Tick-host-Borrelia interaction

Implications for host immunity and vaccination strategies

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Chapter 2

Rapid Outer surface protein C DNA tattoo-vaccination protects against Borrelia afzelii infection

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ABSTRACT

*Borrelia afzelii* is the predominant *Borrelia* species causing Lyme borreliosis in Europe. Currently there is no human vaccine against Lyme borreliosis, and most research focuses on recombinant protein vaccines against *Borrelia burgdorferi* sensu stricto. DNA tattooing is a novel vaccination method that can be applied in a rapid vaccination schedule. We vaccinated C3H/HeN mice with *B. afzelii* strain PKo OspC using a codon optimized DNA vaccine tattoo and compared this with recombinant protein vaccination in a 0-2-4 week vaccination schedule. We also assessed protection by DNA tattoo in a 0-3-6 day schedule. DNA tattoo and recombinant OspC vaccination induced comparable total IgG responses, with a lower IgG1/IgG2a ratio after DNA tattoo. Two weeks after syringe-challenge with 5x10⁵ *B. afzelii* spirochetes most vaccinated mice had negative *B. afzelii* tissue DNA loads and all were culture negative. Furthermore, DNA tattoo vaccination in a 0-3-6 day regimen also resulted in negative *Borrelia* loads and cultures after challenge. To conclude, DNA vaccination by tattoo was fully protective against *B. afzelii* challenge in mice in a rapid vaccination protocol, and induces a favourable humoral immunity compared to recombinant protein vaccination. Rapid DNA tattoo is a promising vaccination strategy against spirochetes.
Lyme borreliosis is caused by *Borrelia burgdorferi* sensu lato (s.l.) spirochetes, which are transmitted by *Ixodes* ticks. The disease usually manifests itself as a local skin lesion, designated erythema migrans, and can disseminate and cause multiple erythema migrans, Borrelial lymphocytoma, acrodermatitis chronica atrophicans, carditis, oligo-arthritis or a polyradiculitis / meningoencephalitis (Lyme neuroborreliosis). In North-America Lyme borreliosis is caused by *Borrelia burgdorferi* sensu stricto (s.s.), while in Europe most cases are caused by *Borrelia afzelii* followed by *Borrelia garinii* and *B. burgdorferi* sensu stricto.(2, 18)

During transmission from the tick to the host *B. burgdorferi* s.l. spirochetes differentially express specific genes to adapt to the environment of the mammalian host. For example, they downregulate Outer surface protein A (OspA) - which facilitates egression from the tick midgut - and upregulate Outer surface protein C (OspC) - which is necessary for migration to the tick salivary glands and dissemination in the mammalian host.(4-6) Moreover, OspC has been shown to bind to an *Ixodes* tick salivary protein (Salp15) providing *B. burgdorferi* s.l. spirochetes protection from antibody-mediated killing at the tick bite site.(58, 59)

Currently, there is no human vaccine against Lyme borreliosis. Human vaccine studies have been mostly based on recombinant OspA as antigen, and a human OspA vaccine was previously on the market for four years but was discontinued for multiple reasons. (40) Other vaccine candidates have been studied, of which OspC vaccines have been most successful. In addition, other Borrelia outer membrane proteins and vector-based vaccines have shown (partial) protection.(48) Compared to conventional protein vaccines DNA vaccines have the advantage of being cheap. Furthermore, they have a long shelf-life at ambient temperatures, do not yield vector specific immune responses in multi-boost regimens and induce potent cellular immune responses in animal models. DNA vaccination based on *Borrelia* OspA delivered intramuscularly and OspC delivered by gene-gun or intradermal needle application was previously shown to be effective against challenge with *Borrelia burgdorferi* s.s. spirochetes in a murine infection model.(67-70)

In an effort to improve immunogenicity and thus facilitate translation to humans, a dermal delivery technique using a tattoo device has been developed. This method injects the DNA into the skin via thousands of skin perforations and hence locally induces an inflammatory milieu that functions as an adjuvant. It allows for faster vaccination regimens and leads to robuster immune responses than intramuscular DNA vaccination. As this technique has not previously been investigated for bacterial
pathogens in general, and Borrelia in particular, in this paper we describe vaccination against *B. afzelii* spirochetes using a codon-optimized DNA vaccine based on *B. afzelii* strain PKo OspC, applied by tattoo in a normal and a rapid vaccination protocol.

