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Cytokines (IL-4 and IFN-γ) and antibodies (IgE and IgG2a) produced in mice infected with *Borrelia burgdorferi* sensu stricto via nymphs of *Ixodes ricinus* ticks or syringe inoculations

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Abstract Mice were tolerant to tick bites during three infestations with nymphs of *Ixodes ricinus* infected with *Borrelia burgdorferi* sensu stricto. To determine whether tick bites influence the immune response against *B. burgdorferi*, we examined the production of cytokines IL-4 and IFN-γ by lymph node cells of BALB/c mice and IL-4 deficient BALB/c mice after tick inoculation versus syringe inoculation of *B. burgdorferi*. We also measured IgG2a anti-borrelial antibodies and total IgE in these mice. Results showed that BALB/c mice developed a Th2 immune response against *B. burgdorferi* after tick inoculation and a mixed Th1/Th2 response after syringe inoculation of *B. burgdorferi*. IL-4 deficient mice produced a Th1 immune response in both cases. IL-4 produced following tick bites greatly decreased the production of anti-borrelial IgG2a antibodies by comparison with the production of anti-borrelial IgG2a antibodies produced following syringe injection of *B. burgdorferi*.

Introduction

The agent of Lyme disease, *Borrelia burgdorferi*, is transmitted by *Ixodes* ticks (Barbour et al. 1983). The tick–host interface is characterized by a complex array of host immune defenses. Ixodid saliva contains a cocktail of pharmacologically active substances with different roles, such as anti-coagulant, anti-platelet or immunosuppressive activities (Titus and Ribeiro 1990). This saliva stimulates host immune regulatory and effector responses involving antigen-presenting cells, cytokines, T lymphocytes, basophils, mast cells, eosinophils, complement and circulating antibodies (Willadsen 1980; Wikel 1982; Brossard et al. 1991; Brossard and Wikel 1997). The immune response developed against the vector may affect transmission of various pathogens carried by that arthropod. BALB/c mice infested four times with pathogen-free *I. scapularis* nymphal ticks were protected against infection with *B. burgdorferi* transmitted with infected ticks (Wikel et al. 1997). Only 16.7% of mice repeatedly infested with pathogen-free ticks prior to infected nymph challenge became infected by *B. burgdorferi* whereas 100% of control mice infested only once with infected ticks were cultured positive for *B. burgdorferi*. These findings contrast with another experiment where prior infestation of mice with nymphs of *I. scapularis* did not protect them from infection by spirochetes (Richter et al. 1998). In both experiments, mice appeared to tolerate the bites of the vector tick.

The influence of the vector on the transmission of parasites is also described in other host–parasite associations. For example, *Phlebotomus papatasi* sand fly salivary gland lysate up-regulated expression of IL-4 mRNA in *Leishmania major*-resistant CBA mice (Mbow et al. 1998). The enhancement of Th2 cytokine IL-4 exacerbated lesion size and parasite burden.

Previous studies in our laboratory showed that BALB/c mice developed a strong Th2 response against nymphs of *I. ricinus*, associated with the tolerance of tick bites (Ganapamo et al. 1995). This immune response was not shifted towards Th1 after injection of anti-IL-4 monoclonal antibodies. In addition, IL-4 deficient mice infested with nymphs did not produced the Th1 cytokine IFN-γ (Christe et al. 1998). Moreover, the polarization of the immune response developed against nymphs of *I. ricinus* in other strains of mice, DBA (H-2d), C57BL/6 (H-2b), C3H (H-2k), CBA (H-2k), SJL (H-2s) and FVB (H-2q), was also polarized towards Th2 (Christe et al. 1999).

The aim of the present study is to compare the immune response developed by mice against *B. burgdorferi* sensu stricto either after tick or syringe inoculation of the Lyme

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disease spirochete. The production of IL-4 versus IFN-γ in in vitro lymphocyte cultures of draining lymph nodes, and two antibody isotypes (anti-borrelial IgG2a and total IgE) produced in vivo have been considered.

Materials and methods

Animals

Eight- to 12-week old BALB/c female mice were obtained from Iffa Credo (Arbresle, France). IL-4 deficient BALB/c mice and Swiss mice were obtained from the Institute of Zoology (Neuchâtel, Switzerland).

