Tabled by Dr Mualla McManus, 15 April 2016

Advances in Integrative Medicine 2 (2015) 81–89

Contents lists available at ScienceDirect

Advances in Integrative Medicine

journal homepage: www.elsevier.com/locate/aimed

Effects of *Borrelia* on host immune system: Possible consequences for diagnostics

Mualla McManus *, Ann Cincotta

Tick Borne Diseases Unit, School of Medical Sciences (Pharmacology), University of Sydney, 2006 NSW, Australia

ARTICLE INFO

Article history:
Received 10 August 2014
Accepted 6 November 2014
Available online 13 January 2015

Keywords:
Lyme disease
Borreliosis
Immune response
Immune dysfunction
Diagnostics
ELISA
Immunoblot
Stage 3 Borreliosis
Borrelia genospecies
Stages of Lyme disease (Borreliosis)

ABSTRACT

Borreliosis, Lyme disease, is the fastest growing tick borne infection in the world. Annually 300,000 (0.094%) people are diagnosed in the USA.

Objective: To clarify and aid in the understanding of the indirect diagnostics of Borreliosis in the light of immune dysfunction.

Diagnosis is difficult not only due to multi-systemic and nonspecific nature of symptoms but also due to the indirect diagnostics assuming immuno-competence in all three stages of Borreliosis. Indirect diagnostics are the most common method of testing for Borreliosis as they are cheap and convenient. However due to wide variation in antigenicity of genospecies, the sensitivity and specificity of diagnostics can be questioned. Evidence is accumulating which suggests that immune dysregulation induced by *Borrelia* (and other tick borne infections) can impact the indirect diagnostics, especially in Stage 3. The direct detection of *Borrelia* using nucleotide amplification method is possible but wider usage of this method is difficult as it has high specificity and narrow sensitivity. In vitro culturing is ideal but difficult as *Borrelia* has fastidious growth requirements.

The immune status of the borreliosis patient needs to be considered, especially in Stage 3 in conjunction with clinical symptoms in the diagnosis. *Borrelia* has the ability to manipulate both the innate and active immunity and alter the cytokines secreted hence alter the path of the immune response. Immune parameters such as IFN-gamma/IL-10, lymphocyte markers, complement C3a, C4a, and total immunoglobulin levels may help to discriminate between stages and monitor treatment outcomes. The level of immune dysfunction in Stage 3 may depend on the number of co-infections delivered by a tick bite, such as Babesia, and Rickettsia, the genospecies of *Borrelia*, other pathogens, the patients' biome and immunogenetics.

Borreliosis has the highest incidence of any tick borne bacterial infection in USA, Europe and Africa [1,2]. *Borrelia* can cause Lyme disease (*B. burgdorferi sensu stricto* (Bbss)), Lyme Borreliosis (*B. burgdorferi senso lato* group e.g. *B. garinii*, *B. afzelii* and Bbss) [3,4] and relapsing fever (e.g. *B. hermsii*, *B. parkeri*, *B. miyamotoi*) [2,5] (Table 1). Borreliosis will be used in this document to encompass all *Borrelia* infections as relapsing fever is often excluded from definition of Borreliosis. Non-specificity of symptoms, difficulty in culturing *Borrelia*, the diversity, the number of genospecies and the lack of sensitivity of indirect diagnostics make diagnosis of Borreliosis difficult. An over reliance on diagnostics or clinical symptoms can result in misdiagnosis. This paper will discuss immune dysfunction induced by *Borrelia* to disseminate and infect the host. Potential impact of immune dysregulation on indirect diagnostics and their limitations will also be discussed but coverage does not intent to be comprehensive.

1. Symptoms

Borrelia infection can result in multi-organ disease and is non-pathognomonic except in subcutaneous presentation such as the erythema migrans (EM) rash. Symptoms range from asymptomatic to debilitating. They can imitate many chronic diseases including motor neurone disease [6], multiple sclerosis [7], Parkinson's disease [8], Alzheimer's [9] and fibromyalgia [10] and CFS [11].

Presentation depends on many factors including the stage of the disease [12] and genospecies (Table 2).

2. Stages of Borreliosis

Stage 1 (early localised) symptoms can begin within 3 days of the tick bite with flu-like symptoms, fever, headache, myalgia, joint

* Corresponding author. Tel.: +61 449838887; fax: +61 291442585.
E-mail addresses: mualla.mcmanus@sydney.edu.au, makinci88@gmail.com (M. McManus).

http://dx.doi.org/10.1016/j.aimed.2014.11.002
2212-9588/

Table 1
Borrelia genospecies, distribution and vectors.

Borrelia genospecies	Distribution	Vectors
Lyme disease borreliae		
B. burgdorferi sensu stricto	North America, Europe, North Africa, China, Taiwan	*Ixodes scapularis, I. ricinus, I. pacificus*
B. garinii	Europe, Russia, China, Japan, Canada, Korea, Mongolia, North Africa, subantartic islands	**I. ricinus, I. persulcatus**, *I. cantsuga, I. hexagonus Boophilus* sp.
B. afzelii	Europe, Russia, China, Japan, Korea, Mongolia	**I. ricinus, I. persulcatus**, *I. cantsuga, I. hexagonus, Boophilus* sp., *Dermacentor* spp., *Haemaphysalis* spp.
B. japonica	Japan	*I. ovatus*
B. lusitaniae	Southwestern Europe, North Africa, Central Europe, Turkey, Scandinavia	**I. ricinus**, *I. uriae, I. hexagonus, D. marginatus*
B. valaisiana	Europe, China, Japan, South Korea, Taiwan	**I. ricinus**, *I. hexagonus, I. uriae, D. merginatus*
B. bissetti	USA (West, Southeast, Upper Midwest), Central Europe	**I. scapularis**, *I. affinis, I. canisuga, I. minor, I. pacificus, I. ricinus*
B. spielmanii	Central Europe, Hungary, Ukraine	**I. ricinus**, *I. hexagonus*
B. bavariensis	Central Europe, Eastern Europe, Russia, Central Asia, China, Japan	*I. ricinus, I. persculatus, I. triangultceps, D. reticulatus*
B. kurtenbachii	USA (East, North), Canada, Europe?	*I. pacificus, I. scapularis, I. spiipalpis*
B. finlandensis	Finland	*I. ricinus*
B. andersoni	North America	*I. dentatus*
B. californiensis	USA	*I. pacificus, I. spinipalpis, D. californicus*
B. americana	USA	*I. pacificus, I. minor*
B. carolinensis	USA	*I. minor*
B. tanukii	Japan	*I. tanuki*
B. turdi	Japan	*I. turdus*
B. sinica	China	*I. ovatus*
B. yangtzee	China	*Haemaphysalis longicornis, I. granulatus*
B. chilensis	Chile	*I. stilesi*
B. burgdorferi sensu lato Haplotype A, B, C, D, E	Uruguay	*I. pararicinus*
Relapsing fever Borreliae – new world		
B. hermsii	USA (West)	*Ornithodoros hermsii*
B. turicatae	USA (Southwestern), Mexico	*O. turicata*
B. pakeri	USA (West)	*O. parkeri*
B. miyamotoi	USA (Northeastern), Russia, Canada	*I. persculatus, I. scapularis, I. pacificus*
B. mazzottii	Central America	*O. talaje*
B. venezuelensis	Central America	*O. rudis*
Relapsing fever Borreliae – old world		
B. duttoni	Sub-Saharan Africa	*O. moubata*
B. crocidurae	North Africa, Middle East	*O. erraticus*
B. persica	Middle East, Central Asia	*O. tholozani*
B. hispanica	Iberian Peninsula, North Africa	*O. marocanus*
B. latyschewii	Iran, Iraq, Eastern Europe	*O. tartakowskyi*
B. caucasia	Iraq, Eastern Europe	*O. asperus*
Reptile Borrelia		
B. turica	Turkey	*Hyalomma aegyptium*
Borrelia sp. *GP*	Zambia	*Amblyomma Sparsum*
Borrelia sp. *BF*	Sri Lanka	*Amblyomma trimaculatem*
Borrelia sp. *ST*	Ghana	*Amblyomma latum*

[78–89].

pain, stiff neck, fatigue and EM rash, their occurrence dependent on the genospecies [13] (Table 2).

