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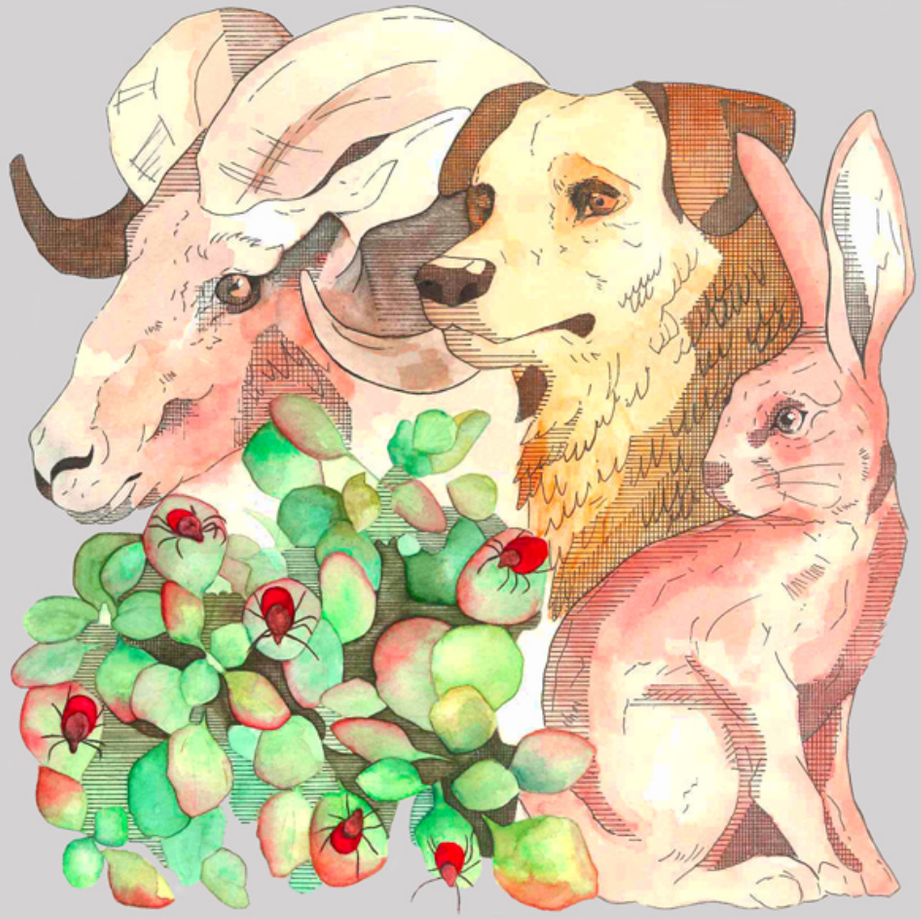
Tick-host-pathogen interactions and vaccine development for the control of tick-borne diseases



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Trabajo presentado por Marinela Contreras Rojo
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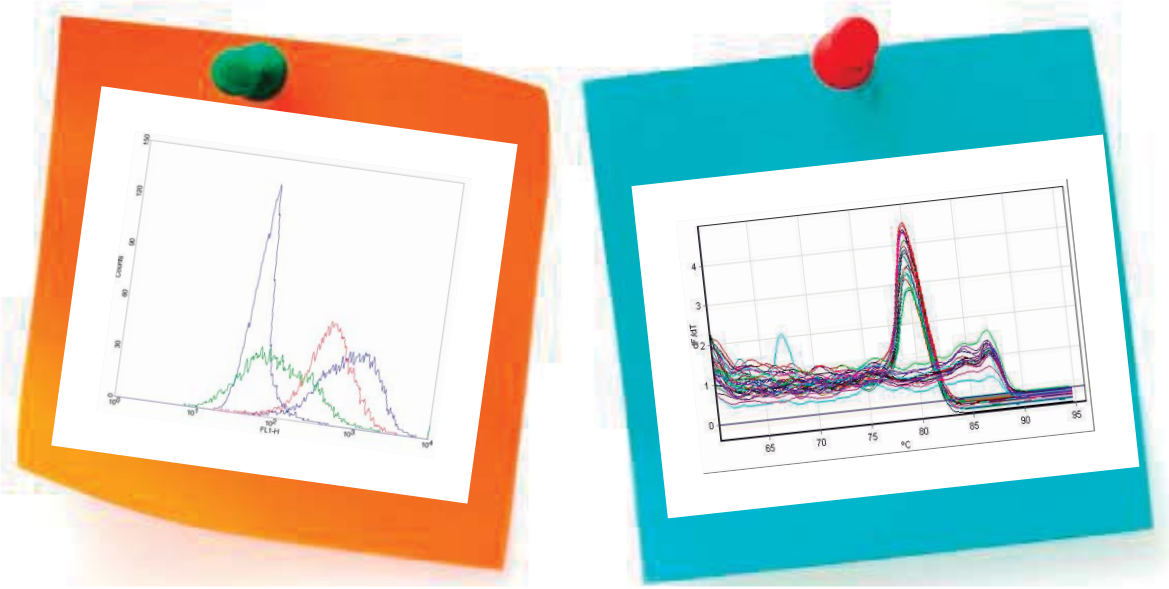
"Si quieres hacer algo en la vida, no creas en la palabra imposible. Nada hay imposible para una voluntad enérgica"
Pío Baroja

Para el mejor compañero, Poker.

CONTENTS

SUMMARY



SUMMARY

Tick-borne diseases have a negative impact on human and animal health worldwide. Ticks are the most important vectors of pathogens that cause different diseases in humans, domestic and wild animals. Traditional tick control methods are primarily based on the use of chemical acaricides, which can result in the selection of resistant ticks, environmental pollution, and contamination of milk and meat products with residues affecting in particular the cattle industry.

Chapter I: General introduction

Content: Chapter I is a review paper [de la Fuente, J., and Contreras, M. (2015). Tick vaccines: current status and future directions. *Expert. Rev. Vaccines*. 14, 1367-1376] that constitute an introduction for this thesis by reviewing the importance of tick vaccines as an alternative environmentally sound approach for the control of tick infestations and pathogen infection and transmission. This chapter also describes critical issues, and recommendations for future directions towards developing improved and effective tick vaccines. The results with commercial vaccines for the control of cattle tick infestations support the efficacy of tick vaccines under field conditions. Future strategies include the vaccinomics pipeline developed by our group for the identification and characterization of tick protective antigens. Vaccinomics is a holistic approach based on the use of genome-scale or omics technologies integrated in a systems biology approach to characterize tick-host-pathogen interactions for the development of next-generation vaccines. In vaccinomics, the integration of "omics" technologies can improve the probability of identifying new candidate protective antigens, therefore advancing the development of effective vaccines. Furthermore, we propose that the combination of tick-derived and pathogen-derived protective antigens could result in more effective vaccines for the prevention and control of tick-borne diseases.

Chapter II: Vaccinomics for the identification and characterization of candidate protective antigens

Content: In chapter II, the intracellular bacterium, *Anaplasma phagocytophilum*, which causes human and animal granulocytic anaplasmosis, and its tick vectors, *Ixodes scapularis* and *I. ricinus* are used as models for the identification of tick-derived and pathogen-derived candidate protective antigens using a vaccinomics approach. The sequence, assembly and annotation of the *I. scapularis* genome were recently released, and transcriptomics studies in *I. ricinus* suggest that these tick species are genetically closely related [Genomic Resources Development Consortium, Contreras, M., de la Fuente, J., Estrada-Peña, A., Grubhoffer, L., and Tobes, R. (2014). Transcriptome sequence divergence between Lyme disease tick vectors, *Ixodes scapularis* and *Ixodes ricinus*. Genomic Resources Notes accepted 1 April 2014 – 31 May (2014). *Mol. Ecol. Resour.* 14: 1095]. The vaccinomics pipeline was developed based on quantitative transcriptomics and proteomics data from uninfected and *A. phagocytophilum*-infected *I. scapularis* nymphs, adult female midguts and salivary glands [Contreras, M., Villar, M., Alberdi, P., and de la Fuente, J. (2016). Vaccinomics approach to tick vaccine development. *Methods. Mol. Biol.* 1404, 275-286]. This approach resulted in the identification of the candidate tick protective antigens, lipocalin

(ISCW005600) and lectin pathway inhibitor (AAY66632) for the control of vector infestations and *A. phagocytophilum* infection [Contreras, M., Alberdi, P., Fernández de Mera, I.G., Krull, C., Nijhof, A., Villar, M., de la Fuente, J. (2017). Vaccinomics approach to the identification of candidate protective antigens for the control of vector infestations and pathogen infection. *Front. Cell. Infect. Microbiol*., 7:360. doi:10.3389/fcimb.2017.00360]. Additionally, the characterization of protein-protein interactions at the host-pathogen interface are characterized in order to discover and design new pathogen-derived protective antigens [Contreras, M., Alberdi, P., Mateos-Hernández, L., Fernández de Mera, I.G., García-Pérez, A.L., Vancová, M., et al. (2017). *Anaplasma phagocytophilum* MSP4 and HSP70 proteins are involved in interactions with host cells during pathogen infection. *Front. Cell. Infect. Microbiol*. 7: 307]. The results show that *A. phagocytophilum* MSP4-HSP70 are involved in interaction and infection of host cells, but were only partially protective against pathogen infection in sheep. However, these antigens may constitute candidate protective antigens for the development of vaccines against tick borne diseases when used in combination with other antigens.

Chapter III: Vaccination trails with tick protective antigens

Chapter III describes vaccination trials that were conducted to evaluate the protective capacity of tick protective antigens for the control of tick infestations. The efficacy of the Q38 chimera, containing Subolesin (SUB) and Akirin (AKR) conserved protective epitopes, was evaluated on *I. ricinus* and *Dermacentor reticulatus*. It was demonstrated that Q38 vaccination had an efficacy of 99.9% and 46.4% on the control of *I. ricinus* and *D. reticulatus* larvae, respectively [Contreras, M., de la Fuente, J. (2016). Control of *Ixodes ricinus* and *Dermacentor reticulatus* tick infestations in rabbits vaccinated with the Q38 Subolesin/Akirin chimera. Vaccine 34: 3010-3013]. In a second trial, vaccination with the *I. ricinus* aquaporin (IrAQP) and a tick aquaporin conserved region (CoAQP) on *I. ricinus* larvae infestations had an efficacy of 32% and 80%, respectively [Contreras, M., de la Fuente, J. (2017). Control of infestations by *Ixodes ricinus* tick larvae in rabbits vaccinated with aquaporin recombinant antigens. Vaccine 35: 1323-1328]. Finally, the characterization, composition and immunogenicity of the membrane-bound SUB-*Anaplasma marginale* MSP1a chimeric antigen is presented [Contreras, M., Moreno-Cid, J.A., Domingos, A., Canales, M., Díez-Delgado, I., Pérez de la Lastra, J.M., Sánchez, E., Merino, O., López Zavala, R., Ayllón, N., Boadella, M., Villar, M., Gortázar, C., de la Fuente, J. (2015). Bacterial membranes enhance the immunogenicity and protective capacity of the surface exposed tick Subolesin-*Anaplasma marginale* MSP1a chimeric antigen. Ticks and Tick-Borne Diseases 6: 820-828]. The SUB-MSP1a chimeric antigen was produced in *Escherichia coli* as a membrane-bound and exposed protein and used to protect vaccinated cattle against tick infestations. In this study, lipidomics and proteomics characterization showed the presence of components with potential adjuvant effect in the bacterial membrane, enhancing the immunogenicity of the SUB-MSP1a antigen in mouse and pig vaccination models. In addition, this system provides a simple and cost-effective approach, to produce tick protective chimera antigens on the *E. coli* membrane, with endotoxin levels within the limits acceptable for recombinant vaccine formulations.

Chapter IV: General discussion

Chapter IV is presented as a general discussion with an overview of the challenges to the control of tick-borne diseases through vaccine intervention, and also the current status of vaccines for the control of tick-borne diseases [de la Fuente, J., Contreras, M., Estrada-Peña, A., Cabezas-Cruz, A. (2017). Targeting a global health problem: Vaccine design and challenges for the control of tick-borne diseases. *Vaccine*, (35) 5089–5094]. These vaccines could constitute the safest and most effective intervention for the control of tick-borne diseases, inducing a long-lasting immunity, and reducing or even preventing tick infestation, pathogen infection and transmission in humans, domestic and wild animals.

CHAPTER I.
General introduction

de la Fuente, J. and Contreras, M. (2015). Tick vaccines: current status and future directions. *Expert Review of Vaccines* 14(10), 1367–1376.

Tick vaccines: current status and future directions

de la Fuente, J. and Contreras, M. (2015). Tick vaccines: current status and future directions. *Expert Review of Vaccines* 14(10), 1367–1376.

EXPERT REVIEWS

Tick vaccines: current status and future directions

Expert Rev. Vaccines 14(10), 1367–1376 (2015)

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Ticks and tick-borne diseases are a growing problem affecting human and animal health worldwide. Traditional control methods, based primarily on chemical acaricides, have proven not to be sustainable because of the selection of acaricide-resistant ticks. Tick vaccines appear to be a promising and effective alternative for control of tick infestations and pathogen transmission. The purpose of this review is to summarize previous tick vaccine development and performance and formulate critical issues and recommendations for future directions for the development of improved and effective tick vaccines. The development of effective screening platforms and algorithms using omics approaches focused on relevant biological processes will allow the discovery of new tick-protective antigens. Future vaccines will likely combine tick antigens with different protective mechanisms alone or pathogen-derived antigens. The application of tick vaccines as a part of integrated control strategies will ultimately result in the control of tick-borne diseases.

KEYWORDS: acaricide • control • tick • vaccine • vaccinomics

Ticks (Acari: Ixodida) are obligate hematophagous arthropod ectoparasites that are distributed worldwide and transmit pathogens causing diseases in humans and animals [1,2]. In the past decades, the continuous human exploitation of environmental resources and increase in human outdoor activities has allowed for the contact with ticks normally present in the field, resulting in increased transmission of tick-borne pathogens (TBP) [3,4]. In addition, tick populations are expanding due to changes in climate and human interventions that affect reservoir host movement and human contact with infected ticks [3–8]. As blood-sucking ectoparasites, ticks inflict great damage to humans, domestic and wild animals in many parts of the world. This damage consists of direct damage to hides, reduction in animal production, secondary infections and diseases caused by TBP [9,10]. Furthermore, despite efforts to implement measures to control tick infestations, ticks and the pathogens they transmit continue to be a serious problem to human and animal health [10–16].

Ticks are difficult to control because they have few natural enemies and traditional control methods, based on chemical acaricides, have been only partially successful [10,17]. Therefore, new strategies are needed for the control of ticks and TBP and tick vaccines appear to be a promising and sustainable approach toward this objective [10–15,18–22]. Recent reviews have focused on the efficacy and limitations of BM86-based vaccines and the discovery and characterization of new candidate tick-protective antigens for the development of vaccines for the control of tick infestations and pathogen infection and transmission [10–15,23–28]. The purpose of this review is to summarize previous tick vaccine development and performance literature and to formulate critical issues and recommendations for future directions for development of improved and effective tick vaccines for control of tick infestations and pathogen transmission.

Traditional tick control methods & associated problems

Traditional tick control methods are primarily based on the use of chemical acaricides, which have had limited efficacy in reducing tick infestations [10]. In addition, the use of acaricides is often accompanied by serious drawbacks, including the selection of acaricide-resistant ticks, environmental contamination and contamination of milk and meat products with residues [17]. The selection of ticks resistant to chemical acaricides is a growing problem, particularly affecting the cattle industry worldwide [29–31]. These facts together with the

high cost of developing new acaricides result in the lack of sustainability for continuous acaricide use for tick control [25].

Alternative control methods based on the use of botanical acaricides and repellents, entomopathogenic fungi and the education of farmers about recommended tick control practices and available options for the management of drug resistance have been proposed to reduce the effect of acaricide use on the selection of acaricide-resistant ticks [24,29–32]. Furthermore, integrated control programs that include habitat management and the genetic selection of hosts with higher resistance to ticks have been also proposed to reduce the use of acaricides for the control of tick infestations [33,34]. Nevertheless, based on the experience obtained with the commercial use of tick vaccines based on the *Rhipicephalus microplus* BM86 recombinant antigen for the control of cattle tick infestations, tick vaccines have been proposed as an effective component of the integrated programs for the control of tick infestations and TBP, while reducing the use of chemical acaricides [10–15,23–28].

Tick vaccines for the control of tick infestations

As proposed by Elvin and Kemp [35], candidate tick-protective antigens should fulfill certain important criteria such as: host antibodies should be able to gain access to the target protein in sufficient quantities; the formation of the antibody–antigen complex should disrupt the function of the target protein and/or induce physiological changes that affect vector biology; and the antigen should share conserved epitopes among several tick species to protect against multiple vector infestations. These criteria are still valid for the selection of candidate tick-protective antigens, considering that the vaccine should also reduce tick vector capacity for TBD [36].

The protective mechanism characterized so far for tick vaccines is based on the development of antigen-specific antibodies in immunized hosts that interact and affect the function of the targeted antigen in ticks feeding on immunized hosts [21,37]. As shown for BM86-based vaccines, tick vaccines reduce the number, weight and reproductive capacity of engorging female ticks, thus reducing tick infestations in subsequent generations [10].

Some tick species parasitize several vertebrate hosts and share habitat and hosts with other tick species [38]. These facts stress the need for developing vaccines effective in different hosts and against several tick species. However, a limited number of tick vaccines have been characterized so far in different hosts and cross-protective against multiple tick species [25,28,39–45].

Due to the importance of tick infestations for the cattle industry worldwide, most of the efforts toward the development of tick vaccines are directed for the control of tick species infecting cattle, particularly *R. microplus* [10–15,23–28] (Table 1). However, recent reports have addressed the effect of tick vaccines on alternative hosts such as sheep [45,46], camels [39], deer [40] and dogs [44].

Recent developments are directed toward the use of *R. microplus* BM86 homologs in other tick species infecting cattle [47–49]. In addition, new candidate tick protective antigens for the control of *R. microplus* infestations include Subolesin, Metalloprotease, Aquaporin, Ribosomal protein P0, Silk and Ferritins (Table 1). Furthermore, antigens protective against multiple tick species have been also characterized [11,27,28,42,43,45,50]. These results support the possibility of developing vaccines effective in different hosts and for the control of multiple tick species. However, new antigens and especially antigen combinations are required to develop more effective vaccines against tick infestations.

The efficacy of antigen combinations on tick infestations was first demonstrated by Allen and Humphreys [51] using tick protein extracts. However, until recently the combination of tick-protective antigens did not result in higher efficacy for the control of tick infestations [52,53]. Merino *et al.* [52] used a chimeric antigen composed of protective epitopes from tick Subolesin and mosquito Akirin with a higher efficacy when compared with tick Subolesin for the control of *R. microplus* infestations in cattle (Table 1). In the patent application by Schetters and Jansen [53], the inventors claim that the combination of the well-characterized tick protective antigens BM86 and Subolesin in a single formulation results in high vaccine efficacy against cattle tick infestations due to a synergy between both antigens (Table 1). The combination of tick-protective antigens is a promising direction to increase the efficacy of tick vaccines against multiple tick species. Other directions to improve tick vaccine efficacy include the use of novel formulations based on more effective adjuvant and antigen presentation and the possibility of developing vaccines with tick knock-down effects (i.e., substantial decrease of tick numbers on animals) as exhibited by chemical acaricides [10,23] and suggested by recent results with the BM86 + Subolesin combined antigen vaccine (Table 1) [53].

Tick vaccines for the control of pathogen infection & transmission

The ultimate goal of tick vaccines is the control of both ticks and TBD. Vaccination with tick-protective antigens such as BM86 among others that were directed toward control of tick infestations has also shown reduction in pathogen prevalence as a result of reducing tick populations [15,54]. Other antigens, such as Subolesin, show a direct effect on affecting pathogen infection and/or transmission while reducing tick infestations (Box 1) [28,41,43,55,54]. Furthermore, recent results have revealed the molecular interactions between ticks and transmitted pathogens with the identification of candidate tick antigens to reduce pathogen infection and transmission while also affecting tick infestations [52,56–66]. These results support the identification of tick-protective antigens with the dual function of reducing tick infestations and pathogen infection and transmission to ultimately protect against TBD. However, the combination of tick-derived and pathogen-derived antigens is probably the best way of achieving high vaccine efficacy for the control of vector-borne diseases.

Antigens from TBP such as *Borrelia burgdorferi* [67], flaviviruses [68], *Ehrlichia chaffeensis* [69], *Anaplasma phagocytophilum* [69–71] and *Anaplasma marginale* [72–78] among others have been proposed as candidate protective antigens for the control of pathogen infection and transmission. The possibility of

Table 1. Recently evaluated candidate tick protective antigens for the control of *R. microplus* infestations in cattle.

N[†]	Recombinant tick antigen	Vaccination conditions	Vaccine efficacy (%)[‡]	Ref.
3–4	Metalloprotease	Dose: 100 μg (doses 1 & 2), 200 μg (doses 3 & 4) Scheme: 4 doses Route: subcutaneous	60	[115]
4	Ribosomal protein P0	Dose: 250 μg Scheme: 4 doses Route: intramuscular	96	[116]
4	Ferritin 2	Dose: 100 μg Scheme: 3 doses Route: intramuscular	64	[102]
5–6	Aquaporin	Dose: 100 μg Scheme: 3 doses Route: intramuscular	68–75	[117]
4–6	Subolesin	Dose: 100 μg Scheme: 3 doses Route: intramuscular	37–44	[118]
3	Q38[§] Silk Subolesin	Dose: 100 μg Scheme: 3 doses Route: intramuscular	75 62 60	[52]
4	BM95-MSP1a Subolesin-MSP1a	Dose: 120 μg Scheme: 3 doses Route: intramuscular	64 81	[81]
5	BM86 BM86 + Subolesin	Dose: 100 μg Scheme: 3 doses Route: subcutaneous	79 97	[53]

[†]Number of animals per group.
[‡]Vaccine efficacy was calculated considering the effect on the reduction of tick infestations, oviposition and fertility (Box 2) but in certain experiments only the effect on some of these parameters was considered.
[§]Q38 is a tick Subolesin and mosquito Akirin chimera.

combining these pathogen-derived antigens with tick-protective antigens should result in new vaccines for the control of vector-borne diseases (Figure 1). In fact, recent results using vaccination with the combination of tick Subolesin with *A. marginale* major surface protein 1a (MSP1a) as a membrane-exposed chimeric antigen [79–81] showed an effect on reducing tick infestations and pathogen infection under field conditions [45].

Tick vaccines & the development of vaccines against other major ectoparasites

Diseases caused by arthropod-borne pathogens account for over 20% of all emerging infectious diseases recorded between 1940 and 2004 [82]. Among ectoparasite arthropod vectors, ticks are considered to be second worldwide to mosquitoes as vectors of human diseases and the most important vectors of diseases that affect the cattle industry worldwide [2,83]. However, other ectoparasites are also relevant for human and animal health and current research efforts are directed toward developing vaccines for their control [84]. In this context, research on tick vaccine development is more advanced than that reported for other major ectoparasites. Therefore, tick vaccine research may provide models for development of vaccines against other arthropod pests [20,83,85–88]. In this direction, recent efforts using tick Subolesin or the Akirin homolog in mosquitoes have shown how vaccination with these antigens protects against multiple ectoparasites and the infection with vector-borne pathogens [42,43,89] (Box 1). These results encourage the use of similar strategies for the identification of protective antigens across different ectoparasite species and suggest the possibility of developing vaccines for the control of multiple ectoparasite infestations.

Conclusions & future directions

The control of TBD is a priority in the current context of the global burden that infectious diseases represent and the one-health approach through integration of physicians, ecologists and veterinarians to monitor and control of zoonotic diseases. To address this priority, tick vaccines have become a major component of strategies for the control of tick infestations and TBP. In spite of the fact that vaccines are among the best achievements in science, past strategies for vaccine development need to be revised to increase possibilities for developing effective vaccines for the control of tick infestations and TBD. The identification of new tick-protective antigens is a critical step toward developing effective vaccines for the control of tick

Box 1. Subolesin: a challenging candidate tick protective antigen.

Tick Subolesin, the ortholog of insect and vertebrate Akirin, was discovered as a tick-protective antigen in *Ixodes scapularis* by expression library immunization in a mouse model of tick infestations [50].

Subolesin/Akirin constitute a recently renamed group of evolutionarily conserved proteins in arthropods and vertebrates [42,43]. Only one *subolesin/akirin* gene has been identified in ticks and insects, which is evolutionarily and functionally related to mammalian *akirin2* [42,43].

Tick Subolesin functions as a transcription factor required for NF-kB-dependent and NF-kB-independent gene expression and regulation of the innate immune response to pathogen infection [42,43,55]. The broad function of Subolesin as a transcription factor explains the profound effect of gene knockdown on tick physiology and reproduction [42,43] and as a protective antigen against infestation with multiple tick species and infection with TBP [41–43,54]. Vaccination with Subolesin/Akirin has shown an effect on the reduction of infestations by soft and hard ticks (*I. scapularis, I. ricinus, R. microplus, R. annulatus, R. sanguineus, Amblyomma americanum, Dermacentor variabilis, Ornithodoros erraticus. O. moubata*), mosquitoes (*Aedes albopictus*), poultry red mites (*Dermanyssus gallinae*), sand flies (*Phlebotomus perniciosus*) and sea lice (*Caligus rogercresseyi*) [42,43]. Recently, vaccination with the membrane-exposed Subolesin-MSP1a chimeric antigen resulted in the reduction of tick infestations and pathogen infection under filed conditions [45]. Furthermore, the combination of Subolesin with BM86 was recently patented as a new and more effective vaccine formulation for the control of cattle tick infestations [53].

Subolesin knockdown by RNA interference (RNAi) produces sterile female and male ticks [37,42,43]. Therefore, a sterile acarine technique for autocidal control of tick populations by release of *subolesin*-knockdown ticks was proposed and proven effective for the control of *R. microplus* in combination with Subolesin-based vaccination in cattle [37].

Vaccination with tick Subolesin reduces tick infection with *Anaplasma marginale*, *Anaplasma phagocytophilum*, *Babesia bigemina* and *Borrelia burgdorferi* [54] and mosquito infection with malaria parasite, *Plasmodium berghei* [89]. However, vaccination did not affect infection with tick-borne encephalitis virus [54]. Because of Subolesin's role in tick innate immune response to pathogen infection [42,43], targeting Subolesin by vaccination or RNAi reduces tick immunity, thereby increasing pathogen infection levels. However, lower pathogen infection levels result from the effect on tissue structure and function and the expression of genes that are important for pathogen infection and multiplication. Both direct and indirect effects of targeting Subolesin result in lower tick infestations, feeding and fertility [54]. These results challenge the paradigm that intracellular proteins are not capable of inducing a protective response against ectoparasite infestations [42]. Host antibodies may interact with arthropod intracellular proteins through a process that has not been fully characterized but results suggests that antibodies may be specifically transported across the midgut barrier into the hemolymph, and then enter into cells to interact with these intracellular proteins [42,54]. Nevertheless, other possibilities should be considered to explain the effect of the vaccination with Subolesin including the effect of a host cell-mediated immune response and antibody responses that are cross-reactive with other proteins [54,94,106].

infestations and TBP and in spite of resent advances in the study of tick biology and tick–host–pathogen interactions, this continues to be the major hurdle toward conducting vaccine animal trials [26]. In this direction, recent developments in last generation omics technologies, including reverse vaccinology and vaccinomics, will play a key role [26,52,59,61,73–75,90,91]. The integration of omics data sets must overcome important challenges such as development of algorithms that will allow for analysis and validation of data produced by the systems biology approach to tick research and development of effective screening platforms for the selection of candidate protective antigens [26]. Systems biology studies for the selection of candidate protective antigens should focus on the characterization of physiological processes such as suppression of host immune responses, blood digestion, embryogenesis, innate immunity and tick–pathogen interactions that are critical for tick feeding, reproduction and vector capacity [26,56,66,92–105].

Recently, Guerrero *et al.* [23] proposed the selection of tick antigens from unique or low copy number genes encoding membrane-associated or membrane-bound antigens that are expressed in gut, ovary and salivary gland tissues or in the saliva. In this regard, they proposed to select molecules with low redundancy and combining properties of 'exposed antigens' (antigens that are in contact with the host immune system during tick infestation and thus hosts immunized with these antigens are boosted by continuous tick exposure) and 'concealed antigens' (antigens that are not exposed to the host immune system and thus ticks are unlikely to have evolved mechanisms to effectively counteract the effect of the host immune system as had occurred with exposed antigens but requiring repeated immunizations to maintain elevated antibody titers) [11,24,106–108]. However, although these concepts are valid for the selection of candidate tick-protective antigens, recent results using tick Subolesin have challenged the *a priori* criteria for selecting membrane-exposed antigens (**Box 1**).

Along with the problems associated with the selection of candidate tick-protective antigens to reduce the need for animal trials, vaccination experiments require standardization to optimize results and make them comparable across different controlled pen and field trials (**Box 2**). In addition, the development of validated models for tick lifecycle under relevant field conditions will provide a valuable tool for the modeling of vaccine efficacy and impact of tick control [108].

In addition to tick vaccines, future directions for the control of tick infestations and vector-borne pathogens could also include tick autocidal control [37], transgenic or paratransgenic ticks resistant to pathogen infection as recently shown in mosquitoes [109], vertebrate hosts genetically modified to confer

Box 2. Tick vaccine trials: general considerations and guidelines.

Vaccination trials. To compare different vaccination trials, it is important to standardize reporting guidelines. Reports should describe among other factors:

- Animal race, sex, age, health status considering major diseases and body condition, previous exposure to ticks and TBP, and previous or ongoing treatments with vaccines, pharmaceuticals and acaricides.
- Tick species properly verified by independent taxonomists and/or molecular tools, developmental stage(s), origin (laboratory colony or field collected), infection with TBP.
- Antigen preparation (sequence, expression system, purification protocol and purity, adjuvant, formulation) and controls applied to the final vaccine preparation.
- Vaccination (schedule, dose and route) and tick challenge (number of ticks and infestation model).
- Monitoring, collection and processing of collected ticks, including approaches for determining tick weight, oviposition and egg fertility with the corresponding statistical analyses.

Tick vaccine efficacy (E). The current standard test for E against cattle ticks was established by Canales *et al*. [113] and recently updated by Aguirre Ade *et al*. [114]. E is calculated considering the effect on the reduction of tick infestations, oviposition and fertility as 100 [l-(CRTxCR0xCRF)], where CRT, CRO and CRF are the reduction in the number of adult female ticks, oviposition and egg fertility compared with the control group [113,114]. Despite the validity of this formula to calculate E, it may be important to make the calculation not only considering all parameters but also with the values showing significant differences between vaccinated and control animals (see e.g., [52]). In this way, the results will reduce the impact of animal-to-animal variations on E.
Correlation between vaccination and tick phenotype. After the vaccination trial, a positive correlation between reduction in tick infestations, weight, oviposition and/or fertility and antibody titers obtained in vaccinated animals will provide additional support to the result obtained with the vaccine (see e.g., [52]). Depending on the vaccine antigen and predicted protective mechanisms, additional analyses could be conducted using different immunological parameters and molecular tools to determine gene expression and protein content in vaccinated hosts and/or ticks.

resistance to tick infestation and/or pathogen infection as proposed using transgenic plants [110], glycoconjugate vaccines based on tick protein glycosylation [111] and the manipulation of the tick microbiota to reduce pathogen infection and transmission rates [112]. Finally, cocktails of tick-derived antigens alone or in combination with pathogen-derived antigens should result in more effective vaccines that could be used in combination with other control methods for the integrated control of tick infestations and TBD (Figure 1).

Expert commentary

In the future, TBD are expected to increase, thus having greater impact on human and animal health worldwide. Ticks are difficult to control because they have few natural enemies and traditional control methods based on chemical acaricides have been only partially successful with some implicit drawbacks such as the selection of ticks resistant to acaricides. New strategies are needed for the control of ticks and TBP and tick vaccines appear to be a promising and sustainable approach toward this objective. The use of BM86-based commercial vaccines for the control of cattle tick infestations demonstrated the possibilities for tick vaccines and encouraged research for the development of improved vaccines. Currently, various candidate tick protective antigens have been identified and tested in controlled pen trials. However, the identification of new tick-protective antigens is the critical step toward developing effective vaccines for the control of tick infestations and TBP.

