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Strain-specific antibodies reduce co-feeding transmission of the Lyme disease pathogen, *Borrelia afzelii*

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Summary

Vector-borne pathogens use a diversity of strategies to evade the vertebrate immune system. Co-feeding transmission is a potential immune evasion strategy because the vector-borne pathogen minimizes the time spent in the vertebrate host. We tested whether the Lyme disease pathogen, *Borrelia afzelii*, can use co-feeding transmission to escape the acquired immune response in the vertebrate host. We induced a strain-specific, protective antibody response by immunizing mice with one of two variants of OspC (A3 and A10), the highly variable outer surface protein C of *Borrelia* pathogens. Immunized mice were challenged via tick bite with *B. afzelii* strains A3 or A10 and infested with larval ticks at days 2 and 34 post-infection to measure co-feeding and systemic transmission respectively. Antibodies against a particular OspC variant significantly reduced co-feeding transmission of the targeted (homologous) strain but not the non-targeted (heterologous) strain. Cross-immunity between OspC antigens had no effect in co-feeding ticks but reduced the spirochaete load twofold in ticks infected via systemic transmission. In summary, OspC-specific antibodies reduced co-feeding transmission of a homologous but not a heterologous strain of *B. afzelii*. Co-feeding transmission allowed *B. afzelii* to evade the negative consequences of cross-immunity on the tick spirochaete load.

Introduction

Pathogens have evolved many strategies to avoid being cleared by the immune system of their hosts (Schmid-Hempel, 2008). Evasion of the host immune system is particularly important for vector-borne pathogens that establish long-lived systemic infections inside vertebrate hosts (Brunham *et al*., 1993). Many vector-borne pathogens use antigenic variation to stay one step ahead of the vertebrate antibody response (Bloom, 1979; Blaxter *et al*., 1992; Roberts *et al*., 1992; Damian, 1997; van der Woude and Baumler, 2004; Frank and Barbour, 2006). Another strategy by which vector-borne pathogens can avoid the vertebrate immune system is to spend less time in the vertebrate host and more time in the arthropod vector. This strategy is most developed in vector-borne pathogens that are capable of co-feeding transmission. In co-feeding transmission, vector-borne pathogens are transmitted between infected and uninfected vectors feeding next to each other on the same vertebrate host at the same time (Randolph *et al*., 1996; Nuttall and Labuda, 2004; Tsao, 2009; Randolph, 2011; Voordouw, 2015). In systemic transmission by contrast, there is a latent phase during which the pathogen establishes a widespread (systemic) infection inside the vertebrate host before achieving host-to-vector transmission. Thus the main difference between co-feeding and systemic transmission is that the former is local and immediate whereas the latter is from anywhere on the host body and delayed (Randolph, 2011; Voordouw, 2015). These two modes of transmission are not exclusive and many vector-borne pathogens use both. Co-feeding transmission has been reported in a variety of vector-borne pathogens including the vesicular stomatitis virus in black flies (Mead *et al*., 2000), the West Nile Virus in mosquitoes (Higgs *et al*., 2005) and a number of tick-borne pathogens including Thogoto virus (Jones *et al*., 1987), Bunyavirus (Labuda *et al*., 1997a), tick-borne encephalitis virus (TBEV) (Alekseev and Chunikhin, 1990; Labuda *et al*., 1993a,b,c), *Anaplasma phagocytophilum* (Levin and Fish, 2000) and *Borrelia burgdorferi* sensu lato (s.l.), the species complex of tick-borne spirochaete bacteria that includes the aetiological agents of human Lyme disease (Gern and Rais, 1996; Sato and Nakao, 1997; Piesman and Happ, 2001; Crippa *et al*., 2002; Richter *et al*., 2002; Hu *et al*., 2003; Tonetti *et al*., 2015).

Co-feeding transmission allows vector-borne pathogens to evade the innate and acquired immune system of their vertebrate hosts (Voordouw, 2015). TBEV causes a

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short-term viremia in mice that induces lifelong sterilizing immunity against future infection (Labuda *et al*., 1997b). However, rodents with acquired immunity against TBEV are still capable of transmitting the virus via co-feeding transmission (Labuda *et al*., 1997b). Thus co-feeding transmission allows TBEV to evade the antibody response of resistant vertebrate hosts (Labuda *et al*., 1997b). Similarly, a study on the intracellular tick-borne bacterium, *A. phagocytophilum*, found that acquired immunity in rodents reduced but did not completely block co-feeding transmission (Levin and Fish, 2000). Co-feeding transmission also allows *B. burgdorferi* s.l. pathogens to obtain some fitness benefits from incompetent vertebrate hosts (Randolph *et al*., 1996; Gern *et al*., 1998; Voordouw, 2015). Ungulate hosts do not develop a systemic infection because their complement system kills *Borrelia* spirochaetes (Kurtenbach *et al*., 1998a; 2002). However, a number of field studies suggest that deer and sheep can amplify *Borrelia* pathogens via co-feeding transmission (Kimura *et al*., 1995; Ogden *et al*., 1997; Pichon *et al*., 2000). Thus co-feeding transmission allows *Borrelia* pathogens to evade clearance by the hostile innate immune system of incompetent reservoir hosts (Voordouw, 2015). The purpose of the present study was to investigate whether the Lyme disease pathogen, *B. afzelii*, can use co-feeding transmission to evade pre-existing acquired immunity in the vertebrate host.

