The *Ixodes ricinus* salivary gland proteome during feeding and *B. Afzelii* infection: New avenues for an anti-tick vaccine

Michelle J. Klouwens [a,b,c,*], Jos J.A. Trentelman [a], Diego Barriales [e], Jasmin I. Ersoz [a], Mikel Azkargorta [e,f], Felix Elortza [e,f], Radek Šíma [d,j], Ondrej Hajdušek [d], José-Luis Lavin [e], Julen Tomás Cortazar [e], Iraide Escobes Corcuera [e,f], Emil Colstrup [a], Abhijeet Nayak [a], Itziar Martín Ruíz [e], Hector Rodriguez [e], Ard M. Nijhof [g,h], Juan Anguita [e,i,1], Joppe W.R. Hovius [a,b,c,1]

[a] *Department of Internal Medicine, Center for Experimental and Molecular Medicine, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands*
[b] *Division of Infectious Diseases, Department of Internal Medicine, Academic Medical Center, Amsterdam, The Netherlands*
[c] *Amsterdam Multidisciplinary Lyme borreliosis Center, Academic Medical Center, Amsterdam, The Netherlands*
[d] *Biology Centre, Institute of Parasitology, Czech Academy of Sciences, Ceske Budejovice, Czech Republic*
[e] *CIC bioGUNE, Basque Research and Technology Alliance (BRTA), Bizkaia Science and Technology Park, Derio, Spain*
[f] *Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), Spain*
[g] *Institute for Parasitology and Tropical Veterinary Medicine, Freie Universität Berlin, Berlin, Germany*
[h] *Veterinary Centre for Resistance Research (TZR), Freie Universität Berlin, Berlin, Germany*
[i] *Ikerbasque, Basque foundation for science, Bilbao, Spain*
[j] *Biopticka laborator s.r.o., Plzen, Czech Republic*

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ABSTRACT

Introduction: *Borrelia burgdorferi sensu lato*, the causative agents of Lyme borreliosis, are transmitted by *Ixodes* ticks. Tick saliva proteins are instrumental for survival of both the vector and spirochete and have been investigated as targets for vaccine targeting the vector. In Europe, the main vector for Lyme borreliosis is *Ixodes ricinus*, which predominantly transmits *Borrelia afzelii*. We here investigated the differential production of *I. ricinus* tick saliva proteins in response to feeding and *B. afzelii* infection.
Method: Label-free Quantitative Proteomics and Progenesis QI software was used to identify, compare, and select tick salivary gland proteins differentially produced during tick feeding and in response to *B. afzelii* infection. Tick saliva proteins were selected for validation, recombinantly expressed and used in both mouse and guinea pig vaccination and tick-challenge studies.
Results: We identified 870 *I. ricinus* proteins from which 68 were overrepresented upon 24-hours of feeding and *B. afzelii* infection. Selected tick proteins were successfully validated by confirming their expression at the RNA and native protein level in independent tick pools. When used in a recombinant vaccine formulation, these tick proteins significantly reduced the post-engorgement weights of *I. ricinus* nymphs in two experimental animal models. Despite the reduced ability of ticks to feed on vaccinated animals, we observed efficient transmission of *B. afzelii* to the murine host.
Conclusion: Using quantitative proteomics, we identified differential protein production in *I. ricinus* salivary glands in response to *B. afzelii* infection and different feeding conditions. These results provide novel insights into the process of *I. ricinus* feeding and *B. afzelii* transmission and revealed novel candidates for an anti-tick vaccine.

1. Background

Ixodes ticks are arthropod parasites with a three-host lifecycle requiring blood ingestion to develop from one stage into the next sequential stage. During a blood meal, *Ixodes* ticks can acquire and transmit bacterial, protozoal and viral pathogens. Lyme borreliosis is the most prevalent vector-borne disease in the Northern hemisphere and is caused by *Borrelia burgdorferi* sensu lato (sl) [1]. *Borrelia afzelii* is the dominant *B. burgdorferi* sl genospecies causing Lyme borreliosis in Europe [2].

Tick-host-*Borrelia* interactions have been studied extensively [3]. During a bloodmeal, *Ixodes* ticks introduce salivary gland pro-

teins into the host skin, facilitating feeding. Through modulation of host defense mechanisms such as host hemostasis, as well as inflammatory- and immune responses, they also aid in the transmission of tick-borne pathogens. Tick saliva contains a mixture of molecules that cluster in multigenic families. The proteins display pleiotropic and redundant functions in order to deal with the different host defense mechanisms. Thus, a single protein can affect different mechanisms or pathways and many different low-immunogenicity proteins can inhibit specific responses at the same time [4]. Interestingly, *B. burgdorferi* sensu stricto has been shown to induce the production of certain tick salivary gland proteins that, either by direct interaction with the spirochete or by interference with the host immune system, facilitate its transmission from the tick to the host [5]. On the other hand, hosts react to tick infestation by activating coagulation and platelet aggregation cascades to inhibit tick feeding. Certain mammals, including humans, are also able to develop both cellular and humoral immune responses directed against tick proteins upon repeated tick infestations, a phenomenon known as tick-immunity. Tick immunity, also known as acquired resistance to tick-bite, impairs tick feeding and partially protects animals from the development of tick-borne diseases, such as Lyme borreliosis [6–10].

Vaccines to prevent B. burgdorferi sl infection could work in different ways [11–14]. This includes targeting the pathogen itself to prevent infection of the mammalian host. An alternative method is targeting the tick vector instead to prevent successful transmission of the pathogen from the tick to the host. Most research focuses on protective antigens derived from the pathogen, B. burgdorferi sl. Indeed, a human vaccine to prevent Lyme borreliosis (LYMErix), based on *B. burgdorferi* OspA antigen, was available in the late 1990 s, but it was withdrawn within years because of poor sales due to alleged side effects and the requirement of annual boosts [15–17]. There is currently no vaccine available to prevent Lyme borreliosis in humans, although human studies are ongoing. Because of the impact of Lyme borreliosis in many parts of the world, we are in need of a protective vaccine.

An alternative to pathogen-directed immunogens is the development of a vaccine targeting the vector and its capacity to transmit the spirochete. Indeed, to date two commercial anti-tick vaccines are or have been available for cattle, TickGard and Gavac, based on recombinant *Rhipicephalus microplus* Bm86/Bm95 antigens [18–21]. There is however no vaccine available against *Ixodes* tick species. The genome of *Ixodes scapularis*, the vector for Lyme disease in Northern America, has been described [22]. This facilitated the characterization of the transcriptome of *I. ricinus*, the highly related and main vector for tick-borne pathogens in Europe [23,24]. However, the transcriptome is an approximation of the final and biologically most relevant product: the proteins that together constitute the proteome. In comparison to the genome and transcriptome, the proteome is more dynamic and varies in response to requirements and conditions of a biosystem [25] and proteomics is therefore a powerful approach to identify candidates for an anti-tick vaccine [26].