The integration of last generation omics datasets is improving the possibilities for identifying candidate tick-protective antigens. However, this approach faces important challenges such as the development of algorithms that allow the analysis and validation of data produced by systems biology and effective screening platforms for the selection of candidate protective antigens. Nevertheless, focusing on the study of physiological processes that are critical for tick feeding, reproduction and vector capacity using a systems biology approach offer great possibilities for the identification of new tick-protective antigens for the development of improved vaccines for the control of tick infestations and pathogen infection and transmission.

Five-year view

In the coming years, TBD are expected to continue expansion, affecting human and animal health. As part of integrated control programs, tick vaccines are a promising and effective intervention for the control of tick infestations and the infection and transmission of TBP. Research on tick vaccines will continue to focus on cattle ticks and pathogens due to the impact of TBD on the cattle industry worldwide. However, due to the fact that some tick species parasitize several vertebrate hosts and share habitat and hosts with other tick species, the development of vaccines effective in different hosts and against several tick species is a growing area of research. In addition, TBD affecting humans, pets and other domestic and wild animals also encourage research into tick vaccines. The application of omics technologies to tick vaccine research will result in effective screening platforms and algorithms for the discovery of new tick-protective antigens. Vaccinomics and reverse vaccinology approaches will be used to identify and fully characterize candidate protective antigens and validate vaccine formulations. New candidate protective antigens will most likely be identified by

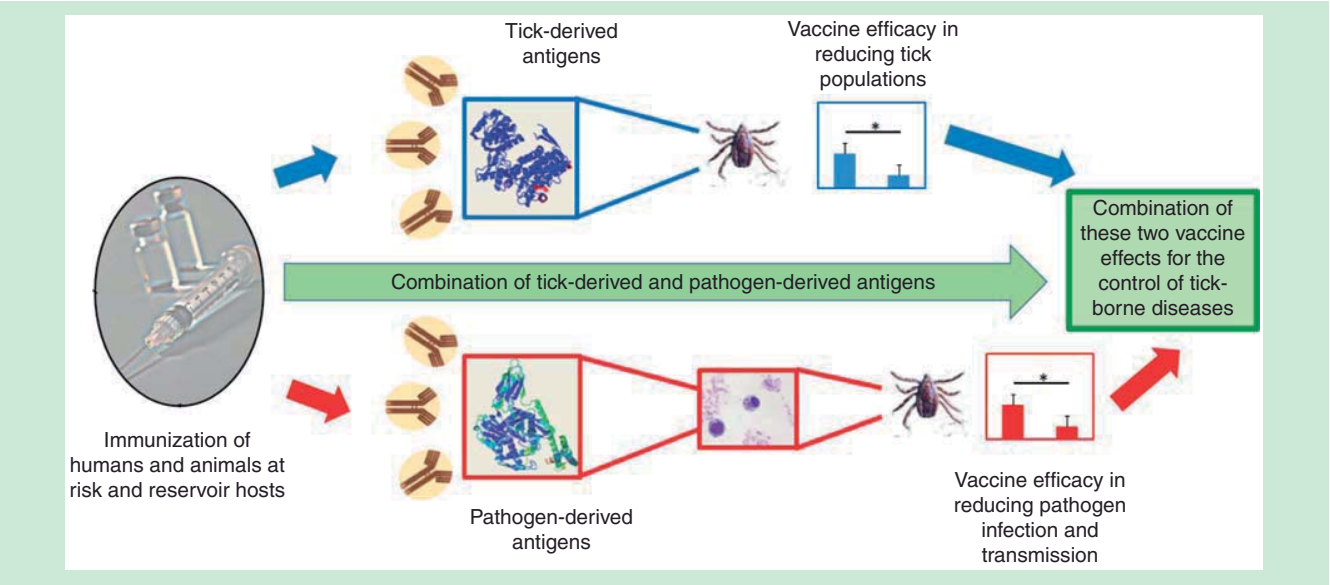

Figure 1. Future directions in tick vaccine development. New tick vaccines will likely combine tick antigens with different protective mechanisms alone or in combination with pathogen-derived antigens to ultimately result in the reduction of tick infestations while affecting pathogen infection and transmission to control TBD. A similar strategy could be applied to develop vaccines for the control of other vector-borne diseases.

focusing on abundant proteins with relevant biological function in tick feeding, reproduction, development, immune response, subversion of host immunity and pathogen infection and transmission. Consequently, tick-protective antigens will be discovered with multiple impacts when used in a vaccine including reductions in: tick infestations and fertility; tick pathogen infection; tick vector capacity for pathogen transmission; and tick response to pathogen infection. These new vaccines will likely combine tick antigens associated with different protective mechanisms alone or in combination with pathogen-derived antigens to have an effect on reducing tick infestations while affecting pathogen infection and transmission to ultimately result in the control of TBD. Finally, the most economical integrated tick control strategies will be those combining tick vaccines with other control methods while reducing acaricide applications to reduce risks for humans, animals and the environment. These integrated tick control strategies should overcome difficulties in the commercialization of tick vaccines due to its new approach for tick control.

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Key issues

- Ticks are obligate hematophagous arthropod ectoparasites that vector pathogens causing diseases in humans and animals.
- TBD are an increasing problem affecting human and animal health worldwide.
- Ticks are difficult to control and traditional control methods based primarily on chemical acaricides have been only partially successful.
- Tick vaccines appear to be a promising and sustainable approach for the control of tick infestations and pathogen transmission.
- Effective screening platforms and algorithms will be required for discovery of new tick protective antigens.
- Vaccinomics and reverse vaccinology approaches will be used to identify and fully characterize candidate protective antigens and validate vaccine formulations.
- Focusing on abundant proteins with relevant biological function will most likely identify new candidate tick-protective antigens.
- New tick vaccines will likely combine tick antigens with different protective mechanisms alone or pathogen-derived antigens.
- Integrated tick control strategies combining tick vaccines with other control methods should be developed.
- The application of tick vaccines will ultimately result in reducing tick infestations while affecting pathogen infection and transmission to control TBD.

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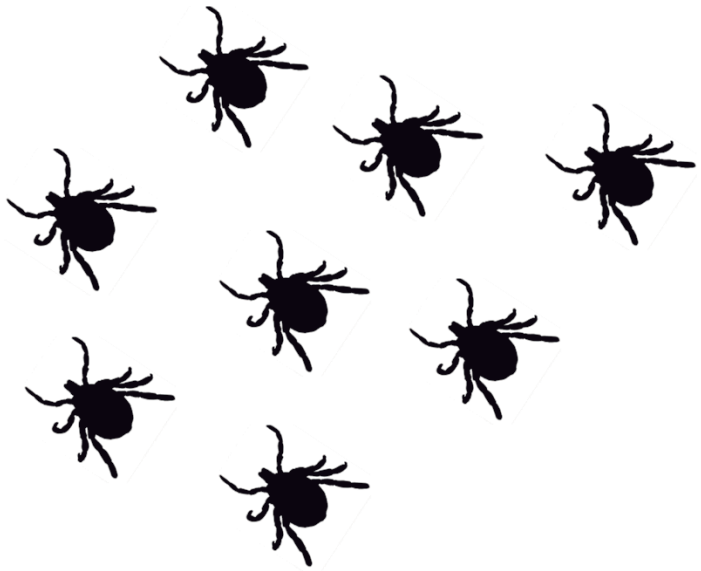
HYPOTHESIS AND OBJECTIVES



HYPOTHESIS

Proteins involved in tick-host-pathogen interactions could be used for vaccine development reducing tick infestations and tick-borne diseases.

OBJECTIVES

Main objective:

Study and characterization of tick-host-pathogen interactions for the identification of candidate antigens and vaccine development.

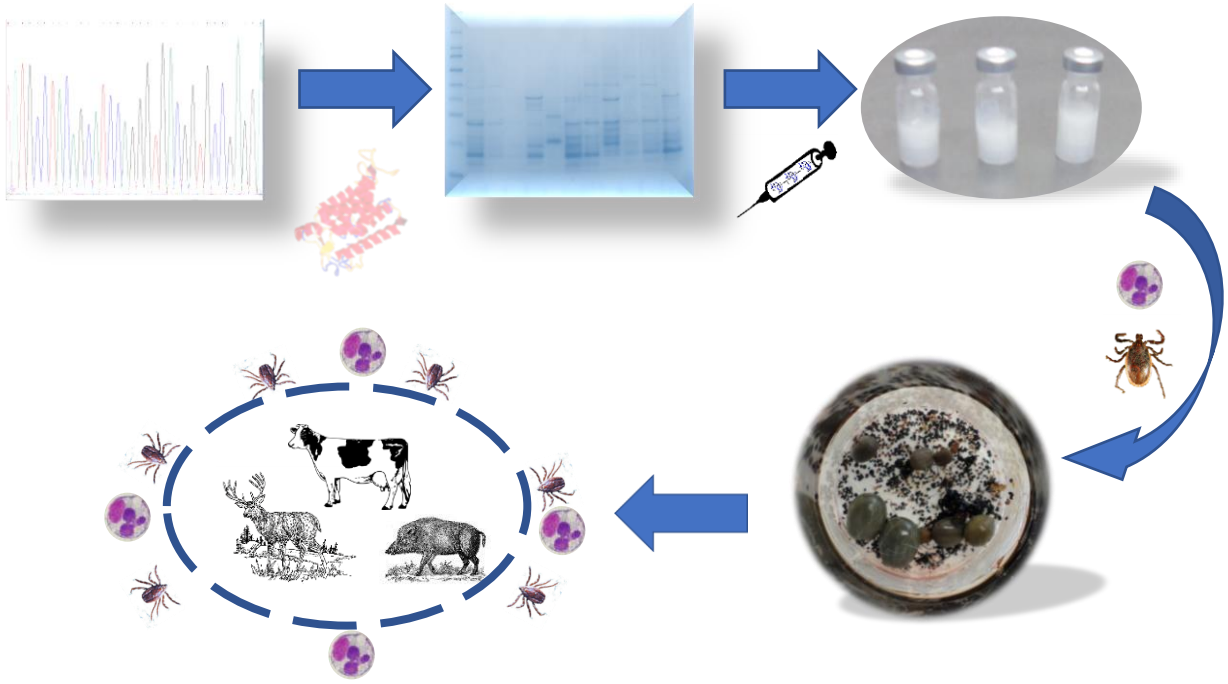
Specific objectives:

1. To develop and apply vaccine strategies for tick vaccine development.

2. To characterize proteins involve in host and tick pathogen interaction to study their potential protective capacity and mechanisms of protection.

3. To characterize the effect of recombinant tick antigens on control of tick infestations.

CHAPTER II
Vaccinomics for the identification and characterization of candidate protective antigens



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Vaccinomics Approach to Tick Vaccine Development

Marinela Contreras, Margarita Villar, Pilar Alberdi, and José de la Fuente

1 Introduction

Ticks are blood-feeding arthropod ectoparasites that transmit disease causing pathogens to humans and animals worldwide [1 – 3].

Tick–host–pathogen interactions have envolved trough dynamic processes involving genetic traits of hosts, pathogens and ticks that mediate their development and survival [2 – 4]. In the early 1990s, a cost-effective alternative for cattle tick (*Rhipicephalus microplus* and *R. annulatus*) control became commercially available with BM86-based tick antigen vaccines reducing the use of acaricides and the problems associated with them such as selection of acaricide- resistant ticks and the contamination of the environment and animal products with pesticide residues [5]. However, new vaccines are needed for effi cient control of vector infestations and pathogen infection and transmission [6]. Vaccinomics is based on the integration of "omics" technologies such as immunogenomics, transcriptomics, and proteomics with systems biology and bioinformatics for the development of next-generation vaccines [7]. As described here, the integration of various omics technologies towards discovering candidate tick protective antigens is important for development of next-generation tick vaccines. As a model we used the deer tick, *Ixodes scapularis* , and the transmitted patho-genic rickettsia, *Anaplasma phagocytophilum* , the causative agent of human, canine, and equine granulocytic anaplasmosis and tick- borne fever of ruminants.

2 Materials

All reagents used for buffer preparations need to be of analytical grade. The solutions are prepared with ultrapure water and stored at 4 ° C, except for the solutions containing SDS that are stored at 20 °C to avoid detergent precipitation.

Reagents for protein digestions and mass spectrometry analysis need to be of liquid chromatography–mass spectrometry (LC-MS) grade.

2.1 Tick Samples

Ticks are collected after feeding on vertebrate hosts, including both domestic and wild animals. After repletion, ticks are processed 1–3 h after collection. In some cases, ticks could be stored at −20 °C or in 70 % ethanol at 4 °C until processed (*see* **Note 1**).

I. scapularis ticks are obtained from laboratory colonies. Larvae and nymphs are fed on rabbits and adults are fed on sheep . Off- host ticks are maintained in a 12 h light: 12 h dark photoperiod at 22–25 °C and 95 % relative humidity. Ticks are infected with *A. phagocytophilum* by feeding on a sheep inoculated intravenously with *A. phagocytophilum* (human NY18 isolate)-infected HL-60 cells [8]. Ticks (N = 100–500) are removed from the sheep 7 days after infestation, held in the humidity chamber for 4 days and dissected for DNA, RNA and protein extraction from (nymphs) or midguts and salivary glands (adult females).

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Uninfected ticks are prepared in a similar way but feeding on an uninfected sheep. For analysis of biological replicates, two independent samples are collected and processed for each tick developmental stage and tissue. These experiments are conducted with the approval and supervision of the Institutional Animal Care and Use Committee.

2.2 Cultured Tick Cells and Anaplasma Phagocytophilum

The *I. scapularis* embryo- derived cell line ISE6 (provided by U. G. Munderloh, University of Minnesota, USA) is maintained in L-15B300 medium. The cells are cultured in sealed containers in ambient air at 31 ° C, medium is changed once a week. The *I. scapu-laris* ISE6 tick cells are inoculated with the *A. phagocytophilum* human isolate NY18 purifi ed from infected HL-60 cells. For purifi cation of *A. phagocytophilum* , infected cells (late infection, 90 % infected cells) are harvested by pipetting and centrifuged at 200 × *g* for 5 min at room temperature. The cell pellet is resuspended in complete L-15B300 medium and, using a syringe, the cell suspension is mechanically disrupted fi ve to ten times through a 26-gauge needle. After centrifugation at 1500 × *g* for 5 min, the supernatant is collected and used for inoculation of ISE6 cells. Uninfected cells are cultured in the same way, except with the addition of uninfected culture medium.

2.3 Reagents, Consumables, Kits, Equipment, and Software

These materials and their origin and use are described in the Subheading 3.

2.4 Buffers

1. Buffer for solubilization of proteins: 50 mM Tris–HCl pH 8.5, 4 % SDS, and 10 mM DTT. Mix 0.303 g of Tris, 2 g of SDS, and 0.077 g of DTT (dithiothreitol), add water to a volume of 25 ml, adjust to pH 8.5 with HCl, and bring up the volume to 50 ml with water.
2. UA buffer: 8 M urea in 0.1 M Tris–HCl (pH 8.5). Mix 24.024 g of urea and 0.606 g of Tris, add water to a volume of 25 ml, adjust to pH 8.5 with HCl, and bring up the volume to 50 ml with water.
3. 50 mM iodoacetamide in UA buffer: Add 0.009 g of iodoacet-amide to 1 ml of UA buffer and mix with vortex until complete solubilization. This buffer should be prepared fresh prior to digestion and stored in the dark.
4. 50 mM ammonium bicarbonate, pH 8.8: Add 0.04 g of ammo-nium bicarbonate to 9 ml of LC-MS grade water, mix and adjust pH to 8.8 with 5 N ammonium hydroxide. Complete to 10 ml with water to obtain a 50 mM fi nal solution.
5. 0.5 M sodium chloride: Add 2.922 g of sodium chloride to 75 ml of water, mix until complete solubilization, and bring up the volume to 100 ml with water.
6. 10 mM phosphate buffered saline (PBS), pH 7.4. Weigh 0.26 g KH_2PO_4, 2.17 g Na_2HPO_4-$7H_2O$, 8.71 g NaCl and add water to a volume of 1000 ml adjust to pH 7.4 with NaOH.
7. Lysis buffer. 50 mM potassium phosphate, pH 7.8, 400 mM NaCl, 100 mM KCl, 7 M urea, 10 mM imidazole. Prepare 1 M stock solutions KH_2PO_4 and K_2HPO_4. Mix 0.3 ml KH_2PO_4, 4.7 ml K_2HPO_4, 2.3 g NaCl, 0.75 g KCl, and 68 mg imidazole, adjust pH to 7.8 with HCl.
8. Elution buffer. 50 mM potassium phosphate, pH 7.8, 400 mM NaCl, 100 mM KCl, 7 M urea, 500 mM imidazole. Prepare 1 M stock solutions KH_2PO_4 and K_2HPO_4. Mix 0.3 ml KH_2PO_4, 4.7 ml K_2HPO_4, 2.3 g NaCl, 0.75 g KCl, and 3.4 g imidazole, adjust pH to 7.8 with HCl.

3 Methods

3.1 General Considerations

Different methodological approaches could be applied to the gen-eration of transcriptomics and proteomics data. However, these methodologies have been optimized for tick samples and are thus described here [9 – 11].

3.2 Extraction of Tick Samples

1. Dissect ticks in PBS and wash adult midguts and salivary glands in PBS after collection to remove hemolymph-related cells.
2. Extract total RNA, DNA, and proteins from uninfected and infected tick samples using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Valencia, CA, USA) and store them at−20 °C until used.

3.3 RNA Sequencing

1. Evaluate total RNA quality using the Agilent 2100 Bioanalyzer RNA Nano Chip (Agilent Technologies, Santa Clara, CA, USA).
2. Prepare samples for RNA sequencing using the TruSeq Stranded mRNA Sample Prep Kit (Illumina, San Diego, CA, USA) according to the manufacturer's protocol.
3. Perform size selection using a 2 % agarose gel to produce cDNA libraries ranging in size from 200 to 500 bp.
4. Enrich the libraries with 15 cycles of PCR and purify them using the QIAquick PCR purifi cation kit (Qiagen, Valencia, CA, USA).
5. Run the enriched libraries on one Illumina Hiseq 2000 lane using 100 bp sequencing (CD BioSciences, Shirley, NY, USA). In the case of paired-end reads, distinct adaptors from Illumina are ligated to each end with PCR primers that allow reading of each end as separate runs. The sequencing reaction is run for 100 cycles. For paired-end reads, data are collected as two sets of matched 100-bp reads.
6. Separate reads for each of the indexed samples using a custom Perl script. Image analysis and base calling are done using the Illumina GA Pipeline software.

3.4 Bioinformatics for the Analysis of RNA Sequencing Data

1. Use TopHat [12] that incorporates the Bowtie algorithm to perform the alignment [13] to align the sequencing reads to the *I. scapularis* reference genome (assembly JCVI_ISG_i3_1.0; http://www.ncbi.nlm.nih.gov/nuccore/NZ_ABJB000000000)(*see* **Note 2**).
2. Estimate the raw counts per gene by the Python script HTSeq count [http://www-huber.embl.de/users/anders/HTSeq/] using the reference genome.
3. Use the raw counts per gene to estimate differential expression at $P<0.05$ using DEGseq [14].

3.5 Proteomics

1. Dissolve the protein pellet obtained as described in Subheading 3.2 , **step 2** in buffer for solubilization of proteins, boil for 10 min, and centrifuge at 12,000 × *g* for 10 min at room temperature. Discard the pellet and quantify the protein content in the supernatant with the Direct Detect system (Millipore, Billerica, MA, USA).
2. Precipitate 150 μg of protein extract to be analyzed by adding four volumes of ice-cold acetone to one volume of sample. Vortex the mixture, incubate at −20 °C for at least 4 h, and centrifuge at 12,000 × *g* for 15 min at 4 ° C. Discard the super-natant and air-dry the pellet.
3. Digest proteins by the fi lter aided sample preparation (FASP) protocol (*see* **Note 3**). For that, dissolve the protein pellet in 200 μl of UA buffer and load onto 30 kDa centrifugal fi lter devices (FASP Protein Digestion Kit, Expedeon, TN, USA). Centrifuge at 14,000 × *g* for 30 min and discard the fl ow- through from the collection tube.
4. Alkylate proteins adding 100 μl of 50 mM iodoacetamide in UA buffer and incubate for 20 min in the dark, Remove the excess of alkylation reagents washing three times with 100 μl UA and three additional times with 100 μl of 50 mM ammo-nium bicarbonate, pH 8.8.

5. Add 75 μl of modifi ed trypsin (Promega, Madison, WI, USA) dissolved in 50 mM ammonium bicarbonate, pH 8.8 at 40:1 protein–trypsin (w/w) ratio. Incubate overnight at 37 °C for protein digestion wrapping the tops of the tubes with Parafi lm to minimize the effects from evaporation.
6. Elute the resulting peptides by centrifugation with 50 μl of 50 mM ammonium bicarbonate, pH 8.8 (twice) followed by 50 μl 0.5 M sodium chloride, centrifuging the Spin Filter at 14,000 × *g* for 10 min after each addition.
7. Add trifl uoroacetic acid (TFA) to a fi nal concentration of 1 %to stop the digestion and desalt the peptides using C18 Oasis-HLB cartridges (Waters, Milford, MA, USA) following the manufacturer instructions. Vacuum-dry and store at −20 °C until the mass spectrometry analysis.
8. For stable isobaric labeling, dissolve the resulting tryptic peptides in triethylammonium bicarbonate (TEAB) buffer (Sigma-Aldrich) and label using the 4-plex iTRAQ Reagents Multiplex Kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's protocol (*see* **Note 4**). After labeling, combine the samples to be analyzed and desalt as described in **step 7** .
9. Resuspend the sample in 0.1 % formic acid, load into the LC-MS/MS system for on-line desalting onto C18 cartridges, and analyze by RP-LC-MS/MS using a C-18 reversed phase nano-column (75 μm I.D. × 50 cm, 3 μm particle size, Acclaim PepMap 100 C18; Thermo Fisher Scientifi c, Waltham, MA, USA) in a continuous acetonitrile gradient consisting of 0–30 % B in 145 min, 30–43 % B in 5 min and 43–90 % B in 1 min (solvent A = 0.5 % formic acid; solvent B = 90 % acetoni-trile, 0.5 % formic acid). A fl ow rate of ca. 300 nl/min is used to elute peptides from the reverse phase nano-

3.6 Bioinformatics for the Analysis of Proteomics Data

Peptide identifi cation from raw data is carried out using the SEQUEST algorithm (Proteome Discoverer 1.4, Thermo Scientifi c).

1. Database search is performed against a compiled database containing all sequences from Ixodida (77,177 Uniprot entries in February 2015) and Anaplasmataceae (64,633 entries in February, 2015)(http://www.uniprot.org) for identification of tick and pathogen proteins.
2. The following constraints may be used for the searches: tryptic cleavage after Arg and Lys, up to two missed cleavage sites, and tolerances of 600 ppm for precursor ions and 1200 mmu for MS/MS fragment ions and the searches were performed allowing optional Met oxidation and Cys carbamidomethyl-ation. For iTRAQ labeled peptides, N-terminal and Lys iTRAQ modifi cation is added as a fi xed modifi cation.
3. Peptide identification is validated using the probability ratio method [15] and false discovery rate (FDR) was calculated using inverted databases and the refi ned method [16] with an additional fi ltering for precursor mass tolerance of 12 ppm. Only peptides with a confi dence of at least 95 % were used to quantify the relative abundance of each peptide.
4. Protein quantification from reporter ion intensities and statistical analysis of quantitative data is performed using QuiXoT [17] .
5. Significant protein-abundance changes to estimate differential expression is obtained from the z values (standardized variable used by the method that expresses the quantitative values in units of standard deviation) at $P < 0.05$, using two replicates per sample.

3.7 Selection of Candidate Tick Protective Antigens

New candidate protective antigens will most likely be identifi ed by focusing on abundant proteins with relevant biological function in tick feeding, reproduction, development, immune response , subversion of host immunity, and pathogen transmission. Tick antigens studied thus far have demonstrated multiple impacts when used in a vaccine including reductions in (a) tick infestations and fertility, (b) tick pathogen infection, (c) tick vector capacity for pathogen transmission, and (d) tick response to pathogen infection. Consequently, several criteria could be used for the selection of candidate tick protective antigens.

1. Select tick gene/protein fulfi lling at least two of the following criteria (Fig. 1):
   (a) Highly differentially regulated gene in at least two samples. (b) Highly differentially represented protein.
   (c) Genes/proteins with a relevant putative biological function in tick–pathogen interactions.
   (d) Secreted or membrane-exposed protein.

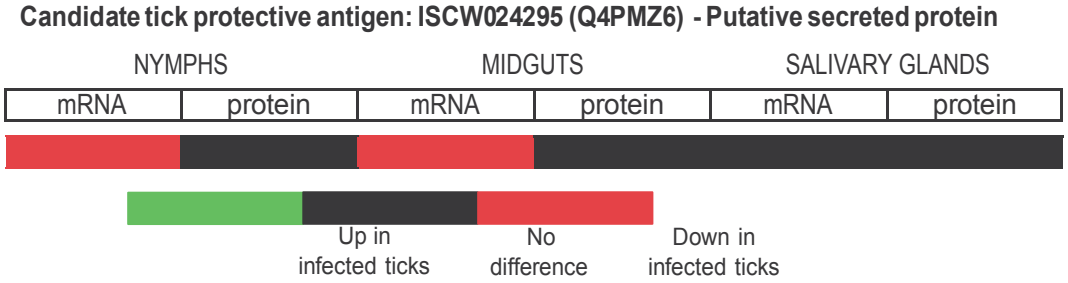

Fig. 1 Selection of candidate tick-protective antigens. Example of a candidate tick-protective antigen selected after transcriptomics and proteomics analysis of *I. scapularis–A. phagocytophilum* interactions and fulfilling the selection criteria of being highly differentially regulated gene in at least two samples and secreted or membrane-exposed protein

3.8 Screening of Candidate Tick-Protective Antigens

The screening of candidate tick- protective antigens is one of the limiting steps in the vaccinomics pipeline [7]. Several methodologies have been used to reduce the number of candidate tick- protective antigens that could be tested in vaccination trials [7]

These methodologies include, but are not limited to, RNA interference (RNAi) in cultured tick cells and ticks , DNA and protein immunization, in vitro tick feeding with anti-tick protein antibodies, and incubation of tick cells with anti-tick protein antibodies (Fig. 2).

A combination of some of these methodologies may become the most effective platform for the screening and characterization of candidate tick-protective antigens.

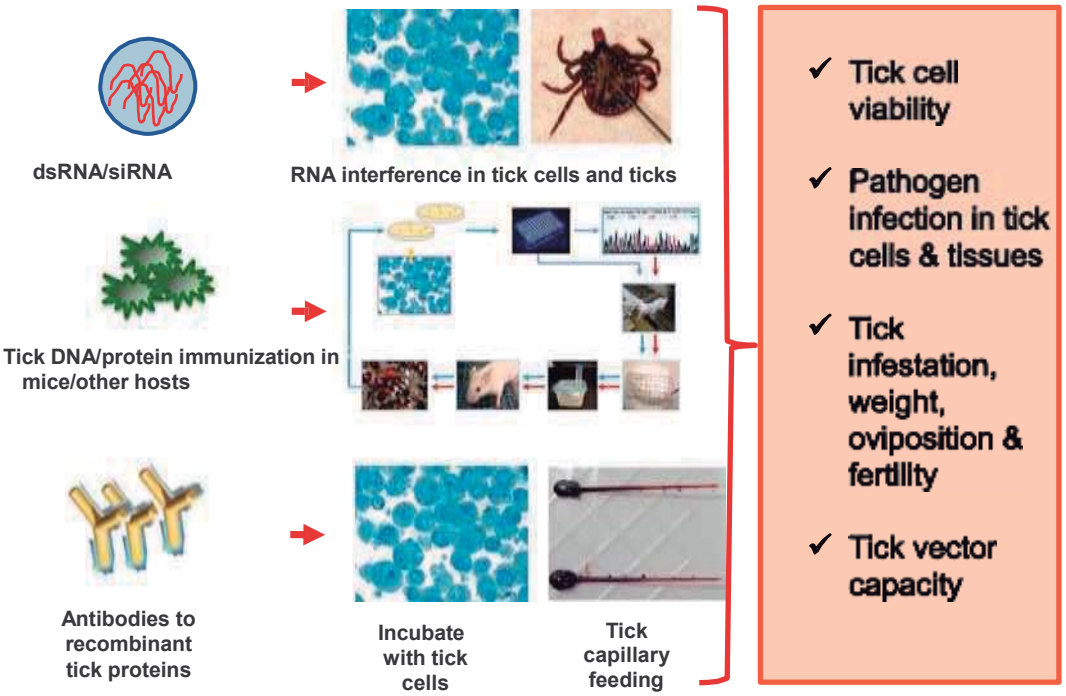

Fig. 2 Screening for candidate tick-protective antigens. Representation of different methodologies used for the screenings and characterization of tick-protective antigens

Table 1
Oligonucleotide primers used for dsRNA synthesis, real-time PCR, and RT-PCR

Gene	GenBank accession no.	Forward and reverse primers (5′–3′)	PCR conditions
Ap 16S rRNA	CP006617	CAGAGTTTGATCCTGGCTCAGAACG GAGTTTGCCGGGACTTCTTCTGTA	55 °C/30 s
Is 16S rDNA	ABJB010000000	GACAAGAAGACCCTA ATCCAACATCGAGGT	55 °C/30 s
Ap msp4	JQ522935	ATGAATTACAGAGAATTGCTTGTAGG TTAATTGAAAGCAAATCTTGCTCCTATG	60 °C/30 s
Is rps4	DQ066214	GGTGAAGAAGATTGTCAAGCAGAG TGAAGCCAGCAGGGTAGTTTG	60 °C/30 s
Is cyclophilin	ISCW008497	GCTTCGGTTACAAGGGCAGCAGCATTT TCGGGTGTGCTTCAGGATGAAGTT	60 °C/30 s
Is secreted protein	ISCW024295	CACCATGCCGAAACAAGGCGAAAC TCCAGAGTCACCACACAAAACG	60 °C/30 s

Ap A. phagocytophilum, *Is I. scapularis*

3.8.1 Productionof TickRecombinant Proteins in Escherichia coli

1. Amplify the coding sequence for the *I. scapularis* protein (GenBank accession no. ISCW024295) by RT-PCR using total RNA from *I. scapularis* with specifi c primers and amplifi -cation conditions (Table 1).
2. Purify and quantify PCR products using the GenClean III kit (MP Biomedicals, Solon, OH, USA).
3. Clone into the expression vector pET101/D-TOPO (Invitrogen, Carlsbad, CA, USA) and transform into *E. coli* strain BL21 (Invitrogen, Carlsbad, CA, USA). Inoculate cells into Luria–Bertani (LB) broth containing 50 μg/ml ampicillin and 0.5 % glucose. Grow cultures at 37 °C to an OD600 nm = 0.8 and add Isopropyl-β- d -thiogalactopyranoside (IPTG) to a fi nal concentration of 0.5 mM, then incubate for 4 h to induce the production of recombinant proteins.
4. Harvest the bacteria and lyse in lysis buffer, containing prote-ase inhibitors (Roche, San Cugat del Vallés, Barcelona, Spain).
5. Disrupt *E. coli* cells with a cell sonicator (Model MS73; Bandelin Sonopuls, Berlin, Germany). Sonicate for 10 min at 20 kHz; fi x the acoustic power to 70 kW.
6. Separate insoluble protein fraction containing the recombinant protein as inclusion bodies by centrifugation at 15,000 × *g* for 15 min at 4 °C and fi lter the supernatant (0.22 μm, Millipore, Billerica, MA, USA).
7. Purify the lysate by Ni affi nity chromatography using 1 ml HisTrap FF columns mounted on AKTA–FPLC system (GE Healthcare, Piscataway, NJ, USA) and elute using elution buffer.
8. Refold the proteins by dialysis against 100 volumes of 10 mM PBS for 12 h at 4 °C.
9. Determine protein concentration using bicinchoninic acid (Pierce™ BCA Protein Assay Kit, Thermo Scientifi c, Rockford, IL, USA).
10. Analyze purified proteins by SDS- PAGE according to standard procedures.