**RESULTS**

*Dose finding for B. afzelii strain PKo challenge*

Prior to our vaccination studies, we performed a dose finding experiment to establish an adequate number of spirochetes for challenge by intradermal needle inoculation. We inoculated 38 mice with a range of spirochetes (5x10^2 to 5x10^6), and assessed infection by qPCR on DNA extracted from ear biopsies after 7 days. Mice were sacrificed after 2 weeks and *Borrelia* loads in heart and bladder were detected by qPCR. In addition, skin biopsies (taken from the inoculation site) and bladder were cultured and finally *Borrelia* antibody titers were assessed by ELISA, using an OspC deficient *B. burgdorferi* s.s. strain as a lysate. After 7 days, most mice inoculated with 5x10^4 spirochetes or higher produced qPCR positive ear tissue (Figure 1A), while after 14 days all mice inoculated with 5x10^4 spirochetes or higher had qPCR positive heart tissue (Figure 1B) and bladder tissue tested positive in 8 out of 9. (Figure 1C). Cultures of skin (inoculation site) demonstrated that an inoculum of 5x10^3 spirochetes yielded viable spirochetes in all mice at 2 weeks after infection, while in the bladder this was observed at an inoculum of 5x10^4 spirochetes or higher (Figure 1D and 1E). Anti-*Borrelia* antibody titers correlated with the height of the inoculum (Figure 1F). Based on these findings, we determined that a challenge of 5x10^5 spirochetes was optimal for our vaccination studies.
Generation of a B. afzelii PKo DNA vaccine and a regular 0, 2, 4 weeks DNA vaccination protocol

A DNA vaccine was constructed based on the cDNA of B. afzelii PKo OspC, with its signal sequence replaced by the hTPA signal sequence and preceded by a Kozak sequence. The resulting sequence was codon-adapted, leading to the recombinant plasmid pVAX-hTPA-OspC (Figure 2A and supplemental data S1).

In the first set of experiments we compared DNA vaccination by tattoo with a subcutaneously administered recombinant protein vaccine (rOspC). The protein vaccine was emulsified in complete Freund’s adjuvant for priming, while the boosters...
after 2 and 4 weeks were emulsified in incomplete Freund's adjuvant. Using this regimen the rOspC vaccine elicited a higher antibody titer ($P = .04$) than the DNA vaccine (Figure 2B) at 4 weeks, which was 2 weeks after the first booster. However, eventually at $t = 6$ weeks the titers of both vaccines plateaued at a comparable level. To determine differences in T-helper-cell polarization between the two vaccination strategies we measured IgG subclasses induced by the vaccination. The DNA vaccine resulted in a higher IgG2a antibody level after the first vaccination than the rOspC vaccine ($P = .005$), indicating a rapid Th1 response (Supplemental data, S2). The OspC-specific IgG1/IgG2a ratio was significantly lower after DNA tattoo compared to rOspC vaccination after 2 weeks ($P = .04$) and 6 weeks ($P = .002$) (Figure 2C). These lower IgG1/IgG2a ratios after DNA tattoo indicate a more Th1-skewed immune response compared to recombinant OspC vaccination.