Anti-tick vaccines could impair tick feeding by targeting tick proteins important for the tick feeding process, which might also impact *Borrelia* transmission. In addition, anti-tick vaccines could also diminish *Borrelia* infection of the host by targeting tick proteins that facilitate *Borrelia* transmission from the tick to the host. As such, the key objective of this study was to identify salivary gland proteins from *I. ricinus* produced during feeding or during infection with *B. afzelii* that can serve as potential anti-tick vaccine candidates interfering with tick feeding, *B. afzelii* transmission or both. We have used quantitative proteomics and *in silico* analysis making use of existing databases to identify tick salivary gland proteins that could be used as anti-tick vaccine candidates by comparing the proteome of unfed, 24 h fed and fully fed ticks and

uninfected and infected ticks. A selection of these proteins was produced in *E. coli* and assessed as vaccine candidates in two independent experimental tick challenge models.

2. Results

2.1. Quantitative proteomics

We used quantitative proteomics to describe the proteome of *I. ricinus* salivary glands during different feeding and infection conditions and in particular for the identification of tick proteins abundantly produced during tick feeding and *Borrelia* infection as potential anti-tick vaccine candidates. The experimental design is depicted in Fig. 1. In the differential production analysis, we only considered proteins identified with at least two peptides with an FDR < 1 %. In total we identified 922 different protein accession numbers accounting for 870 different proteins (Supplemental file 1). From these 870 identified proteins, 68 were overrepresented upon 24-hour feeding and *B. afzelii* infection. We calculated the ratios of the normalized abundance of the identified proteins between 24 h fed and unfed and between fully fed and unfed samples to determine the overrepresentation upon feeding. The relative abundance of a protein was normalized against protein length and total signal. We calculated these ratios for both the infected and uninfected samples and depicted those proteins with more than one $\log_2$ fold change upon infection in unfed, 24 h fed and fully fed samples in a Venn diagram (Fig. 2a). To select possible vaccine candidates, we investigated the relative protein production in the salivary glands at different stages of feeding and infection as shown in Fig. 2 b, c and d. We focused on the significantly overrepresented proteins in salivary glands of *B. afzelii*-infected nymphs after 24 h of feeding in the upper right quadrant in Fig. 2b (in red), as this is thought to be a critical time point for *B. burgdorferi* sl transmission [27]. Among these proteins, we found many uncharacterized proteins, but also proteins with putative functions described earlier for tick salivary gland proteins such as 13 lipocalins, 6 Kunitz domain proteins, 11 basic tail proteins and 16 metalloproteases. Next, we selected *I. ricinus* saliva gland proteins for further study. The selection of proteins was based on the following criteria: proteins significantly overrepresented upon *B. afzelii* infection at the 24 h feeding time point (upper right quadrant Fig. 2b); no similarity with mammalian proteins; complete protein and not a fragment, a low molecular weight (easier to produce as recombinant protein); and the absence of a transmembrane region due to the difficulty to express as recombinant proteins. We selected five proteins that matched all the above criteria (Table 1). In addition, the two salivary gland proteins that were most represented upon feeding (fully fed) were selected to target tick salivary proteins specifically important in the tick feeding process (Supplemental file 2).

2.2. RNA expression

In order to determine whether the differential protein production was associated with transcriptional changes under the different conditions analyzed, qRT-PCRs were performed. Gene-specific primers were designed and used in qRT-PCRs to determine whether the genes coding for the selected proteins were differentially expressed, using cDNA from the salivary glands of infected and uninfected nymphs at different time points. Our results showed RNA expression for all proteins under the different conditions (Supplemental Fig. 1). Interestingly, a large variation was noted in RNA expression and the under- or overrepresentation at the RNA level did not overall correspond to protein production levels as determined by the proteomic analysis, suggesting that
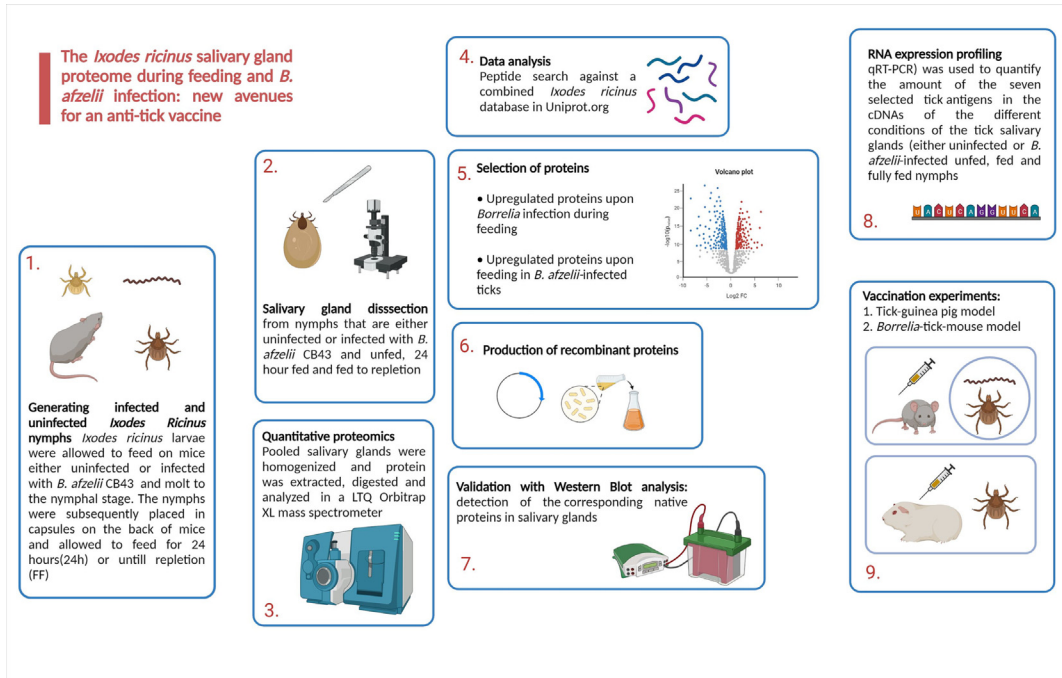
Fig. 1. Overview of the experimental design. Quantitative proteomics was used to describe the proteome of *I. ricinus* salivary glands during different feeding and infection conditions. Tick proteins were identified as potential anti-tick vaccine candidates that are abundantly expressed during tick feeding and *Borrelia* infection: created in BioRender.com.
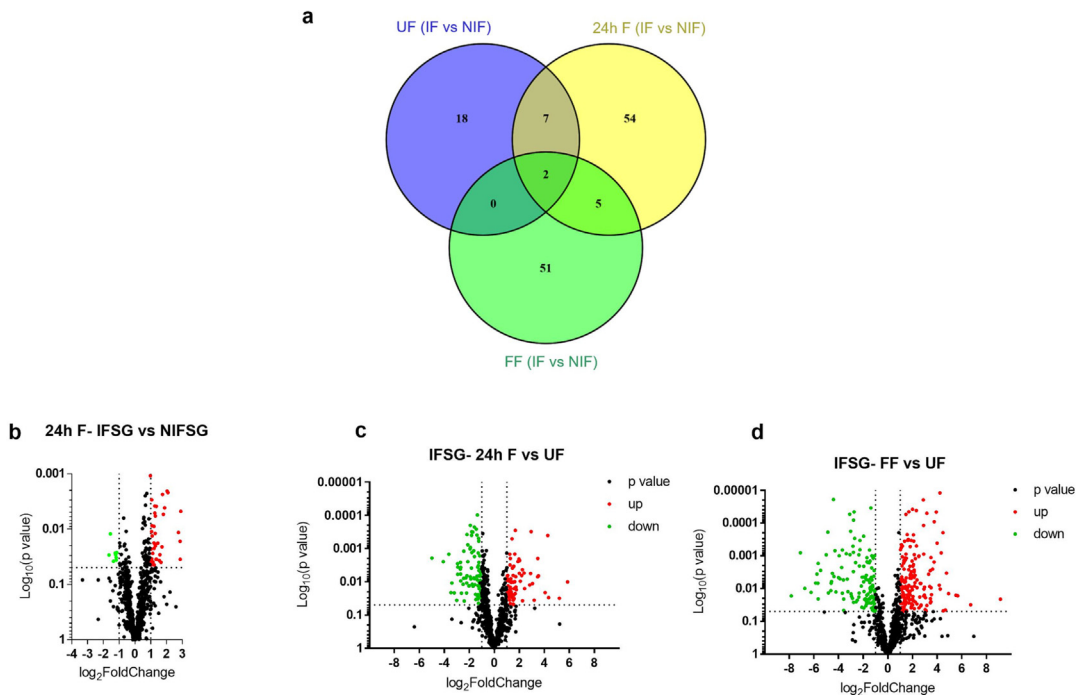