3.8.2 Rabbit Immunization with Tick Recombinant Proteins

1. Mix the recombinant proteins in PBS with anhydromannitole-theroctodecenoate (Montanide ISA 50 V; Seppic, Paris, France) 1:1 batch-by-batch processes using two syringes connected to a T-connector (Braun Discofi x-3, B. Braun Melsungen AG, Germany) to a fi nal protein concentration of 250 μg/ml, then fi ll manually 2 ml glass bottles (Wheaton, Millville, NJ, USA) under sterile conditions.
2. Inject two rabbits/group subcutaneously with three doses (weeks 0, 3, and 6) containing 50 μg/0.2 ml dose of purifi ed recombinant protein formulated as described above.
3. Collect blood samples from each rabbit before injection and 2 weeks after the last immunization to prepare preimmune and immune sera, respectively.
4. Purify IgGs from serum samples using the Montage Antibody purification kit and spin columns with PROSEP-A Media (Millipore, MA, USA) following the manufacturer's recommendations.

3.8.3 Analysis of Tick Cell Viability after Incubation with Anti-tick Protein Antibodies

1. Seed ISE6 tick cells 24 h before the assay at a density of approximately 5–7 × 10^5 cells/well, use three replicates per treatment.
2. Include control wells with (a) inoculum incubated with pre- immune IgG, (b) inoculum incubated with medium only, and (c) uninfected tick cells.
3. Purify *A. phagocytophilum* as previously described in Subheading 2.2.

4. Mix rabbit IgGs at a concentration of 2.2–2.4 mg/ml with the semi-purifi ed bacterial inoculum (1:1) from **step 3.8.2** for 60 min at room temperature.

5. Add 100 µl of the inoculum plus IgG mix to each well andincubate at 34 °C for 30 min.

6. Remove the inoculum-IgG mix and wash the cells three times with PBS.

7. Add 1 ml complete medium to each well and incubate at 34 °C for 7 days.

8. Harvest cells, resuspend in PBS, and proceed to determine the effect of anti-tick protein antibodies on tick cell viability using the Apoptosis Detection kit (Immunostep, Salamanca, Spain).

9. Wash cells harvested in **previous step** twice with PBS, resus-pend in 100µl of 1× Annexin V-binding buffer.

10. Incubate cells simultaneously with 5 µl Annexin V (FITC labeled) and 5 µl of the non-vital dye propidium iodide (PI) for 15 min at room temperature in the dark.

11. Add 400 µl of 1× Annexin binding buffer and analyze by fl ow cytometry withi n 1 h.

12. Gate the viable cell population according to forward-scatter and side-scatter parameters.

13. Determine the percentage of live, apoptotic, necrotic, and dead cells by FACS. Intact cells will be Annexin V-FITC negative, PI negative, whereas early apoptotic cells will appear as Annexin V-FITC positive, PI negative. Necrotic cells will be positive for both while dead cells will be Annexin V-FITC negative, PI positive.

3.8.4 RNAi in Cultured Tick Cells

1. Synthesize siRNAs homologous to *I. scapularis* genes encoding for candidate tick-protective antigens using GE Healthcare (Piscataway, NJ, USA) http://dharmacon.gelifesciences.com/. An unrelated gene (i.e., *Rs86* ; ref. [11] is used as negative control.

2. Conduct RNAi experiments for gene knockdown in cell cul-tures by incubating tick cells with 10 µl dsRNA (5×10^{10} to 5×10^{11} mol/µl) and 90 µl L-15B medium in 24-well plates, using four wells per treatment. Control cells are incubated with the unrelated *Rs86* dsRNA.

3. After 48 h of dsRNA exposure, tick cells are infected with cell- free *A. phagocytophilum* human NY18 isolate or mock infected by adding culture medium alone.

4. Incubate tick cells for 72 h and collect for DNA and RNA extraction using TriReagent (Sigma, St. Louis, MO, USA) fol-lowing manufacturer's recommendations.

5. Determine gene knockdown by real-time RT-PCR with gene specifi c primers (Table 1) using the iScript One-Step RT-PCR Kit with SYBR Green and the iQ5 thermal cycler (Bio-Rad, Hercules, CA, USA) following manufacturer's recommenda-tions. A dissociation curve is run at the end of the reaction to ensure that only one amplicon is formed and that the ampli-cons denatured consistently in the same temperature range for every sample. The mRNA levels are normalized against *ribo-somal protein S4* (*rps4*) and *cyclophilin* using the genNorm method (ddCT method as implemented by Bio-Rad iQ5 Standard Edition, Version 2.0). The results are compared between samples by Student's *t* -test with unequal variance ($P = 0.05$; $N = 3$).

6. Determine tick cell viability as described above.

7. Determine *A. phagocytophilum* DNA levels by major surface protein 4 (*msp4*) real-time PCR normalizing against tick *16S rDNA* (Table 1) with PCR conditions of 5 min at 95 °C and 35 cycles of 15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C.

4 Notes

1. When working with ticks collected in nature and on vaccine trials under field conditions, collected ticks are generally stored in ethanol and not deep-frozen because it is easier under fi eld conditions and makes their shipment from one lab to another cheaper. Processing of these samples has been previously described [9]. However, samples could also be processed 1–3 h after collection when working on experimental tick infesta-tions, which are the main approach used in vaccinomics .

2. The only genome available for ticks is that of *I. scapularis* , which allows aligning reads to this reference genome. However, for the other ticks species, de novo sequencing and assembly of mRNA is needed following a different pipeline [10 , 18].

3. There are other digestion methods that could be also used but the Filter-Aided Sample Prep (FASP) is a technology that allows a complete protein solubilization and complete trypsin digestion in a fast way from any biological material. The result-ing fi ltrate is free of detergents, large molecules, and other sub-stances that would interfere with mass spectrometry analysis of proteomes [19].

4. iTRAQ labeling is a robust approach to absolute quantifi cation of complex proteomes, but there are also other proteomics approaches that could be used depending on the necessities of the research (*see* Ref. [20], for a recent revision of quantitative proteomics in the field of microbiology).

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GENOMIC RESOURCES NOTE

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Abstract

This article documents the public availability of a global transcriptome comparison between Lyme disease tick vectors, *Ixodes scapularis* and *Ixodes ricinus*.

Table 1 contains information on the focal species, data type and resource developed, as well as access details for the data. The authors responsible for this genomic resource are listed in the final column. Full descriptions of how each resource was developed and tested are uploaded as Supplemental Information with the online version of this manuscript.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Full descriptions of how each resource was developed and tested.

Table 1 Information on the focal species, data type and resource developed, as well as access details for the data. The authors responsible for each genomic resource are listed in the final column

Species (no. of individuals)	Data type	Resources	Authors
Ixodes ricinus (30 adult female ticks, 300 larvae)	Transcriptome sequences	*Sequence files*: FASTQ files with reads from *I. ricinus* larvae samples and adult female salivary gland samples available from DRYAD entry doi: 10.5061/dryad.9js92; BAM files containing the Tophat-accepted hits of the *I. ricinus* larvae samples and adult female salivary gland samples available from DRYAD entry doi: 10.5061/dryad.9js92; BAI file corresponding to the BAM file of the *I. ricinus* larvae samples and adult female salivary gland samples Tophat-accepted hits available from DRYAD entry doi: 10.5061/dryad.9js92 *Code*: Commands to map the reads to the reference genome, *I. ricinus* larvae samples (IL-tophat-run) and adult female salivary gland samples (IG-tophat-run) available from DRYAD entry doi: 10.5061/dryad.9js92	Contreras, Marinela; Tobes, Raquel; Grubhoffer, Libor; Estrada-Peña, Agustín; de la Fuente, José

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Title

Transcriptome sequence divergence between Lyme disease tick vectors, *Ixodes scapularis* and *Ixodes ricinus*

Authors

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Keywords:Genomics, transcriptomics, Ixodes, tick, vector, RNAseq

Running title : Transcriptome identity between tick species

Introduction

Tick-borne diseases affect human and animal health worldwide. *Ixodes* spp. Infest humans, pets and other domestic and wild animals and transmit disease-causing pathogens such as *Borrelia* spp., tick-borne encephalitis virus and *Anaplasma phagocytophilum* (de la Fuente *et al.* 2008; Beugnet & Marie 2009). For example, *Borrelia burgdorferi* s.l. is transmitted by *Ixodes scapularis* in the U.S. and *I. ricinus* in Europe causing Lyme disease, the most prevalent vector-borne disease in the U.S. with increasing number of cases also in Europe (Sprong *et al.* 2014).

Recent developments in both ticks and tick-borne pathogen genomics and the application of systems biology to the study of tick-host-pathogen interactions have advanced our understanding of the genetic factors and molecular pathways involved in pathogen infection and transmission, which could be used for the development of new control strategies for tick-borne diseases (de la Fuente & Merino 2013). However, despite the global importance of these blood-feeding arthropod vectors, the *I. scapularis* genome is the only assembled tick genome (GenBank accession ABJB010000000). This is a serious limitation for tick research and the development of new interventions for the control of tick-borne diseases.

Due to difficulties in the sequencing and assembling of tick genomes (Pagel Van Zee *et al.* 2007; Nene 2009) including the interference produced by abundant endocytobionts (Carpi *et al.* 2011), genomics resources could help in providing confidence for the analysis of transcriptomics and proteomics data, particularly when comparing closely related species such as *I. scapularis* and *I. ricinus* (Gibson *et al.* 2013). The analysis of *I. ricinus* de novo sequenced transcriptome suggested high sequence identity with *I. scapularis* (Schwarz *et al.* 2013). Further analysis of 100 randomly selected *I. ricinus* sequences deposited in the GenBank evidenced 82-100% (97.49 ±} 3.76) sequence identity to *I. scapularis* orthologs. Furthermore, for some genes such as those encoding for TROSPA (113 *I. ricinus* sequences analyzed) a 99-100% identity to *I. scapularis* sequences was obtained. Recently, Quillery *et al.* (2014) identified 312,088 putative SNPs in the *I. ricinus* genome with 96% confirmation for selected SNPs, which suggest a low genetic variability for an organism with a 2.31 Gbp genome (Geraci *et al.* 2007). These results suggested that sequence divergence between these two closely related tick species might be due to species, strain or individual specific differences (Quillery *et al.* 2014). In addition, discrepancies in the classification of *I. ricinus* (Meeus *et al.* 2002; Kempf *et al.* 2011) and problems associated with DNA barcoding used for tick identification (Zhang *et al.* 2014) may result in sequence divergence. Therefore, a global transcriptome comparison between *I. scapularis* and *I. ricinus* could contribute to further understand sequence similarity between these two tick species.

Data Access

- *I. scapularis* reference genome sequence assembly JCVI_ISG_i3_1.0 – NCBI reference sequence NZ_ABJB000000000
- (http://www.ncbi.nlm.nih.gov/nuccore/NZ_ABJB000000000).
- BAI file corresponding to the BAM file of the I. ricinus larvae samples Tophat accepted hits – dryad entry doi: 10.5061/dryad.9js92/3
- BAM file containing the Tophat accepted hits of the I. ricinus larvae samples – dryad entry doi: 10.5061/dryad.9js92/6BAI file corresponding to the BAM file of the I. ricinus adult female salivary gland samples Tophat accepted hits – dryad entry doi: 10.5061/dryad.9js92/4

• BAM file containing the Tophat accepted hits of the I. ricinus adult female salivary gland samples – dryad entry doi: 10.5061/dryad.9js92/5
• FASTQ file with reads from I. ricinus larvae samples – dryad entry doi: 10.5061/dryad.9js92/1
• FASTQ file with reads from I. ricinus adult female salivary gland samples – dryad entry doi: 10.5061/dryad.9js92/2
• IG-tophat-run: Commands to map the reads to the reference genome, I. ricinus adult female salivary gland samples – dryad entry doi: 10.5061/dryad.9js92/7
• IL-tophat-run: Commands to map the reads to the reference genome, I. ricinus larvae samples – dryad entry doi: 10.5061/dryad.9js92/8

Meta Information
• Sequencing center - Next Generation Sequencing/Secuenciacion Masiva, Unidad de Genomica Cantoblanco, Fundacion Parque Cientifico de Madrid, Madrid, Spain.
• Platform and model – HiSeq2000/GAIIx (Illumina, San Diego, CA, USA).
• Design Description – The goal of this study was to characterize the transcriptome sequence identity between Lyme disease tick vectors, *.0* and *Ixodes ricinus*. In this study, without the need for reporting specific transcripts identified in *I. ricinus* because de novo assembly or comparative quantitative transcriptomics were not conducted, it was possible to compare transcriptome sequence identity between *I. ricinus* and *I. scapularis*. These results are relevant for the characterization of the source of coding sequence variability and analysis of transcriptomics and proteomics data, particularly when comparing closely related species such as *I. scapularis* and *I. ricinus*. The transcriptome of *I. ricinus* adult female ticks (sample 1) and larvae (sample 2) were characterized by RNAseq and mapped to *I. scapularis* reference genome sequence (assembly JCVI_ISG_i3_1.0; http://www.ncbi.nlm.nih.gov/nuccore/NZ_ABJB000000000).
• Analysis type – RNA sequencing.
• Run date – 26 June 2012.

Library
• Strategy - RNAseq using the TruSeq RNA sample preparation and sequencing of 1x75 bp single-end reads with HiSeq2000/GAIIx (Illumina, San Diego, CA, USA).
• Taxon - *Ixodes ricinus* (Linnaeus, 1758), the castor bean ticks (Acari: Ixodidae) were obtained from the reference laboratory colony maintained at the tick rearing facility of the Institute of Parasitology of the Biology Centre of the Academy of Sciences of the Czech Republic under the supervision of Mr. Jan Erhard, an acarologist-practitioner highly experienced in tick morphology and bionomy. Individuals in the laboratory colony of *I. ricinus* correspond with prototype individuals of the *I. ricinus* tick species deposited in the collection of parasitic organisms at the Institute of Parasitology of the Biology Centre of the Academy of Sciences of the Czech Republic under code IPCAS 2036 (http://www.paru.cas.cz/en/collections/)
• Sex – Unfed larvae and adult females.
• Location - The colony originated and is strengthened/supplied with individuals collected in a selected locality named "U Hada" near to the City of Ceske Budejovice, and checked to be free of tick-borne encephalitis virus, spirochetes of Lyme diseases and *Babesia* spp.
• Tissue – Whole internal tissues and salivary glands from larvae and female ticks, respectively.
• Sample handling – All ticks were washed with a series of solutions composed of tap water, 3% hydrogen peroxide, two washes of distilled water, 70% ethanol and two more washes with distilled water prior to RNA extraction (Kocan *et al.* 2010). Total RNA was

extracted from whole internal tissues and dissected salivary glands from larvae and female ticks, respectively using Tri Reagent (Sigma-Aldrich, St. Louis, MO, USA) according to manufacturer instructions.
RNA was purified with the RNeasy MinElute Cleanup Kit (Qiagen, Valencia, CA, USA) and characterized using the Agilent 2100 Bioanalyzer (Santa Clara, CA, USA) in order to evaluate the quality and integrity of RNA preparations. RNA concentration was determined using the Nanodrop ND-1000 (NanoDrop Technologies Wilmington, Delaware USA).
• Selection – poly-A-capture and subsequent PCR amplification.
• Layout – Illumina 1x75 bp single-end reads.
• Library construction protocol - Purified RNAs were used for library preparation using the TruSeq RNA sample preparation kit v.1 and the standard low throughput procedure (Illumina, San Diego, CA, USA) (Villar et al. 2014).
Briefly, 1 μg total RNA was used as starting material for library preparation. Messenger RNA was captured using poly-dT magnetic beads and purified polyA+ RNA was chemically fragmented and reverse-transcribed. Remaining RNA was enzymatically removed and the second strand generated following an end repair
procedure and preparation of double-stranded cDNA for adaptor ligation. Adaptor oligonucleotides containing the signals for subsequent amplification and sequencing were ligated to both ends and cDNA samples were washed using
AMPure SPRI-based magnetic beads (Beckman Coulter, IZASA, Barcelona,
Spain). Adapters contained identifiers, which allow multiplexing in the
sequencing run. An enrichment procedure based on PCR was then performed to ensure that all molecules in the library conserved the adapters at both ends. The number of PCR cycles was adjusted to 10 for both samples. The final amplified library was checked again on a BioAnalyzer 2100 (Agilent, Santa Clara, CA, USA). For RNAseq, libraries were denatured and seeded on the respective lanes of the in single-nucleotide flowcells at a final concentration after re-naturalization of 10-14 pM.

Processing
• Pipeline - The 1x75 bp single-end reads were mapped to the *I. scapularis* reference genome sequence (assembly JCVI_ISG_i3_1.0; http://www.ncbi.nlm.nih.gov/nuccore/NZ_ABJB000000000). The pipeline used for bioinformatics analysis of RNAseq data was similar to that described by Twine et al. (2011) using a TopHat-Cufflinks-Cuffdiff pipeline (Langmead et al. 2009; Trapnell et al. 2009).
• Runs – One fastq file for each sample type was submitted to dryad (fastq read files).

Results
• Total number of reads, number of reads after filtering, mean length, quality, number of aligned/assembled reads, etc. - Table 1. The results showed that the average ±} S.D. percent sequence identity between *I. ricinus* and *I. scapularis* was 99.232 ±} 0.005 with a very low frequency of INDELs, demonstrating high sequence identity between the transcriptomes of these tick species for aligned reads. However, the main limitation of this analysis was the relatively low proportion (26-27%) of *I. ricinus* reads aligned to the *I. scapularis* genome sequence. This limitation is due to several factors associated with the *I. scapularis* genome assembly. The first factor is that the currently assembled *I. scapularis* genome covers around 78% (1.8 Gbp) of the predicted 2.3 Gbp, thus up to 22% of the *I. ricinus* reads may not be mapped to the *I. scapularis* genome due to lack of sequence information. The second factor is that the *I. scapularis*

genome assembly is highly fragmented, currently including 369,492 scaffolds (http://www.ncbi.nlm.nih.gov/nuccore?term=NW_002505054:NW_002874545[PACC]) and 1,141,595 contigs (http://www.ncbi.nlm.nih.gov/Traces/wgs/?val=ABJB01#contigs). The mapping of reads to a highly ragmented genome causes reads mapping to regions of around 70 bp at both ends of the reference contigs (approximately 140 bp per contig) to be considered as unmapped when they could perfectly map to a completely assembled genome. The number of unmapped *I. ricinus* reads due to the high fragmentation of the *I. scapularis* genome assembly could be then estimated as 9% [100 x (1,141,595 contigs x 140 bp/1,765,382,190 bp)] of the total number of reads obtained. The third factor is gene fragmentation. TopHat is able to map reads to different exons of the same gene but only when exons and introns are located in the same contig. With the *I. scapularis* genome fragmented in 1,141,595 contigs with an average length of 1,546 bp, it is highly probable that many *I. ricinus* transcriptome reads did not map to the *I. scapularis* genome due to the presence of fragmented genes. In fact, although it is difficult to estimate the proportion if unmapped reads due to this factor, a recent RNAseq and mapping of I. scapularis transcriptome using TopHat also resulted in approximately 30% mapped reads (unpublished results). The results support the use of I. scapularis genome sequence as a reference for the analysis of *I. ricinus* transcriptomics and proteomics data. These results also suggested the need to further characterize *Ixodes* spp. tick classification and strain-to-strain sequence variability to fully understand sequence variation in these species. This approach could also be applied to validate the use of *I. scapularis* genome sequence as a reference for the analysis of transcriptomics and proteomics data in other *Ixodes* spp., thus expanding the possibilities for genetic and molecular studies of tick species with little genome sequence information. However, the main limitation for tick system biology is the lack of high quality genome sequence information and thus *de novo* RNAseq and proteomics data are required to improve the quality and coverage of sequence information for these species.

• Quality scoring system – phred.

• Quality scoring ASCII character range – "@" to "i"

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Table 1. Sequence identity between *I. ricinus* and *I. scapularis*.

Sample	Adult female ticks	Larvae
TOTAL NO. READS	27,246,477	27,718,396
ALIGNED READS	7,414,535	7,204,681
PF_READS	7,414,535	7,204,681
PF_ALIGNED READS	7,414,535	7,204,681
PF_HQ_ALIGNED READS	7,024,484	5,731,608
MEAN READ LENGTH	75 bp	75 bp
PF_HQ_ERROR_RATE	0.764506	0.772192
PF_INDEL_RATE	0.002987	0.001297

Abbreviations: PF, pass-Illumina filter; HQ, high quality; PF_HQ_ERROR_RATE: Percentage of *I. ricinus* sequence bases that mismatch the *I. scapularis* reference genome sequence in PF HQ aligned reads; PF_INDEL_RATE: Number of insertion and deletion events per 100 PF aligned bases. It uses the number of events as the numerator, not the number of inserted or deleted bases. Picard metrics definitions (http://picard.sourceforge.net/picard-metric-definitions.shtml) were used.

Vaccinomics approach to the identification of candidate protective antigens for the control of tick vector infestations and *Anaplasma phagocytophilum* infection

Contreras, M., Alberdi, P., Fernández de Mera, I. G., Krull, C., Nijhof, A., Villar, M. and de la Fuente, J. (2017). Vaccinomics approach to the identification of candidate protective antigens for the control of tick vector infestations and *Anaplasma phagocytophilum* infection. *Frontiers in Cellular and Infection Microbiology*. 7:360. doi: 10.3389/fcimb.2017.00360

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Vaccinomics Approach to the Identification of Candidate Protective Antigens for the Control of Tick Vector Infestations and *Anaplasma phagocytophilum* Infection

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Anaplasma phagocytophilum is an emerging tick-borne pathogen causing human granulocytic anaplasmosis (HGA), tick-borne fever (TBF) in small ruminants, and other forms of anaplasmosis in different domestic and wild animals. The main vectors of this pathogen are *Ixodes* tick species, particularly *I. scapularis* in the United States and *I. ricinus* in Europe. One of the main limitations for the development of effective vaccines for the prevention and control of *A. phagocytophilum* infection and transmission is the identification of effective tick protective antigens. The objective of this study was to apply a vaccinomics approach to *I. scapularis-A. phagocytophilum* interactions for the identification and characterization of candidate tick protective antigens for the control of vector infestations and *A. phagocytophilum* infection. The vaccinomics pipeline included the use of quantitative transcriptomics and proteomics data from uninfected and *A. phagocytophilum*-infected *I. scapularis* ticks for the selection of candidate protective antigens based on the variation in tick mRNA and protein levels in response to infection, their putative biological function, and the effect of antibodies against these proteins on tick cell apoptosis and pathogen infection. The characterization of selected candidate tick protective antigens included the identification and characterization of *I. ricinus* homologs, functional characterization by different methodologies including RNA interference, immunofluorescence, gene expression profiling, and artificial tick feeding on rabbit antibodies against the recombinant antigens to select the candidates for vaccination trials. The vaccinomics pipeline developed in this study resulted in the identification of two candidate tick protective antigens that could be selected for future vaccination trials. The results showed that *I. scapularis* lipocalin (ISCW005600) and lectin pathway inhibitor (AAY66632) and *I. ricinus* homologs constitute candidate protective antigens for the control of vector infestations and *A. phagocytophilum* infection. Both antigens are involved in the tick evasion of host defense response and pathogen infection and transmission, but targeting different immune response pathways. The vaccinomics

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pipeline proposed here could be used to continue the identification and characterization of candidate tick protective antigens for the development of effective vaccines for the prevention and control of HGA, TBF, and other forms of anaplasmosis caused by *A. phagocytophilum*.

Keywords: anaplasmosis, immunology, vaccine, tick, *Ixodes*, *Anaplasma phagocytophilum*

INTRODUCTION

The intracellular bacterium, *Anaplasma phagocytophilum* (Rickettsiales: Anaplasmataceae) is an emerging tick-borne pathogen causing human granulocytic anaplasmosis (HGA), which has emerged as a tick-borne disease of humans in the United States, Europe and Asia, and tick-borne fever (TBF) in small ruminants, most notably in sheep in Europe (Gordon et al., 1932; Foggie, 1951; Dumler et al., 2001; Stuen et al., 2013; Bakken and Dumler, 2015; Dugat et al., 2015; Severo et al., 2015). Clinical presentation of *A. phagocytophilum* infection has been also documented in goats, cattle, horses, dogs, cats, roe deer, and reindeer (Severo et al., 2015). The main vectors of this pathogen are *Ixodes* tick species, particularly *I. scapularis* in the United States and *I. ricinus* in Europe (Stuen et al., 2013; Bakken and Dumler, 2015).

Despite the burden that *A. phagocytophilum* represents for humans and animals, vaccines are not available for prevention and control of pathogen infection and transmission (Dumler et al., 2001; Stuen et al., 2013, 2015; Bakken and Dumler, 2015; Severo et al., 2015; Contreras et al., 2017). One of the main limitations for the development of effective vaccines for the prevention and control of *A. phagocytophilum* infection and transmission is the identification of effective tick protective antigens. Recently, different approaches have been developed for the identification and characterization of candidate tick protective antigens (de la Fuente and Contreras, 2015; de la Fuente et al., 2016a). Vaccinomics is one of the approaches that have been used by our group for the identification of tick-derived and pathogen-derived protective antigens (de la Fuente and Merino, 2013; Merino et al., 2013; Antunes et al., 2014; de la Fuente and Contreras, 2015; Contreras et al., 2016, 2017; de la Fuente et al., 2016a; Villar et al., 2017). Vaccinomics is a holistic approach based on the use of genome-scale or omics technologies integrated in a systems biology approach to characterize tick-host-pathogen interactions for the development of next-generation vaccines (de la Fuente and Merino, 2013; Contreras et al., 2016; de la Fuente et al., 2016a; Villar et al., 2017). In this translational approach, basic biological information on tick-host-pathogen interactions translates into the identification and subsequent evaluation of new candidate protective antigens (de la Fuente and Merino, 2013; de la Fuente et al., 2016a; Villar et al., 2017).

The sequence, assembly and annotation of the *I. scapularis* genome were recently released (Gulia-Nuss et al., 2016), and various genomics, transcriptomics and proteomics studies in *I. ricinus* suggest that these tick species are genetically closely related (Schwarz et al., 2013, 2014; Genomic Resources Development Consortium et al., 2014; Cramaro et al., 2015; Kotsyfakis et al., 2015; Weisheit et al., 2015; Chmelař et al., 2016). These results open new opportunities for research on tick-host-pathogen interactions and the possibility of identifying tick protective antigens for both *I. scapularis* and I. *ricinus* major vectors of *A. phagocytophilum* (de la Fuente et al., 2016b).

Recently, transcriptomics, proteomics and metabolomics datasets have been integrated and used for the characterization of *I. scapularis-A. phagocytophilum* molecular interactions (Ayllón et al., 2015; Villar et al., 2015a,b, 2016; Cabezas-Cruz et al., 2016, 2017a,b; de la Fuente et al., 2016c, 2017; Gulia-Nuss et al., 2016; Shaw et al., 2017). Herein, a vaccinomics pipeline was developed based on quantitative transcriptomics and proteomics data from uninfected and *A. phagocytophilum*-infected *I. scapularis* nymphs, adult female midguts and salivary glands, and ISE6 cells (Ayllón et al., 2015; Villar et al., 2015a). The vaccinomics pipeline was then used for the identification of candidate protective antigens for the control of vector infestations and pathogen infection. The results showed that *I. scapularis* ISCW005600 and AAY66632 and *I. ricinus* homologs constitute candidate protective antigens for the control of vector infestations and *A. phagocytophilum* infection.

MATERIALS AND METHODS

Ticks and Cultured Tick Cells

Ixodes scapularis ticks were obtained from the laboratory colony maintained at the Oklahoma State University Tick Rearing Facility. Nymphs and adult female *I. scapularis* were infected with *A. phagocytophilum* by feeding on a sheep inoculated intravenously with approximately 1×10^7 *A. phagocytophilum* (NY18 isolate)-infected HL-60 human cells (90–100% infected cells) (Kocan et al., 2012; Ayllón et al., 2015). Animals were housed and experiments conducted with the approval and supervision of the OSU Institutional Animal Care and Use Committee (Animal Care and Use Protocol, ACUP No. VM1026). *I. ricinus* ticks were obtained from the laboratory colony maintained at the Freie Universität Berlin. Larvae and nymphs were fed on mice and adults on rabbits. The *I. scapularis* embryo-derived tick cell line ISE6, provided by Ulrike Munderloh, University of Minnesota, USA, was cultured in L-15B300 medium as described previously (Kurtti et al., 1996; Munderloh et al., 1999; Villar et al., 2015a). IRE/CTVM20 embryo-derived tick cells, provided by the Tick Cell Biobank, were maintained as described previously (Bell-Sakyi et al., 2007; Alberdi et al., 2015). Tick cells were first inoculated with *A. phagocytophilum* (human NY18 isolate; Asanovich et al., 1997)-infected HL-60 cells and maintained according to Munderloh et al. (1999). Uninfected and infected cultures ($N = 4$ independent cultures with approximately 10^7 cells each) were

sampled at 7 days post-infection (dpi) (75% infected cells). The percentage of cells infected with *A. phagocytophilum* was calculated by examining at least 200 cells using a 100x oil immersion objective.

Transcriptomics and Proteomics Datasets

The quantitative transcriptomics and proteomics data for uninfected and *A. phagocytophilum*-infected *I. scapularis* nymphs, adult female midguts and salivary glands, and ISE6 cells were obtained from previously published results (Ayllón et al., 2015; Villar et al., 2015a) and deposited at the Dryad repository database, NCBI's Gene Expression Omnibus database and ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD002181 and doi: 10.6019/PXD002181.

Sequence Analysis

To find the *I. ricinus* homologs, selected *I. scapularis* sequences were blasted against the *I. ricinus* database using the Blastp tool from BLAST (Altschul et al., 1990; Madden et al., 1996), and the sequences with the lowest *E*-value were selected. Gene ontology (GO) analysis for biological process (BP) was done with Blast2GO software (version 3.0; http://www.blast2go.com) (Villar et al., 2014).

Production of Recombinant Proteins

The coding sequences for *I. scapularis* candidate protective antigens were amplified from synthetic genes optimized for codon usage in *Escherichia coli* (Genscript Corporation, Piscataway, NJ, USA) using sequence-specific primers (**Table 1**). The amplified DNA fragments were cloned into the expression vector pET101 and expressed in *E. coli* strain BL21 using the Champion pET101 Directional TOPO Expression kit (Carlsbad, CA, USA). Recombinant proteins were fused to Histidine tags for purification by affinity to Ni (Merino et al., 2013; Moreno-Cid et al., 2013). Transformed *E. coli* strains were induced with IPTG for 4.5 h to produce recombinant proteins, which were purified to >85% of total cell proteins by Ni affinity chromatography (Genscript Corporation) as previously described (Merino et al., 2013; Moreno-Cid et al., 2013) using 1 ml HisTrap FF columns mounted on an AKTA-FPLC system (GE Healthcare, Piscataway, NJ, USA) in the presence of 7 M urea lysis buffer. The purified antigens were refolded by dialysis against 1,000 volumes of PBS, pH 7.4 (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) for 12 h at 4°C.

Production of Rabbit Polyclonal IgG Antibodies

For each recombinant tick protein and total ISE6 tick cell proteins, two New Zealand white rabbits (*Oryctulagus cuniculus*) were subcutaneously injected at weeks 0, 4, and 6 with 50 μg protein in 0.4 ml Montanide ISA 50 V adjuvant (Seppic, Paris, France). Blood was collected before injection and 2 weeks after the last immunization to prepare pre-immune and immune sera, respectively. Serum aliquots were kept at 4°C for immediate use or at −20°C for long-term storage. The IgG were purified from serum samples using the Montage antibody purification kit and

TABLE 1 | Oligonucleotide primers used in this study for cloning, RNAi and RT-PCR.