Stage 2 (early disseminated) occurs weeks to months after initial infection and can present with generalised lymphadenopathy and fatigue. Neurological manifestations may include encephalitis, cranial neuritis, radiculoneuritis, paresis, carditis and migratory musculoskeletal symptoms (Table 2).

Stage 3 (late disseminated) occurs from months to years after the initial infection in patients who are not treated or inadequately

Table 2
Clinical signs and symptoms of tick borne Borreliosis [64,12].

Borreliosis stage and time frame	Signs and symptoms	Lyme Borreliosis group		Relapsing fever Borreliosis group
Geographical location		North America	Eurasia	Africa, Europe, North, Central, South America, Asia
Main causative agent		*B. burgdorferi sensu stricto*	*B. garinii, B. afzelii*	*B. hermsii, B. duttonii, B. miyamotoi*
Stage 1 Early localised up to 1 month	Constitutional	Fever, chills, fatigue, lethargy, lymphadenopathy	Fever, chills, fatigue, lethargy, lymphadenopathy	Fever, relapsing with rigours and headache Fatigue Lethargy
	Skin	Erythema migrans	Erythema migrans Lymphocytoma cutis	No obvious initial skin involvement

Table 2 (*Continued*)

Borreliosis stage and time frame	Signs and symptoms	Lyme Borreliosis group		Relapsing fever Borreliosis group
Stage 2 Early disseminated 1–4 months	Constitutional	Fever, chills, fatigue, lethargy, lymphadenopathy	Fever, chills, fatigue, lethargy, lymphadenopathy	Fever, relapsing with rigours and headache Fatigue Lethargy
	Skin	Multiple erythema migrans	Multiple erythema migrans that can be associated with lymphocytoma cutis	
	Musculo-skeletal	Myalgia Arthralgia Arthritis-severe asymmetric oligoarticular joint inflammation Muscle weakness, twitches, tremors	Myalgia Arthralgia Arthritis-less intense inflammation	Myalgia Arthralgia Arthritis
	Cardiac	Atrioventricular block and myocarditis	Atrioventricular block and myocarditis	Carditis
	Eyes	Conjunctivitis Photophobia	Conjunctivitis Photophobia	Iritis Iridocyclitis Photophobia
	Neurological	Headache, neck stiffness, Meningismus with or without cranial neuropathy	Fewer meningeal signs, prominent radiculopathy, encephalomyelitis and cranial neuropathy, including facial palsy, optic neuritis, vestibular neuronitis and oculomotor palsy (mostly *B. garinii*) cognitive abnormalities	Meningitis Cranial-nerve palsies Encephalitis Hemiplegia Seizures Coma
	Haemorrhage			Cerebral haemorrhage Petechiae Epistaxis Haemoptysis Haematuria Haematemesis
Stage 3 Late disseminated >4 months	Skin	Rare	Acrodermatitis Chronica Atrophins (ACA – *B. afzelii*)	Rash
	Musculo-skeletal	Treatment resistant arthritis, muscle weakness, abnormal muscle twitches, tremors	Rare	Myalgia Arthralgia Arthritis
	Cardiac	Dilated cardiomyopathy, endocarditis, heart failure, chest pain, conduction and rhythm disturbances, shortness of breath	Congestive cardiomyopathy, endocarditis, cardiomyopathy, angina, arrhythmias, dyspnoea	Carditis, myocarditis Congestive cardiomyopathy, endocarditis, cardiomyopathy, angina, arrhythmias, dyspnoea
	Ophthalmological	Conjunctivitis, keratoconjunctivitis sicca, foggy or flickering vision	Conjunctivitis, keratoconjunctivitis circa, foggy or flickering vision	Iritis Iridocyclitis
	Neurological	Paresthesias, radiculopathy, encephalopathy, sleep disturbance, cognitive deficits such as impaired memory and impaired concentration, dizziness, polyneuropathy, numbness, tingling, Bell's palsy, speech and swallowing difficulties, gait disturbance, depression, paranoia, anxiety, panic attacks, hallucinations, photophobia	Paresthesias, radiculopathy, sleep disturbance, cognitive deficits such as impaired memory and impaired concentration, sensory deficits in areas affected by ACA, encephalopathy, dizziness, polyneuropathy, numbness, tingling, Bell's palsy, speech and swallowing difficulties, gait disturbance, depression, paranoia, anxiety, panic attacks, hallucinations, photophobia	Meningitis, cranial-nerve palsies, encephalitis, hemiplegia, Seizures, coma, paresthesias, radiculopathy, insomnia, hypersomnia, narcolepsy, catalepsy cognitive deficits such as impaired memory and impaired concentration, dizziness, polyneuropathy, extra-pyramidal symptoms, tingling, Bell's palsy, dysarthia and dysphagia, gait disturbance, flaccid paralysis depression, paranoia, anxiety, panic attacks, hallucinations, photophobia
	Gastro-intestinal	Gastritis, nausea, vomiting, diarrhoea, constipation, stomach pain	Gastritis, nausea, vomiting, diarrhoea, constipation, stomach pain	Gastritis, nausea, vomiting, diarrhoea, constipation, stomach pain, jaundice, hepatamegaly, spleenomegaly
	Urogenital	Irritable bladder, interstitial cystitis, testicular or pelvic pain, decreased libido, unexplained menstrual irregularity, unexplained milk production	Irritable bladder, interstitial cystitis, testicular or pelvic pain, decreased libido, unexplained menstrual irregularity, unexplained milk production	Irritable bladder, interstitial cystitis, testicular or pelvic pain, decreased libido, unexplained menstrual irregularity, unexplained milk production?
	Haemorrhage			Cerebral haemorrhage Petechiae Epistaxis Haemoptysis Haematuria Haematemesis
	Fatality rate			
	Spirochaetaemia	Very low density spirochaetaemia in blood, micro-aerophillic and disseminates from hematogenous areas to tissue such as CNS, synovium, skin		High density spirochaetaemia in blood

treated [14]. It can develop with gradual intensity from Stage 1 [15]. Symptoms seen in Stage 3 are usually more severe and include neurological, cardiac, dermatological, cognitive and arthritic presentations (Table 2).

3. Immune evasion

Micro-organisms have developed sophisticated methods of facilitating survival in host. *Borrelia*, a spirochete bacterium, has

evolved many strategies to ensure survival including utilising arthropod salivary proteins (sialostatin, Sal 15) to assist establishment of infection by inhibiting T and dendritic cells [16,17]. *Borrelia* prefers micro-anaerobic environments where the immune surveillance is low such as the CNS, joints and skin. To survive in the host, *Borrelia* has adopted a communication mechanism, quorum sensing to communicate with other bacteria in the colony network [18]. *Borrelia* can inhabit biofilm with other symbiotic pathogens and evade the immune system and antimicrobials [19]. It can create and release blebs, encapsulated bits of *Borrelia* DNA to distract the immune system [20].

