



Cross-reactive acquired immunity influences transmission success of the Lyme disease pathogen, *Borrelia afzelii*



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ABSTRACT

Cross-reactive acquired immunity in the vertebrate host induces indirect competition between strains of a given pathogen species and is critical for understanding the ecology of mixed infections. In vector-borne diseases, cross-reactive antibodies can reduce pathogen transmission at the vector-to-host and the host-to-vector lifecycle transition. The highly polymorphic, immunodominant, outer surface protein C (OspC) of the tick-borne spirochete bacterium *Borrelia afzelii* induces a strong antibody response in the vertebrate host. To test how cross-immunity in the vertebrate host influences tick-to-host and host-to-tick transmission, mice were immunized with one of two strain-specific recombinant OspC proteins (A3, A10), challenged via tick bite with one of the two *B. afzelii* ospC strains (A3, A10), and infested with xenodiagnostic ticks. Immunization with a given rOspC antigen protected mice against homologous strains carrying the same major ospC group allele but provided little or no cross-protection against heterologous strains carrying a different major ospC group allele. There were cross-immunity effects on the tick spirochete load but not on the probability of host-to-tick transmission. The spirochete load in ticks that had fed on mice with cross-immune experience was reduced by a factor of two compared to ticks that had fed on naive control mice. In addition, strain-specific differences in mouse spirochete load, host-to-tick transmission, tick spirochete load, and the OspC-specific IgG response revealed the mechanisms that determine variation in transmission success between strains of *B. afzelii*. This study shows that cross-immunity in infected vertebrate hosts can reduce pathogen load in the arthropod vector with potential consequences for vector-to-host pathogen transmission.

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1. Introduction

Cross-reactive acquired immunity occurs when the antibodies developed against one pathogen strain interfere with the fate of another pathogen strain. Antibodies developed against an earlier, primary infection may prevent the establishment of a later, secondary infection or reduce the density of the secondary strain in the host tissues. Cross-reactive acquired immunity (or cross-immunity) induces indirect competition between strains and is critical for structuring the ecology of mixed infections (Frank, 2002; Read and Taylor, 2001). In vector-borne infections, acquired immunity can reduce pathogen transmission success at two critical steps in the pathogen life cycle: vector-to-host transmission and host-to-vector transmission. Previous work has shown that host-to-vector transmission success often depends on the density of the pathogen in the host tissues at the time of vector attachment (de Roode et al., 2005; Mackinnon et al., 2008; Raberg, 2012). Thus cross-immunity, by reducing the density of competing pathogen strains inside the host, might have important consequences for host-to-vector transmission success.

Borrelia burgdorferi sensu lato (s. l.) is a genospecies complex of tick-borne spirochete bacteria that includes the causative agents of Lyme disease in Europe and North America (Kurtenbach et al., 2006). This zoonotic pathogen is maintained in nature by cycles involving *Ixodes* ticks and vertebrate reservoir hosts such as birds and small mammals. Each *Borrelia* genospecies, in turn, consists of multiple strains that are often differentiated by the single copy, highly polymorphic ospC gene (Andersson et al., 2013b; Brisson and Dykhuizen, 2004; Durand et al., 2015; Earnhart and Marconi, 2007c; Perez et al., 2011; Qiu et al., 2002; Strandh and Raberg, 2015; Theisen et al., 1993; G. Wang et al., 1999; Wilske et al., 1986, 1993). The ospC gene codes for the immunodominant outer surface protein C (OspC), which induces a strong antibody response in the vertebrate host (Dressler et al., 1993; Engstrom et al., 1995; Fung et al., 1994). The anti-OspC IgG response provides protection against secondary infection (Gilmore et al., 1996; Preac-Mursic et al., 1992; Probert and Lefebvre, 1994). A study on the North American genospecies of *B. burgdorferi* sensu stricto (s. s.) showed that immunization with OspC provides protection only against strains carrying that particular ospC allele suggesting that there is no cross-protective immunity (Probert et al., 1997). Similarly, a sequential infection experiment with two strains of *B. burgdorferi* s. s. carrying different ospC alleles found no evidence for cross-protective immunity (Derdakova et al., 2004). In contrast, a recent study on the

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European genospecies of *Borrelia afzelii* in wild rodents found a pattern of co-occurrence between *ospC* strains suggesting that cross-immunity was shaping the community of multiple infections in the rodent reservoir host (Andersson et al., 2013b). Thus despite the fact that the OspC antigen has received extensive study, the pattern of protective cross-immunity between the different *ospC* strains is not well understood for most members of the *B. burgdorferi* s. l. genospecies complex.

Acquired immunity against *Borrelia* pathogens can reduce the efficacy of host-to-tick transmission. Immunization of infected mice with outer surface protein A (OspA) reduced the transmission rate of *B. burgdorferi* s. s. (Bhattacharya et al., 2011; Gomes-Solecki et al., 2006; Richer et al., 2014; Tsao et al., 2001; Voordouw et al., 2013). However, this transmission-blocking acquired immunity does not occur under natural conditions because the spirochetes rarely express the OspA antigen inside the vertebrate host (De Silva and Fikrig, 1997; De Silva et al., 1996). In contrast, the OspC antigen is expressed inside the vertebrate host (Crother et al., 2004; Liang et al., 2004; Zhong et al., 1997) and so OspC-specific antibodies could potentially reduce host-to-tick transmission. In particular, hosts with previous immune experience with the OspC antigen may develop a faster and more effective anti-OspC IgG response against secondary infections carrying a different *ospC* allele. In *B. burgdorferi* s. s., shared epitopes between different OspC antigens can create cross-reactive antibodies (Ivanova et al., 2009). Thus the purpose of the present study was to test whether antibodies against a given OspC antigen can influence the host-to-tick transmission success and tick pathogen load of a strain carrying a different *ospC* allele. To isolate the effect of cross-immunity and avoid direct competition between strains, we used recombinant OspC (rOspC) proteins to induce an OspC-specific antibody response, thereby removing the confounding effect of a resident primary infection. We predicted that immunization with the rOspC antigen would protect mice against infectious challenge (via tick bite) with strains carrying the same *ospC* allele (homologous strain) but not against strains carrying a different *ospC* allele (heterologous strain). We also predicted that cross-immunity would reduce the host-to-tick transmission rate and the tick spirochete load. Specifically, we predicted that these two spirochete phenotypes would be lower in infected mice that had immune experience with the heterologous rOspC antigen compared to infected mice that had no immune experience with the rOspC antigen.

