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Chapter 3

Infectivity of Borrelia burgdorferi sensu lato is unaltered in C3 deficient mice

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Abstract

B. burgdorferi, *B. afzelii* and *B. bavariensis* show resistance to mouse and human complement. *B. garinii* and *B. valaisiana* are sensitive to mouse and human complement. We evaluated whether the absence of C3 in mice influenced infectivity and pathogenicity of different *Borrelia* species.
C3 knockout mice (C3 -/-) and syngeneic C57Bl/6 wild type (WT) mice were challenged with five different *Borrelia* species. After two weeks, quantitative PCR (qPCR), culture, histopathology and immunofluorescence was performed on heart, joint, brain, bladder and skin.
Spirochetes were detected by qPCR after infection with *B. burgdorferi, B. afzelii* or *B. bavariensis* strains. In joints of C3 -/-, but not WT mice challenged with *B. burgdorferi*, spirochetes were detected by qPCR. No other significant differences between C3 -/- and WT mice were seen. Histopathology demonstrated concordance between borrelia load and inflammation score. Only after *B. burgdorferi* and *B. afzelii* infection spirochetes were detected by immunofluorescence microscopy.
B. burgdorferi was cultured from heart, joint, bladder and skin from all mice within 2 weeks. *B. afzelii* and *B. bavariensis* grew only from heart tissue from both C3 -/- and WT mice after 2-6 weeks.
The infectivity and pathogenicity of complement-resistant *Borrelia* strains is unchanged in complement-deficient mice. Complement-susceptible strains do not become infectious in the absence of C3.

Introduction

Lyme disease is a multiorgan tick-borne infection caused by spirochetes belonging to *Borrelia burgdorferi* sensu lato (sl) complex. Several species have currently been identified: *B. burgdorferi* sensu stricto (ss) is present in North America and in Europe, whereas *B. afzelii, B. bavariensis* and *B. garinii* are present only in Eurasia. In Eurasia other *Borrelia* species that have been associated with Lyme disease such as *B. valaisiana and B. spielmanii* have been isolated from ticks [532]. The pathogenicity of *B. valaisiana* in humans is still under discussion [143, 533, 534]. Ticks can carry many other borrelia species of which pathogenicity in humans is not yet described like *B. lusitaniae, B. tanukii, B. japonicum, B. turdi and B. andersonii. B. bavariensis* has recently been proposed to be delineated as a separate species. This species was previously known as *B. garinii* OspA serotype 4 [512].

Infectivity and pathogenicity of the different strains of the *B. burgdorferi* sl complex varies greatly. In mammals for instance the infectious dose of a *B burgdorferi* ss strain was much lower in comparison to that of a reference *B. afzelii* or *B garinii* strain [535]. Moreover, after infection with the *B. burgdorferi* ss strain, arthritis and carditis were much more severe [536]. Pachner et al. also showed that after infections with *B. garinii* or *B. afzelii* spirochete load was lower than in *B. burgdorferi* ss infected mice and non-human primates. Furthermore many *B. garinii* strains were not infective at all in mice [537].

Complement plays an important role in killing of bacteria. Deposition of complement on the membrane of bacteria can lead to opsonization and lysis of the bacteria, but also influences adaptive immune responses directly. Borrelia spirochetes differ in their susceptibility to complement [64]. In vitro, *B. afzelii*, *B. burgdorferi* ss and *B. bavariensis* strains are highly resistant to human and mouse serum, whereas *B. valaisiana* and *B. garinii* strains are readily killed by human and mouse serum [64, 65]. *B. burgdorferi* sl is capable of evading innate immunity by binding of host factor H and factor H-like protein 1 (FHL-1) [66-68]. Borrelia bind factor H/FHL-1 through expression of several proteins such as CspA, CspZ and OspE proteins, also designated Complement Regulatory Acquiring Proteins (CRASPs) on the spirochetal membrane. These CRASPs are upregulated during various parts of the mammalian tick transmission cycle but it is not always clear what roles they play in the protection to complement mediated killing [95, 538, 539]. Primarily *B. burgdorferi* and *B. afzelii*, but also *B. bavariensis* have the ability to bind factor H by CRASPs [77, 84, 540-542].