GenBank accession No.	Oligonucleotide sequence (5′-3′ for forward and reverse primers)
CLONING	
ISCW024685	CACCATGAAAAGCAGCGCACTGCTG
	GCGTTTACCACGAACGCACC
ISCW024295	CACCATGCCGAAACAAGGCGAAAC
	TCCAGAGTCACCACACAAAACG
ISCW022212	CACCATGTGGGGTCAGATTGCGCT
	ACAGATGAATTTTTTCAGGC
ISCW020900	CACCATGAACAAAGCGATCTTCAT
	CACTTCACCGAAAAAGCCGC
ISCW000326	CACCATGCCGGCGTCAATGAAAAG
	CAGAGAACCCAGATTCGGAA
ISCW008146	CACCATGGATTTTGATGACCTGTT
	GAAGCTCAGGGTGTTCTGTT
ISCW008641	CACCATGCAACGTGACATTTTTAG
	CCAACAGCCCGGCTGCGATT
ISCW024499	CACCATGTGCCTGGTGTTTGCAAC
	GCGCAGAAAGGAACTCGTAC
ISCW005600	CACCATGATTCGTCAGGTTCGCGA
	CGAACCTGAGATCGATGAGG
ISCW013709	CACCATGTTTCGTACCAGCTCTGG
	CACAATATAATCCGGTGCAC
ISCW017117	CACCATGCTGAGTGTGCTGCTGGG
	CGTGGTGGCGTCCGGCGGCG
ISCW013574	CACCATGTATCAGCTGCGCGATTT
	GCAACGGGATTTGCGAACAC
ISCW018900	CACCATGGGCCCGTTTATTGGTCT
	GCCGATAATGCGACCGATAA
ISCW023907	CACCATGCCGGTCAATCGCCTGAT
	AACTTTACGAAAGAAAAACA
ISCW024682	CACCATGATTCATGAACCGGTGAT
	ATACGGACAGTACAGTTTGCA
ISCW015453	CACCATGATGAAAAGCCCGCTGTTTAT
	ACCGAAAAAGCCGTGGCCGA
ISCW021670	CACCATGTGGGAACTGCATGCCGA
	CTCCTGGGTAATATTACGCGT
ISCW017271	CACCATGTGCAGCGATTCTAAACC
	CGGCAGATAGGAACCGTGCG
AAY66632	CACCATGGGCCTGACCGGTACCAC
	GTTGTCTTTGGTTTTCTTGG
RNAi AND RT-PCR	
ISCW005600	TCCCCTTCTCAAAGGAGGAT*
	ATCCACAGGCGGATATGAAG*
AAY66632	ACCCGTTCATGGGACAAATA*
	TTCTTGGGCTTCTCAGTTGG*
DQ066214 (*rpS4*)	GGTGAAGAAGATTGTCAAGCAGAG
	TGAAGCCAGCAGGGTAGTTTG

**The same oligonucleotide primers were used to determine gene expression levels by RT-PCR and for the generation of dsRNA for RNAi. To produce dsRNA, the T7 promoter sequence 5′-GAATTAATACGACTCACTATAGGGAGA-3′ was added to the 5′-end of each primer.*

spin columns with PROSEP-A media (Millipore, Billerica, MA, USA) following the manufacturer's recommendations.

Western Blot Analysis

Ten micrograms of each recombinant protein or 20 μg total proteins from ISE6 tick cells were loaded onto a 12% SDS-polyacrylamide pre-cast gel (Life Science, Hercules, CA, USA) and transferred to a nitrocellulose membrane. The membrane was blocked with 5% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MI, USA) for 2 h at room temperature (RT), and washed four times with TBS (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5% Tween 20). Purified rabbit IgG were used at a 1:500 dilution in TBS, and the membrane was incubated overnight at 4°C and washed four times with TBS. The membrane was then incubated with an anti-rabbit IgG-horseradish peroxidase (HRP) conjugate (Sigma-Aldrich) diluted 1:1,000 in TBS with 3% BSA. The membrane was washed five times with TBS and finally developed with TMB (3,3′, 5,5′- tetramethylbenzidine) stabilized substrate for HRP (Promega, Madrid, Spain) according to the manufacturer recommendations.

Immunofluorescence Assay (IFA) in Adult Female Ticks

Adult *I. scapularis* females were infected with *A. phagocytophilum* (NY18) as described above. Female ticks were removed from the sheep 10 days after infestation, held in the humidity chamber for 4 days and fixed with 4% paraformaldehyde in 0.2 M sodium cacodylate buffer, dehydrated in a graded series of ethanol and embedded in paraffin (Ayllón et al., 2015). Sections (4 μm) were prepared and mounted on glass slides. The paraffin was removed from the sections with xylene and the sections were hydrated by successive 2 min washes with a graded series of 100, 95, 80, 75, and 50% ethanol. The slides were treated with Proteinase K (Dako, Barcelona, Spain) for 7 min, washed with PBS and incubated with 3% BSA (Sigma-Aldrich) in PBS for 1 h at RT. The slides were then incubated for 14 h at 4°C with primary rabbit IgG antibodies diluted 1:100 in 3% BSA/PBS and, after 3 washes in PBS, developed for 1 h with goat-anti-rabbit IgG conjugated with phycoerythrin (PE) (Sigma-Aldrich) (diluted 1:50 in 3% BSA/PBS). The slides were washed twice with PBS and mounted in ProLong Antifade with DAPI reagent (Molecular Probes, Eugene, OR, USA). The sections were examined using a Zeiss LSM 800 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany). Sections of uninfected ticks and IgG from pre-immune and anti-ISE6 sera were used as controls.

Antibody Inhibition Assay

The inhibitory effect of rabbit IgG antibodies on *A. phagocytophilum* (NY18) was conducted as described previously (Villar et al., 2015b). ISE6 and IRE/CTVM20 tick cells were pooled and used to seed 24-well plates for each assay. Each well received 1×10^6 cells in L-15B300 (ISE6) or L-15/L-15B (IRE/CTVM20) medium 24 h prior to inoculation with *A. phagocytophilum*. Infected cultures for inoculum were harvested when infection reached 80% and host cells were mechanically disrupted with a syringe and 26-gauge needle. Purified IgG (100 μg/ml) were added to the culture media and incubated with the cells for 48 h. Then, the medium with antibodies was removed and the *A. phagocytophilum* inoculum (100 μl) was added to the cell monolayers and incubated at 31°C for 60 min. The inoculum was removed from the wells and cell monolayers washed three times with PBS. Complete medium (1 ml) was added to each well and the plates were incubated at 31°C. The control included inoculum incubated with rabbit pre-immune and anti-ISE6 IgG. Four replicates were done for each treatment. After 72 h, cells from all wells were harvested and processed for *A. phagocytophilum* detection by real-time PCR after DNA extraction. Results were compared between treatments by the Student's *t*-test with unequal variance ($P = 0.05$; $N = 4$ biological replicates).

Flow Cytometry of Tick Cells Incubated with Rabbit IgG Antibodies

Approximately 5×10^5–1×10^6 of *A. phagocytophilum*-infected ISE6 and IRE/CTVM20 tick cells were collected after incubation with rabbit IgG. Purified IgG (2.2–2.4 mg/ml) were mixed with *A. phagocytophilum* and incubated with tick cells as described above in the antibody inhibition assay. Apoptosis was measured by flow cytometry using the Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Immunostep, Salamanca, Spain) following the manufacturer's protocols. The technique detects changes in phospholipid symmetry analyzed by measuring Annexin V (labeled with FITC) binding to phosphatidylserine, which is exposed in the external surface of the cell membrane in apoptotic cells. Cells were stained simultaneously with the non-vital dye propidium iodide (PI) allowing the discrimination of intact cells (Annexin V-FITC negative, PI negative) and early apoptotic cells (Annexin V-FITC positive, PI negative). All samples were analyzed on a FAC-Scalibur flow cytometer equipped with CellQuest Pro software (BD Biosciences, Madrid, Spain). The viable cell population was gated according to forward-scatter and side-scatter parameters. The percentage of apoptotic cells was determined by flow cytometry after Annexin V-FITC and PI labeling and compared between treated and untreated uninfected cells by Student's *t*-test with unequal variance ($P = 0.05$; $N = 4$ biological replicates).

RNA Interference (RNAi) for Gene Knockdown in Tick Cells

RNAi was used to characterize the effect of gene knockdown on tick cell pathogen infection. Oligonucleotide primers homologous to selected *I. scapularis* ISCW005600 and AAY66632 genes containing T7 promoters (**Table 1**) were used for *in vitro* transcription and synthesis of dsRNA as described previously (Ayllón et al., 2013), using the Access RT-PCR system (Promega, Madison, WI, USA) and the Megascript RNAi kit (Ambion, Austin, TX, USA). The unrelated *Rs86* dsRNA was synthesized using the same methods described previously and used as negative control (Ayllón et al., 2013). The dsRNA was purified and quantified by spectrophotometry. RNAi experiments were conducted in cell cultures by incubating ISE6 tick cells with 10 μl dsRNA (5×10^{10}–5×10^{11} molecules/μl) and 90 μl L15B300 medium in 24-well plates using 5 wells per treatment (Ayllón

et al., 2013). Control cells were incubated with the unrelated *Rs86* dsRNA. After 48 h of dsRNA exposure, tick cells were infected with cell-free *A. phagocytophilum* (NY18) obtained from approximately 5×10^6 infected HL-60 cells (90–100% infected cells) (Thomas and Fikrig, 2007) and resuspended in culture medium to use 1 ml/well. Cells were incubated for an additional 72 h, harvested and used for DNA and RNA extraction. RNA was used to analyze gene knockdown by real-time RT-PCR with respect to *Rs86* control. DNA was used to quantify the *A. phagocytophilum* infection levels by real-time PCR.

Determination of *A. phagocytophilum* Infection by Real-Time PCR

A. phagocytophilum DNA levels were characterized by *major surface protein 4* (*msp4*) real-time PCR normalized against tick *ribosomal protein S4* (*rpS4*) as described previously (Ayllón et al., 2015). Normalized Ct-values were compared between untreated and treated cells by Student's *t*-test with unequal variance ($P = 0.05$; $N = 4$ biological replicates).

Determination of Tick mRNA Levels by Real-Time RT-PCR

Total RNA was extracted from ISE6 tick cell cultures using TriReagent (Sigma-Aldrich) following manufacturer's recommendations. The expression of selected *I. scapularis* ISCW005600 and AAY66632 genes was characterized using total RNA extracted from infected and uninfected ISE6 tick cells. Real-time RT-PCR was performed on RNA samples using gene-specific oligonucleotide primers (**Table 1**) and the Kapa SYBR Fast One-Step qRT-PCR Kit (Kapa Biosystems, Wilmington, MA, USA) and the Rotor-Gene Real-Time PCR Detection System (Qiagen, Madrid, Spain). A dissociation curve was run at the end of the reaction to ensure that only one amplicon was formed and that the amplicons denatured consistently at the same temperature range for every sample. The mRNA levels were normalized against tick *rpS4* using the genNorm method (Delta-Delta-Ct, ddCT) as described previously (Ayllón et al., 2015). Normalized Ct-values were compared between infected and uninfected tick cells by Student's *t*-test with unequal variance ($P = 0.05$; $N = 4$ biological replicates).

Artificial Tick Feeding

Artificial tick feeding was conducted as previously described for *Dermacentor reticulatus* (Krull et al., 2017). Briefly, 17–19 female and 3 male *I. ricinus* ticks were placed on each feeding unit. The feeding unit was subsequently closed by the insertion of a pierced plastic lid (PE-LD Stopfen 26 mm, Brimon Laborbedarf, Hamburg, Germany) wrapped in gauze fabric into the feeding unit, leaving approximately one cm between the silicone membrane and lid. The feeding unit was then hung into a glass beaker (50 ml, Simax, Czech Republic) containing the bovine blood using a rubber ring with an inner diameter of 32 mm (Lux, Wermelskirchen, Germany). Blood was supplemented with ATP and gentamycin (Krull et al., 2017), and 5 ml blood per feeding unit was pipetted into a sterile beaker and preheated to 37°C on a hot plate. The blood was changed twice daily at 12 ± 2 h intervals. During each blood change, the outside of the feeding unit and underside of the silicone membrane were rinsed with sterile 0.9% NaCl solution, pre-heated to body temperature. The number of attached, dead and fed ticks was counted after which the feeding unit was transferred to a new sterile beaker with fresh blood. Males stayed inside the feeding unit until the end of the experiment, to provide them with sufficient opportunity and time to fertilize any females present. Feeding units were placed in an incubator (ICH 256C, Memmert GmbH, Schwabach, Germany), where the blood was maintained at a constant temperature of 37°C using a heating plate (Hot Plate 062, Labotect, Göttingen, Germany). Environmental conditions were set at 20°C, 80% relative humidity, 5% CO_2 and 15 h light/9 h dark. Once ticks were partially engorged, the feeding units were transferred to a six-well plate (Sarstedt, Nümbrecht, Germany) and ticks were fed for 36 h with 3 ml blood supplemented with 1 mg/ml of pre-immune or antigen-specific purified IgG. Dead and detached engorged ticks were removed, and engorged females that detached were weighed and stored individually in 2 ml Eppendorf tubes with pierced lids, which were kept in desiccators with approximately 90% relative humidity at RT. Ticks were assessed for egg mass 8 weeks post-feeding. The number of dead/fed ticks, ticks and eggs weight, and ticks with or without oviposition were compared between groups by a Fisher's exact test ($P = 0.05$; http://www.socscistatistics.com/tests/fisher/Default2.aspx).

RESULTS AND DISCUSSION

Selection of Candidate Tick Protective Antigens

A vaccinomics pipeline was developed for the selection and characterization of candidate tick protective antigens for the control of vector infestations and pathogen infection (**Figure 1**). The vaccinomics pipeline included the use of quantitative transcriptomics and proteomics data from uninfected and *A. phagocytophilum*-infected *I. scapularis* ticks (Ayllón et al., 2015) for the selection of candidate protective antigens based on the variation in tick mRNA and protein levels in response to infection, their putative biological function, and the effect of antibodies against these proteins on tick cell apoptosis and pathogen infection (**Figure 1**). The characterization of selected candidate tick protective antigens included the identification and characterization of *I. ricinus* homologs, functional characterization by different methodologies including RNAi, IFA, gene expression profile, and artificial tick feeding on rabbit antibodies against the recombinant antigens to select the candidates for vaccination trials (**Figure 1**). This process could be repeated as many times as needed to cover all potential candidate antigens or until the desired number of candidate antigens for vaccination trials is reached (**Figure 1**). The vaccinomics pipeline included some of the algorithms previously proposed (de la Fuente and Merino, 2013; Contreras et al., 2016) and validated (Merino et al., 2013; Antunes et al., 2014) for the selection and characterization of candidate protective antigens, but for the first time it was applied to integrated transcriptomics and proteomics data of tick-pathogen interactions.

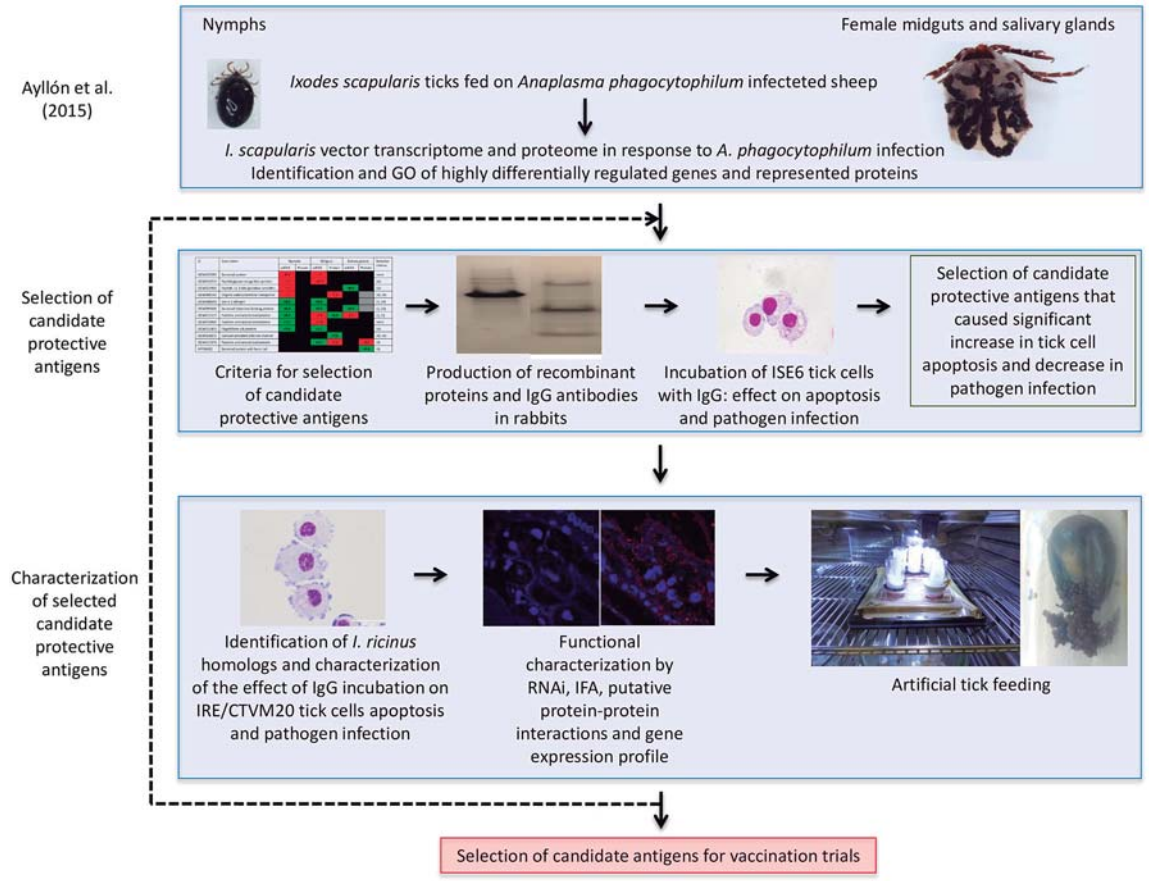


FIGURE 1 | Vaccinomics pipeline for the selection and characterization of candidate tick protective antigens for the control of vector infestations and pathogen infection. The quantitative transcriptomics and proteomics data for uninfected and *A. phagocytophilum*-infected *I. scapularis* nymphs, adult female midguts and salivary glands were obtained from previously published results (Ayllón et al., 2015). The selection of candidate tick protective antigens included criteria based on the variation in tick mRNA and protein levels in response to infection, their putative biological function, and the effect of antibodies against these proteins on tick cell apoptosis and pathogen infection. The characterization of selected candidate protective antigens included the identification and characterization of *I. ricinus* homologs, functional analyses by different methodologies, and artificial tick feeding on rabbit antibodies against the recombinant antigens to select the candidates for vaccination trials.

The characterization of tick-pathogen molecular interactions was based on the previous work by Ayllón et al. (2015) of the *I. scapularis* transcriptome and proteome in response to *A. phagocytophilum* infection in nymphs and female midguts and salivary glands. The highly differentially regulated genes were selected as those with more than 50-fold (log2 normalized fold change >5.64) difference between infected and uninfected tick samples ($P < 0.00003$) (**Figure 2A**). The highly differentially represented proteins were selected as those with more than 15-fold (log2 normalized fold change >3.90) change between infected and uninfected tick samples ($P < 0.00003$) (**Figure 2A**). Of the highly differentially regulated/represented genes/proteins, between 0 and 50% were identified at both mRNA and protein levels in the different samples (**Figure 2B**). The analysis of highly differentially expressed/represented genes/proteins in response to *A. phagocytophilum* infection evidenced tissue-specific differences in response to infection (Ayllón et al., 2015), which were taken into consideration for the selection of candidate protective antigens (**Figures 2A–D**). The candidate protective antigens were selected by using the criteria (i) highly differentially up-regulated genes in at least two samples, (ii) highly down-regulated genes in at least one sample, (iii) highly differentially over-represented proteins and identified in the *I. scapularis* proteome, (iv) highly differentially under-represented proteins and identified in the *I. scapularis* proteome, and/or (v) putative BP in tick-pathogen and tick-host interactions (**Figure 3A**). The rationale behind the selection criteria for candidate protective antigens was based on their putative relevance in (i, iii) tick response to infection (de la Fuente et al., 2016c,d, 2017), (ii, iv) manipulated by *A. phagocytophilum* to decrease tick protective mechanisms and increase infection (de la Fuente et al., 2016c,d, 2017), and (v) tick-pathogen and tick-host interactions (**Figure 3B**).

By using these criteria, a total of 12 candidate tick protective antigens were initially selected, and 7 of them fulfilled two of the selection criteria (**Figure 3A**). The recombinant antigens were

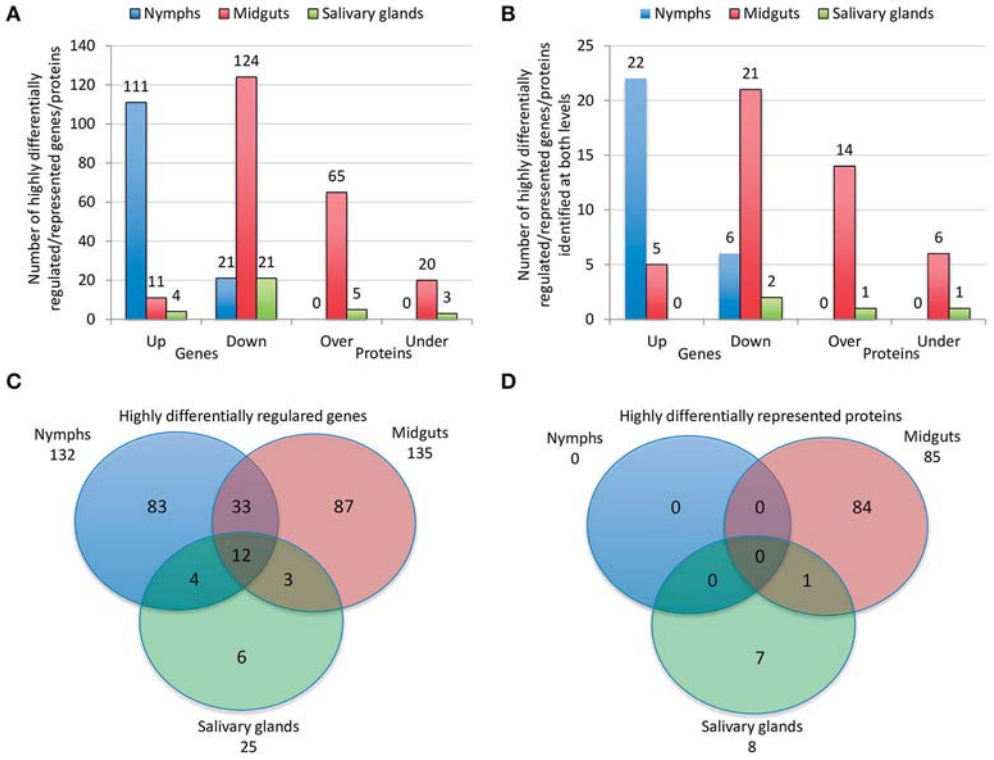


FIGURE 2 | Effect of *A. phagocytophilum* infection on *I. scapularis* highly differentially regulated genes and represented proteins. **(A)** Number of tick highly differentially regulated genes and differentially represented proteins that were up-regulated (Up), down-regulated (Down), over-represented (Over) and under-represented (Under) in response to pathogen infection. **(B)** Number of tick highly differentially regulated genes and differentially represented proteins that were up-regulated (Up), down-regulated (Down), over-represented (Over) and under-represented (Under) in response to pathogen infection, and identified at both mRNA and protein levels. **(C)** Venn diagram with highly differentially regulated genes that were identified in tick nymphs, midguts and salivary glands. **(D)** Venn diagram with highly differentially represented proteins that were identified in tick nymphs, midguts and salivary glands. The highly differentially regulated genes were selected as those with more than 50-fold (log2 normalized fold change > 5.64) difference between infected and uninfected tick samples ($P < 0.00003$). The highly differentially represented proteins were selected as those with more than 15-fold (log2 normalized fold change > 3.90) change between infected and uninfected tick samples ($P < 0.00003$). The quantitative transcriptomics and proteomics data for uninfected and *A. phagocytophilum*-infected *I. scapularis* nymphs, adult female midguts and salivary glands were obtained from previously published results (Ayllón et al., 2015).

produced in *E. coli* and used for the preparation of antigen-specific IgG antibodies in immunized rabbits (**Figures 4A,B**). These IgG antibodies were then used for the incubation with *I. scapularis* ISE6 cells before infection with *A. phagocytophilum* to characterize the effect on cell apoptosis (**Figure 5A**) and pathogen infection (**Figure 5B**). The results showed that anti ISCW005600 and AAY66632 IgG significantly increased the percentage of apoptotic cells when compared to negative control cells incubated with pre-immune IgG (**Figure 5A**). The incubation of ISE6 cells with rabbit IgG against recombinant antigens significantly decreased pathogen infection for 7 antigens when compared to the negative control (**Figure 5B**). The positive control cells were incubated with rabbit IgG antibodies against total ISE6 tick cells proteins, which significantly increased cell apoptosis but did not affect pathogen infection when compared to the negative control (**Figures 5A,B**). The anti-ISE6 antibodies did not affect pathogen infection of tick cells, which as previously discussed (Stuen et al., 2015) was due to the presence of not protective dominant antigens in the protein extract used to immunize rabbits for antibody production. Nevertheless, these results showed that incubation of ISE6 tick cells with IgG antibodies against ISCW005600 and AAY66632 antigens affected both cell apoptosis and pathogen infection, and were therefore selected as the candidate tick protective antigens for further characterization (**Figures 5A,B**).

Characterization of Selected Candidate Tick Protective Antigens

The first step in the characterization of selected candidate tick protective antigens was the identification of *I. ricinus* homologs to evaluate their protective potential in both major tick vector species for *A. phagocytophilum*. The *I. ricinus* homologs for *I. scapularis* ISCW005600 and AAY66632 antigens corresponded to putative salivary gland secreted proteins lipocalins (Beaufays et al., 2008; Schwarz et al., 2013; Valdés et al., 2016) and a lectin pathway inhibitor (Ribeiro et al., 2006; Schuijt et al., 2011), respectively (**Figure 5C**). At the amino acid level, over 70% sequence identity was obtained for both antigens (**Figure 5C**), suggesting that these proteins are highly conserved in *I. scapularis*

A

ID	Description	Nymphs		Midguts		Salivary glands		Selection criteria
		mRNA	Protein	mRNA	Protein	mRNA	Protein	
ISCW024295	Secreted protein	**-8.0**		-2.9				(ii)
ISCW022212	Peptidoglycan-recognition protein	-3.3		**-10.7**				(ii), (v)
ISCW020900	Peptide 11.3 kDa (putative ixostatin)	-1.8				**+8.0**		(v)
ISCW008146	Organic cation/carnitine transporter	-1.5			**-7.9**			(iv), (v)
ISCW008641	Ves G 1 allergen	**+6.0**		**+8.6**				(i), (v)
ISCW005600	Secreted histamine binding protein	**+6.4**		**+5.7**		+0.4		(i), (v)
ISCW017117	Putative uncharacterized protein	**+8.9**		**-7.1**	**+6.4**	**-7.1**		(ii), (iii)
ISCW018900	Putative uncharacterized protein	**+1.6**		**-13.9**				(ii)
ISCW015453	Flagelliform silk protein	**+7.6**		+3.2				(v)
ISCW021670	Calcium activated chlorine channel				**+7.5**			(iii), (v)
ISCW017271	Putative uncharacterized protein			+0.7	**-4.5**		**-4.4**	(iv)
AAY66632	Secreted protein with basic tail						**+4.4**	(iii), (v)

Up in infected ticks (+) | Down in infected ticks (-) | No difference | Not found

B

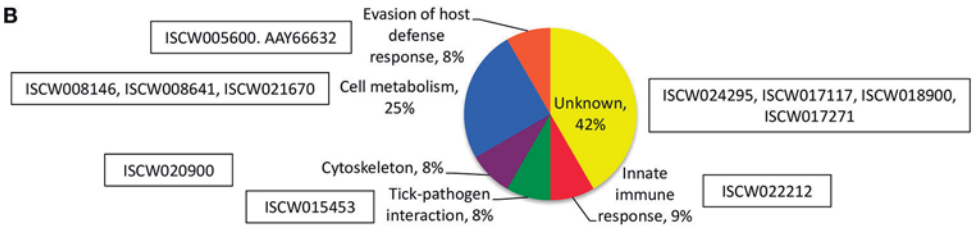


FIGURE 3 | Criteria for the selection of candidate tick protective antigens. **(A)** Annotation and criteria used for the selection of candidate tick protective antigens. The log2 normalized fold change between infected and uninfected tick samples ($P < 0.00003$) is shown. Bold numbers indicate highly differentially regulated genes (log2 normalized fold change > 5.64) and highly differentially represented proteins (log2 normalized fold change > 3.90) between infected and uninfected tick samples. The selection criteria were (i) highly differentially up-regulated genes in at least two samples, (ii) highly down-regulated genes in at least one sample, (iii) highly differentially over-represented proteins and identified in the *I. scapularis* proteome, (iv) highly differentially under-represented proteins and identified in the *I. scapularis* proteome, and/or (v) putative BP in tick-pathogen and tick-host interactions. **(B)** The GO for BP of selected candidate protective antigens was done with Blast2GO software (version 3.0; http://www.blast2go.com).

and *I. ricinus*, and may be protective in vaccine preparations against both tick vector species.

Experiments were then conducted to characterize the effect of rabbit IgG antibodies against ISCW005600 and AAY66632 antigens in heterologous *I. ricinus* IRE/CTVM20 cells as described before in the homologous *I. scapularis* ISE6 cells (**Figures 5D,E**). As in ISE6 tick cells, the results showed that incubation of IRE/CTVM20 tick cells with IgG antibodies against ISCW005600 and AAY66632 antigens affected both cell apoptosis (**Figure 5D**) and pathogen infection (**Figure 5E**), supporting the putative effect of vaccination with these antigens in both tick vector species.

Functional analyses were conducted to gain additional insight into the possible protective mechanisms for these antigens. The expression of ISCW005600 and AAY66632 was determined by RT-PCR and did not change in response to *A. phagocytophilum* infection of ISE6 tick cells (**Figure 6A**), a result that agreed with previous results of transcriptomics analysis (Villar et al., 2015a; **Figure 6B**). The IFA in uninfected and *A. phagocytophilum*-infected *I. scapularis* females showed that as expected, a negative and positive staining was obtained with pre-immune and anti-ISE6 IgG in infected ticks, respectively (**Figures 6Ca–d**). The ISCW017271 antigen, which protein levels were highly under-represented in response to infection in both midguts and salivary glands (**Figure 3A**), was used to validate proteomics results. The IFA using anti-ISCW017271 IgG antibodies showed a positive staining in uninfected (**Figure 6Ce**) but not infected cells (**Figure 6Cf**), thus corroborating the proteomics results.

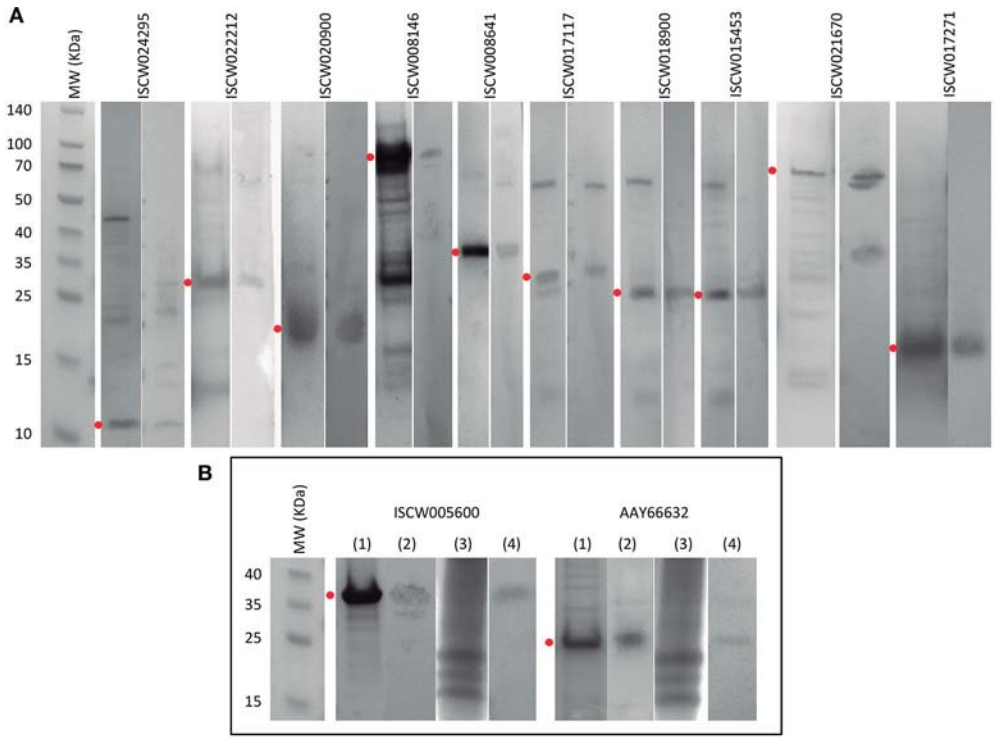


FIGURE 4 | Production of recombinant proteins and rabbit IgG antibodies. **(A)** SDS-PAGE (left panel) and Western blot analysis (right panel) of selected candidate tick protective recombinant antigens. **(B)** Antigens selected for further characterization. (1) SDS-PAGE of recombinant antigens. (2) Western blot analysis of recombinant antigens. (3) SDS-PAGE of total proteins from ISE6 tick cells. (4) Western blot analysis of total proteins from ISE6 tick cells. Western blots were performed with IgG antibodies from rabbits immunized with recombinant antigens. Red dots denote the position of the recombinant antigen. Other protein bands in some of the samples correspond to *E. coli* contamination proteins, and aggregation or degradation products of the recombinant antigens.