Fig. 2. Overview of differentially produced tick salivary gland proteins. 1a. Overlap of the identified proteins upon *B. afzelii* infection in unfed, 24 h fed and fully fed samples at 1 % FDR. Shown here are the identified proteins with a statistically changed protein level between feeding conditions in infected samples compared to uninfected samples. 54 proteins are overrepresented significantly upon *B. afzelii* infection and upon 24 h feeding. 1b-d. Volcano plot showing the identified tick salivary gland proteins according to p-value and fold change. The p-value is plotted on the y-axis and the log2 fold change on the x-axis. Red dots are more than one Log_2fold significantly overrepresented, green dots are more than one Log2fold downregulated. (b) Tick salivary gland proteins expressed in 24 h fed infected *I. ricinus* nymphs compared to non-infected *I. ricinus* nymphs. (c) Tick salivary gland (SG) proteins expressed in 24 h fed *I. ricinus* nymphs infected with *B. afzelii* compared to unfed *B. afzelii* infected ticks. (d) Tick salivary gland proteins expressed in fully fed *I. ricinus* nymphs infected with *B. afzelii* compared to unfed *B. afzelii* infected ticks. 24F = 24 h fed nymphs; IFSG = infected salivary gland; NIFSG = uninfected salivary gland; UF = unfed nymphs; FF = Fully fed nymphs; IF = infected; NIF = not infected. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Selected tick salivary gland proteins.

Uniprot ID	Peptide count	Unique peptides	Putative function	Inducted upon
A0A0K8REN3_IXORI	5	2	Putative metalloprotease	*B.afzelii infection*
A0A147BSD0_IXORI	7	7	Putative fasciclin	*B.afzelii infection*
A0A131XYT7_IXORI	5	5	Obg-like ATPase 1	*B.afzelii infection*
A0A131Y1N1_IXORI	2	2	Polyadenylate-binding protein	*B.afzelii infection*
V5HE54_IXORI	2	2	Metalloendopeptidase	*B.afzelii infection*
V5HWB8_IXORI	3	2	Uncharacterized protein	Tick feeding
V5HV74_IXORI	4	4	Putative grp-2 449 glycine rich family	Tick feeding

the observed protein changes were generally related to post-translational events and further underscoring the importance of the proteomics analysis (Supplemental Fig. 1).

2.3. Protein validation

Next, we performed Western blot analysis as an internal validation experiment for the seven selected proteins. To this end we generated new lysates of salivary glands from pooled fully fed (*B. afzelii*-infected) nymphs. The fully fed condition was used since these lysates contained sufficient amounts of protein for use in Western blots. Fig. 3 shows that all native proteins could be detected in these lysates by Western blot using sera from the mice immunized with the recombinant proteins.

2.4. Vaccination experiments

Next, we performed a vaccination study in both mice and guinea pigs. In both studies, control animals were immunized with PBS and adjuvant. We first measured total IgG antibodies by ELISA in mouse and guinea pig sera after three vaccinations with the recombinant proteins. As expected, high antibody titers were induced in both hosts against all recombinant proteins (Fig. 4 a and b).

In order to assess the effect of vaccination on tick feeding we compared the post-engorgement weights of nymphs fed on vaccinated guinea pigs and control vaccinated hosts. The nymphs were checked daily and were weighed after detachment. Notably, tick weights were significantly lower in the guinea pigs that had been vaccinated with the recombinant proteins compared to controls, indicating that the vaccination formulation using the recombinant proteins was able to impair tick feeding (Fig. 4c). As expected, there was a clear distinction in the weights of the female and male nymphs. Of note, no significant skin reaction was observed in the skin of the animals after tick infestation. In addition, we also tested the effect of the same vaccination formulation and regime on tick feeding in mice. Remarkably, vaccination also induced significantly lower post-feeding tick weights in the vaccinated mice (Fig. 4d), suggesting a similar mechanism of protection among these host species.

Finally, we determined whether the recombinant protein vaccine also provided protection against *B. afzelii* CB43 infection. The vaccinated mice were exposed to tick challenge with ten *B. afzelii*-infected nymphs three weeks after the last vaccination and were killed three weeks later. Post-mortem tissues were subjected to qPCR and culture. *Borrelia afzelii* DNA loads were quantified by qPCR using OspA *Borrelia* primers and compared to mouse *Beta-actin* as a housekeeping gene (Supplemental Fig. 2).