Borrelia is a pleomorphic bacterium (exists as spirochaetal, L- and cyst form) [9], with a very slow replication rate (12–24 h in vitro) [21]. *Borrelia* can employ multiple methods of antigenic variation of its outer surface proteins to evade immune detection. It has 21 or more plasmids which enable it to change antigenicity and adapt its survival in the tick and the host [22,23]. *B. hermsii*, relapsing fever *Borrelia* alters its variable outer membrane protein (Vmp) regularly which elicits waves of spirochaetemia of different antigen type, and as a result prolonged IgM response [24]. Whereas, *B. burgdorferi sensu lato* employs segmental recombination which results in a large number of *Borrelia* strains each with different VlsE (variable membrane protein-like sequence expressed lipoprotein) [24]. These differences can be responsible for symptom variation between genospecies [25,3].

The L-form lacks a cell wall allowing intracellular location and evasion of the immune system cystic forms (intra- or extracellular) that may be dormant, non-metabolising, and non-immunogenic, and may represent persistent *Borrelia* infection [9]. The clinical relevance of pleomorphic forms is not well understood [26]. Brazilian *Borrelia* (Baggio–Yoshinari syndrome) is unusual in that only the L-form has been detected, not a spirochaete [27]. *Borrelia* has the capacity to move faster than a human neutrophil, the fastest moving immune cell [28] which represents another survival mechanism.

With such efficient survival methods it may be difficult to eradicate in the disseminated stage.

4. Immune dysregulation

There is a growing body of evidence in literature that indicate the presence of significant immune dysfunction in tick borne infections including Borreliosis. The level of immune dysfunction is determined by the host's immune system, number of tick bites, pathogen load, genospecies, strains and the number of co-infections delivered by the tick [24,29,30]. Alteration of *Borrelia* outer surface proteins (Osp), A–E expression inhibits complement activation which aids to establish infection [31–33].

4.1. T cell dependent/independent response

Immune response to *Borrelia* involves pattern recognition receptors (PRR)-like Toll-like receptors (TLR) or intracellular nucleotide binding oligomerisation domain ((NOD 1,2) proteins) [34] and C-type lectin receptors such as the mannose receptor [32]. The recognition by dendritic cells initiate the production of immune regulatory cytokines which affect innate and the adaptive immune system. Recognition of *Borrelia* by TLR7 and TLR9 induces IFN-alpha and beta response in human immune cells [35,36]. Generally, IFN-alpha is produced in response to viral infections and IFN-gamma in response to viral and intracellular bacterial infections [29]. In Stage 1 Borreliosis there is no or minimal disruption to IFN alpha, beta, and gamma production [37–39]. However in Stage 3 there may be diminished or no IFN-gamma [40] and increased IFN-alpha production [41]. Normally IFN-gamma induces an efficacious immune response by the development of the Th1, T cell immunity, involving IL-1 and opsonising complement fixing antibodies and activation of NK cell mediated immunity [42]. In contrast, the Th2, T cell independent pathway phenotype, results in IL-4 production and plasma cells producing non-cytolytic antibodies, an inefficient immune response. With diminished IFN-gamma, there is preferential diversion to the Th2 response (Fig. 1). Th1 response addresses intracellular and extracellular infections while Th2 response mainly addresses extracellular infections [34,24]. *Borrelia* can evade the Th2 response by switching to the intracellular L-form. This switch may reflect the transition from Stage 1/2 to Stage 3 Borreliosis.

Borrelia can also induce the production of anti-inflammatory cytokines, IL-10 [43], adrenomedullin [44] and anti-alarmins [45]. This immune dysregulation may be reflected in the ratio of IFN-gamma and IL-10 and may correlate to the Stage of *Borrelia* infection. The normalisation of this ratio may also reflect the recovery of the immune system and the effectiveness of the treatment protocols. However, due to the diversity of the immune dysfunction the standard IFN-gamma/IL-10 ratio may not be applicable to all Stage 3 patients. The clinical symptoms, the length of illness, the co-infections and other immune parameters need to be considered.

Generally, isotype switching from low affinity (IgM) to high affinity (IgG) antibodies results in increased efficacy of the immune system. T cell dependent response expression of CD40 and/or COX 1 receptors enables the secretion of different Ig isotypes, formation of germinal centres and memory B cell establishment [46,47]. Th1 and IFN-gamma can induce the production of IgG1 (high affinity) antibodies. Cytokine release can be manipulated by *Borrelia* and alter the level of Ig isotypes and isotype subclasses. For example IL-4 has a role in IgG4, IgE production. IL-5 and TGF-beta has role in IgA production [48,49]. In Stage 3 Borreliosis total antibody isotypes' levels can vary significantly in mice [50]. IgE levels can alter significantly in the presence of parasites and increase the risk of allergies as documented for red meat following tick bites [51,52] (Fig. 2).

4.2. Modulation of NF-k beta (nuclear factor-k beta) by Borrelia

Borrelia inhibits CD14, a co-receptor of both TLR2 and TLR4 and complement receptor 3 (CR3) [53]. Inhibition of TLR2 and TLR7 receptors by *Borrelia* modifies NF-k beta activation and not only diminishes the antibody mediated T cell cytotoxicity but also determines the types of interferon being released [54,36]. NF-k beta regulates the activation of different cytokine genes thereby controlling which phenotype (Th1or Th2) is activated. The Th2 phenotype results in IL-4 production and plasma cells producing non-cytolytic antibodies, an inefficient immune response (Fig. 1). This is in contrast to the Th1 phenotype response resulting in IFN-gamma, IL-1 and opsonising complement fixing antibodies and activation of NK cell mediated immunity, a more efficacious immune response [42].

Measuring complement proteins C3a, C4a, NK cell marker (CD57), a plasma cell marker (CD19) from germinal centres, T helper cells (CD4), cytotoxic T cells (CD8), and dendritic cells (CD14) involved in Th1 immune response levels can reflect the individual patient's heterogeneity of immune dysfunction [55,56].

5. Diagnostics

Interpretation of diagnostic results needs be in the context of symptomology, risk of tick exposure and/or travel history. Immune status needs to be considered in diagnosis, particularly in suspected Stage 3 Borreliosis. This may impact on the sensitivity of diagnostic tests that are based upon lymphocyte (B or T cell) mediated immune response to *Borrelia*.

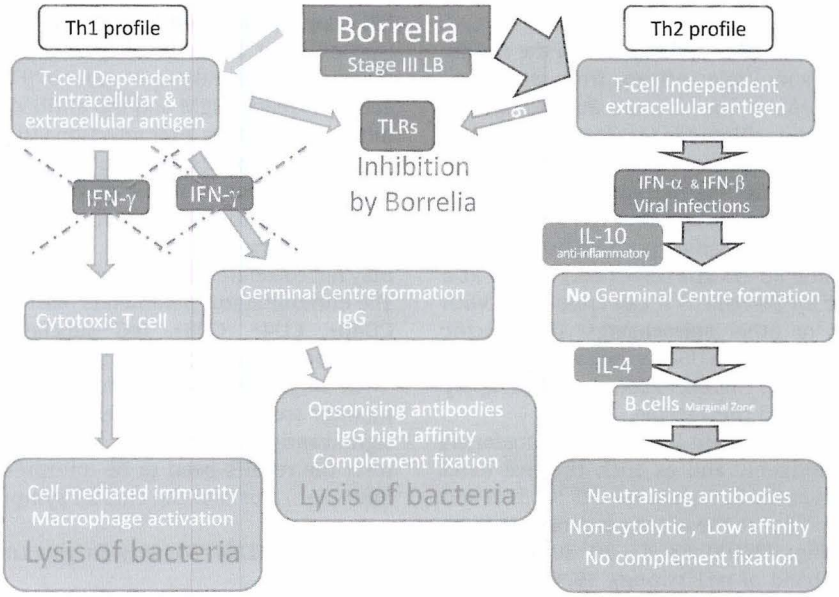

Fig. 1. Summary of immune response to *Borrelia*. Th1 profile is where the immune system is most efficient. Th1 is initiated by *Borrelia* proteins (Stages 1 and 2) and there is recruitment of T cells in antigen processing. Th2 profile in contrast involves immune response to *Borrelia* without or very limited T cell support and involves T cell independent antigens. *Borrelia* cell membrane has different antigens and they are processed differently from other Gram-negative bacteria. Germinal centres are sites in secondary lymphoid organs to differentiate and mature and sites where Ig isotype switching can occur.