For the selected candidate tick protective antigens, the IFA with anti-ISCW005600 IgG did not produce any positive staining (**Figures 6Cg–h**), in accordance with proteomics results (**Figure 3A**). However, for the AAY66632 antigen, a positive staining was obtained in salivary glands from infected ticks after IFA with anti-AAY66632 antibodies (**Figures 6Ci,j**). The positive staining in infected (**Figure 6Cl**) but not uninfected (**Figure 6Ck**) ticks formed a membrane-like structure in salivary glands (arrows in **Figure 6Cl**), and also corroborated the proteomics results for this antigen (**Figure 3A**).

Gene knockdown by RNAi in ISE6 tick cells resulted in significantly lower *A. phagocytophilum* infection levels for both antigens when compared to control cells using the unrelated Rs86 dsRNA (**Figure 6D**). These results suggested that although ISCW005600 and AAY66632 mRNA levels did not change in response to infection of ISE6 tick cells, which constitute a model for tick hemocytes involved in pathogen infection and immune response (Villar et al., 2015a; Alberdi et al., 2016), they may play a role in *A. phagocytophilum* infection.

These results encouraged a final experiment to evaluate the potential effect of ISCW005600 and AAY66632 as vaccination antigens to reduce tick infestations and reproduction. An artificial tick feeding system using silicone membranes was used in this experiment (Kröber and Guerin, 2007; Krull et al., 2017). Although the development of standardized *in vitro* feeding methods for ixodid ticks has been hampered by their complex feeding behavior and the long duration of their blood meal, recent developments provide a valuable tool for the study of tick physiology, tick-host-pathogen interactions and the discovery of drugs and other control interventions without the use of experimental animals (Kröber and Guerin, 2007; Bonnet and Liu, 2012; Sojka et al., 2015; Tajeri et al., 2016; Krull et al., 2017; Trentelman et al., 2017). *I. ricinus* ticks were selected for artificial feeding and the results shown here supported an effect of antibodies against *I. scapularis* antigens (**Figure 4B**) on *I. ricinus* ticks (**Figures 5D,E**).

On the artificial feeding device, the number of attached ticks was similar between groups, but the number of dead ticks increased after feeding on anti-antigen IgG and was significantly higher in ticks fed on anti-AAY66632 antibodies when compared to control ticks fed on pre-immune IgG (**Figure 7**). Significant differences were not observed between groups in tick weight, number of ticks with oviposition and egg weight, but a tendency in the reduction in the number of ticks with oviposition was also observed in ticks fed on anti-AAY66632 IgG (**Figure 7**). Although the number of ticks used for artificial feeding was limited due to experimental conditions, the results suggested an effect of anti-ISCW005600 and anti-AYY66632 antibodies on tick

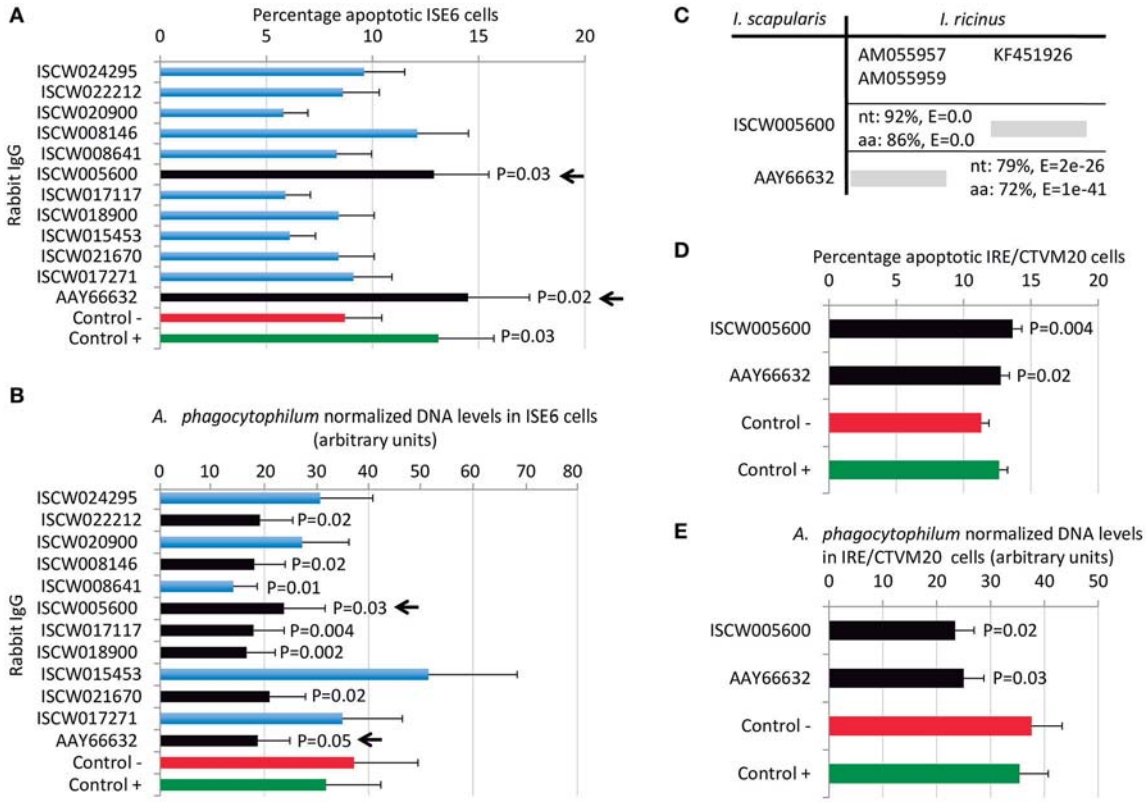


FIGURE 5 | Selection of candidate tick protective antigens. Purified rabbit IgG against recombinant candidate tick protective antigens were incubated with *I. scapularis* ISE6 and *I. ricinus* IRE/CTVM20 tick cells before infection with *A. phagocytophilum*. **(A)** Characterization of the effect of rabbit IgG antibodies on ISE6 tick cells apoptosis. **(B)** Characterization of the effect of rabbit IgG antibodies on pathogen infection of ISE6 tick cells. **(C)** Sequence identity between *I. scapularis* and *I. ricinus* homologs. The accession numbers are shown together with corresponding percent identity for nucleotide (nt) and amino acid (aa) sequences and *E*-values. The selected *I. scapularis* sequences were blasted against the *I. ricinus* database using the Blastp tool from BLAST, and the sequences with the lowest *E*-value were selected. **(D)** Characterization of the effect of rabbit IgG antibodies on IRE/CTVM20 tick cells apoptosis. **(E)** Characterization of the effect of rabbit IgG antibodies on pathogen infection of IRE/CTVM20 tick cells. The percentage of apoptotic cells was determined by flow cytometry after Annexin V-FITC and PI labeling. *A. phagocytophilum* DNA levels were determined by *msp4* real-time PCR normalizing against tick *rpS4*. Control cells were incubated with rabbit pre-immune IgG (negative control, Control -) or rabbit anti-ISE6 IgG (positive control, Control +). Results were presented as average + S.D. normalized Ct-values and compared between each treatment and negative control by Student's *t*-test with unequal variance ($P \leq 0.05$; $N = 4$). The selected candidate protective antigens are shown with arrows.

mortality and a reduction in the number of ticks with oviposition for anti-AYY66632 antibodies.

These results suggested that the selected candidate tick protective antigens might constitute effective vaccine antigens to control tick vector infestations and prevent or control pathogen infection, and therefore could be selected for future vaccination trials.

Putative Mechanisms of Protection for Vaccines Based on Selected Candidate Tick Protective Antigens

After the successful completion of the main objective of this study, which was the identification of tick candidate tick protective antigens for the control of vector infestations and *A. phagocytophilum* infection, a question arose about the putative protective mechanisms of the selected candidate protective antigens. The answer to this question may assist in the selection of additional candidate protective antigens following the vaccinomics pipeline (**Figure 1**), and the evaluation of possible combinations of antigens with different functions to enhance vaccine efficacy (de la Fuente and Merino, 2013).

Both selected candidate tick protective antigens were grouped into the evasion of host defense response BP (**Figure 3B**). The ISCW005600 secreted histamine binding protein appears to be a salivary lipocalin (Beaufays et al., 2008; Schwarz et al., 2013). Lipocalins are a family of salivary gland secreted proteins that play a role in evasion of host immune and inflammatory responses by competing for histamine or serotonin binding (Paesen et al., 2000; Mans, 2005; Beaufays et al., 2008; Valdés, 2014; Valdés et al., 2016). Therefore, these proteins play an important role during tick feeding. The genes encoding for these proteins are up-regulated during tick feeding (Kim et al., 2016; Valdés et al., 2016; Ribeiro et al., 2017) and pathogen infection (Ayllón et al., 2015; Valdés et al., 2016). Additionally, lipocalins were also produced in tick midguts and up-regulated

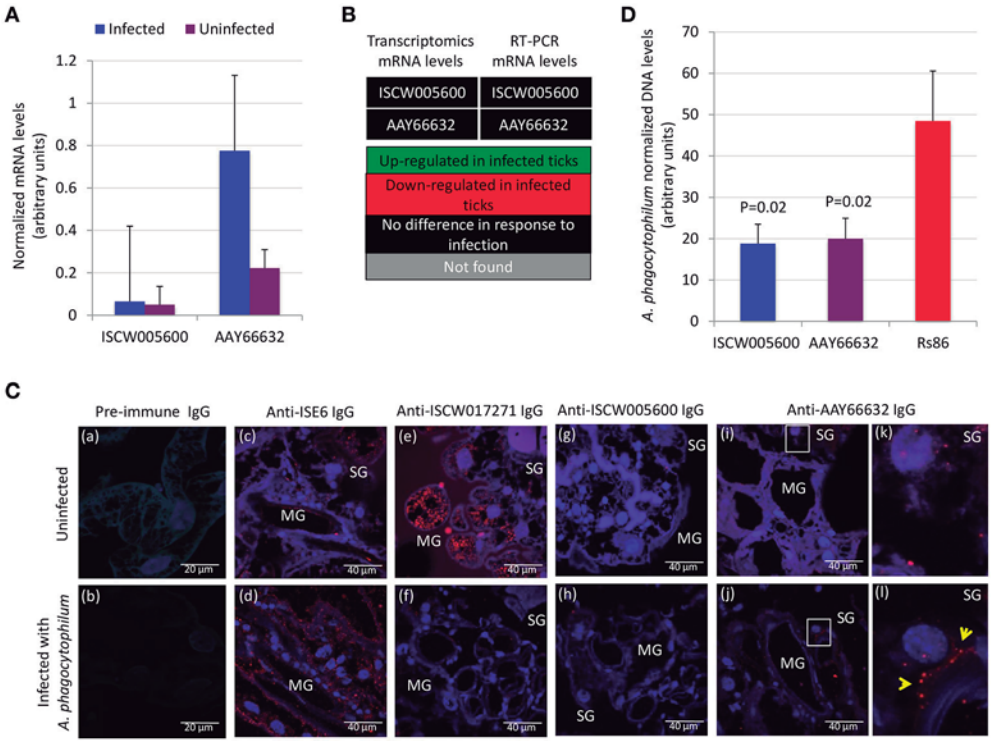
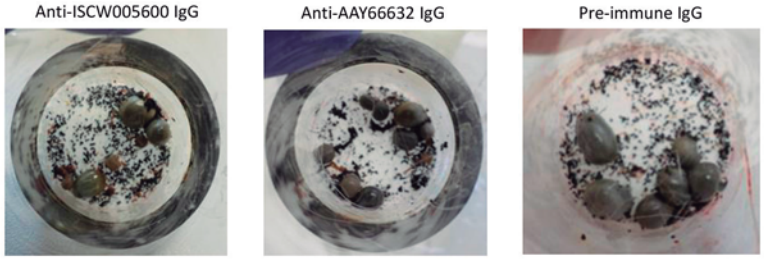


FIGURE 6 | Functional characterization of selected candidate tick protective antigens. **(A)** Results of the RT-PCR analysis of the expression of ISCW005600 and AAY66632 genes in uninfected and *A. phagocytophilum*-infected ISE6 tick cells. Results were presented as average + S.D. normalized Ct-values and compared between infected and uninfected cells by Student's *t*-test with unequal variance ($P \leq 0.05$; $N = 4$). **(B)** Comparison of the transcriptomics and RT-PCR results for mRNA levels of ISCW005600 and AAY66632 genes in ISE6 tick cells in response to *A. phagocytophilum* infection. Transcriptomics results were obtained from Villar et al. (2015a). **(C)** Representative images of imunofluorescence analysis of uninfected **(a,c,e,g,i,k)** and *A. phagocytophilum*-infected **(b,d,f,h,j,l)** adult female *I. scapularis* midguts (MG) and salivary glands (SG). Tick tissues were stained with rabbit pre-immune control IgG **(a,b)**, anti-ISE6 tick cells IgG **(c,d)**, or anti-tick antigens IgG **(e–l)** labeled with RFP (red) and DAPI (blue). Yellow arrows illustrate a positive staining for AAY66632 in the SG sections in white squares in infected **(l)** but not uninfected **(k)** ticks. **(D)** The *A. phagocytophilum* DNA levels were determined after RNAi in infected ISE6 tick cells treated with ISCW005600 and AAY66632 dsRNAs or control *Rs86* dsRNA. *A. phagocytophilum* DNA levels were determined by *msp4* real-time PCR normalizing against tick *rpS4*. Results are shown as average + S.D. normalized Ct-values and compared between treated and control groups by Student's *t*-test with unequal variance ($P < 0.05$; $N = 5$ biological replicates).

in response to *A. phagocytophilum* infection (Ayllón et al., 2015; **Figure 3A**), suggesting as reported in other organisms (Cassidy and Martineau, 2014; Abella et al., 2015) a role for these proteins in tick innate immune response to infection. Therefore, lipocalins may have a dual role in tick-pathogen interactions. These proteins may facilitate pathogen transmission by reducing host inflammatory responses (Valdés et al., 2016), but control tick infection by depleting strategic compounds for pathogens (Ferreira et al., 2015). In humans, lipocalins have also been shown to regulate apoptosis by inducing or inhibiting this process under different physiological conditions (Chakraborty et al., 2012; Abella et al., 2015). Based on the results obtained here with anti-ISCW005600 antibodies and RNAi (**Figures 5A,B,D,E**, **6D**, **7**), ISCW005600 may function to inhibit tick cell apoptosis and facilitate *A. phagocytophilum* infection with a possible role during tick feeding (**Figure 7**). Therefore, the proposed protective mechanisms for vaccines containing this antigen may include reduction of tick infestations by increasing cell apoptosis and reducing protective capacity to host response while reducing pathogen infection and transmission. Tick lipocalins have been proposed before as vaccine antigens for the control of tick infestations (de Castro et al., 2016; Manzano-Román et al., 2016), but only low partial protection have been reported in soft ticks, *Ornithodoros moubata* fed on immunized rabbits (Manzano-Román et al., 2016).

The AAY66632 antigen is a secreted lectin pathway inhibitor (Ribeiro et al., 2006; Schuijt et al., 2011), which is involved in the inhibition of the innate immune response complement lectin pathway (CLP). The CLP is involved in host response to infection with different pathogens (Evans-Osses et al., 2013). The CLP is activated when mannan-binding lectins or ficolins bind to patterns of carbohydrates or acetyl groups on the surface of protozoan, virus, fungi, or bacteria (Runza et al., 2008; Héja et al., 2012; Evans-Osses et al., 2013). In ticks, the inhibition of the complement system during and after blood feeding is critical for tick feeding success and development by minimizing damage to the intestinal epithelium as well as avoiding inflammation and opsonization of salivary molecules at the bite site (Wikel and Allen, 1977; Franco et al., 2016). Therefore, complement inhibitors are present in both tick saliva and midgut (Barros et al.,



	Anti-ISCW005600 IgG	Anti-AAY66632 IgG	Pre-immune IgG
Number of attached ticks	19	17	17
Number of dead ticks	4	6*	0
Number of fed ticks	15	11	17
Tick weight (mg) (average ± S.D.)	193±39	134±81	177±44
Number of ticks with oviposition	13	5	10
Egg weight (mg) (average ± S.D.)	39±25	48±29	24±18

FIGURE 7 | Artificial tick feeding. Artificial tick feeding was conducted as previously described (Krull et al., 2017) with 17–19 female and 3 male *I. ricinus* ticks per feeding unit. Environmental conditions were set at 20°C, 80% relative humidity, 5% CO_2 and 15 h light/9 h dark. Feeding units containing partially engorged ticks were placed on a six well plate and ticks were fed for 36 h with blood supplemented with 1 mg/ml of pre-immune or antigen-specific purified IgG. Dead and detached engorged ticks were removed, and engorged females that detached were weighed and stored individually in 2 ml Eppendorf tubes with pierced lids, which were kept in desiccators with approximately 90% relative humidity at RT. Ticks were assessed for egg mass 8 weeks post-feeding. The number of dead/fed ticks and thus with oviposition were compared between groups by a Fisher's exact test (*$P = 0.02$).

2009; Mendes-Sousa et al., 2013; Ayllón et al., 2015) (**Figure 3A**). The presence and activity of salivary anti-complement molecules has been well characterized in *Ixodes* spp. ticks including the *A. phagocytophilum* vectors, *I. scapularis* (Valenzuela et al., 2000; Tyson et al., 2007, 2008, Schuijt et al., 2011) and *I. ricinus* (Lawrie et al., 1999, 2005, Daix et al., 2007; Couvreur et al., 2008). Moreover, tick lectin pathway inhibitors have been shown to facilitate *Borrelia burgdorferi* pathogen infection and transmission (Schuijt et al., 2011; Wagemakers et al., 2016). Our results supported a role for AAY66632 in tick feeding success (**Figure 7**), the inhibition of tick cell apoptosis (**Figures 5A,D**) and facilitation of *A. phagocytophilum* infection (**Figures 5B,E, 6D**). Therefore, the proposed protective mechanisms for vaccines based on this antigen may include reduction of tick infestations by affecting tick attachment and/or feeding, while reducing pathogen infection and transmission. The protective capacity of vaccines containing this antigen has not been reported.

CONCLUSIONS

The main objective of this study was to apply a vaccinomics approach to the identification and characterization of candidate tick protective antigens for the control of vector infestations and *A. phagocytophilum* infection. The vaccinomics pipeline developed in this study was applied to tick-*A. phagocytophilum* interactions and resulted in the identification of two candidate tick protective antigens that could be selected for future vaccination trials. The results showed that *I. scapularis* ISCW005600 and AAY66632 and *I. ricinus* homologs constitute candidate protective antigens for the control of vector infestations and *A. phagocytophilum* infection. Both lipocalin (ISCW005600) and lectin pathway inhibitor (AAY66632) are involved in the tick evasion of host defense response and pathogen infection and transmission, but targeting different immune response pathways. Therefore, based on the putative function of these antigens, vaccine protective mechanisms were proposed that supported antigen combination to improve vaccine efficacy. The vaccinomics pipeline proposed here could be used to continue the identification and characterization of candidate tick protective antigens for the development of effective vaccines for the prevention and control of HGA, TBF, and other forms of anaplasmosis caused by *A. phagocytophilum*.

AUTHOR CONTRIBUTIONS

JD conceived the study. MC, PA, IF, MV, CK, and AN performed the experiments. MC, PA, MV, CK, AN, and JD performed data analyses. JD, MC, and PA wrote the paper, and

other coauthors made additional suggestions and approved the manuscript.

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Anaplasma phagocytophilum MSP4 and HSP70 proteins are involved in interactions with host cells during pathogen infection

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Anaplasma phagocytophilum MSP4 and HSP70 Proteins Are Involved in Interactions with Host Cells during Pathogen Infection

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Anaplasma phagocytophilum transmembrane and surface proteins play a role during infection and multiplication in host neutrophils and tick vector cells. Recently, *A. phagocytophilum* Major surface protein 4 (MSP4) and Heat shock protein 70 (HSP70) were shown to be localized on the bacterial membrane, with a possible role during pathogen infection in ticks. In this study, we hypothesized that *A. phagocytophilum* MSP4 and HSP70 have similar functions in tick-pathogen and host-pathogen interactions. To address this hypothesis, herein we characterized the role of these bacterial proteins in interaction and infection of vertebrate host cells. The results showed that *A. phagocytophilum* MSP4 and HSP70 are involved in host-pathogen interactions, with a role for HSP70 during pathogen infection. The analysis of the potential protective capacity of MSP4 and MSP4-HSP70 antigens in immunized sheep showed that MSP4-HSP70 was only partially protective against pathogen infection. This limited protection may be associated with several factors, including the recognition of non-protective epitopes by IgG in immunized lambs. Nevertheless, these antigens may be combined with other candidate protective antigens for the development of vaccines for the control of human and animal granulocytic anaplasmosis. Focusing on the characterization of host protective immune mechanisms and protein-protein interactions at the host-pathogen interface may lead to the discovery and design of new effective protective antigens.

Keywords: anaplasmosis, immunology, HL60, tick, vaccine, sheep, *Anaplasma phagocytophilum*

INTRODUCTION

Anaplasma phagocytophilum (Rickettsiales: Anaplasmataceae) is an emerging tick-borne intracellular bacterial pathogen in many regions of the world, but vaccines are not available for prevention of transmission and infection in humans and animals (Dumler et al., 2001; Severo et al., 2013; Stuen et al., 2013, 2015; Bakken and Dumler, 2015). *Anaplasma phagocytophilum* causes

human granulocytic anaplasmosis (HGA), which has emerged as a tick-borne disease of humans in the United States, Europe and Asia (Severo et al., 2013). In Europe, *A. phagocytophilum* is an established pathogen of small ruminants, most notably in sheep, where it was first described as the etiologic agent of tick-borne fever (TBF; Gordon et al., 1932; Foggie, 1951; Dugat et al., 2015). Clinical presentation of *A. phagocytophilum* infection has been also documented in goats, cattle, horses, dogs, cats, roe deer, and reindeer (Severo et al., 2013). Although, *A. phagocytophilum* is recognized as a threat for human and animal health in Europe and the United States, its pathogenic and epidemic potential is neglected in tropical regions of the world (Heyman et al., 2010; Dugat et al., 2015). Prophylactic uses of tetracycline together with acaricide applications for tick control are the main measures to control *A. phagocytophilum* infection in endemic areas (Woldehiwet, 2006; Stuen et al., 2015). However, these control measures raise concerns about their impact on the environment and human health, and the selection of resistant pathogens and ticks (Woldehiwet, 2006; Stuen et al., 2015).

Results using next generation sequencing technologies have advanced our understanding of the mechanisms by which *A. phagocytophilum* infection affects gene expression, protein content and microbiota in the vertebrate host and tick vector (Ge and Rikihisa, 2006; Sukumaran et al., 2006; de la Fuente et al., 2010, 2016a,b,c,d, 2017, Neelakanta et al., 2010; Rikihisa, 2011; Severo et al., 2012; Ayllón et al., 2013, 2015; Hajdušek et al., 2013; Villar et al., 2015a; Cabezas-Cruz et al., 2016, 2017; Gulia-Nuss et al., 2016; Abraham et al., 2017; Mansfield et al., 2017). However, less information is available on the bacterial molecules involved in pathogen infection and multiplication (Ge and Rikihisa, 2007; Huang et al., 2010; Lin et al., 2011; Troese et al., 2011; Mastronunzio et al., 2012; Oliva Chávez et al., 2015; Seidman et al., 2015; Villar et al., 2015b; Truchan et al., 2016). Definition of bacterial proteins involved in host-pathogen and vector-pathogen interactions may provide target antigens for the development of vaccines and therapeutics that interfere with pathogen host infection and transmission by ticks (Gomes-Solecki, 2014; de la Fuente and Contreras, 2015).

Recently, Villar et al. (2015b) demonstrated that *A. phagocytophilum* activates a new mechanism associated with bacterial cell stress and membrane proteins to counteract tick cell response to infection and favor pathogen infection and multiplication. Their results showed that *A. phagocytophilum* proteins, Major surface protein 4 (MSP4) and Heat shock protein 70 (HSP70), are localized on the bacterial membrane where they interact with a possible role during pathogen infection in ticks (Villar et al., 2015b). Furthermore, antibodies against MSP4 and HSP70 inhibited pathogen infection of tick cells, supporting that these proteins are involved in tick-pathogen interactions (Villar et al., 2015b). They proposed that the inhibitory effect of anti-MSP4 and anti-HSP70 antibodies could be the result of the antibodies blocking the interaction between bacterial ligands (e.g., MSP4) and tick receptors or an effect on proteins functionally important for bacterial infection and/or multiplication in tick cells (e.g., HSP70 and those physically and/or functionally interacting with it; Villar et al., 2015b). The results of these experiments suggested that *A. phagocytophilum* MSP4 and HSP70 proteins constitute candidate protective antigens to interfere with pathogen infection in the tick vector, *Ixodes scapularis*.

The characterization of the *A. phagocytophilum* proteome demonstrated that chaperones, surface and stress response proteins are among the most abundant proteins found in *I. scapularis* tick salivary glands (Mastronunzio et al., 2012). HSP70 is a chaperone involved in protein folding and stress response (Johnson, 2012). This protein functions by protecting cells from stress-induced lethal damage and under physiological growth conditions by acting as carriers for immunogenic peptides, assisting in protein export or mediating adherence to host cells and may play an essential role during cell division (Scopio et al., 1994; Susin et al., 2006; Multhoff, 2007; Seydlová et al., 2012). The role of MSPs such as MSP4 in adhesion to tick cells for bacterial infection has been demonstrated in *A. marginale* (de la Fuente et al., 2001) and *A. phagocytophilum* (Villar et al., 2015b).

Anaplasma phagocytophilum infects vertebrate host neutrophils and various tick tissues, where it develops within membrane-bound inclusions in the cell cytoplasm (Severo et al., 2013). However, this pathogen has evolved common molecular mechanisms to establish infection in tick vectors and vertebrate hosts that collectively mediate pathogen infection, development, persistence, and survival (de la Fuente et al., 2016a). These strategies include, but are not limited to, remodeling of the cytoskeleton, inhibition of cell apoptosis, manipulation of the immune response, and the use of rickettsial proteins for infection and manipulation of tick and host gene expression (Cabezas-Cruz et al., 2016; de la Fuente et al., 2016a).

Based on these results, we hypothesized that the use of common strategies by *A. phagocytophilum* to establish infection in ticks and vertebrate hosts resulted in similar functions for MSP4 and HSP70 proteins in host-pathogen and tick-pathogen interactions. To address this hypothesis, in this study we characterized the role of these bacterial proteins in infection of vertebrate host cells and their potential protective capacity in immunized sheep. The results showed that *A. phagocytophilum* MSP4 and HSP70 are involved in host-pathogen interactions during pathogen infection, but were only partially protective against pathogen infection in sheep.

MATERIALS AND METHODS

Ethics Statement

The study was ethically approved by the local Animal Health and Welfare Authority (Diputación Foral de Alava) with reference No. 1820, 12th May 2015, following Spanish ethical guidelines and animal welfare regulations (Real Decreto 53/2013).

Production of Recombinant Proteins and Rabbit Antibodies

The His-tag recombinant *A. phagocytophilum* human NY18 isolate (Asanovich et al., 1997) proteins MSP4 (AFD54597) and HSP70 (KX891324) were produced in *Escherichia coli* BL21 cells (Champion pET101 Directional TOPO Expression kit, Carlsbad, CA, USA), after induction with IPTG and purified using the Ni-NTA affinity column chromatography system (Qiagen Inc.,

Valencia, CA, USA) as previously described (Villar et al., 2015b). Recombinant purified proteins showed purity higher than 85% of total proteins and were used to immunize rabbits to purify IgGs from preimmune and immunized animals (Montage Antibody Purification Kit and Spin Columns with PROSEP-A Media, Millipore, Billerica, MA, USA) for Western blot and antibody inhibition analyses as previously described (Villar et al., 2015b).

Surface Trypsin Digestion of *A. phagocytophilum* from Infected HL60 Human Cells

The *A. phagocytophilum* human NY18 isolate was propagated in cultured HL60 human promyelocytic cells as previously described (de la Fuente et al., 2005). The *A phagocytophilum*-infected cells ($\sim 1 \times 10^7$ cells) were collected when 70–80% of the cells were infected as determined by detection of intracellular morulae in stained cytospin cell smears. Host cell-free bacteria were isolated from cell lysates after five passages through a 27-gauge syringe, followed by differential centrifugation in Percoll gradients as previously described for *A. marginale* to separate bacteria from host cell debris (Lis et al., 2014). The pellet of purified *A. phagocytophilum* was resuspended in 200 µl of SPG buffer (0.25 mM sucrose, 10 mM sodium phosphate, 5 mM L-glutamic acid, pH 7.2), and 5 µl of sequencing-grade trypsin (Promega, Madison, WI, USA) was added to half of the cell reaction mixture. Bacteria were incubated at 37°C for 30 min and then centrifuged at 10,000 × g for 15 min and resuspended in Laemmli protein loading buffer, boiled for 5 min and loaded onto a 12% SDS-PAGE and analyzed by Western blot using rabbit antibodies against recombinant MSP4 and HSP70 proteins as previously described (Villar et al., 2015b).

Tertiary Models and Protein-Protein Docking

The active *A. phagocytophilum* HSP70 and MSP4 proteins were modeled using I-TASSER (Zhang, 2008), and the unbound (apo)-HSP70 protein was modeled using Robetta (Kim et al., 2004). All tertiary models were optimized with the Schrödinger's Maestro Protein Preparation Wizard (Li et al., 2007). All steric clashes were resolved via minimization with the default settings in the Schrodinger's Maestro package. For the tertiary models, the Protein Preparation Wizard clusters at the highest degree of hydrogen bonding in equilibrium were used. Monte Carlo orientations were performed (100,000) for each cluster. The optimized structure is based on electrostatic and geometric scoring functions. The membrane positioning for MSP4 was calculated by the OPM database (Lomize et al., 2006) and generated using the Desmond systems builder (Bowers et al., 2006) as part of the Schrodinger's Maestro package. The protein-protein docking was assessed using the SwarmDock server (Torchala et al., 2013) that incorporates flexible docking by exploring in proximity to the Cartesian center of mass of the target protein. Minimization steps are included for the whole system. The poses are calculated based on the most energy favorable poses, minimized once again and sent to the user. We chose to analyze the top 10 poses since these were highly energy favorable (−41 to −54 kcal/mol). The server also produces the residue contacts made between both proteins. All structures were visualized and analyzed using the Visual Molecular Dynamics (Humphrey et al., 1996).