Similar numbers of control and vaccinated mice were infected upon analysis, and both groups showed similar levels of *B. afzelii*. These results suggested that the partial prevention of tick feeding resulting from the immunization was not able to prevent spirochetal transmission.

3. Discussion

The incidence of Lyme borreliosis and other known tick-borne diseases is increasing and new tick-borne diseases are emerging for many reasons, including climate change. There is currently no human vaccine to prevent Lyme borreliosis or many other tick-borne diseases. An anti-tick vaccine could constitute an innovative and potentially efficient strategy to prevent multiple human tick-borne diseases. In this study, we aimed to identify anti-tick vaccine candidates that are abundantly expressed during the tick feeding process as well as upon *B. afzelii* infection using proteomics on tick salivary gland extracts of *I. ricinus* ticks that were infected with *B. afzelii*.

In general, gene expression can be measured and modulated at many levels: from transcription into translation, but eventually, the protein is the actual functional output of the cell. Quantitative proteomics focuses on identifying and quantifying proteins and provides information about the protein content that may greatly differ from results at the transcriptomic level. This makes proteomics a powerful tool for tick vaccine discovery. Surface-located or secreted proteins are considered to be potentially promising and immunogenic vaccine candidates, as they are likely to come into contact with the host's immune system. On the other hand, concealed antigens have proven to be successful as vaccine candidates. These antigens do not induce an immune response upon tick infestation but are immunogenic when used as extracts or recombinant proteins and protection is mediated through vaccine-induced antibodies [28,29]. In addition, concealed antigens are often conserved across vector species which enables the possibility to develop a universal vaccine for multiple arthropod vectors and the pathogens they transmit [30]. The downside is that tick exposure does not serve as natural booster. For example, Bm86 is not naturally exposed to the immune system and represents a successful example of a concealed antigen [31]. Another example of a concealed protein that protects against different vectors - mosquitos, sandflies and ticks- is Subolesin, a regulatory protein involved in signal transduction [32]. It has been shown that Subolesin is differentially produced in infected ticks and may be important for tick innate immunity to different pathogens [33,34]. Proteomics analysis allows the identification of the subcellular location of proteins under different conditions, providing important information about the possible function of the proteins and the potential efficacy of selected candidate protective antigens.

In the current study, using label-free quantitative proteomics, tick saliva protein production between different experimental conditions was compared, enabling us to identify tick salivary gland proteins abundantly expressed during the tick feeding process as well as upon *B. afzelii* infection. Thus, we have identified a total of 870 proteins. For external validation, we compared the identified and selected proteins with potential tick protein candidates described in the literature. Schwarz et al. also applied proteomics and assessed protein production in nymphal *I. ricinus* midgut and salivary glands at 12 h and 24 h of feeding [24]. They found that the 20 most abundant proteins were conserved across all experi-
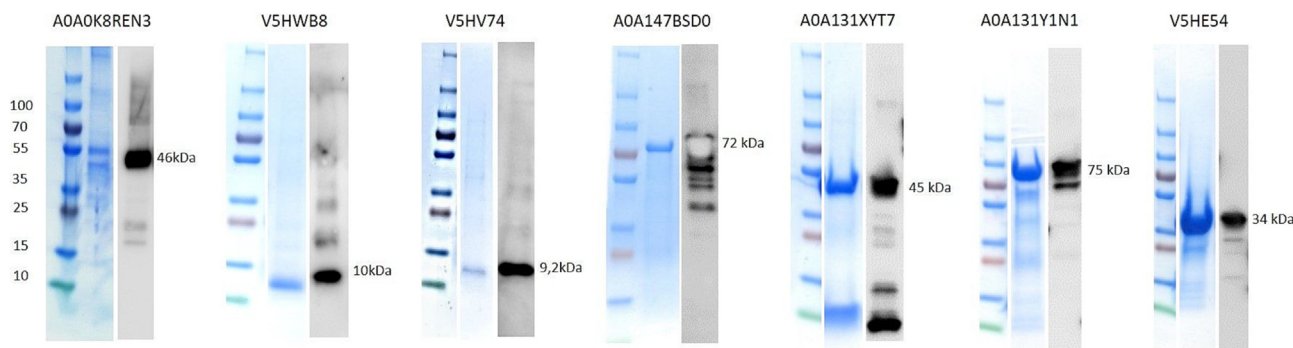
Fig. 3. Western blot showing production of native tick salivary gland proteins. Lysates of fully fed, *B. afzelii*-infected *I. ricinus* nymphs were obtained and subjected to Western blot. The right lane shows the native proteins as detected by Western Blot using polyclonal mouse IgG. The left lane shows the recombinant protein on a Coomassie staining as a control. Blot images were cropped (Image acquisition tools Microsoft Powerpoint). Imaging was performed using ImageQuant LAS 4000 and quantification using Image J (Wayne Rasband, National Institutes of Health, USA, Java 1.8.0_77(32-bit), http://imagej.nih.gov/ij).
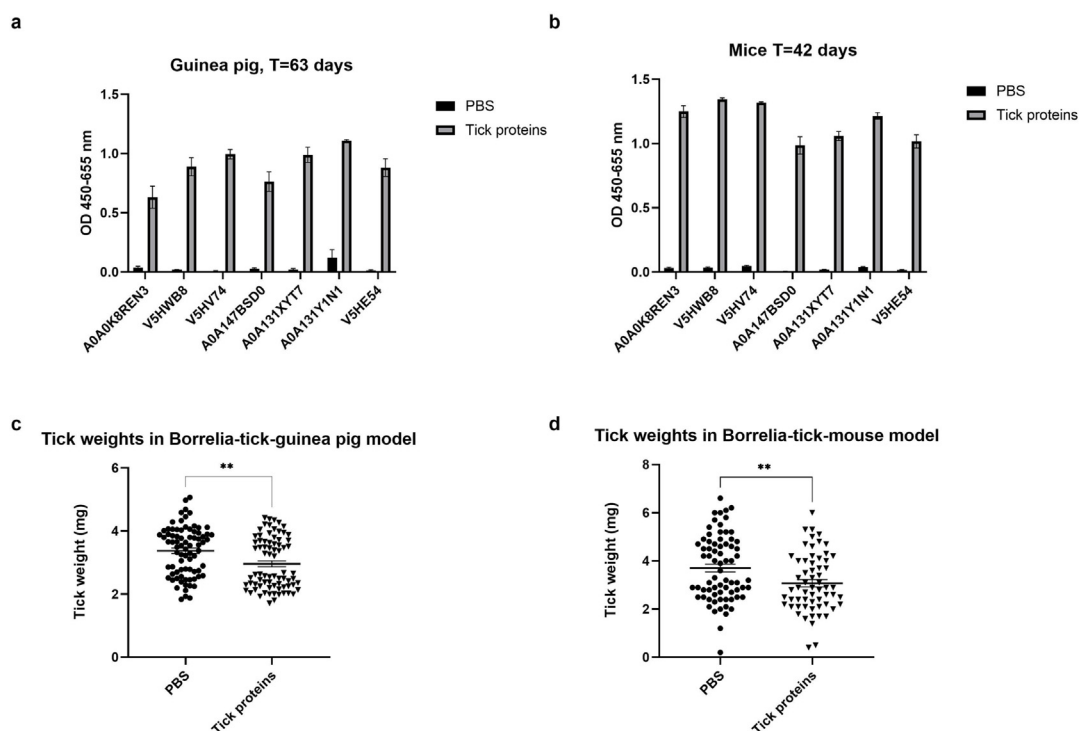