Borrelia afzelii is one of the most common causes of Lyme disease in Europe (Piesman and Gern, 2004; Kurtenbach *et al*., 2006). This tick-borne spirochaete bacterium is vectored by the hard tick *Ixodes ricinus*, and the main reservoir hosts are wild rodents (Humair *et al*., 1995; 1999; Humair and Gern, 1998; Kurtenbach *et al*., 1998b; Hanincova *et al*., 2003). *Borrelia afzelii* can establish long-lived infections in its rodent reservoir hosts with a high rate of systemic (host-to-tick) transmission (Gern *et al*., 1994; Humair *et al*., 1999). This tick-borne pathogen is also capable of co-feeding transmission (Gern and Rais, 1996; Crippa *et al*., 2002; Richter *et al*., 2002; Hu *et al*., 2003; Tonetti *et al*., 2015). We have recently shown that there is genetic variation in the efficacy of co-feeding transmission among strains of *B. afzelii*, suggesting that this trait can evolve under natural selection (Tonetti *et al*., 2015). In nature, rodent reservoir hosts are repeatedly exposed to infected ticks and studies in the United States have shown that wild rodent populations develop high levels of *Borrelia*-specific antibodies (Hofmeister *et al*., 1999; Bunikis *et al*., 2004a). Under these circumstances, co-feeding transmission may allow *Borrelia* pathogens to escape acquired immunity in the rodent host (Voordouw, 2015).

Acquired immunity in the vertebrate host plays an important role in the epidemiology of Lyme disease (Johnson *et al*., 1986a,b; Kurtenbach *et al*., 1994; Piesman *et al*., 1997; Liang *et al*., 2004). One *Borrelia* antigen that is particularly important for the pathogen's interaction with the vertebrate immune system is outer surface protein C (OspC) (Radolf and Caimano, 2008). OspC is expressed during the transmission of *Borrelia* spirochaetes from the tick vector to the vertebrate host (Schwan *et al*., 1995; Gilmore and Piesman, 2000; Grimm *et al*., 2004; Pal *et al*., 2004; Tilly *et al*., 2006; Fingerle *et al*., 2007). The single-copy *ospC* gene is highly polymorphic, and this variability has likely evolved in response to the acquired immune system of the vertebrate host (Wang *et al*., 1999; Baranton *et al*., 2001). For the three *Borrelia* species that have been studied (*B. burgdorferi* s.s., *B. afzelii*, and *B. garinii*), the *ospC* alleles cluster into 14–22 major *ospC* groups, which are defined as > 8% divergent at the DNA sequence level from all other such groups (Wang *et al*., 1999; Baranton *et al*., 2001; Lagal *et al*., 2003; Brisson and Dykhuizen, 2004; Bunikis *et al*., 2004b; Durand *et al*., 2015; Strandh and Raberg, 2015). Each OspC antigen induces a strong IgG antibody response that is protective against strains carrying that particular major *ospC* group allele (Preac-Mursic *et al*., 1992; Probert and Lefebvre, 1994; Gilmore *et al*., 1996) but not against strains carrying different major *ospC* group alleles (Probert *et al*., 1997; Earnhart *et al*., 2005; Jacquet *et al*., 2015). In nature, wild rodents and *Ixodes* ticks are often infected with multiple *ospC* strains of a given *B. burgdorferi* s.l. pathogen (Wang *et al*., 1999; Qiu *et al*., 2002; Brisson and Dykhuizen, 2004; Anderson and Norris, 2006; Pérez *et al*., 2011; Andersson *et al*., 2013; Durand *et al*., 2015; Strandh and Raberg, 2015). A recent study on *B. afzelii* suggested that cross-immunity between OspC antigens determined the pattern of multiple strain infections in wild rodents (Andersson *et al*., 2013). In summary, the OspC protein is a highly polymorphic immunodominant antigen that plays a key role in structuring the strain community of *Borrelia* pathogens in the field.

In a previous study, we showed that immunization with recombinant OspC protein (rOspC) protected mice from a homologous infectious challenge with *B. afzelii* strains carrying the same major *ospC* group allele but not from a heterologous infectious challenge with *B. afzelii* strains carrying a different major *ospC* group allele (Jacquet *et al*., 2015). While there was no cross-immunity effect on systemic transmission, there was a cross-immunity effect on the spirochaete load in 'systemic' ticks (i.e. ticks that had acquired the infection via systemic transmission). The purpose of the present study was to test whether co-feeding transmission allowed *B. afzelii* to evade the negative effects of strain-specific antibodies developed against the homologous or the heterologous rOspC antigen. We predicted that co-feeding transmission would occur on the homologous mice but that transmission success would be reduced compared with the heterologous and control mice. We also predicted that the

cross-immunity effect on the spirochaete load in the 'systemic' ticks, which depends on an enhanced secondary antibody response to *B. afzelii* infection, would not occur in the co-feeding ticks. Co-feeding spirochaetes evade this cross-immunity effect because transmission occurs before the secondary antibody response has time to develop. Of the two strains used in the immunization trial, strain A10 but not A3 is highly competent at co-feeding transmission (Tonetti *et al*., 2015). We chose these two strains to test whether a *B. afzelii* strain capable of co-feeding transmission would have a fitness advantage when faced with hosts that have protective, sterilizing antibodies.

Results

Definitions

Mice that were immunized with a rOspC antigen that matched or did not match the major *ospC* group of the subsequent challenge strain are referred to as 'homologous' or 'heterologous' mice respectively. Larval ticks that had the opportunity to acquire the *B. afzelii* infection via co-feeding or systemic transmission and then molted into nymphs are referred to as 'co-feeding' or 'systemic' ticks respectively.

Prevalence of B. afzelii *in co-feeding challenge nymphs*

There was no evidence for co-feeding transmission among the blood-engorged challenge nymphs. For strain A3, the prevalence of infection in the blood-engorged challenge nymphs was similar between the control (58.8% = 20/34), heterologous (62.1% = 18/29) and homologous (53.4% = 31/58) groups. For strain A10, the prevalence of infection in the blood-engorged challenge nymphs was also similar between the control (55.3% = 21/38), heterologous (54.4% = 31/57) and homologous (72.5% = 37/51) groups. There was no effect of immunization treatment (generalized linear model [GLM]: Δ df = 1, Δ $\chi^2 = 0.972$, $P = 0.615$), strain (GLM: Δ df = 1, $\Delta\chi^2 = 0.608$, $P = 0.436$) and their interaction (GLM: Δ df = 2, Δ $\chi^2 = 4.225$, $P = 0.121$) on the proportion of blood-engorged challenge nymphs that were infected with *B. afzelii*.