2. Materials and methods

2.1. Mice and ticks

Four-week-old, pathogen-free, female *Mus musculus* BALB/cByJ mice (Charles River, l'Arbresle, France) were housed in groups of four or five with ad libitum access to food and water (Protector, Switzerland). The animals were allowed to adjust to their new surroundings for seven days before the start of the experiment. Mice were housed individually following infectious challenge with *B. afzelii* to avoid any direct transmission between animals. The mice were euthanized 28 weeks after entering our animal care facility. The commission that is part of the 'Service de la Consommation et des Affaires Vétérinaires (SCAV)' of Canton Vaud, Switzerland evaluated and approved the ethics of this study. The Veterinary Service of the Canton of Neuchâtel, Switzerland issued the animal experimentation permit used in this study (NE2/2012). *Ixodes ricinus* ticks came from our pathogen-free, laboratory colony that has been maintained for over 33 years at the Institute of Biology, University of Neuchâtel. To ensure that this *I. ricinus* colony remains pathogen-free, no wild-caught ticks have been introduced into the colony since its establishment.

Host-to-tick transmission was recently compared between laboratory and wild *I. ricinus* ticks infected with one of the two strains of *B. afzelii* used in this study (A10) and BALB/c mice. Host-to-tick transmission of strain A10 was 85.5% for the laboratory ticks (Tonetti et al., 2015) and 64.0% (64 infected/100 total) for the wild ticks (unpublished data). This comparison suggests that laboratory ticks are more competent at acquiring *B. afzelii* than wild ticks. One explanation for this difference

is that the laboratory ticks have a reduced microbial symbiont community compared to wild *I. ricinus* ticks (Lo et al., 2006). *Ixodes* ticks with experimentally reduced microbial symbiont communities are more susceptible to infection with *B. burgdorferi* s. l. pathogens (Narasimhan et al., 2014).

2.2. *B. afzelii* isolates and the major *ospC* group allele

B. afzelii isolates E61 and NE4049 were chosen for this study because both isolates are highly infectious to laboratory mice via tick bite (Tonetti et al., 2015). The origins of these isolates and their capacity for tick-to-host transmission and systemic (host-to-tick) transmission were described in a previous study (Tonetti et al., 2015). Both isolates had been passaged fewer than five times to avoid the loss of the virulence genes that are critical for infection (Tonetti et al., 2015). The *ospC* alleles of a given *Borrelia* species are often clustered into what are called major *ospC* groups that are defined as being more than 8% divergent at the DNA sequence level from all other such groups (I.N. Wang et al., 1999). *B. afzelii* contains at least 19 different major *ospC* groups (Strandh and Raberg, 2015). There are currently two different systems of nomenclature for the major *ospC* groups of *B. afzelii*: one developed by Lagal et al. (2003) and the other developed by Bunikis et al. (2004). Using the nomenclature of Bunikis et al. (2004), isolates E61 and NE4049 carried the major *ospC* groups A3 (GenBank accession number: L42890) and A10 (GenBank accession number: JX103488), respectively (Durand et al., 2015; Tonetti et al., 2015). The genetic distance between major *ospC* groups A3 and A10 is intermediate (20.7%) compared to other such pairs (8.9–26.4%; Durand et al., 2015). Thus if cross-immunity effects occur for this intermediately divergent pair of major *ospC* groups, it is likely to exist for pairs that are genetically more similar. Hereafter, we refer to isolates E61 and NE4049 as *B. afzelii ospC* strains A3 and A10, respectively.

Isolates of *B. burgdorferi* s. l. often contain multiple *ospC* strains (Durand et al., 2015; Perez et al., 2011; Qiu et al., 2002). We recently used deep sequencing to confirm that isolates E61 and NE4049 were 100.0% pure for major *ospC* groups A3 and A10, respectively (Tonetti et al., 2015). In the present study, we also used the *ospC* gene as a strain-specific marker to differentiate between strains as numerous other studies have done (Durand et al., 2015; Andersson et al., 2013b; Baum et al., 2012; Brisson and Dykhuizen, 2004; Perez et al., 2011; Tonetti et al., 2015; I.N. Wang et al., 1999). Previous genetic work has shown that the *ospC* locus is in linkage disequilibrium with many other loci in the *Borrelia* genome (Brisson et al., 2012; Bunikis et al., 2004; Hellgren et al., 2011; Qiu et al., 2004). We therefore emphasize that any phenotypic differences between strains A3 and A10 may be due to genetic variation at these other loci.

2.3. Creation of nymphs infected with *B. afzelii ospC* strains A3 and A10

Five mice were infected via nymphal tick bite for each of the two strains of *B. afzelii* (total of 10 mice). The nymphal ticks used to infect the mice were obtained from a previous experiment (Tonetti et al., 2015). Four weeks after infection, each mouse was infested with ~100 larval ticks. Blood-engorged larvae were placed in individual tubes (1.7 ml Eppendorf tubes containing a moistened piece of paper towel) and were allowed to molt to nymphs. These flat pre-challenge nymphs were tested for *B. afzelii* infection using a quantitative polymerase chain reaction (qPCR) at 1 month and 7 months post-molt. The infection prevalence of the 7-month-old nymphs was 80.0% (16 infected/20 total) and 70.0% (14 infected/20 total) for strains A3 and A10, respectively (Table 1).

2.4. Production of recombinant OspC proteins

DNA was isolated from ticks infected with *B. afzelii ospC* strains A3 or A10 using the QIAGEN DNeasy® Blood & Tissue kit according to the

Table 1

The geometric mean spirochete loads are shown for the subset of *Borrelia afzelii*-infected *Ixodes ricinus* nymphs that were used to challenge the immunized mice.

Nymphal state ^a	Nymphal age (months) ^b	<i>B. afzelii</i> strain	rOspC immunogen	Immunization treatment	Infected nymphs/total nymphs	Spirochete load ^c Geometric mean (95% C. L.) ^d
Flat	1	A3	N. A. ^e	N. A.	23/30	1406 (584–3382)
Flat	1	A10	N. A.	N. A.	27/30	11,344 (6912–18,619)
Flat	7	A3	N. A.	N. A.	16/20	743 (375–1472)
Flat	7	A10	N. A.	N. A.	14/20	1537 (471–5014)
Engorged	11	A3	PBS	Control	20/34	3530 (1437–8667)
Engorged	11	A3	rOspC A10	Hetero	18/29	1521 (769–3007)
Engorged	11	A3	rOspC A3	Homo	31/58	3159 (1799–5546)
Engorged	11	A10	PBS	Control	21/38	2896 (1478–5675)
Engorged	11	A10	rOspC A3	Hetero	31/57	2723 (1468–5050)
Engorged	11	A10	rOspC A10	Homo	37/51	2907 (1750–4861)

^a The nymphal state refers to whether the nymphs were flat (pre-challenge) or blood-engorged (post-challenge).

