform responsible for MHCII expression in activated human T cells and murine pDCs (85). Furthermore, in B cells, IFNγ stimulation resulted in enhanced type IV expression and managed to induce pIII expression in some B cell lines (21). It would be interesting to study to what extend and how this differential expression could result in specific inhibition of MHCII related genes across various cell types.
Intriguingly, macrophages from pIII and pIV deficient mice have normal levels of MHCII expression (33,82), suggesting that, although pIII and pIV can be induced by IFNγ stimulation, its promoter expression levels consist of type I, similar to (mo)DCs. pI expression in murine macrophages was also more significantly increased in response to IFNγ stimulation, while pIV was enhanced transiently. However, the pI promoter region does not contain IFNγ response sequences suggesting this upregulation was caused by an indirect consequence of the IFNγ stimulation. pI expression was also maintained for a longer time period (86). PRDI-BF1 or Blimp1 mediated pIV suppression in plasma and multiple myeloma cells (87). Whether the same mechanism can regulate CIITA pIV expression in macrophages is unknown. Though pIV has been shown to regulate antigen presentation in DCs, through the association with the PU.1/IRF8 binding sequence on CIITA pIV by recruitment of histone deacetylases. Blimp-1 is mostly induced through STAT3, IRF4, AP-1 and expression of various TLRs (88). Through a similar mechanism, MHCII is abrogated in plasma cells, mature DCs, trophoblasts and various tumour cells, as a direct consequence of CIITA silencing (80,83,89–94) by BLIMP1 binding to pIII. This also resulted in a loss of terminal differentiation of B cells into plasma cells. IFNγ stimulation can induce MHCII expression in other non-haemopoietic cell types such as endothelial cells, fibroblasts, and keratinocytes (21,95–97). This probably occurs through pIV promoter induction, as was shown in pIV deficient mice studies (82,84,86,98,99).

IFNγ induction of the type IV CIITA promoter is caused by GAS, an E box and an IRF element binding (21,96,97,99,100). IFNγ activates JAK1 and JAK2 (95), leading to phosphorylation of STAT1. A complex of GAS and E box is bound by activated STAT1 and USF1, while the IRF element is occupied by IRF1 and IRF2 (97,101,102), both of which are most likely activated by STAT1. Furthermore, type IV deficient mice showed a reduction in CD4 T cell numbers, from a defect in CD4 T cell positive selection by cortex MHCII+ epithelial cells in the thymus, severely affecting the immune function in these mice (82,103).

In contrast, pIII contains an E box motif, ETS-IRSE motif, activation response element 1 (ARE1) and ARE2. CREB1 and activating transcription factor 1 (ATF1) bind ARE2 (94,104,105) (Figure 2). In turn, the E box has been shown to bind E47 and PU.1 and IRF4, specifically and synergistically activating pIII and pIV in B cells in vivo. PU.1 was also demonstrated to play an important role in inhibition MHCII expression in murine DC, through CIITA pI suppression (85). Moreover, IRF8, Oct1 and NF-1 can interact with CIITA promoters (86). Using human cell lines, Van der Stoep et al. (2004) also demonstrated that IRF-4 binds specifically to the B cell E -
box and EICE motif of CIITA pIII, and it does so synergistically with PU.1 and E47 (105). RUNX3, or AML2, is another regulatory element for pIII and pIV (31,85).

**Figure 2: MHC2TA, or CIITA, gene expression regulation through the various promotor regions.** pI is bound by PU.1, IRF8, SP-1, NF-kB, while pII transcription factors are unknown. pIII is a promotor region that interacts with various transcription factors such as E47, IRF4, PU.1, AML2, CREB, ATF, OCT and NF1. In contrast, STAT1, USF-1 and IRF1 attach to the pIV region of CIITA.

Contrary to pIII and pIV, CIITA pI is differentially regulated and mostly of importance for its expression in myeloid cells. Through CIITA inactivation by histone deacetylation of the regulatory region of MHC2TA, activity of pI is markedly decreased in DCs after maturation. MHCII synthesis, peptide loading, and cellular localization are reorganized, while MHCII stability is increased and de novo MHCII synthesis is shut down. Other than the silencing of pI during DC maturation, probably due to loss of histone acetylation, there are almost no studies of pI regulation available. During differentiation of monocytes into DCs, pI expression was enhanced (83). CIITA pI was observed to contain binding sites for PU.1, NF-kB and ETS/ISRE. In immature DCs, pI transcription required Sp-1, PU.1, NF-kB and IRF-8 binding to the promoter. The latter three sites were crucial for pI transcription (85,106). pI transcriptional regulation is largely unknown despite the importance of its role in myeloid antigen presentation cell. Recent literature also describes several additional CIITA isoforms, formed via posttranslational modifications, though their exact role in antigen presentation regulation is yet to be investigated (107).
Consequences of changes in MHCII and CIITA expression

Permanent loss of CIITA function in humans leads to bare lymphocyte syndrome, whose patients are characterized by severe immunodeficiency. The patient’s lymphocytes lack MHCII expression, are deficient in T-helper cells, and show impaired humoral and cytotoxic responses (108). Of interest, absence of MHCII expression by lymphatic endothelial cells may also enhance auto-immunity, because of the importance of these cells in T cell tolerance and supporting regulatory T cells (109).

In contrast, downregulation of IFNy, or production of TGFβ, IL-1β, IL-4 and IL-10, can interrupt CIITA expression and interfere with MHCII expression (100,110–114). The role of TGFβ is regulated through SMAD3 and SMAD4 activation, while RMND5B involvement inhibited this process, possibly by blocking SMAD activation (34). Downregulation of both CIITA and HLA-DR mRNA levels were also observed. Of interest, PLEKHA4 silencing in a melanoma cell line called MelJuSo resulted in expression of peptide loaded MHCII and other antigen presentation components. The exact regulatory mechanism is unknown since it has only been described as a phosphoinositide-binding protein. Knockdown of EFHD2 and HTATIP resulted in increased expression levels of CD74 and HLA-DR, while only IL-27 receptor silencing affected MHC expression.

In vitro assays by Gourley et al. (1999) showed how CD4+ T cells can express CIITA in a Th1 environment. In contrast, CIITA expression was absent under Th2 conditions (115,116). This may be due to the presence of IL-4 or its regulatory pathways. In contrast, Otten et al. (2003), observed that MHC-II expression is not differentially regulated in activated in human or murine T cells (117). Whereas Gourley et al. suggested CIITA probably suppresses IL-4 in Th1 cells through competition with the IL-4 transcription factor NFAT (118), Otten et al. (2003) suggested that instead of inhibition of IL-4 expression, CIITA induces a Th2 bias during CD4 T cell activation (117). A study with CIITA transfected human Jurkat T cells expressing IL-4 but no IFNy demonstrated how IL-4 expression was increased in Th1 cells expressing CIITA, probably due to enhanced histone H3 acetylation of the IL-4 promoter. CIITA also abrogates binding of coactivator CBP/p300 and STAT6/NFAT1 to IL-4 promoters, repressing IL-4 gene activation in the T cell line and primary human CD4 T cells (119). Not just IL-4 but also, IL-10, FAS ligand, cathepsin E, collagen type Iα2, cyclin D1 are proposed to be repressed through co-
activator CBP (cAMP-responsive-element-binding protein (CREB)-binding protein), a histone acetyl transferase, by CIITA (115,118,120,121). Intriguingly, none of these genes contains the SXY module normally bound by CIITA at MHCII gene promoters.

Another molecule called semaphorin receptor-plexin A1 interaction was also shown to be involved in enhancement of APC-T cell stimulation. Its expression in mature murine DCs was dependent on CIITA through the promoter of Plxna1 (122). This receptor-ligand complex is known to play a role in type I IFN stimulation. Sempahorin receptor plexins induce cytokine production in monocytes and macrophages, through integrins, as a component of the immunological synapse (123).

Silencing of three other genes called KIAA1007 (CNOT1), CDCA3, and MAPK1 enhanced CIITA and HLA-DR transcription, suggesting suppressive role. While MAPK1 is widely known for its role in immunity, this was also the only gene affecting the MHC1 locus. Of CDCA3, the function has yet to be discovered (34). CNOT1 is part of the CCR4 negative on TATA (CCR4-NOT) complex. CCR4-NOT complexes are central regulators of gene expression. These complexes are located within the nucleus where they bind promoters and nascent RNA transcripts, as well as the cytoplasm. Here, CCR4-NOT complexes regulate mRNA metabolism and translation from ribosomes (124–128). Gene regulation by CCR4-NOT may also be induced through interaction with transcription factors AP-1, retinoic acid X receptor or c-Myb (127,129). A recent study using doxycycline-inducible shRNAs that downregulated CCR4-NOT subunits, demonstrated how CNOT2 suppression resulted in elevated MHCII expression, independently of CIITA function and detectable changes of chromatin structure. Neither CIITA, nor RFX complex components were affected by CNOT2 knockdown (124). Furthermore, CNOT3 knockdown did not result in an upregulation of MHCII in T cells or the Raji B cell lymphoma cell line but did affect naïve macrophage MHCII expression. CNOT1 knockdown was not very successful and could only partially knocked out, possibly due to is essential role in the complex formation and functionality. The authors suggested that most of the gene regulation of CCR4-NOT complexes is induced via ubiquitination or phosphorylation.

A recent study by Buxadé et al. (2018) showed how NFAT5 is specifically important for CIITA expression in bone marrow derived macrophages (BMDM). Yet, NFAT5 doesn’t play a role in CIITA expression in DCs. NFAT5 deficient macrophages failed to activate CD4 T cells through MHCII-dependent responses. NFAT5 deficiency affected the various CIITA promoters, MHCII and CD74 expression, but did not
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decrease the expression of enhanceosome components. Active enhancers such as H3K27 acetylation markers in the CIITA pl region have NFAT5 binding characteristics. However, the study shows that even though the expression of CIITA plII and plIV is much lower in BMDMs, NFAT5 deficiency significantly downregulates their expression as well (130). Of interest, NFAT5 shares structural and functional properties with NF-kB and other NFAT proteins (131,132). Its regulatory mechanisms are important during macrophage polarization, pre-TCR-induced T cell development and in mature T cell function. When studying the capacity of the NFAT5 deficient BMDM to activate T cells, Buxadé et al. (2018) observed that the reduced T cell expression of CD25 and CD69 was caused by high numbers of MHCII^{LOW} BMDMs in the macrophage population and not by differences in MHCII function of these macrophages when compared to the wildtype. Also, NFAT deficient macrophages expressed MHCII following IFNγ stimulation, but MHCII molecule expression was lost again as the macrophages returned to their basal conditions. NFAT5 seems to facilitate the formation of a remote enhancer A loop, causing its interaction with CIITA pl in macrophages but not myeloid DCs (130).

Hypoxic conditions are known to severely affect immune responses. Under these conditions, tumour-associated macrophages (TAMs) are polarized into an M2 macrophage phenotype. One of the genes affected by this polarization is Forkhead box protein O1 (FoxO1), a transcription factor important in gluconeogenesis, glycogenolysis and adipogenesis. FoxO1 knockout mice showed a significant reduction in CIITA expression, specifically pl seemed to be affected. Both FoxO1 and MHCII downregulation promoted tumour growth. Intriguingly, their gene suppression was not regulated by HIF1α. In a hypoxic environment, the TAM FoxO1 expression is regulated through phosphorylation and enhanced degradation by E3 ubiquitin ligases such as Skp2 and Cop1 (133).

Both MHCII and CIITA suppression play a major role in the development of tumour tolerant environment. Recent advances in the oncology field are using CIITA to restore these immune functions, especially inside solid tumours, which are harder to target. By re-establishing CIITA expression, MHCII is reintroduced and will start to present tumour relevant antigens, that may be used to optimally stimulate in vivo tumour specific MHC class II restricted CD4 T cells generating specific and long-lasting protective immunity against the tumour (134,135). Of interest, chemotherapeutics may also activate MHCII expression through double DNA strand breaks that enhance CIITA expression (135).
CIITA influence on MHCI expression

Although, CIITA is thought to target MHCII genes specifically, it is known to play a limited role in MHCI activation (136,137) and literature continues to challenge this notion of CIITA specificity, as more genes are observed to be modestly influenced by CIITA expression. In contrast to MHCII, MHCI genes are located at chromosome 6p21 (27). While the NLR caspase recruitment domain (CARD) containing 5 (NLRC5) regulates the transcription of MHCI (25) via STAT1 or STAT3 and the induction of type I and II IFNs (20,138–140). The SYK motif of the MHCII genes are also present in MHCI promoter regions (141). Furthermore, NLRC5-dependent MHCI activation is reliant on components of the enhanceosome (142,143). CIITA is capable of activating MHCI promoters in vitro, but its role in vivo is still enigmatic. CIITA lysine transferase (KAT) activity bypassed TATA box binding protein (TBP)-associated factor 250 kDa (TAFII250) in MHCI promoter activation (144). Contrarily, NLRC5 only associates with MHCI promotors and does not activate MHCII genes (20). Moreover, the numerous MHCI genes are differentially regulated by CIITA. Gobin et al. (2001) demonstrated how HLA-G, TAP1 and TAP2 are not affected by CIITA downregulation, while B2M and HLA-C expression are slightly affected by CIITA deficiency. In contrast, HLA-A, HLA-B and HLA-E expression was almost fully dependent on CIITA expression (32).

Through both MHCI and MHCII pathways, APCs can initiate immune responses by presenting exogenous antigens to T cells. Lysosomal activity is key in modulating the balance between these two pathways. Transcription factor EB (TFEB) is a master gene for lysosomal biogenesis via the regulation of hydrolases, membrane proteins and genes involved in autophagy. Transportation of TFEB from the cytoplasm to the nucleus to induce transcription of its regulated genes is inhibited via serine phosphorylation by ERK2 and mTORC1, protein C kinase is another potential activator through phosphorylation of its carboxy-terminal region (145,146). During DC maturation, upregulation of TFEB enhanced lysosomal proteolytic activity, causing a shift from cross presentation of exogenous antigens to increased MHCII presentation. This shift is probably the result of TFEB acidification induced via enhanced expression of vacuolar ATPase subunits (145). Furthermore, TFEB increased transcription of lysosomal cathepsins D, L and S, some of which are also involved in CD74 degradation. TFEB was upregulated both by TLR4 and TLR2 ligands, while TFEB activation showed no change upon stimulation with TLR9 ligands. Of interest TFEB knockdown had no effect on LPS induced bone marrow DC maturation, but it does influence lysosomal fusion with the plasma membrane and therefore MHC transportation to the cell surface (147).
Another basal MHCI transcription factor is TAF1, its acetyltransferase activity is associated with that of CIITA and required for the initiation of MHCI transcription. Constitutive MHCI expression in the absence of IFN is dependent on canonical transcription factor II D (TFIID), while in the presence of IFN, CIITA is induced and interacts with TATA box binding proteins, as well as several TAFs including TAF7, TAF6 and TAF9. TAF7 regulates the acetyltransferase activities of both CIITA and TAF1 by binding to the molecules and inhibiting their interaction with other proteins. TAF7 is therefore important in inhibiting transcription initiation until the pre-initiation complex is fully assembled. By TAF1 autophosphorylation, TAF7 is released from the TFIID complex, allowing it to bind to the DNA and regulate transcription. The mechanism of TAF7 release from CIITA has not been identified (148).

MHCI and CIITA expression during infection

Pathogens developed various strategies to target the antigen presentation pathway. Through TLR2 signalling, *Mycobacterium tuberculosis* affects the processing of Ag85 and degrade phagosome-associated antigens. Moreover, there are several studies that suggests it impairs the transport of newly synthesized MHCII (149,150). Another study suggested that TLR2 and MAPK signalling induced histone hypoacetylation at CIITA pIV, resulting in suppression of CIITA transcription (151). In contrast, *Listeria monocytogenes* enhanced MHCII expression in mice (152), while another study in bone marrow derived macrophages specifically showed suppression of IFN-γ induced MHCII expression (153).

*Salmonella typhimurium*, in turn, expresses phoP, targetting their processing for antigen presentation (154). *Escheria coli* and *Chlamydia* can decrease MHCII expression, inhibiting antigen processing and presentation (155,156), while the LPS component of Gram-negative bacteria can also increase MHCI and MHCII on macrophages (157,158). Long-term CpG exposure can also decrease MHCII expression on macrophages (159). Of interest, *Helicobacter pylori* exposed macrophages reduced MHCII expression after 48 hours of exposure, showing decreased expression of CIITA mRNA within 6 hours after exposure, compared to untreated cells. After further analysis, Codolo *et al.* (2020) observed that Let-7f-5p, Let-7i-5p, miR-146b-5p, and -185-5p miRNA target CIITA expression in the melanoma cell line M121224 and the cervical cancer cell line HeLa (160).
Human cytomegalovirus (CMV) infects a significant proportion of the human population, and it is well known that during CMV infection, DC undergo downregulation of MHC class I and class II molecules and become unable to efficiently stimulate T-cell responses (161,162). However, in a recent study they demonstrated that macrophage expression of MHCI and MHCII were unaffected by CMV infection, enabling the induction of significant T cell responses (163). Next to CMV, various other viruses including Epstein-Bar virus, *Herpes simplex* virus and adenoviruses are known to interfere with the CIITA-MHCII pathways via STAT1 and IFN-y signalling (164).

MHCII expression was significantly inhibited in *B. burgdorferi* infection (165,166), also known as Lyme Borreliosis (LB). This suppression is probably induced via CIITA inhibition (Brouwer 2021, unpublished). Several major signalling pathways may be involved in suppression of these antigen presentation pathways. Activation of STAT3 cascades may be involved. Moreover, IL-1β and IL-10 are cytokines which are produced in significant amounts during *B. burgdorferi* infection (167–169). Of interest, a study of LB in Rhesus Macaques showed a strong downregulation of the semaphoring plexin signalling pathway after 24h hours of *ex vivo* stimulation with live *Borrelia burgdorferi*, possibly interfering in the presentation of *Borrelia* antigens to T cells (170). Overall, infections have a significant impact on this important recognition and communication pathway.

**Conclusion**

In conclusion, the regulatory pathways of antigen presentation are a major target for invading pathogens, often through CIITA expression and STAT signalling cascades. Recent studies have also observed novel ways of gene silencing, including the expression of specific miRNAs. Further understanding of how these pathways are affected and in which immune cells may provide new opportunities to target the causal diseases.
Regulation of MHC II during infections

References


Regulation of MHCII during infections


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Regulation of MHCII during infections


Chapter 4

*Borrelia burgdorferi* inhibits CIITA transcription through pSTAT3 activation and enhanced SOCS1 and SOCS3 expression leading to limited IFN-γ production

Michelle A.E. Brouwer, Samuel T. Keating, Frank L. van de Veerdonk, Mihai G. Netea, Leo A.B. Joosten
Abstract

Interferons (IFNs) are important signalling molecules in human immune response against micro-organisms. Throughout initial *B. burgdorferi* infection, inadequate IFN-γ production results in the absence of a strong T-helper 1 cell response, hampering the development of an effective antibody responses in Lyme Borreliosis (LB) patients. To determine why IFN-γ production is absent, the STAT activation signature, following exposure of human immune cells to *B. burgdorferi* was investigated. While STAT3 phosphorylation was highly induced in T cells, B cells and NK-(T) cells, STAT1 expression only showed a slight increase upon *B. burgdorferi* exposure. STAT2 phosphorylation remained stable, increasing only in NK-(T) cells, whereas STAT4 signalling was reduced in all *B. burgdorferi* exposed immune cells. Moreover, *B. burgdorferi* significantly increased suppressor of cytokine signalling (SOCS)1 and SOCS3 gene expression in LB patients. Absence of IFN-γ production and STAT4 activation, in combination with STAT3 phosphorylation and upregulated SOCS1 and SOCS3 gene expression, suggests the formation of a more tolerant and anti-inflammatory response, specifically in T cells and B cells, in response to *B. burgdorferi*. *B. burgdorferi* also specifically interfered with CIITA isoforms normally expressed in antigen presenting dendritic cells, while enhancing CIITA isoforms present in adaptive immune cell subsets. Restoring the antigen presentation capacity of innate immune cells and the early production of IFN-γ in LB patients may re-establish immune functions during initial Lyme Borreliosis. These new insights will help advance the development of improved diagnostic assays and lead to novel treatment strategies.
Introduction

Lyme Borreliosis (LB) is the most common human vector borne disease. In 2017, the estimated incidence of LB per year was 22/100,000 in Europe and 9.1/100,000 in the United States, with an increasing number of cases reported each year [1; 2; 3]. The disease is caused by bacterial spirochetes of the B. burgdorferi genus transmitted by tick bites of the genus Ixodes [4; 5]. B. burgdorferi can cause severe symptoms in the human host, ranging from Lyme arthritis to neuroborreliosis and skin complications [4; 6]. One of the major hurdles in early LB development is the hampered T-cell and B-cell responses [7]. Antibody responses are dysregulated and appear late during the disease, preventing early detection of B. burgdorferi infection in the absence of a tick bite or erythema migrans (EM) [7; 8; 9]. While T helper (Th) 2 responses have been described in early LB, prominent Th1 responses towards B. burgdorferi remain nonexistent, often until the development of disseminated LB [7; 10; 11]. The key cytokine driving the Th1 response is interferon-γ (IFN-γ) [12]. In response to B. burgdorferi infection, IFN-γ production is generally not induced, and the lack of IFN-γ production in LB patients is associated with development of chronic symptoms [13; 14]. IFN-γ is an important regulatory cytokine of the immune response [13; 15] that triggers host defense mechanisms in immune cells, and subsequently leads to elimination of a diverse range of pathogens. Adoptive transfer of IFN-γ secreting CD4+ T cells, into B. burgdorferi-infected T cell deficient mice, promoted Lyme carditis resolution [16]. Moreover, absence of IFN-γ, enhanced C3H murine joint swelling in an experimental arthritis model [13].

Another important cytokine in the initiation of a Th1 immune response is IL-12. Whereas IL-12 plays a role in Th1 phenotype induction, IFN-γ is essential for completion and stabilization of Th1 polarization [17]. Though TNFα and IL-6 were secreted in significant amounts by human PBMCs in response to live B. burgdorferi spirochetes, IL-12 production was not detected [18]. Moreover, pre-treatment of mice with monoclonal antibodies against IL-12 caused an increase in B. burgdorferi spirochete burden [19]. When cytokines bind to their receptor, they phosphorylate the receptor’s associated signalling molecules, including the signal transducer and activator of transcription (STAT) family of transcription factors. Upon activation, STATs dimerize and translocate to the nucleus [20; 21], where they activate different immune pathways and induce cytokine production. STAT4 is activated by
IL-12 signalling, and is important for Th1 development from naïve CD4 T cells as well as the initiation of IFN-γ production [22; 23]. Upon phosphorylation, STAT4 binds to the promotor regions of genes encoding IFN-γ (IFNG) and TNFα (TNFα), as well as IL-12 and IL-18 receptor subunits [24]. In contrast, STAT1 is directly induced by IFN-γ signalling through JAK1/2 activation and promotes Th1 responses. In disseminated LB, STAT1-deficient mice could not resolve Lyme carditis and even demonstrated an exacerbation of LB compared to wild-type mice [25]. Different STAT proteins can also regulate each other negatively. STAT3 negative regulation of STAT1 is the most well-known example [26; 27; 28]. In contrast to STAT1 activation, STAT3 is involved in Th2 responses. STAT3 is activated by a broad range of cytokines, including IL-6, IL-10 and type I IFNs, all known to be induced during B. burgdorferi infection, as well as Toll-like receptors (TLRs) such as TLR2, which is one of the two prominent recognition receptors in B. burgdorferi infection [29][30; 31][32; 33; 34; 35]. Moreover, STAT3 is responsible for the anti-inflammatory effects of IL-10 and its associated Th2 responses [36] [37; 38; 39; 40].

Of interest, STAT proteins also regulate MHC class II (MHCII) expression, which is generated upon activation of its regulatory gene class II transactivator (CIITA). CIITA is normally induced through STAT1 phosphorylation [41; 42; 43] [44]. We recently described the molecular mechanism involved in inhibition of antigen presentation by B. burgdorferi spirochetes, interfering with the activation of adaptive immune cells and the development of a more targeted immune response against B. burgdorferi [45]. Antigen presentation was specifically inhibited in innate antigen presenting cells, suppressing T cell responses and several major signalling pathways. As a result, IFN-γ production may remain continually suppressed in LB patients [46]. A more prominent Th1 response with early IFN-γ involvement, via STAT1 and STAT4 activation, may therefore help to control LB early on during B. burgdorferi infection and restore the antigen presentation machinery.

Inhibition of pro-inflammatory T cell responses play a significant role during B. burgdorferi infection. While IFN-γ has been studied in disseminated murine models of LB, little is known about its function early on in patients, before the development of EM [25]. To investigate this further, the IFN-γ response was assessed in different immune cell types. The STAT signature was evaluated to determine the pathways involved in these key responses. Furthermore, we assessed the expression of an
important transcription factor, CIITA, that is regulated through STAT and suppressor of cytokine signalling (SOCS) expression. In this study, we show that IFN-γ is inhibited while *B. burgdorferi* induces STAT3, SOCS1 and SOCS3 activation, and inhibiting antigen presentation in innate immune cells through suppression of specific isoforms of CIITA.

**Materials and Methods**

**Peripheral blood mononuclear cell and monocyte isolation**

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats (Sanquin Blood Bank in Nijmegen) or blood freshly donated by anonymous healthy volunteers. Ethical approval was obtained from the committee on research involving human subjects (CMO) Arnhem-Nijmegen (NL32357.091.10). Blood was diluted 1:1 in PBS. Using Ficoll-Paque density gradient centrifugation, PBMCs were collected from the procured interphase. PBMCs were washed twice in ice-cold PBS and resuspended in RPMI 1640 Dutch Modified; an addition of sodium bicarbonate and 20mM HEPES, supplemented with glutamate, gentamycin, and pyruvate. Cells from different healthy volunteers were used across the various assays. Monocyte-enriched populations were isolated by Percoll density gradient centrifugation following Ficoll isolation. Monocyte interphase was collected and washed three times in ice-cold PBS, followed by resuspension in RPMI 1640, Dutch Modified, supplemented with glutamate, gentamycin and pyruvate. Monocytes were left to adhere for an hour at 5 x 10^5/well, before washing away all non-adherent cells.

**B. burgdorferi spirochetes**

*B. afzelii* ATCC 51567, *B. burgdorferi* s.s., American Type Culture Collection (ATCC) 35210 type strain B31, and *B. garinii* ATCC 51383 were cultured at 24 °C in Barbour–Stoenner–Kelley (BSK)-H medium (Sigma-Aldrich) with 6% rabbit serum. Spirochetes were grown at 34 °C to late-logarithmic phase. Dark-field microscopy was used to check spirochete motility and spirochetes were quantified using a Petroff–Hauser counting chamber. *B. burgdorferi* species were centrifugated at 7000g for 15 min, washed three times with sterile PBS, diluted to the required concentration and stored at -80°C in sterile PBS until use. When thawed for cell stimulation, *B. burgdorferi* s.l. was prepared by mixing equal amounts of *B. afzelii*, *B. burgdorferi*, and *B. garinii*. A final concentration of 10^6 spirochetes/mL, equal to a multiplicity of infection (MOI) of 0.2, was used.
Candida albicans
Heat-killed, at 65°C for 60 minutes, Candida albicans blastoconidia (strain ATCC MYA-3573, UC 820) in a concentration of $10^6$ CFU/mL were used throughout this study.

Pam3Cys
Pam3Cys, a TLR1/2 ligand, was purchased at EMC microcollections (L-2000) and used in a final concentration of 10 μg/mL.

Mycobacterium tuberculosis
Cultures of H37Rv M. tuberculosis were grown to mid-log phase in Middlebrook 7H9 liquid medium (Difco, Becton-Dickinson) supplemented with oleic acid/albumin/dextrose/catalase (OADC) (BBL, Becton-Dickinson), washed in sterile saline and heat-killed for 30 minutes at 110 °C.

Cell stimulations and inhibitors of cell signalling
$5 \times 10^5$ PBMCs and monocytes were seeded in a round bottom 96 well plate. Cells were treated with different stimulants: Pam3Cys 10 μg/mL, IFN-γ (Boehringer Ingelheim), heat-killed Candida albicans $10^6$ CFU/well, MOI of 0.2, heat-killed Mycobacterium tuberculosis $10^6$ CFU/well, MOI of 0.2, heat-killed Mycobacterium tuberculosis $10^6$ CFU/well, MOI of 0.2, live Borrelia burgdorferi sensu lato Mix $10^6$ CFU/well, MOI of 0.2, was used. After addition of the stimuli to the cells the content of the wells were resuspended and cultured for various time periods.

Enzyme Linked Immunoassay
IFN-γ was measured using commercial enzyme-linked immunosorbent assay kits (Sanquin) according to the manufacturer’s instructions.

Quantitative Polymerase Chain Reaction
Cells incubated in TRIzol (ThermoFisher) and RNA was isolated according to the manufacturer’s instructions. RNA purity was checked using nanodrop (ThermoFisher). RNA was reverse transcribed into cDNA using an Iscript cDNA synthesis kit (Biorad). Quantitive PCR was analysis was performed using Power Sybr Green PCR Master Mix (Applied Biosystems) and a 7300 Real-time PCR system (Applied Biosystems). Primers were designed for the various CIITA isoforms known
on the Ensemble database at the time of development. For all primers, see supplementary Table SI.

**Transcriptome data**
Previously published RNA sequencing data of PBMCs from healthy controls (n = 13) and EM patients (n = 29), followed from baseline to 3 weeks and 6 months [46], were obtained from the publicly available National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (accession number GSE63085). Expression of selected genes was compared between EM patients at different time points and healthy controls by Kruskal–Wallis one-way ANOVA using R Software for Statistical Computing, version 3.2.4.

**Intracellular IFN-γ measurement**
For intracellular IFN-γ staining. Cells were primed 4h before analysis using 200 ul of RPMI with 10% human pool serum, 0.2 ul Golgi-plug inhibitor (brefeldin A; BD Bioscience), 50 ng/mL PMA (Sigma Aldrich) and 1 ug/mL Ionomycin (Sigma Aldrich), at 37°C 5% CO₂.

**Flow cytometry**
After a PBS supplemented with 1% BSA buffer wash (PBA), PBMCs were stained for 15 min at RT with for IFN-γ: CD3 ECD (Beckman Coulter), CD4 FITC (BD), CD8 PE-Cy5 (Biolegend), CD56 BV650 (Biolegend), CD14 AlexaFluor 700 (Biolegend), CD19 PE-Cy7 (Biolegend); STAT1, STAT3 and STAT4 with: CD3 ECD (Beckman Coulter), CD4 FITC (BD), CD8 PE-Cy5 (Biolegend), CD56 BV650 (Biolegend), CD14 PcB (Biolegend), CD19 PE-Cy7 (Biolegend) and STAT2 with: CD3 ECD (Beckman Coulter), CD4 APC (Beckman Coulter), CD8 Pacific Blue (Biolegend), CD56 BV650 (Biolegend), CD14 PE (Beckman Coulter), CD19 PE-Cy7 (Biolegend). PBMCs were washed again and permeabilized using a fixation and permeabilization buffer set (eBioscience, 00-5523-00), by incubation for 45 min at 4°C. After washing the cells with the permeabilization buffer kit, half of the cells were analysed for extracellular staining, while remaining cells were stained intracellularly with either IFN-γ PE (Biolegend), STAT1 PE (eBioscience), STAT3 (eBioscience), STAT4 PE (eBioscience), STAT2 FITC (Biolegend) for 15 min at RT. Gating strategy is demonstrated in Figure S1. Marker expression was measured with a CytoFlex (Beckman Coulter) and analysed on Kaluza version 1.5a (Beckman Coulter).
Statistical Analysis
Two or more independent experiments were used. qPCR data was normalized to a housekeeping gene, followed by direct comparison to the delta Ct value of the medium control, the delta delta Ct value was used for the final analysis. Flow cytometry results were normalized to the medium control for each donor at every time point. Statistics for measurements of cytokine levels, protein and qPCR data were performed using GraphPad Prism version 5.03 for Windows (GraphPad Software). Data represent mean ± SEM of different donors. Unless otherwise stated, means were compared using the nonparametric Wilcoxon matched pairs signed ranks test, with two-tailed significance level set as P > 0.05. Statistical significance was accepted at p<0.05 and indicated as follows: * p<0.05 and ** p<0.01. Additional statistical details are stated in the appropriate figure legends.
Results

Figure 1: Absence of extracellular IFN-γ after *B. burgdorferi* stimulation while intracellular reserves increase in different cell types. (A) PBMCs from healthy volunteers were stimulated for 24h (n=8), 48h (n=3) and 7 days (n=10) with a medium control, a live mix of *B. burgdorferi sensu lato* at 10⁶/mL and heat-killed *C. albicans* 10⁶/mL. Supernatants were removed and IFN-γ production was measured by ELISA (pg/ml). PBMCs from healthy volunteers (n=6) were stimulated with a medium control, a live mix of *B. burgdorferi sensu lato* at 10⁶/mL, heat-killed *C. albicans* 10⁶/mL, and Pam3Cys for 24h. (A) After 4 hours of priming with phorbol 12-myristate 13-acetate (PMA) 50 ng/mL, ionomycin 1 µg/mL and Golgi-plug inhibitor (Brefeldin A) 1 µl, intracellular IFN-γ expression was studied and the mean of the medium controls geometric mean fluorescent intensity (MFI) for each cell type is shown. To study the differences in intracellular IFN-γ per condition the MFI was normalized to this medium control and demonstrated as the fold-change in various cell types: (B) CD4⁺ T cells, (C) CD8⁺ T cells, (D) CD56⁺ CD3⁺ NK cells, (D) CD56⁺ CD3⁺ NK T cells and (E) CD19⁺ B cells. The dotted line represents the expression level of the medium control, to which the stimulated expression levels are normalized for each time point. Significance indications shown above the bars were compared to the medium control.
**B. burgdorferi** is a poor inducer of IFN-γ production

To determine the importance of IFN-γ in *B. burgdorferi* infection, extracellular IFN-γ secretion was determined over time (Figure 1A). IFN-γ production induced by *B. burgdorferi* was comparable to the culture medium at all timepoints. In contrast, IFN-γ concentrations were strongly enhanced when stimulated with *C. albicans*.

We explored whether intracellular IFN-γ concentrations were determined in various cell subsets. In unstimulated cells at 24h, the highest intracellular IFN-γ concentration was observed in CD56⁺ CD3⁻ NK cells, showing almost double the mean fluorescent intensity (MFI) of the other immune cells assessed, including CD56⁺ CD3⁺ NK-T cells (Figure 1B). While at 48h this expression stabilized more between cell types, showing more similar intracellular levels between CD4⁺, CD8⁺ and NK cells (Figure S2A). CD19⁺ B cells and CD56⁺ CD3⁺ NK-T cells showed a moderate increase in intracellular IFN-γ at 48h.

At the 24h timepoint, cells exposed to *B. burgdorferi* showed a trend for increased intracellular IFN-γ expression in CD4⁺ and CD8⁺ T cells (Figure 1C and D). Similar IFN-γ expression levels were observed for Pam3Cys. When PBMCs were exposed to *C. albicans* a more robust increase in IFN-γ was apparent. After 48h this increase was no longer observed (Figure S2B and C), suggesting IFN-γ was either secreted by the examined cells (Figure 1A), as observed for *C. albicans*, or degraded. In CD56⁺ CD3⁻ NK cells, *B. burgdorferi* induced no significant enhancement in intracellular IFN-γ production at 24h (Figure 1E). Similar production was observed for Pam3Cys stimulated NK cells. CD56⁺ CD3⁻ NK cells showed a significant increase in intracellular IFN-γ production when stimulated with *C. albicans* and an increasing trend with Pam3Cys at 48h (Figure S2D). At 24h a slight increase was observed in CD56⁺ CD3⁻ NK-T cells when stimulated with *B. burgdorferi* spirochetes. Yet, at 48h there was no change in expression (Figure 1E, S2D). In contrast, CD56⁺ CD3⁺ NK-T cells exposed to *C. albicans* or Pam3Cys showed higher IFN-γ expression levels at 48h. CD19⁺ B cells showed a slight increase for all stimuli (Figure 1F and S2E). Overall, intracellular IFN-γ expression showed an enhancement in CD56⁺ CD3⁻ NK cells at 48h, however, IFN-γ production remains very low or absent in *B. burgdorferi* exposed cells.
B. burgdorferi induces pSTAT3 and inhibits CII TA

A. Baseline MFI

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<tr>
<td>CD8+</td>
<td>8649</td>
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<tr>
<td>CD56+CD3-</td>
<td>10706</td>
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<tr>
<td>CD19+</td>
<td>10770</td>
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B. CD4+ T cells pSTAT2

C. CD8+ T cells pSTAT2

D. NK (T-)cells pSTAT2

E. CD19+ cells pSTAT2

F. Baseline MFI

<table>
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<td>pSTAT3</td>
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<tr>
<td>CD56+CD3-</td>
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<td>CD19+</td>
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G. CD4+ T cells pSTAT3

H. CD8+ T cells pSTAT3

I. NK (T-)cells pSTAT3

J. CD19+ B cells pSTAT3
Figure 2: Stable phosphorylated STAT2 and enhanced active STAT3 in different cell types exposed to *B. burgdorferi*. PBMCs from healthy volunteers (n=6) were stimulated with a medium control, a live mix of *B. burgdorferi sensu lato* at $10^6$/mL, heat-killed *C. albicans* $10^6$/mL, and Pam3Cys for 24h. (A) Phosphorylated STAT2 (pSTAT2) and (F) phosphorylated STAT3 (pSTAT3) expression was studied and the mean of the medium controls geometric mean fluorescent intensity (MFI) for each cell type is shown. To study the differences in pSTAT2 per condition the MFI was normalized to this medium control and demonstrated as the fold-change and studied in various cell types: (B) CD4$^+$ T cells, (C) CD8$^+$ T cells, (D) CD56$^+$ CD3$^-$ NK cells, (D) CD56$^+$ CD3$^+$ NK T cells and (E) CD19$^+$ B cells. STAT3 expression was also shown: CD4$^+$ T cells (G), CD8$^+$ T cells (H), CD56$^+$ CD3$^-$ NK cells (I), CD56$^+$ CD3$^+$ NK T cells (I) and CD19$^+$ B cells (J) using flow cytometry. The dotted line represents the expression level of the medium control, to which the stimulated expression levels are normalized for each time point. Significance indications shown above the bars were compared to the medium control.