Borrelia burgdorferi strain

The European strain *Borrelia burgdorferi* sensu stricto ZS7 used in this study was cultivated and quantified as described previously (Schaible et al. 1989).

Tick colony

Larvae and nymphs of *Ixodes ricinus* ticks were derived from a colony maintained at the Institute of Zoology (Neuchâtel).

Infection of ticks with *B. burgdorferi*

Swiss mice were intradermally inoculated with 10^6 spirochetes. Five weeks after the injection, an ear punch biopsy of each mouse was placed in BSK-H to confirm the infection (Sinsky and Piesman 1989). All mice were infected and larvae of *I. ricinus* were fed on those infected mice. After moulting, nymphs were tested by immunofluorescence to evaluate the prevalence of *B. burgdorferi* as described by Gern et al. (1991). The infection rate of nymphs was 50%.

Tick inoculation of mice with *B. burgdorferi*

Each mouse was infested three times with ten nymphs infected with *B. burgdorferi*. Ticks were placed within a plastic capsule (11 mm diameter) glued onto the shoulders of mice as described by Mbow et al. (1994). No skin site was infested more than once. Each infestation lasted 4–7 days, with intervals of 7 days between infestations. The capsule was removed when all nymphs had completed their bloodmeal. We used five mice per group. Attachment success and weights of engorged nymphs were assessed as previously described (Christe et al. 1999).

Syringe inoculation of mice with *B. burgdorferi*

Spirochetes (2×10^3 in 200 µl of BSK-H) were injected intradermally three times in the shoulder of each mouse. We used five mice per group. The injections and infestations with infected nymphs were made simultaneously for all groups of mice.

Infection of mice with *B. burgdorferi*

To determine whether mice had become infected after the third tick or syringe inoculation, ear punch biopsies were placed in BSK-H. The spirochetal infection was then confirmed by examination of the BSK-H medium by means of dark-field microscopy 10 days after the addition of the ear punch biopsy (Sinsky and Piesman 1989).

Western blot

Sonicated *B. burgdorferi* strain ZS7 were boiled in 5% SDS buffer (Laemmli 1970) for 3 min. Electrophoresis was done on SDS-polyacrylamide gels (12%). The separated proteins were transferred to nitrocellulose. After blocking with 5% milk, the membrane was incubated with the respective sera. Alkaline phosphatase-labeled rat anti-mouse immunoglobulin (rat anti-mouse IgG2a, Pharmingen) was used as a second antibody. Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma) were used as substrates.

Measurement of anti-borrelial IgG2a antibodies by ELISA

Sera were collected before the first infestation and 7 days after drop-off of each tick infestation.

Each well was coated with 1 µg of a spirochetal lysate in 100 µl carbonate buffer (50 mM, pH 9.6). Incubation was done overnight at 4 °C. Blocking was carried out with 2% bovine serum albumin (BSA) in phosphate buffer saline (PBS, 10 mM, pH 7.4) for 1 h at 37 °C. The wells were filled with sera diluted 1:50 in 1% BSA dissolved in PBS (1% BSA-PBS) and incubated for 2 h at 37 °C. Then, alkaline-phosphatase labeled anti-mouse IgG2a (rat anti-mouse IgG2a, Pharmingen) diluted 1:500 in 1% BSA-PBS was added and incubated for 1 h at 37 °C. Three washes of 5 min were performed with 0.05% Tween 20 in PBS after each step. The presence of bound anti-mouse IgG2a antibodies was detected colorimetrically using 40 µg para-nitrophenylphosphate (Aldrich)/well as a substrate in diethanolamine HCl pH 9.8. Plates were incubated with substrate for 1 h at 37 °C and read at 405 nm. Results were recorded as optical density (OD). Three well replicates were done for each analysis.

Measurement of total IgE antibodies by ELISA

Total IgE antibody levels were measured using two kinds of rat anti-mouse IgE mAb as previously described (Christe et al. 1998). Three well replicates were done for each analysis.