Figure 2. Characterization of the humoral immune response after vaccination with a DNA vaccine by tattoo versus vaccination with recombinant OspC in a 0-14-28 day immunization protocol. 
A, pVAX-hTPA-OspC DNA vaccine insert. It contains a Kozak sequence, a codon-optimized human Tissue Plasminogen Activator (hTPA) signal sequence, a codon-optimized OspC gene from B. afzelii strain PKo and a double stop codon, and was cloned into a pVAX1 vector.
B, Mice were vaccinated at 0, 2 and 4 weeks with the DNA vaccine or negative control by tattoo, or with a recombinant OspC vaccine using complete and incomplete Freund's adjuvant. Vaccination time points are indicated by arrow symbols.
C, OspC-specific IgG1/IgG2a ratios in individual mice at $t = 2, 4$ and 6 weeks, after vaccination with rOspC or DNA tattoo at $t = 0, 2$ and 4 weeks. Antibody titers and ratios were compared using a two-tailed student's t-test. Error bars represent mean ± SEM.
After challenging the mice with $5 \times 10^5$ B. afzelii PKo spirochetes we determined B. afzelii PKo loads in the ear by qPCR at 7 days (ear biopsy) and in other tissues at 14 days, when all mice were sacrificed. *Borrelia* DNA loads were negative in all tissues both in the rOspC and in the pVAX-OspC vaccinated mice, except for one positive bladder sample after pVAX-hTPA-OspC vaccination and one positive heart sample in a rOspC vaccinated mouse (Figure 3A-D). Ear biopsies taken 14 days after challenge were also negative in all vaccinated mice (data not shown). In all calculations, negative OspA values were replaced by the value of the OspA detection limit, and the difference in *Borrelia* loads between vaccinated and control mice remained significant in all organs. Importantly, 6-week cultures of the skin and bladder of the animals in both groups remained negative, further underscoring the observed protective effect (Table 1). Hence, using stringent detection methods a clear protective response against *Borrelia* infection in skin and deeper tissues was observed after both vaccination strategies. As another marker of protection we measured anti-*Borrelia* IgG antibodies in both groups after *B. afzelii*-challenge. For this purpose we coated ELISA plates with a mutated *B. burgdorferi* s.s. strain lacking OspC, thus measuring OspC-independent anti-*Borrelia* IgG antibody titers. Upon inoculation with *B. afzelii* we could demonstrate a clear rise in antibody levels in control mice ($P < 0.0001$), but no significant difference was found in either the rOspC or DNA tattoo vaccinated groups ($P = 0.08$ and $P = 0.40$; Figure 3E).

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** $P = 0.0022$, Fisher’s exact test
Figure 3. Protection from challenge with 5x10^5 *Borrelia afzelii* spirochetes after vaccination with recombinant OspC or DNA tattoo vaccination in a 0-14-28 day immunization schedule.

A-D, *Borrelia* DNA loads were determined 7 days (ear biopsy) and 14 days after challenge (skin around the inoculation site, bladder and heart). Black dots depict positive *Borrelia* loads, open diamonds depict negative loads where the OspA detection limit was divided by the sample’s mouse beta actin load. Borrelia loads were compared using a 2-sided non-parametric test (Mann-Whitney).

E, *Borrelia* serology independent of OspC antibodies was compared between mice before *Borrelia* challenge (open circles) and 2 weeks after challenge (closed circles). Serology results were compared using a two-tailed student’s t-test. Error bars represent mean ± SEM.
Vaccination against B. afzelii PKo with two 3-day boosters - a rapid DNA vaccination protocol.