5.1. B cell based Indirect Serology Assays – the 2 tier system

The CDC USA recommends the 2 tier test criteria. The first tier assay is ELISA/IFA, and if either test is positive or equivocal a confirmatory Immunoblot/Western blot (WB) test is performed. This allows for any false positives to be detected by the more specific WB. Indirect assays based on antibody production assume immune-competency and as outlined above, Borreliosis patients (particularly Stage 3) can present with diversity of immune dysfunction [24,40,41,43,44,46,57].

5.1.1. ELISA/IFA – Indirect Immunoassay

ELISA (Enzyme Linked Immuno Absorbent Serology Assay) and IFA (Immuno Fluorescent Assay are simple, inexpensive, fast and multiple patients can be tested in one assay. It is ideal for infections where there is one known pathogenic species and bacterial/viral antigens are relatively stable. Multiple *Borrelia* genospecies, with great antigenic variability, is problematic for ELISA sensitivity. Routinely ELISA is employed for IgG and/or IgM.

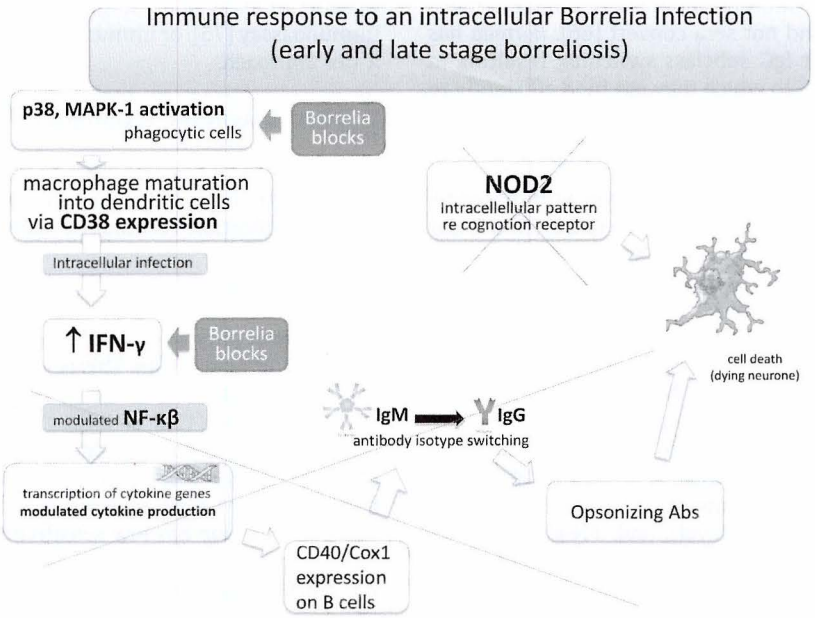

Fig. 2. Mechanism of immune modulation by *Borrelia*. Inhibition of P38, an MAPK-1 (mitogen activated protein kinase 1), an extracellular signal regulating kinase involved in proliferation and differentiation by *Borrelia*, leads to inhibition of CD38 which is crucial in maturation of dendritic cells. Without active dendritic cells *Borrelia* is not phagocytised and intracellular pattern recognition receptors NOD2 are not activated. Inhibition of CD3 and CD14 (co-receptor that activate TLR2) prevents complement activation. There is diminished IFN gamma production and ensuing NF-k beta modulation cause altered cytokine production which is bias towards Th2 immune response and allow *Borrelia* to disseminate uninhibited. This scenario is more likely to occur in Stage 3 Borreliosis.

5.1.2. Immunoblot/Western blot

The immunoblot assay is normally used to confirm the results of ELISA and IFA and employs multiple antigens from whole cell lysates or recombinant antigens of one or more *Borrelia* species increasing specificity [58]. Immunoblots can test for an IgM or IgG response. A positive IgM immunoblot constitutes of two bands from 23–25, 39 and 41 kDa if performed within 1 month of tick bite/symptom development (CDC USA). A positive IgG immunoblot criterion is 5 bands out of 10 to be classified positive from 18, 23–25, 28, 30, 39, 41, 45, 58, 66 and 83–93 kDa (CDC USA). The 41 kDa flagellin band is not specific for *Borrelia* as it can cross-react with antibodies against syphilis or other spirochaetes. The specific bands 31 and 34 kDa are not accepted in USA as they were used in a vaccine against *Borrelia* previously. This exclusion should not apply to patients who have not received the vaccine.

Antigenicity is also species dependent [59], with *B. burgdorferi sensu stricto* being highly antigenic and as such the five band criteria is appropriate; however the European species *B. afzelii* and *B. garinii* are less antigenic and hence the criteria is proposed to be ≥2 IgG bands of the following: 83/100, 58, 43, 39, 30, OspC, 21, Osp17, 14 kDa and ≥1 IgM band of p41(strong), 39, Ospc, DbpA (Osp17). CDC USA criteria need to be re-evaluated when patients present with a Lyme-like illness from an area of indeterminate endemicity. In such cases a detailed travel history for possible exposure in endemic areas (Europe, USA, Asia and Africa) and a broad approach to testing is recommended where a link cannot be made through travel history. The CDC USA criteria may not be suitable for Eurasian Lyme Borreliosis or the relapsing fever *Borrelia* which is endemic in Europe, Asia, Africa, South America, Middle East and the West Coast of USA [60].

5.1.3. Limitations of ELISA/IFA/WB

If tested in early infection, <4 weeks post-tick bite, a false negative may result from insufficient or delayed IgM production [61]. If IgM does not seroconvert to IgG >1 month this is classified as a negative but can be false negative [62]. The role of IgM in intracellular infections and relapsing fever *Borrelia* [63,64] is often not considered neither is IgE [52] nor IgA [65]. Studies in macaque's monkeys have shown that immunosuppressed monkeys have continually produced IgM and not sera convert [66]. *Borrelia* has been demonstrated to affect IgG subclass switching resulting in production of low affinity IgG2b which may not bind efficiently to antigen and may result in a false negative [50]. Sensitivity is also dependent on the type of antigen used (recombinant or whole cell lysate), the *Borrelia* species and the strain used. Antibodies raised against a European species may not be detected on a test based on antigens from USA species reducing sensitivity to 22% in a 2 tier assay [67]. There are multiple serology assay kits available and without standardisation there can be variability between kits [68].

In some situations with antibiotic therapy for 4–6 weeks patients can seroconvert and if retested return positive IgG [69].

The immune status of the patient in conjunction with clinical symptoms needs to be considered in the diagnosis. To assist diagnosis of potential Stage 3 (possibly Stage 2) Borreliosis where WB bands do not meet positive criteria it may be helpful to consider immune status parameters such as CD40+, CD14+, CD4+, CD8+, C57+CD3−, CD19+ other lymphocyte markers, total IgG (subclasses), IgA, IgE, IgM and IgD levels, and IFN-gamma/IL-10 ratio. Any abnormalities in these immune parameters can aid in the diagnosis, monitor therapy and recovery from Borreliosis.