^b The nymphal age is the number of months after the larva-to-nymph molt that the nymphs were killed to check their infection status for *B. afzelii*.

^c The spirochete load is the number of spirochetes per nymph.

^d 95% confidence limits of the geometric mean.

^e N. A. = not applicable.

manufacturer's instructions. The *ospC* gene, corresponding to the full OspC protein without its leader peptide, was amplified using primers modified from Earnhart et al. (2005). The forward primer contained a BamHI restriction site (underlined) in the 5' end (5'-GT ATA GGA TCC AAT AAT TCA GGG AAA GGT GG-3') and the reverse primer contained a HincII restriction site (underlined) in the 5' end (5'-C ATG GTC GAC TTA AGG TTT TTT TGG ACT TTC TGC-3'). DNA was ligated by T/A cloning to a pGEM-T plasmid (PROMEGA) and then digested with BamHI and HincII restriction enzymes. Digested blunt-ended DNA was ligated to the BamHI and HincII sites of the bacterial expression vector pQE30Xa. ImmBiomed GmbH (Pfungstadt, Germany) performed the expression and purification of the rOspC proteins using His-Tag chromatography and gel filtration. The rOspC proteins were dissolved in PBS (pH 7.0) and their concentrations were determined using a Bradford assay.

2.5. Immunization treatments and infectious challenge

Forty-two mice were randomly assigned to one of three immunization treatments: rOspC A3 (n = 16), rOspC A10 (n = 16), or PBS (n = 10). Each mouse was immunized subcutaneously four times at weekly intervals (days 1, 8, 15, and 22). The first immunization contained 20 µg of rOspC mixed with Freund's complete adjuvant (total volume = 100 µl). The second, third and fourth immunizations contained 10 µg of rOspC mixed with Freund's incomplete adjuvant (total volume = 100 µl per immunization). Control mice were inoculated with 100 µl of PBS and adjuvant. Immunized mice were randomly assigned to infectious challenge via tick bite with one of two *B. afzelii* *ospC* strains: A3 or A10. Thus mice immunized with rOspC A3 were challenged with the homologous A3 strain (n = 8 mice) and the heterologous A10 strain (n = 8 mice) and vice versa for the mice immunized with rOspC A10 (Table 2). The control mice were challenged with strain

A3 (n = 5) or strain A10 (n = 5). One of the mice belonging to the rOspC A10/strain A3 group died during the experiment so that the final sample size was 41 mice.

2.6. Infectious challenge with *B. afzelii*-infected ticks

To test whether immunization was protective, we challenged the mice with *B. afzelii* via tick bite two weeks after the last immunization (day 34). To ensure infectious challenge, each mouse was infested with ten randomly selected, putatively infected nymphs. To prevent the challenge nymphs from escaping, they were placed in a plastic cap (15 mm diameter) that was glued to the shaved backs of the mice using a mix of resin and honey wax (4:1). Mice were anesthetized with a mix of xylazine, ketamine and PBS (1:2:9; 5 µl per gram of mouse) during this procedure. The mice were checked daily and any detached, blood-engorged nymphal ticks were removed from the cap and frozen at -20 °C for further analysis.

2.7. Mouse ear skin biopsies

Ear skin biopsies were taken to test whether the immunization treatments had protected the mice from infectious challenge. Ear tissue samples were taken from each mouse four weeks after the nymphal challenge (day 68) and again seven days later (day 75) using a forceps type punch (2 mm in diameter). With respect to another important event in the pathogen life cycle, the two tissue samples were taken on the day of and one week after the infestation with the xenodiagnostic larvae. For simplicity, these two biopsies will be referred to as the pre-xenodiagnosis and the post-xenodiagnosis ear tissue samples.

Table 2

The status of *B. afzelii* infection is shown for the six combinations of the rOspC immunogen and *B. afzelii* strain.

rOspC immunogen	<i>B. afzelii</i> Strain	Immunization treatment	Ear tissue sample ^a	VlsE ELISA ^b	Systemic transmission ^c	Infected ticks All mice ^d	Infected ticks Infected mice ^e
PBS	A3	Control	5/5 (100.0%)	5/5 (100.0%)	5/5 (100.0%)	39/50 (78.0%)	39/50 (78.0%)
rOspC A10	A3	Heterologous	5/7 (71.4%)	5/7 (71.4%)	5/7 (71.4%)	36/70 (51.4%)	36/50 (72.0%)
rOspC A3	A3	Homologous	0/8 (0.0%)	0/8 (0.0%)	0/8 (0.0%)	0/79 (0.0%)	NA
PBS	A10	Control	5/5 (100.0%)	5/5 (100.0%)	5/5 (100.0%)	45/50 (90.0%)	45/50 (90.0%)
rOspC A3	A10	Heterologous	8/8 (100.0%)	8/8 (100.0%)	8/8 (100.0%)	73/80 (91.3%)	73/80 (91.3%)
rOspC A10	A10	Homologous	0/8 (0.0%)	0/8 (0.0%)	0/8 (0.0%)	0/80 (0.0%)	NA

^a Proportion of mice that tested positive for *B. afzelii* infection according to the qPCR of the ear tissue sample at four weeks post-infection.

^b Proportion of mice that tested positive for *B. afzelii* infection according to the ELISA using the VlsE protein at seven weeks post-infection.

^c Proportion of mice that produced at least one *B. afzelii*-infected tick via systemic transmission at four weeks post-infection.

^d Systemic transmission rate for all mice (n = 41). Number of infected ticks/total number of ticks (% of infected ticks).

^e Systemic transmission rate for the subset of infected mice (n = 23). Number of infected ticks/total number of ticks (% of infected ticks).

2.8. Systemic transmission assay

The systemic transmission rate refers to the proportion of xenodiagnostic larval ticks that acquire the spirochete from an infected mouse. To measure systemic transmission, each mouse was infested with 50 to 100 xenodiagnostic larvae four weeks after the nymphal challenge (day 68). The mice were anesthetized during this procedure as described above. Infested mice were placed in individual cages that facilitated the collection of blood-engorged larvae. Blood-engorged larval ticks were placed in individual tubes and were allowed to molt into nymphs. These tubes were stored in plastic cryoboxes at room temperature and high humidity. Four weeks after molting, ten nymphs were randomly selected for each mouse and frozen at -20°C for further analysis (total of 410 nymphs).