Cell culture

Axillary and brachial lymph nodes were removed 9 days after the third infestation. Stimulation of lymph node cells has been described previously (Ganapamo et al. 1995). Briefly, 10^6 cells from pooled lymph nodes in 100 µl complete culture medium [RPMI-1640 supplemented with 10% fetal calf serum (v/v), 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM non-essential amino acids, 0.05 mM mercaptoethanol, 100 U penicillin:streptomycin/ml and 25 µg Fungizone/ml] were stimulated with 4 µg ConA/well or with 5 µg borrelial lysate/well. Supernatants were removed 24 h after stimulation with ConA and 72 h after stimulation with borrelial lysate and stored at −80 °C prior to IL-4 and IFN-γ titration. Incubation times with ConA and borrelial lysate were chosen previously and corresponded to the peak of cytokine production (results not shown).

IL-4 and IFN-γ -specific ELISA

ELISA cytokine tests were performed as described by Ganapamo et al. (1995). Three well replicates were done for each analysis. Dilutions of IL-4 (1250-5 U/ml) or IFN-γ (125-2 U/ml) recombinant proteins (rIL-4, rIFN-γ, Pharmingen) were used as standard curves. The assay for IL-4 has a sensitivity of 10 U/ml and is reproducible in the linear 10–625 U/ml range. For IFN-γ, the assay has a sensitivity of 4 U/ml and is reproducible in the linear 4–125 U/ml range.

Presentation of results

A comparison of the percentage of tick attachment was made with Fischer's exact test. Statistical analysis of the engorged weight of ticks was performed using a Mann-Whitney *U*-test.

For IL-4, IFN-γ and antibodies production, experiments were repeated several times and representative data are shown.

Results

Lymph node cells from control BALB/c or IL-4 deficient mice stimulated either with ConA or borrelial lysate produced baseline levels of IL-4 and IFN-γ (< 10 U/ml and < 4 U/ml respectively), except for lymph node cells from IL-4 deficient mice stimulated with borrelial lysate which produced low levels of IFN-γ (12 U/ml) (Table 2).

Production of cytokines IL-4 and IFN-γ after tick or syringe inoculation of mice with *Borrelia burgdorferi*

BALB/c and IL-4 deficient mice were infested three times with ten nymphs of *Ixodes ricinus* infected with *Borrelia burgdorferi*. The biology of the nymphs was not disturbed, as measured by their percentage of fixation and nymph body weight. These stayed constant for the three infestations (Table 1). In one case only, the tick fixation was less efficacious (third infestation of BALB/c mice, $P < 0.05$).

After stimulation with ConA, lymph node cells from BALB/c mice produced significant levels of IL-4 (232 U/ml). In contrast, IL-4 deficient mice produced no IL-4 (< 10 U/ml) (Table 2). Only a small quantity of IFN-γ was produced by IL-4 deficient mice (5 U/ml). After stimulation with spirochetal lysate, no IL-4 was produced by lymph node cells of either group of mice (< 10 U/ml). IFN-γ was particularly produced by IL-4 deficient mice (47 U/ml; Table 2).

BALB/c and IL-4 deficient mice were injected three times with 2000 spirochetes. After stimulation with ConA, lymph node cells from the BALB/c mice produced low levels of IL-4 (18 U/ml) and did not produce IFN-γ (< 4 U/ml; Table 2). After stimulation with spirochetal lysate, only BALB/c mouse lymph node cells produced low levels of IL-4 (11 U/ml; Table 2). In contrast, IL-4 deficient mice produced higher levels of IFN-γ (respectively 12 U/ml and 37 U/ml).

IgG2a anti-borrelial antibodies detection by ELISA after tick or syringe inoculations of mice with *B. burgdorferi*

Levels of anti-borrelial IgG2a produced by BALB/c mice infected by tick inoculation remained low for the three infestations. After syringe inoculation, they produced more IgG2a anti-borrelial antibodies than when infested by infected nymphs (OD of 0.700 and 0.320 after the third infection, respectively).

IL-4 deficient mice produced increased levels of anti-borrelial IgG2a for each tick infestation, particularly after the third infestation (OD of 0.320 compared to 1.300; Fig. 1).

IL-4 deficient mice also produced higher levels of anti-borrelial IgG2a than BALB/c mice after syringe inoculation (OD of 1.380 and 0.700 after the third inoculation, respectively; Fig. 1).