Correspondence between mice that had co-feeding and systemic transmission

There was a statistically significant association between the two modes of transmission across the 40 mice ($\chi^2 = 4.812$, df = 1, $P = 0.028$). Sixteen mice had both modes of transmission and 12 mice had neither. There were five homologous mice that had co-feeding but no systemic transmission: two challenged with strain A3 and three challenged with strain A10. There were 7 *B. afzelii*-infected mice that had systemic but no co-feeding transmission: six infected with strain A3 (three control, three heterologous) and one infected with strain A10 (heterologous).

Antibodies against rOspC reduced the mouse-specific co-feeding transmission rate

There was no difference in the mouse-specific co-feeding transmission rate between the control and heterologous mice (GLM: Δ df = 2, Δ $\chi^2 = 0.24$, $P = 0.889$; Fig. 1), and these two groups were therefore combined (Table 1). In contrast, there was a highly significant difference in the mouse-specific co-feeding transmission rate between the homologous mice and the combined group of control and heterologous mice (GLM: Δ df = 1, Δ $\chi^2 = 83.74$, $P < 0.001$; Fig. 1). For strain A10, the co-feeding transmission rate of the control and heterologous mice combined (51.6% = 98/190 ticks; 13 mice; Table 1) was 15.6 times higher than the homologous mice (3.3% = 3/90

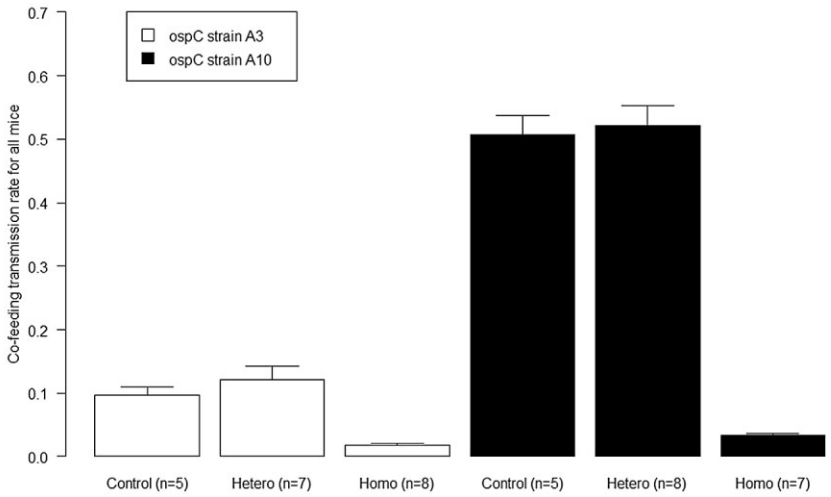

Fig. 1. Co-feeding transmission of *Borrelia afzelii* was blocked by the homologous but not the heterologous immunization treatment. There was no difference in co-feeding transmission between the heterologous and control group. The rate of co-feeding transmission of strain A10 was almost five times higher than that of strain A3. The unit of replication is the mouse-specific co-feeding transmission rate. The sample size includes all the mice from which we recovered co-feeding larval ticks ($n = 40$). Shown are the means and the standard errors.

Table 1. The rate of co-feeding transmission is shown for the six combinations of the antigen used for immunization (rOspC A3, rOspC A10, or PBS) and the *Borrelia afzelii ospC* strain used in the infectious challenge (A3 or A10).

				Co-feeding transmission		
Antigen	Strain	Treatment	Infected mice/ Total mice[a]	Co-feeding mice/ Total mice[b]	Infected ticks/ Total ticks[c]	Infected ticks/ Total ticks[d]
PBS + Adjuv	A3	Control	5/5 (100.0%)	2/5 (40.0%)	5/52 (9.6%)	5/28 (17.9%)
rOspC A10	A3	Heterologous	5/7 (71.4%)	2/7 (28.6%)	9/74 (12.2%)	9/22 (40.9%)
	A3	Control + Hetero[e]	10/12 (83.3%)	4/12 (33.3%)	**14/126 (11.1%)**	14/50 (28.0%)
rOspC A3	A3	Homologous	0/8 (0.0%)	2/8 (25.0%)	**2/111 (1.8%)**	2/29 (6.9%)
PBS + Adjuv	A10	Control	5/5 (100.0%)	5/5 (100.0%)	35/69 (50.7%)	35/69 (50.7%)
rOspC A3	A10	Heterologous	8/8 (100.0%)	7/8 (87.5%)	63/121 (52.1%)	63/119 (52.9%)
	A10	Control + Hetero[e]	13/13 (100.0%)	12/13 (92.3%)	**98/190 (51.6%)**	98/188 (51.1%)
rOspC A10	A10	Homologous	0/8 (0.0%)	3/7 (42.9%)	**3/90 (3.3%)**	3/48 (6.3%)
Total			23/41	21/40		

a. Proportion of mice that were systemically infected ($n = 23/41$). Mouse infection status was determined by three independent criteria: (1) qPCR of ear tissue biopsy, (2) ELISA using the VlsE antigen, and (3) qPCR of xenodiagnostic ticks.
b. Proportion of mice that produced at least one infected tick via co-feeding transmission ($n = 21/40$).
c. Proportion of ticks that were infected via co-feeding transmission for all mice ($n = 40$).
d. Proportion of ticks that were infected for the subset of mice ($n = 21$) that produced at least one infected tick via co-feeding transmission.
e. The rows titled 'Control + Hetero' contain the combined data for the control and heterologous mice for each strain.

ticks; 7 mice; Table 1). For strain A3, the co-feeding transmission rate of the control and heterologous mice combined (11.1% = 14/126 ticks; 12 mice; Table 1) was 6.2 times higher than the homologous mice (1.8% = 2/111 ticks; 8 mice; Table 1). Thus in both strains, co-feeding transmission was drastically reduced but not completely eliminated by antibodies directed against the homologous but not the heterologous rOspC antigen.