2.9. Serum sampling

One week before (day 28) and seven weeks after (day 83) the infectious challenge with *B. afzelii*, blood samples were collected from the tail vein of each mouse. Blood samples were spun at 1500 G for 10 min and the serum was transferred to a new tube.

2.10. Enzyme-linked immunosorbent assay (ELISA)

To determine the specificity of the anti-OspC IgG response, the mice serum samples were tested for their ability to bind both the homologous and the heterologous rOspC antigen. The details for the ELISA protocol are given in the supplementary material. To test whether the mice were systemically infected with *B. afzelii*, an ELISA targeting the VlsE protein was performed on the serum samples taken seven weeks after the infectious challenge (day 83). The VlsE protein is expressed by *B. burgdorferi* s. l. pathogens during systemic infection and is one of the classical antigens used to determine the infection status of a vertebrate host. The full-length VlsE antigen used in this study was a gift from Reinhard Wallich and had been derived from *B. burgdorferi* s. s. strain B31-5A3 (Lawrenz et al., 1999). The ELISA protocol for the VlsE antigen was the same as the one for the OspC antigen.

2.11. DNA extraction of nymphs and mouse ear tissue biopsies

All xenodiagnostic ticks analyzed in this study were killed four weeks after molting into the nymphal stage. Ticks were crushed using the TissueLyser II by shaking them with a stainless steel bead (1.4 mm in diameter) at a frequency of 30 Hz for 1 min. Total DNA was extracted for each tick using the DNeasy 96 Blood & Tissue kit well plates (QIAGEN) and following the manufacturer's instructions. Each DNA extraction plate contained 94 ticks and two negative DNA extraction controls (*Anopheles gambiae* mosquitoes). DNA from the mouse ear tissue samples was extracted using the DNeasy Blood & Tissue kit mini spin column according to the manufacturer's instructions. We measured the DNA concentration of all mouse ear tissue samples using a Nanodrop.

2.12. qPCR to determine spirochete infection

A qPCR amplifying a 132 base pair fragment of the *flagellin* gene (Schwaiger et al., 2001) was used to detect and quantify *Borrelia* DNA. The 20 μl qPCR mixture consisted of 10 μl of $2\times$ Master Mix (FastStart Essential DNA Probes Master, Roche Applied Science), 3 μl of water, 0.4 μl of 20 μM primer FlaF1A, 0.4 μl of 20 μM primer FlaR1, 0.2 μl of 10 μM Flaprobe1, and 5 μl of DNA template. The thermocycling conditions included a denaturation step at 95°C for 10 min followed by 55 cycles of 60°C for 30 s and 95°C for 10 s using a LightCycler® 96 (Roche Applied Science, Switzerland). Each sample (tick or mouse ear biopsy) was run in triplicate. Each qPCR plate contained 28 samples, 3 standards, and one negative control (all in triplicate) for a total of 96 qPCR

reactions. The three standards contained 27,780, 2778 and 278 copies of the *flagellin* gene in 5 μl , respectively (see supplementary material for details). The LightCycler® 96 software (Roche Applied Science, Switzerland) calculated the standard curves and the absolute number of spirochetes present in each positive sample. The total spirochete load for each tick was calculated by multiplying the spirochete load in 5 μl of tick DNA template by the appropriate correction factor.

2.13. Statistical methods

All statistical analyses were done in R version 3.1.0. (R Development Core Team, 2013).

2.13.1. Quantification of the OspC-specific IgG antibody response

To obtain a reliable measure of OspC-specific or VlsE-specific antibody activity, the area under the curve of absorbance versus time was integrated over the first 28 min of measurement (hereafter referred to as the Absorb₂₈ value). The specificity of the anti-OspC IgG antibody response to immunization with one of the two rOspC antigens and to infection with one of the two *B. afzelii* ospC strains is presented in the supplementary material.

2.13.2. Definition of *B. afzelii* infection status for mice and ticks

Mice or ticks were considered infected if at least two of the three qPCR runs tested positive for *B. afzelii*. All mice and the vast majority of ticks were either definitively positive (all three runs tested positive) or definitively negative (all three runs tested negative). Ticks with ambiguous qPCR results (one or two positive runs) were rare ($5.3\% = 90/1697$) and the classification of their infection status did not influence the results.

2.13.3. Effect of rOspC immunization on the mouse-specific systemic transmission rate

The systemic transmission rate was calculated for each infected mouse ($n = 23$ infected mice). The homologous mice were excluded from this analysis because they were not infected. A GLM with binomial errors was used to test whether the immunization treatment (control, heterologous), *B. afzelii* ospC strain (A3, A10), and their interaction had an effect on the mouse-specific systemic transmission rate. As the rodent spirochete load can influence the probability of host-to-tick transmission (Raberg, 2012), the above analysis was repeated using the spirochete load of the pre-xenodiagnosis ear tissue samples as a covariate. Mouse ear spirochete load was divided by the DNA concentration of the ear tissue sample and this ratio was subsequently log-transformed (see supplementary material for more details). This variable is hereafter referred to as the mouse ear spirochete load.

2.13.4. Effect of cross-immunity on spirochete load inside xenodiagnostic ticks infected via systemic transmission

The spirochete load of each xenodiagnostic tick was calculated as the geometric mean of the three replicate runs (negative runs were excluded). Similarly, the average xenodiagnostic tick spirochete load for each infected mouse ($n = 23$) was calculated as the geometric mean of the infected ticks (negative ticks were excluded). This variable was log-transformed to improve normality and then modeled as a linear function of immunization treatment (control, heterologous), *B. afzelii* ospC strain (A3, A10), and their interaction. The homologous mice were excluded from this analysis because they were not infected. The above analysis was repeated using the mouse ear spirochete load as a covariate.

3. Results

In what follows below, the tick spirochete load refers to the total number of *B. afzelii* spirochetes inside a tick. The mouse spirochete load refers to the number of spirochetes inside the ear tissue biopsy.

All means are reported with their standard errors unless otherwise indicated.