Adhesion of Recombinant *E. coli* Strains to HL60 Human Cells

The adhesion of recombinant *E. coli* strains to HL60 human cells was characterized as previously reported (de la Fuente et al., 2001; Villar et al., 2015b). Briefly, *E. coli* strains producing *A. phagocytophilum* MSP4, HSP70, and mutant HSP70 with truncated peptide-binding domains that are involved in protein-protein interactions (Villar et al., 2015b) recombinant proteins were grown and induced as described before. The *E. coli* cells transformed with expression vector alone were used as negative control. Cell densities were determined and adjusted to 10^8 cells per ml in Luria Broth (LB). One hundred microliters (10^7 bacteria) culture were added to 900 µl of 10^6 cells per ml suspensions of HL60 human cells in LB. Human cells and bacteria were incubated for 30 min at 37°C with occasional agitation. Cells were then collected by centrifugation, washed two times in PBS and resuspended in 100 ml of PBS. Elimination of unbound bacteria from human cells with bound bacteria was performed by Percoll (Sigma, St. Louis, MI, USA) gradient separation (de la Fuente et al., 2001). The band containing human cells was removed with a pipette and washed in PBS. The final cell pellet was lysed in 1 ml of sterile water and 5 µl plated onto LB agar plates containing 100 µg of ampicillin per ml. Two replicates were done for each experiment. Adhesive bacteria were quantitated as the number of colony forming units (CFU) recovered from each test and compared to the control values by Chi2 test ($P = 0.001$; $N = 2$).

Transmission Electron Microscopy (TEM)

The HL60 human cells incubated as described above with *E. coli* strains producing *A. phagocytophilum* MSP4 and HSP70 recombinant proteins or transformed with expression vector alone as controls were pelleted and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 1 h at room temperature. Fixed cells were washed three times in 0.1 M phosphate buffer with 4% glucose and embedded in 2% agar at 60°C. Samples were post-fixed using 2% OsO_4 for 2 h at room temperature, three times washed and dehydrated in a graded series of acetone (30–100%) solutions for 15 min at each step. Samples were infiltrated with 25, 50, and 75% solutions of Spi Pon Epoxy resin (Structure Probe, Inc. Supplies, West Chester, PA, USA) diluted in anhydrous acetone for 1 h at each step. Samples were left in 100% resin overnight, transferred to embedding molds and polymerized at 60°C for 48 h. Ultrathin sections were contrasted in ethanolic uranyl acetate and lead citrate, carbon coated and observed in a JEOL 1010 TEM (JEOL Ltd., Akishima, Tokio, Japan) at an accelerating voltage of 80 kV. Images were captured using a Mega View III camera (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

Prediction of B-cell Epitopes

Anaplasma phagocytophilum MSP4 (AFD54597) and HSP70 (AAC31306) amino acid sequences were aligned using MAFFT version 7, applying a gap opening penalty of 3 (range 1–3, default 1.53; Katoh and Standley, 2013). Sequence homology was calculated using Clustal Omega (Sievers et al., 2011). The linear B-cell epitopes on *A. phagocytophilum* MSP4 and HSP70 proteins were predicted using the Bepipred Linear Epitope Prediction tool (http://tools.immuneepitope.org/bcell/; Haste Andersen et al., 2006; Larsen et al., 2006; Ponomarenko and Bourne, 2007). Subsequently, to search for epitope homology, each predicted epitope within each protein was aligned with the full sequence of the other protein.

Antibody Inhibition Assay

The inhibitory effect of rabbit IgG antibodies against MSP4 and HSP70 recombinant proteins on *A. phagocytophilum* human NY18 (Asanovich et al., 1997) and sheep (Alberdi et al., 2015) isolates infection of HL60 human cells was conducted as described previously for tick cells (Villar et al., 2015b). The inhibitory effect of IgG antibodies purified from MSP4 and MSP4-HSP70 immunized and control sheep at 0 and 94 days post-infection (dpi) on *A. phagocytophilum* human NY18 isolate infection of HL60 human cells was conducted using the same experimental approach as for rabbit IgG. The IgGs were purified from sheep sera using the NAb Protein G spin kit (Thermo Fisher Scientific, Waltham, MA, USA) following manufacturer's recommendations. HL60 cells were pooled and used to seed 24-well plates for each assay. Each well received 1×10^6 cells in RPMI 1,640 medium (Gibco, Thermo Fisher, Madrid, Spain) 48 h prior to inoculation with *A. phagocytophilum*. Infected cultures for inoculum were harvested when infection reached 80% and host cells were mechanically disrupted with a syringe and 26-gauge needle. Rabbit or sheep purified IgGs (2.2–2.4 mg/ml) were mixed with inoculum (1:1) for 60 min before being placed on the cell monolayers. Each monolayer then received 100 µl of the inoculum plus IgG mix and plates were incubated at 37°C for 30 min. The inoculum was removed from the wells and cell monolayers washed three times with PBS. Complete medium (1 ml) was added to each well and the plates were incubated at 37°C. The control for each trial included inoculum incubated with rabbit pre-immune IgG or control sheep IgG. Four replicates were done for each treatment. After 7 days, cells from all wells were harvested, resuspended in 1 ml PBS and frozen at −70°C. Samples were thawed and solubilized with 1% Triton-X100 and processed for *A. phagocytophilum* detection by PCR after DNA extraction using TriReagent (Sigma) according to the manufacturer's recommendations. *Anaplasma phagocytophilum* infection levels were determined by *msp4* real-time PCR normalizing against human *β-actin* as described previously (de la Fuente et al., 2005) but using oligonucleotide primers MSP4-L (5′-CCTTGGCTGCAGCACCACCTG-3′) and MSP4-R (5′-TGCTGTGGGTCGTGACGCG3′), with PCR conditions of 5 min at 95°C and 35 cycles of 10 s at 95°C, 30 s at 55°C and 30 s at 60°C. Results were compared between treatments by the Student's *t*-test with unequal variance ($P = 0.05$; $N = 4$).

Protein Inhibition Assay

HL60 cells were incubated with 4 µM MSP4 and HSP70 recombinant proteins or their combination in culture media for 1 h at 37°C and 5% CO2 in a humidified atmosphere. For antigen combination, equal molar ratios of each protein, equivalent to one part of HSP70 and two parts of MSP4, were incubated at 4°C on a rotator overnight. HL-60 cells (4×10^5 cells/well) were fixed in 4% paraphormaldehyde (PFA) in PBS for 1 h at room temperature (RT), and then incubated with a 6x-His epitope tag monoclonal antibody (3 µg/ml mouse IgG1, Thermo Fisher 4A12E4) for 1 h at RT. After washing, cells were incubated with FITC-conjugated goat anti-mouse IgG (1/100, Sigma F2012) for 1 h at RT. Protein binding was assessed by flow cytometry using a FACScalibur® Flow Cytometer, equipped with the CellQuest Pro® software (BD-Biosciences, San Jose, CA, USA) as previously described (Seidman et al., 2015; Hebert et al., 2017). Incubation with PBS was used as negative control. The viable cell population was gated according to forward scatter and side scatter parameters. To determine the effect of MSP4 and HSP70 recombinant proteins on *A. phagocytophilum* infection, HL60 cells were incubated with 4 µM MSP4 and HSP70 recombinant proteins or their combination for 1 h, after which *A. phagocytophilum* human NY18 isolate bacteria purified as described above were added and incubated with the host cells in the continued presence of recombinant protein for 2 h. Unbound bacteria and proteins were removed and the infection was allowed to proceed for 48 h. Then, cells were harvested and infection levels determined by PCR and statistically analyzed as described above. The *Rhipicephalus microplus* Subolesin recombinant protein (SUB; Merino et al., 2011) and PBS were included as controls. Four replicates were done for each treatment.

Lamb Immunization and Infection with *A. phagocytophilum*

The recombinant MSP4 was formulated alone or combined with HSP70. Antigen combination was done as described above in Section Protein Inhibition Assay. Recombinant antigens or saline control were adjuvated in Montanide ISA 50 V2 (Seppic, Paris, France; Merino et al., 2013). Nine 3-month old lambs of the Latxa breed (Basque Country, Spain) were selected from the experimental sheep flock maintained at NEIKER and were kept indoor during the experiment. This flock has no known history of ticks or tick-borne diseases. However, blood of lambs and their dams were analyzed prior to the start of the study to check their status for hemoparasites *Theileria*, *Babesia,* and *Anaplasma* spp. as previously described (Hurtado et al., 2015). All animals were negative for these hemoparasites. Three groups of 3 lambs each with similar live weight were formed. Lambs from each group were injected subcutaneously in the loose skin of the axilla (armpit) using a sterile syringe with removable needle 20 G × 1″ (9.0 × 25 mm), and taking aseptic precautions. Lambs were immunized three times on days 55, 30, and 10 before

experimental infection with 1 ml doses of MSP4 (100 μg/dose), MSP4-HSP70 (100 μg of equal molar ratios of each protein/dose) or adjuvant/saline as control. The strain of *A. phagocytophilum* used for experimental infection originated from an infected lamb in Norway, which suffered TBF but was negative to other tick-borne pathogens (Alberdi et al., 2015; Stuen et al., 2015). The inoculum consisted of *A. phagocytophilum* infected heparinised blood that had been stored at −70°C with 10% dimethyl sulfoxide (DMSO). Once unfrozen, 1 ml of infected blood containing 1.8 × 10^6 infected neutrophils per ml was intravenously inoculated into each experimental lamb through the jugular vein using sterile winged infusion sets with needle 21 G × 3/4″ (0.8 × 19 mm).

Sheep Samples and Analysis

Whole blood and serum samples were collected from the jugular vein of each lamb previous to each immunization, daily starting on infection day during 10 days, and at weekly intervals until the end of the experiment at 94 dpi (Supplementary Table 1). Rectal temperatures were taken daily until 10 dpi and then weekly until 94 dpi (Supplementary Table 1). Lambs were also weighed periodically (Supplementary Table 1). Hematological analyses including leukocyte and erythrocyte cell counts, leukocyte cell differentiation (percent neutrophils, lymphocytes, monocytes and eosinophils), hemoglobin levels, hematocrit, mean cell volume (MCV), and mean corpuscular hemoglobin (MCH) were performed with an electronic counter (Hemavet 950, Drew, USA) in blood samples collected in EDTA-containing tubes (Supplementary Table 1). Blood smears stained with Giemsa stain were examined to investigate the presence of *A. phagocytophilum* in blood cells (Supplementary Table 1). At least 100 neutrophils were counted and examined to calculate the number of infected neutrophils per milliliter blood of each lamb throughout the experiment. The differential percent of *A. phagocytophilum*-infected neutrophils was calculated as the difference between values at different dpi and values at 3 dpi when infected neutrophils were first detected (Supplementary Table 1).

Analysis of the Antibody Response in Lambs

An indirect ELISA test was performed to detect IgG antibodies against MSP4 and HSP70 proteins in immunized and control lambs using serum samples collected before each immunization and at 0, 7, 10, and 94 dpi. High absorption capacity polystyrene microtiter plates were coated with 100 μl (0.01 μg/μl solution of purified recombinant MSP4 or HSP70 protein) per well in carbonate-bicarbonate buffer (Sigma). After an overnight incubation at 4°C, coated plates were blocked with 100 μl/well of blocking solution (5% skim milk in PBS). Serum samples or PBS as negative control were diluted (1:100, v/v) in blocking solution and 100 μl/well were added into duplicate wells of the antigen-coated plates. After an overnight incubation at 4°C, the plates were washed three times with a washing solution (PBS containing 0.05% Tween 20). A donkey anti-sheep IgG-peroxidase conjugate (Sigma) was added (diluted 1:1000 in blocking solution) and incubated at room temperature for 1 h. After three washes with washing solution, 100 μl/well of substrate solution (Fast OPD; Sigma) was added. Finally, the reaction was stopped with 50 μl/well of 3N H_2SO_4 and the optical density (OD) was measured in a spectrophotometer at 450 nm. Antibody titers were expressed as OD_{450nm} ($OD_{lambsera}$ $OD_{PBScontrol}$). The antigen-specific antibody response in immunized lambs was corroborated by ELISA using pooled sera collected at 0 dpi, but incubating sera with *A. phagocytophilum* purified from infected HL60 human cells as described above in Section Antibody Inhibition Assay. Results from rectal temperature and hematological analyses, differential percent of *A. phagocytophilum*-infected neutrophils and antibody titers were compared between immunized and control groups by two-way ANOVA test ($P = 0.05$; $N = 3$).

Analysis of *A. phagocytophilum* DNA Levels in Lambs

For the analysis of *A. phagocytophilum* infection in lambs during the trial, DNA was extracted from 200 μl of blood using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), including negative extraction controls every 9 samples. DNA was stored at −20°C until subsequent analysis. The presence of *Anaplasma* spp. was firstly determined using a real-time PCR assay for the screening of piroplasms and *Anaplasma* spp. (RTi-PCR1) that targets the 16S rRNA gene of *Anaplasma* spp. and the 18S rRNA gene of piroplasms of the genera *Theileria* and *Babesia* (Hurtado et al., 2015). The assay also includes an internal amplification control (IAC) to monitor for possible inhibition of the PCR reaction. All samples positive to *Anaplasma* spp. in the RTi-PCR1 were analyzed with a multiplex PCR assay that specifically amplifies the major surface protein 2 (*msp2*) gene of *A. phagocytophilum*, and the *msp4* gene of *Anaplasma ovis* (RTi-PCR2). Sequences of primers and probes, as well as details on cycling conditions were as reported previously (Hurtado et al., 2015). Analyses were performed in 20 μl volume reactions using an ABI PRISM 7500 Fast Sequence Detection System (Applied Biosystems, Foster City, CA, USA). For the quantitative analysis of *A. phagocytophilum* infection levels, DNA was extracted from 200 μl blood samples using Nucleospin 96 Blood (Machery-Nagel, Düren Germany). A quatitative real-time PCR was conducted on DNA samples using a Quantitect SYBR Green RT-PCR Kit and a Rotor Gene Q thermocycler (Qiagen, Inc. Valencia, CA, USA) following manufacturer's recommendations. A dissociation curve was run at the end of the reaction to ensure that only one amplicon was formed and that the amplicon denatured consistently in the same temperature range for every sample (Ririe et al., 1997). The DNA levels were normalized against sheep *aldolase B* using primers Ovi-ALDOB-F: CCCATCTTGCTATCCAGGAA and Ovi-ALDOB-R: TACAGCAGCCAGGACCTTCT, and the genNorm method (ddCT method as implemented by Bio-Rad iQ5 Standard Edition, Version 2.0; Livak and Schmittgen, 2001). Normalized Ct values were compared between immunized and control groups by Student's *t*-test with unequal variance ($P = 0.05$; $N = 3$).

RESULTS

The *A. phagocytophilum* MSP4 and HSP70 Proteins are Localized on the Bacterial Membrane and Involved in Pathogen Infection of HL60 Human Cells

The subcellular localization of MSP4 and HSP70 proteins was characterized in *A. phagocytophilum* purified from infected HL60 human promyelocytic leukemia cells, mock treated or surface digested with trypsin and loaded onto polyacrylamide gels for Western blot analysis using rabbit antibodies specific against recombinant proteins. The results showed that as a transmembrane protein, MSP4 was partially resistant to trypsin digestion in *A. phagocytophilum* from HL60 cells, while HSP70 was extracellular and exposed to protease digestion (**Figure 1A**).

The apo and bound *A. phagocytophilum* HSP70 models showed that the major structural difference between the two HSP70 tertiary structures is at the C-terminus (residues 400–533), with a 40–90 Å α-carbon backbone root mean square deviation (RMSD; Supplementary Figure 1A). The *A. phagocytophilum* MSP4 showed a 25° ± 1° tilt from the membrane with its N/C-terminus oriented toward the cytosol, and the β-sheets buried within the membrane with remaining β-hairpin loops exposed extracellularly (Supplementary Figure 1B). The models suggested a limited number of possible MSP4-HSP70 binding positions due to the membrane orientation of MSP4 (Supplementary Figure 1C). The energy score of the optimum MSP4-HSP70 bound state (Supplementary Figure 1C) was calculated at −46 kcal/mol. Furthermore, although the residue map showed that the majority of protein-protein contacts are formed between the β-sheets of MSP4 buried within the membrane and the C-terminus of HSP70, several contacts between the β-hairpin loops of MSP4 and the N-terminus of HSP70 are exposed extracellularly, and therefore these residues may act as markers for mutational studies and antibody targeting (Supplementary Figure 1C). These models supported the interaction between *A. phagocytophilum* MSP4 and HSP70 proteins when localized on the bacterial membrane.

The binding of HSP70 and MSP4 to HL60 human cells was characterized using recombinant proteins and *E. coli* producing surface-exposed *A. phagocytophilum* proteins. The results demonstrated that MSP4 and HSP70 are involved in binding to human promyelocytic leukemia cells (**Figures 1B,C**). Furthermore, *E. coli* producing the mutant HSP70 with truncated peptide-binding domains that are involved in protein-protein interactions did not bind to human HL60 cells, thus supporting the role of this protein in interactions with host cells. The interaction of recombinant *E. coli* producing *A. phagocytophilum* MSP4 (**Figure 1D**) and HSP70 (**Figure 1E**) proteins with HL60 human cells was also characterized by electron microscopy in comparison with control *E. coli* cells to provide additional evidence for the role of these proteins in the interaction with vertebrate host cells.

To provide additional support for the role of *A. phagocytophilum* MSP4 and HSP70 proteins in the interaction with and infection of vertebrate host cells, recombinant proteins and antibodies against these proteins were used to evaluate their effect on pathogen infection of HL60 human cells. Anti-MSP4 and anti-HSP70 or recombinant MSP4 and HSP70 proteins were incubated with HL60 cells prior to infection with *A. phagocytophilum*. The results showed an inhibitory effect of anti-MSP4 and anti-HSP70 antibodies on infection of human cells with *A. phagocytophilum* human NY18 (**Figure 2A**) and sheep (**Figure 2B**) isolates when compared to cells treated with pre-immune serum. Furthermore, incubation with HSP70 and MSP4-HSP70 but not MSP4 recombinant proteins inhibited infection of human cells with *A. phagocytophilum* human NY18 isolate when compared to cells incubated with PBS or SUB controls (**Figure 2C**).

These results evidenced a role for MSP4 and HSP70 proteins in *A. phagocytophilum* adhesion to vertebrate host cells, and suggested a role for HSP70 during pathogen infection. These results also suggested that these proteins might constitute candidate protective antigens to prevent or control pathogen infection.

Experimental Infection with *A. phagocytophilum* Correlates with TBF in Lambs

To gain additional information on the role of *A. phagocytophilum* MSP4 and HSP70 proteins in host-pathogen interactions, sheep that are natural hosts for this pathogen were selected for immunization with recombinant proteins followed by experimental infection with *A. phagocytophilum*. Groups of three lambs each were immunized with recombinant MSP4, MSP4-HSP70 combination or adjuvant/saline control and infected with a sheep isolate of *A. phagocytophilum*. Then, several parameters including rectal temperature, animal weight, hemoglobin content, and hematological variables were evaluated in immunized and control *A. phagocytophilum*-infected lambs to correlate with TBF main clinical signs (Supplementary Table 1).

The results showed signs of TBF in lambs infected with *A. phagocytophilum*. Evidence of *A. phagocytophilum* in neutrophils was obtained for all animals (Supplementary Table 1). Fever was evident in animals from all groups, primarily between 3 and 9 dpi (**Figure 3A** and Supplementary Table 1). Although immunized lambs tend to gain more weight, differences with controls were not significant (Supplementary Table 1). Control sheep showed evidence of anemia at 4 dpi, and between 8 and 10 dpi with all animals being anemic at 9 dpi, a result that correlated with low erythrocyte counts at 8–10 dpi (**Figure 3B** and Supplementary Table 1). The percent neutrophils in the leukocyte population increased in all animals after *A. phagocytophilum* infection between 4 and 9 dpi (**Figure 3C** and Supplementary Table 1). A severe neutropenia was evident in all animals after 59 dpi and lasted until the end of the experiment at 94 dpi (**Figure 3C** and Supplementary Table 1). Although monocyte levels were within normal values throughout the experiment, an increase was observed in all animals after infection between 2–10, 38–45, and 59–94 dpi (**Figure 3D** and Supplementary Table 1).

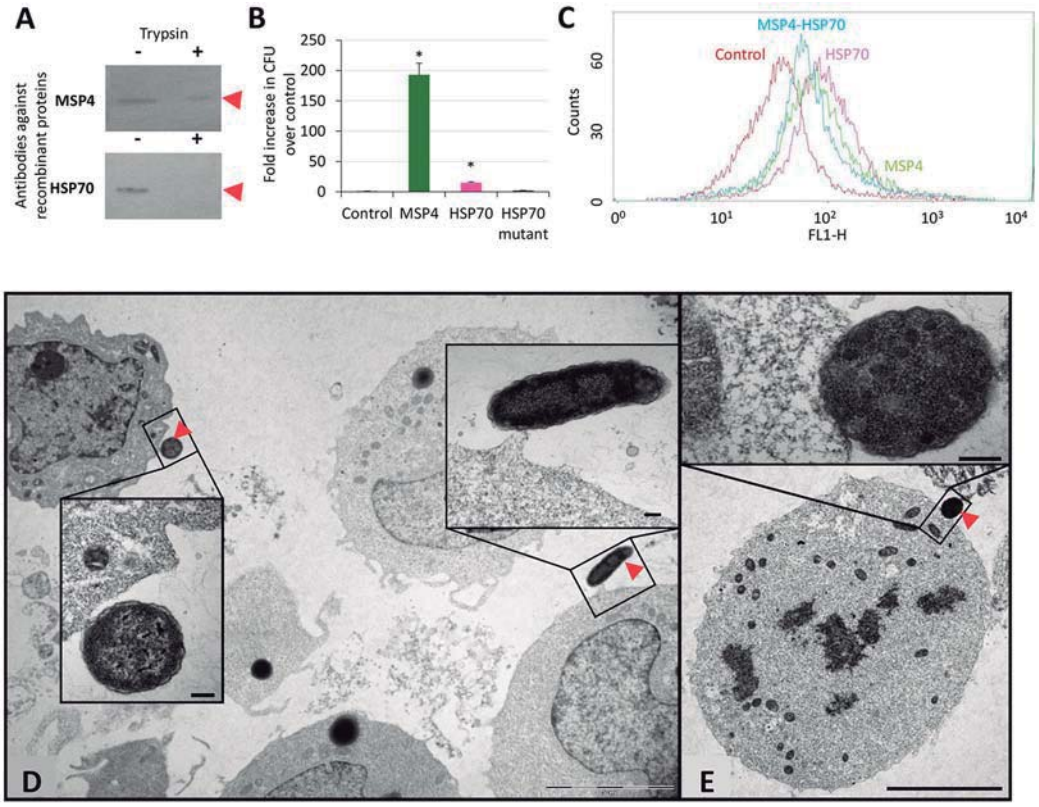

FIGURE 1 | Role of *A. phagocytophilum* MSP4 and HSP70 proteins in interactions with HL60 human cells. **(A)** *A. phagocytophilum* (NY18) purified from infected HL60 human cells were mock treated (–) or surface digested with trypsin (+) and 10 μg protein loaded onto polyacrylamide gels for Western blot analysis using rabbit antibodies produced against recombinant proteins. These experiments have been repeated three times with similar results. **(B)** *E. coli* strains were grown and induced for the production of recombinant proteins. *E. coli* cells transformed with expression vector alone were used as negative control (Control). Bacteria adhesion to human HL60 cells was quantitated as the number of colony forming units (CFU) recovered from each test and compared to the control values by Chi2 test. Asterisks denote statistical significant differences between CFU recovered from *E. coli* transformed with MSP4 or HSP70 and the control ($P < 0.001$; $N = 2$ replicates per treatment). **(C)** HL-60 cells were incubated with *A. phagocytophilum* human NY18 isolate in the presence of recombinant MSP4, HSP70 and their combination. HL60 cells incubated with PBS served as negative controls. After washing to remove unbound bacteria and proteins, host cells were incubated for 48 h and binding of recombinant proteins to human host cells was analyzed by flow cytometry. The viable cell population was gated according to forward scatter and side scatter parameters. **(D,E)** Representative images of the adhesion of recombinant *E. coli* producing membrane exposed *A. phagocytophilum* proteins to human HL60 cells. **(D)** *E. coli* producing MSP4 or **(E)** HSP70 were incubated with HL60 cells and revealed by TEM to show adhesion to human cells (arrows). Cells incubated with control bacteria did not show adhesion to HL60 cells. Details of both interacting cells are shown in insets. Scale bars: 5 μm **(D,E)** and 200 nm (insets).

The Antibody Response in Immunized Lambs Is Specific for *A. phagocytophilum* MSP4 and HSP70 Recombinant Proteins

The results showed that anti-MSP4 (**Figure 4A**) and anti-HSP70 (**Figure 4B**) IgG antibody titers were higher in MSP4-immunized than in control animals from second immunization until 94 dpi. In MSP4-HSP70 immunized lambs, anti-MSP4 IgG antibody titers were significantly higher until 10 dpi (**Figure 4A**), while anti-HSP70 IgG antibody titers remained higher then in control animals until 94 dpi (**Figure 4B**). The increase in the IgG antibody response to *A. phagocytophilum* MSP4 and HSP70 proteins after experimental infection was higher in immunized than in control animals (**Figures 4A,B**), suggesting an anamnestic response that may be protective against pathogen infection.

The IgG antibody response in MSP4 and MSP4-HSP70 immunized lambs was specific for MSP4 and HSP70 proteins as supported by the dilution effect observed after incubating sera collected before infection (0 dpi) with *A. phagocytophilum* purified from infected HL60 human cells (**Figure 4C**). However, the antibody response in MSP4-immunized lambs raised a question regarding the anti-HSP70 response in these animals. Possible explanations to this question are the production of polyreactive antibodies, and the existence of common B-cell epitopes between *A. phagocytophilum* MSP4 and HSP70 proteins. The MSP4 and HSP70 proteins were aligned and the linear B-cell epitopes were predicted and aligned to both protein sequences (Supplementary Figure 2A). A total of 14 and 32 linear B-cell epitopes were predicted for MSP4 and HSP70, respectively. Only epitopes longer than 8 amino acids were included in further analysis, resulting in 5 and 12 linear B-cell epitopes identified in MSP4 and HSP70, respectively. Only one epitope of MSP4 (DGATGYAI) aligned without gaps to a region of HSP70 (DGQTAVTI)

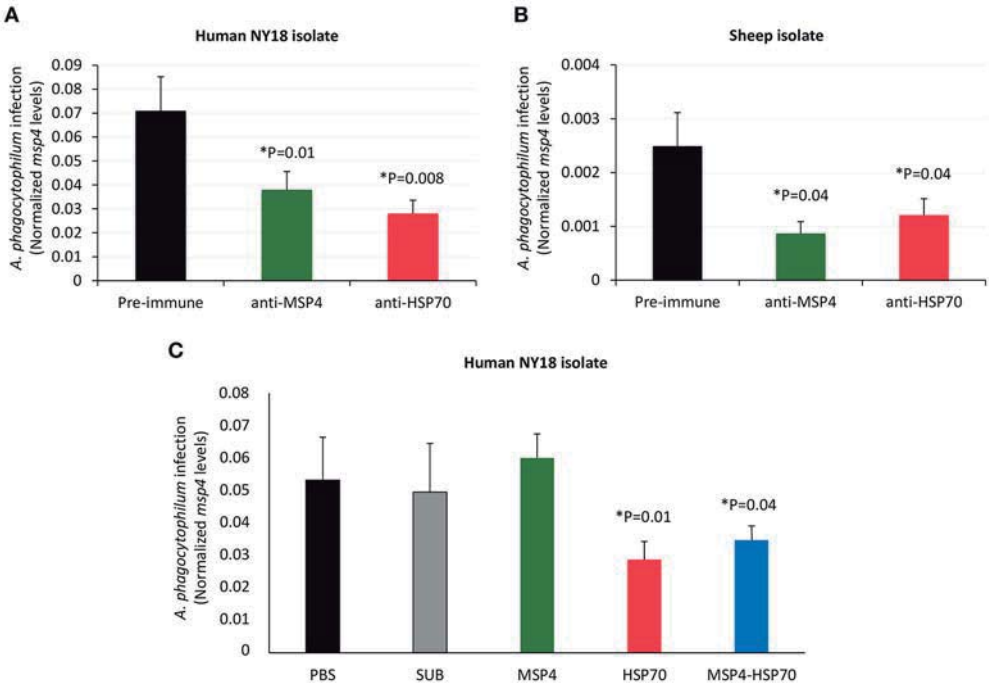

FIGURE 2 | Role of *A. phagocytophilum* MSP4 and HSP70 proteins in infection of HL60 human cells. **(A,B)** Rabbit antibodies against *A. phagocytophilum* MSP4 and HSP70 recombinant proteins or **(C)** MSP4, HSP70 and MSP4-HSP70 recombinant proteins were used to characterize the inhibition of pathogen infection of HL60 human cells. Rabbit purified IgGs or recombinant proteins were mixed with *A. phagocytophilum* inoculum of human NY18 or sheep isolates for 60 min before being placed on the cell monolayers. Treatments included rabbit pre-immune serum, PBS and SUB as negative controls. *A. phagocytophilum* infection levels were determined by *msp4* real-time PCR normalizing against human *β-actin*. Results were compared between groups treated with pre-immune and anti-MSP4/HSP70 antibodies **(A,B)** or between groups treated with PBS or SUB and recombinant proteins **(C)** by the Student's *t*-test with unequal variance (*$P < 0.05$; $N = 4$ replicates per treatment).

with 50% identity (Supplementary Figure 2A). Three epitopes from HSP70 (FNDAQRQATKDAGTI, AGIKDNSKV and SNCSTDTLQQ) aligned without gaps to regions of MSP4 (FVAVGRDATLTPDNF, AGIPASNRV and AVCACSLLIS), with 26, 44, and 20% identity, respectively. These results suggested that antibodies against MSP4 epitopes (i.e., DGATGYAI) could cross-react with a region of HSP70 (DGQTAVTI), thus explaining the anti-HSP70 response in MSP4-immunized lambs. Furthermore, these epitopes were highly conserved because *A. phagocytophilum* MSP4 and HSP70 protein sequences show a high homology between different strains (Supplementary Figures 2B–D). In 56 of the MSP4 sequences available containing this region, the B-cell epitope was conserved (Supplementary Figure 2D). This region was conserved in all HSP70 sequences available in GenBank (Supplementary Figure 2D).

Immunization of Lambs with *A. phagocytophilum* MSP4 and MSP4-HSP70 Recombinant Proteins Is Only Partially Protective against TBF

To address the role of *A. phagocytophilum* MSP4 and HSP70 proteins as potential targets for the development of vaccines for the control of pathogen infection in vertebrate hosts, their potential protective capacity was characterized in immunized lambs.

The IgG antibody levels to MSP4 and HSP70 antigens remained higher after infection in immunized animals when compared to controls (**Figures 4A,B**). Although all animals showed signs of fever after infection with similar fever relapses, rectal temperature decreased faster in lambs immunized with MSP4-HSP70 (**Figure 3A**). The anemia typical of TBF was evident in control sheep at 8–10 dpi, while in immunized animals it did not occur (MSP4 group) or was observed at 9 dpi only (MSP4-HSP70 group; **Figure 3B**). Erythrocyte counts were not affected in immunized animals (Supplementary Table 1). The analysis of leukocytes, lymphocytes and eosinophils showed lower values at various dpi in immunized animals when compared to controls (Supplementary Table 1). In contrast, neutrophil and monocyte levels were higher in immunized animals when compared to controls at different dpi (**Figures 3C,D**; Supplementary Table 1). These results showed that while immunized animals presented evidence of TBF such as fever and neutropenia, the response to immunization resulted in less severe anemia in response to infection.