Fig. 4. (a–d) Guinea pig-tick model and Mouse-tick model. Enzyme-linked immunosorbent assay (ELISA) of specific IgG antibodies induced in immunized guinea pigs (a) and mice (b). Recombinant protein specific total IgG responses were measured in guinea pig sera from two vaccination groups with 3 guinea pigs each, vaccinated at three time points. Recombinant protein specific total IgG responses were measured in mice sera from four vaccination groups with 8 mice each. The horizontal line represents the mean and error bars standard error of the mean (SEM). IgG responses are presented as optical density (OD) 450–655 nm for sera dilution of 1:50.000. The horizontal line represents the mean and error bars standard error of the mean (SEM). The first group was immunized with PBS and the second group was immunized with a cocktail of all proteins. Plates were coated with each recombinant protein in triplicate and incubated with sera. Statistical significance between each protein and PBS control was calculated with a 2way ANOVA with multiple comparisons, GraphPad Prism software version 5.0, San Diego, CA, USA and was statistically significant for each protein (**** P < 0.0001 in both mice (a) and guinea pig experiments (b)).Post-engorgement tick weights in the guinea pig model (c) and in the *Borrelia*-tick-mouse model (d). After 3 vaccinations each guinea pig (T = 63) was infested with 30 uninfected *I. ricinus* nymphs and each mouse (T = 42) was infested with ten *I. ricinus* nymphs infected with *B. afzelii* CB43. On the Y-axis the individual weight of each tick is plotted with the X-axis displaying the two experimental groups: animals immunized with PBS or with a cocktail of all seven recombinant proteins. Error bars in all figures demonstrate mean ± SEM. Statistical significance of cumulative data of the experimental groups compared to the PBS group was calculated with a two-sided nonparametric test (Mann-Whitney, GraphPad Prism software version 5.0, San Diego, CA, USA. Distribution of data was non-normal and was assessed both by D'Agostino & Pearson normality test and Shapiro-Wilk normality test). Comparison between both guinea pigs and mice immunized with PBS and those immunized with all selected proteins was statistically significant (* P < 0.05, ** P < 0.01, ***P < 0.001).

mental samples suggesting a conserved proteome upon tick attachment. Interestingly, these 20 most abundant proteins published by Schwarz et al, both from 12 h fed nymphs and 24 h fed nymphs are all among the proteins that we determined to be differentially expressed upon tick feeding. Secondly, we have compared our identified proteins with the contigs - with minimally 1 normalized read – identified in the Massive Analysis of cDNA Ends

(MACE) database from the ANTIDotE project [35] by translating the nucleotide sequences from contigs into protein sequences using tBLASTn (NCBI; https://blast.ncbi.nlm.nih.gov/). Out of the 870 identified proteins in our proteomics project, 869 were present in the MACE database.

From the differentially expressed proteins we selected two that were highly represented upon feeding (both 24 h and FF) and five

tick proteins showing a more than $\log_2$ fold change upon *B. afzelii* infection in 24 h fed samples for downstream experiments. As transmission of *B. burgdorferi* sl from tick to host usually starts around 24 h post attachment, tick saliva is likely to contain the most interesting vaccine candidates at this time. The native counterparts of the *I. ricinus* salivary gland proteins we selected could all be verified by Western blots in salivary gland lysates, validating the presence of these proteins in tick salivary glands. More importantly, it also underscored the ability to induce antibodies by vaccination with *E. coli*-expressed antigens, which were able to bind to their native counterparts. Western blot incubated with sera specific for protein A0A131XYT7 showed, in addition to the recognized protein at the expected weight, a reactive fragment around 12 kDa. This might be explained by a nonspecific binding or the presence of a degraded protein fragment. We also set out to investigate whether each candidate is robustly expressed in different tick lineages at RNA level in five different cDNA pools each deriving from their own egg mass. We were able to identify the antigens corresponding to the selected proteins in all cDNA pools. Although we showed RNA expression, the expression levels were not comparable to the production levels for the proteins among the different conditions used. This comes as no surprise as transcript levels by themselves are seldom sufficient to predict protein levels as they are dependent on many dynamic processes such as temporal mRNA variation and availability of resources for protein synthesis [36].

Targeting *Ixodes* tick salivary gland proteins by vaccination has been described to prevent transmission of *B. burgdorferi* sl. For example a Salp15-based vaccine was shown to partially block *B. burgdorferi* ss infection [5,37]. In addition a vaccine targeting tick histamine release factor (tHRF), resulted in significantly diminished tick feeding as well as transmission of *B. burgdorferi* ss [38]. Finally, we and others identified Tick Salivary Lectin Pathway Inhibitor (TSLPI), which was shown to impair complement-mediated killing of *B. burgdorferi* sl, and impaired *B. burgdorferi* ss transmission in mice that were injected with rabbit TSLPI-antiserum [39]. In contrast, the anti-tick vaccine based on the selected salivary gland proteins in the current study did not prevent transmission of *B. afzelii* to the murine host. This could be due to the fact that the functions associated with the selected tick proteins are not critical for the transmission of *B. afzelii* from the tick to the murine host, are not neutralized by the elicited antibody response or the vaccine has a different mechanism of action altogether [40]. Indeed, ticks were in general able to feed to completion, albeit with reduced efficiency, yet allowing *B. afzelii* to be transmitted [40].

Sajid et al. recently showed protection against tick bites as well as *B. burgdorferi* transmission by using an mRNA vaccine encoding 19 *I. scapularis* salivary gland proteins in guinea pigs [41]. In terms of recombinant vaccines, there are previous examples of tick salivary gland proteins used as recombinant vaccines, showing decreased feeding ability of nymphs in vaccinated guinea pigs [42]. Interestingly, Kotsyfakis et al. also showed that repeated nymphal infestation of guinea pigs did not lead to decreased ability to feed on control guinea pigs, but only in those that had been vaccinated with recombinant proteins, which leads to the term: 'silent' or 'concealed' antigens.