3.1. Immunization with rOspC induced a strong IgG response against the rOspC antigen

Immunization with the rOspC antigen induced a strong IgG response in the mice one week after the last immunization (Fig. S1; Supplementary material). For the pre-infection serum samples, the mean Absorb₂₈ value of the mice immunized with rOspC A3 (2105 ± 119.3 units) was 26 times higher than that of the control mice (81 ± 2.8 units). Similarly, the mean Absorb₂₈ value of the mice immunized with rOspC A10 (2942 ± 99.9 units) was 33 times higher than that of the control mice (89 ± 2.4 units).

3.2. Infection status of the challenge nymphs

An average of 6.5 blood-engorged nymphs were recovered per mouse (range = 1–10). For strains A3 and A10, each mouse was challenged with an average of 3.5 infected ticks (range = 2–10) and 4.2 infected ticks (range = 1 to 9), respectively. Analysis of the blood-engorged nymphs confirmed that all the mice in the study had been challenged with at least one *B. afzelii*-infected nymph. The mean spirochete load inside the pre-challenge flat nymphs decreased over time (compare month 1 versus month 7 in Table 1). For strains A3 and A10, the mean spirochete load decreased by 47.2% ($p = 0.283$) and 86.5% ($p < 0.001$), respectively. The spirochete load inside the challenge nymphs increased over the blood meal (compare pre-challenge flat nymphs at 7 months versus post-challenge engorged nymphs fed on the control mice at 11 months in Table 1). Blood feeding increased the spirochete load of the challenge nymphs for strains A3 and A10 by 375.1% ($p = 0.444$) and 88.4% ($p = 0.067$), respectively. We note here that a previous study on *B. burgdorferi* s. s. in *I. scapularis* found that the nymphal spirochete load increased six-fold over the blood meal (Piesman et al., 2001). There was no effect of immunization treatment ($p = 0.681$), strain ($p = 0.399$), and their interaction ($p = 0.342$) on the mean spirochete load inside the post-challenge engorged nymphs (Table 1).

3.3. Infection status of mice following the infectious challenge

Of the 41 mice, 18 individuals (16 homologous, 2 heterologous) were protected from the infectious challenge with *B. afzelii* (Table 2). The remaining 23 individuals (10 controls, 13 heterologous) became infected with one of the two strains of *B. afzelii* (Table 2). The infection status of the mice was determined using three independent tests: (1) the ear tissue biopsies one month after infectious challenge, (2) the VlsE ELISA seven weeks after infectious challenge (Fig. S3; supplementary material), and (3) the xenodiagnostic assay one month after infectious challenge (Table 2). Importantly, there was 100% agreement between these three independent lines of evidence (Table 2).

3.4. Antibodies against rOspC provides specific protection against *B. afzelii*

All of the ten control mice immunized with PBS became infected with either strain A3 or strain A10 following the infectious challenge (Table 2). This result shows that the challenge nymphs were infectious to immunologically naive mice. The effect of the immunization treatment was highly significant (GLM with binomial errors, $p < 0.001$). Immunization with rOspC induced strong protection against infectious challenge with the homologous strain but not the heterologous strain. All of the 16 homologous mice were protected from infectious challenge (Table 2) whereas only 2 of the 15 heterologous mice were protected from infectious challenge (Table 2). These two mice had been immunized with rOspC A10 and challenged with strain A3. The cross-protective immunity of the rOspC A10 antigen against strain A3

(28.6% = 2/7) was therefore broader than that of the rOspC A3 antigen against strain A10 (0.0% = 0/8) but the difference was not significant.

3.5. Antibodies against rOspC had no effect on the mouse-specific systemic transmission rate

For the subset of infected mice ($n = 23$), the GLM analysis of the mouse-specific systemic transmission rate found a significant effect of strain ($p = 0.001$; Fig. 1) but not of the immunization treatment (control versus heterologous, $p = 0.678$; Fig. 1) or the interaction ($p = 0.545$). The systemic transmission rate of strain A10 (90.7% = 118/130 ticks; $n = 13$ mice) was 1.2 times higher than strain A3 (75.0% = 75/100 ticks; $n = 10$ mice).

The previous analysis was repeated using mouse ear spirochete load as a covariate. The main effect of strain remained statistically significant ($p = 0.019$). There was a significant interaction between immunization treatment and mouse ear spirochete load ($p = 0.033$). The relationship between mouse ear spirochete load and systemic transmission was therefore examined separately for the control and heterologous mice (Fig. 2). There was a significant positive relationship between mouse ear spirochete load and systemic transmission in the heterologous mice ($p = 0.035$) but not in the control mice ($p = 0.667$; Fig. 2).

3.6. Effect of immunization treatment and *B. afzelii* ospC strain on the mouse ear spirochete load

The repeatability of the mouse ear spirochete load was 0.513 (see supplementary material for details). For the subset of infected mice ($n = 23$ mice), a two-way ANOVA found no significant interaction between immunization treatment and strain on the mouse ear spirochete load ($p = 0.065$). The immunization treatment was not statistically significant ($p = 0.918$) but there was a significant effect of strain ($p = 0.004$). The mean mouse ear spirochete load (in a 2 mm diameter biopsy) for strain A10 ($34,716 \pm 4732$ spirochetes) was 1.9 times higher than strain A3 ($18,172 \pm 3300$ spirochetes).

3.7. Effect of cross-immunity on spirochete load of xenodiagnostic ticks infected via systemic transmission

The repeatability of the log-transformed spirochete load inside the xenodiagnostic ticks was 0.972 (see supplementary material for details). The linear model of the log-transformed spirochete load of the xenodiagnostic ticks found a significant effect of immunization treatment ($p = 0.009$) and of strain ($p = 0.040$) but not for the interaction ($p = 0.535$). For strain A3, the mean spirochete load of the

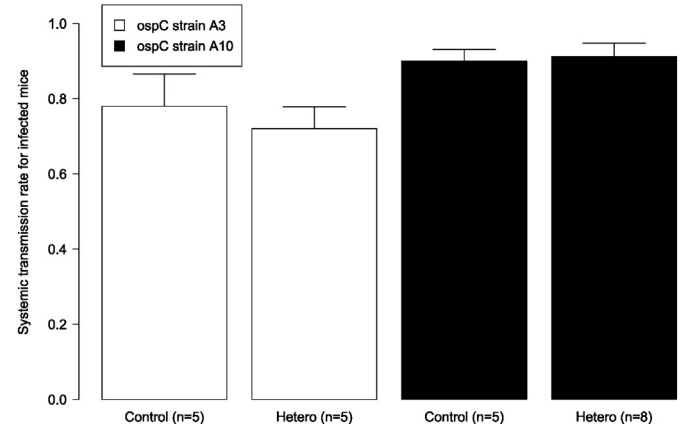


Fig. 1. Cross-reactive acquired immunity in the mouse had no effect on the systemic transmission rate of *B. afzelii*. Strain A10 had significantly higher systemic transmission than strain A3. The sample size was the subset of infected mice ($n = 10$ control and 13 heterologous). Shown are the means and the standard errors.