5.2. T cell based assays

5.2.1. ELISPOT – Enzyme Linked Immunoabsorbent Spot Assay

A common way of measuring T cell response to *Borrelia* is ELISPOT – Lymphocyte Transformation Test which relies on the PBL (peripheral blood lymphocyte) response to *Borrelia* antigens (recombinant, or whole cell lysate). Response is measured either as ^{3}H-thymidine uptake or number of IFN-gamma producing cells. In acute Borreliosis IgM and IgG antibodies are not easily detectable until several weeks after infection [70] and any significant immune dysfunction would be negligible. Therefore ELISPOT test would be useful in Stages 1 and 2 of Borreliosis. If there is an immune response shift to Th2 state with decreased levels of IFN-gamma and T cell response (CD14, TLR2, TLR4 inhibition) the ELISPOT result may not correlate with clinical symptoms, most relevant in Stage 3. The immune status parameters like the IFN-gamma/IL-10 ratio and CD40+, CD4+, CD8+, IgG subclasses especially IgG3 would corroborate the negative ELISPOT results.

In Stage 1 and 2 Borreliosis ELISPOT can be a useful tool to monitor treatment outcomes as positive ELISPOT results decline significantly post-antibiotic treatment [71,72]. Like other indirect tests the results need to be interpreted in conjunction with the immune status and the clinical symptoms of the patient.

5.2.2. LTT-MELISA (Memory Enzyme Linked Immunostimulation Assay)

MELISA is also a lymphocyte transformation assay that measures T cell response to *Borrelia* [73]. MELISA uses well defined recombinant *Borrelia* antigens, not whole cell lysates, higher number of PBL and claim to have high reproducibility and clinical use as it measures the activation of memory lymphocytes, that is, the state of active infection. Like other indirect tests the results need to be interpreted in conjunction with the immune status and the clinical symptoms of the patient.

6. C6 antigen assay – VlsE C6 peptide assay

The C6 antigen assay (26 mer peptide from the sixth invariable region of VlsE) of *B. burgdorferi* [61] should be sensitive and specific as this region is conserved across the genus. In USA C6 assay failed to detect 1 out 3 ribosomal spacer defined genotypes of *Borrelia* [74]. This would translate to 33% failure of the C6 antigen assay in the USA East Coast the centre of *B. burgdorferi sensu stricto* infection. As a result a single C6 test approach recombinant VslE immunoassay [75], or immunoblot [60], was not as sensitive as the 2 tier approach.

7. Direct Diagnostic Techniques

Commonly employed Direct Diagnostic Techniques include culture from tissue specimens, microscopy techniques and Nucleic Acid Amplification Techniques (NAAT).

7.1. Culture and microscopy

Culture of micro-organisms has long been considered the "gold standard" of diagnostics. This is also true for *Borrelia* but there are limitations. Culture can take weeks to months due to prolonged generation time (12 h or longer) [76,21]; it is expensive, labour intensive; and cannot be used once antimicrobial therapy has been initiated [7]. Skin biopsy samples (EM rash or ACA skin lesions and lymphocytoma) show reasonable sensitivity; however CSF, blood or synovial tissue has low sensitivity [77].

7.2. Nucleic Acid Amplification Techniques (NAAT)

In regions where epidemiological, clinical or serological data are available it is possible to develop specific molecular tools for the detection of *Borrelia*. NAAT employ Polymerase Chain Reaction (PCR) a sensitive, specific and fast method of detection to allow for early treatment. The target gene ideally is sufficiently conserved to

allow amplification of multiple species of *Borrelia* but sufficiently different to allow discrimination between species [78]. Primers define the specificity of the PCR and the sensitivity is influenced by reaction parameters such as annealing temperatures, concentration of primers, quality and quantity of template DNA and the presence of inhibitors. Currently there are 18 species within the Lyme Borreliosis (LB) group [79] and 18 species within the Relapsing fever group [80,81] (Table 1) and endemic regions are expanding in both Northern and Southern hemispheres and clinicians need to consider carefully the patient information in regards to travel to possible endemic regions. If tick bite occurred in a country of indeterminate endemicity then a more conserved approach to gene targets would be warranted.

NAAT has limitations (Table 3); PCR results show reasonable sensitivity with cutaneous presentations of Borreliosis but low sensitivity in extra-cutaneous presentations such as neuroborreliosis. Skin biopsies have higher sensitivity than CSF, blood, plasma and synovial tissue [77]. False negatives (inappropriate choice of primers or PCR conditions or presence of inhibitors) and false positives (due to contamination) are possible. Numbers of spirochaetes in tissue, storage and transport of tissue affect the outcome [82]. In cases of suspected relapsing fever, blood collection should occur at the time of fever, when the bacteraemia is high [2].

Real time PCR (rtPCR) is a more sensitive, quantitative method [83]. Multiplex rtPCR can be conducted on either multiple genospecies of *Borrelia* or in addition multiple tick pathogens [83].

8. Summary

Understanding the immune dysregulation induced by *Borrelia* and co-infections can aid the interpretation of diagnostics and improve diagnosis of Borreliosis. Present diagnostics do not discriminate between different stages of Borreliosis. In addition indirect diagnostics reliant on the immune response assume immuno-competence of the host in all stages of Borreliosis. This document has tried to highlight that apart from immune evasion aided by tick saliva in Stages 1 and 2, there is ongoing immune dysfunction in the established disseminated Stage 3 Borreliosis. The limitations of indirect diagnostics are highlighted in the context of these assumptions. Immune status parameters such as IFN-gamma/IL-10 ratio are suggested as an objective method of discriminating between stages, monitor efficacy of treatment, restoration of T cell dependent, and a more efficacious immune response. The possible reason for the prolonged IgM response observed in Stage 3 Borreliosis may be explained either by genospecies involved in symptomology is relapsing fever *Borrelia*

Table 3
Comparison of diagnostic tests for Borreliosis. Indirect tests that rely on an immune response are contraindicated in immunocompromised individuals.

	Advantages	Limitations
Indirect Diagnostic Test		
ELISA – Enzyme Linked Immuno-sorbent Assay	• Inexpensive • Gives an indication of whether IgM or IgG immunoglobulins can be detected against *Borrelia* antigens	• Sensitivity species dependent • Cross-reactivity of some antigens (Flagellin) • Not distinguish from active and past infection clearly • A specific prolonged IgM response for relapsing fever can be interpreted as false positive
Western blot	• Higher specificity than ELISA • Allows discrimination between genus and species specific antigens	• Sensitivity can be species dependent e.g.; relapsing fever *Borrelia* vs Lyme *Borrelia* • Not distinguish between active or past infection • Prolonged IgM response for relapsing fever may be interpreted as a false positive • Immunogenic diversity in genospecies makes it difficult to use one criterion (>5 bands) for positive response.
ELISPOT – Lymphocyte Transformation Test – LTT	• Earlier detection of T cell response compared to IgG • Can measure treatment outcomes	• T cell response may not be specific
LTT-MELISA (Memory Enzyme Linked Immuno Stimulation Assay)	• Earlier detection of T cell response compared to IgG • Can measure treatment outcomes	• T cell recognition may not be specific
C6 antigen assay – VlsE C6 peptide assay	• C6 antigen is highly immunogenic • Inexpensive	• Sensitivity is dependent on the C6 antigen expressed in VlsE. Segmental recombination adds greater diversity and sensitivity varies with genospecies
Direct Diagnostic Test		
Culture	• Detects active infection • Growth and better detection using PCR, and labelling and microscopy • Highest sensitivity with skin biopsy • 40% EM, 22% ACA, 24% lymphocytoma	• Long incubation time due slow replication time (12 h or longer) • Fastidious growth requirements difficult to culture • Low levels in CSF, blood, synovium (<10%) • EM rash may not occur, depended on genospecies • Only for patients who have not had antibiotic therapy
Microscopy	• Detects active infection • Direct visualisation • Can be confirmed monoclonal antibody or DNA confirmation with PCR)	• Specimen collection during periods of high activity e.g. high spirochaetaemia in Relapsing fever • Confirmation with PCR or monoclonal fluorescent antibody required.
Nucleic Acid Amplification Techniques – NAAT (PCR)	• Sensitive, specific and is a fast • Detects recent infection • Narrow sensitivity and high specificity • DNA sequences can be obtained • Quantification using rtPCR • Monitoring levels of	• Not detecting all genospecies due to high diversity among genospecies • Inhibition of PCR process due to sample contents • Possible contamination if control/strict procedures are not abided to. Sequencing of all amplicons would detect contamination

or due to immunosuppression isotype switching is inhibited so IgM to IgG switch may not occur.