There was also a significant effect of *B. afzelii* strain on the mouse-specific co-feeding transmission rate (GLM: Δ df = 1, $\Delta \chi^2 = 58.16$, $P < 0.001$; Fig. 1). For the control and heterologous mice combined ($n = 25$), the co-feeding transmission rate of strain A10 (51.6% = 98/190 ticks; summed over 5 control and 8 heterologous mice; Table 1) was 4.6 times higher than that of strain A3 (11.1% = 14/126 ticks; summed over 5 control and 7 heterologous mice; Table 1).

Efficacy of co-feeding versus systemic transmission

Larval ticks were more likely to acquire spirochaetes via systemic transmission than co-feeding transmission. For strain A10, systemic transmission (90.7% = 118/130; summed over 5 infected control and 8 infected heterologous mice) was 1.75 times higher than co-feeding transmission (51.6% = 98/190) and this difference was statistically significant (paired *t*-test: $t = 4.67$, $df = 12$, $P < 0.001$). For strain A3, systemic transmission (75% = 75/100; summed over 5 infected control and 5 infected heterologous mice) was 5.8 times higher than co-feeding transmission (13.3% = 14/105), and this difference was statistically significant (paired *t*-test: $t = 8.58$, $df = 9$, $P < 0.001$). Thus systemic transmission was more efficient than co-feeding transmission for both strains.

Effect of co-feeding versus systemic transmission on the tick spirochaete load

Nymphs infected as larvae via co-feeding transmission had significantly lower spirochaete loads than nymphs infected as larvae via systemic transmission (analysis was restricted to the subset of mice that had both modes of transmission for strain A10 ($n = 12$); paired *t*-test: $t = 3.30$, $df = 11$, $P = 0.007$; Fig. 2). For the control mice ($n = 5$), the spirochaete load of the systemic ticks (32 557 ± 4590 spirochaetes per nymph) was 6.1 times higher than the co-feeding ticks (5337 ± 1221 spirochaetes per nymph). For the heterologous mice ($n = 7$), the spirochaete load of the systemic ticks (16 809 ± 3133 spirochaetes per nymph) was 1.9 times higher than the co-feeding ticks (8940 ± 2267 spirochaetes per nymph). There was no difference in the spirochaete load of co-feeding ticks between control and heterologous mice (independent samples *t*-test: $t = 1.52$, $df = 10$, $P = 0.161$).

Correlations between co-feeding transmission rate, systemic transmission rate, co-feeding tick spirochaete load, and the systemic tick spirochaete load

None of the six pairwise correlations were statistically significant between the following four variables: the co-feeding transmission rate, the systemic transmission rate, the co-feeding tick spirochaete load and the systemic tick spirochaete load (Table S1).

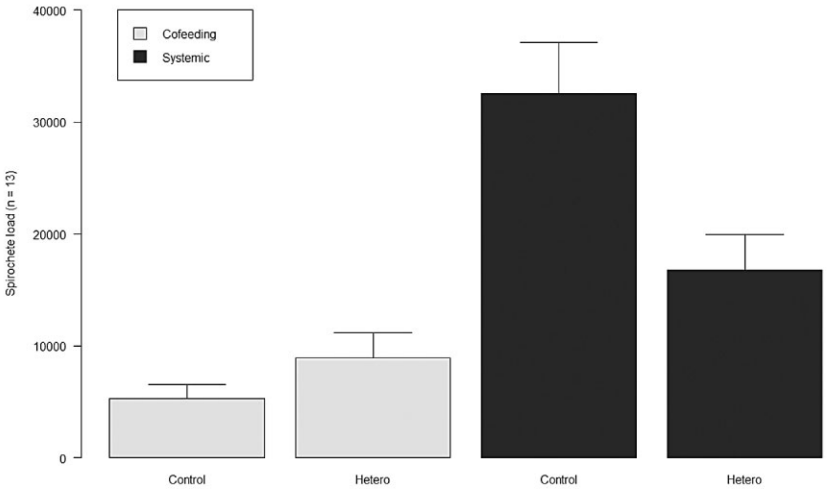

Fig. 2. The mode of transmission (co-feeding or systemic) influenced the spirochaete load of *Borrelia afzelii ospC* strain A10 inside *Ixodes ricinus* nymphal ticks. Nymphs infected as larvae via systemic transmission had a higher spirochaete load than nymphs infected as larvae via co-feeding transmission. The effect of the immunization treatment (control versus heterologous) depended on the mode of transmission. For the co-feeding nymphs, the immunization treatment had no effect on the tick spirochaete load. For the systemic nymphs, immune experience with the heterologous rOspC antigen reduced the tick spirochaete load relative to the control group (Jacquet *et al*., 2015). The unit of replication is the mouse-specific geometric mean spirochaete load. The sample size is the subset of systemically infected mice that produced at least one A10-infected tick via co-feeding transmission ($n = 12$). Shown are the means and the standard errors.