As part of the current study and as a proof of concept, the seven selected proteins were used in vaccination studies in both mice and guinea pigs. We show that vaccination of guinea pigs with our cocktail recombinant protein vaccine significantly impaired tick feeding, as opposed to ticks feeding on control mice. Interestingly, it has been demonstrated that tick glycoproteins in tick saliva are important tick vaccine targets to protect against tick feeding [43], but here we show that *E. coli* expressed non-glycosylated antigens can also result in a robust anti-tick feeding

phenotype. Decreased *Ixodes* tick feeding upon vaccination with a recombinant tick protein vaccine has only been shown once before in mice [38], which has subsequently been challenged [37]. Notably, the fact that we observed the same protective phenotype when vaccinating guinea pigs and mice, suggests that the mechanism of protection is conserved among both species. The mechanism does not appear to involve skin inflammatory reactions noted in other protection studies [44]. Indeed, we did not observe any sign of skin inflammation during or after tick feeding in vaccinated guinea pigs, suggesting a specific effect on the tick rather than on the local skin response. It could be that (a selection of) these seven antigens (and their associated functions) are important for the tick feeding process and the observed diminished tick feeding after vaccination may be due to interference with these functions. Alternatively, lower weights of the nymphs may be caused by vaccine-induced non-specific bystander damage to the tick salivary glands or guts, for instance by antibody-mediated complement deposition.

A combination of proteins involved in different biological processes could potentially increase vaccine efficacy. In that regard it should be noted that previously published work revealed 20 genes at least two fold more abundantly produced in salivary glands of adult *I. scapularis* ticks after attachment [45]. The most represented protein families among these were Kunitz domain containing proteins, Salp15, lipocalins, metalloproteases and several proteins of unknown function [4,22,46]. Interestingly, two of the selected proteins in the current study included metalloproteases. Of further interest, some of the selected tick proteins were annotated as potential intracellular proteins and previous work by others has established intracellular tick proteins as promising vaccine targets, such as Subolesin [30,34]. How antibodies against intracellular tick proteins lead to impaired tick feeding is unknown. Nevertheless, intracellular tick proteins constitute interesting vaccine candidates, as these are likely to be more conserved and to suffer less from the well-established redundancy of secreted tick saliva proteins [4]. Although as part of the current study we did not select tick proteins with a transmembrane domain, for future purposes one could also consider using extracellular regions of transmembrane proteins as vaccine antigens as well, since these come into contact with the host immune system similar to secreted or surface located proteins. Based on the above, which of the seven tick proteins contributes most to the tick feeding process, the contribution of the intracellular tick proteins to the observed phenotype, the exact biological functions of the tick proteins, and the exact mechanism of action of our experimental vaccine remains to be investigated.

In conclusion, an anti-tick vaccine based on the recombinant forms of seven selected tick saliva proteins impaired nymphal tick feeding in two independent experimental tick-challenge models. This is of great interest, as nymphs are the most important life stage of *Ixodes* ticks for human disease. The results represent the identification of a cross-species protective vaccine formulation against tick feeding. This could aid tick control and set the basis to reformulate the vaccine antigens used in this study to refine and identify the mechanisms of protection that could serve to generate improved vaccine formulations. Such approaches could include innovative approaches that, for example, combine tick and *Borrelia* proteins in a vaccine, as shown before [5,47]. Future vaccination experiments could therefore investigate whether combining a selection of our newly identified antigens with other antigens from either the tick or tick-borne pathogens could impair transmission of *B. burgdorferi* sl, and other tick-borne pathogens, from the tick to the host. Novel vaccination platforms, including nanoparticle mRNA vaccines, could facilitate generating and investigating such multivalent vaccines [41].

4. Materials and methods

4.1. Spirochetes, ticks, guinea pigs and mice

4.1.1. Ethics statement

Experiments have been conducted according to European and national guidelines. All mouse experiments were reviewed and approved by the Animal Research Ethics Committee of the Academic Medical Center, Amsterdam, the Netherlands (protocols DIX208AH, DIX208AI, DIX208AJ). All guinea pig experiments were carried out according to the animal protection law of the Czech Republic (§17, Act No. 246/1992 Sb). The animal experimental protocol was approved by the Czech Academy of Sciences Animal Care and Use Committee (protocol permit number 102/2016). The experiments with *Borrelia* were performed in BSL2 conditions.

4.1.2. Generation of infected and uninfected ticks

Low passage *B. afzelii* strain CB43 spirochetes were cultured in Modified Kelly-Pettenkofer (MKP) medium and counted using a Petroff-Hausser counting chamber and dark-field microscopy. An inoculum of $1x10^6$ spirochetes in 200 µl was injected subcutaneously in the midline of the back between the shoulders of six-to-eight-weeks-old female C3H/HeN mice (Charles River). Mice were checked for *Borrelia* infection positivity by quantitative PCR (qPCR) 14 days post-inoculation. After infection was confirmed, approximately 500 *I. ricinus* larvae (kindly provided by the Centers for Disease Control and Prevention, BEI Resources, NIAID, NIH) were placed on each mouse. In the following 6 days, detached, fully-fed larvae were collected and allowed to molt into the nymphal stage, which took approximately 6–8 weeks. Ticks were housed in an incubator (Panasonic, MIR-154-PE) at room temperature and at a constant relative humidity of 90 %. Once molted, nymphal infection rates were assessed by qPCR. To establish tick infection rate, DNA was extracted from a minimum of ten ticks using the DNeasy Blood and Tissue kit (QIAGEN). qPCR was used to quantify *B. afzelii* DNA in ticks and mouse tissues following a previously described protocol [48,49]. The infection rate of the tick batches used in these studies was >85 %.

4.2. Quantitative proteomics

4.2.1. Salivary gland dissection

Ten *B. afzelii*-infected or uninfected nymphal *I. ricinus* ticks were placed in a containing capsule on six-to-eight-weeks-old female C3H/HeN mice. The nymphs were allowed to feed for either 24 h or until repletion. For salivary gland dissection we used 3 groups of nymphal ticks: unfed, 24 h fed and fully fed ticks. We used 4 mice and 10 nymphal ticks per mouse per feeding condition. The nymphs were washed in 70 % ethanol for 30 s after which the salivary glands were dissected under a dissection microscope at 40x magnification (Zeiss, 475052). The salivary glands from the ticks that had fed on one single mouse were pooled in 200 µl PBS. The pooled salivary glands were homogenized on ice using an ultrasound sonicator (Vibra-Cell High Intensity ultrasonic processor) and centrifuged for 30 min at 15,000g at 4 °C. Subsequently, the supernatant of each pellet was 1:1 diluted with CLB buffer (7 M urea, 2 M thiourea, 4 % CHAPS and 200 mM DTT). The samples were stored at −80 °C until further analysis.