The salient points:

- Interpretation of indirect diagnostics of Borreliosis can be complicated due to immune dysregulation by *Borrelia* and other tick borne pathogens.
- Serology testing of Borreliosis patients can result in false negatives (ELISA and Western blot) due to production of low affinity IgG subclasses and reduced total IgG.
- Prolonged IgM response observed could be due to relapsing fever *Borrelia* infection or inhibition of isotype switching prevention of the IgG response.

Additional immune-markers may help to determine the extent of the immune dysregulation and better interpretation of diagnostics.

Acknowledgment

Extremely grateful for the contributions of Ann Mitrovic to the compilation and editing of this manuscript.

References

[1] Radolf JD, Caimano MJ, Stevenson B, Hu LT. Of ticks, mice and men: understanding the dual-host lifestyle of Lyme disease spirochaetes. Nat Rev Microbiol 2012;10(January (2)):87–99.
[2] Larsson C, Andersson M, Bergstrom S. Current issues in relapsing fever. Curr Opin Infect Dis 2009;22:443–9.
[3] Nau R, Christen HJ, Eiffert H. Lyme disease – current state of knowledge. Dtsch Arztebl Int 2009;106(5):72–82.
[4] Franke J, Hildebrandt A, Dorn W. Exploring gaps in our knowledge on Lyme borreliosis spirochaetes – updates on complex heterogeneity, ecology, and pathogenicity. Ticks Tick Borne Dis 2013;4(1/2):11–25.
[5] Cutler SJ. Relapsing fever – a forgotten disease revealed. J Appl Microbiol 2010;108:1115–22.
[6] Harvey WT, Martz D. Motor neuron disease recovery associated with IV ceftriaxone and anti-Babesia therapy. Acta Neurol Scand 2007;115(2):129–31.
[7] Brinar VV, Habek M. Rare infections mimicking MS. Clin Neurol Neurosurg 2010;112(September (7)):625–8.
[8] Cassarino DS, Quezado MM, Ghatak NR, Duray PH. Lyme-associated Parkinsonism: a neuropathologic case study and review of the literature. Arch Pathol Lab Med 2003;127(September (9)):1204–6.
[9] Miklossy J. Alzheimer's disease – a neurospirochetosis. Analysis of the evidence, following Koch's and Hill's criteria. J Neuroinflamm 2011;8:90. http://dx.doi.org/10.1186/1742-2094-8-90.
[10] Zimering JH, Williams MR, Eiras ME, Fallon BA, Logigian EL, Dworkin RH. Acute and chronic pain associated with Lyme borreliosis: clinical characteristics and pathophysiologic mechanisms. Pain 2014;155(August (8)):1435–8. http://dx.doi.org/10.1016/j.pain.2014.04.024.
[11] Gustaw K. Chronic fatigue syndrome following tick-borne diseases. Neurol Neurochir Pol 2003;37(November–December (6)):1211–21.
[12] Bhate C, Schwartz RA. Lyme disease: Part 1. Advances and perspectives. J Am Acad Dermatol 2011;64(4):619–36.
[13] Tijsse-Klasen E, Pandak N, Hengeveld P, Takumi K, Koopmans MP, Sprong H. Ability to cause erythema migrans differs between *Borrelia burgdorferi sensu lato* isolates. Parasit Vectors 2013;6(January):23. http://dx.doi.org/10.1186/1756-3305-6-2.
[14] Klempner MS, Baker PJ, Shapiro ED, Marques A, Dattwyler RJ, Halperin JJ, et al. Treatment trials for post-Lyme disease symptoms revisited. Am J Med 2013;126(August (8)):665–9.
[15] Soloski MJ, Crowder LA, Lahey LJ, Wagner CA, Robinson WH, Aucott JN. Serum inflammatory mediators as markers of human Lyme disease activity. PLOS ONE 2014;9(4):e93243. http://dx.doi.org/10.1371/journal.pone.0093243.
[16] Ribeiro JM, Weis JJ, Telford 3rd SR. Saliva of the tick *Ixodes dammini* inhibits neutrophil function. Exp Parasitol 1990;70(May (4)):382–8.
[17] Hovius JW, van Dam AP, Fikrig E. Tick–host–pathogen interactions in Lyme borreliosis. Trends Parasitol 2007;23(September (9)):434–8.
[18] Stevenson B, Babb K. Lux-S mediated quorum sensing in *Borrelia burgdorferi* Lyme disease spirochete. Infect Immun 2002;70:4099–105.
[19] Sapi E, Bastian SL, Mpoy CM, Scott S, Rattelle A, Pabbati N, et al. Characterization of biofilm formation by Borrelia burgdorferi in vitro. PLoS One 2012;7(10):e48277. http://dx.doi.org/10.1371/journal.pone.0048277.
[20] Beermann C, Wunderli-Allenspach H, Groscurth P, Filgueira L. Lipoproteins from *Borrelia burgdorferi* applied in liposomes and presented by dendritic cells induce CD8(+) T-lymphocytes in vitro. Cell Immunol 2000;201(2):124–31.
[21] Jutras BL, Chenail AM, Stevenson B. Changes in bacterial growth rate govern expression of the *Borrelia burgdorferi* OspC and Erp infection-associated surface proteins. J Bacteriol 2013;195(4):757–64.
[22] Bunikis I, Kutschan-Bunikis S, Bonde M, Bergström S. Multiplex PCR as a tool for validating plasmid content of *Borrelia burgdorferi*. J Microbiol Methods 2011;86(2):243–7.
[23] Rupprecht TA, Koedel U, Fingerle V, Pfister HW. The pathogenesis of Lyme neuroborreliosis: from infection to inflammation. Mol Med 2008;14(3/4):205–12.
[24] Dickinson GS, Alugupalli KR. Deciphering the role of Toll Like receptors to Borreliae. Front Biosci 2012;4:699–712.