Discussion

OspC-antibodies reduced co-feeding transmission of B. afzelii

OspC-specific antibodies in laboratory rodents greatly reduced the efficacy of co-feeding transmission of the homologous but not the heterologous strain of *B. afzelii*. For strain A10, immunization with the homologous rOspC A10 antigen reduced the co-feeding transmission rate 15-fold compared with the control and heterologous groups. There were a number of homologous mice that infected larval ticks via co-feeding transmission despite being protected from systemic infection. This important result shows that co-feeding transmission can occur independently from and is not inevitably followed by systemic infection. However, the co-feeding transmission rate of *B. afzelii* on these homologous mice was so low that this strategy is unlikely to make a significant contribution to pathogen fitness (Hartemink *et al*., 2008). Other studies have shown that acquired immunity in the vertebrate host can reduce the efficacy of co-feeding transmission of tick-borne pathogens (Jones and Nuttall, 1989; Labuda *et al*., 1997b; Levin and Fish, 2000). For the tick-borne bacterium *A. phagocytophilum*, acquired immunity reduced the co-feeding transmission rate 10-fold (10.8% versus 1.1%) in a natural rodent host (Levin and Fish, 2000). For TBEV, acquired immunity reduced the co-feeding transmission rate threefold (72% versus 24%) in field mice and 1.4-fold (42% versus 29%) in bank voles (Labuda *et al*., 1997b). Finally, acquired immunity against the Thogoto virus in guinea pigs completely eliminated co-feeding transmission (Jones and Nuttall, 1989). Thus acquired immunity in the vertebrate host generally reduces co-feeding transmission of vector-borne pathogens, but there is substantial variation in the magnitude of the effect size. In summary, co-feeding transmission did not allow *B. afzelii* to escape the protective strain-specific antibody response of the vertebrate host.

Co-feeding transmission allows B. afzelii *to escape the negative effects of cross-immunity on tick spirochaete load*

Cross-reactive acquired immunity (or cross-immunity) refers to differences in infection phenotype between the heterologous and control groups. Heterologous mice had previous experience with a different (heterologous) OspC antigen whereas control mice were completely naïve at the time of the infectious challenge. Our study found no effects of cross-immunity on the co-feeding transmission rate or on the co-feeding tick spirochaete load (Figs 1 and 2). By contrast, we showed in a previous study that there were strong effects of cross-immunity on the spirochaete load of both strains in 'systemic' ticks (Jacquet *et al*., 2015). The mean spirochaete load of the systemic ticks that had fed on the infected heterologous mice was half that of the systemic ticks that had fed on the infected control mice (Jacquet *et al*., 2015). This result suggests that previous immune experience with a different OspC antigen allowed the heterologous mice to develop a faster secondary antibody response against the *B. afzelii* infection than the control mice. The efficacy of this secondary antibody response would have peaked at 3–4 weeks after the infectious challenge, which is when the mice were infested with the second batch of larval ticks to measure systemic transmission. In contrast, co-feeding transmission was measured 48 h after the infectious challenge, which was insufficient time for the heterologous mice to develop the enhanced secondary antibody response. Thus the difference in timing between co-feeding and systemic transmission explains the difference in the cross-immunity effect on tick spirochaete load between these two modes of transmission. Co-feeding transmission is instantaneous and therefore escapes the negative consequences of the cross-immunity-enhanced

secondary antibody response, which is time-lagged. Systemic transmission is delayed and is therefore vulnerable to this time-lagged, cross-immunity-enhanced secondary antibody response. In summary, co-feeding transmission allowed *B. afzelii* to evade the negative effects of cross-immunity on tick spirochaete load.

The mechanism of co-feeding transmission

The mechanism of co-feeding transmission in *Borrelia* pathogens is not well understood (Voordouw, 2015). During the blood meal, infected nymphs inoculate about 100 spirochaetes into the feeding lesion (Kern *et al*., 2011). These spirochaetes replicate locally around the site of the tick bite before disseminating to other host tissues (Shih *et al*., 1992; Hodzic *et al*., 2003). Larval ticks attached near the feeding lesion of infected nymphal ticks could subsequently imbibe these locally replicating spirochaetes (Randolph *et al*., 1996; Tsao, 2009). A study on *B. afzelii* in laboratory mice showed that co-feeding transmission has both a spatial and a temporal component (Richter *et al*., 2002). Co-feeding transmission was most efficient (55.3%) when the larvae fed in close proximity (<1 cm) to the nymphs and when the larval ticks attached 2–3 days after the nymphs (Richter *et al*., 2002). Previous studies have shown that nymph-to-host transmission of *B. afzelii* increases over time and reaches ~100% after 48 h (Kahl *et al*., 1998; Crippa *et al*., 2002). This time delay in nymph-to-host transmission is caused by the migration of the *B. afzelii* spirochaetes from the tick midgut to the tick salivary glands. The duration of this spirochaete migration explains why co-feeding transmission is highest when the larvae attach > 48 h after the nymphs (Richter *et al*., 2002).

The saliva of ticks is believed to play an important role in the co-feeding transmission of tick-borne pathogens (Nuttall and Labuda, 2004). Tick saliva contains substances that modulate the inflammatory and immune response of the vertebrate host (Ribeiro *et al*., 2006; Bowman and Nuttall, 2008; Kazimirova and Stibraniova, 2013). For example, tick saliva inhibits or interferes with the vertebrate complement response (Lawrie *et al*., 1999; 2005), the activity of chemokines and cytokines (Hajnicka *et al*., 2001; 2005; Brossard and Wikel, 2004) and macrophage function (Kopecky and Kuthejlova, 1998). The immunosuppressive properties of tick saliva help tick-borne pathogens, including *B. burgdorferi* s.l., to evade the host immune system (Kuthejlova *et al*., 2001; Ramamoorthi *et al*., 2005). Tick salivary glands also contain substances that stimulate spirochaete growth *in vitro* (Rudolf and Hubalek, 2003; Rudolf *et al*., 2010) and in laboratory mice (Zeidner *et al*., 2002; Macháčková *et al*., 2006). In summary, we expected that co-feeding ticks inside the capsule would create a local immunosuppressed environment in the rodent skin that is propitious for spirochaete replication and transmission. However, this local immunosuppression was not sufficient to suppress the protective capacity of the OspC-specific antibodies.