*B. burgdorferi* exposure strongly activates STAT3, whereas STAT2 was not phosphorylated in T-cells

Since it is well-known that phosphorylated STAT2 (pSTAT2) and STAT3 (pSTAT3) expression is crucial for the regulation of IFN-$\gamma$ production and activation of specific IFN-$\gamma$ induced genes during an infection, we investigated STAT2/3 phosphorylation during *B. burgdorferi* recognition by human PBMCs.

Of interest, basal pSTAT2 was highest in CD56$^+$CD3$^+$ NK-T and CD19$^+$ B cells (Figure 2A). While the adaptive immune cells were not significantly affected by *B. burgdorferi* exposure (Figure 2B-E), STAT2 was only slightly phosphorylated in CD8$^+$ T-cells in response to *C. albicans* (Figure 2C). CD56$^+$CD3$^-$ NK cells showed no change in pSTAT2 to any of the stimuli (Figure 2D). In contrast, *C. albicans* and Pam3Cys showed an enhancing trend in pSTAT2 expression in CD56$^+$CD3$^+$ NK-T cells (Figure 2D). *B. burgdorferi* induced no STAT2 phosphorylation.

Contrary to levels of pSTAT2, basal pSTAT3 levels were specifically elevated in CD56$^+$CD3$^+$ NK-T cells (Figure 2F). Moreover, the level of pSTAT3 was significantly enhanced in CD4$^+$ and CD8$^+$ T cells after 24h of *B. burgdorferi*, *C. albicans* and Pam3Cys stimulation (Figure 2G and H). STAT3 phosphorylation levels increased to a lesser extent in CD56$^+$CD3$^-$ NK cells, CD19$^+$ B cells and CD56$^+$CD3$^+$ NK-T cells (Figure 2I and J), even though basal expression levels were high in CD56$^+$CD3$^+$ NK-T cells (Figure 2F). Except for CD56$^+$CD3$^+$ NK-T cells, no differences in pSTAT3 were
observed between *B. burgdorferi*, *C. albicans* and Pam3Cys. In contrast to pSTAT2, *B. burgdorferi* significantly induced STAT3 phosphorylation.

![Figure 3: Levels of *B. burgdorferi* activated STAT1 in different cell types.](image)

PBMCs from healthy volunteers (n=6) were stimulated with a medium control, a live mix of *B. burgdorferi sensu lato* at 10⁶/mL, heat-killed *C. albicans* 10⁶/mL, and Pam3Cys for 24h. (A) Phosphorylated STAT1 (pSTAT1) expression was studied and the mean of the medium controls geometric mean fluorescent intensity (MFI) for each cell type is shown. To study the differences in pSTAT1 per condition the MFI was normalized to this medium control and demonstrated as the fold-change and studied in various cell types: (B) CD4⁺ T cells, (C) CD8⁺ T cells, (D) CD56⁺ CD3⁻ NK cells, (D) CD56⁺ CD3⁺ NK T cells and (E) CD19⁺ B cells. The dotted line represents the expression level of the medium control, to which the stimulated expression levels are normalized for each time point. Significance indications shown above the bars were compared to the medium control.
STAT1 phosphorylation is induced in NK (T-)cells by *B. burgdorferi* spirochetes

STAT1 often forms heterodimers with other STATs to induce varying signalling cascades in immune cells, which could lead to activation or suppression of IFN-γ production. Therefore, studying pSTAT1 expression and activation in relation to pSTAT3 and pSTAT2 could reveal new insights into the mechanism of IFN-γ suppression by *B. burgdorferi*.

The highest basal expression of pSTAT1 was observed in CD56⁺ CD3⁺ NK-T cells (Figure 3A). At 24h, *B. burgdorferi* significantly induced STAT1 phosphorylation in CD56⁺ CD3⁺ NK-T cells, showing a robust increase (Figure 3D). CD56⁺ CD3⁺ NK-T cells showed high levels in pSTAT1 following *C. albicans* and Pam3Cys stimulation, while *B. burgdorferi* spirochete stimulation marginally enhanced pSTAT1. Of particular interest, in CD56⁺ CD3⁺ NK-T cells, Pam3Cys alone showed higher levels of STAT1 activation than *B. burgdorferi* or *C. albicans* stimulation. CD56⁺ CD3⁻ NK cells, CD4⁺ T cells and CD19⁺ B cells exhibited a moderate enhancement in pSTAT1 when exposed to *B. burgdorferi*, especially compared to *C. albicans* (Figure 3B, D and E). CD8⁺ T cells showed no modulation of STAT1 activation (Figure 3C).
**B. burgdorferi** induces pSTAT3 and inhibits CIITA

### Baseline MFI
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### CD4+ T cells pSTAT4

### CD8+ T cells pSTAT4

### NK (T-)cells pSTAT4

### SOCS1

### SOCS3
Figure 4: *B. burgdorferi* reduced phosphorylated STAT4 and increases SOCS1 and SOCS3 gene expression in different cell types. PBMCs from healthy volunteers (n=6) were stimulated with a medium control, a live mix of *B. burgdorferi sensu lato* at $10^6$/mL, heat-killed *C. albicans* $10^5$/mL, and Pam3Cys for 24h. (A) Phosphorylated STAT4 (pSTAT4) expression was studied and the mean of the medium controls geometric mean fluorescent intensity (MFI) for each cell type is shown. To study the differences in pSTAT1 per condition the MFI was normalized to this medium control and demonstrated as the fold-change and studied in various cell types: (B) CD4$^+$ T cells, (C) CD8$^+$ T cells, (D) CD56$^+$ CD3$^-$ NK cells, (D) CD56$^+$ CD3$^+$ NK T cells and (E) CD19$^+$ B cells. The dotted line represents the expression level of the medium control, to which the stimulated expression levels are normalized for each time point. Significance indications shown above the bars were compared to the medium control. (F-G) PBMCs were stimulated with *B. burgdorferi* species for 24h. SOCS1 (F) and SOCS3 (G) expression was determined by quantitative PCR analysis in response to a medium control, RPMI (n=8), a positive control, IFN-γ (n=5), *C. albicans* (n=8) and *B. burgdorferi* (n=8) stimulation. Relative expression was assessed using the B2M housekeeping gene. qPCR data was normalized to the housekeeping gene, followed by direct comparison to the delta Ct value of the medium control, the delta delta Ct value was used to determine the fold-change. The dotted line represents the medium control.

STAT4 phosphorylation was significantly inhibited by *B. burgdorferi* in CD8$^+$ T cells, while *B. burgdorferi* enhanced SOCS1 and SOCS3 gene expression in PBMCs

IFN-γ production may also be induced via another pathway through IL-12 signalling. IL-12 activates STAT4 transduction pathways which in turn causes IFN-γ transcription and secretion [47]. Compared to other cell types, basal pSTAT4 expression was twice as high in CD56$^+$ CD3$^+$ NK-T cells (Figure 4A). STAT4 phosphorylation was significantly reduced by *B. burgdorferi* in CD8$^+$ T cells (Figure 4C). No change or a decreasing trend was observed for pSTAT4 expression in CD4$^+$ T cells and CD19$^+$ B cells (Figure 4B and E). STAT4 was also not activated by *C. albicans* or Pam3Cys in CD4$^+$ and CD8$^+$ T cells or CD19$^+$ B cells (Figure 4B, C and E). CD56$^+$ CD3$^+$ NK-T cells stimulated with *B. burgdorferi* show suppressed STAT4 phosphorylation compared to the medium control (Figure 4D). In contrast, Pam3Cys and *C. albicans* demonstrated STAT4 activation in both NK and CD56$^+$ CD3$^+$ NK-T cell types. Pam3Cys showed a significantly high increase of pSTAT4 in CD56$^+$ CD3$^+$ NK-T cells. CD56$^+$ CD3$^-$ NK cells are the only STAT4 responders to *B. burgdorferi* exposure, as they showed an increasing trend instead of a reduction. However, both *C. albicans* and Pam3Cys show more robust pSTAT4 expression. In conclusion,
STAT4 was not activated upon *B. burgdorferi* exposure, suggesting an absence of IL-12 secretion. In combination with STAT3 activation, this lack of IL-12 induced STAT4 expression may play a role in *B. burgdorferi* inhibition of IFN-γ.

SOCS1 and SOCS3 are important negative regulators of the immune response induced by phosphorylated STAT expression [48; 49]. Therefore, SOCS1 and SOCS3 gene expression analysis was performed in PBMCs after 24h of stimulation with *B. burgdorferi*, IFN-γ or *C. albicans*. 
**Figure 5: Inhibited STAT4, while STAT3 and STAT1 expression significantly increased.** Transcriptome data of PBMCs of 29 physician-documented erythema migrans (EM) patients at diagnosis (T=0), 3 weeks and 6 months, and 13 matched healthy controls (HC). (A) SOCS1 expression was enhanced in patients, 3 weeks after diagnosis of infection (p = 0.03461). (B) SOCS3 expression is significantly increased (p = 0.02038). (C) STAT2 expression shows no change in patients compared to HCs (p = 0.2215) (D) STAT3 was significantly upregulated in the patient cohort (p = 0.002954), as was (E) STAT1 expression did not change significantly in EM patients compared to HCs (p = 0.005513). (F) STAT4 expression showed a significant decrease in expression compared to HCs (p=0.02801). FPKM = fragments per kilobase of transcript per million fragments mapped. HCs = healthy controls. Significance was calculated using a Kruskal–Wallis one-way ANOVA to compare HC and Lyme disease patient cohort expression levels during *B. burgdorferi* infection.

Both SOCS1 and SOCS3 expression were increased in *B. burgdorferi* stimulated cells, when compared to the medium control, *C. albicans* and IFN-γ (Figure 4F and G). Interestingly, IFN-γ exposure showed a decreasing trend in SOCS3 expression (Figure 4G). *B. burgdorferi* significantly increased SOCS3 and SOCS1 expression.

**STAT3 and SOCS3 expression is significantly enhanced in the PBMC transcriptome of Lyme borreliosis patients**

To confirm our findings in patients, RNA sequencing data of PBMCs from 29 physician-documented erythema migrans (EM) LB patients were assessed at diagnosis (T=0), 3 weeks and 6 months, and 13 matched healthy controls (HC). These results were obtained from the publicly available National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (accession number GSE63085) [46].

Similar to our *in vitro* findings, STAT2 gene expression did not change in Lyme disease patients (Kruskal-Wallis chi-squared = 4.3985, df = 3, p-value = 0.2215; Figure 5A). In contrast, STAT3 (Kruskal-Wallis chi-squared = 13.964, df = 3, p-value = 0.002954; Figure 5B) and STAT1 (Kruskal-Wallis chi-squared = 12.629, df = 3, p-value = 0.005513; Figure 5C) mRNA expression increased significantly upon EM diagnosis. Of particular interest, STAT4 mRNA expression was significantly decreased (Kruskal-Wallis chi-squared = 9.0986, df = 3, p-value = 0.02801; Figure 5D). STAT1, STAT4 and STAT3 gene expression slowly returned to baseline levels over the course of 6 months. Both SOCS1 (Kruskal-Wallis chi-squared = 8.6316, df =
B. burgdorferi induces pSTAT3 and inhibits CIITA

3, p-value = 0.03461; Figure 5E) and SOCS3 mRNA expression (Kruskal-Wallis chi-squared = 9.7958, df = 3, p-value = 0.02038; Figure 5F) were induced significantly. SOCS1 expression only begins to revert to the original expression levels after 3 weeks, while SOCS3 expression is the highest at T=0. In conclusion, STAT1, STAT3, SOCS1 and SOCS3 gene expression significantly increased during onset of Lyme borreliosis, while STAT4 mRNA expression was suppressed, showing normalization towards baseline levels 6 months after B. burgdorferi infection.

Figure 6: B. burgdorferi inhibits the antigen presentation transcriptional regulator CIITA. In (A) PBMCs (n=8) and (B) monocytes (n=6), at 24h, class II transactivator (CIITA) expression determined by quantitative PCR analysis in response to a medium control, RPMI, a positive control, IFN-γ, C. albicans and B. burgdorferi stimulation. Relative expression was assessed using the B2M housekeeping gene. qPCR data was normalized to the housekeeping gene, followed by direct comparison to the delta Ct value of the medium control, the delta-delta Ct value was used to determine the fold-change. The dotted line represents the medium
control. Data shown is mean ± SEM. Statistical significance was accepted at p<0.05 and indicated as follows: * p<0.05. (C) Transcriptome data of PBMCs of 29 physician-documented erythema migrans (EM) patients at diagnosis (T=0), 3 weeks and 6 months, and 13 matched healthy controls (HC). CIITA expression showed a significant decrease in expression compared to HCs (p=0.0003073). FPKM = fragments per kilobase of exon model per million reads mapped. HCs = healthy controls. Significance was calculated using a Kruskal–Wallis one-way ANOVA to compare HC and Lyme disease patient cohort expression levels during *B. burgdorferi* infection.

**B. burgdorferi**-induced inhibition of antigen presentation was mediated by CIITA expression

Both STAT activation and IFN-γ production can affect CIITA expression. Through a positive feedback loop, CIITA expression may in turn induce STAT phosphorylation and IFN-γ signalling. CIITA is also the major transcriptional regulator of antigen presentation genes. To determine whether CIITA expression is downregulated by *B. burgdorferi*, inhibition CIITA RNA expression was studied during *B. burgdorferi* exposure in vitro.

CIITA gene expression was slightly enhanced in PBMCs stimulated by IFN-γ for 24h, while *B. burgdorferi* exposure significantly downregulated CIITA mRNA expression by 2-fold (Figure 6A). A previous study showed that monocytes were the most affected cell type by *B. burgdorferi* regarding antigen presentation [45]. Indeed, CIITA expression in monocytes was inhibited to 25% of its original levels after 24h of incubation with *B. burgdorferi* spirochetes (Figure 6B). IFN-γ slightly increased CIITA expression at 24h of stimulation, though at 24h mRNA expression in monocytes (Figure 6B) showed a lower trend than observed in the whole PBMC fraction (Figure 6A), suggesting a more pronounced presence of CIITA expression in B cells or activated T cells. IFN-γ is often used as the golden standard positive control for MHCII expression [50; 51; 52], therefore it was used as a positive control in further experiments.

To confirm our findings, an existing transcriptome database [46] was used to study CIITA expression in LB patients over time. In LB patients, CIITA gene expression was significantly inhibited (Kruskal-Wallis chi-squared = 18.755, df = 3, p-value = 0.0003073; Figure 6C), demonstrating a crucial role of CIITA in Lyme disease both in vitro and in vivo.
B. burgdorferi induces pSTAT3 and inhibits CIITA
Figure 7: *B. burgdorferi*-induced inhibition of CIITA in PBMCs occurs specifically through pi and IsoV isoforms. At 4 and 24h, various isoform expression levels were studied of the gene called class II transactivator (CIITA) in PBMCs. PBMCs were treated with *B. Burgdorferi* (n=8) and incubated with positive control IFN-γ (n=6), determined by quantitative PCR analysis in response to a medium control (n=8). Relative expression was assessed using the B2M housekeeping gene. qPCR data was normalized to the housekeeping gene, followed by direct comparison to the delta Ct value of the medium control, the delta-delta Ct value was used to determine the fold-change. The dotted line represents the medium control.

Both CIITA pi and IsoV isoforms were significantly inhibited by *B. burgdorferi*

Previous studies have shown that various CIITA promotors can be more active in certain cell types. However, several CIITA isoforms are known to lead to protein expression which are the result of post-translational changes. To study whether these various isoforms of CIITA show differences in expression in response to *B. burgdorferi* exposure, the expression was determined at earlier timepoints before the previously observed inhibition of antigen presentation was initiated [45].

CIITA isoform pi (Figure 7A) and CIITA IsoV (Figure 7E) showed a significant decrease of expression in PBMCs. CIITA pi, is the isoform that is described as the antigen presentation cell CIITA promotor [42]. Several other isoforms; CIITA pii, CIITA IsoVII, CIITA pIV, CIITA IsoVIII, demonstrated stable expression (Figure 7B, D, G and H). CIITA IsoVI showed an increased trend at 4h and a similar level of expression at 24h compared to the medium control (Figure 7F). Also, following 24h of IFN-γ stimulation the pi and IsoVI isoforms decreased in expression, while other isoforms were enhanced by IFN-γ (Figure 7E). However, after 4h of IFN-γ incubation, all isoforms showed increased in expression compared to the medium control (Figure 7). This suggests a short increase in CIITA protein production, specifically for CIITA isoforms pii and IsoVI.

In general, monocytes showed more widespread and continued inhibition of CIITA isoforms (Figure S3). In monocytes, pi, piiI and IsoV demonstrated a decrease in expression upon *B. burgdorferi* exposure, along with an increase in their expression upon stimulation with the positive control IFN-γ (Figure S3A, C and E). Why CIITA expression in monocytes decreases upon IFN-γ stimulation in the other isoforms is unclear. piiI is an isoform which is normally associated with high IFN-γ production, suggesting a lack thereof, further reducing CIITA expression in monocytes. When
monocytes and PBMCs were exposed to *B. burgdorferi*, CIITA IsoVI showed an increase in expression at 4h, followed by degradation at 24h in both cell types (Figure 7F and S3F). In contrast, CIITA IsoV decreases in both PBMCs and monocytes with the same trend at 4 and 24h (Figure 7E and S3E). CIITA pl shows the most robust and specific trend in CIITA inhibition and more specifically in monocytes (Figure 7A and S3A). Overall, *B. burgdorferi* specifically inhibits antigen presentation in PBMCs and antigen presenting cells through reduction of CIITA isoforms pl, pIII and IsoV expression.

**Figure 8:** *B. burgdorferi* inhibits HLA-A, -B and -C expression in monocytes. (A) PBMCs and (B) monocytes were stimulated with *B. burgdorferi sensu lato* for 24h. HLA-A, -B, and -C expression was studied in these cells using flow cytometry. PBMCs from healthy volunteers were stimulated with a medium control, a live mix of *B. burgdorferi sensu lato* at 10^6/mL (n=10), IFN-γ 1 µg/mL (n=3), and heat-killed *Mycobacterium tuberculosis* 10^6/mL (n=3). The dotted line represents the expression level of the medium control, to which the stimulated expression levels are normalized for each time point. Data were normalized by dividing the geometric mean fluorescent intensity (MFI) of each condition to the MFI of the medium control per time point and demonstrated as the fold-change. Significance indications shown above the bars demonstrate whether there is a significant change compared to the medium control.

**CIITA suppression partially inhibits HLA-A, -B and -C expression in monocytes**
CIITA is also a transcription factor of MHC class I expression. Because of the inhibition of CIITA expression by *B. burgdorferi*, we investigated whether *B. burgdorferi* also inhibited MHCI expression in PBMCs and CD14^+^ monocytes. PBMCs exhibited a decreasing trend in HLA-A, -B and -C protein expression (combined
staining, Figure 8A). Of interest, *Mycobacterium tuberculosis* showed an increase in extracellular MHCI expression. The decreasing trend observed with *B. burgdorferi* stimulation in PBMCs became more apparent in CD14⁺ monocytes. CD14⁺ monocytes showed a significant reduction in both intra- and extracellular HLA-A, -B and -C expression (Figure 8B). In contrast, *Mycobacterium tuberculosis* enhanced extracellular MHCI expression, decreasing intracellularly located complexes. The positive control IFN-γ, enhanced both intra- and extracellular HLA-A, -B and -C protein expression. Here, we noted that *B. burgdorferi* induced inhibition of both CIITA (mRNA) and MHCI (protein) expression in human monocytes.

### Discussion

In this study, we showed that *B. burgdorferi* impaired the adaptive immune response in human PBMCs. *B. burgdorferi* did not induce the production of extracellular IFN-γ, although the different immune cell subsets still show the intracellular capacity to produce IFN-γ (Figure 1 and S2). However, in response to *B. burgdorferi* exposure, intracellular IFN-γ concentrations did not increase significantly, compared to the control. Production of IFN-γ is essential to control Lyme Borreliosis, via the induction of an early Th1 response, as was shown by the promotion of Lyme carditis resolution through the adoptive transfer of IFN-γ secreting CD4⁺ T cells into *B. burgdorferi* infected T cell-deficient mice [16]. Of interest, phagocytosed *B. burgdorferi* was previously shown to induce IFN-γ production in NK cells [18], in contrast to the human PBMCs used in this study. This indicates that how important it is to study *B. burgdorferi* induced IFN-γ production in different settings.

Similar to prior studies in a murine Lyme carditis model [53] and human chondrocytes [54], *B. burgdorferi* significantly increased active STAT3 in human PBMCs (Figure 2). STAT3 was also the only STAT protein strongly induced by *B. burgdorferi*. STAT3 is well-known for its inhibition of IL-12 and, IFNγ-related STAT4 induction [55]. *B. burgdorferi*-induced STAT3 may also promote STAT4 inhibition (Figure 4 and S2) and IFNγ production. STAT4 is indispensable for IL-12/IL-18-induced production of IFNγ by human NK cells and murine macrophages [56; 57; 58; 59; 60], suggesting that the suppression of STAT4 that we observed may be involved in delayed or absent IFN-γ production [56]. Combined signalling of STAT4-
dependent IL-12 and IL-18 may help restore Th1 responses [61] and signalling during *B. burgdorferi* infection.

Together with STAT4, STAT1 is a central mediator in IFN-γ signalling cascades [62]. However, when STAT1 and STAT3 are both activated they can form heterodimers. STAT1:STAT3 heterodimers induce different signalling pathways through various mechanisms. Heterodimerization may remove certain STATs from the pool of signalling molecules, competing with homodimer signals [31]. Another mechanism involves differential signalling through heterodimers because they bind a more diverse gene spectrum that induces different transcription programs from those of homodimers, while inducing diminution homodimer-specific pathways [63]. Here, we observed a relatively low induction of phosphorylated STAT1 expression compared to STAT3 expression during *B. burgdorferi* infection. Therefore, *B. burgdorferi* induced a STAT3 and STAT1:STAT3 phenotype, which explains the absence of STAT1 induced IFN-γ production. This caused a significantly higher status of STAT3 activation than STAT1 (Figure 3 and S2), steering STAT1 signalling towards a more tolerant STAT3 phenotype in T cells, B cells and NK(-T) cells, away from the necessary Th1 responses [64; 65; 66; 67].

SOCS are important inhibitors of STAT signalling. Without the presence of the SOCS3 protein, IL-6-induced STAT3 will switch to STAT1 signatures [49; 68; 69]. However, since both SOCS1 and SOCS3 were significantly induced by *B. burgdorferi* (Figure 4, 5 and S3), they play a key role in immune suppression during *B. burgdorferi* infection. Of interest, SOCS3 inhibits STAT3 activation, by some, but not all STAT3-activating cytokine receptors [70; 71]. STAT3 and high levels of SOCS3 activation have been strongly correlated with IL-10 production in human macrophages, and less so with IL-6 [72]. Both IL-10 and IL-6 are significantly produced upon *B. burgdorferi* infection [73]. Therefore, the enhanced *B. burgdorferi* SOCS3 activation was probably generated through IL-10 signalling. IL-10 production is induced via STAT3, p38, and possibly CD14 activation, which we recently showed to be enhanced during *B. burgdorferi* infection [45]: [34]. IFN-γ is important in disrupting this IL-10-STAT3 inhibitory feedback loop, through suppressing IL-10 production via enhancement of GSK3β activity [74; 75; 76]. Of interest, IL-10 not only inhibits IFN-γ production but is also involved in suppression of MHCII expression through its transcription factor CIITA [32; 77].
In this study, CIITA, an essential co-activator in MHCII transcription, was severely inhibited both in patients and in vitro by *B. burgdorferi* infection (Figure 7 and S3). Isoform pl, which has been linked to regulation of the MHCII antigen presentation machinery in innate immune cells, was specifically inhibited by *B. burgdorferi*. Of interest, a CIITA isoform whose biological role remains unknown, namely IsoV, was also significantly affected, suggesting it may play a significant role in antigen presentation in innate immune cells. In monocytes, all isoforms were inhibited at 24h of *B. burgdorferi* exposure. This suggests *B. burgdorferi* specifically interferes in innate immune cell recognition and antigen presentation. In contrast, IFNγ stimulation only activated specific isoforms after 24h, while at the 4h timepoint, IFN-γ activated all isoforms, suggesting it induces a short peak in CIITA expression.

**Figure 9: STAT3, SOCS1 and SOCS3 are activated following *B. burgdorferi* recognition.**

*B. burgdorferi* is recognized via NOD2 and TLR1/2 signalling. In response, immune cells start producing inflammatory factors such as TNFα, IL-6 and IL-10. These cytokines induce STAT3, SOCS1 and SOC3 signalling in other immune cells, resulting in CIITA and antigen presentation inhibition in innate immune cells.

| 140 |
STAT1 is an important inducer of CIITA expression. Previous studies have shown that reduced MHCII expression, frequently observed in tumours, is often the result of STAT1 suppression [78]. In contrast, STAT3 mediates tumour immune-evasion and partially inhibits antigen presenting cell (APC) maturation, through the production of factors such as VEGF and IL-10, and the reduction of pro-inflammatory responses [79]. Moreover, STAT3-mediated suppression impaired APC induction of T cell responses [29]. Activation of STAT3 in APCs can also induce DC anergy, triggering T cell tolerance [80], which we observe during B. burgdorferi infection. STAT1 knockout mice also showed reduced expression of MHCI molecules, which is thought to lead to hyporesponsive, unlicensed NK-cells [81; 82]. Previous studies have shown that SOCS3 is an inhibitor of STAT4 [83] and STAT1 activation [68; 84]. Overall, we show that CIITA expression is strongly inhibited by B. burgdorferi, possibly through the high STAT3 and SOCS1/3 signalling by B. burgdorferi, quenching of the STAT1 and STAT4 pathways, severely affecting its downstream pathways MHCI and MHCII (Figure 9). The exact relation between all these pathways and how this activation may be steered in a different direction during B. burgdorferi infection to resolve infection more effectively should be further explored in future studies.

In this study, B. burgdorferi significantly induced STAT3 and SOCS1/3 signalling, while inhibiting IFNγ production and STAT4 activation, resulting in a more tolerant phenotype in both adaptive and innate immune cells. CIITA expression was downregulated, specifically the isoforms normally expressed in dendritic cells. Here, we also demonstrated that MHCI expression was also inhibited in human monocytes during B. burgdorferi exposure. Through these different pathways, B. burgdorferi inhibits the inflammatory Th1 response, steering the host immune response towards an anti-inflammatory profile. Targeting antigen presentation and related signalling pathways early after B. burgdorferi infection may help to control LB rapidly and avoid the development of severe symptoms observed in disseminated Lyme Borreliosis.
Acknowledgements

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References


[28] R. Wakahara, H. Kunimoto, K. Tanino, H. Kojima, A. Inoue, H. Shintaku, and K. Nakajima, Phospho-Ser727 of STAT3 regulates STAT3 activity by enhancing dephosphorylation of


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### Supplemental Information

**Table 1: Primer sequences**

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Figure S1: Gating strategy changes phosphorylated STAT expression in various CD4+ and CD8+ T cells, B cells and NK-(T) cells.
Figure S2: Absence of extracellular IFN-γ after *B. burgdorferi* stimulation while intracellular reserves increase in different cell types. PBMCs from healthy volunteers (n=6) were stimulated with a medium control, a live mix of *B. burgdorferi sensu lato* at 10⁶/mL, heat-killed *C. albicans* 10⁶/mL, and Pam3Cys for 48h. (A) After 4 hours of priming with phorbol 12-myristate 13-acetate (PMA) 50 ng/mL, ionomycin 1 ug/mL and 1 µl Golgi-plug inhibitor (Brefeldin A), intracellular IFN-γ expression was studied and the mean of the medium controls geometric mean fluorescent intensity (MFI) for each cell type is shown. To study the differences in intracellular IFN-γ per condition the MFI was normalized to this medium control and demonstrated as the fold-change in various cell types: (B) CD4⁺ T cells, (C) CD8⁺ T cells, (D) CD56⁺ CD3⁻ NK cells, (E) CD56⁺ CD3⁺ NK T cells and (F) CD19⁺ B cells. The dotted line represents the expression level of the medium control, to which the stimulated expression levels are normalized for each time point. Significance indications shown above the bars were compared to the medium control. Statistical significance was accepted at p<0.05 and indicated as follows: * p<0.05.
B. burgdorferi induces pSTAT3 and inhibits CIITA
Figure S3: *B. burgdorferi*-induced inhibition of CIITA in monocytes occurs specifically through pl, pIII and IsoV isoforms. In monocytes, at 4 and 24h, various isoform expression levels were studied of the gene called class II transactivator (CIITA) expression, n=6 for monocytes, determined by quantitative PCR analysis in response to a medium control, RPMI, a positive control, IFN-γ, and *B. burgdorferi* stimulation. Relative expression was assessed using the B2M housekeeping gene. qPCR data was normalized to the housekeeping gene, followed by direct comparison to the delta Ct value of the medium control, the delta delta Ct value was used to determine the fold-change. The dotted line represents the medium control.
Chapter 5

*Borrelia burgdorferi* is a poor inducer of interferon gamma: amplification induced by interleukin 12


Submitted

# both authors shared first authorship
Abstract

Background
Laboratory diagnosis of Lyme borreliosis (LB) is mainly based on serology, which has limitations, particularly in the early stages of the disease. In recent years there have been conflicting reports concerning a new diagnostic tool using the cytokine interferon-gamma (IFN-γ). Previous studies have generally found low concentrations of IFN-γ in early LB infection. The goal of this study is to investigate IFN-γ regulation during early LB and provide insights into the host response to *B. burgdorferi*.

Methods
We performed in vitro experiments with whole blood assays and peripheral blood mononuclear cells (PBMCs) of LB patients and healthy volunteers exposed to *B. burgdorferi* and evaluated the IFN-γ response using ELISA and related interindividual variation in IFN-γ production to the presence of single nucleotide polymorphisms.

Results
IFN-γ production of *B. burgdorferi*-exposed PBMCs and whole blood was amplified by the addition of IL-12 to the stimulation system. This effect was observed after 24 hours of *B. burgdorferi* stimulation in both healthy individuals and LB patients. The effect was highly variable between individuals but was significantly higher in LB patients six weeks since the start of antibiotic treatment compared to healthy individuals. IL-12 p40 and IL-18 mRNA was upregulated upon exposure to *B. burgdorferi*, whereas IL-12 p35 and IFN-γ mRNA expression remained relatively unchanged. SNP Rs280520 in the downstream IL-12 pathway, Tyrosine Kinase 2, was associated with increased IFN-γ production.

Conclusions
This study shows that IL-12 evokes an IFN-γ response in *B. burgdorferi* exposed cells, and LB patients and healthy controls respond differently to this stimulation.
Introduction

Lyme borreliosis (LB) is caused by the tick-borne spirochete *Borrelia burgdorferi* sensu lato (*B. burgdorferi* s.l.) and has a broad clinical spectrum, ranging from early localized disease to severe disseminated manifestations. The most common early manifestation is erythema migrans (EM) and, more rarely, *B. burgdorferi* s.l. infection can result in disseminated disease, including Lyme arthritis and Lyme neuroborreliosis (1).

Until today, the readily available and generally used method for diagnostic confirmation of LB is serology (2). The sensitivity of serology increases in weeks to months after infection but is limited early in the disease (3). Also, serology cannot be used to monitor disease activity, as antibodies can remain detectable for years, even after antibiotic treatment and resolution of infection (2, 4). Furthermore, cross-reactivity and differences between commercial test kits may complicate clinical interpretation of serological results (5-7). Specifically, diagnostic parameters such as sensitivity and specificity can vary between tests. This can be due to the difference in antigens that are used. These include sonicated whole-cell, whole-cell combined with recombinant or exclusively recombinant antigens.

Although the vast majority of LB patients recover completely after antibiotic treatment, signs and symptoms may persist and cause a significant decrease in quality of life (8-10). Persistent symptoms include fatigue, arthralgia, myalgia, and neurocognitive problems. After confirmed LB, these clinical manifestations are often referred to as post-treatment Lyme disease syndrome (PTLDS) (11). Similar symptoms are prevalent in up to 20% of the general population (10, 12-14), and proper identification of patients with LB is necessary to be able to initiate appropriate treatment. Therefore, additional understanding of immune responses in patients with active LB compared to those with past infection is warranted.

*B. burgdorferi* infection triggers both innate and adaptive immune responses, including a Th1 response leading to IFN-γ release (15-19). Previous studies have demonstrated IFN-γ in the skin, cerebrospinal fluid, and synovial fluid of LB patients (19-28). Interestingly, in a cohort of 500 healthy subjects the IFN-γ production upon *B. burgdorferi* s.l. stimulation of human peripheral blood mononuclear cells (PBMCs) was limited (29). In contrast to IL-1β being rapidly
produced, IFN-γ production was only detectable after 96 hours of \textit{B. burgdorferi} s.s. exposure of PBMCs in healthy volunteers (30). Therefore, in the present study, we aim to further unravel the mechanisms of IFN-γ production by human PBMCs upon \textit{B. burgdorferi} s.l. exposure.

\textbf{Materials and methods}

\textbf{Study participants and blood samples}

Blood samples from several groups of volunteers were obtained after written informed consent, in accordance with the principles of the Declaration of Helsinki. Ethical approval was obtained from the medical ethics committee (METC) Arnhem-Nijmegen (NL32357.091.10) and CMO Noord-Holland (NL50227.094.14).

First, peripheral blood mononuclear cells (PBMCs) from healthy volunteers were isolated from buffy coats (Sanquin Blood Bank, Nijmegen, the Netherlands) and freshly obtained blood samples. PBMCs were isolated immediately after blood collection, or the day after the collection in case of buffy coats. Data on experiments with freshly isolated PBMCs from six individuals were published previously (30). Second, whole blood and freshly isolated PBMCs were obtained from the 200FG cohort, a cohort of 200 foresters in The Netherlands (29). Blood samples for \textit{B. burgdorferi} s.l. serology, PBMC isolation, and whole blood stimulation were acquired from 149 foresters in 2016 and 201 foresters in 2018 and 2019. Third, isolated PBMCs were available from 46 adult patients with physician-confirmed LB included in the LymeProspect study (10, 31). For this group, the baseline blood sample was drawn within seven days after initiation of antibiotic therapy. Blood samples of EM patients were processed within 24 hours after collection, while blood samples of patients with disseminated LB were isolated directly. Case definitions were based on the European Society of Clinical Microbiology and Infectious Diseases study group for Lyme borreliosis (ESGBOR) criteria (32). This group included patients with confirmed localized disease (EM) (n=40), Lyme arthritis (n=1), acrodermatitis chronica atrophicans (ACA) (n=4), and Lyme neuroborreliosis (n=1). From 35 patients (76%), a follow-up blood sample was acquired six weeks after the first.
Isolation of human peripheral blood mononuclear cells

Blood was diluted 1:1 in phosphate-buffered saline (PBS), after which the PBMC fraction was obtained by density centrifugation over Ficoll-Paque (Pharmacia Biotech). PBMCs were isolated, washed three times in cold PBS, and resuspended in RPMI 1640 (Dutch modification, including Phenol Red, HEPES (4-(2-HydroxyEthyl)-1-PiperazineEthaneSulfonic acid), sodium bicarbonate 1 g/L, Life Technologies, Nieuwekerk, The Netherlands) supplemented with 1% gentamicin, 2 mM L-glutamine, and 1 mM pyruvate. In experiments with an incubation time longer than 24 hours, 20% serum, autologous if available, was used.

*B. burgdorferi* cultures

*B. burgdorferi* s.s., ATCC strain 35210 (B31), *B. afzelii* PKO (low passage), *B. garinii*, ATCC strain 51383 (20047) were cultured in MKP (modified Kelly-Pettenkofer)-II medium with 6% rabbit serum at 33⁰C. Spirochetes were grown to the mid-logarithmic phase and dark-field microscopy was used to check for motility. The number of spirochetes was determined using a Petroff-Hauser counting chamber. After harvesting, the bacteria were washed in PBS thrice, and divided to use directly in stimulation experiments as viable *B. burgdorferi* and stored at -80⁰C until use as live attenuated *B. burgdorferi*.

Stimulation experiments

Whole blood (100 μl per well in a 48-wells plate) or PBMCs (5x10^5 cells per well in a round-bottom 96-wells plate) were stimulated with either RPMI medium as a negative control, *B. burgdorferi* spirochetes, or various other stimuli as positive controls. For the whole blood assay and the PBMC experiments, 400 μL and 100 μL of stimulus were used, respectively. Both a mix of *Borrelia* species indicated as *B. burgdorferi* s.l., and *B. burgdorferi* sensu stricto (s.s.) were used in the experiments. *Borrelia* s.l. mix was prepared by combining equal amounts of *B. burgdorferi* s.s., *B. afzelii* and *B. garinii*. Various concentrations were used depending on the experiment. Multiplicity of infection (MOI) of 1 (5x10^6 spirochetes/mL), 0.2 (1x10^6 spirochetes/mL), 0.1 (5x10^5 spirochetes/mL) were used for PBMC stimulation and MOI 30 (7.5x10^6 spirochetes/mL), MOI 10 (2.5x10^6 spirochetes/mL) and MOI 4 (1x10^6 spirochetes/mL) for whole blood assays. Positive controls included heat-killed *Candida albicans* (HKCA) 1x10^6/ml, lipopolysaccharide (LPS) 100 ng/ml in case of experiments with cases included through the LymeProspect study (31) and 10 ng/ml otherwise,
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Pam3Cys (P3C) 10 μg/ml, and *Mycobacterium tuberculosis* lysate (MTB) 1 μg/ml. The acquisition and preparation of these positive controls have been described earlier (29, 33). The addition of stimuli was preceded by pre-incubation of cells for 1 hour with either recombinant human IL-12 (R&D Systems), recombinant human IL-7 (Fisher Scientific), and/or IL-18 binding protein (BP) (R&D Systems). After incubation for 24, 48, 72, or 7 days at 37⁰C with 5% CO₂, plates were centrifuged, and cell-free supernatants were collected and stored at -20⁰C until cytokine measurement.