Table 1 Percentage of attachment and weights (mean ± SD) of nymphs of *Ixodes ricinus* fed on five BALB/c mice or five IL-4 deficient BALB/c mice for three infestations. No significant difference was noted between the groups of mice and between infestation 1 versus infestations 2 or 3

	Mice	Infestation 1	Infestation 2	Infestation 3
% of attachment	BALB/c	92	78	74*
	IL-4 deficient BALB/c	94	90	90
Weight of engorged females (mg)	BALB/c	4.27 ± 0.32 (n = 25)	4.32 ± 0.11 (n = 22)	4.35 ± 0.37 (n = 18)
	IL-4 deficient BALB/c	4.14 ± 0.43 (n = 22)	4.25 ± 0.46 (n = 21)	4.33 ± 0.42 (n = 24)
Weight of engorged males (mg)	BALB/c	2.52 ± 0.34 (n = 21)	2.62 ± 0.21 (n = 17)	2.72 ± 0.30 (n = 19)
	IL-4 deficient BALB/c	2.48 ± 0.27 (n = 25)	2.57 ± 0.35 (n = 24)	2.54 ± 0.45 (n = 21)

*Only tick attachment between the first and third infestation of BALB/c was significantly different ($P < 0.05$)

Table 2 Three tick inoculations or three syringe inoculations of mice with *Borrelia burgdorferi*: measurement of the in vitro production of IL-4 and IFN-γ by a pool of lymph node cells stimulated with ConA or with borrelia lysate. Pooled results are from five BALB/c mice or five IL-4 deficient BALB/c mice. Results show the mean of triplicate wells ± SD

Mice	Pool	IL-4 (U/ml)	IFN-γ (U/ml)	IL-4 (U/ml)	IFN-γ (U/ml)
		+ ConA[a]		+ borrelia lysate[b]	
BALB/c	Control	< 10	< 4	< 10	< 4
IL-4 deficient BALB/c	Control	< 10	< 4	< 10	12 ± 0.66
BALB/c	Tick inoculation	232 ± 12.5	< 4	< 10	13 ± 0.2
IL-4 deficient BALB/c	Tick inoculation	< 10	5 ± 0.3	< 10	47 ± 1.9
BALB/c	Syringe inoculation	18 ± 6.3	< 4	11 ± 1.4	12 ± 2.3
IL-4 deficient BALB/c	Syringe inoculation	< 10	< 4	< 10	37 ± 1.8

[a] Stimulation with 4 μg ConA/well for 24 h
[b] Stimulation with 5 μg borrelia lysate strain ZS7/well for 72 h

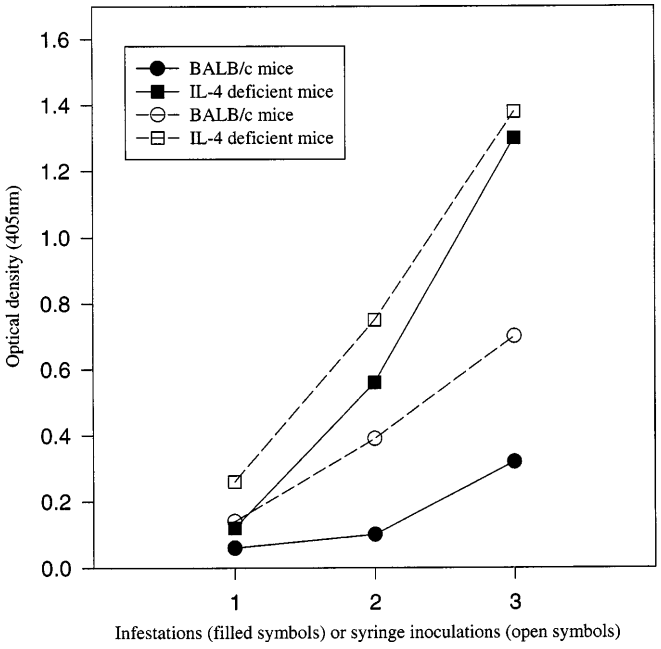

Fig. 1 Measurement by ELISA of specific IgG2a to *Borrelia burgdorferi* contained in a pool of sera from five BALB/c mice (●) or five IL-4 deficient BALB/c mice (■) after each infestation with 10 nymphs of *Ixodes ricinus* infected with *B. burgdorferi*, or after each syringe inoculation of 2000 spirochetes in five BALB/c mice (○) or five IL-4 deficient BALB/c mice (□). Results show the mean of triplicate wells. The SD was always inferior to 7% of the observed value. The optical density (OD) obtained with blood taken before infestations or inoculations was always less than 0.08