Protection of OspC-specific antibodies

The mechanism by which the OspC-specific antibodies reduced co-feeding transmission is not completely understood. *Borrelia* spirochaetes express OspC during their migration from the tick midgut to the tick salivary glands (Schwan *et al*., 1995; De Silva and Fikrig, 1997). There is some controversy regarding the functional role of the OspC protein during tick-to-host transmission (Radolf and Caimano, 2008). Some studies suggest that the OspC protein allows the *Borrelia* spirochaetes to invade the salivary glands of *Ixodes* ticks (Pal *et al*., 2004; Fingerle *et al*., 2007). However, the research by Rosa and colleagues shows that the OspC protein allows the spirochaete to disseminate from the site of the tick bite and establish infection inside the vertebrate host (Grimm *et al*., 2004; Stewart *et al*., 2006; Tilly *et al*., 2006; 2008; Seemanapalli *et al*., 2010; Kenedy *et al*., 2012). Thus OspC-specific antibodies can target spirochaetes in either the tick vector or the vertebrate host (Gilmore *et al*., 1996; Gilmore and Piesman, 2000). Regardless of the underlying mechanism, the present study clearly shows that OspC-specific antibodies reduce co-feeding transmission of homologous strains of *B. afzelii* carrying the same major *ospC* group allele.

Co-feeding transmission among nymphs

The results do not allow us to conclude whether co-feeding transmission occurs between nymphs or not. In the present study, we found no effect of the immunization treatment on the proportion of infected nymphs, which suggests that co-feeding transmission did not occur between nymphs. However, the blood-engorged nymphs were frozen immediately after dropping off the host. Hence, a likely explanation is that any spirochaetes transmitted by co-feeding between nymphs did not have enough time to replicate to a detectable abundance. In contrast, an experimental infection study on songbirds that allowed blood-engorged nymphs to molt into adults showed that nymphs can acquire *Borrelia* pathogens via co-feeding transmission (Heylen *et al*., 2014). Regardless of its existence or not, theoretical models have shown that nymph-to-nymph co-feeding transmission makes a negligible contribution to the reproductive number (R_0) of *Borrelia* pathogens (Hartemink *et al*., 2008; Harrison *et al*., 2011; Harrison and Bennett, 2012). Thus from an epidemiological perspective, nymph-to-larva co-feeding transmission is much more important than nymph-to-nymph transmission (Voordouw, 2015).

The mode of transmission and tick spirochaete load

The spirochaete load in co-feeding nymphs was up to six times lower than in systemic nymphs 2 months after the larval blood meal (Fig. 2). This result suggests that larval ticks acquire fewer spirochaetes via co-feeding transmission than systemic transmission and/or that co-feeding spirochaetes are not able to increase their growth rate to reach the same population size as spirochaetes acquired via systemic transmission. A study on *B. burgdorferi* s.s. in *Ixodes scapularis* has shown that the spirochaete population is highly dynamic during this period (Piesman *et al*., 1990). The spirochaete population grows rapidly after the blood meal and then declines dramatically during the larva-to-nymph molt (Piesman *et al*., 1990). The detection of spirochaetes after the larva-to-nymph molt is therefore proof of transstadial transmission and that the nymph contains a viable population of spirochaetes (Richter *et al*., 2002; Heylen *et al*., 2014). Additional evidence of the viability of co-feeding spirochaetes comes from studies that have cultured spirochaetes from co-feeding ticks (Piesman and Happ, 2001; Hu *et al*., 2003). Whether ticks infected via co-feeding transmission are capable of infecting competent reservoir hosts is currently unknown and should be addressed in future research (Voordouw, 2015).

Strain-specific differences in co-feeding transmission and fitness

There were strain-specific differences in the efficacy of co-feeding transmission (Table 1). The co-feeding transmission rate of strain A10 was 4.6 times higher than strain A3 confirming our previous study (Tonetti *et al*., 2015). The rate of systemic transmission of strain A10 is also higher than strain A3 (Jacquet *et al*., 2015; Tonetti *et al*., 2015). We recently used next-generation matrices to estimate the reproductive number (R_0) for six different *ospC* strains of *B. afzelii* (Tonetti *et al*., 2015). This analysis found 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). This strain-specific difference in fitness is associated with strain-specific differences in spirochaete load in both the vertebrate host and the tick vector. Compared with strain A3, the spirochaete load of strain A10 is 1.9 times higher in the mouse tissues and 1.34 times higher in systemic nymphs (Jacquet *et al*., 2015). These results suggest that strain A10 has higher co-feeding and systemic transmission success than strain A3 because it establishes a higher spirochaete load in the mouse tissues. Importantly, the strain-specific differences in co-feeding and systemic transmission success were not caused by differences in the infectious challenge because the prevalence of infection in the challenge nymphs was the same between strains A3 and A10, both before and after the infectious challenge.

Contribution of co-feeding transmission to fitness of B. afzelii

The importance of co-feeding transmission to *Borrelia* pathogens is controversial (Richter *et al*., 2002; 2003; Randolph and Gern, 2003; Voordouw, 2015). Theoretical models suggest that co-feeding transmission makes a modest contribution to the reproductive number of *Borrelia* pathogens and is not necessary for the maintenance of Lyme disease in nature (Hartemink *et al*., 2008; Harrison *et al*., 2011; Harrison and Bennett, 2012). However, these models ignore the reality that *Borrelia* infections in the vertebrate host and the tick vector frequently consist of multiple strains (Wang *et al*., 1999; Qiu *et al*., 2002; Brisson and Dykhuizen, 2004; Anderson and Norris, 2006; Pérez *et al*., 2011; Andersson *et al*., 2013; Durand *et al*., 2015; Strandh and Raberg, 2015). A recent study showed that *B. afzelii ospC* strains compete with each other inside wild rodent reservoir hosts although the underlying mechanism remains unknown (Strandh and Raberg, 2015). Studies on other vector-borne diseases, namely, rodent malaria, have demonstrated that competition between parasite strains inside the rodent host is common and can influence host-to-vector transmission success (de Roode *et al*., 2005; Bell *et al*., 2006; Alizon *et al*., 2013). Assuming that competition exists in multiple-strain infections of *B. afzelii*, strains capable of co-feeding transmission may have an important competitive advantage over strains that are not capable of this mode of transmission.