Since it was previously shown that DNA vaccine delivery by tattoo is able to induce protection against influenza and subcutaneous tumors using a fast vaccination protocol, we were interested to see whether this would also be the case for a bacterial pathogen.(71) Therefore, we vaccinated mice with pVAX-hTPA-OspC or a negative control at $t = 0$, 3 and 6 days and challenged the mice at $t = 21$ days with $5 \times 10^5$ B. afzelii PKo spirochetes. As could be expected, assessment of OspC-specific antibody titers before challenge showed that the rise in anti-OspC IgG did not occur any faster after rapid DNA vaccination than after the standard DNA vaccination protocol (Figure 4A). At the time of challenge the anti-OspC antibody titer was lower than in the mice vaccinated in a regular protocol ($P = .04$). Next, we assessed the protection induced by the fast regimen by qPCR and culture. QPCR of ear DNA 7 days after Borrelia challenge as well as of ear DNA (data not shown), skin DNA (at the inoculation site) bladder DNA and heart DNA 14 days after challenge were all negative for Borrelia in the mice vaccinated with the pVAX-hTPA-OspC DNA vaccine administered by tattoo in a 0-3-6 day regimen (Figure 4B-E). Significant differences between mice receiving rapid DNA tattoo versus controls were found in all organs except skin, even using the OspA detection limit in negative samples. Moreover, cultures of bladder and skin (inoculation site) remained negative, whereas cultures from mice tattooed with a negative control plasmid were all positive (Table 2). Finally, similar to our observation using the normal DNA vaccination schedule, mice vaccinated with pVAX-hTPA-OspC by the rapid protocol did not develop anti-Borrelia antibody responses after B. afzelii challenge (Figure 4F). No significant difference was found when comparing pre- and post- challenge serum ($P = .50$) while control mice showed a rise in anti-Borrelia antibodies after challenge ($P < .0001$).

Table 2. Culture positivity for B. afzelii six weeks after challenge. Mice were vaccinated three times at 3-day intervals with the pVAX-hTPA-OspC construct (Rapid DNA tattoo) or an empty pVAX vector (Rapid tattoo control).

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<td>Rapid DNA tattoo</td>
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** $P = 0.0022$, Fisher’s exact test
Figure 4. Effects of DNA tattoo vaccination using pVAX-hTPA-OspC in a rapid vaccination protocol (0, 3, 6 days).

A, total IgG antibody titers after DNA vaccination comparing a rapid vaccination protocol (solid lines) with a regular vaccination protocol (broken lines). Vaccination timepoints are indicated by solid arrows (rapid protocol) and by broken arrows (regular protocol). Antibody titers were measured prior to *B. afzelii* challenge (2 weeks after the second booster vaccination). B-D, *Borrelia afzelii* DNA loads in mice organs. Samples were analysed by q-PCR in triplicate. When OspA signal was not detected in all any of three reactions the detection limit for OspA was used (open diamonds). Borrelia loads were compared using a 2-sided non-parametric test (Mann-Whitney). E, Anti-*Borrelia* IgG was measured in pre- and post-challenge serum as indicated by open and solid circles, respectively. Serology results were compared using a two-tailed student’s t-test. Error bars represent mean ± SEM.
DISCUSSION

In this study we describe the successful application of a rapid DNA tattoo method as a vaccination technique against *Borrelia* and show that it induces a similar humoral immune response as recombinant protein vaccination, yet yields a more favourable IgG1/IgG2a ratio. Furthermore, we performed the first published dose-finding study for *B. afzelii* strain PKo and using the selected inoculum in our vaccination and challenge experiments none of the mice vaccinated with either recombinant OspC or with a DNA tattoo vaccine targeting OspC were infected as determined by qPCR, culture and serology. Finally, when the DNA tattoo was administered in a rapid vaccination protocol (0, 3 and 6 days), full protection was also obtained, to our knowledge for the first time in the setting of a DNA tattoo vaccination against a bacterial pathogen.

Humoral immunity to OspC is highly important for *Borrelia* clearance.\(^{(42, 72)}\) Therefore, it was our goal to maximize the humoral immune response by adding an hTPA signal sequence as keratinocyte-derived OspC antigen will be secreted, probably skewing the immune response from a CD8\(^+\) T-cell response towards CD4\(^+\) T-cell and B-cell activation.\(^{(69)}\) IgG1 in mice does not activate complement due to its relative inflexibility and is indicative of a Th2 polarized immune response.\(^{(73, 74)}\) In contrast, IgG2a is indicative of a Th1-skewed response, which has been shown to be important in clearance of *Borrelia*.\(^{(75-77)}\) In our study, a DNA tattoo elicited an immune response with a lower IgG1/IgG2a ratio than a recombinant OspC vaccine, while maintaining high total IgG levels. This indicates a more Th1-polarized immune response and is in line with the lower IgG1/IgG2a ratio’s observed after needle-based DNA vaccination, as opposed to gene-gun immunization.\(^{(70, 74)}\) Presumably, in our case the danger signals elicited by over 5x10\(^4\) needle injections in the skin skew the immune response away from Th2-associated IgG1 production. Importantly, total IgG levels after 6 weeks were similarly elevated after DNA tattoo vaccination compared to recombinant OspC vaccination, which shows that DNA tattoo is also able to induce prolonged presence of transmission-blocking antibodies.