**Cytokine measurements**

Concentrations of human IFN-γ (Sanquin, Amsterdam), TNF-α, IL-1β, IL-1Ra, IL-6, IL-10, IL-17, IL-22, IL-18, and IL-12 (R&D Systems, Minneapolis) in the cell culture supernatants were measured using commercial ELISA kits, according to the manufacturer’s protocol.

**Serological testing**

For the detection of *B. burgdorferi* s.l. antibodies in the 200FG cohort, ELISA (Serion/Virion GmbH, Wurzburg, Germany *Borrelia* IgM; ER-121-M and *Borrelia* IgG; ER-121-G for samples before 2017 and DiaSorin/LIAISON Saluggia, Italy *Borrelia* IgM 310010, *Borrelia* IgG 310880 for samples from 2017 on) were performed on all samples, and in case of equivocal or positive results, IgM and/or IgG immunoblot analysis was performed (Eurolmmun, Lubeck, Germany; *Borrelia* IgG and IgM, DY-2131-3001-1G and DY-2131-3001-1M for samples before 2019 and Mikrogen GmbH, Neuried, Germany; recomLine *Borrelia* IgM; 4273 (4277) and *Borrelia* IgG; 4272 (4276) from 2019 on). All assays were performed according to the manufacturer’s instructions.

**Single nucleotide polymorphism analysis**

DNA was isolated from whole blood of the 200FG cohort, as was described in a previous study (34). Single nucleotide polymorphisms (SNPs) were selected based on literature and gnomAD (https://gnomad.broadinstitute.org/) (Suppl. Table 1).

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism (Version 5.03, San Diego, California, USA). Data were analysed using Mann-Whitney U-test for
Results

(A+B) IFN-γ production of *B. burgdorferi*-stimulated PBMCs of healthy controls (HCs) is minimal compared to stimulation with heat-killed *Candida Albicans* (HKCA). PBMCs produce more IFN-γ when exposed to viable *B. burgdorferi* than to frozen/live-attenuated *B. burgdorferi* s.s.. (C) Stimulation for 7 days with viable *B. burgdorferi* results in comparable IFN-γ levels as with HKCA stimulation. (D+E) PBMCs, thawed from liquid nitrogen, of a different cohort of healthy individuals were used as a validation experiment. (F) PBMCs of HCs produce more IFN-γ after *B. burgdorferi* s.s. exposure for 7 days in comparison to 24 or 48 hours of stimulation, however there are still many non-responders. (G) IFN-γ production by PBMCs from 11 erythema migrans (EM) patients following *B.
**burgdorferi** s.l. stimulation is limited compared to HKCA stimulation. **(H)** Robust production of IL-1β, IL-6, IL-10 and IL-1Ra in contrast to IFN-γ by PBMCs of EM patients after 24 hours stimulation. **(I)** The production of IFN-γ was not related to **B. burgdorferi** antibody status in healthy individuals. PBMCs = Peripheral Blood Mononuclear Cells; IFN-γ = interferon gamma; HCs = healthy controls; ACA = acrodermatitis chronic atrophicans; Bb ss = **B. burgdorferi** sensu stricto; Bb sl = **B. burgdorferi** s.l.; HKCA = heat-killed *Candida albicans*; EM = erythema migrans; LB = Lyme borreliosis; ns = not statistically significant, * p < 0.05, ** p < 0.01, *** p < 0.0001 tested with Wilcoxon signed-rank test for panels A-E and Mann-Whitney U test for panel I.

### **B. burgdorferi** s.l. is a poor IFN-γ inducer

Previously, we have shown that IFN-γ production by PBMCs obtained from healthy subjects and stimulated with **B. burgdorferi** for 24 hours to 48 hours was limited or even absent. In the present study, IFN-γ production by **B. burgdorferi**-stimulated PBMCs was evaluated in a cohort of healthy individuals. After 24 or 48 hours of incubation with live attenuated and viable **B. burgdorferi** s.s., minimal production of IFN-γ was detectable (Figures 1A and 1B), whereas PBMCs exposed to heat-killed *C. albicans* (HKCA), a known inducer of memory T-cell responses, induced high production of IFN-γ. Seven days after initial **B. burgdorferi** s.s. exposure IFN-γ production in healthy individuals was observed (Figure 1C and 1F). Viable **B. burgdorferi** s.s. induces low amounts of IFN-γ by itself following 24 and 48 hours of PBMC stimulation, however, HKCA is the more potent IFN-γ inducer (Figure 1A and B). As a validation, these experiments were repeated in EM patients, using PBMCs thawed from liquid nitrogen (Figure 1G), and compared to thawed cells from healthy individuals (Figure 1D and E). PBMCs from EM patients also showed minimal IFN-γ production after **B. burgdorferi** exposure (Figure 1G). In contrast to IFN-γ, innate proinflammatory cytokines were effectively produced by PBMCs of these EM patients upon stimulation with **B. burgdorferi** s.l. for 24 hours (Figure 1H). In the forester’s cohort, consisting of healthy subjects with high tick exposure, high concentrations of IL-1b, IL-6 and IL-1Ra were produced after 24h of **B. burgdorferi** s.s. exposure as well (Suppl. Figure. 1). Lastly, IFN-γ production was comparable in seropositive healthy individuals, defined by the presence of either **B. burgdorferi** IgM or IgG antibodies based on standard two-tiered testing, and seronegative individuals (Figure 1I).
**B. burgdorferi** is a poor inducer of IFN-γ

**Figure 2:** Transcriptome PBMCs of LB patients and healthy volunteers. Transcriptome of unstimulated PBMCs of 29 physician-confirmed LB patients, including 17 with a single EM and 12 with multiple EM, compared to 13 matched healthy controls (HCs). The expression of cytokines is displayed as fragments per kilobase million (FPKM). (A) There was no difference in IL-12 p35 expression between HCs and LB patients. (B+E) IL-12 p40 and IL-18 are upregulated in PBMCs of LB patients at baseline and after three weeks compared to HCs. (C+D) IL-12Rβ1 and 2 expression was not different between the groups. (F) IFN-γ mRNA was upregulated in several LB patients at the time of diagnosis, however most individuals showed no upregulation. (G) Expression of IL-7 was upregulated at the 3 weeks timepoint compared to HCs. (H) TYK2 mRNA was elevated in LB infection after 6 months compared to 3 weeks and baseline. (I) In the WBA condition, individuals with the Rs280520 SNP had higher IFN-γ production upon stimulation with *B. burgdorferi* in combination with IL-12. This SNP was present in 21 of 65 subjects investigated. *B. burgdorferi* s.l. = *Borrelia burgdorferi* sensu lato; FPKM = fragments per kilobase of exon model per million reads mapped; HCs = healthy controls; LB t0 = Lyme borreliosis patients on baseline; LB t3wk = Lyme borreliosis patients 3 weeks after inclusion; LB t6m = Lyme borreliosis patients 6 months after inclusion; IL-12RB1
IL-12 p35 and IFN-γ gene expression is not upregulated upon *B. burgdorferi* infection

Next, expression of IFN-γ and related cytokines upon *B. burgdorferi* recognition was evaluated at the transcriptional level. It is well-known that IL-12 and IL-18 synergize IFN-γ production (35, 36). Bioactive IL-12 p70, which can induce IFN-γ production, is a heterodimer formed by subunits p35 (IL-12a) and p40 (IL-12b) (37, 38). In a previously published study (39), blood samples of 29 LB patients, including 17 individuals with a single EM lesion and 12 with multiple EM, were collected before the start of treatment, and three weeks and six months later. Furthermore, 13 healthy controls, matched by age, sex, ethnicity, and comorbidity, were included. Transcriptome data of unstimulated PBMCs showed an upregulation of IL-12 p40 mRNA in patients at three weeks (Figure 2B). However, no change in expression of the IL-12 p35 gene was observed (Figure 2A). The IL-12 receptor subunits, IL-12Rβ1 and IL-12Rβ2, were not differentially expressed at any time point (Figure 2C and 2D), suggesting there is no increased IL-12 signalling. Expression of IL-18 mRNA was significantly increased in EM patients compared to healthy controls (Figure 2E) at baseline and after three weeks. IFN-γ expression was upregulated in some EM patients (Figure 2D). We could validate these findings in transcriptome analysis of *B. burgdorferi* stimulated PBMCs isolated from 36 healthy individuals from another previously published dataset (40) (Figure 3). Similar to transcriptome data of EM patients, we observed upregulation of IL-12 p40 and IL-18 following 24 hours of stimulation, however no change in IL-12 p35 and IFN-γ expression. This may indicate that, although IL-12 p40 and IL-18 mRNA are upregulated after *B. burgdorferi* infection, IFN-γ expression remains unchanged for most individuals.
B. burgdorferi is a poor inducer of IFN-γ

Figure 3: Transcriptional responses PBMCs of healthy volunteers to B. burgdorferi
Gene expression of relevant genes by micro-array of 36 healthy individuals stimulated for 4 (A) and 24 hours (B) with RPMI (medium control), heat-killed B. burgdorferi s.s. (Bb), Mycobacterium tuberculosis (MTB) and heat-killed Candida Albicans (HKCA). The data are log2 transformed and in case of multiple transcripts per gene the mean is displayed, and scaling was performed per gene. IL-12 p35 expression after 4 and 24 hours of B. burgdorferi stimulation is lacking compared to HKCA. IL-12 p40 is increased after 24 hours of B. burgdorferi stimulation. These IL-12 p35 and p40 responses are in agreement with figure 2A+B. IFN-γ is not upregulated following B. burgdorferi stimulation, in contrast to IL-18 expression. After B. burgdorferi exposure TYK2 and IL-7 expression are upregulated for both timepoints compared to the other stimuli. IL-12Rβ1 and IL-12Rβ2 expression increased after 4 hours of B. burgdorferi stimulation, however this could not be observed after 24 hours. Bb = Borrelia burgdorferi sensu stricto; HCs = healthy controls; IL-12Rβ1 and 2 = Interleukin 12 Receptor Subunit Beta 1 and 2; PBMC = peripheral blood mononuclear cell; TYK2 = Tyrosine Kinase 2.

IL-12 is crucial for B. burgdorferi induced IFN-γ production by human PBMCs
Next, we evaluated whether IL-12 and IL-18 are present following B. burgdorferi stimulation of PBMCs. In contrast to IL-1β, IL-12 was not detectable after 24h or 48h of B. burgdorferi s.s. stimulation in vitro, while IL-18 concentration was measurable in low concentrations (Figure 4A). When PBMCs were exposed to B. burgdorferi...
s.s. and a low concentration of IL-12 a significant increase of IFN-γ production was observed compared with stimulation with either stimulus alone (Figures 4B-H). IFN-γ production appeared to be dose-dependent of IL-12 concentrations, and could be observed in both EM patients and healthy controls (Figure 4B+C+G+H). IFN-γ was already detectable after 24 hours of incubation (Figure 4B+D+E+G+H). This effect was inducible by stimulation with frozen/live attenuated and viable *B. burgdorferi* s.s. in combination with IL-12 (Figure 4E and F). There was marked interindividual variability in IFN-γ induction upon IL-12 addition in both healthy individuals and EM patients.

The IL-12 induced IFN-γ response was observed in multiple settings, including in PBMCs of healthy controls isolated within 24 hours and PBMCs isolated directly after blood collection (Suppl. Figure 2). Interestingly, IFN-γ production by *B. burgdorferi* s.s. and IL-12 stimulated PBMCs from LB patients was higher at 6 weeks after the diagnosis of EM compared to healthy individuals, however, with considerable overlap between groups (Figure 4I). In previous studies, IFN-γ production was observed this early in experiments with mitogens, heat-killed *C. albicans* or anti-CD3/CD28 antibodies (Figure 1A and 1B) (41-43). Altogether, our findings demonstrated that the addition of IL-12 induces early IFN-γ production upon *B. burgdorferi* exposure.
B. burgdorferi is a poor inducer of IFN-γ

Figure 4: IFN-γ production can be induced by addition of IL-12 to PBMCs of healthy individuals and EM patients. (A) PBMCs exposed to B. burgdorferi s.s. produce neither IL-12 nor IFN-γ, in contrast to IL-1β. IL-18 is produced in modest amounts upon stimulation with B. burgdorferi s.s. and is measurable after 48 hours incubation. (B+C) Addition of IL-12 induces the production of IFN-γ by B. burgdorferi s.s.-stimulated PBMCs of 21 healthy volunteers in a dose-dependent manner after 24 and 48 hours of stimulation. (D) IL-18 binding protein (bp) partially reverses the IFN-γ production upon IL-12 and B. burgdorferi s.s. stimulation in a dose-dependent manner in 6 healthy donors. (E+F) Induction of IFN-γ was observed with both frozen/live-attenuated and viable B. burgdorferi in two different concentrations in combination with IL-12 (10ng/ml). IFN-γ production was higher following 48 hours compared to 24 hours of stimulation. (G+H) PBMCs from patients with physician-confirmed EM produced IFN-γ upon stimulation with B. burgdorferi s.s. and IL-12. This could be observed at baseline (t0) and 6 weeks later (t6wk) (I) Six weeks after diagnosis IFN-γ
concentrations upon PBMC stimulation were higher than at baseline (n=16 at baseline and n=9 after 6 weeks). HC = healthy control; EM = erythema migrans; *B. burgdorferi* s.s. = *B. burgdorferi* sensu stricto; Bb mix = *B. burgdorferi* s.l.; IL-18bp = IL-18 binding protein; LB = Lyme borreliosis; PBMC = peripheral blood mononuclear cell, ns = not statistically significant; * p < 0.05, ** p < 0.01, *** p < 0.0001 calculated by Wilcoxon signed-ranked test for paired testing in panel B-H and Mann-Whitney U test for comparing groups in panel I.

**Genetic variations in TYK2 are associated with *B. burgdorferi*-induced IFN-γ production**

Further evaluation of downstream proteins of the IL-12 pathway showed that tyrosine kinase 2 (TYK2) mRNA expression was upregulated following *B. burgdorferi* exposure (Figure 3A and B), whereas in PBMCs of EM patients, TYK2 mRNA was decreased at baseline compared to 3 weeks and 6 months later (Figure 2H). TYK2 together with Janus Kinase 2 (JAK2) is tyrosine phosphorylated by induction of the IL-12 receptor and activate signal transducer and activator of transcription (STAT) 1, 3, 4, and 5 (37). At the genomic level, single nucleotide polymorphisms (SNPs) in the IFN-γ signalling pathway were evaluated in data from 65 individuals from the 200FG cohort (29). SNP rs280520 in the gene encoding TYK2 (Figure 2I) was significantly associated with increased IFN-γ production in a whole blood assay (WBA). The association of this SNP with IFN-γ production upon IL-12 stimulation further argues for a role for the IL-12 pathway in the impaired IFN-γ induction upon *B. burgdorferi* exposure.

**IL-18 production is important for the induction of IL-12 and *B. burgdorferi* IFN-γ production**

To assess the role of IL-18 in *B. burgdorferi* induced IFN-γ production, PBMCs were pre-incubated with IL-18 binding protein (IL-18bp) and IL-12 before *B. burgdorferi* stimulation. The induction of IFN-γ was partially reversed in 6 healthy donors (Figure 4D). Thus, although IL-18 was not strongly enhanced by *B. burgdorferi in vitro* (Figure 4A), the low amounts induced appeared to be bioactive. This shows that IL-12-induced IFN-γ production by *B. burgdorferi* is partially dependent on IL-18.
Figure 5: Addition of IL-12 and IL-7 further enhances the production of IFN-γ in PBMCs. 
(A+B, D+E) IL-7 and IL-12 enhance IFN-γ production compared to IL-12 alone in both HCs and EM patients. (C+F) IFN-γ levels were highest in EM patients 6 weeks after diagnosis compared to baseline and healthy controls. (G-I) Validation experiments in 200FG cohort. (G) 48 hours of *B. burgdorferi* s.l. stimulation in the presence of IL-12 and IL-7 showed a significant upregulation of IFN-γ production compared to *B. burgdorferi* s.s. or *B. burgdorferi* s.l. alone. (H) Seven days of *B. burgdorferi* s.l. stimulation showed the same effect as 48 hours of stimulation. (I) IFN-γ production was highest in individuals with positive Bb serology of whom PBMCs were stimulated with *B. burgdorferi* s.l. 5*10^6/ml, compared to individuals with negative serology (p=0.0292), and compared to a lower concentration of *B. burgdorferi* s.l. Bb ss = *Borrelia burgdorferi* sensu stricto; Bb
mix = *Borrelia burgdorferi* sensu lato; HKCA = heat-killed *Candida albicans*; Neg/Pos ser = Negative/positive *B. burgdorferi* s.l. serology according to two-tier testing; PBMC = peripheral blood mononuclear cell. * p < 0.05 ** p < 0.01 *** p < 0.0001 by Wilcoxon signed-rank test for paired testing for all panels, expect for panel C and F where the Mann-Whitney U test was used to compare healthy controls with LB patients.

The addition of IL-12 and IL-7 enhances IFN-γ production

To optimize IFN-γ production capacity of PBMCs after *B. burgdorferi* exposure *in vitro*, IL-7 in combination with IL-12 and *B. burgdorferi* was used to stimulate PBMCs thawed from liquid nitrogen. IL-7 is a well-known T-cell growth factor and acts synergistically with IL-12 (44-47). IL-7 mRNA of PBMCs was enhanced upon *B. burgdorferi* stimulation in healthy volunteers and IL-7 mRNA was slightly upregulated in LB patients 3 weeks after infection (Figure 2G and Figure 3). We observed a cumulative effect of IL-7 to IL-12 and *B. burgdorferi*-stimulated PBMCs of both healthy individuals (Figure 5A+B) and EM patients (Figure 5D+E, Suppl. Figure 3), with higher IFN-γ concentrations in the EM group, compared to the healthy control group. However, similarly to stimulation with IL-12 alone (Figure 4I), there was substantial overlap between groups (Figure 5F).

Next, we explored the combination of IL-12, IL-7, and *B. burgdorferi* in the 200FG cohort, which consists of healthy foresters, most of whom report numerous tick bites each year (Figure 5G+H). To maximize the effect on IFN-γ production, we stimulated PBMCs with a mix of *B. burgdorferi* s.s., *B. garinii*, and *B. afzelii* (Figure 5G+H). We confirmed the added effect of IL-12 and IL-7, after 48 hours and 7 days of *B. burgdorferi* s.l. stimulation compared to *B. burgdorferi* s.l. alone. Also, IL-22 and IL-10 production was significantly induced by the combination of IL-12 and IL-7, while IL-17 production was inhibited (Suppl. Figure 3).

Moreover, IFN-γ production did not correlate with the reported number of tick bites in the last year (Suppl. Figure 3). In contrast, positive standard two-tiered *B. burgdorferi* s.l. serology, indicating previous exposure to the spirochete, was associated with a higher IFN-γ response (Figure 5I). This could be observed when PBMCs were stimulated with the highest concentration of *B. burgdorferi* s.l. for either 48 hours or 7 days of incubation, and exclusively in the presence of IL-12 and IL-7. Interestingly, in patients with active LB who had antibodies against *Anaplasmaphagocytophilum* (Ap) the number of IL-12 secreting cells was reduced (48). As we could not measure IL-12 with an ELISA, we compared
IFN-γ production of PBMCs stimulated with IL-12 and *B. burgdorferi*. We did not find a difference in IFN-γ production between IL-12/*B. burgdorferi* stimulated PBMCs of EM patients with or without Ap antibodies (Suppl. Fig. 5). The reported seroprevalence was 8.1% in European foresters (49) and therefore we cannot rule out that previous Ap exposure influenced our results regarding IFN-γ production. Taken together, IL-7 in combination with IL-12 further enhances the ability of PBMCs to produce IFN-γ in response to *B. burgdorferi* s.l. *in vitro*, especially in individuals with previous *B. burgdorferi* exposure.

Figure 6: IFN-γ production in whole blood assays.
(A) Addition of IL-12 to whole blood of healthy volunteers (n=9) showed some induction of IFN-γ production in the presence of *B. burgdorferi* s.s., although it did not reach statistical
significance compared to IL-12 alone (p = 0.1484). (B) In the forester cohort (n=149) this effect was statistically significant compared to B. burgdorferi s.s. alone (p < 0.0001). (C+D) These experiments were performed with WB obtained from foresters. (C) Addition of IL-7 and IL-12 further enhances the production of IFN-γ in whole blood, especially when stimulated with a higher concentration of B. burgdorferi s.l.. (D) After 48 hours the effect is significantly greater in the higher B. burgdorferi s.l. concentration compared to 24 hours. WBA = whole blood analysis; Bb ss = B. burgdorferi sensu stricto; Bb mix = B. burgdorferi 1012 sensu lato; LPS = lipopolysaccharide; * p < 0.05. ** p < 0.01, *** p < 0.0001 calculated by 1013 Wilcoxon signed-ranked test for paired testing for all panels.

IFN-γ production in 24h and 48h-stimulated whole blood

Lastly, we assessed the effect of IL-12 on IFN-γ production in a whole blood assay (WBA) (Figure 6). In PBMCs, the added effect of IL-12 on the induction of IFN-γ was B. burgdorferi s.s.-specific (Suppl. Figure 2A and 2B). However, in WBA, IFN-γ was also produced after stimulation with Pam3Cys, a TLR2 agonist, and HKCA in combination with recombinant IL-12 (Figure 6A). Interestingly, the enhancing effect of IL-12 upon B. burgdorferi s.l. exposure was not exclusively observed for IFN-γ, but for other cytokines as well, including TNF-α, IL-1b (in whole blood stimulation), IL-10, and IL-22 (in PBMC stimulation) (Suppl. Fig. 3, 4, 5).

Similar to PBMCs, the addition of IL-7 to IL-12 to WBA amplified the induction of IFN-γ. This effect depended on incubation time and the concentration of B. burgdorferi s.l. (Figure 6C and 6D). In WBA IL-1β and TNF-α production were also induced upon addition of IL-12 addition (Suppl. Figure 4B), as well as IL-12 combined with IL-7 to B. burgdorferi s.l. stimulation for 24 hours (Suppl. Figure 3I). Overall, we demonstrate here that IL-12 and IL-7 in addition to B. burgdorferi s.l. can elicit an IFN-γ response in a WBA.

Discussion

This study demonstrates that B. burgdorferi alone is a poor early IFN-γ inducer in PBMCs isolated from healthy subjects, possibly due to the incapacity to induce bioactive IL-12, a crucial step for the activation of Th1 responses. Transcriptome analysis of B. burgdorferi-exposed PBMCs reveals that the IL-12 p35 mRNA was not upregulated. We show that the addition of recombinant IL-12 promotes B. burgdorferi-induced IFN-γ production in PBMCs, both in healthy individuals and
EM patients. This effect is dose-dependent for both IL-12 and *B. burgdorferi* s.l. in PBMCs. Simultaneous addition of IL-7 and IL-12 to *B. burgdorferi* s.l. stimulation further enhances IFN-γ production. Of note, the IFN-γ response induced in patients at six weeks after the diagnosis of EM is higher than at baseline, and their IFN-γ production is much higher than in healthy individuals. Lastly, we demonstrate that the addition of recombinant IL-12 also amplifies IFN-γ production in whole blood exposed to *B. burgdorferi* s.l. for 24 hours.

*B. burgdorferi* was unable to initiate IFN-γ production by PBMCs of healthy controls, EM patients, and even patients with disseminated LB following 24 or 48 hours of stimulation. In an earlier study, IFN-γ production by PBMCs was slightly elevated in 36 LB patients compared to controls, however there was a wide variety of disseminated LB manifestations, longer incubation times and usage of sonicated *B. burgdorferi* (50). For the stimulation experiments, we used whole live *B. burgdorferi* spirochetes. Others have found that whole live *B. burgdorferi* elicited a comparable transcriptional profile as heat-killed *B. burgdorferi* (51) and whole spirochetes led to enhanced immune responses compared to lysates (52). However, memory T cells recognize peptides, whereas we used the whole bacterium in the PBMC stimulation, and therefore we could have missed (part of) the specific T cell response in our model. Furthermore, limited contact of *B. burgdorferi* with infiltrating T cells in the skin might also explain poor IFN-γ production by PBMCs.

Another potential mechanism for the limited IFN-γ production by *B. burgdorferi* is the ability to interfere with mammalian immune responses by suppression of antigen presentation by host immune cells (53). Antigen presentation is vital for a sufficient adaptive immune response. Significant alterations in gene expression and protein production have previously been observed in human PBMCs by *B. burgdorferi* stimulation (53). Specifically, the antigen presentation pathway, and its proteins HLA-DM, MHC-II, and CD74 via TNF receptor I and RIP1 signalling, were severely downregulated, in monocyte subsets, monocyte-derived macrophages, and dendritic cells. Inhibition of antigen presentation was specific for *B. burgdorferi*, since the exposure of CD14+ monocytes to other pathogens resulted in a significantly different protein expression and caused impaired T-cell recognition of *B. burgdorferi*. This might explain the delayed adaptive immune response in LB patients and *ex vivo* *B.
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*B. burgdorferi* stimulations (22, 54-56). Interestingly, a decreased expression of IFN-γ in EM biopsies was associated with the presence of persisting symptoms suggesting a relevant clinical consequence of this cytokine (22).

To produce the IFN-γ-inducing IL-12 p70 heterodimer, both IL-12 p35 and p40 genes need to be expressed (37, 57). We showed that while mRNA of both IL-18 and IL-12 p40 was upregulated, IL-12 p35 was unchanged and IFN-γ gene expression was only minimally induced in PBMCs from EM patients (39). Other studies have shown that in LPS-stimulated human monocytes, IL-12 p40 production was higher than IL-12 p70, therefore lack of IL-12 p35 was thought to be the limiting factor for IL-12 production. mRNA expression confirmed that IL-12 p40 was produced in excess of IL-12 p35 (58). Moreover, IL-12 p40 can form homodimers, and IL-12 p80 has been shown to inhibit IFN-γ production by competitively binding to IL-12Rβ1 (38, 59-63). Additionally, IL-12 p40 can be paired with the p19 chain to form IL-23, which has previously been described to be induced in response to *B. burgdorferi* (18, 30). Thus, during *B. burgdorferi* s.l. infection, a lack of IL-12 p35 production combined with high IL-12 p40 levels produced by myeloid cells is likely to explain the poor IFN-γ induction.

The SNP rs280520 in the IL-12 downstream protein TYK2 was associated with higher concentrations of IFN-γ. This SNP is located in an intron region of the TYK2 gene and changes adenine to guanine (64). IL-12 signalling is disrupted in TYK2-deficient mice, rendering them more susceptible to viruses (65, 66). In addition, IL-12/IL-18 synergistic IFN-γ induction was reduced in TYK2-deficient murine NK and T cells (67). Inherited TYK2 deficiency in humans impairs IL-12 dependent IFN-γ signalling and may facilitate tuberculosis and viral infections (68-71). In contrast, TYK2 variants (I684S and P1104A) are associated with autoimmune diseases, including systemic lupus erythematosus, multiple sclerosis, Crohn’s disease, psoriasis, type 1 diabetes (72-85). In our study, this SNP was associated with increased IFN-γ production when stimulated with *B. burgdorferi* and IL-12. These studies show that TYK2 is involved in IL-12 mediated signalling of IFN-γ and confirmed an important role for IL-12 signalling in *B. burgdorferi* induction of IFN-γ.

Next, we observed that IL-12 was not produced following *B. burgdorferi* stimulation for 24 and 48 hours and that the addition of IL-12 could induce the IFN-γ signal. Several studies have described that IL-12 stimulates T- and NK-cells
to secrete IFN-γ (86-90). Interestingly, this includes one study of 17 seropositive LB patients, including 12 patients with chronic neuroborreliosis and five Lyme arthritis patients (91). They found that whole mononuclear cells from LB patients produced IL-12 in contrast to controls and if the authors blocked IL-12 with a monoclonal antibody the IFN-γ producing cell population was inhibited. In C3H mice, IL-12 induced the production of IFN-γ through activation of the p38 MAP kinase (92) and treatment with anti-IL-12 reduced arthritis but elevated B. burgdorferi load (93). Altogether, our findings together with these studies suggest that IFN-γ production in B. burgdorferi infection is partially under control of IL-12. This is important as IFN-γ does not only reduce B. burgdorferi load but is also involved in inflammatory tissue damage (94).

When drafting a new diagnostic assay, a whole blood assay is preferred above PBMC-based experiments. We found that co-stimulation with B. burgdorferi and IL-12 also induced IFN-γ production in whole blood. For latent tuberculosis, the IGRA is widely accepted as a diagnostic tool (95) and studies have suggested similar techniques to discriminate active from past Q-fever using whole blood IFN-γ assays (96, 97). Two studies performed in Northern America reported a higher sensitivity for a whole blood IFN-γ assay in EM patients compared to serology (98, 99). However, we could not reproduce these findings in a European cohort (100), where B. afzelii is the most common cause of EM, in contrast to B. burgdorferi s.s. in Northern America. A validation study for other cellular tests for LB based on IFN-γ production among others is currently ongoing (101). Intriguingly, a recent study showed that stimulation of whole blood of 22 Lyme neuroborreliosis patients provided lower IL-12 and IFN-γ concentrations compared to healthy controls, which indicates a limited Th1 response (102). Although these authors did not stimulate with B. burgdorferi in their whole blood assay, they stimulated with various pathogens to study a broad function of different signalling pathways. This resulted in decreased IL-12 and IFN-γ production, which is in line with our results. Our findings were predominantly based on healthy volunteers and erythema migrans patients, however this report expands on this group with a disseminated form of LB, Lyme neuroborreliosis.

Our study has several limitations. Firstly, since erythema migrans is a clinical diagnosis, other (infectious) diseases presenting as a red skin lesion could have
been misinterpreted as EM in study participants. Strict case definitions and physician confirmation of the diagnosis were used, to limit the chance of including patients with signs and symptoms that were not caused by *B. burgdorferi* s.l. infection (10, 31). Secondly, we observed a highly variable effect size of IL-12 on IFN-γ production between individuals, and overlapping IFN-γ concentrations between LB patients and healthy individuals. However, we were able to distinguish IFN-γ production by *B. burgdorferi* exposed PBMCs from unstimulated PBMCs by adding a combination of IL-12 and IL-7. Thirdly, our experimental study was not powered beforehand. Fourth, it remains unknown whether IFN-γ induction after the addition of IL-12 (and IL-7) can be used to differentiate active *B. burgdorferi* infection from past infection. Also, the observed large interindividual differences in IFN-γ response, and their potential clinical consequences, are of interest and should be further investigated. IFN-γ producing cells, such as T cells and NK cells, could be studied in high responders compared to low responders to see if these cells respond differently to IL-12/IL-7. A validation study with large, well-defined groups of patients and healthy individuals is required to confirm the potential and clinical relevance of a diagnostic test.

In conclusion, we show that *B. burgdorferi* is a weak inducer of Th1 responses early during the infection and point toward an obstruction in the IL-12 pathway as a potential mechanism, supported by the association of a SNP in the IL-12 downstream protein TYK2 with increased IFN-γ production. The effects may be due to a lack of IL-12 p35 production and high IL-12 p40 levels. This study is not only relevant to unravel the pathogenesis of LB infection, but can also contribute to the search for a new diagnostic test for LB.

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**References**


Chapter 5


B. burgdorferi is a poor inducer of IFN-γ


B. burgdorferi is a poor inducer of IFN-γ


B. burgdorferi is a poor inducer of IFN-γ


Suppl figure 1: Cytokine profile of PBMCs of healthy volunteers. Stimulation of PBMCs with *B. burgdorferi* s.s. induces relatively low amounts of pro-inflammatory cytokines, even after 7 days of incubation, compared to other stimuli in the 200FG cohort. Evaluated
B. burgdorferi is a poor inducer of IFN-γ for 24 and 48 hour timepoints (n = 188), and for 7 days timepoint (n = 201). Bb ss = B. burgdorferi sensu stricto; HKCA = heat-killed Candida albicans; LPS = lipopolysaccharide; MTB = Mycobacterium tuberculosis lysate; PBMC = peripheral blood mononuclear cell.

Suppl. Figure 2: Addition of IL-12 induces the production of IFN-γ in B. burgdorferi s.s.-stimulated PBMCs after 24 hours. IFN-γ production by PBMCs, isolated from blood taken from (A) 14 healthy blood donors the previous day, (B) 6 healthy blood donors the same day (p = 0.0625) and (C) 149 foresters from the 200FG cohort the same day. The increase in IFN-γ production after addition of IL-12 is a B. burgdorferi specific effect. (D) PBMCs isolated from disseminated LB patients were stimulated with B. burgdorferi and IL-12. There was a large variation in IFN-γ production by PBMCs of this small cohort of patients with disseminated LB manifestations (n=4). Bb ss = B. burgdorferi sensu stricto; LPS = lipopolysaccharide; HKCA = heat-killed Candida albicans. * p < 0.05. *** p < 0.0001 calculated by Wilcoxon-signed ranked test for paired testing.
Suppl. Figure 3: Combination of IL-12 and IL-7 enhances the production of IFN-γ, IL-10, IL-22 and decreases IL-17 production. (A-C) Addition of IL-12 and IL-7 further enhances the production of IFN-γ in PBMCs, but also induces production of other cytokines such as IL-10 and IL-22, and inhibits the production of IL-17. This was evaluated in 114 healthy individuals of the 200FG cohort with the Bb mix concentration 5x10^5 sp/ml and in 50 individuals with Bb mix concentration of 5x10^6 sp/ml. (D) PBMCs of seropositive individuals produced more IFN-γ compared to individuals with negative serology after 48 hours of stimulation. (E-F) The number of reported tick bites in the last year showed no correlation with the ability of PBMCs to secrete IFN-γ after stimulation with B. burgdorferi in addition of IL-12 and IL-7 for 48 hours and 7 days. (G-H) 24h stimulation of PBMCs of EM patients with IL-12 and combination of IL-12 and IL-7 induced IFN-γ at baseline and 6 weeks later. (I) Addition of IL-12 and IL-7 further enhances the production of IL-1β in WBA. This effect is stronger when a higher concentration of B. burgdorferi s.l. is used (n=117 vs n=47). Bb ss = B. burgdorferi sensu stricto; Bb mix = B. burgdorferi sensu lato; HKCA = heat-killed Candida albicans; Neg/Pos ser = Negative/positive B. burgdorferi s.l. serology according to two-tier testing; PBMC = peripheral blood.
B. burgdorferi is a poor inducer of IFN-γ

mononuclear cell. * p < 0.05, ** p < 0.01, *** p < 0.0001 calculated by Wilcoxon-signed ranked test for paired testing.

Suppl. Figure 4: Pro-inflammatory cytokine profile of B. burgdorferi s.s.-exposed PBMCs after IL-12 addition in 149 healthy volunteers of 200FG cohort. (A + B) IL-1β concentration was higher in both PBMC and WBA conditions. (C + D) In WBA, TNF-α concentration was elevated. This was not observed in supernatants of PBMCs after IL-12 addition. (E + F) IL-6
production did not change in WBA or PBMC condition. WBA = whole blood analysis; Bb ss = B. burgdorferi sensu stricto; HKCA = heat-killed Candida albicans. ns = not statistically significant; PBMC = peripheral blood mononuclear cell; WBA = whole blood assay. ** p < 0.01. *** p < 0.0001 calculated by Wilcoxon-signed ranked test for paired testing.

Suppl. Figure 5: Anaplasma phagocytophilum serology and IFN-γ.
IFN-γ production by IL-12/B. burgdorferi stimulated PBMCs isolated from EM patients at baseline (t0) and after 6 weeks (t6w) compared between individuals with and without Anaplasma phagocytophilum (Ap). WE did not observed a difference in IFN-γ production between Ap seropositive and seronegative EM patients. Statistical testing was performed with Mann Whitney U tests.
**Suppl. Table 1:** SNPs investigated for associations with IFN-γ production.

Il-12 p40 = Interleukin 12 Subunit of 40kDa; IL-12RB1/2 = IL-12 Receptor Subunit Beta 1/2; TYK2 = Tyrosine Kinase 2; STAT4 = Signal Transducer and activator of transcription 4; IL18R1 = Interleukin 18 Receptor 1.