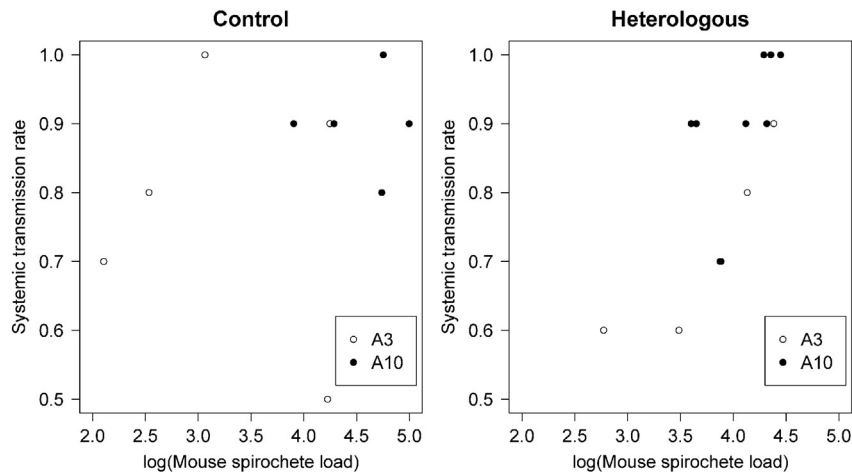


Fig. 2. The systemic transmission rate of *Borrelia afzelii* increases with the spirochete load in the mouse ear tissues. The sample size was the subset of infected mice ($n = 10$ control and 13 heterologous) and each data point represents a single mouse.

xenodiagnostic ticks infected by the control mice ($24,284 \pm 7384$ spirochetes/nymph) was 2.3 times higher than the heterologous mice ($10,348 \pm 5044$ spirochetes/nymph). For strain A10, the mean spirochete load of the xenodiagnostic ticks infected by the control mice ($32,552 \pm 4589$ spirochetes/nymph) was 1.9 times higher than the heterologous mice ($16,809 \pm 3133$ spirochetes/nymph). Thus acquired cross-immunity (in the heterologous mice) reduced by half the spirochete load inside the xenodiagnostic ticks for both strains of *B. afzelii* (Fig. 3). Strain A10 established a mean spirochete load in the xenodiagnostic ticks that was 1.34 times higher than strain A3 (for the control mice in Fig. 3).

Including mouse ear spirochete load as a covariate did not change the conclusions of the previous analysis. None of the 3- or 2-way interactions between immunization treatment, strain, and mouse ear spirochete load had a significant effect on the xenodiagnostic tick spirochete load. The mouse ear spirochete load itself had no significant effect on the xenodiagnostic tick spirochete load ($p = 0.953$).

4. Discussion

4.1. Antibodies against rOspC provides specific protection against *B. afzelii*

Immunization with rOspC antigen protected mice from infection with the matching homologous *ospC* strain. Our results are consistent

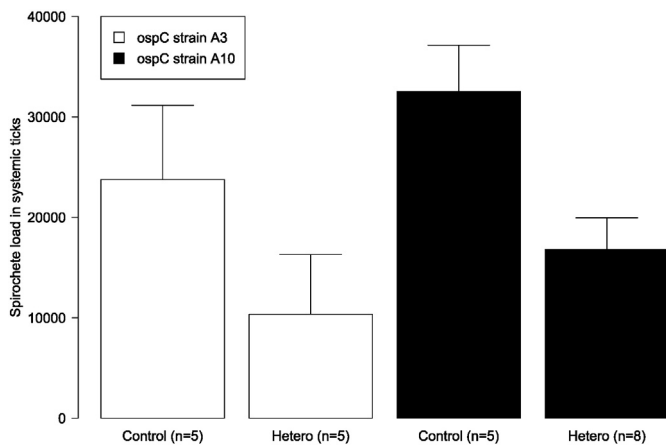


Fig. 3. Cross-reactive acquired immunity reduced the mean spirochete load of *Borrelia afzelii* inside the xenodiagnostic ticks. Strain A10 had a significantly higher mean tick spirochete load than strain A3. The sample size was the subset of infected mice ($n = 10$ control and 13 heterologous). Shown are the means and the standard errors.

with previous studies on *B. afzelii* and *B. burgdorferi* s. s., which showed that immunization with rOspC protects mice from infection (Gilmore et al., 1996; Preac-Mursic et al., 1992; Probert and Lefebvre, 1994). Our study is the first demonstration in *B. afzelii* that immunization with a given rOspC antigen provided little or no cross-protection against a strain carrying a different major *ospC* group allele. There are surprisingly few studies showing the pattern of cross-protection of the anti-OspC antibody response against strains carrying different major *ospC* group alleles (Earnhart and Marconi, 2007a; Probert et al., 1997). The study by Probert et al. (1997) demonstrated the absence of cross-protection of the anti-OspC antibody response in *B. burgdorferi* s. s. by showing that immunization with the rOspC antigen from strain SON188 protected mice from homologous challenge but not heterologous challenge (strains CA4 and 297). Infection experiments that demonstrate that mice can be sequentially infected with strains carrying different major *ospC* group alleles also demonstrate the specificity of the anti-OspC antibody response (Derdakova et al., 2004). More generally, the observation that wild reservoir hosts are frequently infected with multiple *ospC* strains is further evidence that there is limited cross-immunity between the major *ospC* groups (Anderson and Norris, 2006; Andersson et al., 2013b; Brisson and Dykhuizen, 2004; Perez et al., 2011; Strandh and Raberg, 2015).