Although the percent of infected neutrophils was apparently higher in immunized than in control animals at some

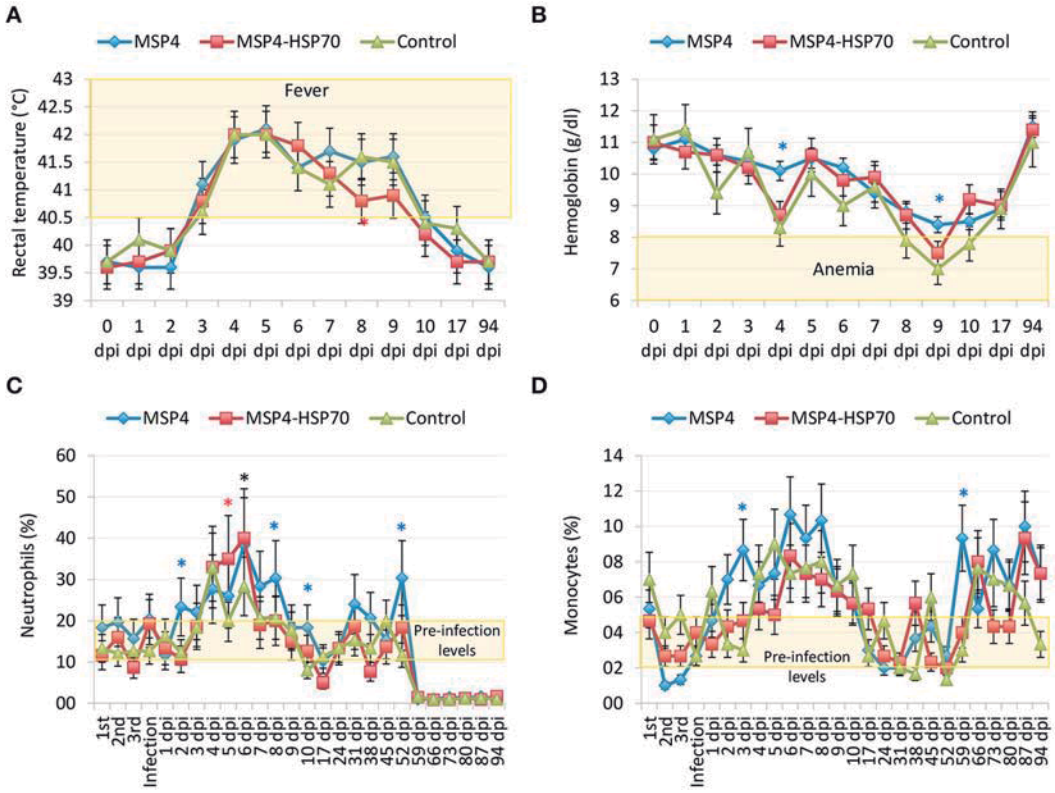

FIGURE 3 | Evidence of TBF in lambs experimentally infected with *A. phagocytophilum* sheep isolate. Groups of three lambs each were immunized with recombinant MSP4, MSP4-HSP70 combination or adjuvant/saline control and experimentally infected with a sheep isolate of *A. phagocytophilum*. **(A)** Rectal temperatures were taken daily until 10 dpi and then weekly until 94 dpi. **(B–D)** Whole blood was collected in EDTA-containing tubes from the jugular vein of each lamb at different time points for different hematological analyses including **(B)** hemoglobin, and percent **(C)** neutrophils and **(D)** monocytes for leukocyte cell differentiation using an electronic counter (Hemavet 950, Drew, USA). Results from rectal temperature and hematological analyses were compared between immunized and control groups by two-way ANOVA test (*$P < 0.05$; $N = 3$ replicates per treatment). Red and blue asterisks denote statistical significant differences between MSP4-HSP70 and MSP4 immunized animals and controls, respectively.

dpi, the results suggested differences in the initial infection rate despite the injection of the same amount of unfrozen infected blood (Supplementary Table 1). These differences could be explained by variations in cell viability between different inoculums, resulting in animal-to-animal variations in the initial infection rate. Therefore, the differential percent of infected neutrophils with respect to the initial value at 3 dpi was used to characterize the effect of vaccination to normalize for these differences. The results showed a significant decrease in *A. phagocytophilum*-infected neutrophils in animals immunized with the MSP4-HSP70, but not MSP4 antigen at 8–10 dpi when compared to controls (**Figure 5A**). Furthermore, the *A. phagocytophilum* normalized DNA levels were significantly lower in lambs immunized with MSP4 and MSP4-HSP70 antigens at 17 dpi (**Figure 5B**). Taken together, these results suggested that the number of infected neutrophils decreased at 8–10 dpi in response to immunization with MSP4-HSP70, while pathogen levels per cell were lower in immunized lambs when compared to controls at 17 dpi.

The Antibodies against Recombinant Proteins in Immunized Lambs Do Not Inhibit the *A. phagocytophilum* Infection of HL60 Human Cells

An antibody inhibition assay using IgG from immunized sheep at 0 and 94 dpi was conducted to further characterize the antibody response in immunized lambs in relation with the protective capacity of MSP4 and MSP4-HSP70 antigens (**Figure 6**). While rabbit IgG antibodies against *A. phagocytophilum* MSP4 and HSP70 recombinant proteins inhibited pathogen infection of HL60 human cells (**Figures 2A**, **7**), sheep IgG collected from immunized animals before infection (0 dpi) and after infection at the end of the experiment (94 dpi) did not affect pathogen infection (**Figure 6**). These results evidenced differences in the IgG response between immunized rabbits and lambs, and provided support for the limited protection against *A. phagocytophilum* infection observed in sheep immunized with MSP4 and MSP4-HSP70.

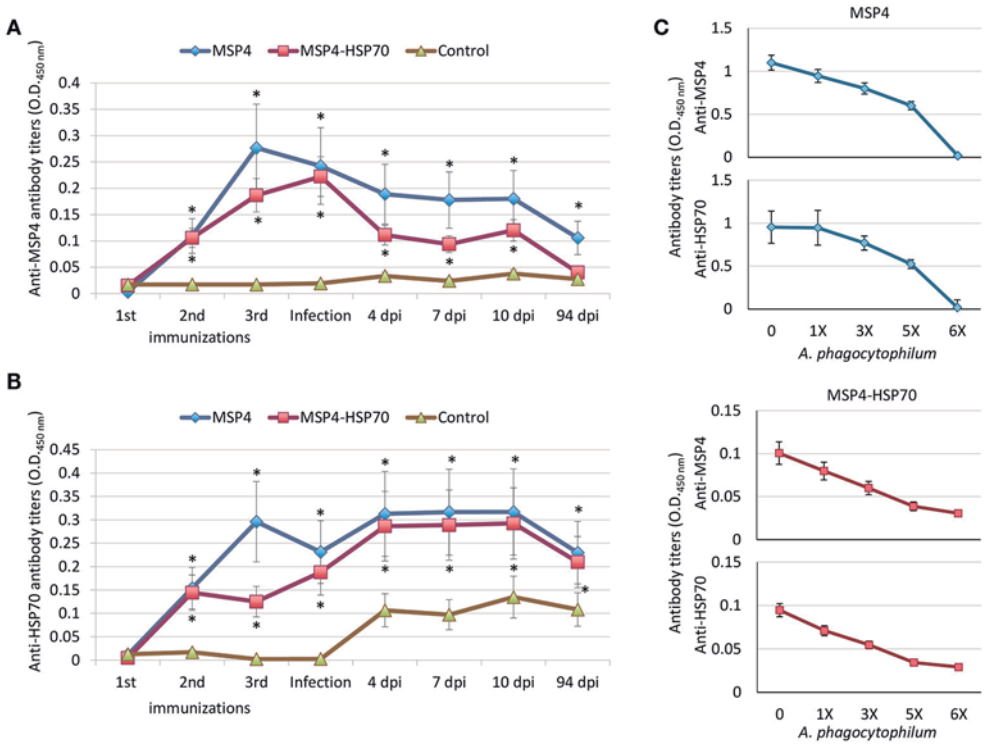

FIGURE 4 | Antibody response in lambs immunized with *A. phagocytophilum* MSP4 and MSP4-HSP70 proteins. Groups of three lambs each were immunized with recombinant MSP4, MSP4-HSP70 combination or adjuvant/saline control and experimentally infected with a sheep isolate of *A. phagocytophilum*. An indirect ELISA test was performed to detect IgG antibodies against **(A)** MSP4 and **(B)** HSP70 proteins in immunized and control lambs using serum samples collected before each immunization and at 0, 7, 10, and 94 dpi. Antibody titers were expressed as OD_{450nm} ($OD_{lambsera} - OD_{PBScontrol}$). The results were compared between immunized and control groups by two-way ANOVA test (*$P < 0.05$; $N = 3$ replicates per treatment). **(C)** The antigen-specific IgG antibody response in immunized lambs was corroborated by ELISA using pooled sera collected at 0 dpi, but incubating sera with different concentrations of *A. phagocytophilum* purified from infected HL60 human cells.

DISCUSSION

Anaplasma phagocytophilum transmembrane and surface proteins are involved in infection of vertebrate host cells (Seidman et al., 2015; Truchan et al., 2016). The *A. phagocytophilum* MSP4 and HSP70 proteins were previously shown to interact when localized on the bacterial membrane, with a possible role during pathogen infection of tick cells (Villar et al., 2015b). These results, together with the finding that *A. phagocytophilum* evolved common molecular mechanisms to establish infection in tick vectors and vertebrate hosts (de la Fuente et al., 2016a), suggested the hypothesis that MSP4 and HSP70 proteins have similar functions in host-pathogen and tick-pathogen interactions with possible implications as potential targets for the development of vaccines for the control of pathogen infection in both ticks and vertebrate hosts.

To address this hypothesis, we first characterized the role of these bacterial proteins in the infection of vertebrate host cells. The results using *A. phagocytophilum* derived from infected HL60 human cells corroborated those previously obtained with *A. phagocytophilum* derived from ISE6 tick cells (Villar et al., 2015b). The results showed that MSP4 is a transmembrane protein in *Anaplasma* spp. (de la Fuente et al., 2001), while HSP70 was probably translocated to the cell surface by still unknown mechanisms in which the bacterial type IV secretion system (T4SS) may be involved (Niu et al., 2006; Lin et al., 2007; Villar et al., 2015b). The binding of HSP70 and MSP4 to HL60 human cells was characterized using two alternative models based on recombinant proteins and *E. coli* producing surface-exposed *A. phagocytophilum* proteins with similar results, therefore supporting their role in the interaction with host cells. Although it is possible that the production of *A. phagocytophilum* proteins in *E. coli* may alter bacterial surface to cause binding to HL60 human cells not mediated by MSP4 and HSP70 proteins, previous results using this system with *A. marginale* MSP1a and MSP1b (de la Fuente et al., 2001) and with *A. phagocytophilum* proteins in tick cells (Villar et al., 2015b) makes this possibility unlikely. Furthermore, *E. coli* producing the mutant HSP70 with truncated peptide-binding domains that are involved in protein-protein interactions (Villar et al., 2015b) did not bind to human HL60 cells, therefore supporting the role of this protein in interactions with host cells.

Protein models supported the interaction between *A. phagocytophilum* MSP4 and HSP70 proteins when localized on the bacterial membrane, which was previously demonstrated in tick cells and may be functionally relevant for pathogen

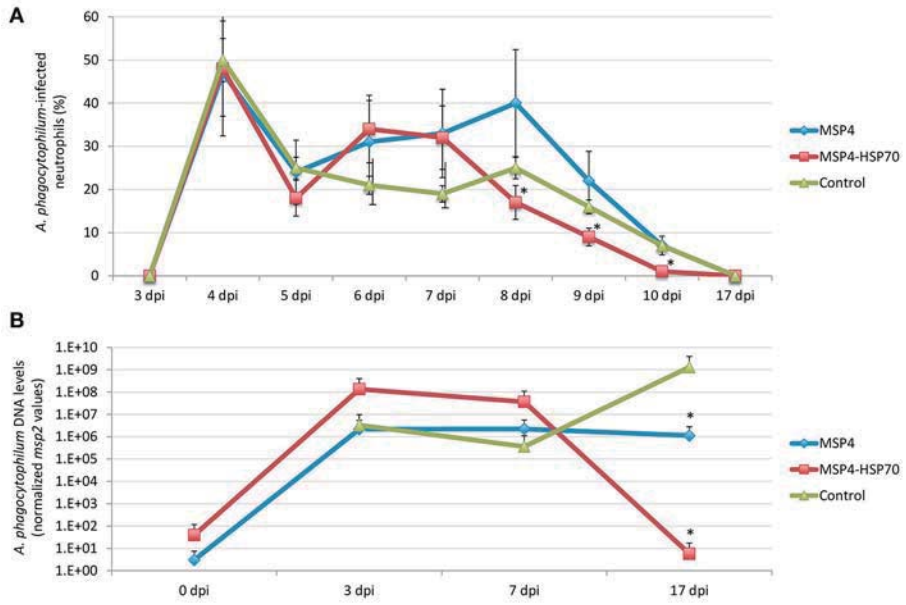

FIGURE 5 | *Anaplasma phagocytophilum* infection levels in immunized and control lambs. **(A)** Blood smears stained with Giemsa stain were examined to investigate the presence of *A. phagocytophilum* in blood cells. At least 100 neutrophils were counted and examined to calculate the number of infected neutrophils per milliliter blood of each lamb. The differential percent of *A. phagocytophilum*-infected neutrophils was calculated as the difference between values at different dpi and values at 3 dpi when infected neutrophils were first detected. The results were compared between immunized and control groups by two-way ANOVA test (*$P < 0.05$; $N = 3$ replicates per treatment). **(B)** For the quantitative analysis of *A. phagocytophilum* infection levels, a quatitative real-time PCR was conducted. The *A. phagocytophilum* DNA levels were normalized against sheep *aldolase B* using the genNorm method (ddCT method as implemented by Bio-Rad iQ5 Standard Edition, Version 2.0). Normalized Ct values were compared between immunized and control groups by Student's *t*-test with unequal variance (*$P < 0.05$; $N = 3$ replicates per treatment).

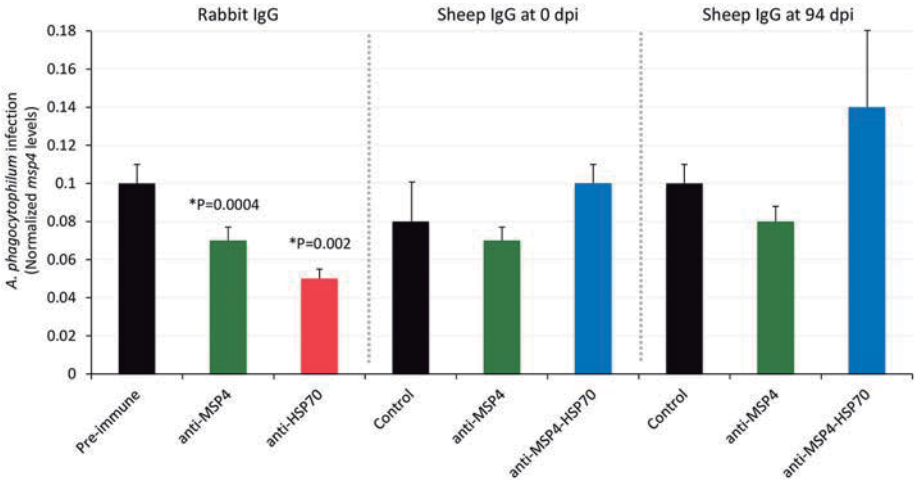

FIGURE 6 | Role of antibodies against recombinant proteins from immunized lambs in the inhibition of *A. phagocytophilum* infection of HL60 human cells. Sheep IgG antibodies against *A. phagocytophilum* MSP4 and MSP4-HSP70 recombinant proteins were obtained from control and immunized sheep at 0 and 94 dpi and used to characterize the inhibition of pathogen infection of HL60 human cells. Treatments included purified IgGs from rabbit pre-immune, anti-MSP4 and anti-HSP70 sera. Purified IgGs were mixed with *A. phagocytophilum* inoculum of human NY18 isolate for 60 min before being placed on the cell monolayers. *A. phagocytophilum* infection levels were determined by *msp4* real-time PCR normalizing against human *β-actin*. Results were compared between groups treated with pre-immune/control and anti-MSP4/HSP70 antibodies by the Student's *t*-test with unequal variance (*$P < 0.05$; $N = 4$ replicates per treatment).

infection of both tick and vertebrate host cells (Villar et al., 2015b). Antibody inhibition assays showed that as previously discussed in the experiments using ISE6 tick cells (Villar et al., 2015b), antibodies against MSP4 and HSP70 proteins could affect the interaction between bacterial ligands and tick receptors to interfere with infection or affect the interaction with

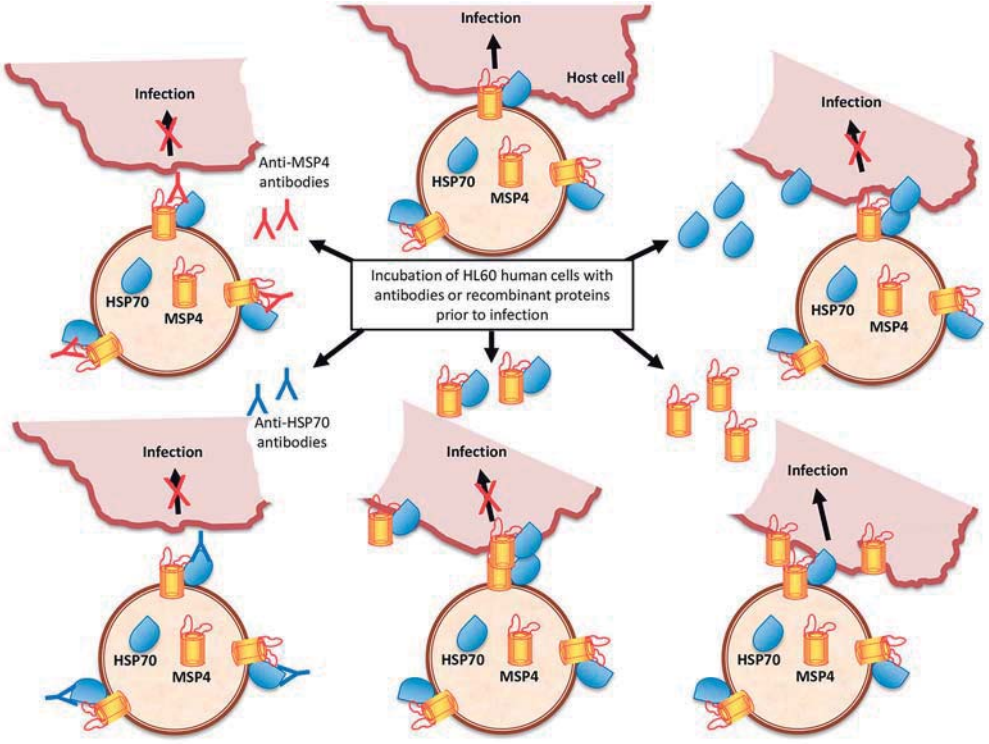

FIGURE 7 | *Anaplasma phagocytophilum* HSP70 and MSP4 are necessary for pathogen infection of host cells. Based on the results of this study, a model was developed to explain the role of HSP70 and MSP4 during pathogen infection of host cells. HSP70 and MSP4 form a complex on the bacterial membrane where MSP4 probably acts a doking protein for HSP70. The incubation of HL60 human cells with recombinant HSP70 or MSP4-HSP70 interacting proteins inhibits infection by interfering with pathogen interaction with host cells mediated by HSP70, which is necessary for infection. However, the addition of recombinant MSP4 does not affect infection because the interaction of bacterial HSP70 with host cells occurs and is sufficient for infection. The anti-MSP4 antibodies probably inhibit infection through binding to MSP4 at the MSP4-HSP70 interaction site, thus preventing HSP70 adhesion to host cells. The anti-HSP70 antibodies bind to HSP70 and prevent the interaction with host cells required for pathogen infection.

proteins functionally important for bacterial infection and/or multiplication in host cells. However, the inhibition assay using recombinant proteins suggested different roles for HSP70 and MSP4 during pathogen infection of host cells (**Figure 7**). While HSP70 seems to be directly involved in the pathogen interaction with host cells, MSP4 may acts as a doking protein for HSP70 to form the MSP4-HSP70 complex on the bacterial membrane (**Figure 7**). These results extended previous findings in tick cells (Villar et al., 2015b), supporting the role of MSP4 and HSP70 proteins in *A. phagocytophilum* infection and/or adhesion to vertebrate host cells.

The role of *Anaplasma* MSPs and other outer membrane proteins and invasins in adhesion to tick and vertebrate host cells for bacterial infection has been demonstrated in *A. marginale* and *A. phagocytophilum* (de la Fuente et al., 2001; Garcia-Garcia et al., 2004; Ge and Rikihisa, 2007; Rikihisa, 2011; Ojogun et al., 2012; Severo et al., 2012, 2013; Kahlon et al., 2013; Seidman et al., 2015; Truchan et al., 2016; Hebert et al., 2017). This mechanism appears to be conserved in other tick-borne pathogens, and in pathogen interactions with other arthropod vector species (de la Fuente et al., 2017). HSP70 was shown to relocate to the *Bacillus subtilis* membrane to restore membrane structure and function after ethanol stress (Seydlová et al., 2012), and to function in the molecular processing of *Borrelia burgdorferi* flagellin (Scopio et al., 1994). This protein may be functionally relevant at the *A. phagocytophilum*-host interface, and may interact with other membrane proteins for its function during pathogen infection (Susin et al., 2006; Multhoff, 2007).

To evaluate the potential protective capacity of these proteins, lambs that are natural hosts for this pathogen were immunized with recombinant MSP4, MSP4-HSP70 combination or adjuvant/saline control and infected with a sheep isolate of *A. phagocytophilum*. The MSP4-HSP70 combination was included based on evidence of protein-protein interactions, suggesting a physical and/or functional connection between these proteins (Villar et al., 2015b).

The results evidenced signs of TBF such as fever, anemia, and neutropenia in lambs infected with *A. phagocytophilum*, therefore validating the model for the comparative analysis between immunized and control animals. In sheep and dogs, *A. phagocytophilum* infection is accompanied by fever of approximate 7 days duration, which is the main clinical sign of TBF (Eberts et al., 2011; Stuen et al., 2011; Severo et al., 2013). The severe leukopenia and especially the prolonged neutropenia that

accompanies the disease are also evident with TBF (Eberts et al., 2011; Stuen et al., 2011; Severo et al., 2013). Immune suppression by impaired antibody and lymphocyte response and reduced oxidative burst, together with anemia and monocytosis have been also reported in animals infected with *A. phagocytophilum* (Whist et al., 2003; Eberts et al., 2011). Weaning weight is also affected in lambs infected with *A. phagocytophilum* (Grøva et al., 2011).

The immunized lambs raised an antibody response that was specific for *A. phagocytophilum* MSP4 and HSP70 recombinant proteins. However, MSP4-immunized lambs developed an anti-HSP70 response. One possible explanation to this finding was the production of polyreactive antibodies, which constitute a major component of the natural antibodies that bind with low affinity to structurally unrelated antigens with broad antibacterial activity (Gunti and Notkins, 2015). Additionally, the analysis of protein sequences showed the existence of common B-cell epitopes between *A. phagocytophilum* human isolate MSP4 and HSP70 proteins that may also contribute to serum cross-reactivity. The B-cell epitopes are protein regions that bind to antibodies. Most epitopes are composed of different parts of the polypeptide chain that are brought into spatial proximity by the three-dimensional structure of the protein. These discontinuous epitopes can also react with continuous peptide fragments (i.e., linear epitopes) within the protein (Larsen et al., 2006). Epitopes can be understood as "antigenic determinants" within proteins and homology between linear epitopes can results in antibody cross-reactivity (Terajima et al., 2013).

Despite the effect of *A. phagocytophilum* infection on the impairment of antibody response in sheep (Whist et al., 2003), the results showed that IgG antibody levels to MSP4 and HSP70 antigens remained higher after infection in immunized animals when compared to controls. In contrast to the results reported in lambs immunized with inactivated *A. phagocytophilum* (Stuen et al., 2015), the number of fever relapses was similar between immunized and control animals, supporting that antigen-specific response is different from that obtained with whole organisms. The immunization with MSP4-HSP70 resulted in a decrease in the percent of infected neutrophils and pathogen levels per cell, supporting that immunization with MSP4-HSP70 was only partially protective for the control of *A. phagocytophilum* infection of neutrophils.

A previous experiment using a crude *A. phagocytophilum* protein extract for immunization did not protect against pathogen infection in sheep, but immunized lambs had reduced levels of infection (Stuen et al., 2015). The authors discussed that the lack of protection was probably due to the presence of not protective dominant antigens in the vaccine preparation, stressing the need for the identification of protective antigens conserved among different strains (Stuen et al., 2015). The results obtained here were similar to those reported previously by Stuen et al. (2015), but using two proteins shown to be highly conserved and involved in pathogen infection and/or interaction with host cells. The failure to protect lambs from *A. phagocytophilum* infection after immunization with MSP4 and MSP-HSP70 antigens may be due to several factors. Although these proteins seem to be involved in host-pathogen interactions and infection, other proteins may be also necessary for infection within this mechanism or as part of alternative mechanisms of infection. The results showed that IgG antibodies rose in immunized lambs did not inhibit *A. phagocytophilum* infection of HL60 human cells, suggesting differences between rabbit and sheep IgG responses that may be associated with epitope recognition in MSP4 and HSP70 proteins. These differences in the immune response between rabbits and sheep could be used to identify candidate protective regions or epitopes in MSP4 and HSP70 proteins to increase vaccine efficacy. Additionally, the intravenous inoculation of infected blood is different from natural infection after tick bite, and may affect the evaluation of the protective response after immunization.

CONCLUSIONS

The *A. phagocytophilum* transmembrane and surface proteins play a crucial role during infection and multiplication in host neutrophils (Ge and Rikihisa, 2007; Rikihisa, 2011; Severo et al., 2012, 2013; Seidman et al., 2015; Truchan et al., 2016). However, the results reported here provided the first evidence for the role of *A. phagocytophilum* MSP4 and HSP70 proteins in this process. These results suggested that while membrane-localized MSP4 and HSP70 were involved in *A. phagocytophilum* interaction with host cells, HSP70 was directly implicated in pathogen infection. As for other intracellular pathogens, cellular immunity is essential for an effective protection against infection by *Anaplasma* spp. (Palmer et al., 1999; Hajdušek et al., 2013; de la Fuente et al., 2016a; Shaw et al., 2017). However, previous experiments have provided evidence that antibodies to bacterial proteins have a protective effect on infected hosts (Kaylor et al., 1991; Messick and Rikihisa, 1994; Sun et al., 1997; de la Fuente et al., 2003; Gomes-Solecki, 2014; Stuen et al., 2015). The results obtained here showed that the *A. phagocytophilum* MSP4-HSP70 antigen was only partially protective against pathogen infection in sheep. This limited protection may be associated with several factors, including the recognition of non-protective epitopes by IgG from immunized lambs. Nevertheless, these antigens may constitute candidate protective antigens for the development of vaccines against TBF in combination with other antigens. Focusing on the characterization of host protective immune mechanisms and protein-protein interactions at the host-pathogen interface may lead to the discovery and design of new protective antigens (de la Fuente et al., 2016c,d). Additionally, proteins involved in tick-pathogen and host-pathogen interactions such as *A. phagocytophilum* MSP4 and HSP70 may be used to develop double effect vaccines targeting infection in both vertebrate hosts and tick vectors (de la Fuente and Contreras, 2015).

AUTHOR CONTRIBUTIONS

Jd and CG conceived the study. MC, PA, LM, IF, MVa, MVi, and NA performed the experiments. MC, AG, and SS performed the vaccine trial. AC, MC, JV, and Jd performed

data analyses. Jd and MC wrote the paper, and other coauthors made additional suggestions and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcimb.2017.00307/full#supplementary-material

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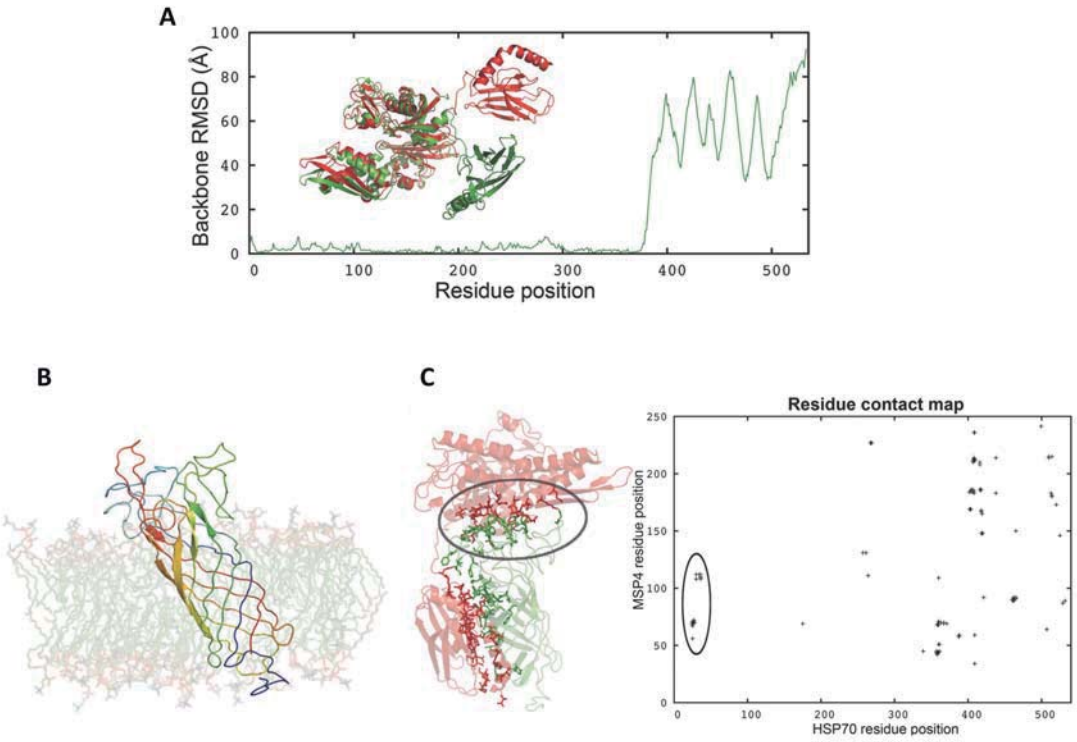
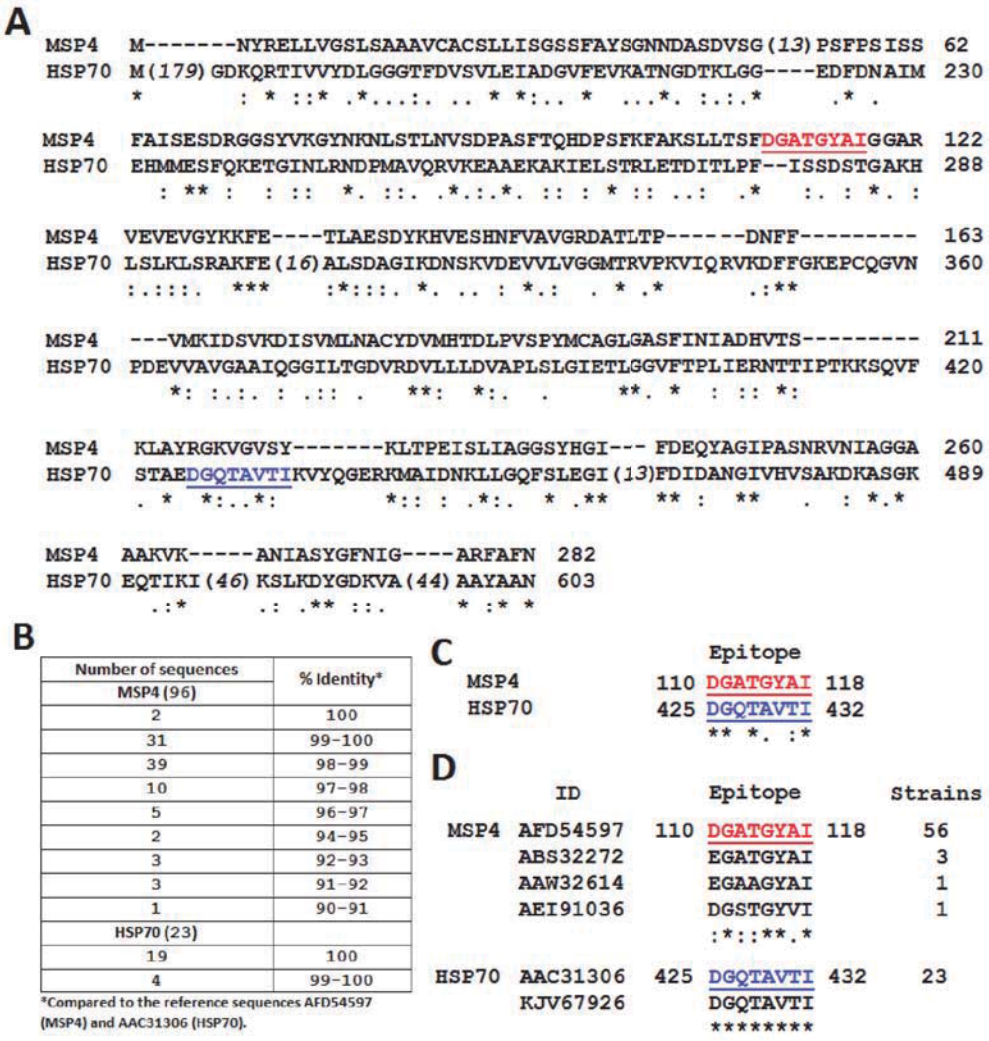
Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Figures



Supplementary Figure 1. Structural analysis and interactions of *A. phagocytophilum* MSP4 and HSP70 proteins. (A) Line graph on the α-carbon (backbone; y-axis) root mean square deviation (RMSD), for each residue (x-axis), between the tertiary structures of the apo-AHSP70 (red) and bound HSP70 (green). (B) Membrane-bound position and orientation of MSP4 that is color-coded from the N-terminus (blue) to the C-terminus (red). The membrane is shown as sticks and is atom color-coded (carbon = green; nitrogen = blue; oxygen = red; phosphorus = purple); hydrogen atoms are not shown. (C) HSP70 (red) bound to MSP4 (green) with their contact residues shown as predicted by SwarmDock. The Cartesian plot to the right is the all-reside contact map between the bound structures. The circles correspond to the tertiary (structure) and residue (plot) positions making contacts between the β-hairpin loops of MSP4 and the N-terminus of HSP70 that are exposed extracellularly, and therefore may act as markers for mutational studies and antibody targeting.