In the current study we show that DNA tattoo vaccination is able to induce a favourable humoral immune response that is capable of effectively preventing infection with an extracellular bacterial pathogen. However, vaccination against OspC is limited by the large sequence heterogeneity between OspC serotypes.\(^{(47, 78, 79)}\) The immunodominant OspC epitopes are believed to reside in variable regions,
making it unlikely for a monovalent OspC vaccine to be cross-protective against all naturally occurring *Borrelia burgdorferi* s.l. strains. Indeed, upon challenge of PKo-OspC vaccinated mice (using the normal protocol) with $1 \times 10^4$ *B. burgdorferi* strain N40 spirochetes we did not observe full protection (data not shown). In this respect an advantage of using DNA vaccines could be that one can easily combine multiple OspC sequences to prevent this issue. This is subject of our current and future investigations.

We have challenged mice with *in-vitro* cultured spirochetes and not by *Borrelia*-infected ticks. *In-vitro* cultured *B. burgdorferi* s.l. spirochetes lack the differential antigen expression and interaction with immunomodulating tick salivary proteins. It also has been shown that fewer spirochetes are required to infect mice, when these are derived from ticks or mice compared to *in vitro*-grown spirochetes. Our vaccine candidate OspC is up regulated during migration of the spirochetes from the tick midgut to the tick salivary glands and OspC expression is necessary for the first stage of mammalian infection. OspC vaccination has previously been found to protect against both *B. burgdorferi* infection through tick challenge and syringe-inoculation. Apart from its detrimental effects on spirochetes that infect the mammalian host, OspC vaccination was also found to diminish OspC-expressing *B. burgdorferi* spirochetes in the midgut of biting ticks, reducing their presence in the tick salivary glands. Since DNA vaccination by tattoo induces similar IgG levels compared to recombinant OspC vaccination, and even a more favourable subclass distribution, we postulate that our technique will similarly protect *B. burgdorferi* infection through tick challenge. However, more research and the establishment of a robust *Borrelia afzelii* PKo tick challenge model are required to confirm this hypothesis.

DNA tattooing is a promising vaccination technique. Previous studies have shown that the DNA tattoo vaccination approach is far more effective in inducing cellular immune responses in mice and non-human primates compared to intramuscular DNA vaccination, despite the lower transfection efficiency and lower and shorter antigen expression. Superiority of the DNA tattoo has also been demonstrated in a 0-14-28 day vaccination schedule in which DNA tattoo outperformed intramuscular delivery both in inducing humoral and cellular immunity. Improving immunogenicity of DNA vaccines is of paramount importance for successful translation to humans. Importantly, by using the DNA tattoo it is also possible to apply a compact vaccination strategy where boosters are delivered after 3 and 6...
days.

DNA tattoo vaccination could provide the key to vaccination strategies targeting pathogens in settings where quick vaccination schedules can either boost adherence or when epidemics demand quick vaccination coverage and effectiveness. Moreover, DNA vaccines can be developed quickly and can be easily deployed in developing countries due to the low cost and the long shelf-life. Since we have shown that rapid DNA tattoo vaccination can elicit protection against an extracellular spirochetal pathogen, one could speculate that DNA vaccination by tattoo could also protect against other spirochetal diseases such as syphilis, relapsing fever and leptospirosis. Moreover, the adequate humoral immune responses we show here, added to the previously described rapid induction of $\text{CD8}^+$ T-cells, makes DNA tattoo a very interesting technique to prevent other extra- and intracellular bacterial pathogens.