4.2. Limited cross-immunity favors strain A10 over strain A3

We found evidence of some cross-protective acquired immunity between the two strains of *B. afzelii*. Previous studies on North American strains of *B. burgdorferi* s. s. found no evidence of cross-protection between rOspC antigens (Earnhart and Marconi, 2007a; Probert et al., 1997). A recent field study suggested that cross-immunity was structuring the community of *B. afzelii* *ospC* strains in a population of wild rodents (Andersson et al., 2013b). That study found a positive relationship between the genetic distance between two major *ospC* groups and their degree of association in the rodent host (Andersson et al., 2013b). Our study found evidence of asymmetric cross-immunity because previous immune experience with rOspC type A10 protected 28.6% (2/7) of the mice from infection with strain A3 but the reverse was not true. Asymmetric cross-immunity gives the dominant strain a two-fold competitive advantage over the weaker strain (Frank, 2002; Read and Taylor, 2001). First, the dominant strain induces an acquired immune response that blocks the weaker strain from super-infecting the same host. Second, the dominant strain is not affected by cross-immunity and is therefore capable of super-infecting hosts carrying the weaker strain. The genetic distance between major *ospC* groups A3 and A10 is intermediate (20.7%) with respect to the range of genetic distances

(8.9–26.4%) between other pairs of major *ospC* groups (Durand et al., 2015). Thus the limited cross-protective immunity observed in this study might exist for other pairs of major *ospC* groups. Whether the observed cross-immunity effect also occurs under natural conditions remains to be determined.

4.3. Mechanism of how OspC-specific antibodies protect mice from infection

The mechanism of how OspC-specific antibodies protect mice from infection is not completely understood. We found that the immunization treatment had no effect on the load of spirochetes inside the blood-engorged challenge nymphs. This result is consistent with previous work showing that OspC-specific antibodies are not borreliacidal inside the challenge nymphs (Gilmore et al., 1996). In contrast, OspA-specific antibodies are known to reduce the prevalence and load of spirochetes inside the tick vector (Fikrig et al., 1992). Expression of the OspC protein is controlled during spirochete transmission from the tick vector to the vertebrate host (De Silva and Fikrig, 1997; Tilly et al., 2008). Following tick attachment to the host, the spirochetes in the tick midgut start expressing OspC (Fingerle et al., 1998; Ohnishi et al., 2001; Schwan and Piesman, 2000; Schwan et al., 1995). Some studies suggest that OspC is critical for spirochetes to migrate from the tick midgut to the tick salivary glands (Fingerle et al., 2007; Pal et al., 2004). Other studies have shown that OspC is critical for dissemination inside the vertebrate reservoir host (Grimm et al., 2004; Seemanapalli et al., 2010; Tilly et al., 2006). Gilmore et al. (1996) proposed that OspC-specific antibodies could act in either the tick vector or the vertebrate host to protect the latter from infection. The OspC-specific antibodies can act inside the tick vector to block the migration of the spirochetes from the tick midgut to the tick salivary glands (Gilmore and Piesman, 2000). Alternatively, the vertebrate immune system can kill the spirochetes once they are injected into the host tissues by the tick vector. Heterogeneous expression of the OspC protein suggests that spirochetes will be targeted at different times during their transition from the tick vector to the vertebrate host (Ohnishi et al., 2001) and so the two mechanisms are not mutually exclusive.

4.4. Acquired cross-immunity reduces spirochete load in xenodiagnostic ticks

There was no effect of acquired cross-immunity on systemic (host-to-tick) transmission (Fig. 1). In contrast, we found cross-reactive acquired immunity effects on the tick spirochete load. The spirochete load of the ticks that had fed on the infected heterologous mice was two-fold lower than the ticks that had fed on the infected control mice (Fig. 3). This result suggests that previous immune experience with the OspC antigen allowed the heterologous mice to develop a more effective antibody response, which ultimately reduced the spirochete load inside the xenodiagnostic ticks, compared to the PBS-immunized control mice. The OspC antigen is not believed to play an important role in host-to-tick transmission because its expression is generally suppressed inside the vertebrate reservoir host to facilitate long-term persistence (Crother et al., 2004; Liang et al., 2004; Zhong et al., 1997). However, the regulation of gene expression is not 100% perfect (Gilmore and Piesman, 2000; Ohnishi et al., 2001) and OspC-specific antibodies could clear any spirochetes that accidentally expressed the OspC antigen. We found no effect of the immunization treatment on mouse ear spirochete load suggesting that this infection phenotype did not mediate the observed cross-immunity effect on tick spirochete load. This result suggests that the OspC-specific antibodies transmitted with the blood meal reduced the spirochete load inside the tick vector. Previous work has shown that the spirochete load increases inside the larval tick following the blood meal before declining dramatically during the molt from larva to nymph (Piesman et al., 1990). Given these dynamic changes in spirochete abundance, we were surprised to find an effect of the anti-OspC IgG antibodies two

months after the host-to-tick transmission event. A recent field study suggested that the innate immune system of the vertebrate reservoir host plays an important role in structuring the spirochete load inside *I. ricinus* nymphs (Herrmann et al., 2013). The present study extends this work by showing that the acquired immune system of the vertebrate host can also influence the spirochete load inside *I. ricinus*.

Cross-immunity effects on tick spirochete load are only relevant if they influence spirochete fitness. Higher spirochete load might increase the probability of spirochete persistence in the tick vector and/or the probability of tick-to-host transmission in the next step of the Lyme disease life cycle. A recent study on *I. scapularis* ticks infected with *B. burgdorferi* s. s. found that the proportion of infected ticks decreased from 90% to 15% as the spirochete infection aged inside the ticks over a period of six months under laboratory conditions (Voordouw et al., 2013). In the present study, we found that the spirochete load of *B. afzelii* decreased dramatically over a period of 6 months in the flat pre-challenge *I. ricinus* nymphs for both strains A3 (47.2% decrease) and A10 (86.5% decrease). In contrast, the proportion of infected nymphs over the same period was stable: from 90% to 70% for strain A10 and from 77% to 80% for strain A3. Thus the spirochete population declines over time inside the nymphal midgut under laboratory conditions and future studies should investigate whether this phenomenon occurs under natural conditions.

4.5. Mechanism underlying fitness variation between strains of *B. afzelii*

We found a positive relationship between the spirochete load inside the mouse ear tissues and the systemic transmission rate (heterologous mice in Fig. 2). A positive relationship between the spirochete load in the mouse tissues and the probability of host-to-tick transmission makes intuitive sense and was previously shown in a study on two species of wild rodents (Raberg, 2012). Strains of *B. afzelii* are probably under strong selection to maintain a high density in transmission-relevant tissues like the skin of the ears where ticks are likely to feed and acquire spirochetes.