The role of complement in murine Lyme borreliosis has been addressed by Bockenstedt et al, who found that C5-deficient mice infected with *B. burgdorferi ss* ran a similar clinical course as wild type mice [543]. Lawrenz et al. demonstrated that infection of C3 deficient mice with *Borrelia burgdorferi* ss resulted in a higher spirochete load and higher histopathology scores in ear, ankles and heart, compared to wildtype (WT) mice, but only early in infection [191]. This was confirmed for skin tissue in another study [98]. Very little research has been done on the effect of C3-deficiency on the infectivity of other *Borrelia* spp. Lawrenz et al. described that all three C3 -/- mice infected with 10^4 spirochetes of a *B. garinii* strain developed a positive PCR on bladder tissue, whereas this was the case in only 1/3 WT mice. However, they found no infection in WT and C3 -/- mice after an inoculum of 10^3 spirochetes versus detectable infection of all mice after an inoculum of 10^5 spirochetes.
In the present study, WT and C3 -/- mice were injected with spirochetes belonging to different *B. burgdorferi* sl species to determine whether complement deficiency in mice leads to increased susceptibility to Lyme borreliosis. The infectivity and pathogenicity of the different species was assessed by culture, quantitative PCR and histology.

Materials and methods

Bacterial and mouse strains

The experimental protocol was approved by the Animal Ethical Committee of the Leiden University Medical Center. Mice were housed in an animal facility, placed in groups of 2 to 5 in polycarbonate cages with wide bar lids and micro-isolator tops and provided with food and water ad libitum.
C57Bl/6 C3 deficient mice were generated by replacing the 5'-flanking region of the C3 gene with a neomycin resistance marker [544]. These C3 (-/-) mice produce no detectable C3 protein and have no complement activity.
For infectivity testing *B. burgdorferi* ss N40 was used, for all other species a mix of 5 different *Borrelia* strains per species was used in order to correct for the per strain pathogenicity: for *B. afzelii* A17S, A20S, A57T, A63T, pKo; for *B. garinii* A77S, PBr, 20047, A87S, VSBM; for *B. bavariensis* A01C, A19S, A91S, A94S, PBi; for *B. valaisiana* IDP3, M19, M49, M53 and UK2 (Table1). The origin of the strains is shown in table 1. An uninfected C57Bl/6 mouse was also sacrificed to serve as a negative control. Before injection in the mice, fresh medium was added to the cultures and cultures were incubated at 37°C for 4 hours. Borrelia were counted and rinsed with 37°C PBS directly before injection. All mice were injected with $2*10^5$ spirochetes per strain in a total volume of 100 µl PBS, intradermally in the

scapular region of 6 weeks old mice [535, 545]. The high load was chosen due to the previous report that *B. garinii* have low infectivity rates under a needle inoculation dose of 10^5 [191]. Each group consisted of three mice. Mice were sacrificed two weeks post inoculation by asphyxiation. Heart, kidney, bladder, brain, joint and skin were collected. The period of two weeks was elected due to previous studies where the peak of the infection takes place two weeks after syringe inoculation [191, 459, 546].

Strain	Species	Biological source	Geographical origin	Serum resistance*
N40	*B. burgdorferi* ss	*I. scapularis*	US	Resistant
A17S	*B. afzelii*	Human Skin	The Netherlands	Resistant
A20S	*B. afzelii*	Human Skin	The Netherlands	Resistant
A57T	*B. afzelii*	*I. ricinus*	The Netherlands	Resistant
A63T	*B. afzelii*	*I. ricinus*	The Netherlands	Resistant
PKo	*B. afzelii*	Human Skin	Germany	Resistant
20047	*B. garinii*	*I. ricinus*	France	Sensitive
A77S	*B. garinii*	Human Skin	The Netherlands	Sensitive
A87S	*B. garinii*	Human Skin	The Netherlands	Sensitive
PBr	*B. garinii*	Human CSF	Germany	Sensitive
VSBM	*B. garinii*	Human CSF	Switzerland	Sensitive
A01C	*B. bavariensis*	Human CSF	The Netherlands	Resistant
A19S	*B. bavariensis*	Human Skin	The Netherlands	Resistant
A91S	*B. bavariensis*	Human Skin	The Netherlands	Resistant
A94S	*B. bavariensis*	Human Skin	The Netherlands	Intermediate
PBi	*B. bavariensis*	Human CSF	Germany	Intermediate
IDP3	*B. valaisiana*	*I. ricinus*	The Netherlands	Sensitive
M19	*B. valaisiana*	*I. ricinus*	The Netherlands	Sensitive
M49	*B. valaisiana*	*I. ricinus*	The Netherlands	Sensitive
M53	*B. valaisiana*	*I. ricinus*	The Netherlands	Sensitive
UK2	*B. valaisiana*	*I. ricinus*	The Netherlands	Sensitive

Table 1: Strains used in this study. *Human and mouse serum sensitivity as determined in literature and remaining strains (B. valaisiana) were determined as described [64, 66, 513].