[25] Cadavid D, Barbour AG. Neuroborreliosis during relapsing fever: review of the clinical manifestations, pathology and treatment of infections in humans and experimental animals. Clin Infect Dis 1998;26:151–64.
[26] Lantos PM, Auwaerter PG, Wormser GP. A systematic review of *Borrelia burgdorferi* morphologic variants does not support a role in chronic Lyme disease. Clin Infect Dis 2014;58(March (5)):663–71.
[27] Yoshinari NH, Mantovani E, Bonoldi VL, Marangoni RG, Gauditano G. Brazilian Lyme-like disease or Baggio–Yoshinari syndrome: exotic and emerging Brazilian tick-borne zoonosis. Rev Assoc Med Bras 2010;56(3):363–9.
[28] Malawista SE, de Boisfleury Chevance A. Clocking the Lyme spirochete. PLoS ONE 2008;3(February (2)):e1633.
[29] Berende A, Oosting M, Kullberg BJ, Netea MG, Joosten LA. Activation of innate host defense mechanisms by Borrelia. Eur Cytokine Netw 2010;21(March (1)):7–18. http://dx.doi.org/10.1684/ecn.2009.00179 [Review].
[30] Cadavid D, Londoño D. Understanding tropism and immunopathological mechanisms of relapsing fever spirochaetes. Clin Microbiol Infect 2009;15(May (5)):415–21. http://dx.doi.org/10.1111/j.1469-0691.2009.02785.x.
[31] Kraiczy P, Hartman K, Hellwage J, Skerka C, Kirschwink M, Brade V, et al. Immunological characteristics of the complement regulation factor H binds CRASP and Erp proteins of *Borrelia burgdorferi*. Int J Med Microbiol 2004;293:152–7.
[32] de Taeye SW, Kreuk L, van Dam AP, Hovius JW, Schuijt TJ. Complement evasion by *Borrelia burgdorferi*: it takes 3 to tango. Trends Parasitol 2010;117:1–10.
[33] Kennedy M, Lenhart TR, Akins DR. The role of *Borrelia burgdorferi* outer surface proteins. Immunol Med Microbiol 2012;66:1–26.
[34] Kean IR, Irvine KL. Lyme disease aetiopathogenesis factors for disease development and control. Inflammopharmacology 2013;21:101–11.
[35] Petzke MM, Brooks A, Krupna MA, Mordue D, Schwartz I. Recognition of *Borrelia burgdorferi* the Lyme disease spirochete by TLR7 and TLR9 induces a type 1 IFN response by human immune cells. J Immunol 2009;183:5279–92.
[36] Love AC, Schwartz I, Petzke MM. *Borrelia burgdorferi* RNA induces type I and III interferons via Toll-like receptor 7 and contributes to production of NF-κB-dependent cytokines. Infect Immun 2014;82(June (6)):2405–16.
[37] Miller JC, Ma Y, Bian J, Sheehan KC, Zachary JF, Weis JH, et al. A critical role for type I IFN in arthritis development following *Borrelia burgdorferi* infection of mice. J Immunol 2008;181(12):8492–503.
[38] Olson Jr CM, Bates TC, Izadi H, Radolf JD, Huber SA, Boyson JE, et al. Local production of IFN-gamma by invariant NKT cells modulates acute Lyme carditis. J Immunol 2009;182(6):3728–34. http://dx.doi.org/10.4049/jimmunol.0804111.
[39] Salazar JC, Duhnam-Ems S, La Vake C, Cruz AR, Moore MW, Caimano MJ, et al. Activation of human monocytes by live *Borrelia burgdorferi* generates TLR2-dependent and -independent responses which include induction of IFN-beta. PLoS Pathog 2009;5(5):e1000444. http://dx.doi.org/10.1371/journal.ppat.1000444.
[40] Sonderegger FL, Ma Y, Maylor-Hagan H, Brewster J, Huang X, Spangrude GJ, et al. Localised production of IL-10 suppresses early inflammatory cell infiltration and subsequent development of IFN-gamma mediated Lyme arthritis. J Immunol 2012;188(3):1381–93. http://dx.doi.org/10.4049/jimmunol.1102359.
[41] Jacek E, Fallon BA, Chandra A, Crow Mk, Wormser GP, Alaedini A. Increased IFN-alpha activity and differential antibody response in patients with history of Lyme disease and persistent cognitive deficits. J Neuroimmunol 2013;255(1/2):85–91.
[42] Alexopoulou L, Thomas V, Schnare M, Anguita J, Schoen RT, Medzhitov R, et al. Hyper responsiveness to vaccination with *Borrelia burgdorferi* OspA in humans and TLR1 and TLR-2 deficient mice. Nat Med 2002;8:878–84.
[43] Embers ME, Ramamoorthy R, Philipp MT. Survival strategies of *Borrelia burgdorferi*, the etiologic agent of Lyme disease. Microbes Infect 2004;6:312–8.
[44] Marre ML, Darcy C, Yinh J, Akira S, Uematsu S, Steere AC, et al. Role of adrenomedullin in Lyme disease infection. Infect Immun 2010;78(12):5307–13.
[45] Marchal C, Schramm F, Kern A, Luft BJ, Yang X, Schuijt T, et al. Antialarmins effect of tick saliva during the transmission of Lyme disease. Infect Immun 2011;79(2):774–85.
[46] McHeyzer-Williams LJ, Driver DJ, McHeyzer-Williams MG. Germinal centre reaction. Curr Opin Haematol 2001;8:52–9.
[47] Blaho A, Buczynski MW, Dennis EA, Brown CR. Cyclooxygenase-1 orchestrates germinal centre formation and antibody class switch via regulation of IL-17. J Immunol 2009;183(9):5644–53.
[48] Male, Brostoff J, Roth DB, Roitt I. Immunology. 7th ed. Philadelphia: Mosby Elsevier; 2006.
[49] Janeway Jr A, Travers P, Walport M, Shlomchik MJ. Immunobiology. 5th ed. NY: Garland Publishing; 2001.
[50] Tunev SS, Hastey CJ, Hodzic E, Feng S, Barthold SW, Baumgarth N. Lymphadenopathy during Lyme Borreliosis is caused by spirochete migration induced specific B cell activation. PLoS Pathog 2011;5:e1002066.