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<td>0.0011</td>
<td>***</td>
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<tr>
<td>Figure 4E</td>
<td>Rs 280520 vs no rs280520</td>
<td>Mann Whitney test</td>
<td>0.0011</td>
<td>***</td>
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<tr>
<td>Figure 4F</td>
<td>Rs 280520 vs no rs280520</td>
<td>Mann Whitney test</td>
<td>0.0011</td>
<td>***</td>
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<tr>
<td>Figure 4G</td>
<td>Rs 280520 vs no rs280520</td>
<td>Mann Whitney test</td>
<td>0.0011</td>
<td>***</td>
</tr>
<tr>
<td>Figure 4H</td>
<td>Rs 280520 vs no rs280520</td>
<td>Mann Whitney test</td>
<td>0.0011</td>
<td>***</td>
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<tr>
<td>Figure 4I</td>
<td>Rs 280520 vs no rs280520</td>
<td>Mann Whitney test</td>
<td>0.0011</td>
<td>***</td>
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<tr>
<td>Figure 4J</td>
<td>Rs 280520 vs no rs280520</td>
<td>Mann Whitney test</td>
<td>0.0011</td>
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**Note:** ns = not significant, * = p < 0.05, ** = p < 0.01, *** = p < 0.001.
Figure 4F
Bb frozen MOI 0.2 vs Bb frozen MOI 0.2 + L-12
Wilcoxon matched pairs signed rank test 0.0005 ***

Bb viable MOI 0.2 vs Bb viable MOI 0.2 + IL-12
Wilcoxon matched pairs signed rank test 0.0005 ***

Bb frozen MOI 1 vs Bb frozen MOI 1 + L-12
Wilcoxon matched pairs signed rank test 0.0005 ***

Bb viable MOI 1 vs Bb viable MOI 1 + L-12
Wilcoxon matched pairs signed rank test 0.0005 ***

Figure 4G
RPMI vs Bb + IL-12 (10 ng/ml)
Wilcoxon matched pairs signed rank test 0.0002 ***

RPMI + IL-12 (10 ng/ml) vs Bb + IL-12 (10 ng/ml)
Wilcoxon matched pairs signed rank test 0.0002 ***

Bb ss + IL-12 (10 ng/ml) vs Bb ss + IL-12 (1 ng/ml)
Wilcoxon matched pairs signed rank test 0.0009 ***

Bb ss + IL-12 (1 ng/ml) vs Bb ss + IL-12 (0.1 ng/ml)
Wilcoxon matched pairs signed rank test 0.0002 ***

Figure 4H
RPMI vs Bb ss + IL-12 (10 ng/ml)
Wilcoxon matched pairs signed rank test 0.0071

RPMI + IL-12 (10 ng/ml) vs Bb ss + IL-12 (10 ng/ml)
Wilcoxon matched pairs signed rank test 0.0081 **

Bb ss + IL-12 (10 ng/ml) vs Bb ss + IL-12 (1 ng/ml)
Wilcoxon matched pairs signed rank test 0.0039 **

Bb ss + IL-12 (1 ng/ml) vs Bb ss + IL-12 (0.1 ng/ml)
Wilcoxon matched pairs signed rank test 0.0038 **

Figure 4I
HC vs EM 10
Mann Whitney test 0.6621 ns

HC vs EM 16
Mann Whitney test 0.0003 ***

EM 10 vs EM 16
Wilcoxon matched pairs signed rank test 0.9433 ns

EM 10 vs EM 16
Wilcoxon matched pairs signed rank test 0.1517 ns

EM 10 vs EM 16
Mann Whitney test 0.0128 *

EM 10 vs EM 16
Wilcoxon matched pairs signed rank test 0.6406 ns

Figure 5A
Bb MOI 1 vs Bb MOI 1 + IL-12
Wilcoxon matched pairs signed rank test 0.0375 ns

Bb MOI 1 vs Bb MOI 1 + IL-12
Wilcoxon matched pairs signed rank test 0.0395 **

Bb MOI 0.2 vs Bb MOI 0.2 + IL-12
Wilcoxon matched pairs signed rank test 0.0386 *

Figure 5B
Bb ss MOI 0.2 vs Bb MOI 1 + IL-12
Wilcoxon matched pairs signed rank test 0.0386 *

Figure 5C
Bb MOI 1 vs Bb MOI 1 + IL-12
Wilcoxon matched pairs signed rank test 0.0001 ***

Bb MOI 0.2 vs Bb MOI 0.2 + IL-12
Wilcoxon matched pairs signed rank test 0.0004 ***

Bb MOI 0.2 vs Bb MOI 0.2 + IL-12
Wilcoxon matched pairs signed rank test 0.0006 ***

Figure 5D
EM vs HC 0
Mann Whitney test 0.1484 Ns

EM vs HC 16
Wilcoxon matched pairs signed rank test 0.0738 *

EM 10 vs EM 16
Wilcoxon matched pairs signed rank test 0.9543 Ns

EM 10 vs EM 16
Mann Whitney test 0.0253 Ns

EM 10 vs EM 16
Wilcoxon matched pairs signed rank test 0.6486 ns

EM 10 vs EM 16
Mann Whitney test 0.8692 ns

EM 10 vs EM 16
Wilcoxon matched pairs signed rank test 0.9610 ns

Figure 5E
Bb MOI 1 vs Bb MOI 1 + IL-12
Wilcoxon matched pairs signed rank test 0.0302 ***

Bb ss MOI 1 vs Bb ss MOI 1 + IL-12
Wilcoxon matched pairs signed rank test 0.0003 ***

Bb ss MOI 1 vs Bb ss MOI 1 + IL-12
Wilcoxon matched pairs signed rank test 0.0001 ***

Bb ss MOI 1 vs Bb ss MOI 1 + IL-12
Wilcoxon matched pairs signed rank test 0.0004 ***

Figure 5F
HC vs EM 10
Mann Whitney test 0.0978 *

HC vs EM 16
Mann Whitney test 0.6578 ns

HC vs EM 16
Mann Whitney test 0.0253 ns

HC vs EM 16
Wilcoxon matched pairs signed rank test 0.4686 ns

HC vs EM 16
Mann Whitney test 0.8932 ns

HC vs EM 16
Wilcoxon matched pairs signed rank test 0.8631 ns

HC vs EM 16
Mann Whitney test 0.9230 ns

Figure 5G
Bb viable MOI 1 vs Bb viable MOI 1 + IL-12
Wilcoxon matched pairs signed rank test 0.0003 ***

Bb viable MOI 0.2 vs Bb viable MOI 0.2 + IL-12
Wilcoxon matched pairs signed rank test 0.0003 ***

Bb viable MOI 1 vs Bb viable MOI 1 + IL-12
Wilcoxon matched pairs signed rank test 0.0003 ***

Bb viable MOI 0.2 vs Bb viable MOI 0.2 + IL-12
Wilcoxon matched pairs signed rank test 0.0001 ***

Figure 5H
Bb viable MOI 1 vs Bb viable MOI 1 + IL-12
Wilcoxon matched pairs signed rank test 0.0003 ***

Bb viable MOI 0.2 vs Bb viable MOI 0.2 + IL-12
Wilcoxon matched pairs signed rank test 0.0001 ***

Bb viable MOI 1 vs Bb viable MOI 1 + IL-12
Wilcoxon matched pairs signed rank test 0.0003 ***

Bb viable MOI 0.2 vs Bb viable MOI 0.2 + IL-12
Wilcoxon matched pairs signed rank test 0.0001 ***

Figure 5I
HC vs HC 0
Mann Whitney test 0.1484 Ns

HC vs HC 12
Wilcoxon matched pairs signed rank test 0.0078 **

HC vs HC 12
Wilcoxon matched pairs signed rank test 0.0015 **

Figure 5J
HC vs HC 12
Mann Whitney test 0.0003 ***

HC vs HC 12
Wilcoxon matched pairs signed rank test 0.0001 ***

HC vs HC 12
Wilcoxon matched pairs signed rank test 0.0001 ***

Figure 6A
Bb MOI 10 vs Bb MOI 10 + IL-12
Wilcoxon matched pairs signed rank test 0.0001 ***

Figure 6B
Bb MOI 10 vs Bb MOI 10 + IL-12
Wilcoxon matched pairs signed rank test 0.0001 ***

Figure 6C
Bb MOI 10 vs Bb MOI 10 + IL-12
Wilcoxon matched pairs signed rank test 0.0001 ***

Figure 6D
Bb MOI 10 vs Bb MOI 10 + IL-12
Wilcoxon matched pairs signed rank test 0.0001 ***

Figure 6E
Bb MOI 10 vs Bb MOI 10 + IL-12
Wilcoxon matched pairs signed rank test 0.0001 ***

Figure 6F
Bb MOI 10 vs Bb MOI 10 + IL-12
Wilcoxon matched pairs signed rank test 0.0001 ***

Figure 6G
Bb MOI 10 vs Bb MOI 10 + IL-12
Wilcoxon matched pairs signed rank test 0.0001 ***
Borrelia burgdorferi is strong inducer of IFN-γ production by human primary CD56⁺ NK cells

Marije Oosting*, Michelle Brouwer*, Hedwig D. Vrijmoeth, Rosa Pascual Domingo, Anna Greco, Hadewych ter Hofstede, Ellen H. van den Bogaard, Joost Schalkwijk, Mihai G. Netea, Leo A.B. Joosten

Submitted, # both authors shared first authorship
Abstract

Natural Killer (NK) cells belong to the innate lymphoid lineage and are highly present in the human skin. NK cells can produce a range of pro-inflammatory mediators, including cytokines and chemokines. The role of NK(-T) cells in the immune response towards Borrelia burgdorferi infection was studied. The production of interleukin (IL)-6, IL-1β and interferon-gamma (IFN-γ) by human primary peripheral blood mononuclear cells (PBMCs) exposed to B. burgdorferi was assessed. Interestingly, CD56+ (NK + NK-T) cells were the only cells within the PBMC-fraction that produced IFN-γ during the first 24 hours of stimulation. Within the NK(-T) cell fraction, NK cells seemed to be responsible for the IFN-γ production. Since it was previously demonstrated that both TLR2 and NOD2 receptors are involved in the recognition of B. burgdorferi, the expression of both TLR2 and NOD2 mRNA on NK cells was determined. In contrast to TLR2, NOD2 mRNA was upregulated on CD56+ (NK + NK-T) cells after Borrelia exposure. Finally, to unravel the mechanisms underlying erythema migrans (EM) development, crosstalk between CD56+ (NK + NK-T) cells and keratinocytes was investigated. CD56+ (NK + NK-T) cells activated by B. burgdorferi produced soluble mediators strongly inducing the expression of antimicrobial peptides, such as β-defensin-2 and psoriasin in human keratinocytes. In conclusion, CD56+ (NK + NK-T) cells produced IFN-γ shortly after exposure to B. burgdorferi and released soluble mediators that were able to activate keratinocytes. These observations underscore the important role of CD56+ (NK + NK-T) cells during early host defence when Borrelia burgdorferi enters the human skin during a tick bite.
Introduction

Natural Killer (NK) cells are part of the innate immune system and comprise 5–15% of human peripheral blood lymphocytes [1]. In contrast, the percentage of NK-T cells (CD3⁺/CD56⁺) in peripheral blood is only 0.1% to 1% [1]. The ability of NK cells to kill tumour cells and virally infected cells is well established. Nevertheless, innate immune function of NK cells is not restricted to host defence responses against viral infection but also to bacteria [2,3]. In bacterial infections, NK cells can contribute in two ways: activation due to cross-talk with other leukocytes or direct recognition of pathogen-associated molecular patterns (PAMPs) [3][1]. There is evidence that NK cells express pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and nucleotide oligomerization domain (NOD)-like receptors [3–6].

The skin is a key organ in arthropod-borne diseases, such as Lyme borreliosis. NK cells are abundantly present in the skin, interacting closely with the resident tissue cells. Within this reciprocal relationship, keratinocytes may release CCL5 and CXCL10, thereby recruiting NK cells to a site of infection in the skin. In turn, skin-resident NK cells can produce great amounts of IFN-γ, further activating the keratinocytes [7]. Inflammatory cells in plaques of psoriasis patients consisted out of 5-8% NK cells. Nowadays, there is increasing evidence, both cellular and genetic, for NK and NK-T cell involvement in psoriasis [8]. NK cells may therefore be pivotal cells, orchestrating the beginning of the innate immune response in the human skin, where *Borrelia* spirochetes primarily encounter the human host.

Lyme borreliosis (LB) is the most common vector-borne disease in North America, Europe and Asia and it is caused by spirochetes of *Borrelia burgdorferi sensu lato* [9]. LB is distinguished by multifaceted clinical manifestations. The first stage is characterized by a red expanding skin rash at the site of the tick bite, called erythema migrans (EM). The etiology of the EM remains largely unknown [9,10][2,3]. A better understanding of the mechanisms underlying the development of EM may lead to novel insights in the pathogenesis of LB. This may be crucial to face the serious consequences of disseminated Lyme borreliosis, such as acrodermatitis chronic atrophicans, neuroborreliosis, or Lyme arthritis, and long-term symptoms attributed to the infection [11]. The main cytokines produced by human peripheral blood mononuclear cells (PBMCs) exposed to *Borrelia* spirochetes, are IL-1β, IL-6, IL-17 and only very low levels of IFN-γ [12]. Production of IFN-γ is regulated through
production of IL-18 (caspase-1 and inflammasome-dependent) [12], IL-12[13], and type I IFNs [14], and it has been strongly associated with NK cells[15,16]. In this study, direct recognition and antigen presentation of B. burgdorferi spirochetes by peripheral blood CD56+ (NK + NK-T) cells was explored. In contrast to other immune cells, (purified) CD56+ CD3- NK cells produced IFN-γ rapidly after Borrelia exposure and enhanced MHCII expression. In addition, CD56+ (NK + NK-T) cells exposed to Borrelia spirochetes produced soluble factors that promoted the induction of antimicrobial peptides by keratinocytes, which are seen as proinflammatory mediators and chemotactic factors [17].

Materials and methods

Borrelia burgdorferi cultures
B. burgdorferi species were cultured at 25°C (and in a later time-point 34°C) in Barbour-Stoenner-Kelley (BSK)-H medium (Sigma-Aldrich) supplemented with 6% rabbit serum. Spirochetes were grown to late-logarithmic phase and motility was examined by dark-field microscopy. Their count was assessed by fluorescence microscopy using a Petroff-Hauser counting chamber. After centrifugation at 7000 x g for 15 minutes, bacteria were harvested, and washed three times with sterile PBS (pH 7.4), and diluted in the specified medium to required concentration. Spirochetes were stored at -80°C until use.

Isolation of human peripheral blood mononuclear cells (PBMCs)
PBMCs were isolated from buffy coats from healthy human donors (Sanquin bloodbank, Nijmegen, The Netherlands). PBMC fraction was obtained by density centrifugation of blood diluted 1:1 in PBS over Ficoll-Paque (Pharmacia Biotech). Cells were washed twice in PBS and suspended in RPMI 1640 supplemented with 50 µg/mL gentamicin, 1 mM pyruvate and 2 mM L-glutamine. Cells were counted in a Coulter Counter and the number was adjusted to 5x10^6 cells/mL.

Isolation of human NK/NK-T cells
Human CD56+ (NK + NK-T) cells were isolated from PBMCs by MACS-based positive selection (CD56+ microbeads, Miltenyibiotec) or negative selection (NK isolation kit, Miltenyibiotec), according to instructions of the manufacturer. Concerning the positive selection, PBMCs were incubated with bead-conjugated antibodies specific
for CD56 for the collection of NK/NK-T cells. Cells were passed through ferromagnetic columns (MS or LS) held in a magnetic field. The flow-through collected in the presence of the magnetic field was named as “Rest” (fraction depleted of CD56+ (NK + NK-T) cells). With regard to the negative selection, PBMCs were incubated first with biotin-conjugated antibodies against DCs, monocytes, erythrocytes, T- and B-cells. Subsequently, after 10 minutes, cells were incubated with the “NK cells Microbead cocktail”. Non-NK cells were depleted by magnetic separation on LS columns.

**In vitro stimulation**

PBMCs, CD56+ (NK + NK-T) selected cells, and CD56+ (NK + NK-T) depleted PBMCs (“Rest” cells) were cultured in round-bottom 96-wells plates (Corning, NY, USA) at a concentration of 5x10^5 cells/well. Cells were incubated with either culture RPMI medium (negative control), *B. burgdorferi* (1x10^6 sp/mL, MOI = 0.2), Pam3Cys (10 µg/mL) or MDP (10 µg/mL). When mentioned, 10% human pool serum was added. Cells were incubated 24 hours, 48 hours or 7 days at 37°C with 5% CO₂. Then, supernatants were collected and stored at -20°C until being assayed.

**Keratinocytes cell culture**

Primary human epidermal keratinocytes cells were provided by the Department of Dermatology, Radboudumc, Nijmegen. Briefly, keratinocytes were isolated from skin biopsies from healthy volunteers and cultured in keratinocyte growth medium (KGM, Lonza) as previously described [18]. At 100% confluence, cells were stimulated with either supernatants of the CD56+ (NK + NK-T) cells or control stimuli. All stimuli were diluted 1:3 in KGM depleted from growth factors to induce keratinocyte differentiation. A cytokine mixture of IFN-γ (100 U/mL) and IL-1β (30 ng/mL) was used as positive control. Cells were harvested after 48 hours and stored at -80°C in Trizol for RNA extraction.

**RNA isolation and quantitative real-time PCR**

Total RNA from cultured cells was isolated according to the Trizol protocol (Invitrogen). Specific qPCR primers were designed with Primer Express 1.0 Software (Applied Biosystems, Carlsbad, CA) and purchased from Biolegio (Nijmegen, The Netherlands). Primer sequences are described in supplementary table 1. Real-time PCR using SYBR Green was performed on AB7300 (Applied Biosystems): two initial steps of 2’ 50°C and 10’ 95°C were followed by 40 cycles at 95°C for 15’’ and 60°C for 25’’. Relative mRNA expression levels were calculated using the comparative 2^Δ-
(ΔCt) method and β2-microglobulin (B2M) or hARP as reference genes (for PBMC and keratinocytes, respectively). Data analysis was performed using 7300 System SDS Software.

**Cytokine measurements.**

Concentrations of human IL-1β, IL-6 or IFN-γ were determined using commercial ELISA kits (Sanquin, Amsterdam; or R&D Systems, Minneapolis) in accordance with manufacturer’s instructions. Sensitivity of the assays was 39 pg/mL, except for IFN-γ ELISA (detection limit of 8 pg/mL).

**Flow cytometry**

Intracellular IFN-γ was analysed using stimulated or unstimulated PBMCs. At 4-6 hours prior to the end of incubation, Golgi Plug inhibitor (BD Pharmingen), Ionomycin (Sigma Aldrich) and PMA (Sigma Aldrich) were added to the culture according to the protocol supplied by the manufacturer. When indicated the standard concentration of PMA (50 ng/mL) and Ionomycin (1 µg/mL) was reduced 5 times. Cells were stained with anti-CD3 PE-CY5 (Biolegend), anti-CD56 PE-CY7 (Beckman Coulter), anti-CD8 PE-CY7 (Biolegend), anti-CD4 PE-CY7 (BD Biosciences) and/or anti-TLR2 Al647 (eBioscience).

PBMCs were stained for 15 min at RT with CD45 APC (Biolegend), CD3 PE (Biolegend), CD14 AlexaFluor 700 (Biolegend), CD56 PE-Cy7 (Biolegend), HLA-A, -B and -C Pacific Blue (BPE (Biolegend) or HLA-DR BV421 (BD Bioscience) and CD74 FITC (Biolegend).

In case of intracellular staining, cells were fixed and permeabilized with Cytofix/cytoperm (eBioscience) 45 minutes at 4ºC followed by one wash in Perm/wash buffer (eBioscience). Thereafter, cells were stained for intracellular IFN-γ levels with either anti-IFN-γ FITC (eBioscience), HLA-A, -B and -C Pacific Blue (BPE (Biolegend) or HLA-DR BV421 (BD Bioscience) and CD74 FITC (Biolegend), according to the manufacturer’s protocol.

Cells were measured on a FC500 flowcytometer (Beckcam Coulter) and CytoFleX (Beckman Coulter) analysis of the data was performed using CXP analysis software v2.2 (Beckman Coulter) and Kaluza version 1.5a (Beckman Coulter).
Statistical Analysis
Data are expressed as mean ± SEM unless stated otherwise. Differences between experimental groups were tested using the two-sided Mann-Whitney $U$ test and a paired Wilcoxon signed rank test performed on GraphPad Prism 4.0 and 8.0 software (GraphPad). $P$ values ≤ 0.05 were considered significant.

Results
Dose-response cytokine induction in NK/NK-T cells after Borrelia exposure.
To determine the cytokine profile of NK/NK-T cells after Borrelia exposure, positive selection of CD56$^+$ (NK + NK-T) cells was performed by isolation from human PBMCs. These cells were stimulated for 24 hours with live Borrelia burgdorferi, $1\times10^6$ spirochetes/mL. Moreover, two bacterial ligands were used as positive controls: Pam3Cys lipopeptide (TLR2 agonist) and MDP (NOD2 agonist). Isolated CD56$^+$ (NK + NK-T) cells produced IL-$1\beta$, IL-$6$ and a relatively high concentration of IFN-$\gamma$ in a dose-dependent fashion upon Borrelia stimulation (Fig. 1A-C). Interestingly, the cell fraction depleted of CD56$^+$ (NK + NK-T) cells (Rest) and whole PBMCs fraction (including CD56$^+$ NK + NK-T cells) failed to produce detectable levels of IFN-$\gamma$. In accordance with measured protein concentrations, IFN-$\gamma$ mRNA expression was strongly upregulated in CD56$^+$ (NK + NK-T) cells after B. burgdorferi exposure and only moderately upregulated after Pam3Cys stimulation (Fig. 1D).
Figure 1. IFN-γ production by CD56+ (NK + NK-T) cells is suppressed by the PBMC fraction during B. burgdorferi exposure. CD56+(NK + NK-T) -cells, fraction depleted of CD56+ (NK + NK-T) cells (Rest) or PBMCs were stimulated with B. burgdorferi spirochetes 10^6 /mL, Pam3Cys (10µg/mL), MDP (10µg/mL) or culture medium (RPMI, negative control). Production of (A) IL-1β and (B) IL-6 assessed by ELISA 24h after stimulation. (C) Production of IFN-γ assessed by ELISA and (D) fold change IFN-γ mRNA setting unstimulated PBMCs as reference. (E) Production of IFN-γ in pg/mL in cells exposed to Borrelia for 24, 48 and 7 days. +S indicates that 10% Human Pool Serum (HPS) was added to the culture (See Figure S1). *P<0.05 **P<0.01 using the nonparametric Wilcoxon matched pairs signed ranks test. N≥6.
Furthermore, the kinetics of the release of IFN-γ by either CD56+ (NK + NK-T) cells, Rest or whole PBMCs fraction were investigated (Fig. 1E). At 24h, IFN-γ production was only visible in the NK/NK(-T) fraction, whereas other cells became a source of IFN-γ at 48h and 7 days (Fig. 1E). However, IFN-γ concentrations remained lower than produced by CD56+ (NK + NK-T) cells. For cells cultured for 48 hours or 7 days, 10% of human pool serum in the culture medium was required. No significant differences were detected in the absence or presence of serum at 24 hours (Fig. S1A). To determine whether the difference in IFN-γ concentrations between 24h of incubation and later time points was a result of the addition of serum, IFN-γ concentrations were measured after stimulation of cells with B. burgdorferi with and without serum during 24h. Both the Rest and PBMCs fractions did not show any detectable levels of IFN-γ (Fig. S1B). This confirmed that these cell types only release IFN-γ at later time points, independent of the presence of human pooled serum at 24h.

**CD56+ CD3- natural Killer cells have the capacity to produce high concentrations of IFN-γ after B. burgdorferi exposure.**

The CD56+ (NK + NK-T) population isolated after magnetic labelling consists of a mixture of NK (CD56+CD3-) and NK-T (CD56+CD3+) cells. NK-T cells represent a subpopulation of T cells that possess properties of innate NK cells. At least two subpopulations of NK-T cells can be distinguished: lineage of T cells expressing a semi-variant TCR (Vα24Jα18), often referred as “classical” NK-T cells and a subset of conventional α/β T cells named “NK-T-like” cells that expresses NK associated receptors such as CD56[19].

To determine the involvement of NK cells as robust producers of IFN-γ after recognition of *Borrelia* spirochetes, a negative selection procedure was performed to isolate pure NK cells. A comparison between a mix of NK cell and NK-T cells (CD56+CD3+/−), and a pure NK cell (CD56+CD3-) population, when stimulated with *B. burgdorferi* or Pam3Cys, showed no differences regarding IFN-γ production (Fig 2C and 2D, respectively). Of interest, the production of IL-6 and IL-1β was absent in pure NK cells (Fig. 2A and 2B).
Figure 2. CD56+CD3− NK cells produce IFN-γ upon B. burgdorferi exposure. Comparison between NK + NK-T-cells (CD56+ cells) and pure NK cells (NK isolation kit). Cells were stimulated 24h with 10^6 sp/mL live B. burgdorferi without human pool serum. (A) IFN-γ production measured by ELISA. (B) IFN-γ production after stimulation with Pam3Cys (10 µg/mL). (C) IL-6 and (D) IL-1β production measured by ELISA. Median is depicted as a straight line; **P<0.01, ***P<0.001 using the nonparametric Wilcoxon matched pairs signed ranks test. N≥7.

In addition, flow cytometry lineage sorting was performed to further study the contribution of NK and NK-T-like cells to the release of IFN-γ. NK-T-like cells
NK cells produce IFN-γ in response to Borrelia

(CD56+CD3+) accounted for a small population (around 10%) of the total CD56+ (NK + NK-T) population (Fig. 3A). To shed further light on the cell types involved in IFN-γ production, the different lineages were stained for IFN-γ. After gating for IFN-γ, cells were identified as either cytotoxic T lymphocytes (CD8+), T helper lymphocytes (CD4+), NK-T-like cells (CD56+CD3+) or NK cells (CD56+CD3-). NK cells were responsible for a higher percentage of total IFN-γ production than NK-T cells (Fig. 3B). This may be related to their recognition and presentation of B. burgdorferi peptides via major histocompatibility complexes (MHC). Intracellular staining for IFN-γ showed that NK-T-like cells had greater potential than NK cells to produce IFN-γ, because the majority of these cells were IFN-γ positive after PMA and ionomycin activation (Fig. 3C).

Figure 3. CD8+ T cells have the highest expression of intracellular IFN-γ upon B. burgdorferi exposure. Fluorescence-activated cell sorting (A) Representative donor for percentage of CD3+ cells within the CD56+ (NK + NK-T) population. PBMCs were stained and gated for CD56+ (NK + NK-T) cells (N=2). (B) Portion of each cell type within the PBMC fraction capable
of IFN-γ production upon *B. burgdorferi* stimulation. (C) Percentage of IFN-γ+ cells within the lineage of NK cells or NK-T-like cells, pre-incubated with PMA and ionomycin. PBMCs were stimulated for 24h with human pool serum plus RPMI, *B. burgdorferi* 10⁶ sp/mL, or MDP 10 µg/mL.

**NOD2 mRNA was upregulated in NK/NK-T-like cells upon *B. burgdorferi* stimulation.**

CD56+ (NK + NK-T) cells, and specifically NK cells, can recognize *B. burgdorferi* as well as pure PRR agonists like Pam3Cys (TLR2 agonist) and MDP (NOD2 agonist) (Fig. 1A and 1B). Therefore, the expression of TLR2 and NOD2 on basal and *Borrelia*-stimulated NK cells was determined. mRNA expression of TLR2 and NOD2 receptors in NK/NK-T-like cells could be detected, confirming previous results [4,5] (Fig. 4A-B). NOD2 mRNA was significantly upregulated upon *B. burgdorferi* stimulation (Fig. 4B). Despite the high response to Pam3Cys, TLR2 was not upregulated in CD56+ (NK + NK-T) cells. Since NK/NK-T cells respond adequately to TLR2 agonists, it was hypothesized that CD56+ (NK + NK-T) cells already express a high number of TLR2 proteins on their surface. To test this hypothesis, we stained the cells for CD56 and TLR2, to analyse expression by FACs. Interestingly, only 1-3% of CD56+ (NK + NK-T) cells expressed TLR2 on their surface, and their TLR2 expression was not upregulated upon recognition of *B. burgdorferi* (Fig. 4C and D). Therefore, activation of NK/NK-T cells upon *B. burgdorferi* stimulation was thought to occur through the TLR1 pathway, as both *B. burgdorferi* and Pam3Cys are also TLR1 ligands. Moreover, NOD2 expression might play a role, since its mRNA expression was upregulated upon *B. burgdorferi* exposure.
Figure 4. NOD2 expression is upregulated in *B. burgdorferi* exposed NK and NK-T cells. CD56+ (NK + NK-T) cells were stimulated for 24h with *B. burgdorferi* spirochetes $10^6$ sp/mL (Bb), Pam3Cys (10 µg/mL), MDP (10 µg/mL), or culture medium (RPMI, negative control), without human pool serum. Fold change expression of TLR2 (A) and NOD2 (B) mRNA, setting unstimulated CD56+ (NK + NK-T) cells as reference. Significance is depicted as *P<0.05, using a two-tailed Mann-Whitney test. N=5 except for MDP stimulation (N=2). (C and D) FACS analysis gating on CD56+ (NK + NK-T) cells positive for TLR2. PBMCs were stimulated for 24h with *B. burgdorferi* spirochetes $10^6$ sp/mL or RPMI, without human pool serum. Figures show representative donor (N=2).
Activation of the keratinocytes by NK and NK-T cell derived mediators.
Towards a better understanding of the key mechanisms that takes place during the initiation phase of an EM, we investigated whether soluble mediators released by NK/NK-T cells are able to activate keratinocytes (KCs). Therefore, supernatants of isolated CD56⁺ (NK + NK-T) cells (cultured with or without Borrelia) were used to activate human keratinocytes. As read-out for this activation, we determined the upregulation of antimicrobial peptides (AMPs), which are part of the humoral skin immune system (SIS)[20]. AMPs have an alarmin effect acting as natural antibiotic, and they also induce a positive feedback on human KCs to release cytokines, chemokines and more AMPs [21]. mRNA levels of β-defensins 2 and 3 (hBD-2 and hBD-3), psoriasin (S100A7), and DAMPs (danger-associated molecular patterns) S100A8 and S100A9 were measured, since expression of these AMP genes was shown to positively correlate with skin inflammation [22,23]. Although psoriasin is not as well characterized as β-defensin, it has been described to be induced in several dermatoses (psoriasis, atopic dermatitis, and mycosis fungoides) [24]. Upregulation of the mRNA levels of human β-defensin-2 (hBD-2), Psoriasin (S100A7), S100A8 and S100A9 were detected in the keratinocytes of three different donors, when NK/NK-T cells were previously exposed to B. burgdorferi, in contrast to hBD-3 (Fig. 5).
Figure 5. Keratinocytes show upregulation in hBD-2, psoriasin, S100A8 and S100A9 expression upon exposure to *B. burgdorferi*-induced CD56+ (NK + NK-T) cell responses. Keratinocytes (KCs, 2x10^5 cells/well) were cultured and stimulated during 48h. Fold change expression of mRNA setting the value with medium as reference. The negative controls
(grey bars) were Medium (RPMI), Medium with *B. burgdorferi* (10⁶ sp/mL) overnight and supernatants from unstimulated CD56⁺ (NK + NK-T) cells. The positive control (red bar) was IFN-γ (100 U/mL) plus IL-1β (30 ng/mL). KCs were also stimulated with supernatants from CD56⁺ (NK + NK-T) cells previously stimulated with *B. burgdorferi* spirochetes 10⁶ sp/mL; N=3.

**Proteins associated with MHC class II antigen presentation are suppressed in Bb-stimulated NKT cells, but not NK cells.**

Previous studies have demonstrated that antigen presentation molecules are inhibited in several innate immune cell subsets by pathogens, including *B. burgdorferi* [25,26]. While MHCI has a central role in NK cell antigen presentation, MHCII presentation by NK cells is also possible and often a sign of NK cell activation [27–30]. MHCII antigen presentation was not inhibited in NK cells after exposure to *B. burgdorferi* for 72h, and expression of HLA-DR and its chaperone CD74 were not significantly different from the medium control (Fig.6A and B). MHCI expression in NK cells also remained constant when compared to the medium control (Fig. 6C). NK cell antigen presentation through HLA-DR, HLA-A, HLA-B and HLA-C expression remained consistent after 72h of exposure to *B. burgdorferi*.

In contrast to NK cells, NK-T cells expressed higher basal expression levels of HLA-DR and CD74. Both HLA-DR and CD74 expression were significantly inhibited in NK-T cells when stimulated with *Borrelia burgdorferi* (Fig.6A and B). Interestingly, *B. burgdorferi* did not affect HLA-A, -B and -C expression (Fig. 6C) in NK-T cells, similarly to NK cells.
Figure 6. During *B. burgdorferi* stimulation, intracellular and total CD74 and HLA-DR expression is downregulated in NK-T cells, while NK cells HLA-DR and CD74 expression shows no change. PBMCs (5x10^5 cells/well) were cultured and stimulated during 72h. (A) CD74, (B) HLA-DR and (C) HLA-A, -B and -C protein expression on NK and NK-T-cells was measured by flow cytometry. Fold change expression of HLA-A,-B and -C was set and normalized using the medium as reference (shown by the dotted line), significance is depicted as following; *P<0.05, using the nonparametric Wilcoxon matched pairs signed ranks test, N=6.
Discussion

In the present study, we investigated the role of natural killer (NK) cells in the immune responses towards *Borrelia burgdorferi*, the causative microbe of Lyme borreliosis. We demonstrated that purified primary human NK cells can directly recognize *B. burgdorferi* and are the most potent producers of IFN-γ after *B. burgdorferi* recognition. However, after 24h or 48h of incubation, IFN-γ production by *B. burgdorferi*-exposed human PBMCs (containing approximately 5-15% of NK cells) was undetectable. Interestingly, after 7 days of *B. burgdorferi* exposure, PBMCs still produce very low amounts of IFN-γ. These results are in line with previous reports showing very low levels of IFN-γ production by human PBMCs upon *B. burgdorferi* exposure [15]. In contrast, several other pathogens, such as *Candida albicans*, *Mycobacterium Tuberculosis* and *Listeria monocytogenes*, were able to activate PBMCs to produce IFN-γ [31–35]. Of interest, Moore *et al* (2007) demonstrated low levels of IFN-γ production by human PBMCs within 6 hours of *B. burgdorferi* exposure. However, much higher MOIs were used in this study [36]. The synergistic effect of IL-12 in NK cells was essential to produce IFN-γ in infections with other pathogens [3,5,37]. Although the synergy between IL-12 and *Borrelia* to induce IFN-γ in PBMCs was not investigated in this study, IL-12 co-stimulation was not a requirement for production of IFN-γ by NK cells *in vitro*. Furthermore, the response elicited by *Borrelia* spirochetes was not mediated by accessory cells. In this sense, our study challenges previous studies in which IFN-γ production (in response to *B. burgdorferi*) by purified NK cells culture was only shown when co-cultured with dendritic cells (DCs) [15].

IFN-γ production is normally induced through STAT activation by cytokines such as, IL-12 and IL-18, type I IFNs and IL-10 [38,39]. Of interest, IFN-γ production and STAT activation in NK cells was also associated with antigen presentation ability of these cells via MHCII during *B. burgdorferi* exposure. While NK-T cells showed a reduction in MHCII expression, NK cells have a stable expression, making them able to present new peptides to T cells, inducing NK and CD4+ T cell activation. In contrast, MHCI expression remained the same in both cell types, suggesting MHCI pathways are not affected by *B. burgdorferi*.

Use of both positive and negative selection to obtain purified NK cells, which has led to similar results, strongly underscores our findings. The advantage of positive selection was that it allowed us to make a clear comparison between CD56+ (NK +
NK cells produce IFN-γ in response to Borrelia

NK (NK-T) cells and the untouched depleted fraction. However, after negative selection, IFN-γ was detectable in the unstimulated Rest fraction (PBMCs minus CD56⁺ (NK + NK-T) cells) for some donors, probably caused by unspecific cell activation after interacting with multiple antibodies during the procedure. This problem was overcome by using CD56 beads because only one specific antibody was used. This could explain why the depleted fraction (Rest) showed higher IFN-γ levels in an experimental setup compared to the positive selection (Fig. 2A). In contrast, negative selection was crucial as well, especially for the validation of previous results and for further investigation into the two CD56⁺ (NK + NK-T) populations present in the mixture.

Another remarkable result was the high IL-1β, IL-6 and IFN-γ response to Pam3Cys compared to the low expression of TLR2 on the surface of NK cells in both unstimulated and stimulated cells. Our results are in line with a study using Helicobacter pylori, in which only a small population of NK cells expressed TLR2 on its surface [4]. However, when TLR2 was neutralized using an anti-TLR2 antibody, IFN-γ production was impaired. Therefore, further experiments are necessary to rule out the effect of the TLR2 on NK cells regarding Borrelia recognition. Nevertheless, it might be that upon B. burgdorferi exposure there was an upregulation of TLR2 by other cell types, depicted by a shift of the population to higher intensity within the TLR2⁺ axis. According to the Forward Scatter (FS) and Side Scatter (SS), monocytes were the ones that upregulated TLR2, and this observation fits previous literature [40,41]. The role of the NOD2 receptor in the recognition of B. burgdorferi by NK cells is not fully understood. mRNA of NOD2 receptor was upregulated upon B. burgdorferi stimulation, but also upon Pam3Cys stimulation. Our results suggest that NOD2 may be important for B. burgdorferi recognition by NK/NK-T-like cells, although future experiments are necessary to investigate the role of NOD2 signalling in NK cells further.

Interestingly, the relationship between the number of NK cells and clinical outcome has been studied in chronic Lyme borreliosis. For example, there has been controversy about CD57, an NK cell maturation marker. Stricker et al. claimed that patients with chronic symptoms attributed to Lyme borreliosis have a below-normal count of CD57⁺ NK cells [42]. In contrast, another study showed that the number of CD57⁺ NK cells was not different between patients with post-LB syndrome and healthy controls [43]. Of interest, it was demonstrated that patients
with antibiotic-resistant Lyme borreliosis had higher baseline numbers of circulating NK cells when compared to patients that did respond to antibiotics [44]. NK cells can activate human fibroblasts [45]; it is likely that NK cells play a relevant role in vivo, both in the epidermis and the dermis. That NK cells activate keratinocytes once they have been exposed to B. burgdorferi raises important questions; what is the key mediator driving this activation? And is it Borrelia specific? Further research will be addressed to unravel the molecular mechanisms underlying the activation of keratinocytes by NK cells.

Based on our findings, we hypothesize that NK cells play a key role during the initiation of erythema migrans. Once spirochetes have entered the skin, they are recognized by NK cells, which are potent producers of inflammatory cytokines and chemokines. Subsequently, keratinocytes are activated and aggravate the local skin inflammation. Local expression of IFN-γ was described to decrease in EM lesions from patients with persisting symptoms in comparison with patients without persisting symptoms [46], suggesting that regulation of the early cytokine response in the infected skin might be crucial for resolution of the infection. Genetic variations affecting the proposed mechanisms could be involved in the development of erythema migrans or disseminated Lyme borreliosis. Further investigation to elucidate the role of NK cells in patients with EM and in the persistence of LB-attributed symptoms.