Real-time quantitative PCR

For quantification of bacterial loads a 5x5 mm biopsy of abdominal wall skin, one third of the heart, one third of the bladder, one quarter of the brain, and half of the tibiotarsal joint were collected and stored at –80 °C. Nucleic acids from organs were extracted with a QiaAmp *Mini Blood DNA* kit (Qiagen, Hilden, Germany) All samples were extracted according to the manufacturer's instructions, resulting in 200 µl of purified nucleic acids, which were stored at –20°C. Quantitative DNA analysis was performed using the iCycler PCR system. The relative starting copy number was determined by cycle threshold detection

using iCycler relative quantification software (Roche). Primers and probe for FlaB were designed from an interspecies conserved region of *FlaB* using the Beacondesigner software. Sequences used for the PCR are FlaB forward 5'-GCT TCT GAT GAT GCT GCTG-3', FlaB reverse 5'-TCG TCT GTA AGT TGC TCT ATT TC-3' and FlaBProbe 5'-GAATTRGCAGTAACGG-FAM 3'. The assay was optimized using a TA vector into which the complete FlaB gene from strain B31 had been cloned and had an analytical sensitivity of 1 copy per PCR in saline 0.9% *w/v*. The oligonucleotide primers used to quantify murine β -actin were β -act forward (5'-CAA TAG TGA TGA CCT GGC CGT-3') and β -act reverse (5'-GA GGG AAA TCG TGC GTG AC -3'). Amplification reactions were performed in a 50-µL final volume, containing 25 µL IQ Supermix (Bio-Rad, Veenendaal, the netherlands), 15 pmol forward primer, 15 pmol reverse primer, 2.5 mM $MgCl_2$, 0.3 µM probe or Sybrgreen (Molecular Probes inc.), and 10 µL DNA extract. Following an enzyme activation step for 3 min at 95°C, amplification comprised 50 cycles of 30 s at 95°C, 30 s at 55°C and 30 s at 72°C in an iCycler IQ real-time detection system (Bio-Rad). Total load was expressed in copies FlaB/1000 copies β -actin. Amplification of joint tissue initially resulted in negative results of the β -actin PCR due to the presence of inhibitory substances, therefore all joint samples were additionally tested in a 1/10 dilution. Minimal detection limit per PCR reaction for spirochete load was 0.1 copies FlaB/1000 copies β-actin.

Pathology

Histopathology was performed on multiple sections of all organs fixed using 4% neutral buffered formalin, embedded in paraffin, sectioned at 3 µm thickness and stained with hematoxylin/eosin (HE). Joints were decalcified before embedding. Sections were evaluated in a blinded fashion. Inflammation was scored from zero to three, zero denoting normal tissue and three being severe inflammation, based on infiltration of granulocytes, exudate and synovial proliferation for joints [547]. Inflammation in the other organs was based on infiltration of lymphocytes, macrophages, plasma cells and necrosis [548]. Immunofluorescence (IF) staining was performed on multiple sections of all organs fixed using 4 % neutral buffered formalin, embedded in paraffin and sectioned at 3 µm thickness. Sections were dried on Starfrost (Klinipath) slides, deparaffined, dehydrated, and trypsinized for 20 minutes at 37 °C. Subsequently slides were rinsed with demineralised water, washed three times in sterile PBS and incubated with 1:100 dilution of a rabbit anti-*B. burgdorferi* serum (ImmunoLogic). Slides were washed three times with PBS and incubated with a 1:50 dilution of FITC-conjugated swine anti-rabbit Ig (DakoCytomation), washed again three times in sterile PBS, and coverslips were mounted using Vectorshield mounting medium (Vector Laboratories, Burlingame, USA). Recordings were made using an Axioplan 2 microscope. The assay was

validated with clinical materials from patients with an EM, borrelia culture and borrelia negative tissue samples and used in a clinical setting. Spirochete load was scored according to the amount of spirochetes per low power field (20x), from zero (none), one (1-10), two (10-50) and three (>50). This was performed in a blinded fashion.