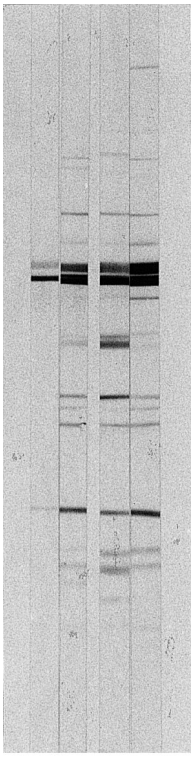

Fig. 2 Detection by Western blot of IgG2a anti-borrelial antibodies in a pool of sera from five BALB/c mice or five IL-4 deficient mice (*1* and *3* respectively) infested three times with ten nymphs of *I. ricinus* infected with *B. burgdorferi*, or from five BALB/c mice or five IL-4 deficient mice (*2* and *4* respectively) syringe-inoculated three times with *B. burgdorferi*

IgG2a anti-borrelial antibodies detection by Western blot after tick or syringe inoculations of mice with *B. burgdorferi*

BALB/c mice developed IgG2a anti-borrelial antibodies against a greater number of antigens after syringe inoculation with *B. burgdorferi* than after tick inoculation with *B. burgdorferi* (Fig. 2, compare 2 with 1). For IL-4 deficient mice, the IgG2a banding pattern was nearly the same after either tick or syringe inoculation with *B. burgdorferi* (Fig. 2, compare 3 with 4).

Total IgE detection by ELISA after tick or syringe inoculations of mice with *B. burgdorferi*

Increasing quantities of total IgE were measured after infestations with infected nymphs (OD of 0.900 after the third infestation; Fig. 3).

BALB/c mice produced less IgE after syringe inoculations than after tick inoculations (OD of 0.500 after the third syringe inoculation compared to 0.900; Fig. 3).

Whatever the mode of infection, IL-4 deficient mice did not produce IgE (OD < 0.070).

Discussion

Antigen-stimulated murine $CD4^+$ T-cells are divided into two subsets according to the profile of cytokines they produce (Mosmann et al. 1986). Murine Th1 cells produce essentially IL-2, IFN-γ and TNF and induce the production of IgG2a and cell-mediated immune responses (delayed type hypersensitivity; Paul and Ohara 1987; Snapper et al. 1988). Th2 cells secrete IL-4, IL-5 and IL-10 and assist in humoral responses, including the switching of IgM-producing B-cells to IgG1 and IgE (Mandler et al. 1993). The expansion of Th1 cells is promoted by IL-2 and IFN-γ and Th2 cells by IL-4 (Swain 1991). Both cells could not be activated at the same time because IL-10 blocks the production of cytokines by Th1 lymphocytes (Fiorentino et al. 1991) whereas IFN-γ inactivates the production of cytokines by Th2 cells (Coffman and Carty 1986). To determine the polarization of an immune response, IL-4 and IFN-γ cytokines and the different antibody isotypes can be analyzed.

The present study shows that mice are tolerant of infestations with nymphs of *Ixodes ricinus* infected with *Borrelia burgdorferi* as previously observed by Mbow et al. (1994) for non-infected nymphs. In our study, the biology of the infected ectoparasites is not disturbed, as measured by their percentage of fixation and nymph body weight. These stay constant for the three infestations. Regarding the production of isotype antibodies