Strain A10 outperformed strain A3 on the three infection phenotypes. The mouse ear spirochete load, the systemic transmission rate, and the spirochete load inside the ticks were 1.9, 1.2, and 1.34 times higher for strain A10 than for strain A3. Interestingly, a field study on *B. afzelii* in populations of wild rodents and *I. ricinus* in Switzerland found that A10 was one of the most common strains (Durand et al., 2015; Perez et al., 2011; Tonetti et al., 2015). In a previous experimental infection study, we estimated the reproductive number (R_0) for six *ospC* strains of *B. afzelii* including strains A3 and A10 (Tonetti et al., 2015). This study showed that strain A10 had one of the highest R_0 values, which was 1.6 times higher than that of strain A3 (Tonetti et al., 2015). The present study suggests that strain A10 is more successful than strain A3 because it maintains a higher spirochete density in both the rodent host and the tick vector. This study has therefore enhanced our understanding of the mechanisms that determine variation in fitness between strains of *B. afzelii* (Tonetti et al., 2015). However, we emphasize that most of the phenotypic differences between strains A3 and A10 are not necessarily caused by the *ospC* gene but by other loci that are in linkage disequilibrium with the *ospC* locus (Brisson et al., 2012; Bunikis et al., 2004; Hellgren et al., 2011; Qiu et al., 2004).

4.6. Specificity of the anti-OspC IgG response differs between OspC antigens

Infection with *B. afzelii* produced an anti-OspC IgG response that was highly specific for that particular OspC antigen (Fig. S2; Supplementary material). The OspC-specific IgG antibodies of the infected control mice were 3.5–9.8 times more likely to bind the homologous rOspC antigen than the heterologous rOspC antigen (Fig. S2). A previous study on *B. burgdorferi* s. s. used a panel of seven rOspC proteins (major *ospC* groups A, B, C, D, H, K, N) to show that the antiserum developed against infection with one of three major *ospC* group strains (A, B, or D) was

specific for that particular rOspC protein (Earnhart et al., 2005). Interestingly, *B. afzelii* strain A10 induced an OspC-specific IgG response that was twice as strong as strain A3 (Fig. S2). Strain A10 had a spirochete load in the mouse tissues that was almost twice as high as strain A3. Thus one possible explanation is that the higher density of strain A10 in the mouse tissues induced a stronger OspC-specific IgG antibody response. Another explanation for the difference in the strength of the OspC-specific immune response is that strain A10 produces more OspC on its surface than strain A3.

The structure of the OspC protein and the locations of the protective epitopes are critical for understanding how the pattern of cross-protective acquired immunity can influence the community structure of *B. afzelii* ospC strains in the field. The OspC protein is a dimer where each monomer consists of five α -helices ($\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$) and two β -strands ($\beta 1$, $\beta 2$) (Eicken et al., 2001; Kumaran et al., 2001). Most of the variable regions are found on the β -strands and the two loops (L4, L5) connecting helix $\alpha 2$ with $\alpha 3$ and helix $\alpha 3$ with $\alpha 4$. Earnhart et al. (2005) found linear epitopes on the $\alpha 5$ helix (residues 168 to 203) and on loop 5 (residues 136 to 150) of the rOspC protein of *B. burgdorferi* s. s. strain B31. Subsequent work showed that antibodies developed against the $\alpha 5$ helix and loop 5 epitopes were bactericidal (Earnhart et al., 2007). Gilmore and Mbow (1999) using the same strain found a conformational epitope involving either the N- or C-terminal of the rOspC protein. Mathiesen et al. (1998) found one linear epitope within the C-terminal seven residues of the OspC protein of *Borrelia garinii*. Future studies should investigate whether the protective epitopes of the OspC antigen in *B. afzelii* are the same as the ones found in *B. burgdorferi* s. s. and *B. garinii*.

The diversity of the ospC gene and the lack of cross-protection between the different OspC antigens complicate the development of an OspC-based vaccine. In the United States, researchers have developed a multivalent vaccine that combines the epitopes of up to eight different OspC antigens (Earnhart et al., 2007; Earnhart and Marconi, 2007b). However, an octavalent vaccine would not be sufficient in Europe where a single population of *I. ricinus* ticks can carry as many as 22 different major ospC group alleles (Durand et al., 2015). In addition, there are concerns regarding the public interest in a Lyme disease vaccine given the previous failure of the OspA-based Lymerix vaccine in the United States (Embers and Narasimhan, 2013; Nardelli et al., 2009; Plotkin, 2011). In summary, an OspC-based Lyme disease vaccine for humans faces both technical and sociological hurdles.

4.7. The diversity and complexity of tick-borne infections in nature

The present experimental infection study is an oversimplification of the situation in nature. In the field, infections with multiple ospC strains are common in both ticks and reservoir hosts (Andersson et al., 2013b; Brisson and Dykhuizen, 2004; Durand et al., 2015; Heylen et al., 2014; Perez et al., 2011; Strandh and Raberg, 2015; I.N. Wang et al., 1999). The present study investigated indirect competition between ospC strains mediated by the host immune system but did not consider direct competition between strains over limited tick or host resources (Derdakova et al., 2004; Strandh and Raberg, 2015). In addition to the ospC strain diversity within a *Borrelia* genospecies, ticks and reservoir hosts are often infected with multiple *Borrelia* genospecies (Gern et al., 2010; Herrmann et al., 2013; Hovius et al., 2007; Perez et al., 2011; Rauter and Hartung, 2005) and with different species of tick-borne pathogens (Aleksiev et al., 2003; Andersson et al., 2013a, 2014; Burri et al., 2014; Levin and Fish, 2000). Mixed infections can result in facilitation or inhibition where one pathogen strain or species has positive or negative effects on the transmission of another pathogen strain or species (Ginsberg, 2008; Macaluso et al., 2002; Mixson et al., 2006). The potential number of interactions between multiple tick-borne pathogen strains and species is therefore overwhelming. However, a recent study on the ospC strains of *B. afzelii* found that laboratory estimates of strain fitness could explain a surprisingly large amount of the variation

in the strain-specific frequencies in the field (Tonetti et al., 2015). Thus there is hope that studies that ignore most of the interspecific diversity of tick-borne pathogens can still shed light on the factors that maintain a complex of pathogen strains (Tonetti et al., 2015).

5. Conclusions

In summary, our study found that acquired immunity against a given OspC antigen provides limited cross-protection against *B. afzelii* strains carrying a different major ospC group allele. Cross-reactive acquired immunity in the vertebrate host influenced the spirochete load in ticks that fed on those hosts with potentially important consequences for spirochete persistence inside the tick vector and tick-to-host transmission. The spirochete load in the rodent host influenced the probability of host-to-tick transmission, thereby illuminating the mechanisms underlying the variation in fitness between strains of *B. afzelii*.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.meegid.2015.09.012>.

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