Acknowledgements

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References


NK cells produce IFN-γ in response to *Borrelia*


Figure S1. (NK + NK-T) cells (CD56+ cells), fraction depleted from CD56+ (NK + NK-T) cells (Rest) or PBMCs stimulated with *Borrelia burgdorferi* spirochetes $1\times10^6$ sp/mL or culture medium (RPMI, negative control). (A) Production of IFN-γ by NK(T) cells was measured by ELISA after 24h or 48h in presence or absence of HPS. (B) Production of IFN-γ after 24h measured by ELISA with HPS. **P<0.01 two-tailed Mann Whitney test. 24h (-HPS) N=7; 24h (+HPS) N=3; 48h (-HPS) N=4; 48h (+HPS) N=6. HPS=10% human pool serum.
**Supplementary Table 1. Primer sequences**

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Chapter 7

Interleukin-12 restores *B. burgdorferi*-induced suppression of antigen presentation molecules and IFN-γ production in human cells

Michelle A.E. Brouwer, Vera Mourits, Leonie Helder, Hedwig Vrijmoeth, Heidi L.M. Lemmers, Helga Dijkstra, Mihai G. Netea, Mihaela Lupse, Leo A.B. Joosten

Manuscript in preparation
Abstract

The incidence of Lyme borreliosis is steadily increasing, and yet successful diagnostic strategies are still missing. Serology, interferon-gamma release, and T-cell proliferation assays all have insufficient sensitivity and specificity. Poor antigen presentation and IFN-γ production in early immune responses against *B. burgdorferi* infection may play a role in the lack of induction of potent memory responses that can be easily measured. In this study, we investigated the role of T cell activation in initial *B. burgdorferi* infection and whether these responses could be restored. Different cytokine combinations were assessed to study the effect on *B. burgdorferi*-induced suppression of antigen presentation and IFN-γ production. IL-12 combined with IL-18 was the most effective combination for restoring HLA-DR expression, while IL-12 combined with IL-7 enhanced CD74 to normal expression levels. IL-12 and IL-15 also helped restore IFN-γ production in response to *B. burgdorferi*, and they enhanced antigen presentation in monocytes to re-establish immune functions lost during initial infection. Overall, addition of IL-12 was the most effective method to evaluate *in vitro* whether human PBMCs were previously exposed to *B. burgdorferi*. 
**Introduction**

Lyme borreliosis is one of the most prevalent human vector borne diseases, which is transmitted through tick bites by the genus *Ixodes*\(^1\),\(^2\), with an increasing number of cases reported every year\(^3\). Lyme borreliosis is caused by bacterial spirochetes of the *B. burgdorferi* genus. One of the major hurdles in Lyme borreliosis (LB) is early diagnosis and treatment. Severe disseminated manifestations in patients can range from Lyme carditis, arthritis, neuroborreliosis and acrodermatitis chronica atrophicans (ACA)\(^1,\)\(^4\)\(^5\)\(^6\).

During initial infection, *B. burgdorferi* suppresses various host immune functions, preventing detection and clearance from the body. However, the activation status of T cells by markers such as CD38, CD69, anti-programmed cell death-1 (PD-1), anti–cytotoxic T lymphocyte antigen 4 (CTLA4) and HLA-DR have been poorly explored. Previous studies have shown how antigen presentation molecules as well as critical adaptive immune responses may be specifically suppressed by *B. burgdorferi*\(^7,\)\(^8\)\(^9,\)\(^10\). One of these responses includes the production of interferon gamma (IFN-\(\gamma\)). IFN-\(\gamma\) is important in both the development of the immune response, more specifically in T-helper 1 (Th1) cell responses, and its role as a diagnostic marker for Lyme borreliosis is a topic for ongoing research\(^11,\)\(^12,\)\(^13,\)\(^14\). IFN-\(\gamma\) activates immune cells to produce more cytokines, while enhancing the phagocytosis and killing of a range of pathogens. However, *B. burgdorferi* fails to induce IFN-\(\gamma\) production by human PBMCs after exposure *in vitro*\(^11\).

Interferon-gamma release assays (IGRAs) are well-known for their success in regard to detection of infections such as *Mycobacterium tuberculosis*\(^15\). This assay consists of re-stimulation with mycobacterial antigens of whole blood from a suspected patients infected with *M. tuberculosis*. This re-stimulation induces significant amounts of IFN-\(\gamma\) if the individual was previously exposed to *M. tuberculosis*. Theoretically, if a *B. burgdorferi*-infected individual would undergo an IGRA test in the form of re-stimulating immune cells with *B. burgdorferi* instead of *M. tuberculosis*, the test should provide a positive signal. However, such assays proved not to be reliable because they stay negative for proven patients with erythema migrans (EM). We wanted to investigate whether we can improve this methodology by stimulating immune cells with *B. burgdorferi* in combination with pro-inflammatory cytokines, that in turn may induce IFN-\(\gamma\) production, compared to *B. burgdorferi* stimulation alone\(^16,\)\(^17\). This method could help improve the read-out of
an IFN-γ release assay for *B. burgdorferi* in the form of a higher sensitivity and specificity.

IL-12 is a well-known enhancer of IFN-γ production\(^{18, 19, 20, 21}\). Furthermore, a combination of IL-12 and IL-18 is an even stronger inducer of IFN-γ than IL-12 alone \(^{22}\). Of high interest, IL-12 production was not detected in response to live *B. burgdorferi* spirochetes, while TNFα and IL-6 were secreted in significant levels \(^{23}\).

Recently, our group showed that antigen presentation molecule expression is inhibited by *B. burgdorferi* spirochetes, interfering with the activation of adaptive immune cells and the development of a more targeted immune response against *B. burgdorferi* and other infections \(^{7}\). Antigen presentation molecules were specifically inhibited in APCs, suppressing several major signalling pathways and possibly T cell activation. In the current study, we explored T cell activation early on during *B. burgdorferi* infection, and various strategies to restore IFN-γ production and antigen presentation machinery expression. Moreover, we assessed IFN-γ, IL-12, IL-18 and IFN-α profiles in sera of patients with EM, as well as in vitro assays, to determine T cell activation profiles further.

**Materials and Methods**

**Serum samples from patients with erythema migrans and healthy controls**

Serum samples were available from a Romanian cohort of 22 patients with physician-confirmed EM, before start of antibiotic treatment (baseline) and at follow-up (2, 6 and 12 weeks from baseline). As a control, 10 serum samples from healthy individuals were collected once. Both cohorts were included in an observational study at the University Hospital of Infectious Diseases, Cluj-Napoca, that was approved by the local medical ethics committee (2013/01).

**Human PBMC, CD4⁺ T cells and monocyte isolation**

Peripheral blood mononuclear cells were isolated from buffy coats (Sanquin Blood Bank in Nijmegen) or blood freshly donated by anonymous healthy volunteers. Ethical approval was obtained from the committee on research involving human subjects (CMO Arnhem-Nijmegen (NL32357.091.10)). Blood was diluted 1:1 with PBS. Utilizing Ficoll-Paque density gradient centrifugation, PBMCs were collected from the procured interphase. PBMCs were washed twice in ice-cold PBS. After the
last wash, PBMCs were resuspended in RPMI 1640 Dutch Modified supplemented with glutamate, gentamycin and pyruvate.

CD4+ T cells were isolated using negative selection by magnetic beads according to the manufacturer’s instructions (Miltenyl Biotech).

**Cell stimulations**

PBMCs, CD4 T cells, and monocytes were seeded at 5 x 10^5/well in a round bottom 96 well plate and stimulated with 10^5 *B. burgdorferi* species/well and a medium control for various time periods. Cells were treated with different stimulants and inhibitors: IFN-γ-1β (Immunkine, Boehringer Ingelheim), 10 ng/ml IL-12 (R&D), IL-18 (R&D), IL-7 (Gibco, Fisher Scientific), IL-15 (Bio-connect), IL-10 (Gibco, Fisher Scientific), CD3/CD28 Dynabeads, anti-human CD210 (IL-10 receptor; Biolegend), as well as vehicle controls: DMSO (Invitrogen, Life Technologies), purified Rat IgG2a (Biolegend).

**B. burgdorferi spirochetes**

*B. garinii* American Type Culture Collection (ATCC) 51383, *B. burgdorferi* s.s., ATCC 35210 type strain B31, and *B. afzelii* ATCC 51567 were cultured at 24 °C in Barbour–Stoenner–Kelley (BSK)-H medium (Sigma-Aldrich) with 6% rabbit serum. Once growth commenced, spirochetes were grown at 34 °C to late-logarithmic phase. Dark-field microscopy was used to check spirochete motility and spirochetes were quantified using a Petroff–Hauser counting chamber.

*B. burgdorferi* species were centrifugated at 7000g for 15 min, washed three times with sterile PBS, diluted to the required concentration and stored at -80°C in sterile PBS until use. When thawed for cell stimulation, *B. burgdorferi* s.l. was prepared by mixing equal amounts of *B. afzelii*, *B. burgdorferi*, and *B. garinii*. A final concentration of 10^6 spirochetes/mL, equal to a multiplicity of infection (MOI) of 0.2, was used. The decision to use a mix of these species was made because this way the results could be extrapolated to the three most prevalent *B. burgdorferi* strains.

**Candida albicans**

Heat-killed, at 65°C for 60 minutes, C. albicans blastoconidia (strain ATCC MYA-3573, UC 820) in a concentration of 1 x 10^6 CFU/mL were used as a positive control stimulus throughout this study.
Enzyme Linked ImmunoAssay and Luminex Multiplex Bead Assay
According to the manufacturer’s instructions, IFN-γ was measured using a Sanquin ELISA and IL-2 production was determined by Luminex Multiplex Bead Assay (Thermo Fischer Scientific).

Quantitative Polymerase Chain Reaction
Cells incubated in TRIzol (ThermoFisher) and RNA were isolated according to the manufacturer’s instructions. RNA purity was checked using nanodrop (ref). RNA was reverse transcribed into cDNA using an Iscript cDNA synthesis kit (Biorad). Quantitative PCR analysis was performed using Power Sybr Green PCR Master Mix (Applied Biosystems) and a 7300 Real-time PCR system (Applied Biosystems). qPCR data was normalized to a housekeeping gene, followed by direct comparison to the delta Ct value of the medium control, the delta delta Ct value was used for the final analysis.

Flow cytometry
PBMCs were washed with PBS supplemented with 1% BSA buffer wash (PBA). Thereafter, PBMCs were stained for 15 min at RT with CD45 APC (Biolegend), CD14 AlexaFluor 700 (Biolegend), CD19 PE-Cy7 (Biolegend), CD74 FITC (BD Bioscience), HLA-DM PE (Biolegend), HLA-DR BV421 (BD Bioscience). T cells were stained with CD45 APC (Biolegend), CD3 FITC (Biolegend), CD4 APC-Cy7 (Biolegend), HLA-DR BV421 (BD Bioscience), CD8 PE-Cy5 (Beckman Coulter), CD69 PE (BD Bioscience) or with CD3 APC-Cy7 (Biolegend), CD4 PE (Beckman Coulter), CD8 PE-Cy7 (Biolegend), CD38 FITC (Biolegend), CTLA-4 AF700 (Biolegend), PD-1 BV421 (Biolegend). T cells were only stained extracellularly.

PBMCs were washed again and permeabilized using a fixation and permeabilization buffer set (eBioscience, 00-5523-00), by incubation for 45 min at 4°C. After washing the cells with the permeabilization buffer kit, half of the cells were analyzed for extracellular staining, while remaining cells were stained intracellularly with CD74 FITC (Becton Dickinson), HLA-DM PE (Biolegend), HLA-DR BV421 (Biolegend) for 15 min at RT. Gating strategy is demonstrated in Figure X and the isotype controls are displayed in Figure X. Marker expression was measured with a CytoFleX (Beckman Coulter) and analyzed on Kaluza version 1.5a (Beckman Coulter). Flow cytometry results were normalized to the medium control for each donor at every time point.
Ultrasensitive protein detection assay
An ELLA customized kit for IL-12p70, IL-18, IFNα2 and IFN-γ was used according to the manufacturer’s instructions (Protein Simple).

Statistical Analysis
Statistics for measurements of cytokine levels and protein expression data were performed using GraphPad Prism version 5.03 for Windows (GraphPad Software). Data represent mean ± SEM of n different donors. Unless otherwise stated, means were compared using the nonparametric Wilcoxon matched pairs signed ranks test, with two-tailed significance level set as P > 0.05. Additional statistical details are stated in the appropriate figure legends.

Results
B. burgdorferi drives CD4- and CD8- T cells towards a tolerant and regulatory phenotype
After 72h stimulation of human PBMCs with B. burgdorferi, CD38, CD69 and HLA-DR expression on CD4 and CD8 T cells remained unchanged or were slightly activated, compared to the control medium (Figure 1A-C). C. albicans, used as positive control, activated the T cells, increasing HLA-DR, CD38 and CD69 expression. In contrast, the regulatory T cell markers PD-1 and CTLA-4 were upregulated on both CD4 and CD8 T cells exposed to B. burgdorferi (Figure 1D/E). IL-2 production by CD4 T cells incubated with B. burgdorferi and co-stimulated with CD3/CD28 beads was comparable to control levels, while C. albicans produced increased IL-2 levels (Figure 1F). B. burgdorferi showed no change in IL-2 production or significant differences in T cell activation markers, while a slight elevation was observed in PD-1 and CTLA4 expression.
Figure 1: Lack of T cell activation by *B. burgdorferi*. PBMCs were stimulated with *B. burgdorferi sensu lato* for 72h. (A) CD38 (Bb n=10, Ca n=3), (B) CD69 (Bb n=10, Ca n=6), (C) HLA-DR (Bb n=6, Ca n=6), (D) PD-1 (Bb n=6, Ca n=6) and (E) CTLA-4 (Bb n=7) expression was studied in CD4+ T cells and CD8+ T cells using flow cytometry. PBMCs from healthy volunteers and two independent experiments were stimulated with a medium control, a live mix of *B. burgdorferi sensu lato* at 10⁶/mL and heat-killed *C. albicans* 10⁶/mL. Data shown is mean ± SEM. The dotted lines represent the expression levels of the medium control, to which the stimulated expression levels are normalized for each time point. Data were normalized by dividing the geometric mean fluorescent intensity (MFI) of each condition to the MFI of the medium control per time point and demonstrated as the fold-change. Significance indications shown above the bars demonstrate whether there is a significant change compared to the medium control. (F) CD4 T cells were incubated with no beads or CD3/CD28 beads, in combination with a medium control, a live mix of *B. burgdorferi sensu lato* at 10⁶/mL and heat-killed *C. albicans* 10⁶/mL.

**Pre-treatment with IFN-γ restores antigen presentation machinery expression during *B. burgdorferi* infection**

Since we noted that *B. burgdorferi* exposure inhibits the expression of antigen presentation molecules, HLA-DR and CD74, resulting in suppression of pro-inflammatory T cell responses, we aimed to restore this expression and T cell responses during *B. burgdorferi* infection. PBMCs were pre-treated using different
combinations of cytokines that are well-known from literature to increase expression of antigen presentation proteins and/or enhance IFN-γ responses.

IFN-γ itself is an important inducer of antigen presentation. Therefore, PBMCs were incubated and treated with IFN-γ before and following, *B. burgdorferi* addition (Figure 2). 24 hours pre-incubation with IFN-γ enhanced CD74 (Figure 2A), HLA-DR (Figure 2B) and HLA-DM (Figure 2C) expression significantly. HLA-DM expression increased only after at least 48h of IFN-γ treatment and therefore showed a longer recovery period. When PBMCs were treated with IFN-γ after the cells were exposed to *B. burgdorferi* for 24h, HLA-DR and CD74 did not increase compared to the medium control but instead returned to normal levels of expression (Figure 2A, B). HLA-DM was less affected or showed a delayed response to IFN-γ stimulation. In summary, IFN-γ treatment before and after *B. burgdorferi* restored HLA-DR and CD74 to normal or high levels in human monocytes, while HLA-DM expression remained low.

**Exposure to IL-12 and IL-18 enhances the immune response of human PBMCs against *B. burgdorferi***

We explored strategies to restore IFN-γ production in Lyme borreliosis patients. To determine whether we could use additional stimuli to enhance the IFN-γ production during infection, PBMCs were stimulated with *B. burgdorferi* and various cytokine combinations at the same time. Previous studies have shown how IFN-γ may be produced in response to IL-12, IL-18 and type I IFN production \(^{24,25,26,27}\). Therefore, we exposed PBMCs to *B. burgdorferi* in the presence of IL-12 and/or IL-18 (Figure 3A and 4A). IL-12 treatment enhanced CD74 and HLA-DR expression significantly compared to the medium control. IL-18 treatment alone showed no significant changes in CD74 or HLA-DR expression. While IL-18 enhanced IL-12 treatment effects on HLA-DR expression, no significant increase was observed for CD74 (Figure 3A and 4A).

IL-18 gene expression was suppressed by *B. burgdorferi* in both PBMCs and monocytes after 24h of stimulation (Figure 3B). In contrast, IL-18 was induced upon IFN-γ stimulation. Of interest, IL-18 expression upon *B. burgdorferi* exposure was lower in the PBMC population than in monocytes alone. When PBMCs were stimulated with IL-18 alone there was no increase in intracellular CD74 expression on CD14 monocytes and a slight increase in extracellular expression. Combination of IL-12 and IL-18 partially restored monocyte CD74 expression, but compared to
IL-12 alone, the combination showed decreased expression. IL-18 may therefore not be a necessary addition.

**Figure 2: Pre-treatment with IFN-γ restores *B. burgdorferi* induced inhibition of antigen presentation molecules.** PBMCs from healthy volunteers were stimulated in two or three independent experiments. PBMCs were cultured with a medium control or *B. burgdorferi* and 1 μg/ml IFN-γ to restore CD74 expression levels to normal levels. Cells were treated with IFN-γ for 24h before or after 24h of *B. burgdorferi* exposure. (A) CD74, (B) HLA-DM, (C) HLA-DR expression was studied in the CD14+ monocyte population of PBMCs after a total of 48h of culture. The dotted line represents the expression level of the medium control, to which the stimulated expression levels were normalized for each time point. Data were normalized by dividing the geometric mean fluorescent intensity (MFI) of each condition to the MFI of the medium control per time point and demonstrated as the fold-change. Significance indications shown above the bars demonstrate whether there is a significant change compared to the medium control (RPMI for 48h). Statistical significance was accepted at p<0.05 and indicated as follows: * p<0.05, ** p<0.01 and *** p<0.001.

IL-7 and IL-12 enhance antigen presentation molecules on CD14 monocytes

Another well-known inducer of T cell activation is IL-7. PBMCs incubated with IL-7 and IL-12 incubated in addition to *B. burgdorferi* enhanced both intra- and extracellular expression of CD74 compared to *B. burgdorferi* only but did not restore the expression to the levels of the medium control (Figure 3C). HLA-DR expression did not change significantly in response to *B. burgdorferi* and IL-7 (Figure 4B).
Combined treatment of IL-12 and IL-7 during *B. burgdorferi* infection enhanced HLA-DR expression in some donors and significantly restored CD74 levels. In summary, IL-7 and IL-12 restored CD74 expression in CD14 monocytes during *B. burgdorferi* infection.

**IL-15 and IL-12 combination upregulate antigen presentation molecules and induce IFN-γ production during *B. burgdorferi* stimulation**

IL-15 is a key cytokine in NK cell activation and the production of antigen-specific terminally differentiated effector memory T cells \(^{29, 30}\). To explore its role in promotion of antigen presentation, PBMCs were stimulated with IL-12, IL-15 and *B. burgdorferi* (Figure 2D and 3C). IL-15 induced an increase in intra- and extracellular CD74 and HLA-DR expression, but could not restore *B. burgdorferi*-induced inhibition of CD74 or HLA-DR expression to normal levels. When IL-15 was combined with IL-12, a significant increase in antigen presentation proteins was observed (Figure 3D and 4C). Addition of *B. burgdorferi* spirochetes to IL-15 combined with IL-12 enhanced CD74 expression further, partially restoring *B. burgdorferi*-induced inhibition to the normal levels.

Increased HLA-DR and CD74 expression was similar, and therefore we studied IFN-γ production upon addition of IL-12 and IL-15 (Figure 3E). A significant increase in IFN-γ levels was observed when IL-12 and IL-15 were used in combination with *B. burgdorferi*.

**B. burgdorferi induces secretion of HLA-DR and CD74 inhibiting factors**

To determine the role of endo- and exogenous factors on *B. burgdorferi* induced inhibition of antigen presentation machinery expression in CD14 monocytes, supernatant from *B. burgdorferi* exposed PBMCs was added to *C. albicans* stimulated PBMCs. *C. albicans* exposed PBMCs usually show an increase or stable intra- and enhanced extracellular expression of HLA-DR and CD74. However, after 48h of incubation with *B. burgdorferi* conditioned medium CD14⁺ monocytes of the *C. albicans* exposed PBMC fraction showed a significant decrease in intracellular CD74 and HLA-DR expression.
Figure 3: Restoring *B. burgdorferi* induced inhibition of CD74. PBMCs from healthy volunteers were stimulated in two or three independent experiments. (A) PBMCs were stimulated with *B. burgdorferi sensu lato* and C. albicans for 24h, after 24h supernatant was transferred from *B. burgdorferi* stimulated PBMCs to C. albicans stimulated PBMCs and incubated for another 48h. CD74 expression was studied in the CD14+ monocyte population of PBMCs after a total of 72h of culture. (B, C, D, E, F) PBMCs were cultured with a medium control or *B. burgdorferi* and various stimuli: IL-12 10 ng/mL, IL-7 1 ng/mL, IL-15 10 ng/mL, IL-18 100ng/mL to try and restore CD74 expression levels to normal levels and PBMCs were also incubated with IL-10 and IL-10R antagonist to determine whether IL-10 production by *B. burgdorferi* may play a role in *B. burgdorferi*-induced inhibition of antigen presentation molecules. (G) Transcriptional data observed using quantitative PCR on IL-18 expression following 24h stimulation with either a medium control, *B. burgdorferi* or IFN-γ 1 ug/mL. (H) IL-12 and IL-15 enhanced IFN-γ production in early *B. burgdorferi*-infection. The dotted line represents the expression level of the medium control, to which the stimulated expression levels are normalized for each time point. Data were normalized by dividing the geometric mean fluorescent intensity (MFI) of each condition to the MFI of the medium control per time point and demonstrated as the fold-change. Significance indications shown above the bars demonstrate whether there is a significant change compared to *B. burgdorferi* alone. Statistical significance was accepted at p<0.05 and indicated as follows: * p<0.05, ** p<0.01.

IL-10R inhibition does not affect *B. burgdorferi*-induced antigen presentation molecule inhibition IL-10 is a well-known inhibitor of antigen presentation and is produced in relatively high amounts during *B. burgdorferi* infection. To evaluate whether it may play a role in *B. burgdorferi* induced inhibition of antigen presentation molecules, PBMCs were incubated with various concentrations of IL-10 in the presence of *B. burgdorferi*. IL-10 decreased both CD74 and HLA-DR expression on the cell surface but had no significant effect on intracellular CD74 expression, while HLA-DR expression decreased significantly. To assess whether *B. burgdorferi* induction of IL-10 may inhibit antigen presentation molecule expression on innate immune cells, the IL-10 signalling was blocked by pre-incubation with an anti-IL-10 receptor antibody. Upon treatment with this latter anti-IL-10R antibody, no change in HLA-DR or CD74 was observed compared to stimulation with *B. burgdorferi* alone (Figure 3F and 4F). Overall, *B. burgdorferi* induced IL-10 production alone did not inhibit antigen presentation molecules in CD14+ monocytes.
Figure 4: Restoring *B. burgdorferi* induced inhibition of HLA-DR. PBMCs from healthy volunteers were stimulated in two or three independent experiments. (A, B, C, E, F, G) PBMCs were cultured with a medium control or *B. burgdorferi* and various stimuli: 10ng/mL IL-12, 1 ng/mL IL-7, 10 ng/mL IL-15, 100 ng/mL IL-18 to try and restore HLA-DR expression levels to normal levels and PBMCs were also incubated with IL-10 and IL-10R antagonist to determine whether IL-10 production by *B. burgdorferi* may play a role in *B. burgdorferi* induced inhibition of HLA-DR. (D) PBMCs were stimulated with *B. burgdorferi sensu lato* and *C. albicans* for 24h, after 24h supernatant was transferred from *B. burgdorferi* stimulated PBMCs to *C. albicans* stimulated PBMCs and incubated for another 48h. HLA-DR expression was studied in the CD14+ monocyte population of PBMCs after a total of 72h of culture. The
dotted line represents the expression level of the medium control, to which the stimulated expression levels are normalized for each time point. Data were normalized by dividing the geometric mean fluorescent intensity (MFI) of each condition to the MFI of the medium control per time point and demonstrated as the fold-change. Significance indications shown above the bars demonstrate whether there is a significant change compared to B. burgdorferi alone. Statistical significance was accepted at p<0.05 and indicated as follows: * p<0.05, ** p<0.01, *** p<0.005.

**IL-12p70, IL-18 and IFNα are not produced during in vitro exposure to B. burgdorferi**

IFN-γ may be induced through various signalling pathways. Previous studies have shown how different combinations of IL-12p70, IL-18 and IFNα signalling may induce IFN-γ transcription and production. To study whether these cytokines are induced during Lyme borreliosis, PBMCs were incubated with B. burgdorferi for 24h to 7 days. Over this period, IL-12p70 was not produced, showing concentrations below 1.5 pg/mL comparable to the medium control (Figure 5A). IL-18 was produced at low levels (<60 pg/mL) in the first 24h, compared to 25 pg/mL in the medium control. During following timepoints, IL-18 production remained relatively stable, below 40 pg/mL (Figure 5B). IFNα production showed no differences between B. burgdorferi exposed cells and incubation with a medium control at all timepoints with levels of < 2 pg/mL (Figure 5C). IFN-γ was not produced at any of the early timepoints, but it was induced in several donors after 7 days of incubation, showing production levels up to 400 pg/mL. In summary, IL-12p70, IL-18 and IFNα were not produced or secreted in limited amounts in response to B. burgdorferi. IFN-γ was only produced after seven days in part of the donors, suggesting that induction of IFN-γ transcription is limited during B. burgdorferi infection.
Figure 5: Role of IL-12p70, IL-18 and IFNα in IFN-γ production. PBMCs were stimulated with a medium control or *B. burgdorferi* for 24 (n=7), 48 (n=7) 72 hours (n=8) and 7 days (n=6) following which supernatant was collected. Supernatant was analysed using the ELLA for concentrations of: (A) IL-12p70, (B) IL-18, (C) IFNα and (D) IFN-γ.

Low concentrations of IL-12p70, IL-18, IFNα and IFN-γ in sera from patients with erythema migrans

To determine whether our *in vitro* *B. burgdorferi* responses differ from the *in vivo* situation, we studied serum samples collected from patients with EM and healthy controls. No significant IFN-γ circulating concentrations were observed, though in some patient sera a slight enhancement was measured (Figure 6A). IL-12p70 release was not observed in patients or healthy controls (Figure 6B). In contrast, IL-18 was consistently present at around 150pg/mL in both healthy control and EM patient sera (Figure 6C). IFNα was not detected in either patients or controls (Figure 6D). In conclusion, serum IL-12p70, IL-18, IFNα and IFN-γ concentrations did not differ between patients with EM and healthy controls.
Figure 6: IL-12p70, IL-18, IFNα and IFN-γ concentrations in sera from patients with erythema migrans and healthy control. Patient and healthy control serum was analysed using the ELLA for concentrations of: (A) IFN-γ (B) IL-12p70, (C) IL-18 and (D) IFNα. n.s. indicates there is no significant change in concentration between patients and healthy controls.
Discussion

In this study, we explored the stimulation of T cells by *B. burgdorferi* and novel ways of restoring *B. burgdorferi*-inhibited immune function. First, the activation status of T cells was explored. While activation markers remained low, the immune tolerant marker CTLA-4 was upregulated. Upon *B. burgdorferi* s.l. exposure, IFN-γ production did not increase neither in *in-vitro* assays nor in *in-vivo* in patients with EM compared to healthy controls. Therefore, we assessed novel strategies to re-establish immune responses during *B. burgdorferi* s.l. infection by exposing PBMCs to *B. burgdorferi* in combination with various cytokines.

First, we explored the possibility of adding IFN-γ to PBMCs before or after exposure to *B. burgdorferi*. IFN-γ was successful in restoring the expression of antigen presentation proteins during *B. burgdorferi* infection (Figure 2), especially when cells were pre-incubated with IFN-γ. Second, since IFN-γ production is also a potential read out strategy for diagnostic methods, such as IGRA, the role of other cytokines that may induce IFN-γ during *B. burgdorferi* infection was considered.

IL-12 is composed of two disulfide-linked subunits encoded by two separate genes, whose translation results in two proteins with a mass of 35 and 40 kDa respectively. These proteins in turn form a heterodimeric protein of 70kDa. IL-12 is normally produced by macrophages, monocytes, dendritic cells (DCs) and B lymphocytes. It is also a key regulator for directing naïve CD4 T cell differentiation towards a Th1 phenotype and mediates its function by binding to the IL-12 receptor β1 and β2 chains. Of interest, IL-12Rβ2 chains are known to be induced via IL-12 and antigen stimulation. Whereas IL-12 initiates the Th1 phenotype, IFN-γ is necessary for completion and stabilization of Th1 polarization. Thus, induction of IL-12 may promote a long-term and stable Th1 response. In our studies, IL-12 restored immune function to some extent, but antigen presentation machinery expression and IFN-γ production were not re-established. Therefore, combined treatments of IL-12 with other cytokines were evaluated (Figure 3 and 4).

IL-12 is widely known to cooperate and synergize the pro-inflammatory effects of other cytokines. Originally IL-18 is described as an IFN-γ-inducing factor, secreted by activated macrophages. Here, IL-12 and IL-18 could restore HLA-DR and IFN-γ production (Figure 3), but not CD74 expression (Figure 2). Of interest, addition of IL-18 during *B. burgdorferi* exposure already induced low IFN-γ levels. Next to IFN-
IL-18 activates Th1 proliferation and production of IL-2. IL-2 was not produced in response to B. burgdorferi (Figure 1). IL-12 and IL-18 worked synergistically for the development of T cells into IFN-γ-producing cells, as we observed in this study, possibly without the necessity of TCR engagement.

Human monocytes have previously been shown to produce low IL-18 levels in response to live B. burgdorferi spirochetes at a high MOI. IL-18 is produced via activation of pro-IL-18, constitutively expressed in resting monocytes and macrophages, by active caspase 1. However, here we show that in response to B. burgdorferi alone, no IL-18 was produced, both in in vitro and in EM patients (Figure 4 and 6). Moreover, IL-18 mRNA expression was reduced in B. burgdorferi exposed PBMCs and monocytes. Of interest, this inhibition was stronger in PBMCs than in B. burgdorferi exposed monocytes (Figure 3B), suggesting specific cell types are more affected by this inhibition or may enhance suppression of IL-18 in other cell types. In contrast to IL-18, IL-10 is significantly produced during B. burgdorferi infection, yet it played no significant role in B. burgdorferi-induced inhibition of antigen presentation machinery expression.

IL-18 is also part of the IL-1 family of cytokines, therefore, it may not be surprising that also IL-1β may act synergistically with IL-12 to induce IFN-γ production in T cells. In this study we observed that exposure to IL-18 alone could induce IFN-γ production. In contrast, a previous study showed IL-1β by itself did not induce T cell IFN-γ production. Similar to our previous studies, where the presence of B. burgdorferi-induced IL-1β alone is not enough to trigger IFN-γ secretion. Therefore, in the absence of IL-18, IL-1β combined with IL-12 may induce IFN-γ production. Another study in NK- and T-cells demonstrated how either IFNα or IL-12, combined with IL-18, upregulated IFN-γ expression. In contrast to IFNα, IL-12 elevated these gene expression levels for an extended time period. Whereas STAT4 activation was transient in cells stimulated with IFNα, IL-12 induced long-lasting activation of this transcription factor. IL-12, therefore, may result in more efficient IFN-γ production than other cytokines.

IL-12 and IL-18 synergy, with regards to IFN-γ induction, coactivates STAT4, upregulating IFN-γ promoter activation. Of interest, previous data demonstrated how STAT4 expression was inhibited by B. burgdorferi, while STAT3 activation was significantly increased, whereas STAT4 was not phosphorylated. Therefore, early
STAT4 activation may an important factor in the immune response against *B. burgdorferi* infection in humans.

IL-15 is normally produced by macrophages, DCs, keratinocytes and epithelial cells \(^{48,49}\), but not lymphocytes \(^{50}\). It shares its activity with IL-2 that is produced by T cells and engages with the same receptors \(^{50,51}\). Dissimilar to IL-2 function, IL-15 can induce its pleiotropic effects on multiple cell types, including those of nonlymphoid origin \(^{50}\). T cell proliferation and cytokine responses are induced upon IL-15 binding \(^{51,52,53,54}\) and promoting CTL memory \(^{55,56}\). IL-15 also induces B cell proliferation and isotype switching \(^{57}\). IL-12 and IL-15 have been known to synergize an IFN-γ production in murine NK cells \(^{58}\). The cytokine is also associated with the development of Th1 responses \(^{59}\). In this study, by addition of IL-15 IFN-γ production was induced, both in the presence and absence of IL-12 (Figure 3E). Moreover, it enhanced CD74 and HLA-DR expression in *B. burgdorferi* exposed PBMCs (Figure 3D and 4D). Of high interest, a previous study showed how IL-15 induced STAT1, STAT3, STAT4 and STAT5 binding to the IFN-γ regulatory site \(^{26}\). Similar to our findings, previous studies demonstrated how IL-15 production by monocytes enhanced IFN-γ secretion by NK cells through the IL-2 receptor and in synergy with IL-12 \(^{60,61,62}\). IL-15 is commonly present in MHCII positive cells. However, so far, no literature has demonstrated its role in enhancing MHCII expression \(^{63}\). A recent study showed that in IL-15 deficient mice and in IL-2R beta deficient mice but not in IL-2 deficient mice there was a significant decrease in IL-12 production and upregulation of MHCII and CD40 was impaired \(^{64}\). Here, we show that IL-15 alone can indeed enhance MHCII expression in CD14 monocytes (Figure 3D and 4D). Of interest, IL-15Ra and IL-152Ra also co-localize with MHCII \(^{65}\).

It is well-known that IL-7 is an important cytokine in T cell proliferation and activation and promotes DC-T cell interactions. By itself, IL-7 alone is not able to induce IFN-γ or IL-4 production in human immune cells. However, IL-7 may synergize the effect of other (co-stimulatory) molecules such as CD3 or CD28 \(^{28}\). Here, we demonstrated that IL-7 alone enhanced CD74 expression significantly and HLA-DR expression showed an increasing trend. In combination with IL-12, IL-7 can enhance these levels further. IL-7 remains a crucial regulator for naïve T cell proliferation, especially in the periphery, decreasing the threshold for activation with low-affinity antigens \(^{66}\). Therefore, IL-7 may enhance T cell responses against *B. burgdorferi* as its presence increased IFN-γ responses (Figure 6). IL-7 and IL-12
have also previously been used as a combined therapy for intra-tumoral injection in mice. The treated mice showed more immune cell activation and anti-tumour activity, especially when combined with anti-PD-1 or anti–CTLA4 antibodies⁶⁷.

In conclusion, we observed an absence of IFN-γ production, inhibition of CD74 and HLA-DR expression, and suppression of T cell responses upon *B. burgdorferi* exposure. This may result in an immune tolerant environment during infection. Addition of IL-12, in combination with other cytokines, could largely restore CD74 and HLA-DR expression and IFN-γ production by PBMCs during *B. burgdorferi* exposure *in vitro*, thereby enhancing the chance of clearance of the bacterium from the human host. This knowledge improves our understanding of the host immune response during *B. burgdorferi* infection. Whether other soluble mediators produced after *B. burgdorferi* recognition or cell-cell interactions are responsible for the initial inhibition of the immune response against *B. burgdorferi* remains to be investigated in future studies.
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Chapter 8

*Borrelia burgdorferi* inhibits NADPH-mediated reactive oxygen species production through the mTOR pathway

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Abstract

Redox metabolism is crucial in host defense. Previously, it was shown that *Borrelia burgdorferi* induces the antioxidative metabolism in primary human monocytes. In this study, we explore how *B. burgdorferi* affects the anti-oxidative arm of redox metabolism, i.e. the generation of reactive oxygen species (ROS). Peripheral blood mononuclear cells (PBMCs) were exposed to *B. burgdorferi* and generation of ROS was determined both after acute stimulation and after re-stimulation with a secondary stimulus. Though the spirochete induces very low levels of ROS itself, it dramatically decreases the long-term capacity of PBMCs to generate ROS in response to serum-opsonized zymosan (SOZ). This was followed by a compensatory overshoot in ROS generation at later time points. The PI3K/Akt pathway and intracellular levels of methionine play an important regulatory role in this process. Dysregulation of oxidative metabolism may be a novel mechanism by which the spirochete modulates the human immune system and evades killing.
Introduction

*Borrelia burgdorferi* is the causative agent of Lyme disease and the most common vector borne disease in the northern hemisphere (Bacon et al., 2008; Rizzoli et al., 2011). The first symptom of an infection with *B. burgdorferi* is most often a migrating skin rash called erythema migrans (EM). If left untreated, the infection gives rise to more severe inflammatory complications in the joints (Weinstein and Britchko, 2002), skin (Steere, 1989), heart (Sigal, 1995), or central nervous system (Pachner and Steiner, 2007). Although the bacterium is considered very susceptible to antibiotics, a small percentage of patients experience persisting symptoms after extensive treatment (Berende et al., 2016; Cairns and Godwin, 2005). It is thought that this may not be due to persistent infection or toxic factors released by *B. burgdorferi* (Barbour and Hayes, 1986), but due to aberrant inflammatory responses (Berende et al., 2016; Carlson et al., 1999; Steere et al., 2006).