**MATERIALS AND METHODS**

*Generation of the recombinant and DNA OspC vaccines and vaccination protocols*

Groups of 6 mice were vaccinated at $t = 0$ weeks, $t = 2$ weeks and $t = 4$ weeks, with either recombinant OspC (rOspC), a DNA vaccine coding for the human Tissue Plasminogen Activator signal sequence fused to OspC in a pVAX vector (pVAX-hTPA-OspC) or with a negative control plasmid. *B. afzelii* PKo rOspC was produced as described elsewhere and used as a positive control.(58) Ten µg of rOspC was emulsified 1:1 in 50 µl Complete Freund’s Adjuvant at $t = 0$ weeks and in Incomplete Freund’s Adjuvant at $t = 2$ and 4 weeks, and injected subcutaneously in two 50 µl dosages at the back of the mice. The pVAX-hTPA-OspC DNA vaccine was designed based on the OspC gene sequence in *Borrelia afzelii* PKo plasmid cp27 (CP000402.1) in which we replaced the 23aa signal sequence (predicted by SignalP 4.0 software) with the human Tissue Plasminogen Activator signal sequence (genbank AAA61213.1).(84) Both the OspC sequence and as the hTPA signal sequence were codon-optimized to mouse tRNA usage with Java Codon Adaptation tool. (85) At the 5’ end a BamH1 and a Kozak sequence were added and at the 3’end a sequence encoding a double stop codon and an Xho1 was added. The insert was synthesized (Biobasic Inc, Ontario, Canada) and ligated into a BamH1/Xho1 restricted empty pVAX vector (Invitrogen, Carlsbad, CA, USA). As a negative control, empty circular pVAX was used. Both plasmids were amplified using a Nucleobond Xtra EF kit (Macherey-Nagel, Düren, Germany) and resuspended in DNase-free water. Both in the pVAX-hTPA-OspC and in the negative control groups hair was removed from the mice abdomens using hair removal cream.
Next, 20 µg of DNA vaccine was applied on the hairless abdominal skin. Subsequently a Cheyenne Hawk tattoo machine carrying a Cheyenne 13-magnum tattoo needle (both MT.DERM, Berlin, Germany) was placed on the abdominal skin and the DNA vaccines were tattooed 0.5-1 mm into the skin for 45 seconds at 100Hz under isofluorane anaesthesia. In a separate experiment, groups of 6 mice were vaccinated with pVAX-hTPA-OspC or negative control at t = 0, 3 and 6 days.

**OspC-specific total IgG, IgG1 and IgG2a:**
High-binding ELISA plates (Greiner Bio-one, Kremsmünster, Austria) were coated overnight at 4°C with 1 µg/ml rOspC PKo, washed with PBS-Tween and blocked with 1% BSA in PBS (blocking buffer) for 2 hours. Mouse sera derived from either mandibular puncture or tail bleed (pre-immune, before each booster and before sacrifice) were diluted in blocking buffer and incubated for 1 hour. Plates were washed and incubated for one hour with either HRP-linked anti-mouse IgG (Cell signaling) diluted 1:1000 in blocking buffer, or HRP-linked goat anti-mouse IgG2a /rat anti-mouse IgG1 (Southern biotech) diluted 1:3000 in blocking buffer. Plates were washed and developed in a Biotek ELISA plate reader at 450 nm-655nm. IgG titers were defined as the last dilution where OD450-655 nm was > 3 S.D. above baseline signal.

**B. afzelii challenge**
Low-passage *Borrelia afzelii* strain PKo spirochetes were cultured and counted as described before and 5x10^5 spirochetes in 100µl PBS were needle-inoculated subcutaneously in the midline of the back of mice 2 weeks after the third vaccination (t = 48 days in the regular- and t = 21 days in the rapid vaccination protocol).(86) The inoculation dose was established based on a dose-finding experiment in which 38 mice received a range of doses, i.e. 5x10^2 spirochetes (n=10), 5x10^3 spirochetes (n=10), 5x10^4 spirochetes (n=9), 5x10^5 spirochetes (n=5), 5x10^6 spirochetes (n=4) and 2 mice received only PBS as a negative control. More mice were used in low-inoculum groups due to anticipated variability of the infection read-outs in individual animals. Mice were sacrificed 2 weeks after challenge. In a separate experiment mice were infected with 1x10^4 *Borrelia burgdorferi* sensu stricto strain N40 spirochetes in 100µl PBS.