Culture

For borrelia culture a 5x5mm biopsy of abdominal wall skin, one third of the heart, one third of the bladder, one quarter of the brain, half a kidney and half of the tibiotarsal joint were harvested and incubated in BSK-H medium (Sigma) supplemented with fosfomycin (100ng/ml) and rifampicin (500ng/ml). Cultures were kept at 33 ˚C and checked under darkfield microscopy weekly for six weeks. All cultures were passaged into fresh medium after two weeks. Visual confirmation of live motile spirochetes was considered a positive culture.

Arthritis score

After 10 days arthritis score was done by visual confirmation of swelling of the right ankle. Swelling was scored zero to three, zero being no swelling and three being severe swelling of the ankle.

Statistical analysis

All statistical analyses were done using SPSS 16.0 and Microsoft Excel software. The two-tailed Student t-test was used to analyze quantitative PCR results. Values of $p < 0.05$ were considered to be significant.

Results

Culture

Spirochetes grew from all organs from mice injected with *B. burgdorferi* ss within 2 weeks (Table 2). From the animals injected with *B. afzelii*, spirochetes could be cultured from the heart of 2/3 WT and 1/3 C3-/- mice after 4 weeks. All other cultures from these mice remained negative. From mice injected with *B. bavariensis*, spirochetes grew only from the heart of 1/3 C3-/- mice and 1/3 WT mice both after two weeks. None of the cultures of mice challenged with *B. garinii* and *B. valaisiana* strains became positive.

Real time PCR

At two weeks of infection *B. burgdorferi* ss N40 was detectable by qPCR in bladder, heart, skin and ankles (Table 2).The average number of *B. burgdorferi* ss spirochetes was only significantly higher in the ankles of C3 -/- mice ($p<0.05$),

since spirochete DNA was not detectable in the ankles of WT mice. Spirochetes were not detected by qPCR in the brain of WT or C3 -/- mice.
Regarding the mice injected with *B. afzelii* strains; bladder, heart, skin and ankle tissue had a detectable load of spirochetes in all mice. Loads of spirochetes were slightly, but not significantly higher in bladder, heart and skin of C3 -/- mice. No effect was seen on the load of *B. afzelii* in the ankles. Only one mouse in the wild type group had a weakly positive qPCR signal in brain tissue.
In heart, skin and ankle tissue of mice injected with *B. bavariensis* strains a low, but detectable load of spirochetes was found. Loads were comparable in the WT and C3-/- mice. All mice injected with *B. bavariensis* were infected at two weeks, because at least one of the organs of all mice was positive by PCR. However, all bladder samples and six of nine other organ samples (joint, skin and heart) from C3 -/- mice and three of nine organ samples from WT mice were negative in the PCR. None of the mice injected with *B. garinii* or *B. valaisiana* strains had detectable spirochete DNA in any of the organs.

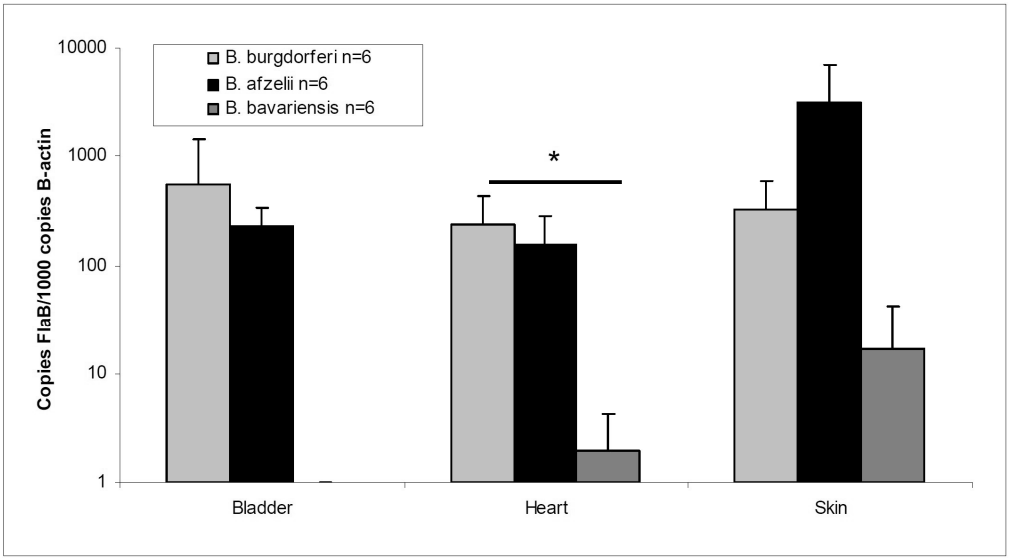

Figure 1: Loads of Borrelia/1000 copies β-actin in tissues from infected mice. Real time PCR results grouped by species and per organ, expressed in copies FlaB/1000 copies β-actin. Borrelia loads are significantly higher in heart B. burgdorferi compared to B. bavariensis strains. Each group consisted of n=6 mice. (* $p<0.05$)