Our group has previously shown the role of glucose (Oosting et al., 2016) and glutathione metabolism (Kerstholt et al., 2018) in the host inflammatory response against *B. burgdorferi*. Kerstholt et al. (2018) showed that *B. burgdorferi* exposure lowered the capacity to generate reactive oxygen species (ROS). Low ROS production was hypothesized to be mediated by altered glutathione metabolism. Of interest, inhibiting glutathione metabolism was unable to restore ROS generation, indicating the involvement of a glutathione-independent mechanism.

The generation of ROS is an important defence mechanism against pathogens. But superfluous ROS generation can lead to significant damage to the host itself. In addition, ROS are increasingly recognized as important signalling molecules in the inflammatory response. Neutrophils are generally the first among the infiltrating responders and primary ROS producers during infection (Antonara et al., 2010). However, previous studies have shown that *B. burgdorferi* (Hartiala et al., 2008; Xu et al., 2007) and tick saliva (Menten-Dedoyart et al., 2012) inhibit neutrophil function, affecting a main source of ROS production during infection. Moreover, complement and specific antibodies can be important in *B. burgdorferi*-induction of oxidative bursts. Yet, complement activation is also severely suppressed by *B. burgdorferi* and tick saliva (Suhonen et al., 2000). Therefore, it is central to investigate the impact of *B. burgdorferi* on the release of ROS by immune cells.
In the present study, we explored how *Borrelia burgdorferi* modulates the generation of reactive oxygen species by human peripheral blood mononuclear cells (PBMCs) and how this may affect the inflammatory response.

**Methods**

**Peripheral blood mononuclear cells (PBMCs) and monocytes**

PBMCs were isolated from buffy coats from healthy volunteers obtained from Sanquin blood bank (Nijmegen, the Netherlands) after informed consent. Briefly, blood was diluted with sterile PBS (1:1) and a density centrifugation was applied over Ficoll-Paque (Pharmacia Biotech). Next, the interphase containing PBMCs was collected, washed with ice-cold PBS and cells were resuspended in medium (RPMI 1640, w/o glucose, w/o glutamine; MP Biomedicals, Illkirch Cedex, France) supplemented with 5.5mM D-glucose (Sigma-Aldrich, Zwijndrecht, the Netherlands), 0.2mM glutamine (glutaMAX™; Thermo Fisher Scientific, Breda, the Netherlands) and 0.1mM pyruvate (Sodium pyruvate; Thermo Fisher Scientific).

For experiments using monocytes, PBMCs were further subjected to a density centrifugation using the hyper-osmotic density medium Percoll® (Sigma). The interphase was collected and washed with ice cold PBS, after which cells were resuspended in RPMI and counted. Monocytes were left to adhere for 1 h before washing away any remaining non-adherent cells.

**Culture of Borrelia burgdorferi spirochetes**

*B. burgdorferi*, ATCC strain 35210 (ATCC, Wesel, Germany) was cultured at 24°C in Barbour-Stoenner-Kelley (BSK)-H medium (Sigma-Aldrich) supplemented with 6% rabbit serum until spirochete growth commenced. Cells were then grown at 34°C to late logarithmic phase, at which point the spirochetes were checked for motility by dark-field microscopy and harvested. Spirochetes were quantified using a Petroff-Hauser counting chamber, washed with PBS and stored at -80°C. For experiments using serum-opsonized *B. burgdorferi*, spirochetes were incubated for 30 min at 37°C in the presence of pooled human serum after which cells were washed twice with PBS to remove unbound material.

**Stimulation experiments**

For measurements of cytokines and metabolic parameters in PBMCs, cells were seeded in duplicate in round-bottom 96-wells plates (5×10⁵ cells/well). Cells were
pretreated with one of the following inhibitors: 5′-Deoxy-5′-(methylthio)adenosine (MTA, Sigma-Aldrich); 3-Deazaneplanocin A (DZNep, Cayman Chemical, Michigan, USA); 5-Aza-2′-deoxycytidine (Sigma-Aldrich); 2′,3′-dideoxyadenosine (ddAdo, Sigma-Aldrich); P1,P5-Di(adenosine-5′) pentaphosphate pentasodium salt (PPdPP, Sigma-Aldrich); Wortmannin (Invivogen, Toulouse, France), Rapamycin (LC Laboratories, Woburn, MA, USA), Torin1 (Tocris, Abingdon, United Kingdom) or vehicle control i.e. RPMI or dimethyl sulfoxide (DMSO, WAK-Chemie Medical GmbH, Steinbach, Germany) for 1 h and then stimulated with *B. burgdorferi* s.s. or a mix of *B. burgdorferi* s.s., *B. afzelii* and *B. garinii* for 24 h at a multiplicity of infection (MOI) of 0.2. *B. burgdorferi* s.l. was prepared by mixing equal amounts of *B. burgdorferi* s.s., *B. afzelii*, and *B. garinii*.

**Measurement of reactive oxygen species (ROS)**

Generation of reactive oxygen species by PBMCs was measured using a luminol-based chemiluminescent assay. For direct *B. burgdorferi* stimulation, PBMCs were seeded in white 96-well plate (5x10⁵ cells/well) in the presence of 10 mM luminol. Cells were stimulated with different concentrations of *B. burgdorferi* or serum-opsonized zymosan as described and immediately transferred to a plate reader where ROS-induced luminescence was measured continuously for up to 4 h. For indirect *B. burgdorferi* stimulations, cells were first stimulated and treated as described above. After incubation (24 h, 48 h or 72 h), cells were collected and replated in six-fold to a white 96-well plate (1x10⁵ cells/well). ROS production was induced in four wells by the addition of 3 mg/ml serum-opsonized zymosan in HBSS, the two remaining wells served as controls. Luminol (10 mM) was added to all wells, which is oxidized by ROS to produce the luminescent intermediate luminophore. Luminescence, correlating to total ROS production (both intra- and extracellular) was measured at 425 nM continuously for 1 h.

**Candida albicans killing assay**

To determine the killing capacity of monocytes against pathogens after exposure to *B. burgdorferi*, Percoll-isolated monocytes were seeded in 96-wells flat bottom plates (1x10⁵ cells/well) and left to adhere for 1 h. After 1 h, non-adherent cells were washed away with warm PBS and cells were exposed to *B. burgdorferi* or RPMI for 24 h. After 24 h incubation at 37°C, the wells were again washed to remove the stimuli and the cells were exposed for 24 h to 1x10⁶/ml *Candida albicans*, ATCC strain UC820 (ATTC). After 24 h incubation, plates were spun down and cells were
lysed in sterile water. The lysate was collected and serial diluted and subsequently plated onto BD™ Sabouraud Glucose Agar plates (Becton Dickinson, Vianen, the Netherlands). Agar plates were incubated for 24 h at 37°C, after which the CFU were counted.

Western blots
After stimulation, 5x10^6 PBMCs were lysed in 100 μL lysis buffer (50 mM Tris, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 10% glycerol, 1% Triton X-100, 40 mM α-glycerophosphate, 50 mM sodium fluoride, 200 μM sodium vanadate, 10 μg/mL leupeptin, 423 10 μg/mL aprotinin, 1 μM pepstatin A, and 1 mM phenylmethylsulfonyl fluoride) and frozen at -80°C until assayed. After thawing, the lysates were centrifuged (10 min, 14000 rpm, 4°C) to eliminate cell debris and the supernatants were used for Western blot analysis. Equal amounts of protein were loaded on 4-15% polyacrylamide gels (Bio-Rad). Separated proteins were transferred to Nitrocellulose (Bio-Rad) membranes. The membrane was blocked for 1 h with 5% (w/v) BSA (bovine serum albumin, Sigma) in TBS-Tween buffer (TBS-T) and then incubated overnight with a primary antibody (phospho-435 Akt, Cell Signaling; phospho-4EBP1, Cell Signaling or rabbit anti-actin, Sigma) at a dilution of 1:1000 in blocking buffer (TBS-T with 5% BSA). After overnight incubation, blots were washed in TBS-T three times and incubated with HRP-conjugated anti-rabbit antibody (1:5000; Sigma) in 5% (w/v) milk in TBS-T for 1 h. After washing the blots, they were developed with ECL (Bio-Rad) according to the manufacturer’s instructions.

Quantitative Polymerase Chain Reaction
PBMCs and monocytes were incubated in TRIzol (ThermoFisher). RNA was isolated according to the manufacturer’s instructions. RNA purity was assessed and reverse transcribed into cDNA using an Iscript cDNA synthesis kit (Biorad). Quantitive PCR analysis was performed using Power Sybr Green PCR Master Mix (Applied Biosystems) and a 7300 Real-time PCR system (Applied Biosystems). Primers used are:

- **B2M** (Housekeeping gene) FW: TGACCGTGATCTTTCTGGTG; B2M (Housekeeping gene) RV: ATTTGAGGTGGGTGGAACTG
- **mTOR** FW: TCCGAGAGATGAGTCAAGAGG; mTOR RV: CACCTTCACCTCTATGAGGC
- **S6** FW: AGAACTTCTGGCTCGAAAGGT; S6 RV: CGACAGGTGTCTGACGTGTA.
Results

Figure 1. *B. burgdorferi* exposure does not induce high levels of ROS directly but modulates ROS production in response to a secondary stimulus.  
(A) PBMCs (n=3 different donors, measured in triplicate) were stimulated with increasing concentrations of *B. burgdorferi* (*B. burgdorferi* 10⁵, 10⁶ or 10⁷ sp/ml), serum-opsonized zymosan (SOZ) or medium control (RPMI) in the presence of luminol. Directly after addition of the stimuli, luminescence was measured continuously for 200 min to determine the kinetics of ROS generation.  
(B) PBMCs were stimulated with *B. burgdorferi*, serum-opsonized *B. burgdorferi* (Bb_ops) or serum-opsonized zymosan in the presence of luminol and luminescence was measured for 1 h. Data represent mean ± SEM area under the curve (AUC) of n=3 different donors measured in triplicate.  
(C) PBMCs were stimulated with increasing concentrations of *B. burgdorferi* (*B. burgdorferi* 10⁵, 10⁶ or 10⁷ sp/ml) for 24 h, 48 h and 72 h. After stimulation, cells were harvested, and ROS generation was induced by serum-opsonized zymosan in the presence of luminol. Data represent mean ± SEM area under the curve (AUC) of n=3 different donors measured in triplicate.  
(D) Remaining C. albicans (C.A.) CFU after 24 h of stimulation of primary monocytes previously exposed to *B. burgdorferi* or RPMI.
**B. burgdorferi** are poor inducers of ROS production by human PBMCs

To determine how exposure to *B. burgdorferi* affects the generation of ROS in PBMCs, we first analyzed the ROS generation by PBMCs in response to *B. burgdorferi* stimulation. As shown in Figure 1a, stimulation with *B. burgdorferi* induced a dose-dependent increase in ROS within 3 hours. However, even at the highest (supraphysiological) MOI of 20:1, *B. burgdorferi*-induced ROS amounts remained much lower than those induced by serum-opsonized zymosan (SOZ), a yeast cell-wall component known to be a potent ROS activator. This indicates that direct stimulation with *B. burgdorferi* induces relatively small amounts of ROS which are far below the maximum capacity of the cell. To boost *B. burgdorferi*-induced ROS generation, we performed serum-opsonization of the spirochetes prior to stimulation. However, as shown in Figure 1b, opsonization of spirochetes only marginally increased ROS generation above non-opsonized *B. burgdorferi*.

As we noted that *B. burgdorferi* induced ROS generation is limited, we investigated the effects of *B. burgdorferi* exposure on ROS production by other, unrelated, microorganisms. PBMCs were first exposed to *B. burgdorferi* for up to 72 h and the capacity to generate ROS was determined by exposing the cells to SOZ. As shown in Figure 1c, exposure to *B. burgdorferi* for 24 h, dose-dependently decreased the capacity of PBMCs to generate ROS, confirming previously reported results (Kerstholt et al., 2018). Interestingly, after 48 h exposure to *B. burgdorferi*, the balance appears to shift and PBMCs exposed to the lowest dose of *B. burgdorferi* actually produced more ROS than unexposed cells (Figure 1c). Nevertheless, cells exposed to the higher dose of *B. burgdorferi* still show a clear defect in ROS generation. After 72 h, both cells exposed to the low- and the intermediate dose of *B. burgdorferi* produced more ROS than unexposed cells and only the high dose of *B. burgdorferi* was able to suppress ROS generation. These data indicate that *B. burgdorferi* exposure may lead to a temporary defect in ROS generation, followed by a compensatory increase in ROS production after longer exposure. The duration of the defect appears to be dependent on the dose of *B. burgdorferi*, with high dose *B. burgdorferi* showing longer-lasting defects that low-dose *B. burgdorferi*.

As the generation of ROS is an important antimicrobial defense mechanism, we investigated whether *B. burgdorferi*-induced suppression of ROS production interferes with microbial killing. Therefore, primary monocytes, the main ROS producers in the PBMC fraction, were first exposed for 24 h to *B. burgdorferi* and
then stimulated with the commensal fungus *Candida albicans*. After 24 h incubation, we determined the amount of remaining *C. albicans* colony forming units (CFU) to measure the killing capacity of the monocytes. As seen from Figure 1d, significantly higher CFU counts were obtained from cells that had been previously exposed to *B. burgdorferi*, compared to cells that had received mock treatment. This indicates that *B. burgdorferi* exposure negatively affects the killing capacity of primary human monocytes.

Taken together, these data indicate that, even though *B. burgdorferi* induces very low levels of ROS immediately after exposure, it induces a prolonged state of tolerance, with effects on the ROS generation in response to a secondary stimulus. Subsequently, these effects hamper pathogen killing in a secondary infection; on the other hand, the overshoot in ROS generation at later time point may be an additional source of tissue damage and inflammation.

**MTA effectively restores *B. burgdorferi*-induced defect in ROS generation by inhibiting the Akt-mTOR pathway**

Having found that *B. burgdorferi* induces long-lasting changes in ROS generation which may have profound consequences for disease development, we set out to elucidate the mechanism behind this. Previously, we have shown that stimulation with *B. burgdorferi* induces a dramatic increase in intracellular levels of glutathione, a potent antioxidant. Therefore, we hypothesized that increased levels of glutathione could be a causative factor for the defect in ROS generation. However, we observed that depleting glutathione was unable to restore ROS generation (Kerstholt et al., 2018). As part of these efforts, we used 5′-Deoxy-5′-(methylthio)adenosine (MTA), an inhibitor of S-Adenosylhomocysteine hydrolase (SAHH), one of the naturally occurring enzymes involved in glutathione biosynthesis as an alternative to direct glutathione depletion. Medium controls with treatments are shown in Figure S1. Surprisingly, pretreatment with MTA completely prevented the *B. burgdorferi*-induced defect in ROS generation (Figure 2a). As this was contradictory to the data with direct glutathione-inhibition, we examined whether this effect was due to SAHH-inhibition or due to some other effect by MTA.
MTA relieves the inhibitory effect of *B. burgdorferi* on ROS production, partly mediated by inhibiting the Akt/mTOR pathway. A-F. PBMCs were stimulated for 24 h with *B. burgdorferi*, in the presence of (A) different concentrations of methylthioadenosine (MTA, n=12), (B) 3-deazaneplanocin (DZNep, n=3), (C) 5-azadeoxycytidine (Aza-cyt, n=6), (D) MTA in combination with inhibitors of adenosine signaling (n=3), (E) wortmannin (n=10) or (F) inhibitors of mTOR signaling (n=3). After incubation, cells were harvested, and ROS generation was induced by serum-opsonized zymosan (SOZ). Data represent AUC of ROS-induced luminescence, measured for 1 h, of *n* different donors assayed in triplicate. G. Western blot of phospho-4E-BP1 and Actin expression in PBMCs after 2 h stimulation with *B. burgdorferi* in the presence or absence of MTA.

First, we made use of another, more specific SAHH-inhibitor, 3-deazaneplanocin. As shown in Figure 2b, this inhibitor had no effect on ROS generation at all, indicating that the effect of MTA is not due to its effect on glutathione metabolism. MTA is also reported to be an adenosine analogue and thereby activates adenosine signaling, therefore we combined MTA treatment with inhibitors of adenosine signaling. As seen from Figure 2b, inhibition of adenosine signaling did not affect the MTA-mediated protection. Next, we investigated whether the effect of MTA could be mediated by epigenetic mechanisms, as MTA is also known as a potent inhibitor of DNA methylation. Therefore, we made use of another inhibitor of DNA-methylation, 5-Aza-2′-deoxycytidine. However, again no effect of this inhibitor on ROS generation was observed (Figure 2c). Finally, a recent paper reported the Akt pathway as a target of MTA (Henrich et al., 2016). As we have previously shown upregulation of the Akt-mTOR pathway in human PBMCs after *B. burgdorferi* exposure (Oosting et al., 2016), we investigated whether we could replicate the effect of MTA by using the Akt-inhibitor wortmannin or the mTOR inhibitors torin1 and rapamycin. As shown in Figure 2d, both Akt- and mTOR inhibition partly alleviated the *B. burgdorferi*-induced decrease in ROS-generation. To confirm the link between MTA and the Akt-mTOR pathway, we analyzed protein expression of phospho-4eBP1, a downstream target of mTOR. Confirming our previous study, we saw upregulation of both p-Akt and p-4eBP1 after stimulation with *Borrelia* and substantial downregulation of both proteins in the presence of MTA. This indicates that Akt activation may indeed play a role in the *Borrelia*-induced downregulation of ROS generation.
mTOR inhibition is specific per cell population

mTOR may be differentially regulated between cell types, especially within the PBMC population. Therefore, we assessed mTOR expression in PBMCs and monocytes. mTOR expression was determined after 24h stimulation with *B. burgdorferi* and compared to culture medium control. *B. burgdorferi* enhanced mTOR expression in PBMCs, while reducing expression in monocytes (Figure 3a). S6 kinase is a signaling molecule directly regulated by mTOR. Therefore, we evaluated whether not just mTOR but also its downstream cascades were affected by *B. burgdorferi* stimulation. Compared to the medium control, *B. burgdorferi* reduced S6 kinase expression in monocytes, while remaining stable in stimulated PBMCs (Figure 3b).

Role of L-methionine

As Akt-inhibition only partially restored ROS-generation, we examined the metabolites in serum of acute patients with erythema migrans. Were we observed decreased levels of L-methionine in patients compared to healthy controls, as well as patients with other bacterial infections (Figure 4a). *B. burgdorferi* lacks the metabolic enzymes to resynthesize methionine itself (von Lackum et al., 2006) and is dependent on the uptake of methionine from the host. This may affect the supply
of L-methionine to host cells. Therefore, we investigated if *Borrelia*-induced methionine deficiency may induce decreased ROS-generation. Increasing levels of L-methionine were simultaneously added to PBMCs with *B. burgdorferi* and ROS generation was analyzed after 24 h. Methionine supplementation dose-dependently increased ROS-generation, though it could not completely restore it to baseline values (Figure 4b). The doses of L-methionine used were supraphysiological, and therefore L-methionine deficiency does not fully explain *B. burgdorferi*-induced down regulation of ROS production.

L-methionine and MTA are both part of the methionine salvage pathway (Figure 4c). Indeed, it has been shown that addition of MTA increased methionine levels (Backlund Jr and Smith, 1981). There are two different mechanisms by which MTA might restore ROS-generation, hence, we tested whether combining these strategies would completely mimic the effect of MTA. The combination of wortmannin-treatment with L-methionine supplementation increased ROS regeneration to concentrations above the MTA-treated cells (Figure 4d). The two strategies had a higher combined effect than both effects individually.

**Discussion**

In this study, we describe how the Lyme borreliosis-causing pathogen *B. burgdorferi* modulates the capacity of PBMCs to generate ROS, without inducing substantial amounts of ROS itself. By chance, we observed that the *B. burgdorferi*-induced defect in ROS generation can be restored by pretreating the cells with MTA and that this is mediated by effects on the Akt/mTOR pathway and L-methionine concentrations.

ROS generation in response to an infection is one of the most important antimicrobial defense mechanisms but it is also associated with collateral damage to surrounding structures. Proper control of the amount of ROS generated is therefore crucial for the proper resolution of infection. Our findings indicate that *B. burgdorferi* is not a strong inducer of ROS.
Figure 4. Restoring L-methionine levels rescues the defect in ROS generation, possibly through interaction with the MTA pathway. A. Serum levels of L-methionine were measured in healthy controls (HC), patients with erythema migrans (EM) and patients with Gram-positive (Gpos) or Gram-negative (Gneg) sepsis. Data was obtained from previously published study (Kerstholt et al., 2018), publicly accessible through the Metabolights database (accession code: MTBLS625). B. ROS induction by serum-opsonized zymosan in PBMCs stimulated for 24 h with RPMI or B. burgdorferi, with or without L-methionine supplementation. C. Schematic representation of how B. burgdorferi affects the methionine pathway. D. ROS induction by serum-opsonized zymosan in PBMCs stimulated for 24 h with RPMI or B. burgdorferi with or without L-methionine supplementation combined with Akt-inhibition in low concentrations (methionine 1 mM, 0.1 µM wortmannin) or high concentrations (methionine 10 mM, 1 µM wortmannin).

This is in line with previous studies, suggesting that the spirochete actively prevents ROS-mediated damage to enhance its chances of survival (Boylan et al., 2008). The generation of ROS might not be an important defense mechanism against B. burgdorferi itself: indeed, patients with chronic granulomatous disease that have defective ROS production are not more susceptible to Lyme disease. Nevertheless,
it is noteworthy that despite to low levels of direct ROS induction, exposure to \textit{B. burgdorferi} shuts down ROS generation in response to a subsequent stimulation with a potent stimulus. This is accompanied by a decreased antimicrobial killing capacity. These data are in accordance with previous findings, indicating that exposure to \textit{B. burgdorferi} leads to a long-term decrease in the inflammatory response, i.e. cytokine production, in response to secondary stimulation (Brouwer et al., 2021; Buffen et al., 2016; Elsner et al., 2015; Hastey et al., 2012; Oosting et al., 2016; Sellati and Barberio, 2020). This well-known phenomenon is called innate immune tolerance (Seeley and Ghosh, 2017). \textit{In vivo}, this may render patients temporary more susceptible to infection.

However, we also noted that this temporary effect is followed by a compensatory increased production of ROS. This mechanism may explain some observations in previous studies, such as an increased oxidative state observed in serum of patients with erythema migrans (Pancewicz S.A. et al., 2001) and a suggested role for oxidized lipids in the pathogenesis of disseminated Lyme borreliosis (Luczaj et al., 2011; Moniuszko-Malinowska et al., 2016).

We observed that treatment with MTA restores the \textit{B. burgdorferi}-induced defect in ROS generation. Subsequently, we ruled out that the effects of MTA were mediated by effects on the glutathione pathway, adenosine signaling pathway or DNA methylation. Instead, the effects of MTA could be partially mimicked by Akt/mTOR inhibition. Indeed, MTA functions as an inhibitor of mTOR signaling. Activation of Akt after \textit{B. burgdorferi} stimulation has previously been shown in multiple studies. In addition, it has been shown that Akt is essential for phagocytosis of \textit{B. burgdorferi} (Shin et al., 2009).

As Akt-inhibition only partly restored ROS-generation, we investigated additional mechanisms. Supplementation of L-methionine was able to partially restore the \textit{B. burgdorferi}-induced defect in ROS generation. There have been several previous studies linking methionine levels directly to ROS production (Caro et al., 2008; Sanz et al., 2006). In our study, circulating levels of L-methionine were also remarkably low in patients with erythema migrans compared to both healthy controls and patients with other infections. This suggests that infection with \textit{B. burgdorferi} depletes L-methionine levels in the circulation of the host. Though, \textit{B. burgdorferi} produces DPD and homocysteine through Pfs and LuxS, \textit{B. burgdorferi} lacks the ability to salvage homocysteine and is unable to resynthesize methionine from
other amino acids and is therefore completely reliant on the host supply of methionine (Babb et al., 2005). Increasing methionine levels may be another mechanism explaining the effect of MTA, as MTA can be used as a precursor in methionine synthesis.

The finding that combining both strategies, Akt-inhibition and methionine-supplementation, induced synergy was surprising. However, there have been some previous studies that support this, as methionine restriction has been found to increase Akt phosphorylation (Stone et al., 2014) and Akt inhibition has been reported to decrease the activity of methionine synthase (Waly et al., 2004).

Taken together, we show that *B. burgdorferi* is weak inducer of ROS production itself and able to modulate ROS generation in response to subsequent stimulation. This may have several consequences; firstly, the temporary decrease in ROS generation and defect in antimicrobial killing may leave the host more susceptible to secondary infections. This may be relevant in the context of the frequent co-infections observed during *B. burgdorferi* transmission through tick bites. Secondly, the compensatory overshoot in ROS generation observed at later time points may lead to tissue damage during infection. These findings provide us with clues for potential targeting strategies that can be beneficial during early *B. burgdorferi* infection and disseminated Lyme borreliosis. Restoring the methionine salvage pathway during the initial phase of *B. burgdorferi* infection may modify the host immune response, while preventing overproduction of ROS later on may avoid unnecessary tissue damage and the resulting symptoms in Lyme borreliosis.
B. burgdorferi inhibits ROS production

References


Supplemental Data

A. SOZ-induced ROS
   MTA

B. SOZ-induced ROS
   DZNep

C. SOZ-induced ROS
   Aza-cyt

D. SOZ-induced ROS
   Ado-inhibition

E. SOZ-induced ROS
   Wortmannin

F. SOZ-induced ROS
   mTOR inhibition

G. SOZ-induced ROS
   L-meth

H. SOZ-induced ROS
   L-methionine Wortmannin
Figure S1: MTA relieves the inhibitory effect of B. burgdorferi on ROS generation, partly mediated by inhibiting the Akt/mTOR pathway. A-F. PBMCs were stimulated for 24 h with B. burgdorferi, in the presence of (A) different concentrations of methylthioadenosine (MTA, n=12), (B) 3-deazaadenosine (DZNep, n=3), (C) 5-azacytidine (Aza-cyt, n=6), (D) MTA in combination with inhibitors of adenosine signaling (n=3), (E) wortmannin (n=10) or (F) inhibitors of mTOR signaling (n=3). After incubation, cells were harvested, and ROS generation was induced by serum-opsonized zymosan (SOZ). Data represent AUC of ROS-induced luminescence, measured for 1 h, of n different donors assayed in triplicate. G. Western blot of phospho-4E-BP1 and Actin expression in PBMCs after 2 h stimulation with B. burgdorferi in the presence or absence of MTA. G. ROS induction by serum-opsonized zymosan in PBMCs stimulated for 24 h with RPMI or B. burgdorferi, with or without L-methionine supplementation. H. ROS induction by serum-opsonized zymosan in PBMCs stimulated for 24 h with RPMI or B. burgdorferi with or without L-methionine supplementation combined with Akt-inhibition in low concentrations (methionine 1 mM, 0.1 µM wortmannin) or high concentrations (methionine 10 mM, 1 µM wortmannin).
Chapter 9

A joint effort: the interplay between the innate and the adaptive immune system in Lyme arthritis

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Abstract

Articular joints are a major target of Borrelia burgdorferi, the causative agent of Lyme arthritis. Despite antibiotic treatment, recurrent or persistent Lyme arthritis is observed in a significant number of patients. The host immune response plays a crucial role in this chronic arthritic joint complication of Borrelia infections. During the early stages of B. burgdorferi infection, a major hinder in generating a proper host immune response is the lack of induction of a strong adaptive immune response. This may lead to a delayed hyper-inflammatory reaction later in the disease. Several mechanisms have been suggested that might be pivotal for the development of Lyme arthritis and will be highlighted in this review, from molecular mimicry of matrix metallopeptidases and glycosaminoglycans, to autoimmune responses to live bacteria, or remnants of Borrelia spirochetes in joints. Murine studies have suggested that the inflammatory responses are initiated by innate immune cells, but this does not exclude the involvement of the adaptive immune system in this dysregulated immune profile. Genetic predisposition, via HLA-DR and microRNA expression, has been associated with the development of antibiotic-refractory Lyme arthritis. Yet the ultimate cause for (antibiotic-refractory) Lyme arthritis remains unknown. Complex processes of different immune cells and signalling cascades are involved in the development of Lyme arthritis. When these various mechanisms are fully been unravelled, new treatment strategies can be developed to target (antibiotic-refractory) Lyme arthritis more effectively.
Introduction and clinical background

In 1976, Lyme disease was first described in the town of Old Lyme, Connecticut, USA. The disease was recognized as a tick-transmitted disease due to the geographical clustering of children with what was initially thought to be juvenile rheumatoid arthritis \(^1\). A couple of years later in 1981, the causal pathogen was identified as *Borrelia burgdorferi* \(^3\). The early localized form of the disease is diagnosed by identifying the pathognomonic erythema migrans (EM), often described as a bull’s eye rash around the tick bite \(^3,4\). In a longitudinal observational study, about 60% of 55 untreated EM patients developed at least one attack of arthritis over the course of 4 years \(^5\). Since these cases, Lyme arthritis has been characterized as a mono- or oligoarthritis, typically presenting as a monoarthritis of the knee. Other signs of Lyme arthritis include: joint swelling, synovial hypertrophy, vascular proliferation and infiltration of immune cells \(^6\).

The occurrence of Lyme arthritis differs between geographical regions due to the presence of various species of *Borrelia burgdorferi sensu lato*, which are more likely to disseminate to a certain organ depending on their surface protein expression \(^7\). Since *Borrelia garinii* and *Borrelia afzelii* are more common in Europe, and *Borrelia burgdorferi sensu stricto*, the strain more likely to disseminate to the joints, is predominantly observed in North America, Lyme arthritis is more common in this latter region \(^8\)-\(^13\).

Lyme disease can be difficult to diagnose when clear pathognomonic signs, such as a typical EM, or the presence of a tick bite, have not been observed. Also, serological testing for Lyme disease has its limitations. Shortly after infection, antibodies can still be undetectable, and serological responses can be broken off due to antibiotic therapy \(^14,15\). On the other hand, once formed, IgG antibodies can be detectable for years, even after the infection has passed \(^16,17\). Incorrect diagnosis increases the risk of further advancement of Lyme disease in the patient while the infection could be effectively treated with antibiotics such as doxycycline \(^18,19\). However, in a small percentage of patients, symptoms persist, even after antibiotic treatment, probably due to differences in disease development and recurrent inflammation (Figure 1) \(^1\).
Figure 1: Schematic representation of the possible course of (antibiotic-refractory) Lyme arthritis symptoms over time. In the majority of patients, arthritis symptoms resolve when antibiotic therapy is given. However, arthritis can persist in a subset of cases. Usually, these symptoms are present intermittently as is presented in this graph. A depiction of the (maladaptive) immune response is given below.

Lyme arthritis can manifest itself as early as 4 days or as late as 4 years after an EM. In untreated patients, it not only affects the knee joint but also other large or small joints can be involved. If left untreated, synovitis can continue for months up to years. Usually, Lyme arthritis symptoms resolve after appropriate antibiotic therapy. In one study, resolution of arthritis has been observed in 80% of patients treated with doxycycline. However, more recent studies describe residual synovitis after the first course of antibiotics in 34% and even up to 40% in patients treated with doxycycline. Even after repeated courses of antibiotic therapy, symptoms persist in some patients. This condition is called antibiotic-refractory Lyme arthritis.

The most imperative question for both health care providers and patients remains if this is due to persistent infection and if additional courses of antibiotics should be prescribed. Persistence of *B. burgdorferi* infection as a cause for antibiotic-
refractory Lyme arthritis seems unlikely based on several observations. Firstly, PCR for *B. burgdorferi* DNA in the synovial fluid is often negative in antibiotic-refractory Lyme arthritis patients, while it was positive at the onset of disease. Likewise, a study on synovial samples collected by arthroscopic synovectomy in 26 antibiotic-refractory Lyme arthritis patients found negative PCR results in all samples. Secondly, in most of the cases cultivation of *B. burgdorferi* in synovial fluid cannot be performed or shows non-motile spirochetes. Finally, recurrent or persistent Lyme arthritis often improves upon anti-inflammatory therapy.

In this review, we will discuss the role and interaction of *Borrelia burgdorferi* with the innate and the adaptive immune response. We will describe this relationship during early infection, dissemination and the development of persistent inflammatory reaction in some patients, resulting in antibiotic-refractory Lyme arthritis. This overview may generate directions for future research on the pathogenesis of Lyme arthritis.

**From skin invasion to the articular joints: initiation of Lyme arthritis**

The dermis is the first tissue that the *B. burgdorferi* bacteria encounter once they enter the skin after tick inoculation. It consist of a broad range of extracellular matrix (ECM) proteins, polysaccharides components and are particularly rich in collagen type I. Tick saliva supports the spirochete to survive in the host tissues. Various tick salivary factors accommodate in the localized disruption of host tissues and immune responses. Outer surface proteins (Osps) are widely expressed by the spirochete once established in the host and support the bacteria to evade the immune system through suppression of several immune recognition pathways.

Following skin inoculation, in some patients *Borrelia burgdorferi* may spread throughout the human body, targeting specific tissue sites. This can cause a diverse range of symptoms, from chronic neurological complications to carditis, skin abnormalities and damage to the host’s joints.

*Borrelia* species dissemination may occur through two routes: hematogenous and non-hematogenous. Non-hematogenous spread transpires via the lymphatic system. Upon hematogenous dissemination, *Borrelia* species translocate through the endothelium and extracellular matrix (ECM) of the vasculature, crossing the last
remaining tissue barriers to arrive at the target site \(^{44-47}\). *Borrelia* species preferentially target the heart, articular joints, or cross the blood-brain barrier \(^{1,7}\).

Microvascular interactions of *Borrelia* spirochetes in murine skin were visualized using epifluorescence and spinning disk confocal intravital microscopy. These images showed that following inoculation the bacterium was localized in the capillaries, postcapillary venules and large veins. After encountering the vascular endothelium, *Borrelia* bacteria tethered to the endothelial cells and underwent dragging interactions in the direction with the blood flow, followed by stationary adhesion \(^{45}\). Plentiful *Borrelia* lipoproteins bind to ECM components and may be involved in the tethering of *Borrelia* bacteria to the vascular endothelium \(^{46-48}\). Fibronectin and glycosaminoglycans (GAGs) potentially mediate these interactions \(^{49}\). Spirochaetal lipoproteins have also been observed to interact with integrins of primary human chondrocytes, triggering myeloid differentiation primary response 88 (MyD88)-independent inflammatory pathways and eventually leading to the development of Lyme arthritis \(^{50,51}\).

Fibronectin binding may generate enough time for integrins to bind to the vascular wall, thereby supporting the contact of pathogen and host \(^{52}\). BBK32 is a *B. burgdorferi* surface protein that binds to fibronectin \(^{44,49,53,54}\), possibly through the utilization of lectin binding sites or other molecules. Several other *B. burgdorferi* proteins have also been identified as fibronectin binding proteins, including RevA, RevB, and BB0347 \(^{55,56}\). *Borrelial* lipoprotein decorin-binding protein A (DbpA) can bind decorin, heparin and dermatan sulphate GAGs \(^{48,57,58}\), and are widely expressed in skin and cartilage tissue \(^{59,60}\). *B. burgdorferi* dissemination to the joints has been correlated to the expression of this DbpA gene and the presence of spirochetes has been associated with the amount of decorin found in host tissues \(^{61}\). Moreover, decorin in the host was observed to be required for the development of Lyme arthritis in mice \(^{62}\).

While decorin is important in *B. burgdorferi* adhesion and invasion, it has also been suggested that the spirochete is able to directly bind to intact collagen. A study using hydrated collagen type I lattices of *B. burgdorferi* adhesion without decorin showed that the bacteria were still able to bind to the collagen matrix, even if glycosaminoglycan chain degrading enzymes were added to the matrix or if an agar matrix, bovine serum albumin, gelatin or pepsinized type I collagen were utilized.
In contrast, *B. burgdorferi* adhesion to collagen was shown to diminish in the absence of flagella and by proteinase K treatment, demonstrating that *B. burgdorferi* surface proteins play an important role in collagen binding. GAGs seem to further promote *Borrelia* spirochete adhesion to the collagen. Therefore, these above mentioned molecules and structures may be important in *B. burgdorferi* association to intact type I collagen matrices. *B. burgdorferi* also expresses peptidoglycans, which may be recognized by the human immune system. Recently, human IgG responses against *B. burgdorferi* peptidoglycan have been related to worsening of Lyme arthritis on the long term.

After adhering to the endothelium, *B. burgdorferi* traverses the endothelial cell monolayers through tight junctions. *Borrelia* P66 protein can form an OM β-barrel porin that adheres to host β1 and β3 chain integrins. The β1 chain is crucial in *Borrelial* internalization and transmigration across cellular junctions. P66 may assist in the intracellular invasion of *B. burgdorferi* as well. Furthermore, P66 is known to associate with OspA and OspB. High-temperature requirement protease A is a well-known family of ATP-independent serine proteases that can breakdown ECM components. *B. burgdorferi* encodes such a protease, supporting *Borrelial* tissue invasion through ECM degradation.

Another *Borrelia* host target is aggrecan, a proteoglycan commonly found in cartilage ECM. The enzymes responsible for the degradation of aggrecan, called aggrecanases, have been shown to be induced in human chondrocytes infected with *B. burgdorferi*. One of these aggrecanases is ADAMTS-4. Active forms of ADAMTS-4 were significantly increased in the synovial fluid samples of patients with active Lyme arthritis and *Borrelia*-infected mice. ADAMTS-5 was not elevated in Lyme arthritis, but is prominent in osteoarthritis. ADAMTS-4 may therefore be an aggrecanase induced differentially from ADAMTS-5. This differential expression may be specifically associated with cartilage damage in Lyme arthritis.