4.2.2. In solution digestion

Protein was extracted using 7 M urea, 2 M thiourea, 4 % CHAPS. Samples were incubated for 30 min at RT room temperature under agitation and digested following the filter-aided FASP protocol as described by Wisniewski and colleagues [50]. Trypsin was added to a trypsin:protein ratio of 1:10, and the mixture was incubated

overnight at 37 °C, dried out in a RVC2 25 speedvac concentrator (Christ), and resuspended in 0.1 % formic acid (FA).

4.2.3. Mass spectrometry analysis

Approximately 500 ng of each sample was submitted to LC-MS label-free analysis. Peptide separation was performed on a nanoACQUITY UPLC System (Waters) on-line connected to an LTQ Orbitrap XL mass spectrometer (Thermo Electron). An aliquot of each sample was loaded onto a Symmetry 300 C18 UPLC Trap column (180 µm × 20 mm, 5 µm (Waters). The precolumn was connected to a BEH130 C18 column (75 µm × 200 mm, 1.7 µm (Waters), and equilibrated in 3 % acetonitrile and 0.1 % FA. Peptides were eluted directly into an LTQ Orbitrap XL mass spectrometer through a nanoelectrospray capillary source (Proxeon Biosystems), at 300 nl/min and using a 120 min linear gradient of 3–50 % acetonitrile. The mass spectrometer automatically switched between MS and MS/MS acquisition in DDA mode. Full MS scan survey spectra (m/z 400–2000) were acquired in the orbitrap with mass resolution of 30,000 at m/z 400. After each survey scan, the six most intense ions above 1000 counts were sequentially subjected to collision-induced dissociation (CID) in the linear ion trap. Precursors with charge states of 2 and 3 were specifically selected for CID. Peptides were excluded from further analysis during 60 s using the dynamic exclusion feature.

4.2.4. Progenesis LC-MS software analysis

Progenesis LC-MS (version 2.0.5556.29015, Nonlinear Dynamics) was used for the label-free differential protein production analysis. One of the runs was used as the reference to which the precursor masses in all other samples were aligned to. Only features comprising charges of 2+ and 3+ were selected. The raw abundances of each feature were automatically normalized and logarithmized against the reference run. Samples were grouped according to the comparison being performed, and an ANOVA analysis was performed. A peak list containing the information of all the features was generated and exported to the Mascot search engine (Matrix Science Ltd.). This file was searched against a Uniprot/Swissprot database, and the list of identified peptides was imported back to Progenesis LC-MS. Protein quantitation was performed based on the three most intense non-conflicting peptides (peptides occurring in only one protein), except for proteins with only two non-conflicting peptides. The significance of production changes was tested at protein level, and proteins with an ANOVA p-value ≤0.05 were selected for further analyses.

4.2.5. Functional analysis

GO enrichment analysis was carried out using the DAVID online tool (https://david.abcc.ncifcrf.gov/summary.jsp) [51]. DAVID is a GO Term annotation and enrichment analysis tool used to highlight the most relevant GO terms associated with a given gene list. A Fisher Exact test was used to determine whether the proportion of genes considered into certain GO term or categories differed significantly between the dataset and the background. A false discovery rate (FDR)-corrected version of the Fisher's test p-value was obtained and used for more conservative selection. Biological Process (BP), Molecular Function (MF) and Cellular Component (CC) categories were assessed, and only GO Terms enriched with a FDR <5 % were considered for comparison and discussion. Additionally, KEGG Pathways, keywords, sequences, and Interpro and Smart databases were also analyzed, considering terms with an enrichment p-value <0.05.

4.2.6. RNA isolation

Salivary gland lysates were used for isolation of RNA using the Nucleospin RNA isolation kit (Macherey-Nagel), according to the manufacturer's instructions. RNA samples were treated with

DNase for a second time using the RNase-Free DNase Set (#79254, QIAGEN) and then cleaned up using the RNeasy MinElute Cleanup Kit (#74104, QIAGEN). Isolated RNA was used to generate cDNA as previously described [52].

4.2.7. Production of recombinant proteins

A total of seven proteins were selected for further studies and were expressed recombinantly. To this end, RNA was extracted from salivary glands from *I. ricinus* ticks as described above and cDNA was generated. For three selected candidates (UniProt IDs A0A0K8REN3; V5HWB8 and V5HV74) the corresponding genes were amplified from the cDNA using the newly-designed primers listed in Supplemental Table 1 and Phusion high-fidelity PCR mix (New England Biolabs, Ipswich, MA) using the following PCR protocol: 1 cycle 95 °C 3 min, followed by 35 cycles of 98 °C 20 s, 58 or 60 °C 15 s and 72 °C 45 s and finally 1 cycle of 72 °C for 1 min. The genes were subsequently cloned into pHISparallel2 (Invitrogen). Four other recombinant tick salivary gland genes (A0A147BSD0; A0A131XYT7; A0A131Y1N1 and V5HE54) were ordered from Genscript USA Inc. as plasmids in pET-21b (Supplemental Table 2). All proteins were produced in *E. coli*. Because the proteins were all present in the insoluble fraction after induction with IPTG, the proteins were solubilized in lysis buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM DTT) with 7 M Urea pH 8. The soluble proteins were then purified using Ni-NTA as detailed elsewhere [53]. Purity was checked using SDS-PAGE, and protein concentrations were measured using a Bradford assay. Pooled fractions were dialyzed in a dialysis membrane (Slide-A-Lyzer™ Dialysis Cassettes, ThermoFisher) overnight at 4 °C in 500 ml of 50 mM Tris pH 7.5, 300 mM NaCl, 1 mM EDTA, 10 % glycerol to remove imidazole, to diminish the concentration of Urea to 2 M and to refold the protein.

4.3. RNA expression profiling

4.3.1. RNA expression

Real-time quantitative polymerase chain reaction (qRT-PCR) was used to quantify the amount of the seven selected tick antigens in the cDNAs of the different conditions of the tick salivary glands (either uninfected or *B. afzelii*-infected unfed, fed and fully fed nymphs). The specific primers for each gene are depicted in Supplemental Table 3. Five different cDNA pools of nymphs were used, each pool originating from the same egg mass of field-collected I. ricinus females. Expression levels were normalized using the *I. ricinus cytochrome c* housekeeping gene. The cDNA was diluted 100-fold and one µl was used as template per reaction. qPCRs were performed using a LightCycler480 (Roche, Nutley, NJ, USA) and LC480 SYBR Green I Master (Roche) in triplicate. The PCR protocol was 95 °C 6 min, and 60 cycles of 95 °C 10 s, 60 °C 20 s and 72 °C 20 s. Results were analyzed using LinRegPCR software (Amsterdam, The Netherlands).