Since no significant difference in spirochete DNA loads between C3 -/- and WT mice was found in heart, skin and bladder tissue and only in the mice infected with strains that are already serum resistant we pooled the data to increase power and compared DNA loads in different tissues for all mice infected with *B. burgdorferi* ss, *B. afzelii* or *B. bavariensis* (Figure 1). It is clear that loads in mice

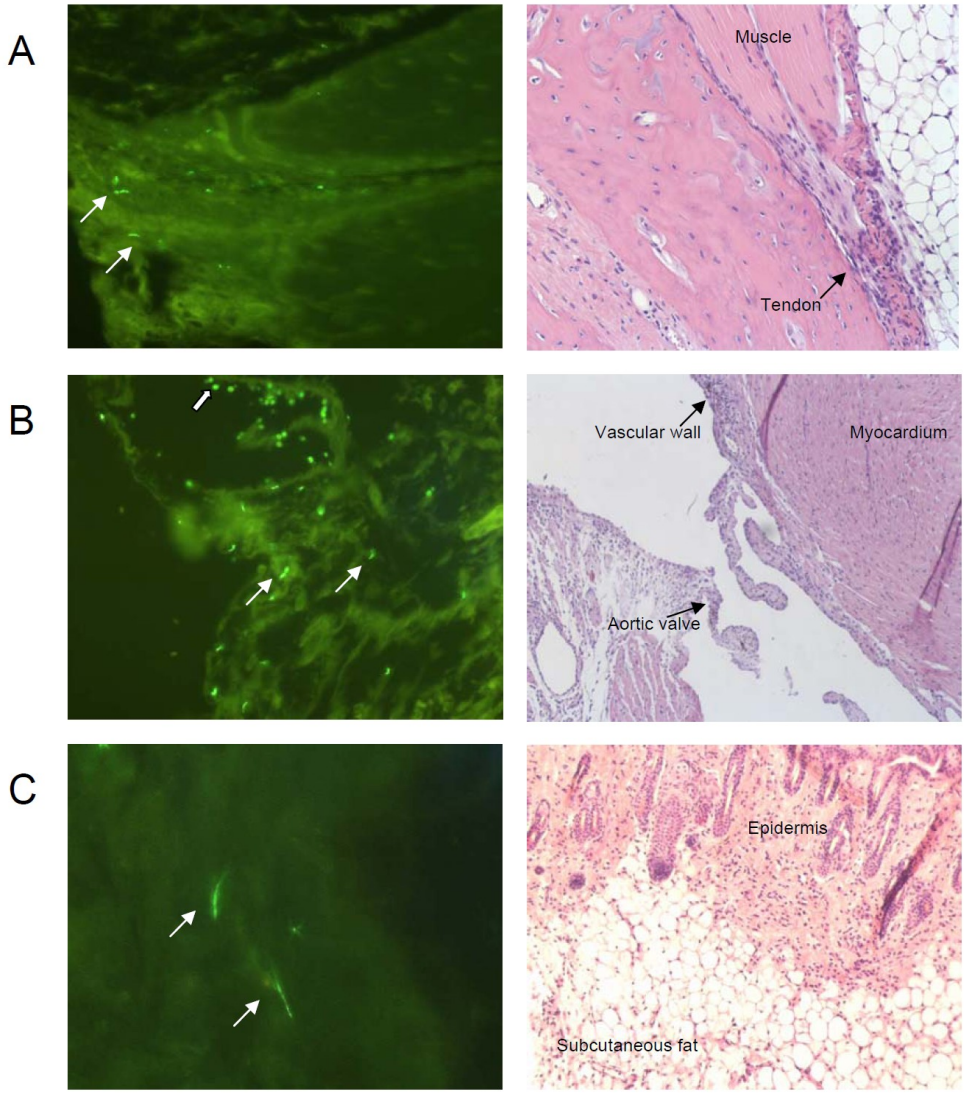

Figure 2: Pathology and IF results. IF and HE slides of organs from a C3 -/- mouse inoculated with B. afzelii. A: Joint: IF (40x), spirochetes in the tendon connected to the tibia (white arrow). HE (20x), Infiltration of plasma cells, lymphocytes and sporadically a granulocyte in the tendon. B: Heart: IF (63x), spirochetes located in the epicardium (white arrow), intravascular erythrocytes also fluoresce (fat arrow). HE (10x), aortal base with valve. Infiltration of macrophages in the adventitia, vascular wall and in a lesser amount in the myocardium. C: Skin: IF (63 x): spirochetes mainly localized on the lining of the dermis with the epidermis (white arrow). HE (10x), Infiltration of plasma cells, lymphocytes and histiocytes in the dermis and subcutaneous fat. As control an uninfected mouse was sacrificed. No spirochetes were detected in the tissue of the control mouse.

infected with *B. burgdorferi* ss are comparable in all organs and are high, ranging from a mean of 240 copies to 850 copies FlaB/1000 copies β-actin. *B. afzelii* infected mice showed a wider range of loads, ranging from 150 to 3000

copies FlaB/1000 copies β-actin. Highest loads of *B. afzelii* were reached in skin. There were no significant differences in loads between *B. burgdorferi* and *B. afzelii*. Loads in mice infected with *B. bavariensis* strains differ little between organs and are low, ranging from 2 to 20 copies FlaB/1000 copies β-actin. Spirochete loads in heart tissue of *B. bavariensis* infected mice were significantly lower than in *B. burgdorferi* ss infected mice.

Pathology

By IF staining, spirochetes were only demonstrated in tissue samples from C3 -/- and WT mice injected with *B. burgdorferi* ss or *B. afzelii* (Table 2).