*B. burgdorferi* also activates the host enzyme plasmin via outer surface protein binding. By binding plasminogen, *B. burgdorferi* promotes its conversion to plasmin by a host-derived activator, thereby further promoting the degradation of ECM components, through the activation of matrix metalloproteinases (MMPs). Plasmin activates pro-MMP forms of MMP-1, MMP-3, MMP-10, and MMP-13.
enzymes involved in degrading the ECM\textsuperscript{81-86}. This degradation may support the host to clear the infection but conversely, it may also promote spirochete dissemination or the development of Lyme arthritis\textsuperscript{78,87-89}. The intricate balance in the immune and tissue repair responses seem to play an important role in whether the infection may develop into a hyperinflammatory response that damages the joints or helps the host in tackling the infection. \textit{B. burgdorferi} can directly induce the production of several MMPs by host cells as well. In human chondrocyte cultures with the bacterium an increased production of MMP-1, MMP-3, MMP-13 and MMP-19 was demonstrated\textsuperscript{87,90-92}, whereas \textit{Borrelia}-stimulated monocytes produced MMP-9 and MMP-10\textsuperscript{78,93,94}. In blister fluid from human EM lesions MMP-9 expression was selectively increased compared with blister fluid from normal-appearing skin\textsuperscript{95}. Moreover, antibiotic-refractory Lyme arthritis patients have higher levels of MMP-3 in their serum and synovial fluid, though antibodies against these enzymes were less common. Furthermore, synovial fluid concentrations of IFNγ and TNF-α, as well as T cell chemo-attractants CXCL9 and CXCL10 significantly higher\textsuperscript{96-98}. The expression of these cytokines and chemokines may stimulate tissue resident cells to express degrading enzymes such as MMP-10.

These studies give an overview of the broad range of host and \textit{B. burgdorferi} molecules, proteins and tissues involved early in infection, further complicating \textit{Borrelia} infection and the immune responses against this invading pathogen. Some of these host and bacterial factors have been shown to be involved in the initiation of Lyme arthritis or even lead to such a hyperinflammatory reaction that antibiotic therapy is no longer an effective treatment strategy.

\textbf{Initiation and development of antibiotic refractory Lyme arthritis}

Over the last years, several hypotheses on the precise mechanism behind the development of antibiotic-refractory Lyme arthritis have been introduced. Both auto-immune responses and remaining bacteria or bacterial antigens have been suggested to contribute to this process\textsuperscript{25,99}.

\textit{Borrelia} DNA has been shown to be present in the synovial tissue of Lyme arthritis patients\textsuperscript{25}. A recent study described persistence of flagellin B DNA and uncultivable \textit{B. burgdorferi} in a range of murine heart muscle, joint, and muscle tissues and at
the inoculation site up to 12 months after antibiotic treatment. Also, spirochete remnants were found within joint entheses and adjacent to cartilage after ceftriaxone and doxycycline treatment. This was studied in MyD88 knockout mice, that lack the crucial adaptor protein for several innate recognition receptors called Toll-like receptors (TLRs), thereby allowing Borrelia spirochetes to survive. Interestingly, these spirochete remnants induced IgG antibodies to B. burgdorferi in naïve mice and triggered macrophages to produce TNFα in vitro. In contrast to mice, pathogen loads in human Lyme arthritis are low. However, these studies suggest that the persistence of dead spirochetes in the joint may cause an immunogenic response, resulting in prolonged inflammation in these tissues.

Autoimmune responses have been suggested to contribute to the pathogenesis of Lyme arthritis by the observation that specific HLA-DR alleles such as DRB1*0401, playing a role in binding of B. burgdorferi OspA, are more frequent in patients with antibiotic-refractory Lyme arthritis than in those responding to treatment.

Molecular mimicry is another autoimmune mechanism that is potentially involved in the development of antibiotic-refractory Lyme arthritis. Partial sequence homology has been observed between OspA and human lymphocyte function-associated antigen 1 (LFA-1), MAWD-BP and human cytokeratin-10. Other possible targets for molecular mimicry with Borrelia bacteria are matrix metalloproteinases (MMP) as well as several non-protein antigens and neural proteins.

Endothelial cell growth factor (ECGF) is an autoantigen, found to be targeted by T and B cell responses in around 20% of the antibiotic-refractory Lyme arthritis patients and in 15% of EM patients. These ECGF antibody responses correlated directly with the extent of obliterative lesions and the amount of vascularity in the tissue. ECGF is an IFN-inducible protein and is highly expressed in the synovial fluid of patients suffering from antibiotic-refractory Lyme arthritis compared to antibiotic-responsive Lyme arthritis. In archival serum samples from 27 untreated patients with Lyme arthritis, 26% of the samples showed ECGF antibody responses. The total duration of active arthritis attacks in these patients was significantly longer than those who lacked ECGF reactivity. Compared to other rheumatological chronic inflammatory diseases, synovial tissue from Lyme arthritis patients consisted of significantly greater layer thickness and cellular infiltration.
Obliterative microvascular lesions were observed in patients with Lyme arthritis only, whereas these lesions were not reported in rheumatoid arthritis. The presence of these lesions was correlated with auto-antibody responses to ECGF. ECGF autoantibodies are produced early in Lyme infection, months before the onset of arthritis and obliterative microvascular lesions. This implies that autoantibodies play a role in the development of these microvascular lesions and thereby in antibiotic-refractory Lyme arthritis. Non-human primate studies have shown that obliterative microvascular lesions may not just be specific for Lyme arthritis, but may be a more general consequence of spirochaetal infections.

Lyme arthritis patients are prone to enhanced inflammatory responses. In patients carrying a particular TLR1 polymorphism develop a strong innate and adaptive immune reaction, resulting in high TNFα, IL-1β and IFNγ concentrations in the joints. IFNγ stimulates ECGF production by macrophages and other cells, leading to vascular proliferation and fibroblast activation. As mentioned above, T and B cell responses to ECGF were found in only a small number of EM patients. However, if these ECGF responders were more prone to develop Lyme arthritis was not further investigated.

Not only ECGF, but also apolipoprotein B-100, annexin A2, N-acetylglucosamine-6-sulfatase and filamin A derived peptides may induce T cell responses in Lyme arthritis patients, usually already early in the infection, when EM is apparent. Antibiotic-refractory Lyme arthritis patients have higher levels of MMP-3 in their serum and synovial fluid, though antibodies against these enzymes were less common. Furthermore, synovial fluid concentrations of IFNγ and TNF-α, as well as T cell chemo-attractants CXCL9 and CXCL10 significantly higher. The expression of these cytokines and chemokines may stimulate tissue resident cells to express degrading enzymes such as MMP-10.

Furthermore, an MMP-10 derived peptide was isolated from an antibiotic-refractory Lyme arthritis patient’s synovial tissue. This peptide could also be bound by HLA-DR sequences. The role of MMP-10 was subsequently studied in various Lyme patient groups, a marked T cell response was observed in 25% of the antibiotic-refractory patients MMP-10 auto-antibody levels were correlated to a worse phenotype of Lyme arthritis. However, as the authors suggest, this single
auto-antibody response is likely not solely responsible for the development of (antibiotic-refractory) Lyme arthritis.

The role of the innate immune system during the early stages of *B. burgdorferi* infection

After initial *Borrelia* infection of the skin, an influx of immune cells from the peripheral blood occurs. Polymorphonuclear leukocytes (PMNs) play an important role in infections. Freshly isolated human PMNs are shown to eradicate spirochetes mostly through antibody independent extracellular killing processes such as ROS production, whereas differentiated monocyte-derived macrophages ingest the bacteria and kill the spirochetes without the necessity of opsonization. When the tick feeds on a human host, both the bacteria and the tick saliva are released, and encounter the accumulating immune cells, influencing the host defence responses.

Neutrophils are recruited very early at the site of infection. They are able to kill pathogens by a range of defence mechanisms such as phagocytosis, ROS production and extrusion of their nuclear DNA with elastase and myeloperoxidase (MPO), leading to formation of neutrophil extracellular traps (NETs), to capture invading pathogens. In vitro incubation of human neutrophils and in vivo infection of mice with *B. burgdorferi* combined with saliva or salivary gland extracts, induced NET formation. NETs can trap and kill *Borrelia* spirochetes. Human neutrophils are directly activated by *B. burgdorferi* through interaction with OspA. *B. burgdorferi* is sensitive to ROS and granule enzymes in an in vitro setting, in the absence of tick saliva. However, tick salivary factors impair neutrophil actions at the site of infection and there is a lack of neutrophils in EM lesions. Moreover, *Borrelia* spirochetes have developed a range of neutrophil evasion mechanisms, including the inhibition of IL-8 secretion, a cytokine involved in neutrophil recruitment, by Evasin-E3, inhibition of neutrophil chemotaxis through N-Formylmethionyl-leucyl-phenylalanine (fMLP) activation, and interference of OspB with neutrophil phagocytosis, possibly through direct interaction with the complement receptor 3 (CR3) and oxidative bursts via unknown mechanisms. Using rapid movements, the spirochetes can also escape neutrophil interaction. Neutrophil migration is inhibited by binding of the
tick saliva leukotriene B4 (LTB4), enhancing bacterial propagation and formation of EM. Tick saliva further interferes with neutrophil function through downregulation of neutrophil integrin expression, inhibition of superoxide anions production and elastase inhibition. Previous studies have shown that a diminished neutrophil response may increase the hosts’ susceptibility to *Borrelia* infection, whereas increased recruitment of neutrophils early in infection may reduce dissemination of *Borrelia* to the joint.

During infections, neutrophils produce IL-1β, TNFα, IL-8 and IL-6 and IL-15 in vitro. IL-15 is involved in adaptive responses and vital in NK cell homeostasis. Depletion of IL-15 in *Borrelia*-vaccinated and -challenged mice hampered the development of arthritis. Furthermore, anti-IL-15 antibody or recombinant IL-15 receptor alpha reduced infiltration of neutrophils into the synovium.

A role for NK cells in the initiation of Lyme arthritis is postulated by several studies. C57BL/6 mice genetically deficient in granulocytes and NK cells developed less severe arthritis than their wild-type counterparts. However, depletion of NK cells did not influence the course of arthritis, suggesting NK cells augment the development of Lyme arthritis. OspA augments the activation of NK cells which can drive the induction of various inflammatory mediators, including TNFα. Anti-TNFα therapy has been described to be effective in antibiotic-refractory Lyme arthritis. Therefore, these NK cell-derived mediators may contribute to the development and persistence of Lyme arthritis.

Mast cells are also important for the development of immune response in early infection, while the spirochetes still reside in the skin. They patrol the skin to detect new pathogens and play a role in other hyperinflammatory reactions such as allergies. The primary mast cell function in the early stages of Lyme disease was recently studied in C57BL/6 mice. While OspC induced degranulation of mast cells, this degranulation was not observed upon stimulation with whole *Borrelia* spirochetes. In contrast, the addition of tick salivary gland extract (SGE) to the spirochetes, enhanced the degranulation. The absence of mast cells in KitWsh−/− mice did not significantly affect the replication rate of *B. burgdorferi* in the skin but promoted *Borrelia* dissemination to the joints. Moreover, the expression of cytokines production by *B. burgdorferi*-activated mast cells was significantly inhibited in the presence of SGE. The authors postulated that the modulation of
mast cell function by *B. burgdorferi* in combination with tick saliva may affect long-term and/or repeated infections and protect the host against Lyme borreliosis.\(^{149}\)

Spread of *B. burgdorferi* is hampered by the ability of macrophages and dendritic cells to bind and phagocytize *B. burgdorferi*\(^{150-152}\). Spirochetes ingested by macrophages are quickly localized in endosomes and lysosomes.\(^{153}\) However, in severe combined immunodeficiency (SCID) and C3H/HeN murine studies with peritoneal macrophages, OspC was shown to protect the spirochete against phagocytosis\(^ {154}\). Depletion of mononuclear phagocytes in these mice permitted non-functional-OspC *Borrelia* mutants to initiate infection *in vivo*. Increased OspC expression also reduced spirochete uptake by murine peritoneal macrophages, suggesting a role for OspC in anti-phagocytic function.\(^ {154}\) Furthermore, OspC was shown to interfere in plasminogen function and promote spirochete invasiveness.\(^ {75,155,156}\) Thus, *B. burgdorferi* effectively uses its outer surface proteins to avoid phagocytosis.

In EM lesions, large populations of monocytes, macrophages, and dendritic cells are observed.\(^ {157}\) Interaction of murine macrophages with *Borrelia* antigens induces production of nitric oxide (NO), IL-1, TNF\(\alpha\), IL-6 and IL-12, and MMP-9.\(^ {95}\) Intriguingly, LSH hamsters injected with macrophages that were previously exposed to *B. burgdorferi*, developed severe, destructive arthritis. In contrast, animals that received unprimed macrophages did not develop arthritis.\(^ {158}\) Furthermore, the combination of primed macrophages and T cells accelerated the development of arthritis in *Borrelia*-infected ICR mice compared to either one alone.\(^ {159,160}\) This highlights the importance of immune cells capable of presenting *Borrelia* antigens to T cells for the progression to Lyme arthritis. Moreover, this suggests that previous exposure to *B. burgdorferi* might increase pro-inflammatory responses of macrophages.

Type I IFN production by macrophages and myostatin expression, has been correlated to *B. burgdorferi* arthritis–associated locus 1 (Bbaa1), in a study on loci associated to Lyme arthritis. Monoclonal antibody blockade of IFN\(\beta\) resulted in reduced severity of Lyme arthritis in infected B6.C3-Bbaa1 mice. Bone marrow-derived macrophages stimulated with *B. burgdorferi* enhanced IFN\(\beta\) expression. Inhibition of myostatin *in vivo* suppressed Lyme arthritis in reduced interval congenic Bbaa1 mice, implicating that myostatin is a downstream mediator in Lyme
Receptor-blocking antibodies and receptor ablation showed the crucial role of type I IFN production by macrophages in Lyme arthritis severity.

Increased IFNγ signalling in macrophages also enhances indoleamine-pyrrole 2,3-dioxygenase levels. This is a molecule regulating both ROS, collagenase, elastase and stromelysin activity, thereby increasing ECM degradation. Destabilization in the membrane structure of murine cells results in adherence of spirochetes remnants to the cartilage. These Borrelia remnants may, in turn, initiate an inflammatory reaction and stimulate TNFα production by macrophages.

Many studies of Lyme arthritis are performed in mice, due to the limited number of Lyme arthritis patients and available tissue specimens. Murine models are not completely comparable to human Lyme arthritis, and large differences in disease expression are observed between different mouse strains. C3H/HeN mice, for example, are highly susceptible to Borrelia-induced arthritis once the bacteria are injected into the joint. These mice have a defect in IL-12 production, which hampers the induction of IFNγ through IL-12/IL-18 signalling. This possibly explains why these mice are more susceptible to invading bacteria. In contrast, C57Bl/6 mice do produce IFNγ upon Borrelia exposure.

TLRs are cell receptors that recognize conserved molecular patterns on microbial components, such as lipoproteins, lipopolysaccharide, proteoglycans, flagellin, and nucleic acids. Binding of TLRs with these components results in NF-κB-mediated expression of proinflammatory cytokines. TLR2 deficient mice were shown to have a significant increase in the number of spirochetes in their joints after infection compared to WT mice. TLR2 deficient C3H mice demonstrated a T cell dependent increase in mononuclear cell infiltration. The number of T cell attracted to the joint tissue was enhanced, possibly through augmented production of T cell chemokines CXCL9 and CXCL10. These T cells, in turn, enhanced mononuclear cell migration via the increased release of other inflammatory factors. A recent study in C3H/HeN mice showed that this increase may be attributed to CD8 T cell activation of synoviocytes. Moreover, transcripts for the IFN-inducible gene IFNγ induced GTPase (igtp) were elevated. Intriguingly, recent studies have shown that TLR2 can function as a co-receptor on activated T cells, influencing their responses and function. However, when compared to wildtype control mice, TLR2-deficient mice had higher Borrelial load but no significant difference in joint...
swelling, while increases of swelling and arthritis have been reported in *Borrelia* infected TLR2-deficient C57BL/6 mice in another study.

The co-receptor CD14 is crucial in *Borrelia* recognition in collaboration with TLR2. Interestingly, a study by Sahay *et al.* suggests that CD14 may be important in *Borrelia* clearance and tolerization of macrophages, through SOCS1 and 3 signalling, whose pathways have also been linked to MMP production and degradation of collagen. CD14 deficiency in mice reduced these effects significantly, led to higher bacterial burdens and more severe and persistent inflammation. However, the murine studies demonstrated a possible link between CD14 signalling and persistent inflammation in Lyme arthritis. Yet, it is still unclear whether this is caused by inhibition of TLR2 signalling or by the direct function of CD14 itself.

The intracellular NOD2 receptor has also been shown to be involved in *Borrelia* recognition. Remarkably, *B. burgdorferi* infection of NOD2 deficiency C57BL/6 mice led to increased inflammation in joints and cardiac tissue when compared to wild type mice, suggesting that NOD2 tolerization normally protects these mice against dissemination, possibly through the induction of multiple cytokines and interferons. A study by Oosting *et al.* (2012) demonstrated that ASC/caspase-1 induction was crucial for IL-1β production of in murine Lyme arthritis. The activation and secretion of IL-1β occurred independently from NOD2/RICK, as well as NOD-like receptor-family member protein 3 (NLRP3) activation, while the TLR2-MyD88 pathway was crucial for IL-1β production. *Borrelia* exposed NOD1-, NOD2-, and RICK-deficient murine cells still produced IL-6 and TNF-α. In contrast to previous *in vitro* data, NLRP3 did not seem to be involved in Lyme arthritis development in a murine model. ASC was pivotal in Lyme arthritis initiation as an important adaptor protein in the inflammasome, involved in antigen presentation, lymphocyte migration, NF-κB activation, mRNA stability and via Dock2 expression and Rac activation. Lyme arthritis patients with high levels of IL-1Ra and a low concentration of IL-1β in their synovial fluid demonstrated rapid resolution of the disease. The crucial role of IL-1Ra in the attenuation of inflammatory diseases was supported by the observation that IL-1Ra deficient mice spontaneously developed arthritis (Figure 3), while IL-1 receptor deficient mice had significantly less inflammatory disorders, such as Lyme arthritis. Caspase 1 also plays a role in the induction of an anti-*Borrelia* response in the joint, but over time becomes less important in controlling the progression of the disease. It would be interesting
to study whether NOD signalling is involved in IL-1Ra production and thereby in the attenuation of Lyme arthritis.

Figure 2: Uncontrolled inflammation in the knee joint of a 16-wk-old IL-1Ra−/− mouse. Depicted from left to right: (A) inflamed ankle joints of the IL-1Ra-deficient mice (swelling and redness) indicated by arrows; (B) HE staining of the knee joint—note the pannus-like tissue destroying the bone and cartilage; and (C) Safranin O staining of the same knee joint, showing the severe cartilage destruction (loss of red staining in the cartilage layer). HE, hematoxylin and eosin; IL-1RA, interleukin-1 receptor antagonist

Another innate regulatory mechanism in Lyme arthritis is the expression of microRNAs (miRNAs). miRNAs are small, noncoding RNA molecules that can bind mRNA and inhibit its translation. They are expressed by various innate immune cells, such as monocytes, macrophages DCs, granulocytes, and NK cells. miRNAs are crucial in the development and function of innate immune cells and play a role in adaptive immune cell regulation. Dysfunction of miRNAs can induce inflammatory diseases and autoimmunity. In mouse studies, several miRNAs are reported to modulate experimental models of arthritis, including miR-155, miR-223, miR-146a. This is consistent with the role of miRNAs in rheumatoid arthritis (RA). Intriguingly, mice lacking either miR-146a or miR-155 were shown to develop more severe Lyme arthritis or carditis.

Recently, in groups of patients with different stages of Lyme arthritis alternate miRNAs profiles were observed. Elevated levels of miR-223 and low levels of miRNAs such as miR-146a, miR-155, miR-142 were found in synovial fluid of Lyme arthritis patients prior to antibiotic therapy (n=5). Also, elevated levels of white blood cells, specifically polymorphonuclear leukocytes were found in SF of these patients.
In contrast to patients with post-antibiotic persistent Lyme arthritis (n=13), who had higher percentages of lymphocytes and mononuclear cells, and high expression of miR-146a, miR-155, miR-142, miR-233 and miR-17-92. Let-7a family levels were reduced. Of note, arthritis in two of these 13 patients resolved after intravenous administration of antibiotics. miRNA expression in patients with postinfectious Lyme arthritis was most similar to the expression in RA patients, where miR-155, miR-146a, miR-223 were prominent in the synovial fluid, synovial tissue and synovial fibroblasts. Higher levels of miR-146a, miR-17 and miR-233 are also correlated with increased duration of Lyme arthritis. Thus, the miRNA pattern in SF changes during stages of Lyme arthritis, possibly representing the altering nature of Lyme arthritis after bacterial killing.

Strle et al. (2012) studied SNPs with a significant effect on the host immune cell function. In general, SNPs in TLR1 (1805GG), TLR2 (2258GA) and TLR5 (1174CT) seem to be crucial in immune function. However, when studying the frequency of these SNPs in a Lyme arthritis cohort, TLR1-1805GG was enhanced in antibiotic-refractory Lyme arthritis patients compared to responsive patients. EM patients carrying this SNP had higher serum levels of CXCL9 and CXCL10, especially if infected with the B. burgdorferi 16S–23S ribosomal spacer RNA intergenic type 1 (RST1) strains. CXCL9 and CXCL10 are well-known chemo-attractants for CD4 and CD8 effector T cells, the most prominent infiltrating cells in the joint lesions of antibiotic-refractory Lyme arthritis patients. EM patients were also more likely to have symptomatic infection and had greater inflammatory responses than EM patients carrying the 1805TG/TT polymorphism, despite having similar number of spirochetes in EM skin lesions and similar frequency of disseminated infection. Furthermore, antibiotic-refractory Lyme arthritis patients with the 1805GG polymorphism had higher CXCL10 concentrations in joint fluid than antibiotic-responsive patients. The frequency and function of TLR2 and TLR5 polymorphisms did not vary between the patient groups. However, previous murine studies have suggested that TLR2 may play a role in the severity of Lyme arthritis. This may be partially explained by the fact that this TLR2 polymorphism does not abolish TLR2 function, as only one allele is affected. Moreover, this allele is present in only a low proportion of patients with a wide range of manifestations of Lyme disease, including EM, ACA and Lyme arthritis. Nevertheless, this is of interest, since the TLR1/TLR2 heterodimer recognizes Borrelia lipopeptides as a complex. It is
unclear how *Borrelia* infection in humans with the 1805GG polymorphism or in mice with TLR2 deficiency results in a heightened Th1 response. The cells with the SNP might recognize different peptides, activate the adaptive immune cells using a different mechanism, or affect a whole recognition pathway. In murine macrophages stimulated with *B. burgdorferi*, TLR1/2 complex deficiency resulted in persistent augmented levels of inflammatory cytokines and chemokines due to a significant reduction in p38 activation and SOCS expression. Interestingly, IFN responsive gene induction was shown to occur independently of TLR2, TLR4, TLR9, and MyD88, suggesting that *B. burgdorferi* activates another type I IFN transcription pathway through a different receptor or sensing molecule.

**Figure 3: Lyme arthritis: a joint effort.** A) The initiation of Lyme arthritis through enhanced cytokine and chemokine production by both macrophages and dendritic cells, as well as neutrophil formation of extracellular traps (NETs) and tissue-resident cells such as chondrocytes producing matrix metalloproteinases (MMPs). Of these factors IL-1β, IFN-γ, IL-17, CXCL9, CXCL10, and MMPs are shown in red because they play a major role in...
worsening the disease development and whose levels are often enhanced in patients with antibiotic-refractory Lyme arthritis. B) The progression of Lyme arthritis, causing a more chronic phenotype with high numbers of (effector) T cells, initiated via dendritic cell stimulation of T-cell receptors using either Borrelia or self-antigens, without the necessary presence of live Borrelia spirochetes, and antibody production from activated B cells. The continued presence of activated innate immune cells further heightens the number of activated adaptive immune cells and cytokines and chemokine levels.

**From innate initiation to adaptive dysregulation**

The innate immune system is the key initiator of Lyme arthritis (Figure 2). It recruits, activates and drives the adaptive immune response, leading to further dysregulation and worsening of the disease. Studies in SCID mice have shown that, in absence of the adaptive immune system, innate immune responses are the primary drivers for *Borrelia burgdorferi* tissue damage and Lyme arthritis. In contrast, the adaptive responses control the pathogen, primarily using antibody production and may in a later stage contribute to immune dysregulation in the joints. The predominant innate immune cells seem to differ in several manifestations of Lyme disease, and so the innate pathways may differ. The importance of innate immune cells was studied in mice without both B and T cells. The authors observed that innate immune cells are crucial in Lyme arthritis commencement and adaptive immune cells are not the direct cause of Lyme arthritis initiation and probably only worsen the disease symptoms. Moreover, innate immune cells are responsible for the production of MMPs, HLA-DR subsets and signalling involved in the disease, as well as for induction of IFN production, through other IFNs, IL-12 and IL-18. In synovial tissue from patients with Lyme arthritis, DCs were observed in areas with T and B cell aggregates. This demonstrates the collaboration of these immune cell subsets in the joints. While adaptive immune cells in turn further contribute either by formation of auto-antibodies or excessive inflammation. The same study showed how plasma cells were abundantly present around the lymphoid aggregates, demonstrating the local production and secretion of (auto-)antibodies as well as a joint effort of both innate and adaptive immune cells in the development of Lyme arthritis.

Interestingly, T cell independent antibodies have been shown to be protective against Lyme arthritis. Splenic B cell compartments expanded marginal zone B
cells during *B. burgdorferi* infection. No changes were observed in the follicular B cell subset \(^{206}\). In contrast, peripheral blood plasmablasts and CD27\(^+\) memory B cell populations were elevated in untreated Lyme arthritis. Higher plasmablast numbers and robust serum reactivity were also correlated with a more rapid resolution of the disease \(^{207}\). Consequently, the balance in B cell and T cell independent antibody responses may affect disease development and patient prognosis of Lyme arthritis.

Beyond these observations on how innate immune recognition, cytokine and antibody production lead to the activation of adaptive immune responses resulting in a more severe phenotype of Lyme arthritis. The initiation of Lyme arthritis in *Borrelia*-infected C3H SCID mice occurred independently of both B and T cell infiltration in the joint tissue. Moreover, the severity of Lyme arthritis seemed directly related to type I IFN induction in innate immune cells, since blocking its receptor, IFNAR1 reduced the ankle swelling in mice significantly \(^{162}\), while anti-IFN\(\gamma\) had no effect. In addition, other studies in mice have shown that IFN\(\gamma\) production was not required for the development of Lyme arthritis \(^{144,208-210}\). However, the synovial fluid of patients with antibiotic-refractory Lyme arthritis contains significant levels of IFN\(\gamma\) and related chemokines. Thus, IFN\(\gamma\) may be crucial in further development of persistent arthritis and/or as a consequence of initiator of the disease \(^{96-98}\).

Next to Th1 cell oriented responses, Th17 cell responses have been shown to play a role in Lyme arthritis. The Th1 and Th17 cell phenotype points towards the involvement of both IFN\(\gamma\) and IL-17 in Lyme arthritis. The role of IL-17 has been elaborated in several studies. In an experimental mouse study, IL-17 inhibition prevented the development of Lyme arthritis \(^{211}\). In patients with Lyme arthritis, Th17 cells were observed in the synovial fluid \(^{212}\). Moreover, *B. burgdorferi* or its lipoproteins were shown to be able to induce the production of IL-17 *in vitro* \(^{213,214}\). In addition, synovial T cells, extracted from patients with Lyme arthritis, produced IL-17 in response to *Borrelia* antigen neutrophil activating protein A \(^{215}\). IL-17 production was shown to be caspase-1 and IL-1\(\beta\) dependent, through the activation of IL-17 producing T cells. In the absence of IL-1\(\beta\), a defective IL-17 production was observed, while IL-18, a cytokine which is also caspase-1 dependent, was vital for IFN\(\gamma\) production in Lyme arthritis. Caspase-1 dependent IL-33 did not play a role in *Borrelia*-induced IL-1\(\beta\), IFN-\(\gamma\) or IL-17 secretion \(^{167}\).
Patients with antibiotic-refractory Lyme arthritis have remarkably high levels of the innate mediator IL-6 in their synovial fluid, which is vital for Th17 lineage specification. IL-23 levels are also enhanced, which may polarize Th17 cells towards a more pathogenic phenotype throughout the course of the disease, due to enhanced infiltration of inflammatory myeloid cells into the tissue. In some patients, a chronic inflammatory environment, with particularly elevated IL-23 levels and autoantigens present in the joints, contributes to the development of pathogenic and Th17 related autoimmune responses. In mice, neutralizing antibodies against both IL-17 and IL-23 delayed the onset of joint swelling, reduced IL-17 producing CD4 T cells, and enhanced regulatory CD4 CD25 T cell numbers.

IL-23 is vital for the development and maintenance of Th17 cells. Oosting et al. (2011) showed that both IL-17 and IL-23 were produced upon *B. burgdorferi* exposure. However, in contrast to murine studies, when the IL-23 bioactivity was inhibited in human PBMCs cultures or when PBMCs from human subjects who carried a loss of function SNP in the gene coding for the IL-23 receptor were stimulated with *B. burgdorferi*, IL-17 production was significantly suppressed, while IFNγ production was not affected. Though IL-17 production was decreased, the IL-23R gene polymorphism had no consequences for the development and pathogenesis of persistent symptoms attributed to Lyme disease. Both IL-17 and IL-22 were produced upon PBMC stimulation with *B. burgdorferi*. However, whereas IL-17 levels remained quite low, IL-22 was significantly upregulated within 10 hours of exposure, related to monocyte-induced caspase-1 and IL-1 bioactivity. Thus, Bachmann et al. (2010) suggest IL-1β as a single stimulus for the induction of IL-22 but not for IL-17 in human PBMCs. Of high interest, *Borrelia*-induced IFNγ and IL-22, but not IL-17, production in humans is shown to be age-dependent. This might explain why elderly subjects are more susceptible to infection, including Lyme disease.

In collaboration with antigen presenting cells, T cells function between the innate and adaptive immune responses, promoting each other’s function and manifesting cytolytic activity. Caspases are also involved in cell proliferation and death. Their signalling in T cells is more efficient if T cell receptors (TCRs) are activated. Analysis of active caspases in human T cells showed that caspase 3, but not its upstream initiator caspase 8 or caspase 9, is elevated in Lyme arthritis. These active caspases seem to appear in lipid rafts at the cell membrane during T cell cycling.
thereby their access to cytoplasmic substrates involved in cell death is denied. The prominent caspase 3 activity may be caused by more intense TCR signalling in T cells, resulting in a rapid effector T cell response. Moreover, this activation may be related to the Fas-associated death domain-like IL-1 expression. Of high interest, recent research proposes that Fas:FasL signalling between CD4 T cells, DCs, and γδ T cells may be part of the inflammatory reaction in (antibiotic-refractory) Lyme arthritis.

The synovium from Lyme arthritis patients contains high levels of CXCL10, a chemokine specific for Th1 cells, and CCL2, important for both Th1 and Th17 recruitment via CXCRI expression. PMNs are well-known for producing these chemokines in response to LPS or IFNγ and may therefore be involved in T helper cell recruitment. Codolo et al. (2013) demonstrated that CCL20 is also present in the synovial cavity of Lyme arthritis patients, which attracts lymphocytes and possibly dendritic cells through its association with CCR6, showing the vital cytokine and chemokine signals involved in attracting various immune cells subsets to the joint.

In the synovial fluid of patients with Lyme arthritis, rheumatoid arthritis and other types of inflammatory arthritis Th1 cells are prominent. Previous studies have shown that the Th1/Th2 cell ratio directly correlates to the severity of Lyme arthritis, suggesting a protective effect of Th2 cells on the Th1 driven inflammatory reaction. Some of the Th1 cells identified were specifically targeting Borrelia OspA. Active CD4 regulatory T cells (Tregs) were more abundant in the synovial fluid of antibiotic-refractory Lyme arthritis than in the peripheral blood. An increase in CD4 effector/Treg cell ratio is known to directly correlate with the development of antibiotic-refractory Lyme arthritis. Patients with fewer Tregs had suboptimal responses to DMARDs, required longer antibiotic treatment and often required synovectomies for successful resolution of their Lyme arthritis. Another study showed that the synovial fluid of antibiotic-refractory Lyme arthritis patients consisted of a CD4+CD25hi population with fewer FOXP3+ Treg cells and more FOXP3-effector T cells compared to patients with antibiotic-responsive Lyme arthritis. Antibiotic-refractory Lyme arthritis patient cells also showed significantly greater expression of glucocorticoid-induced TNFR-related protein (GITR) and OX-40, two co-receptors that augment T cell function. Their T cells did not effectively suppress T cell activation and proliferation. They showed enhanced secretion of
IFNγ or TNF-α. Moreover, in the refractory patients, higher ratios of CD25hiFOXp3−/CD25hiFOXp3+ cells were associated with longer post-treatment duration of Lyme arthritis symptoms.  

**Therapy for Lyme arthritis**

As is the case in localized early Lyme disease, timely antibiotic treatment is recommended for Lyme arthritis. This is based on several double-blinded randomized trials. Efficacy was first investigated in a parental penicillin trial. In this study, 35% of patients had complete resolution compared to none of the placebo treated patients. The percentage of patients with resolution of Lyme arthritis was higher in trials on doxycycline therapy. Oral therapy was shown to be as effective as IV therapy, but safer, and less expensive. Furthermore, antibiotic treatment shortens the duration of Lyme arthritis considerably. The clinical course of 21 patients with erythema migrans and Lyme arthritis treated with NSAIDs (non-steroidal anti-inflammatory drugs) and intraarticular steroids without antibiotic treatment was described in the 1980s. These individuals had attacks of arthritis, ranging from short attacks of arthritis to continuous synovitis, for a median total time of 43 months, whereas the duration of arthritis in antibiotic treated patients ranged from a median of 4 months in an antibiotic responsive group (n=50) to 16 months in an antibiotic-refractory disease group (n=62). In most patients with Lyme arthritis, symptoms resolved after a single course of antibiotic therapy, but this proportion varies from 48-90% in both Europe and North America. In the case of persistent arthritis, a second course of antibiotics could be given. Often, in persistent severe or worsening cases IV therapy with ceftriaxone is chosen because of its reported effectiveness. Another option is to wait for 3 months before retreatment is commenced, because resolution of Lyme arthritis might occur months after a first course of antibiotic therapy. Other therapeutic approaches for antibiotic-refractory Lyme arthritis may be considered. For example, to employ strategies used in other forms of inflammatory arthritis (i.e., rheumatoid arthritis). Unfortunately, randomized trials with Disease Modifying Anti-Rheumatic Drugs (DMARDs) are lacking. In vitro studies suggest an
effect of hydroxychloroquine against *B. burgdorferi* \(^{239,240}\) and there are studies reporting the potential benefits of this drug as an antirheumatic drug \(^{241,242}\), however, no data are available in patients with Lyme arthritis. While randomized controlled clinical trials are lacking for systemic anti-inflammatory regimens, DMARDs have shown to reduce symptoms of inflammation and patients benefit from this therapy in clinical practice. TNF inhibitors (i.e. tocilizumab) are suggested to have a clinical beneficial effect as well, although there have been concerns about the risk of infection with such immune suppressive therapy \(^{243-245}\). Arthroscopic synovectomy is another therapeutic option, when the response to a DMARD is incomplete and the arthritis is limited to one joint \(^{246}\). Intra-articular corticosteroids are not recommended during the second period of antibiotic therapy \(^{230}\), since contradicting reports on steroid efficacy in Lyme arthritis have been published \(^{23,247-249}\). Prognosis of Lyme arthritis is generally favorable after antibiotic therapy. However, in case of antibiotic-refractory Lyme arthritis systemic anti-inflammatory regimens seem to have a positive effect on the course of the disease in clinical practice.

**Future Perspectives**

Over the last few years, our knowledge of Lyme disease has massively increased. The current research shows that the induction of a proper immune response towards *Borrelia* bacteria early in infection may fail, resulting in hyper-inflammatory reaction in different tissues. The interaction of *Borrelia* with different immune cell subsets shows the diverse range of immune evasion mechanisms: from suppressing early immune cell recruitment to reduction of DC migration, and lack of T cell activation. Later, severe immune responses are strongly correlated with the development of Lyme arthritis.