4.4. Validation

4.4.1. Immunization of mice: Generating antibodies for validation experiments

For each protein, two mice were injected subcutaneously in the midline of the back between the shoulder blades with 100 µl of recombinant protein mixed with complete Freund's adjuvant (CFA) or incomplete Freund's adjuvant (IFA). Each mouse was immunized three times at two-week intervals. Three weeks after the last immunization, mice were sacrificed and sera were collected. The sera were used in the validation assays.

4.4.2. Western blot analysis: Detection of the corresponding native proteins in salivary glands

Ixodes ricinus salivary glands from fully fed, *B. afzelii*-infected nymphs were dissected and processed as described above. Protein concentrations were measured using the Pierce BCA protein assay (#23225 from Thermo Scientific). Subsequently, lysates (4,8 µg protein per sample) were mixed in a 5:1 ratio with 5x SDS sample reducing buffer (1.5 % SDS, 10 % glycerol, 62.5 mM Tris-HCl (Ph 6.8), 2 %-mercapto-ethanol and 0.0025 % bromophenol blue) and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using a precast SDS 4–20 % polyacrylamide gel (Bio-Rad Mini-PROTEAN TGX) with PageRuler Plus Prestained Protein Ladder (Thermo Scientific). The corresponding recombinant proteins were used as loading controls at approximately 100 ng per sample. All samples were then transferred to a 99 % ethanol-activated PVDF membrane. The membrane was incubated with a 1:100 dilution of murine sera derived from mice immunized with the corresponding recombinant protein. Immunoblots were labeled with horse-anti-mouse IgG-HRP 1:2500 as secondary antibody (#7076, Cell Signaling, Beverly, MA, USA) and developed using Pierce ECL Western Blotting Substrate (#32106 Thermo Scientific).

4.5. Vaccination studies

4.5.1. Tick-guinea pig model

Laboratory guinea pigs reared in the animal facility of the Institute of Parasitology were used for tick feeding experiments. The tick feeding experiments were performed in five-week-old guinea pigs with two different experimental groups consisting of three guinea pigs each. The first group was vaccinated with a cocktail of all 7 selected proteins containing 30 µg of each protein in a total volume of 383 µl. To this volume an equal amount of CFA at t = 0, or IFA at t = 21 and 42, was added and the amount of 766 µl was injected subcutaneously divided over four injection sites. The second group was vaccinated with the same amount of PBS combined with CFA/IFA as a control. The guinea pigs were vaccinated three times with three-week intervals (t = 0, 21 and 42 days). Blood withdrawal to collect sera for use in ELISA experiments was performed on day 0 and 63 to minimize stress for the guinea pigs. At day 63 each guinea pig was infested with 30 uninfected *I. ricinus* nymphs that were placed in a capsule on the back of the guinea pig. The nymphs were checked daily from day 64 until they were fully fed. At the time the nymphs detached they were weighed, and the number of feeding days was noted. Ticks were housed in an incubator (Panasonic) at room temperature and at a constant relative humidity of 90 %. The guinea pigs were sacrificed at day 84.

4.5.2. Borrelia-tick-mouse model

Six to eight weeks old female C3H/HeN mice were purchased from Charles River Laboratories. In this vaccination study we assessed two different experimental groups consisting of eight mice each: control mice vaccinated with PBS and mice vaccinated with a pool of all seven proteins. Mice were vaccinated at t = 0, t = 14 and t = 28 days and sera were collected at each time point. For the recombinant vaccines 10 µg protein (in total) was emulsified with CFA at t = 0 and 5 µg in IFA at t = 14 and t = 28 days. For the control mice PBS and either CFA or IFA was used. All vaccinations were administered subcutaneously. Two weeks after the third vaccination (t = 42) mice were challenged with *I. ricinus* nymphs infected with *B. afzelii* strain CB43. These *B. afzelii*-infected *I. ricinus* nymphs were placed in a containing capsule and fed on the mice until repletion. Nymphs were then collected and weighed. Additional sera were collected at t = 42 (pre-challenge, 2 weeks after the third immunization) and at t = 63 days (3 weeks post-challenge) mice were sacrificed and

ear, skin, ankle, heart, and bladder were collected for analysis for *B. afzelii* infection. Murine bladder and skin were cultured and checked weekly for the presence of motile spirochetes under dark field microscopy as described previously [52]. DNA was extracted from ear, skin, ankle, bladder and heart tissue using the DNeasy Blood and Tissue kit (QIAGEN) and qPCR was performed to quantify *B. afzelii* DNA according to a previously described protocol [48,52,54]. Mice were considered to be infected when there was at least one positive tissue sample in either qPCR or culture. Differences in *B. afzelii* loads between experimental groups in qPCR were statistically tested by two-sided nonparametric tests (Mann-Whitney, GraphPad Prism software version 5.0, San Diego, CA, USA).

4.5.3. ELISA (mouse and guinea pig)

To measure IgG directed against the recombinant proteins, ELISA was performed according to previous described protocols [48,52]. High-binding 96-well ELISA plates (Greiner Bio-one, Kremsmünster, Austria) were coated overnight at 4 °C with 1 µg/ml of recombinant protein, washed with PBS–Tween (phosphate-buffered saline–0.05 % Tween) and incubated with blocking buffer (1 % BSA in PBS for mouse and 3 % BSA in PBS for guinea pig) for 2 h at room temperature. Mouse sera or guinea pig sera (collected before tick challenge) were diluted in blocking buffer, added to the wells and incubated for 1 h at room temperature. Plates were washed and incubated for 1 h with horseradish peroxidase (HRP)-linked anti-mouse IgG (Cell Signaling, Beverly, MA, USA) diluted 1:1000 or goat anti-guinea pig IgG (Biorad, Hercules, CA, USA) diluted 1:1000 in blocking buffer. The plates were washed again and developed using TMB substrate (50 µl TMB chromogene in 5 ml TMB substrate buffer [8,2 g NaAc and 21 g citric acid mono-hydrate dissolved in 1 L H_2O + 10 µl 3 % H_2O_2]) and optical density was measured in a Biotek (Winooski, VT, USA) ELISA plate reader at 450–655 nm.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Data availability

Data will be made available on request.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [Joppe Hovius reports a relationship with Intravacc that includes: funding grants. Joppe Hovius reports a relationship with Pfizer that includes: funding grants.].

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2023.02.003.

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