In *B. burgdorferi* ss injected mice the highest numbers of spirochetes were seen in heart and skin tissues. Bacteria were visually more abundant in the C3 -/- mice. In the C3 -/-, as well as the WT mice injected with *B. burgdorferi ss*, lower numbers of spirochetes were seen in the bladder and in the kidney.
In mice injected with *B. afzelii* spirochetes were seen in joint, skin, heart, kidney and bladder. No differences were seen between WT and C3 -/- mice.
In the heart, most spirochetes were seen in the epicardium surrounding the base of the vessels of the truncus arteriosus. In ankle tissues spirochetes were most abundant in the tendons and in the skin along the dermal epidermal junction. In organs from mice injected with *B. bavariensis*, *B. garinii* or *B. valaisiana,* no spirochetes were detected by IF staining.
Inflammation was present in the heart and skin of *B. burgdorferi* ss injected mice and *B. afzelii* injected mice (Figure 2). Tissue inflammation was related to the amount of spirochetes seen after IF staining. There was no difference in inflammation scores between WT and C3-/- mice.
In general IF microscopy had a very low sensitivity for detecting infection with borrelia. Borrelia were only visualized and detected by IF when the quantified load in the real time PCR was more than 100 borrelia/1000 copies β-actin

Arthritis score

After 10 days inflammation scores of the ankle joints were evaluated. None of the *B. burgdorferi* ss injected mice showed thickening of the ankle joint, regardless of the presence of C3 in the mice.

Table 2: Results from the real-time qPCR, pathology scores and culture. *Real time PCR results expressed in copies FlaB/1000 copies β-actin (Standard deviation). Pathology scores performed as described. Immunofluorescence (IF) score is a score based on the amount of visible spirochetes by IF. The hematoxylin eosin (HE) staining is scored on amount of inflammation as described. No spirochetes were seen by IF in any of *the B. garinii* and *B. valaisiana* infected mice. No inflammation was seen in *B. garinii* and *B. valaisiana* infected mice except for an inflammation score of 1 in the bladder of one WT mouse infected with *B. garinii* (mean score 0.3). None of the cultures, or qPCRs from *B. garinii* or B. valaisiana was positive.