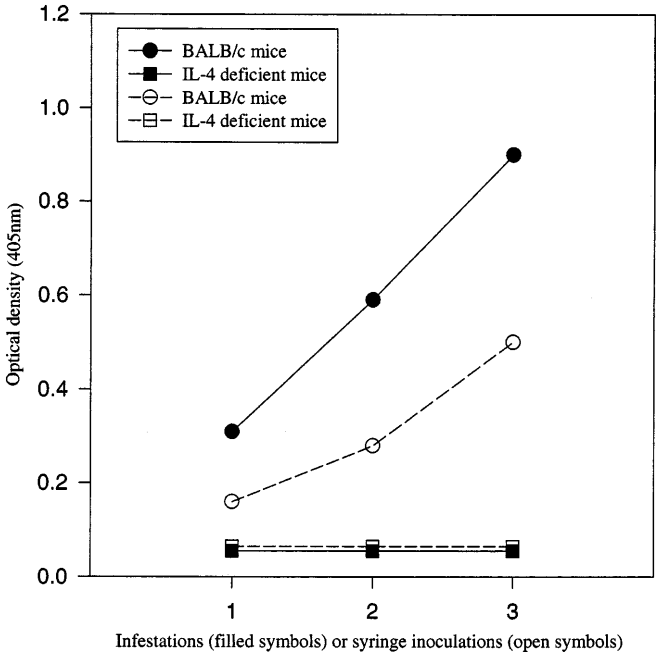

Fig. 3 Measurement by ELISA of total IgE contained in a pool of sera from five BALB/c mice (●) or five IL-4 deficient BALB/c mice (■) after each infestation with ten nymphs of *I. ricinus* infected with *B. burgdorferi*, or from five BALB/c mice (○) or five IL1-4-deficient BALB/c mice (□) after each syringe inoculation of 2000 spirochetes. Results show the mean of triplicate wells. The SD was always inferior to 8% of the observed value. The OD obtained with blood taken before infestations or inoculations was always less than 0.07

and cytokines, we conclude that BALB/c mice develop a Th2 immune response against *B. burgdorferi* after tick inoculation of the spirochetes and a mixed Th1/Th2 response after a syringe inoculation of *B. burgdorferi*. IL-4 produced following *I. ricinus* infestation (Ganapamo et al. 1995) greatly influences the immune response developed against *B. burgdorferi* and particularly decreases the levels of IgG2a anti-borrelial antibodies. This fact is confirmed by results obtained with IL-4 deficient mice which produce high levels of IgG2a antibodies even after tick inoculation of the spirochetes.

To feed successfully, Ixodid ticks secrete saliva containing bioactive proteins and other chemicals into the feeding lesion formed in the host's skin (Bowman et al. 1996). Pathogens entering a site that has been profoundly modified by the pharmacological activities of their vector's saliva can take advantage of this situation to enhance their dissemination (Nuttall 1998). Nevertheless, the response of guinea pigs to repeated *I. scapularis* infestation decreases the transmission of *B. burgdorferi* (Nazario et al. 1998). Two hypothesis are described to explain the mechanisms by which *I. scapularis* immunity interferes with *B. burgdorferi* transmission. First, immunity, by reducing duration of tick attachment and size of the blood meal, could decrease the opportunity for spirochetes to multiply in the tick midgut and to reach the salivary gland. Second, spirochetes could be directly affected by the immune process. In the present study, the first hypothesis can be ruled out because repeated infestations of mice with *I. ricinus* do not disturb the tick feeding. Our work demonstrates that IL-4 produced following tick bites strongly influences the immune response developed against *Borrelia*. But as it is not yet well established which cytokine (IL-4 or IFN-γ, and consequently Th2 or Th1 polarization of the immune response) protects the host against dissemination of the spirochetes, we do not know whether the influence of the IL-4 is favorable or not for transmission of the pathogen. Two observations are in direct contrast. Zeidner et al. (1997) showed that Th1 cytokines are down-regulated during the initial spirochete transmission period in C3H mice infested with *I. scapularis* ticks infected with *B. burgdorferi*. Zeidner et al. (1996) observed also that IFN-γ and IL-2 given at the time of tick feeding could suppress spirochetes transmission by *I. scapularis*. They concluded that the down-regulation of Th1 cytokine is favorable for the transmission of spirochetes. In contrast, Keane-Myers et al. (1996) showed that administration of rIL-4 to susceptible C3H mice during the first week of infection with *B. burgdorferi* leads to early control of their infections, as evidenced by significant reductions in joint swelling and in the numbers of spirochetes recovered from their joints and skin when compared with sham-treated mice.

In conclusion, we have demonstrated that IL-4 produced during infestations with nymphs of *I. ricinus* influences the immune response developed against *B. burgdorferi*, especially the production of IgG2a antibodies specific to *B. burgdorferi*. This shows the importance of taking the vector influence into account when the immune response against a vector-borne pathogen is studied. We have now to determine whether this influence is favorable or not for the transmission and development of the infection in the host.

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