However, there are still gaps in our understanding of the weak induction of both innate and adaptive immune responses and the development of the disease, during early infection and in the late stages of Lyme disease. Most of the studies have only been performed in mice, and give potential clues to the human in vivo situation, but findings may not be translatable directly to the human with Lyme arthritis. Furthermore, many murine studies assess only one or a few aspects of Lyme disease, and therefore lack the complete overview of what is occurring during *B.*
burgdorferi infection in humans. Therefore, models that closely resemble the in vivo situation in humans during Borrelia-induced inflammation need to be elaborated to further investigate the interaction of B. burgdorferi with the innate and adaptive immune networks. When a better understanding of the mechanisms involved in Lyme arthritis is achieved, both innate and adaptive immune responses could be targeted. This might improve treatment strategies for (antibiotic-refractory) Lyme arthritis.
Abbreviations

*B. burgdorferi: Borrelia burgdorferi; s.l.* = sensu lato; *s.s.* = sensu stricto; MMPs: matrix metalloprotidases; GAGs: Glycosaminoglycans; EM: erythema migrans; LA: Lyme arthritis; IgG: immunoglobulin G; PCR: polymerase chain reaction; IFN-γ: interferon-gamma; Radboudumc: Radboud university medical center; PCR: polymerase chain reaction; PBMC: peripheral blood mononuclear cell; Th: T helper; Osp: outer surface protein; ECM: extracellular matrix; IVM: intravital microscopy; MyD88: myeloid differentiation primary response 88; BBK32: *B. burgdorferi* fibronectin-binding protein; DbpA: decorin-binding protein A; ATP: adenosine triphosphate; TLRs: Toll-like receptors; HLA-DR: Human Leukocyte antigen – DR isotype; LFA-1: Lymphocyte function-associated antigen 1; ECGF: Endothelial cell growth factor; IL: Interleukin; APCs: antigen-presenting cells; TNFα: tumor necrosis factor alpha; CXCL: CXC chemokine ligand; PMNs: polymorphonuclear leukocytes; ROS: Reactive oxygen species; NETs: neutrophil extracellular traps; MPO: myeloperoxidase; fMLF: N-Formylmethionyl-leucyl-phenylalanine; CR3: Complement receptor 3; LTB4: Leukotriene B4; C57BL/6: C57 black 6; NK cell: natural killer cell; SCID: severe combined immunodeficiency; NO: nitric oxide; NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells; Igtp: IFNγ induced GTPase; CD: cluster of differentiation; NOD2: Nucleotide-binding oligomerization domain-containing protein 2; NLRP: Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing; ASC: Apoptosis-associated speck-like protein containing CARD; Dock2: Dedicator of cytokinesis 2; mRNA: messenger RNA; miRNAs: microRNAs; RA: rheumatoid arthritis; Bbaa1: *B. burgdorferi* arthritis-associated locus 1; SNPs: Single nucleotide polymorphisms; SOCS: suppressors of cytokine signaling; TCRs: T cell receptors; CXCR: CXC chemokine receptor; CCL2: (C-C motif) ligand 2; FOXP3: forkhead box P3; Tregs: regulatory T cells; GITR: glucocorticoid-induced TNFR-related protein.
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Chapter 9


Chapter 10

General Discussion
Summary and discussion

The vector-borne disease Lyme borreliosis (LB) has a significant impact on human populations living in the north-western hemisphere. With its prevalence progressively increasing and new strains spreading across Europe, understanding the behaviour of this pathogen and the resulting disease becomes more important. *B. burgdorferi* has a diverse repertoire of evasion mechanisms to circumvent immune cell recognition in humans. This often results in low or delayed immune responses in humans, affecting the standard immunology driven diagnostic assays such as serology and interferon gamma release assays (IGRA). Moreover, live *B. burgdorferi* is very difficult to detect in blood and human tissues, even in the presence of symptoms. Thus, it is crucial to develop novel diagnostic tools and strategies to detect and treat the disease early on in LB patients. While specific disseminated forms have been studied extensively over the years, little is known about the pathogenesis of initial phase of LB and how this may develop into more (acute) disseminated forms of the disease. Therefore, in this thesis, we studied the early interplay between the innate and adaptive immune system and *B. burgdorferi*.

Antigen presentation is an important tool for innate immune cells to instruct the adaptive immune system about invading pathogens. Although, previous studies have observed differences in antigen presentation molecules overtime during *B. burgdorferi* infection, relatively few studies have investigated this further. *B. burgdorferi* infection occurs when a tick bites a human host releasing both bacteria and saliva into the skin. Tick saliva also contains many factors suppressing the immune response (Chapter 1). *B. burgdorferi* is also well-known to use various other evasion strategies, were it targets for example the complement system, neutrophil recruitment and dendritic cell function\(^1,2,3,4,5\).

In the present thesis, we showed that *B. burgdorferi* inhibits the expression of one of the most important communication molecules for innate and adaptive immunity: the major histocompatibility complex class II (MHCII) HLA-DR (Chapter 2). Here, *B. burgdorferi* inhibited expression of antigen presentation molecules through Receptor-interaction serine / threonine-protein kinase 1 (RIP1) signalling, probably via suppression of its main transcriptional regulator called class II transactivator (CIITA). This led to a significant inhibition of T cell responses. Upon re-exposure to...
another pathogen well-known to induce these responses, *B. burgdorferi* downregulated anti-*C. albicans* immune responses significantly. In another study, they investigated the role of peripheral blood monocytes and murine microglia, the macrophages of the central nervous system which are not present in the peripheral blood, in *B. burgdorferi* infection\(^6\). Of interest, the modulation of several MHC class II-related genes differed greatly between the two cell types. At 48 hours, there was significant downmodulation of numerous HLA-associated genes in monocytes, whereas their expression was not significantly altered in microglial cells. HLA-DR and HLA-DQ, two MHC molecules expressed on the cell surface, were specifically downregulated in monocytes and upregulated in microglia. Additionally, these monocytes produced increased levels of IL-10 and TNF compared to microglia. These cytokines may increase the expression of CXCL13, Suppressor van cytokine signalling 3 (SOCS3) and downregulate CIITA, causing a more of a tolerized immune response\(^7,8,9\). Another paper, focused on changes in gene expression patterns upon exposure to *B. burgdorferi* with or without IFN-γ in human umbilical cord endothelial cells (HUVEC)\(^10\). *B. burgdorferi* significantly inhibited CIITA expression. In contrast, *B. burgdorferi* was combined with IFN-γ significantly enhanced CIITA expression in HUVECs.

RIP1 is involved in TNF and death pathways\(^11\). Upon inhibition of caspase-8, we observed no increase in antigen presentation molecule expression. However, when caspase-8 was suppressed, it might have resulted in cell death in the cells were antigen presentation was restored\(^12,13\), as higher doses tested induced significant cell death in the stimulated PBMCs. Of interest, RIP1 has also been suggested to play a role in the development of the M2 phenotype in macrophages, showing it may induce wider tolerogenic effects than just antigen presentation and cell death pathways\(^14\). This may be of interest for future studies.

To study the possible pathways involved in downregulation of antigen presentation molecule expression, the regulation of these antigen presentation molecules was further explored (Chapter 3). This research showed the importance of various gene expression patterns, especially of signal transducers such as signal transducers and activator of transcription (STAT)s. Transcription of MHCII and MHCII-related proteins is induced by CIITA, whose coding gene is called MHC2TA. However, to dock and recognize these gene sequences, CIITA is assisted by another complex called the MHCII enhanceosome. Moreover, the role of specific CIITA isoforms are
discussed, as well as mechanisms to inhibit CIITA or MHCII (-related) gene expression.

Following observations recently published, we investigated why IFNγ production is absent in the primary immune response against *B. burgdorferi* in Chapter 4. To investigate this, several different STAT signalling pathways were assessed in various immune cell subsets of peripheral blood mononuclear cells (PBMCs) who were exposed to *B. burgdorferi*. Phosphorylated STAT (pSTAT) signatures showed a prominent role for pSTAT3 during the primary immune response. Furthermore, significant upregulation of SOCS1 and SOCS3 gene expression was observed. We demonstrated this occurred not just in *in vitro* studies with primary cells, but also in LB patients followed over time. Of interest, a previous study showed a possible connection between STAT3 and RIP1 signalling. This study showed that RIP1 signalling cause subsequent activation of STAT3 similar to what we observed in our studies. If RIP1 may play a role in STAT3 phosphorylation induction in *B. burgdorferi* exposed and unexposed cells this would be valuable for our understanding of the phenotype of STAT3 signalling occurring in the various immune cells.

The activation of STAT3, SOCS1, SOCS3 and inhibition of STAT4 pathways may influence the expression of the antigen presentation machinery by interfering with specific CIITA isoforms. CIITA has four different promoters which can induce its transcription. These promoters are to an extent (immune) cell specific and can be induced through various mechanisms, including IFNγ signalling. We showed that during *B. burgdorferi* infection, specifically the isoforms expressed in innate immune cells were suppressed by the pathogen. These findings demonstrate on how these promoters may be differentially induced or inhibited via specific pathways and are vulnerable to influences from outside the cell and nucleus, through induction of certain signalling cascades and inflammatory mediators. Finally, as CIITA is not only a transcriptional regulator of MHCII, but also MHC1, we investigated whether *B. burgdorferi*-induced CIITA inhibition interferes with MHC1 expression. Our study showed that *B. burgdorferi* exposed immune cells downregulate MHC1 surface expression.

Both, CIITA and the resulting expression of antigen presentation proteins are dependent on intracellular signalling pathways. CIITA suppression may be related
to early IL-10, TNF and IL-6 production. While TNF may be involved in RIP1 inhibition of the antigen presentation machinery, IL-6 and IL-10 may activate STAT3, SOCS1 and SOCS3 cascades. Through these cascades *B. burgdorferi* interferes in the pro-inflammatory responses, leading to tolerance and antigen presentation molecule suppression during initial exposure.

In Chapter 5, the absence of IFN-γ, and possible upregulation of its production by IL-12, was further explored in a large cohort of foresters. Of interest, the immune responses were highly variable between individuals, as is often observed within the human population. However, in LB patients 6 weeks after the commence of antibiotic treatment IFN-γ levels were significantly higher. In Chapter 7, we show IL-12 and IL-18 production is absent upon exposure to *B. burgdorferi*, both in patient serum levels and *in vitro* studies. This may be related to the unchanged mRNA expression levels of IL-12p35 (Chapter 5), resulting in the STAT4 inhibition observed in Chapter 3.

In contrast to PBMCs, NK cells showed their ability to produce and release IFNγ in response to *B. burgdorferi* (Chapter 6). However, this was no longer the case in the presence of other immune cells from the PBMC fraction. NK cell activation may therefore be inhibited via direct or indirect immune cell interactions. How this phenotype is induced via the interaction with other immune cells will have to be investigated further. Moreover, antigen presentation pathways were inhibited in all innate immune cells studied, except for NK cells. Therefore, these cells may either not produce the same factors, produce less of the anti-inflammatory proteins produced by other immune cells or *B. burgdorferi* may induce different signalling pathways in these cells.

NK and NKT cells can infiltrate the skin upon infection or skin inflammation. NK cells are an innate immune cell subset important in the detection of intracellular infection, while NKT cells are a subset of T cells with a restricted α/β T cell receptor. Unlike other T cells, NKT cells do not recognize MHCI and MHCI/II peptide complexes but they can detect a diversity of glycolipids presented by CD1d. To assess their responses upon infiltration in *B. burgdorferi* infected skin and possible role in EM development NK(T) cells were incubated with *B. burgdorferi* and the factors they produced upon exposure were incubated with human keratinocytes to assess the response of these primary skin cells to their infiltration. NK(T) cells
significantly induced the expression of antimicrobial peptides, such as β-defensin-2 and psoriasin in human keratinocytes.

The knowledge on T and B responses during initial LB is very limited in humans. Whereas disseminated disease forms have been studied, data on the initial infection is often lacking. This is something we explored further in our in vitro systems and hoped to study in further detail in vivo in a clinical study, and while the design of the study has been finalized the study is yet to commence. In vitro observations demonstrated that T cell activation and function during LB was severely affected by *B. burgdorferi* (Chapter 2, Chapter 4 and Chapter 7). Whether this is also the case in patients will need to be investigated further.

Various approaches to restore production of IFNγ and the expression of HLA-DR during infection were explored in Chapter 5 and 7. IL-12 successfully restored IFNγ inhibition and HLA-DR expression, especially when combined with associated cytokines such as IL-18, IL-15 or IL-7, each inducing their own signature on the resulting immune response. In Chapter 7, we also demonstrated that IL-10R signalling is not significantly involved in antigen presentation inhibition. Moreover, transfer of supernatant 24h after exposure to *B. burgdorferi* did not result in the inhibition of responses against *C. albicans* as we witnessed in Chapter 2. This suggests that direct contact in the hours immediately after infection cause *B. burgdorferi*-induced inhibition of antigen presentation.

The restoration effect of IL-12 combined with IL-18, IL-7 and IL-15 helps us understand LB better and advance the development of new treatment and diagnostic strategies. However, it also raises new questions as to how *B. burgdorferi* specifically induces this antigen presentation inhibition. Furthermore, it will have to be assessed whether similar results are really witnessed in patients who have already been exposed for more than 8 days, due to the difference in time between infection, EM development and the actual doctor’s appointment. Moreover, IL-12 as a treatment has been explored previously and remains problematic due to the potential for rapid development of lethal inflammatory syndrome in patients. New measures, such as local treatment options, are currently being explored to enhance its safety in vivo.

While cytokines and chemokines are important signalling proteins in the immune response, signalling through reactive oxygen species play a key role in phagocytosis.
of bacteria. We demonstrated how *B. burgdorferi* downregulates phagocytotic pathways to induce a more anti-inflammatory environment in immune cells, resulting in decrease ROS production to a secondary stimulus (Chapter 8).

In Chapter 9 we reviewed the literature to describe the pathophysiological mechanisms involved in Lyme borreliosis patients, and specifically one of the most common forms of disseminated disease: Lyme arthritis. The consequences of disseminated *B. burgdorferi* infection showed the importance of the innate immune response. Often the initial immune response to the infection, can steer the course of the infection to a disseminated state. The presence of vital cytokines such IL-1Ra can make a major difference. Also, restoration of DC migration and well-balanced T helper cell responses may help clear the infection early on in the disease. Furthermore, the role of MMP production early on in infection, and the induction of these proteins through signalling cascades such as CD14. The molecular mimicry of these MMPs and GAGs that may trigger hyperinflammation in patients. Moreover, genetic pre-disposition with regards to the expression of specific HLA-DR polymorphisms and microRNA expression were discussed. Exploring all these pathways further may help understanding the disease and targeting the bacterium more successfully.
Figure 1: The interplay between the innate and adaptive immune system in Lyme Borreliosis: Possible pathways involved in inhibition of antigen presentation molecule expression during Lyme Borreliosis, resulting in an absence of T cell responses.

Conclusions

LB is a dynamic disease that can be caused by various genospecies creating a broad spectrum of symptoms, either during initial acute infection, acute disseminated or chronic disease. In my thesis, I have shown how *B. burgdorferi* may circumvent regular recognition pathways and clearance by downregulating primary immune responses, creating a tolerogenic environment in which innate and adaptive immune cell activation is inhibited. The present thesis described the key innate and adaptive immune responses during initial *B. burgdorferi* infection, as well as the consequences in later stages of the disease. The role of the inhibited expression antigen presentation machinery through RIP1 activation, specific suppression of CIITA isoforms in PBMCs, absence of IFN-γ production *in vitro* and possibly also in patients, as well as the STAT3, SOCS1 and SOCS3 signalling is crucial in the
pathophysiology of the disease. As we discussed in our Lyme Arthritis review, innate immunity is a major component in the dissemination of the pathogen and the systemic character of the disease. Therefore, further understanding of the interplay of the innate and adaptive immune system in early LB could be key in resolving and preventing development of disseminated disease in patients later on.

**Future perspectives**

LB is a complex disease with many different aspects and translation of the *in vitro* observations to *in vivo* studies is important to determine whether similar developments occur in the human body and how this develops over time. Further exploration of the method of CIITA inhibition is warranted. Moreover, antigen presentation molecule expression and presentation of *B. burgdorferi* peptides should be investigated during early infection stages in humans. Enhancement of STAT4 expression and specific signalling pathways related to pSTAT4 reduction may be assessed, as well as the role of STAT3 in inhibition of a strong immune response. As is often the case in infectious diseases, there is not a simple solution or a marker that is solely responsible. Various signalling pathways seem to be involved in *B. burgdorferi*’s evasion of the host immune response. In addition, large follow studies, following LB patients over time, such as LymeProspect 24, will hopefully give researchers a better indication of the changes that occur during initial infection that could be key in the development to disseminated forms of LB later on.

By exploring how innate and adaptive responses are manipulated during Lyme disease, this knowledge can be used to promote host recognition and clearance of the pathogen and help treat patients suffering from disseminated disease more effectively. Moreover, diagnostic tools to determine whether a patient has recently been exposed to the pathogen are important to treat the disease on time and prevent the development of disseminated disease.

**References**


Chapter 11

Nederlandse Samenvatting
Samenvatting

De door vectoren overgedragen ziekte Lyme Borreliose (LB) heeft een aanzienlijke invloed op de menselijke populatie van het noordwestelijk halfrond. Nu de prevalentie ervan geleidelijk toeneemt en er nieuwe stammen door Europa verspreiden, wordt het belangrijker om het gedrag van deze ziekteverwekker en de resulterende ziekte te begrijpen. *B. burgdorferi* heeft een divers repertoire van ontwijkingsmechanismen om herkenning van immuuncellen bij mensen te omzeilen. Dit resulteert vaak in lage of vertraagde immuunreacties bij mensen, wat invloed heeft op de standaard immunologische diagnostische tests zoals serologie en interferon-gamma-release-assays (IGRA). Bovendien is levende *B. burgdorferi* zeer moeilijk te detecteren in bloed en menselijke weefsels, zelfs bij aanwezigheid van symptomen. Daarom is het cruciaal om nieuwe diagnostische methodes te ontwikkelen om de ziekte vroeg te detecteren en te kunnen behandelen bij LB-patiënten. Hoewel de specifiek gedissemineerde vormen in de loop der jaren uitgebreid bestudeerd zijn, is er Weinig bekend over de pathogenese van de beginfase van LB en hoe deze zich kan ontwikkelen tot meer (acute) gedissemineerde vormen van de ziekte. Daarom hebben we in dit proefschrift het samenspel tussen het aangeboren en adaptieve immuunsysteem en *B. burgdorferi* bestudeerd tijdens de initiële infectie.

Antigeen presentatie is een belangrijk middel voor aangeboren immuuncellen om het adaptieve immuunsysteem te informeren over binnen gedrongen pathogenen. Eerdere *B. burgdorferi* studies hadden al verschillen in antigeen presentatie moleculen waargenomen, hebben relatief Weinig studies dit verder onderzocht. *B. burgdorferi* infectie treedt op wanneer een geïnfecteerde teek een mens bijt, waarbij zowel bacteriën als speeksel in de huid vrijkomen. Het tekenspeeksel bevat ook veel factoren die de immuunrespons onderdrukken (Hoofdstuk 1). Van *B. burgdorferi* is ook bekend dat het verschillende ontwijkingsstrategieën gebruikt, waar het zich bijvoorbeeld richt op het onderdrukken van het complementsysteem, de rekrutering van neutrofielen en de functie van dendritische cellen 1,2,3,4,5.

In dit proefschrift hebben we laten zien dat *B. burgdorferi* de expressie remt van een van de belangrijkste communicatie middelen voor aangeboren en adaptieve immuniteit, de meest voorkomende vorm van major histocompatibility complex klasse II (MHCII): HLA-DR (Hoofdstuk 2). Hier remde *B. burgdorferi* de expressie van antigeenpresentatiemoleculen door middel van receptor interaction serine/
threonine-proteine kinase 1 (RIP1)-signalering, waarschijnlijk via onderdrukking van de belangrijkste transcriptionele regulator, klasse II-transactivator (CIITA) genoemd. Dit leidde tot een significante remming van verschillende T-cel reacties. Bij hernieuwde blootstelling aan een ander pathogeen waarvan bekend is dat het deze reacties induceert, verlaagde *B. burgdorferi* anti-*C. albicans* immuunreacties aanzienlijk. In een studie waarbij er werd gekeken naar de rol van perifere bloedmonocyten en microglia, de macrofagen van het centrale zenuwstelsel die niet aanwezig zijn in het perifere bloed, in muizen tijdens *B. burgdorferi*-infectie, bevonden de onderzoekers dat de modulatie van verschillende MHC klasse II-gerelateerde genen sterk verschilde tussen de twee celtypen. Na 48 uur observeerde ze een significante inhibietie van talrijke HLA-geassocieerde genen in monocyten, terwijl hun expressie niet significant was veranderd in de microglia. HLA-DR en HLA-DQ, twee MHC-moleculen die op het celoppervlak tot expressie worden gebracht, werden specifiek onderdrukt in monocyten terwijl hun expressie juist toenam in microglia. Bovendien produceerden deze monocyten meer IL-10 en TNF in vergelijking met microglia. Deze cytokines kunnen de expressie van CXCL13, suppressor of cytokine signaling 3 (SOCS3) verhogen en CIITA expressie onderdrukken, en hierdoor een tolerante immuunrespons veroorzaken. Een ander artikel, gericht op veranderingen in gen expressie patronen gedurende *B. burgdorferi* blootstelling met of zonder IFN-γ in human umbilical cord endothelial cells (HUVEC). *B. burgdorferi* remde de CIITA-expressie significant. Daarentegen verbeterde *B. burgdorferi* gecombineerd met IFN-γ aanzienlijk de CIITA-expressie in HUVEC's.

RIP1 is betrokken bij TNF en cel dood signalering routes. Na remming van caspase-8 zagen we geen toename in expressie van antigeen presentatie moleculen. Wanneer caspase-8 echter wordt onderdrukt, leidt dit mogelijk tot celdood in de cellen waar de antigeen presentatie was hersteld, omdat hogere geteste doses significante celdood induceerden in de gestimuleerde PBMC's. Van belang is dat RIP1 ook een rol speelt bij de ontwikkeling van het M2-fenotype in macrofagen, wat aantoont dat het bredere tolerogene effecten teweeg brengen dan alleen antigeen presentatie en cel dood. Dit kan interessant zijn voor toekomstige studies.

Om de mogelijke routes die betrokken zijn bij onderdrukking van antigeen presentatie eiwit expressie verder te bestuderen, werd de regulatie van deze
antigeen presentatie moleculen verder onderzocht (Hoofdstuk 3). Dit onderzoek toonde het belang aan van signaaltransducers zoals signal transducers and activator of transcription (STAT)s. Transcriptie van MHCII en MHCII-gerelateerde eiwitten wordt geïnduceerd door CIITA, waarvan het coderende gen MHC2TA wordt genoemd. Om deze gen sequenties te koppelen en te herkennen, wordt CIITA echter bijgestaan door een ander complex, het MHCII-enhanceosome. Verder bespraken we de rol van specifieke CIITA-isoformen, evenals mechanismen om CIITA of MHCII (-gerelateerde) gen expressie te remmen.

Naar aanleiding van recent gepubliceerde observaties hebben we in Hoofdstuk 4 onderzocht waarom de productie van IFNγ afwezig is in de initiële immuunrespons tegen B. burgdorferi. Sinds de gefosforyleerde STAT (pSTAT) expressie een grote rol speelt in IFN-γ productie, hebben we de pSTAT expressie van verschillende STAT vormen bestudeerd. Hier toonden we een prominente rol voor pSTAT3 aan tijdens de primaire immuunrespons. Verder werd een significante toename van SOCS1- en SOCS3-gens expressie waargenomen. We observeerden dat dit niet alleen optrad in in vitro studies met primaire cellen, maar ook bij LB-patiënten die over langere tijd werden gevolgd. Een eerdere studie heeft een mogelijk verband aangetoond tussen STAT3 en RIP1 signalering. Dit onderzoek toonde aan dat RIP1 signalering de daaropvolgende activering van STAT3 veroorzaakt, vergelijkbaar met wat we in onze studies hebben waargenomen. Als RIP1 een rol zou kunnen spelen bij de inductie van STAT3 fosforylatie in aan B. burgdorferi blootgestelde en niet-blootgestelde cellen, zou dit ons begrip bevorderen van de STAT3 signalering in verschillende immuuncellen.

De activering van STAT3, SOCS1, SOCS3 en remming van STAT4 routes kan de expressie van de antigeen presentatie moleculen beïnvloeden door het odnerdrukken van specifieke CIITA isovormen. CIITA heeft vier verschillende promotoren waardoor de transcriptie ervan geïnduceerd kan worden. Deze promotoren zijn tot op zekere hoogte (immuun)celspecifiek en kunnen worden gecactiveerd via verschillende mechanismen, waaronder IFNγ signalering. We observeerden aan dat tijdens B. burgdorferi infectie, specifiek de isovormen die tot expressie worden gebracht in aangeboren immuuncellen werden onderdrukt door de ziekteverwekker. Deze bevindingen laten zien hoe deze promotoren op verschillende manieren aangezet of geremd kunnen worden, via specifieke routes. Daarnaast, zijn deze routes kwetsbaar voor invloeden van buiten de cel en de
celkern, door middle van activatie van signaalcascades en ontstekingsmediatoren. Aangezien CIITA niet alleen een transcriptionele regulator is van MHCII, maar ook van MHCI, hebben we onderzocht of door *B. burgdorferi* via remming van CIITA transcriptie ook MHCI expressie verstoort. Onze studie toonde aan dat aan *B. burgdorferi* blootgestelde immuuncellen de MHCI expressie aan het cel-oppervlak onderdrukt werd (Hoofdstuk 4).

Zowel CIITA als de resulterende expressie van antigeen presentatie eiwitten worden gereguleerd via intracellulaire signaal routes. CIITA onderdrukking is mogelijk afhankelijk van IL-10, TNF en IL-6 productie \(^7,8,9\). Terwijl TNF betrokken kan zijn bij RIP1 remming van de antigeen presentatie moleculen, kunnen IL-6 en IL-10 STAT3, SOCS1 en SOCS3 cascades activeren. Via deze cascades onderdrukt *B. burgdorferi* de pro-inflammatoire immuun reacties, wat leidt tot tolerantie en onderdrukking van antigeen presentatie moleculen tijdens de blootstelling.

In Hoofdstuk 5 werd de afwezigheid van IFN-\(\gamma\), en mogelijke toename van IFN-\(\gamma\) productie via IL-12 en IL-7 stimulatie verder onderzocht in een groot cohort van boswachters. Van belang was dat de immuunresponsen zeer variabel waren tussen individuen, zoals vaak wordt waargenomen bij immuunreacties binnen de menselijke populatie. Bij LB-patiënten waren echter 6 weken na het begin van de antibiotica behandeling de IFN-\(\gamma\)-spiegels significant hoger. In Hoofdstuk 7, laten we zien dat de productie van IL-12 en IL-18 afwezig is bij blootstelling aan *B. burgdorferi*, zowel in serum spiegels van patiënten als in *in vitro* studies. Dit kan te maken hebben met de onveranderde IL-12p35 mRNA expressie (Hoofdstuk 5), wat resulteert in de STAT4 remming die is waargenomen in Hoofdstuk 3.

In tegenstelling tot PBMC’s hadden NK cellen het vermogen om IFNy te produceren en af te geven in reactie op *B. burgdorferi* (Hoofdstuk 6). Dit was echter niet meer het geval in de aanwezigheid van andere immuun cellen uit de PBMC-fractie. NK-cel activering kan waarschijnlijk worden geremd via directe of indirecte interacties met andere immuun cellen. Hoe dit fenotype zich ontwikkeld via de interactie met andere immuun cellen zal nog nader moeten worden onderzocht. Bovendien werden antigeen presentatie routes geremd in alle bestudeerde aangeboren immuun cellen, behalve NK cellen. Daarom kunnen deze cellen ofwel niet dezelfde factoren produceren, minder van de ontstekingsremmende eiwitten produceren die door andere immuuncellen geproduceerd kunnen worden, of *B. burgdorferi* verschillende signaalroutes in deze cellen kan induceren.
NK en NKT cellen kunnen de huid infiltreren bij infectie of ontsteking\textsuperscript{17,18}. NK cellen zijn een subset van aangeboren immuuncellen die belangrijk zijn bij de detectie van intracellulaire infectie, terwijl NKT cellen een subset zijn van T cellen met een beperkte $\alpha/\beta$ T cel receptor\textsuperscript{19}. In tegenstelling tot andere T cellen herkennen NKT cellen geen MHCI en MHCII peptid complexen, maar kunnen ze een diversiteit aan glycolipiden detecteren die worden gepresenteerd door CD1d\textsuperscript{20}. Om hun reacties na infiltratie in met \textit{B. burgdorferi} geïnfecteerde huid en mogelijke rol in erythema migrans (EM) ontwikkeling te beoordelen, werden NK(T) cellen geïncubeerd met \textit{B. burgdorferi} en werden de factoren die ze produceerden bij blootstelling later toegevoegd aan menselijke keratinocyten om de respons van deze primaire huidcellen te beoordelen. NK(T) cellen veroorzaakten een significante expressie van antimicrobiële peptiden, zoals $\beta$-defensine-2 en psoriasin in de keratinocyten.

De kennis over T en B cel reacties in LB patiënten tijdens de initiële infectie is zeer beperkt. Terwijl gedissemineerde ziektevormen veel zijn bestudeerd, ontbreken vaak gegevens over de initiële \textit{B. burgdorferi} infectie. Daarom hebben we dit verder onderzocht in onze \textit{in vitro} systemen en we hoopten het daarna in een klinische studie in meer detail te bestuderen, terwijl de opzet en aanvraag van de studie is afgerond, moet de studie nog beginnen. \textit{In vitro} studies lieten zien dat de activatie en functie van T cellen tijdens de ziekte ernstig werd aangetast door \textit{B. burgdorferi} (Hoofdstuk 2, Hoofdstuk 4 en Hoofdstuk 7). In de klinische studie hopen we hier in de toekomst verder naar te kunnen kijken.

Verschillende manieren om IFN$\gamma$ productie en HLA-DR expressie tijdens \textit{B. burgdorferi} infectie te herstellen werden onderzocht in Hoofdstuk 5 en 7. IL-12 herstelde met succes de IFN$\gamma$ remming en HLA-DR expressie, vooral in combinatie met geassocieerde cytokines zoals IL-18, IL-15 of IL-7, die elk een verschillend effect hadden op de resulterende immuunrespons. In Hoofdstuk 7 toonde we ook aan dat IL-10R signalering niet significant betrokken was bij remming van antigeen presentatie. Bovendien resulteerde de overdracht van geproduceerde factoren na 24 uur \textit{B. burgdorferi} blootstelling niet in dezelfde remming van antigeen presentatie moleculen in reactie op de tweede stimulus: \textit{C. albicans}, zoals we in Hoofdstuk 2 hadden gezien. Dit suggereert dat direct contact in de uren direct na \textit{B. burgdorferi} infectie belangrijk is voor de remming van antigeen presentatie.

Het herstellende effect van IL-12 in combinatie met IL-18, IL-7 en IL-15 helpt ons LB beter te begrijpen en de ontwikkeling van nieuwe behandelingen- en diagnostische
strategieën te bevorderen. Het roept echter ook nieuwe vragen op over hoe *B. burgdorferi* specifiek deze onderdrukking van antigeen presentatie moleculen induceerde. Verder zullen we moeten beoordelen of vergelijkbare resultaten ook daadwerkelijk worden waargenomen bij patiënten die al langer dan 8 dagen zijn blootgesteld, vanwege het tijdsverschil tussen infectie, de EM ontwikkeling en de daadwerkelijke afspraak met de arts. Bovendien is IL-12 als behandeling eerder onderzocht en blijft het problematisch voor snelle ontwikkeling van dodelijk inflammatoir syndroom bij patiënten. Nieuwe maatregelen, zoals lokale behandelingsopties, worden momenteel onderzocht om de veiligheid van de behandeling te verbeteren.

Hoewel cytokinen en chemokinen belangrijke signaaleiwitten zijn in de immuunrespons, speelt signalering via reactieve zuurstof soorten een sleutelrol bij fagocytose van bacteriën. We hebben laten zien hoe *B. burgdorferi* fagocytose-routes remt om te overleven, en onder andere lagere ROS productie in reactie op een secundaire stimulus veroorzaakt (Hoofdstuk 8).

In Hoofdstuk 9 hebben we de literatuur bestudeerd om de pathofysiologische mechanismen te beschrijven die betrokken zijn bij Lyme-borreliosepatiënten, en in het bijzonder een van de meest voorkomende vormen van uitgezaaide ziekte: Lyme artritis. De gevolgen van gedissemineerde *B. burgdorferi* infectie toonden het belang van de aangeboren immuunrespons aan. Vaak kan de initiële immuunrespons op de infectie het verloop van de infectie aansturen en leiden naar een gedissemineerde toestand. De aanwezigheid van vitale cytokinen zoals IL-1Ra kan een groot verschil maken. Ook kan herstel van DC migratie en een goede T helper cel reactie helpen om de infectie vroeg op te ruimen. Verder spelen MMP productie in een vroeg stadium van infectie en de inductie van deze eiwitten door signaalcascades zoals CD14 een rol in de ontwikkeling van gedissemineerde LB. De moleculaire nabootsing van deze MMP's en GAG's kunnen hyperinflammatie veroorzaken bij LB patiënten. Bovendien wordt genetische predispositie met betrekking tot de expressie van specifieke HLA-DR-polymorfisme en microRNA-expressie besproken. Verder onderzoek hiernaar kan helpen om de ziekte beter te begrijpen en de bacterie uit te roeien.
Nederlandse Samenvatting

Conclusies

LB is een dynamische ziekte die veroorzaakt kan worden door verschillende genospecies met een breed spectrum aan symptomen, zowel tijdens een initiële acute infectie, acute gedissemineerde of chronische ziekte. In mijn proefschrift heb ik laten zien hoe B. burgdorferi de reguliere herkenningsroutes en klaring kan omzeilen door primaire immuun reacties te onderdrukken, waardoor er een tolerogene omgeving ontwikkeld waarin activatie van het aangeboren en adaptieve systeem wordt geremd. Dit proefschrift beschrijft belangrijke aangeboren en adaptieve immuun responsen tijdens de initiële B. burgdorferi infectie, evenals de gevolgen hiervan in latere stadia van de ziekte. De rol van de geremde antigeen presentatie molecuul expressie door RIP1 activering, specifieke onderdrukking van CIITA isovormen in PBMCs, afwezigheid van IFN-γ productie in vitro en mogelijk ook bij patiënten, evenals de activatie van STAT3-, SOCS1- en SOCS3 signalering is cruciaal in de pathofysiologie van de ziekte. Zoals we in onze Lyme Arthritis review hebben besproken, is aangeboren immuniteit een belangrijke component in de verspreiding van de ziekteverwekker en het systemische karakter van de ziekte. Daarom zou meer begrip van het samenspel van het aangeboren en adaptieve immuunsysteem in vroege LB de sleutel kunnen zijn bij het oplossen en voorkomen van gedissemineerde ziekte bij patiënten.

Toekomstperspectieven

LB is een complexe ziekte met veel verschillende aspecten en de vertaling van in vitro observaties naar in vivo studies is belangrijk om te bepalen of soortgelijke ontwikkelingen zich voordoen in het menselijk lichaam en hoe dit zich verder ontwikkelt over de tijd. Verdere studies naar van de methode van CIITA onderdrukking is belangrijk. Bovendien moeten de expressie van antigeen presentatie moleculen en de presentatie van B. burgdorferi-peptiden worden onderzocht tijdens vroege infectiestadia bij mensen. Versterking van STAT4 expressie en specifieke signaal routes gerelateerd aan pSTAT4 onderdrukking worden bestudeerd, evenals de rol van STAT3 in het remmen van de pro-inflammatoire immuun respons. Zoals vaak het geval is bij infectieziekten, is er geen eenvoudige oplossing of een marker die verantwoordelijk is. Verschillende signaalroutes lijken betrokken te zijn bij het ontwijken van de host immuun respons.
door *B. burgdorferi*. Daarnaast zijn er grote onderzoeken, waarbij LB-patiënten in de loop van de tijd gevolgd worden, zoals LymeProspect 24, die belangrijk zijn voor een beter begrip van de veranderingen die optreden tijdens de ontwikkeling van een lokale initiële infectie naar gedissemineerde LB.

Door te onderzoeken hoe aangeboren en adaptieve reacties worden gemanipuleerd tijdens de ziekte van Lyme, kunnen we de gastheerherkenning en klaring van de ziekteverwekker bevorderen en patiënten die lijden aan gedissemineerde LB effectiever behandelen. Bovendien zijn diagnostische hulpmiddelen om te bepalen of een patiënt recentelijk is blootgesteld aan de ziekteverwekker belangrijk om de ziekte op tijd te behandelen en de ontwikkeling van gedissemineerde LB te voorkomen.

**Referenties**


Chapter 12

Appendices
Acknowledgements

Ineens is het zover en moet je een hoofdstuk afsluiten en wordt het tijd om weer aan een nieuwe te beginnen. Deze laatste paar jaar heb ik zoveel geleerd, ervaringen opgedaan en heel veel lieve mensen leren kennen.

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### Portfolio

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<tr>
<td>01-10-2017</td>
<td>Radboudumc - Introduction day</td>
<td>6</td>
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<tr>
<td>01-09-2017</td>
<td>Reporting results to collaborators, patient representatives</td>
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<td>01-09-2017</td>
<td>Cytokine meeting</td>
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<td>01-06-2017</td>
<td>RIMLS PhD Council Member</td>
<td>98</td>
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<tr>
<td>02-03-2017</td>
<td>Radboud Research Rounds Guy Thwaites</td>
<td>4</td>
</tr>
<tr>
<td>01-02-2017</td>
<td>New Frontiers</td>
<td>60</td>
</tr>
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</table>

### Research data management

The data obtained in this thesis were managed according to the Findable, Accessible, Interoperable and Reusable (FAIR) principles (1). Raw and processed in vitro and in vivo data were stored on a local server of the department of internal medicine, which is backed up daily on the Radboud Server. Human studies were conducted according to the principles of the declaration of Helsinki and were approved by the Medical Ethics Committee of the Radboudumc. All participants gave written informed consent before participation. The privacy of participants in these studies is warranted by use of encrypted and unique individual subject codes. Data generated in this thesis are part of published articles and additional files are available from the associated corresponding authors upon request. The GWAS data discussed in Chapter 2 are publicly available at the gene expression omnibus (GEO) data repository, respectively.

List of publications


Curriculum Vitae

Michelle Brouwer was born on 12 december 1993 in Utrecht. In 2012 she finished her secondary school at Sint Maartenscollege in Voorburg. The same year she started her University study Biomedical Sciences at the Radboud University in Nijmegen.

In 2017, she finished her Master Biomedical Sciences Cum Laude at the Radboud University. She did her final internship at the Westmead Institute in Sydney, Australia. Here, she investigated the relationship between IFNα producing plasmacytoid dendritic cells and dendritic cells/macrophages in the establishment and transfer of HIV to T cells, supervised by Dr. Najla Nasr en Prof. Dr. Anthony Cunningham. During her studies she also researched the pre-erythrocytic stage of malaria and the synergy between mucosal adjuvant cholera toxin B and innate immune receptor agonists in newborns and adults.

After finalizing her Masters in 2017, she immediately started her PhD at the department of Internal Medicine at the Radboudumc in Nijmegen. Under supervision of Professor Leo Joosten and professor Mihai Netea she did a three year PhD on the interplay between the innate and adaptive immune response in Lyme borreliosis. Her PhD included both lab work and patient studies, of which part is described in this thesis and some were performed outside the scope of this thesis. During her PhD, Michelle also did the SMBWO course under supervision of Arnold van der Meer to become a registered Immunologist. After her PhD, she started working on the development of immune (-oncology) models at the organ-on-a-chip company MIMETAS.