	B. burgdorferi		*B. afzelii*		*B. bavariensis*		*B. garinii*		*B. valaisiana*	
	WT (n=3)	C3KO (n=3)	WT (n=3)	C3KO (n=3)	WT (n=3)	C3KO (n=3)	WT (n=3)	C3KO (n=3)	WT (n=3)	C3KO (n=3)
Bladder										
Real time PCR*	10 (17)	1102 (1101)	100 (82)	357 (377)	-	-	-	-	-	-
Pathology IF	0	1,3	0,7	1,3	0	0	0	0	0	0
Pathology HE	0,5	0	0,7	1,3	0	0	0,3	0	0	0
Culture	3/3	3/3	-	-	-	-	-	-	-	-
Heart										
PCR*	137 (109)	345 (215)	77 (54)	226 (138)	3 (3)	1 (1)	-	-	-	-
Pathology IF	0,7	2,3	1,7	2,0	0	0	0	0	0	0
Pathology HE	0,7	2,7	2,0	2,3	0	0	0	0	0	0
Culture	3/3	3/3	2/3	1/3	1/3	1/3	-	-	-	-
Brain										
PCR*	-	-	6 (11)	-	-	-	-	-	-	-
Pathology IF	0	0	0	0	0	0	0	0	0	0
Pathology HE	0	0	0	0	0	0	0	0	0	0
Culture	3/3	3/3	-	-	-	-	-	-	-	-
Skin										
PCR*	304(405)	341 (107)	1454 (1222)	4687 (5473)	28 (32)	6 (11)	-	-	-	-
Pathology IF	1,0	2,3	2,7	3,0	0	0	0	0	0	0
Pathology HE	2,0	2,3	2,3	3,0	1,0	0,7	0	0	0	0
Culture	3/3	3/3	-	-	-	-	-	-	-	-
Ankle										
Real time PCR*	-	844 (523)	1918 (1248)	1880 (1563)	6 (3)	3 (4)	-	-	-	-
Pathology IF	0	0	0,7	1,3	0	0	0	0	0	0
Pathology HE	0	0	0	0,3	0	0	0	0	0	0
Culture	3/3	3/3	-	-	-	-	-	-	-	-
Kidney										
Pathology IF	0,7	1,0	0,7	1,3	0	0	0	0	0	0
Pathology HE	0,3	0	0	0	0	0	0	0	0	0
Culture	3/3	3/3	-	-	-	-	-	-	-	-

Among B. afzelii injected mice, 2/3 mice in the C3-/- group showed a slight swelling of the ankles. None of the three mice in the WT group demonstrated any swelling. In the mice injected with B. garinii 1/3 mice from the WT and 1/3 mice from the C3-/- group had slight swelling of the ankle joints.In B. bavariensis injected mice slight swelling was seen in 1/3 C3-/- mice and in 3/3 WT mice; two of those mice had slightly and one moderately swollen ankle joints. A slight swelling in the ankles of 3/3 WT mice infected with B. valaisiana was also seen whereas ankles of C3 -/- mice injected with this species appeared normal.

Discussion and conclusions

In vitro, some *Borrelia* species can evade lysis by complement components. In this study we evaluated the capacity of different *B. burgdorferi* sl species with different levels of complement resistance to induce infection and inflammation in WT and complement-deficient mice in vivo. Infection was not detected in mice after challenge with complement-susceptible spirochetes belonging to *B. garinii* or *B. valaisiana* species. The absence of complement in C3 -/- mice did not enhance the infectivity of complement-susceptible strains.
All cultures from mice inoculated with the *B. burgdorferi* strain N40 were positive, in contrast only a few heart samples from mice infected with the *B. afzelii* strains, despite the fact that spirochete loads in tissue were comparable between mice infected with either of these species. A limited amount of culture positivity in *B. afzelii* infections in mice has previously been reported [537]. This suggests that *B. burgdorferi* ss strains are much easier to culture in vitro and that the results of spirochete cultures from infected humans or mice is dependent on the spirochete genetic background.

Since the infectivity and pathogenicity of the individual isolates that were used in this study was unknown, mixtures of different isolates belonging to the same *Borrelia* species for experiments were applied. Infection could be detected after infection of WT and C3 -/- mice with *B. burgdorferi* ss, *B. afzelii* and *B. bavariensis;* in contrast none of the mice injected with complement sensitive strains became infected. In mice infected with complement-susceptible *B. garinii* strains none of the strains were able to sustain infection. Four of the five strains of the complement sensitive *B. garinii* that we used had been isolated from either human skin or human CSF and have been able to sustain infections in humans. Of the strains used in this study, *B. garinii* PBr has been used in mouse experiments before, but was unable to establish an infection in wild type Swiss Webb mice [537]. In the same study, it was shown that only 4/9 *B. garinii* strains were infectious for mice; complement-sensitivity of the infectious