Previous authors have suggested that co-feeding may allow *Borrelia* pathogens to obtain some transmission on vertebrate hosts that are otherwise refractory to systemic infection (Randolph *et al*., 1996; Gern *et al*., 1998). A recent field study suggested that co-feeding transmission enhances the diversity of *ospC* strains in *B. afzelii* (Pérez *et al*., 2011). The authors speculated that some *ospC* strains are better at the classic life cycle (systemic infection followed by systemic transmission) whereas other strains are better at co-feeding transmission (Pérez *et al*., 2011). However, in a recent experimental infection study on six different *ospC* strains of *B. afzelii*, we found no such trade-off between co-feeding transmission and systemic transmission (Tonetti *et al*., 2015). Instead, strains with high co-feeding transmission also had high systemic transmission, and these strains had the highest values of R_0 (Tonetti *et al*., 2015). *Borrelia afzelii ospC* strains with high co-feeding transmission (and thus a high value of R_0) were also the most common strains in a local population of *I. ricinus* ticks over a period of 11 years (Tonetti *et al*., 2015; Durand *et al*., 2015). Thus co-feeding transmission

is correlated with spirochaete phenotypes that lead to high fitness in mice and high frequency in tick populations in nature.

Conclusions

OspC-specific antibodies in the vertebrate host reduced the efficacy of co-feeding transmission of a homologous but not a heterologous strain of *B. afzelii*. Immunization with a heterologous OspC antigen had no effect on co-feeding transmission compared with naive control mice. While co-feeding transmission occurred in homologous mice that were protected from systemic infection, the efficacy was too low to make an epidemiologically relevant contribution to the fitness of *B. afzelii*. Thus *Borrelia* pathogens cannot use co-feeding transmission to evade host antibodies specific for their OspC antigen. However, in comparison with systemic transmission, co-feeding transmission did allow *B. afzelii* to evade the negative consequences of the secondary antibody response on tick spirochaete load. The two strains of *B. afzelii* (A3 and A10) differed almost fivefold in their efficacy of co-feeding transmission. Co-feeding ticks had a spirochaete load that was six times lower than systemic ticks. Future studies should investigate whether these co-feeding ticks are infectious to vertebrate hosts.

Materials and methods

Immunization trial

We used an immunization trial followed by infectious challenge via tick bite to test whether OspC-specific antibodies in laboratory rodents blocked co-feeding transmission of *B. afzelii*. The details of this immunization trial were previously described in Jacquet and colleagues (2015). Briefly, BALB/c mice were immunized with adjuvant and one of two different recombinant OspC (rOspC) proteins: rOspC A3 ($n = 16$ mice) and rOspC A10 ($n = 16$ mice). The control mice were immunized with phosphate-buffered solution (PBS) and adjuvant ($n = 10$ mice). Mice were subsequently challenged via tick bite with one of two *B. afzelii* strains that carried either the A3 or A10 major *ospC* group allele (hereafter referred to as strain A3 and strain A10). Thus there were six combinations of antigen (rOspC A3, rOspC A10, PBS) and infectious challenge (strain A3, strain A10). In what follows, the terms homologous and heterologous refer to whether the major *ospC* allele of the challenge strain matched the rOspC antigen or not (see Table 1). 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. These 41 mice were distributed as follows: rOspC A3-immunized mice challenged with strain A3 (homologous; $n = 8$), rOspC A3-immunized mice challenged with strain A10 (heterologous; $n = 8$), rOspC A10-immunized mice challenged with strain A3 (heterologous; $n = 7$), rOspC A10-immunized mice challenged with strain A10 (homologous; $n = 8$), control mice challenged with strain A3 ($n = 5$), and control mice challenged with strain A10 ($n = 5$).

In a previous study, Jacquet and colleagues (2015) showed that the 16 homologous mice were protected from the infectious challenge whereas the 10 control mice became infected with *B. afzelii*. Two of the mice immunized with rOspC A10 were protected from infection with strain A3 whereas the remaining 13 heterologous mice became infected with *B. afzelii*. Thus there were 23 mice that became infected with *B. afzelii*: 5 heterologous mice with strain A3, 8 heterologous mice with strain A10, 5 control mice with strain A3, and 5 control mice with strain A10. The systemic infection status of all 41 mice was determined using three independent criteria: (i) IgG antibody response against the VlsE antigen (blood sample taken 21 days after infectious challenge), (ii) qPCR of mouse ear tissue biopsy (taken 34 days after infectious challenge), and (iii) qPCR of xenodiagnostic ticks (larval ticks were fed on mice 34 days after infectious challenge). The correspondence between these three independent measures of systemic infection with *B. afzelii* was 100% (Jacquet *et al.*, 2015).

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

The creation of the infected nymphs used in the infectious challenge (hereafter the 'challenge' nymphs) was previously described in Jacquet and colleagues (2015). Briefly, 50–100 larval ticks from our pathogen-free *I. ricinus* colony were fed on each of ten BALB/c mice that had been previously infected via nymphal tick bite with either strain A3 or strain A10. Blood-engorged larval ticks were placed in individual tubes and allowed to molt into the challenge nymphs. For each of the 10 mice, we randomly sampled four challenge nymphs and tested them for *B. afzelii* infection using qPCR. The mean proportion of infected challenge nymphs for strain A3 was 80.0% (16/20; 95% confidence interval = 55.7–93.4%) and for strain A10 was 70.0% (14/20; 95% confidence interval = 45.7–87.2%). The remaining challenge nymphs were used in the infectious challenge of the rOspC-immunized and control mice (see below).