[51] Mills T. Delayed anaphylaxis, angioedema or urticaria after consumption of red meat in patients with IgE antibodies specific for galactose-alpha-1,3-galactose. J Allergy Clin Immunol 2009;123(2):426–33.
[52] Bluth MH, Robin J, Ruditsky M, Norowitz KB, Chice S, Pytlak E, et al. IgE anti-*Borrelia burgdorferi* components (p18, p31, p34, p41, p45, p60) and increased blood CD8+CD60+ T cells in children with Lyme disease. Scand J Immunol 2007;65(April (4)):376–82.
[53] Hawley KL, Olson Jr CM, Iglesias-Pedraz N, Navasa JL, Cervantes MJ, Caimano H, et al. CD14 cooperates with complement receptor 3 to mediate MyD88 independent phagocytosis of *Borrelia burgdorferi*. PNAS 2012;109(4):1228–32.
[54] Hawley KL, Olson Jr CM, Iglesias-Pedraz JM, Navasa N, Cervantes JL, Caimano MJ, et al. CD14 cooperates with complement receptor 3 to mediate MyD88-independent phagocytosis of Borrelia burgdorferi. Proc Natl Acad Sci U S A 2012;109(January (4)):1228–32. http://dx.doi.org/10.1073/pnas.1112078109.
[55] Rahembulla A, Fung-Leung WP, Schilham MW, Kundig TM, Sambhara SR, Narendran A, et al. Normal development and function of CD8+ cells but markedly decreased helper cell activity in mice lacking CD4. Nature 1991;353:180–4.
[56] Hartiala P, Hytönen J, Yrjänäinen H, Honkinen M, Terho P, Söderström M, et al. TLR2 utilization of *Borrelia* does not induce p38- and IFN-beta autocrine loop-dependent expression of CD38, resulting in poor migration and weak IL-12 secretion of dendritic cells. J Immunol 2010;184(May (10)):5732–42. http://dx.doi.org/10.4049/jimmunol.0803944.
[57] Cadavid D. The mammalian host response to *Borrelia* infection. Wienklin Wochensch 2006;118(21/22):653–8.
[58] Ma B, Christen B, Leung D, Vigo-Pelfrey C. Serodiagnosis of Lyme borreliosis by western immunoblot: reactivity of various significant antibodies against *Borrelia burgdorferi*. J Clin Microbiol 1992;30(February (2)):370–6.
[59] Wilske B. Diagnosis of Lyme borreliosis in Europe. Vector Borne Zoonotic Dis 2003;3(Winter (4)):215–27 [review].
[60] Assous MV, Wilamowski A. Relapsing fever borreliosis in Eurasia – forgotten but certainly not gone. Clin Microbiol Infect Dis 2009;15(5):407–14.
[61] Branda JA, Aguero-Rosenfeld ME, Ferraro MJ, Johnson BJ, Wormser GP, Steere AC. 2-Tiered antibody testing for early and late Lyme disease using only an immunoglobulin G blot with the addition of a VlsE band as the second-tier test. Clin Infect Dis 2010;50(January (1)):20–6. http://dx.doi.org/10.1086/648674.
[62] Branda JA, Linskey K, Kim YA, Steere AC, Ferraro MJ. Two tiered antibody testing for Lyme Disease with use of 2 enzyme immunoassay, a cell sonicate enzyme assay followed by VlsE, C6 peptide enzyme immunoassay. Clin Infect Dis 2011;53(6):541–7.
[63] Racine R, Mc Laughlin M, Jones DD, Wittmer ST, MacNamara ICC, Woodlands DL, et al. IgM production by bone marrow plasma blasts contribute to long term protection against intracellular bacterial infections. J Immunol 2011;86(2):1011–21.
[64] Connolly SE, Benach JL. The versatile roles of antibodies in *Borrelia* infections. Nat Rev Microbiol 2005;3(May (5)):411–20.
[65] Roberg M, Forsberg P, Tegnell A, Ekerfeldt K. Intrathecal production of specific IgA antibodies in CNS infections. J Neurol 1995;242(June (6)):390–7.
[66] Cadavid D, O'Neill T, Schaefer H, Pachner AR. Localization of *Borrelia burgdorferi* in the nervous system and other organs in a nonhuman primate model of Lyme disease. Lab Invest 2000;80(July (7)):1043–54.
[67] Krupka I, Knauer J, Lorentzen L, O'Connor TP, Saucier J, Straubinger RK. *Borrelia burgdorferi sensu lato* species in Europe induce diverse immune responses against C6 peptides in infected mice. Clin Vaccine Immunol 2009;16(November (11)):1546–62.
[68] Ang CW, Notermans DW, Hommes M, Simoon-Smit AM, Herremans T. Large differences between test strategies for detection of anti *Borrelia* antibodies are revealed by comparing eight ELISAs and five immunoblots. Eur J Clin Microbiol Infect Dis 2011;30(8):1027–32.
[69] Rebman AW, Crowder LA, Kirkpatrick A, Aucott JN. Characteristics of serroconversion and implications for diagnosis of post-treatment Lyme disease syndrome: acute and convalescent serology among a prospective cohort of early Lyme disease patients. Clin Rheumatol 2014. http://dx.doi.org/10.1007/s10067-014-2706-z [Epub ahead of print].
[70] Franz JK, Krause A. Lyme disease (Lyme Borreliosis). Best Pract Res Clin Rheumatol 2003;17(April (2)):241–64.
[71] von Baehr Doebis C, Volk HD, von Baehr R. The lymphocyte transformation test for *Borrelia* detects active Lyme Borreliosis and verifies effective antibiotic treatment. Open Neurol J 2012;6(Suppl. 1-M5):104–12.
[72] Dessau RB. Diagnostic accuracy and comparison of two assays for *Borrelia*-specific IgG and IgM antibodies: proposals for statistical evaluation methods, cut-off values and standardization. J Med Microbiol 2013;62(December (Pt 12)):1835–44.
[73] Valentine-Thon E, Ilsemann K, Sandkamp M. A novel lymphocyte transformation test (LTT-MELISA) for Lyme borreliosis. Diagn Microbiol Infect Dis 2007;57(1):27–34.
[74] Wormser GP, Liveris D, Hanincová K, Brisson D, Ludin S, Stracuzzi VJ, et al. Effect of *Borrelia burgdorferi* genotype on the sensitivity of C6 and 2-tier testing in North American patients with culture-confirmed Lyme disease. Clin Infect Dis 2008;47(October (7)):910–4. http://dx.doi.org/10.1086/591529.
[75] Ledue TB, Collins MF, Young J, Schriefer ME. Evaluation of the recombinant VlsE-based liaison chemiluminescence immunoassay for detection of Borrelia burgdorferi and diagnosis of Lyme disease. Clin Vaccine Immunol 2008;15(December (12)):1796–804. http://dx.doi.org/10.1128/CVI.00195-08.
[76] Barbour AG. Immunochemical analysis of Lyme disease spirochetes. Yale J Biol Med 1984;57(July–August (4)):581–6.
[77] Aguero-Rosenfeld ME, Wang G, Schwartz I, Wormser GP. Diagnosis of lyme borreliosis. Clin Microbiol Rev 2005;18(July (3)):484–509.
[78] Nolte O. Nucleic acid amplification based diagnostic of lyme (neuro-)borreliosis – lost in the jungle of methods, targets, and assays? Open Neurol J 2012;6:129–39. http://dx.doi.org/10.2174/1874205X01206010129.
[79] Rudenko N, Golovchenko M, Grubhoffer L, Oliver Jr JH. Updates on *Borrelia burgdorferi sensu lato* complex with respect to public health. Ticks Tick Borne Dis 2011;2(September (3)):123–8.
[80] Rebaudet S, Parola P. Epidemiology of relapsing fever borreliosis in Europe. FEMS Immunol Med Microbiol 2006;48(October (1)):11–5.
[81] Schwan TG, Raffel SJ, Schrumpf ME, Schrumpf ME, Webster LS, Marques AR, et al. Tick-borne relapsing fever and *Borrelia hermsii*, Los Angeles County, California, USA. Emerg Infect Dis 2009;15(July (7)):1026–31. http://dx.doi.org/10.3201/eid1507.090223.
[82] Noda AA, Rodríguez I, Mondeja B, Fernández C. Design, optimization and evaluation of a polymerase chain reaction for detection of *Borrelia* spp.. Adv Clin Exp Med 2013;22(September–October (5)):639–53.
[83] Chan K, Marras SA, Parveen N. Sensitive multiplex PCR assay to differentiate Lyme spirochetes and emerging pathogens *Anaplasma phagocytophilum* and *Babesia microti*. BMC Microbiol 2013;13(December):295. http://dx.doi.org/10.1186/1471-2180-13-295.
[84] Güner ES, Watanabe M, Hashimoto N, Kadosaka T, Kawamura Y, Ezaki T, et al. *Borrelia turcica* sp. nov., isolated from the hard tick *Hyalomma aegyptium* in Turkey. Int J Syst Evol Microbiol 2004;54(September (Pt 5)):1649–52.
[85] Ivanova LB, Tomova A, González-Acuña D, Murúa R, Moreno CX, Hernández C, et al. *Borrelia chilensis*, a new member of the *Borrelia burgdorferi sensu lato* complex that extends the range of this genospecies in the Southern Hemisphere. Environ Microbiol 2014;16(April (4)):1069–80. http://dx.doi.org/10.1111/1462-2920.12310.
[86] Barbieri AM, Venzal JM, Marcili A, Almeida AP, González EM, Labruna MB. *Borrelia burgdorferi sensu lato* infecting ticks of the *Ixodes ricinus* complex in Uruguay: first report for the Southern Hemisphere. Vector Borne Zoonotic Dis 2013;13(March (3)):147–53. http://dx.doi.org/10.1089/vbz.2012.1102.
[87] Diatta G, Souidi Y, Granjon L, Arnathau C, Durand P, Chauvancy G, et al. Epidemiology of tick-borne borreliosis in Morocco. PLoS Negl Trop Dis 2012;6(9):e1810. http://dx.doi.org/10.1371/journal.pntd.0001810.
[88] Takano A, Goka K, Une Y, Shimada Y, Fujita H, Shiino T, et al. Isolation and characterization of a novel *Borrelia* group of tick-borne borreliae from imported reptiles and their associated ticks. Environ Microbiol 2010;12(January (1)):134–46. http://dx.doi.org/10.1111/j.1462-2920.2009.02054.x.
[89] Chu CY, Liu W, Jiang BG, Wang DM, Jiang WJ, Zhao QM, et al. Novel genospecies of *Borrelia burgdorferi sensu lato* from rodents and ticks in southwestern China. J Clin Microbiol 2008;46(September):3130–3. http://dx.doi.org/10.1128/JCM.01195-08. No. 90095-1137/08/$08.000.