strains was not assessed. Escudero et al. found that 8 out of 9 tested *B. garinii* strains from well-characterized different genetic backgrounds were infectious for mice; these 9 strains were all non-OspA serotype 4 strains [549]. A possible explanation of the lack of infectivity of the non-OspA serotype 4 *B. garinii* in WT and C3-/- mice could be that they have lost virulence factors, such as plasmids, by previous excessive in vitro culture. However, most strains had been passaged in vitro less than 8 times before inoculation, which renders loss of plasmids less likely. Apparently, absence of the complement-mediated killing pathway in the C3 -/- mice did not result in increased infectivity of these strains in mice.
The five *B. valaisiana* strains had all been obtained from ticks, since this species has never been cultured from human patients and only once from laboratory mice [549]. Therefore, a low virulence of *B. valaisiana* strains was expected and confirmed by this study.

In nature, *B. garinii* and *B. valaisiana* are more often cultured from birds [507, 550-555]. In concordance with this finding some *B. garinii* are resistant to killing by pheasant serum in vitro, but not to mammalian sera [65]. Removing complement activity did not alter infectivity, thus this study underscores that infectivity is not dependent on complement resistance. Other factors must be more crucial for infectivity of non-OspA serotype 4 *B. garinii* and *B. valaisiana*.

Although C3 -/- mice generally had higher spirochete loads than WT mice by qPCR, this was not statistically significant. The only exception was the ankle joints from mice infected with *B. burgdorferi* ss, where C3 -/- mice contained significantly more spirochetes, in comparison to the negative qPCR results for joints from the WT mice. The low load of spirochetes in *B. burgdorferi* ss infected WT mice can be due to the C57/Bl6 mouse model. It has been described that *B. burgdorferi* ss in C57/Bl6 mice can give low loads of spirochetes in comparison to C3Hen mice [556]. These reports also describe that a low load of spirochetes was accompanied by a relatively low rate of inflammation in the C57/Bl6 mice.

When grouping the WT and the C3 -/- mice together we can conclude that *B. burgdorferi* and *B. afzelii* show comparable loads, while loads in mice infected with *B. bavariensis* strains were much lower. This is in concordance to what has been reported previously using different *B. burgdorferi* ss, *B afzelii* and *B. garinii* strains, where *B. burgdorferi* and *B. afzelii* frequently were strongly PCR positive, while *B. garinii* infected mice differ in infectivity and generally are present with lower loads [537].

We can generally conclude that the absence of C3 does not lead to major differences in infectivity of spirochetes in C57/Bl6 mice. This is in agreement with findings of Woodman et al., who found no major effects of the infectivity of *B. burgdorferi* ss injected in factor H deficient mice concluded that mice lacking factor H were as efficiently infected by *B. burgdorferi* as WT mice [98]. A potential problem with that model is the fact that factor H deficient mice practically do not have C3, compensating for their factor H deficiency and can not kill invading spirochetes by complement activation [99]. Knocking out CRASP2 in a *B. burgdorferi* ss strain did not alter infectivity in a mouse model, though in that model one can still argue that other CRASPs can complement factor H binding [86].
An additional explanation for the apparent lack of involvement of complement-mediated killing in this study is the use of syringe inoculation. Syringe inoculation is not a natural route of infection and tick inoculation is a more efficient method for infection. In the process of transmission from tick to host many proteins are involved. Borrelia proteins but also tick salivary proteins are of importance; SALP 15 binding to OspC can aid *B. burgdorferi* ss to enter the mammalian host, but also other SALPs might be crucial in efficient transmission [46, 48, 557]. In the transmission cycle from tick to host a meticulous up and down regulation of several proteins takes place [79, 558]. Syringe inoculation might bypass essential steps necessary for efficient borrelia infection. For the complement resistant strains however needle inoculation did not seem to influence infectivity in C3 -/- nor in WT mice. It is doubtful whether tick inoculation of spirochetes would make the complement sensitive strains more infectious.

The results from this study suggest that complement evasion strategies are not critical for effective infection and dissemination. Other components play a crucial role especially in infection with *B. garinii*. Developing a model that can properly mimic natural entry of spirochetes in the mammalian host will aid in future research. More studies on *B. burgdorferi* sl, the hosts and the European tick *I. ricinus* are required to fully understand the complex interaction involved in transmission, infection and disease.

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