Co-feeding transmission assay

With respect to the purpose of the present study, *B. afzelii* strains A3 and A10 were chosen because they differ in the efficacy of co-feeding transmission. Strain A10 has high

co-feeding transmission (66.2%) whereas strain A3 has low co-feeding transmission (0.0%) (Tonetti *et al*., 2015). The infectious challenge consisted of infesting each mouse with 10 *B. afzelii*-infected challenge nymphs (Jacquet *et al*., 2015), which had been randomly selected from a pool of nymphs for which the infection rate of strain A3 (80.0%) and strain A10 (70.0%) was known (see above). These challenge nymphs were placed in a plastic capsule that was glued to the backs of the mice to prevent the nymphs from escaping (Jacquet *et al*., 2015). To measure co-feeding transmission, mice were infested with 80 larval ticks at 48 h after the nymphal infestation. To enhance co-feeding transmission, the larvae were placed in the same capsule as the nymphs, and the mice were anaesthetized with isoflurane during this procedure. The larvae were introduced through a small hole in the capsule surface that was covered with tape for 48 h to prevent the ticks from escaping. All nymphal and larval ticks in the capsules were allowed to feed to repletion. Infested mice were placed in individual cages that facilitated the collection of blood-engorged ticks. Blood-engorged nymphs were frozen at −20°C and tested for *B. afzelii* using qPCR to confirm that each mouse had been infested with at least one infected challenge nymph (Jacquet *et al*., 2015). Blood-engorged larvae were placed in individual tubes and were allowed to molt into nymphs. These tubes were stored at room temperature with high humidity to avoid tick dehydration. Four weeks after molting, the nymphs were frozen at −20°C. One month after the infectious challenge, all the mice were infested with a batch of 50–100 xenodiagnostic larvae to measure systemic (host-to-tick) transmission (Jacquet *et al*., 2015). The nymphs infected as larvae via systemic transmission were processed the same way as the nymphs infected as larvae via co-feeding transmission. These two types of nymphs will hereafter be referred to as co-feeding ticks and systemic ticks. For each mouse, we analysed a maximum of 20 co-feeding ticks and 10 systemic ticks.

DNA extraction and qPCR to test ticks for spirochaete infection

DNA extraction of all the ticks was performed following a protocol described by Jacquet and colleagues (2015). A quantitative PCR amplifying a 132 base pair fragment of the flagellin gene was used to detect and quantify *Borrelia* DNA following a protocol described by Jacquet and colleagues (2015). Each qPCR plate contained 28 samples, 3 standards (that also functioned as positive controls), and 1 negative control (all run in triplicate) for a total of 96 qPCR reactions (see Jacquet *et al*., 2015 for details).

Statistical methods

Effect of rOspC immunization on the mouse-specific co-feeding transmission rate

The co-feeding transmission rate was calculated for each mouse for which we recovered at least one co-feeding larval tick (mean = 13.9, range = 2–20). There was one mouse for which we did not recover any co-feeding larval ticks so the final sample size was 40 mice. A GLM with binomial errors was used to test whether immunization treatment, *B. afzelii ospC* strain, and their interaction had an effect on the mouse-specific co-feeding transmission rate. Model simplification was used to test whether the control and heterologous mice could be combined into a single group.

Efficacy of co-feeding versus systemic transmission

The mouse-specific rates of co-feeding and systemic transmission represent paired data. A paired *t*-test was therefore used to determine whether co-feeding transmission was less efficient than systemic transmission for the subset of infected mice ($n = 23$ mice).

Calculation of the nymphal tick spirochaete load

The spirochaete load refers to the number of spirochaetes in the nymph at 4 weeks after the larva-to-nymph molt (when the nymph was killed by freezing). The spirochaete load of each nymphal tick was calculated as the geometric mean of the three replicate runs by the Roche software (negative runs were excluded). Similarly, the average nymphal tick spirochaete load for each mouse was calculated as the geometric mean of the ticks that had acquired the infection after feeding on that mouse (negative ticks were excluded). The estimates of tick spirochaete load were calculated separately for the co-feeding ticks and the systemic ticks. We had previously shown that the spirochaete load in the systemic ticks is a highly repeatable phenotype (Jacquet *et al*., 2015).

Effect of co-feeding versus systemic transmission on the tick spirochaete load

The geometric mean spirochaete load of the infected co-feeding ticks and of the infected systemic ticks was calculated for a subset of 16 infected mice that had both modes of transmission. The analysis was subsequently restricted to the 12 mice infected with strain A10 because only 4 mice were infected with strain A3. A paired *t*-test was used to determine whether the spirochaete load of the co-feeding ticks was different from the spirochaete load of the systemic ticks. An independent two-sample

t-test was used to test whether the immunization treatment affected the spirochaete load in the co-feeding ticks.

Correlations between co-feeding transmission rate, systemic transmission rate, co-feeding tick spirochaete load, and systemic tick spirochaete load

The six pairwise correlations between the co-feeding transmission rate (proportion of ticks infected via co-feeding transmission), the systemic transmission rate (proportion of ticks infected via systemic transmission), the log-transformed spirochaete load in ticks infected via co-feeding transmission, and the log-transformed spirochaete load in ticks infected via systemic transmission, were calculated separately for strain A3 ($n = 4$ mice) and strain A10 ($n = 12$ mice) and for both strains combined ($n = 16$). These tests were done on the subset of systemically infected mice that produced at least one tick infected via co-feeding transmission ($n = 16$ mice).

All statistical analyses were done in R version 3.2.0. (R Development Core Team, 2009).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. The pairwise correlations between the four infection phenotypes of *B. afzelii* are shown separately for strains A3 and A10 and for the two strains combined. The four infection phenotypes include systemic transmission, co-feeding transmission, the log-transformed spirochaete load in ticks infected via systemic transmission (systemic spirochaete load), and the log-transformed spirochaete load in ticks infected via co-feeding transmission (co-feeding spirochaete load). The six pairwise Pearson's correlation coefficients and their p-values (italics) are shown above and below the diagonal respectively.