**Borrelia serology**
We developed an ELISA to quantitatively measure OspC-independent antibodies directed against *Borrelia afzelii* PKo after infection, by measuring cross-reactivity to an OspC-deficient *Borrelia burgdorferi* sensu stricto strain. OspC deficient *Borrelia burgdorferi* 297 (courtesy of Erol Fikrig, Yale University, New Haven, CT, USA) was
cultured from at 33°C until mid-log phase and lysed using a sonicator. High-binding ELISA plates (Microlon) were coated overnight at 4°C with 1 µg/ml of lysate, washed three times in PBS-T and blocked for 2 hours with blocking buffer. Mouse sera derived from mice 2 weeks after inoculation with *B. afzelii* were diluted to 1:100 in PBS and 50 µl was added and incubated for one hour at room temperature. Pooled pre-challenge sera from triple vaccinated mice were used as a negative control. Plates were washed and incubated for one hour with HRP-linked anti-mouse IgG (Cell signaling) diluted 1:1000 in blocking buffer, developed and read at OD 450-655 nm.

**Borrelia DNA loads**

A quantitative (q-)PCR was used to quantify *Borrelia* DNA in mouse tissues after inoculation with *B. afzelii* PKo. Seven days after inoculation an ear biopsy was taken, and 14 days after inoculation an ear biopsy, skin biopsy (around the inoculation site), half of the bladder and the apex of the heart were taken. Tissues were lysed overnight and DNA was extracted using the Blood and Tissue kit (Qiagen). A q-PCR was performed with the *Borrelia*-specific OspA primers Forward AAAAATATTTATGGAATAGGTCT and Reverse CACCAGGCAAATCTACTGAA, and with mouse beta actin primers Forward AGCGGGAAATCGTGCATTG and Reverse CAGGTTACATGGTGGTGC to correct for amount of mouse tissue in the DNA sample. Q-PCR’s were performed using the Lightcycler480 (Roche) and SYBR green dye (Roche), and reactions were performed in triplicate. PCR protocol was 95°C 6 min, and 60 cycles of 95°C 10 seconds, 60°C 20 seconds and 72°C 20 seconds. Results were analyzed using LinregPCR software.(87) Negative and positive controls were included in each qPCR run. In case of 3x negative values for OspA in a sample, the OspA value was replaced by the value of the OspA detection limit in the assay.

**Borrelia cultures**

Blinded samples (half of the bladder and a skin biopsy from the inoculation site) were cultured in Modified Kelly-Pettenkofer Medium with rifampicin (50 µg/ml) and phosphomycin (100 µg/ml) at 33°C and 5µl was checked weekly by dark-field microscopy for the presence of spirochetes for 6 weeks, as described before (58).

**Statistics**

*Borrelia* loads were given their detection limit when negative to exclude the possibility of low qPCR sensitivity and were compared to control mice by 2-sided nonparametric tests (GraphPad Prism Software version 5.0, San Diego, CA, USA). Antibody titers and optical densometry data were analysed by a two-tailed student’s t-test. Culture
positivity was analyzed using a Fisher’s exact test as compared to control. Statistical significance is depicted by the following: \( P < 0.05 (*) \), \( P < 0.01 (**) \) and \( P < 0.001 (***) \). Error bars in all figures illustrate mean ± SEM.

**Ethics**

All experiments were reviewed and approved by the animal research ethics committee of the Academic Medical Center, Amsterdam, The Netherlands.

**ACKNOWLEDGEMENTS**

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**CONFLICT OF INTEREST**

Authors declare no competing financial interests in relation to the work described.
REFERENCES