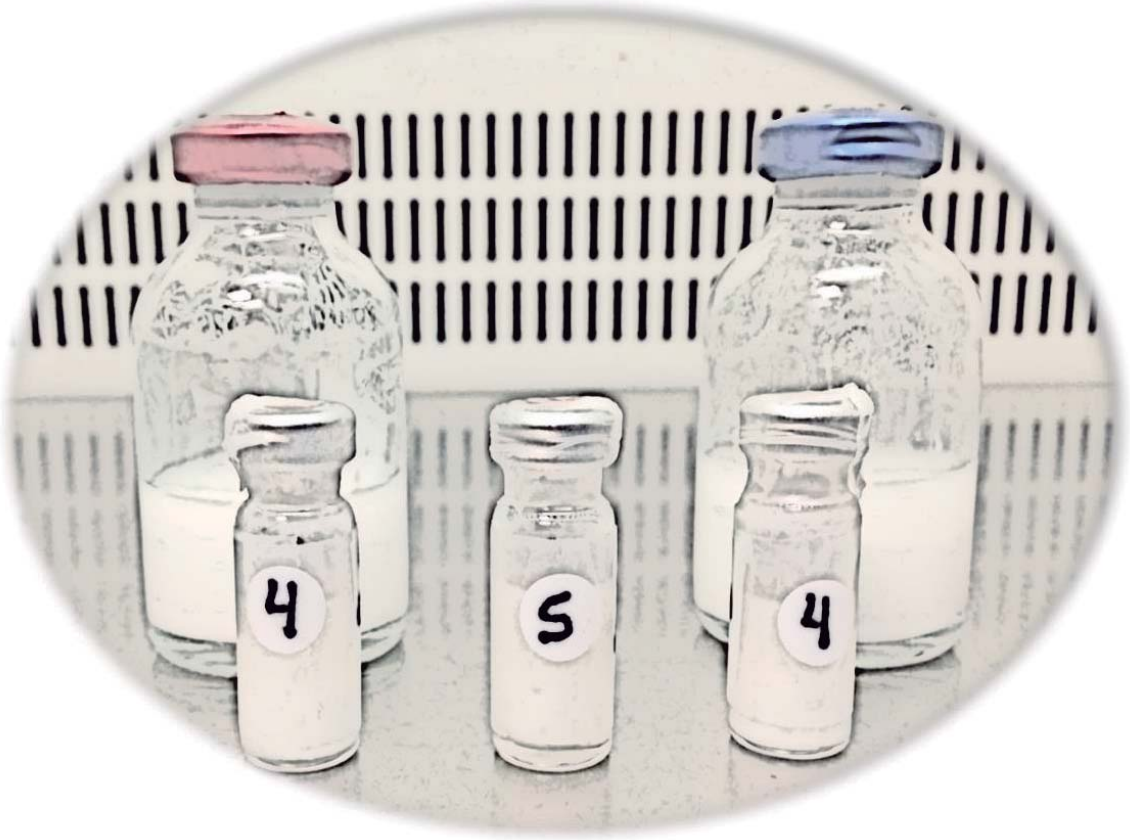


Number of sequences MSP4 (96)	% Identity*
2	100
31	99-100
39	98-99
10	97-98
5	96-97
2	94-95
3	92-93
3	91-92
1	90-91
HSP70 (23)	
19	100
4	99-100

*Compared to the reference sequences AFD54597 (MSP4) and AAC31306 (HSP70).

Supplementary Figure 2. Comparison between MSP4 and HSP70 sequences. (A) Alignment between *A. phagocytophilum* MSP4 and HSP70 amino acid sequences. The number of amino acids in gap regions is shown in parenthesis. The B-cell epitope of MSP4 is underlined in blue. The region in HSP70 with the highest identify to the B-cell epitope of MSP4 is underlined in red. (B) Percent identity between MSP4 (N=96) and HSP70 (N=23) sequences available in GenBank. (C) Alignment between the B-cell epitope of MSP4 and the homolog region in HSP70. (D) Comparison of the B-cell epitope in MSP4 (N=61) and HSP70 (N=23) sequences available in GenBank that contain this region. For each variant, an ID of a reference sequence is provided.

CHAPTER III.
Vaccination trials with tick protective antigens



Contreras, M. and de la Fuente, J. (2016). Control of *Ixodes ricinus* and *Dermacentor reticulatus* tick infestations in rabbits vaccinated with the Q38 Subolesin/Akirin chimera. *Vaccine.* 34 (27): 3010-3013.

Contreras, M. and de la Fuente, J. (2017). Control of infestations by *Ixodes ricinus* tick larvae in rabbits vaccinated with aquaporin recombinant antigens. *Vaccine.* 35 (9): 1323-1328.

Contreras, M., Moreno-Cid, J.A., Domingos, A., Canales, M., Díez-Delgado, I., Pérez de la Lastra, J.M., Sánchez, E., Merino, O., Zavala, R.L., Ayllón, N., Boadella, M., Villar, M., Gortázar, C. and de la Fuente, J. (2015). Bacterial membranes enhance the immunogenicity and protective capacity of the surface exposed tick Subolesin--*Anaplasma marginale* MSP1a chimeric antigen. *Ticks and Tick Borne Diseases.* 6 (6): 820-828.

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Brief report

Control of *Ixodes ricinus* and *Dermacentor reticulatus* tick infestations in rabbits vaccinated with the Q38 Subolesin/Akirin chimera

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ABSTRACT

Diseases transmitted by ticks greatly impact human and animal health and their control is important for the eradication of tick-borne diseases. Vaccination is an environmentally friendly alternative for tick control. Recent results have suggested that Subolesin/Akirin (SUB/AKR) are good candidate antigens for the control of arthropod vector infestations. Here, we describe the effect of vaccination with the Q38 chimera containing SUB/AKR conserved protective epitopes on *Ixodes ricinus* and *Dermacentor reticulatus* tick larval mortality, feeding and molting. We demonstrated that Q38 vaccination had an efficacy of 99.9% and 46.4% on the control of *I. ricinus* and *D. reticulatus* larvae by considering the cumulative effect on reducing tick survival and molting. The effect of the Q38 vaccine on larval feeding and molting is essential to reduce tick infestations and supports that Q38 might be a candidate universal antigen for the control of multiple tick species that can infest the same host.

1. Introduction

Diseases transmitted by arthropod vectors greatly impact human and animal health [1]. Among arthropod vectors, ticks represent a growing burden for the transmission of a variety of pathogens with increasing worldwide prevalence [2,3]. Tick species such as *Ixodes ricinus* and *Dermacentor reticulatus*, infest humans, pets and other domestic and wild animals and transmit disease-causing pathogens such as *Borrelia* spp. (Lyme disease and various borreliosis), TBEV (tick-borne encephalitis), *Anaplasma phagocytophilum* (human and animal anaplasmosis), *Francisella tularensis* (tularemia), *Rickettsia* spp. (human and animal rickettsiosis), OHFV (Omsk hemorrhagic fever), and *Babesia canis* (canine babesiosis) [2,4].

Despite the impact of vector-borne diseases (VBD) on human and animal health, yellow fever is one of the few VBD with an effective vaccine [5]. Therefore, the control of arthropod vectors is important for the eradication of VBD [6–8]. Vaccination is an environmentally friendly alternative for vector control that allows control of several VBD by targeting their common vector [9,10]. Recently, vaccination with arthropod vector antigens in combination with pathogen-derived antigens has been proposed as an alternative for the control of vector populations and reduction in pathogen infection and transmission [6].Subolesin (SUB) was discovered as a tick protective antigen in the mouse model for *Ixodes scapularis* infestations [11]. Akirin (AKR) is the SUB ortholog in insects [12–14]. Both proteins act as transcriptional regulatory factors affecting the expression of signal transduction and innate immune response genes [14–18]. Experiments with recombinant SUB/AKR have shown the effect of vaccination on the control of several arthropod vectors including hard and soft ticks, mosquitoes, sand flies, poultry red mites and sea lice and the infection/transmission of vector-borne pathogens (recently reviewed in de la Fuente and Contreras [6]). Recently, SUB/AKR chimeras were designed containing protective epitopes from both proteins [19,20]. The vaccine containing the chimeric antigens, Q38 and Q41, showed an antibody-dependent effect on the control of *I. ricinus*, *Aedes albopictus* and *Phlebotomus perniciosus* infestations in vaccinated mice [20]. Furthermore, *Rhipicephalus microplus* infestations were reduced by 69% and infection DNA levels with transmitted pathogens, *Anaplasma marginale* and *Babesia bigemina* were reduced by 40–50% in cattle vaccinated with Q38 when compared to controls [21]

These results suggested that Q38 might be a candidate universal antigen for the control of multiple arthropod ectoparasites and infection with vector-borne pathogens. To characterize further the

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0264-410X/

efficacy of the Q38 chimeric vaccine, here we provide evidence showing the effect of vaccination on the control of *I. ricinus* and *D. reticulatus* in rabbits. These results expanded the efficacy of vaccination with Q38 to a new host (rabbits) and a new tick species (*D. reticulatus*).

2. Materials and methods

2.1. Ethics statement

Animal experiments in this study were conducted in strict accordance with the recommendations of the European Guide for the Care and Use of Laboratory Animals. Animals were housed and experiments conducted at LLC ACRO Vet Lab (Pylipovichi village, Kiev region, Ukraine) with the approval and supervision of the Ukrainian Commission for Bioethics and Biosafety for animals (abstract from the minutes #43-a from April 30th 2014) under the study "Tick vaccine experiment on rabbits" number 000369.

2.2. Ticks

D. reticulatus unfed larvae were obtained from a Dutch colony maintained at the Utrecht Centre for Tick-borne Diseases (UCTD), Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands [22]. *I. ricinus* unfed larvae were obtained from the reference laboratory colony maintained at the tick rearing facility of the Institute of Parasitology of the Biology Centre of the Academy of Sciences of the Czech Republic [23].

2.3. Vaccine formulations

The recombinant SUB/AKR chimera Q38 was expressed in *Escherichia coli* from synthetic genes (JX193856) optimized for codon usage in *E. coli* and purified by Ni affinity chromatography (Genscript Corporation, Piscatway, NJ, USA) as previously described [20,21]. After purification by Ni affinity chromatography, recombinant chimeric Q38 protein showed purity higher than 85% of total proteins [20,21]. Recombinant antigens or saline control were adjuvated in Montanide ISA 50 V2 (Seppic, Paris, France) [21].

2.4. Rabbit vaccination and infestation

Two-year-old white rabbits (*Oryctolagus cuniculus*) were immunized at days 0 (T1) and 14 (T2) with 0.2 ml subcutaneous doses (dorsum between shoulders) using a syringe with removable needle (0.45 × 13 mm). Three rabbits per group were each injected with the recombinant Q38 vaccine (50 μg/dose) and adjuvant/saline, respectively and infested at day 28 (T3) with 200 tick larvae of approximately 21 days after hatching placed on bags located on

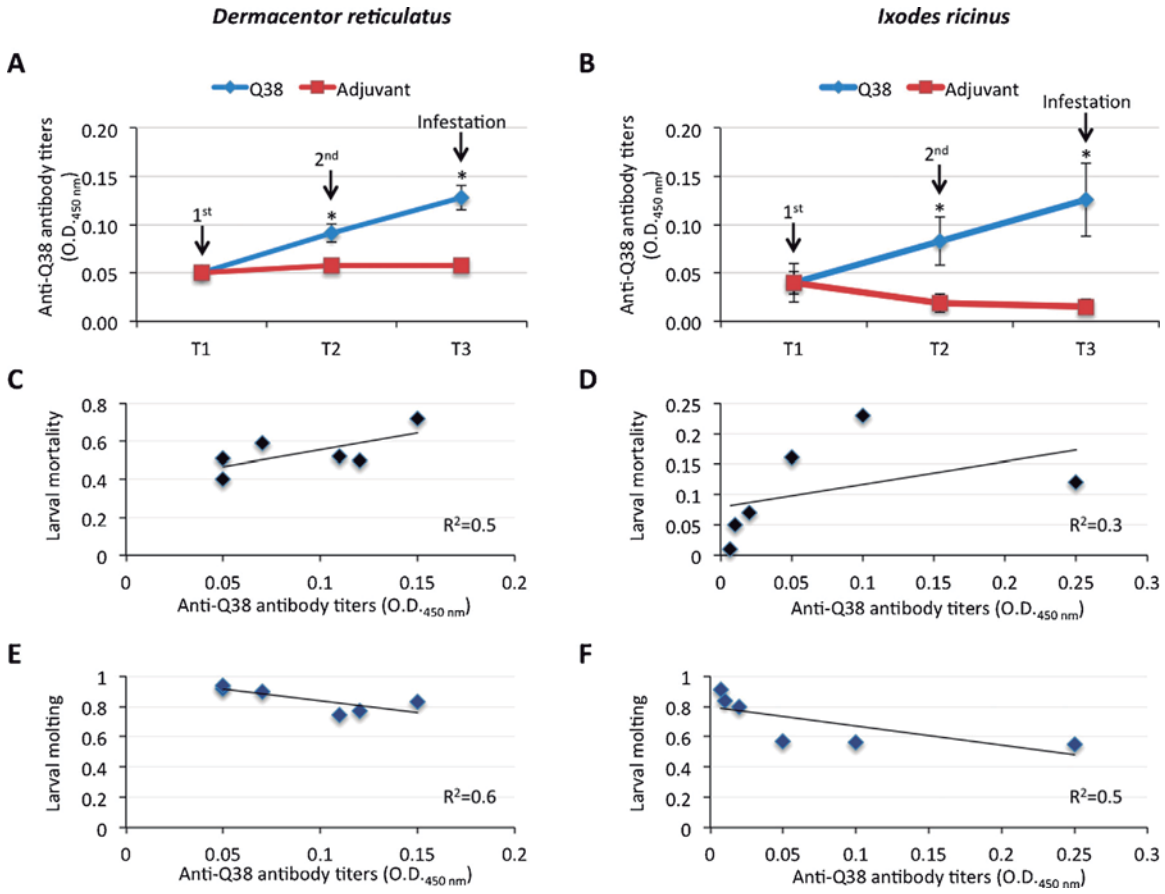

Fig. 1. Antibody response and vaccination effect in rabbits infested with tick larvae. (a,b) Antibody titers were determined by ELISA in vaccinated and control rabbits against the Q38 chimeric protein used for vaccination. Serum samples were collected before 1st (T1) and 2nd (T2) immunizations and vector infestations (T3) with (a) *D. reticulatus* or (b) *I. ricinus* larvae. Antibody titers in vaccinated mice were expressed as the average ± S.D. OD_{450nm} ($OD_{rabbit\ sera}$-$OD_{PBS\ control}$) and compared between vaccinated and control groups by ANOVA test (*$p < 0.05$). (c,d) Antibody titers positively correlated with vaccine effect on larval mortality in (c) *D. reticulatus* and (d) *I. ricinus* infested rabbits. (e,f) Antibody titers negatively correlated with vaccine effect on larval molting in (e) *D. reticulatus* and (f) *I. ricinus* infested rabbits. A correlation analysis was conducted using Microsoft Excel (version 12.0) to compare the vaccine effects on tick biology after feeding on vaccinated and control rabbits with antibody titers at time of tick infestation (T3). The linear correlation coefficients (R^2) are shown ($n = 6$).

Table 1
Effect of Q38 chimera on the control of tick infestations after feeding on vaccinated rabbits.

Parameter	*Ixodes ricinus*		*Dermacentor reticulatus*	
	Fed on control rabbits	Fed on Q38 vaccinated rabbits	Fed on control rabbits	Fed on Q38 vaccinated rabbits
Larval mortality	0.05	0.16	0.51	0.72
(No. dead/Total No. larvae)	0.07	0.12	0.59	0.49
	0.01	0.23	0.40	0.52
	(0.04 ± 0.03)	(0.17 ± 0.06)*	(0.50 ± 0.09)	(0.58 ± 0.13)
Larval feeding (weight/larvae) (mg)	0.41	0.41	0.35	0.28
	0.41	0.51	0.39	0.29
	0.40	0.50	0.35	0.25
	(0.41 ± 0.00)	(0.47 ± 0.05)	(0.36 ± 0.02)	(0.27 ± 0.02)*
Larval molting	0.84	0.57	0.92	0.83
(No. nymphs/No. replete larvae)	0.80	0.55	0.90	0.77
	0.91	0.56	0.94	0.74
	(0.85 ± 0.05)	(0.56 ± 0.01)**	(0.92 ± 0.02)	(0.78 ± 0.05)*

Results are shown for each infested rabbit ($n = 3$) with average ± S.D. in parenthesis. Data were analyzed statistically to compare results between ticks fed on vaccinated and control rabbits by Student's *t*-test (*$p < 0.05$, **$p < 0.005$).

each rabbit shaved ear. Immunizations, tick larval infestations, collections and evaluations were done blinded and the key to the experimental groups was not opened until the end of the experiment. Engorged tick larvae were collected, counted and weighted as they dropped off between days 31 to 34. Fed larvae were incubated at 21 °C, 80–82% humidity with 7 h dark and 17 h light photoperiod until molting. The tick larvae successfully molting to nymphal stage were collected and counted between days 51 to 65. Tick mortality (No. of dead larvae/Total No. larvae used for infestation), feeding (weight/larvae) and molting (No. nymphs/No. replete larvae) were evaluated. Vaccine efficacy (E) was calculated as $E = 100\left(1 - \prod_{k=1}^{n} a_k\right)$, where a_k represent the reduction in the studied developmental processes (k) in tick fed on vaccinated rabbits as compared to the controls fed on adjuvant/saline injected rabbits [11,20]. Data were analyzed statistically to compare results for each tick species between individuals fed on vaccinated and control mice by Student's *t*-test with unequal variance ($p = 0.05$).

2.5. Analysis of rabbit antibody response by ELISA

An indirect ELISA test was performed to detect antibodies against Q38 protein in vaccinated and control rabbit serum samples collected at T1-T3 [20]. High absorption capacity polystyrene microtiter plates were coated with 50 μl (0.02 μg/ml solution of purified recombinant Q38 protein) per well in carbonate–bicarbonate buffer (Sigma, St. Louis, MI, USA). After an overnight incubation at 4 °C, coated plates were blocked with 200 μl/well of blocking solution (5% skim milk in PBS). Serum samples or PBS as negative control were diluted (1:10, v/v) in blocking solution and 50 μl/well were added into duplicate wells of the antigen-coated plates. After an overnight incubation at 4 °C, the plates were washed three times with a washing solution (PBS containing 0.05% Tween 20). A goat anti-rabbit IgG-peroxidase conjugate (Sigma) was added (diluted 1:500 in blocking solution) and incubated at room temperature for 1 h. After three washes with washing solution, 200 μl/well of substrate solution (Fast OPD, Sigma) was added. Finally, the reaction was stopped with 50 μl/well of 3 N H_2SO_4 and the optical density (OD) was measured in a spectrophotometer at 450 nm. Antibody titers in vaccinated and control rabbits were expressed as the OD_{450nm} ($OD_{rabbit\ sera}$-$OD_{PBS\ control}$) and compared between vaccinated and control groups by ANOVA test ($p = 0.05$). A correlation analysis was conducted in Microsoft Excel (version 12.0) to compare the significant effects of vaccination on tick larval mortality and molting after feeding on vaccinated and control rabbits with antibody titers at time of tick infestations (day 2; T3).

3. Results and discussion

The objective of this study was to evaluate the protective capacity of the SUB/AKR Q38 chimeric antigen for the control of *D. reticulatus* tick infestations, in comparison with *I. ricinus* for which results were previously obtained [20]. Larvae were selected for infestations because in these tick species, larvae are the first developmental stage to infest hosts and acquire infection or transmit pathogens that are transovarially transmitted (Fig. 1).

The antibody response against Q38 in vaccinated rabbits increased after successive immunizations in both groups (Fig. 1a and 1b). *I. ricinus* but not *D. reticulatus* larval mortality significantly increased in 4.25 fold in vaccinated rabbits when compared to controls (Table 1). However, the weight of fed larvae showed a significant 25% decrease in *D. reticulatus* only (Table 1). As in a previous report [20], larval molting was significantly reduced by 38% and 15% in *I. ricinus* and *D. reticulatus*, respectively (Table 1). Furthermore, as in previous experiments [20,21], a correlation was shown between the effect of vaccination on larval mortality (positive correlation) and molting (negative correlation) and antibody titers in vaccinated and control mice (Fig. 1c–1f). These results provided additional support for the effect of Q38 vaccination on larval infestations in both tick species. Vaccine E is a parameter that accounts for all effects on vector infestation observed for a given antigen [6]. The vaccine E against larval infestations was 46.4% and 99.9% for *D. reticulatus* and *I. ricinus*, respectively.

The results of this study confirmed the efficacy of the Q38 chimeric antigen for the control of *I. ricinus* tick infestations in vaccinated rabbits. Furthermore, the efficacy of this antigen was extended to include another hard tick species, *D. reticulatus*. The prevalence of tick-borne diseases in companion animals is growing in Europe, where *I. ricinus* and *D. reticulatus* coinfestations are commonly found [4]. The effect of the Q38 vaccine on larval feeding and molting is essential to reduce tick infestations and supports that Q38 might be a candidate universal antigen for the control of multiple tick species that can be found on the same host.

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Conflict of interest statement: The authors declare that there are no conflicts of interest.

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Control of infestations by *Ixodes ricinus* tick larvae in rabbits vaccinated with aquaporin recombinant antigens

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ABSTRACT

Background: Tick-borne diseases greatly impact human and animal health worldwide, and vaccines are an environmentally friendly alternative to acaricides for their control. Recent results have suggested that aquaporin (AQP) water channels have a key function during tick feeding and development, and constitute good candidate antigens for the control of tick infestations.
Methods: Here we describe the effect of vaccination with the *Ixodes ricinus* AQP1 (IrAQP) and a tick AQP conserved region (CoAQP) on *I. ricinus* tick larval mortality, feeding and molting.
Results: We demonstrated that vaccination with IrAQP and CoAQP had an efficacy of 32% and 80%, respectively on the control of *I. ricinus* larvae by considering the cumulative effect on reducing tick survival and molting.
Conclusions: The effect of the AQP vaccines on larval survival and molting is essential to reduce tick infestations, and extended previous results on the effect of *R. microplus* AQP1 on the control of cattle tick infestations. These results supports that AQP, and particularly CoAQP, might be a candidate protective antigen for the control of different tick species.

1. Introduction

Ticks are obligate hematophagous ectoparasites of vertebrate hosts that transmit a variety of pathogens causing life-threatening diseases in humans and animals [1–3]. In particular, *Ixodes ricinus*, the castor bean tick, is one of the most prominent European species of hard-bodied ticks [4–6]. This tick species infest humans, pets and other domestic and wild animals and transmit disease-causing pathogens such as *Borrelia* spp. (Lyme disease and various borreliosis), TBEV (tick-borne encephalitis), louping ill virus (ovine encephalomyelitis), *Anaplasma phagocytophilum* (human and animal anaplasmosis), *Francisella tularensis* (tularemia), *Rickettsia* spp. (human and animal rickettsiosis), OHFV (Omsk hemorrhagic fever), and *Babesia* spp. (canine and bovine babesiosis) [3–6].

Ticks endure enormous osmoregulatory stress following blood ingestion that increases their initial body weight by up to 100 fold [7]. Salivary glands play a major dual role in water management of ticks as they help to absorb atmospheric water vapor via secretion of hygroscopic droplets [8,9] at off-host non-feeding periods between meals, while returning ingested blood-meal water (~75%) back to the host via the saliva [10]. This water passage, driven by osmotic gradients, is facilitated by aquaporins (AQPs) or water transmembrane channels, which are largely crucial for water homeostasis and cryoprotection [11,12]. AQPs are evolutionarily highly conserved members of a larger family of major intrinsic proteins (MIP) that form pores in the membrane of biological cells and are capable of transporting water or other solutes [12–14]. AQPs are not only involved in transporting water and small neutral solutes, but they are also involved in a number of physiological processes such as lipid metabolism, cell migration, cell adhesion, functional epidermal biology and neural signal transduction [15]. In ticks, AQPs have been detected in the digestive track and its associated Malpighian tubules, and also in salivary glands [16].

Due to the growing impact of tick-borne disease worldwide, effective control methods including vaccines for the control of vector infestations and pathogen infection and transmission are needed [17]. Vaccination is an environmentally friendly approach for tick control that allows control of several diseases by targeting their common vector [17].

One strategy in tick vaccine development involves the selection of candidate protective antigens with an important biological function that share conserved structural/sequence motifs for the simultaneous control of different tick species [17–20]. One of these tick

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antigens is the MIP family member, AQPs [7,19,21], which show several highly conserved regions across different species [11,12,14,22]. Recently, the knockdown of genes coding for *I. ricinus* AQP1 [7] and *Rhipicephalus microplus* AQP2 [21] showed that these proteins are essential for tick blood feeding, and consequently affect body weight, blood volume ingested and haemolymph osmolarity. In addition, cattle vaccination with recombinant *R. microplus* AQP1 showed an effect on the reduction of cattle tick infestations and weight with a 68–75% overall vaccine efficacy (E) [19].

These results suggested that AQPs have important biological functions in ticks, and are a promising candidate vaccine antigen for the control of multiple vector infestations. To characterize further the efficacy of the tick AQP vaccine, here we showed that vaccination with recombinant *I. ricinus* AQP (IrAQP) and a conserved region in different tick AQPs (CoAQP) reduces tick larvae infestations and molting in vaccinated rabbits infested with *I. ricinus*. These results support the inclusion of AQPs as a promising candidate protective antigen in vaccines for the control of multiple tick species.

2. Materials and methods

2.1. Ethics statement

In this study, animal experiments were conducted in strict accordance with the recommendations of the European Guide for the Care and Use of Laboratory Animals. Animals were housed and experiments conducted at LLC ACRO Vet Lab (Pylipovichi village, Kiev region, Ukraine) with the approval and supervision of the Ukrainian Commission for Bioethics and Biosafety for animals under the study "Tick vaccine experiment on rabbits" number 000369.

2.2. Ticks

I. ricinus were originally obtained from the reference laboratory colony maintained at the tick rearing facility of the Institute of Parasitology of the Biology Centre of the Academy of Sciences of the Czech Republic [23], and maintained at the LLC ACRO Vet Lab from which unfed larvae were obtained.

2.3. Sequence analysis of tick AQPs

Protein sequences for tick AQPs were searched in the NCBI database (http://www.ncbi.nlm.nih.gov/), and a multiple amino acid sequence alignment was performed with Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) using sequences from *Amblyomma maculatum* AQP (AEO36644), *Amblyomma variegatum* AQP (DAA34147), *I. ricinus* AQP1 (CAX48964), *Ixodes scapularis* AQP (EEC04800), *Dermacentor variabilis* AQP9 (ABI53034), *Rhipicephalus sanguineus* AQP (CAR66115), *Rhipicephalus pulchellus* AQP (JAA60417), *R. microplus* AQP (AIT69684), and *R. microplus* AQP2 (ALJ75650). Conserved protein regions were identified after sequence alignment, and then a topographical analysis was performed using the TMHMM server (http://www.cbs.dtu.dk/services/TMHMM/) [24] to determine transmembrane and extracellular exposed regions.

2.4. Cloning of tick AQPs and production of recombinant proteins

The sequence coding for *I. ricinus* AQP1 (IrAQP; CAX48964) was obtained from adult female tissues. Total RNA was extracted using TRI Reagent (Sigma-Aldrich, St Louis, MO, USA) following the manufacturer's instructions. Then, cDNA was synthesized using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, CA, USA) and the IrAQP coding sequence was amplified by PCR using oligonucleotide primers IrAQP-F: 5′-CACCATGCAGCTTTTCGG CAACACAG-3′ and IrAQP-R: 5′-GGGTATGGCTCCTGTGTACCC-3′. The sequence coding for the selected AQP conserved region (CoAQP; Fig. 1A) was also amplified by PCR using oligonucleotide primers CoAQP-F: 5′-CACCATGCAGCTTTTCGGCAACACC-3′ and CoAQP-R: 5′-GCCAGCGGTGCCGTTGACGCC-3′). The amplified DNA fragments were cloned into the expression vector pET101 and expressed in *Escherichia coli* strain BL21 using the Champion pET101 Directional TOPO Expression kit (Carlsbad, CA, USA). Recombinant proteins produced using this expression system were fused to Histidine tags for purification by affinity to Ni [25,26]. Transformed *E. coli* strains were induced with IPTG for 4.5 h to produce recombinant proteins, which were purified to >95% of total cell proteins by Ni affinity chromatography (Genscript Corporation, Piscataway, NJ, USA) as previously described [25,26] using 1 ml HisTrap FF columns mounted on an AKTA–FPLC system (GE Healthcare, Piscataway, NJ, USA) in the presence of 7 M urea lysis buffer.

2.5. Vaccine formulations

The purified denatured IrAQP and CoAQP were refolded by dialysis against 1000 volumes of PBS, pH 7.4 (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) for 12 h at 4 °C. Recombinant proteins were then concentrated using an Amicon Ultra-15 ultrafiltration device (cut off 10 kDa) (Millipore-Merck, Darmstadt, Germany), adjusted to 0.5 mg/ml. For vaccine formulation, recombinant IrAQP and CoAQP proteins or saline control were adjuvanted in Montanide ISA 50 V2 (Seppic, Paris, France) to a final protein concentration of 250 μg/ml [26].

2.6. Rabbit vaccination and tick infestation

Three two-year-old white rabbits (*Oryctolagus cuniculus*) per group were injected subcutaneously (dorsum between shoulders) at days 0 (T1) and 14 (T2) with 0.2 ml (50 μg) doses of IrAQP, CoAQP or adjuvant/saline using a syringe with removable needle (0.45 × 13 mm). Each vaccinated group had its own control group. Two weeks after the last immunization (day 28; T3), rabbits in vaccinated and control groups were infested with 200 tick larvae of approximately 21 days after hatching placed on bags located on each rabbit's shaved ear as described previously [27]. Immunizations, tick larval infestations, collections and evaluations were done blinded and the key to the experimental groups was not disclosed until the end of the experiment. Engorged tick larvae were collected, counted and weighted as they dropped off between days 31 to 34. Fed larvae were incubated at 21 °C, 80–82% humidity with 7 h dark and 17 h light photoperiod until molting. The tick larvae successfully molting to nymphal stage were collected and counted between days 51 to 65. Tick mortality (No. of dead larvae/Total No. larvae used for infestation), feeding (weight/larvae) and molting (No. nymphs/No. replete larvae) were evaluated. Vaccine efficacy (E) was calculated as $E = 100\ (1 - \prod_{k=1}^{n} a_k)$, where a_k represent the reduction in the studied developmental processes (k) in tick fed on vaccinated rabbits as compared to the controls fed on adjuvant/saline injected rabbits [25,27]. Data were analyzed statistically to compare results for each tick species between individuals fed on vaccinated and control rabbits by Student's *t*-test with unequal variance ($p = 0.05$).

2.7. Analysis of rabbit IgG antibody response by ELISA

Blood samples were collected from each rabbit before each immunization (T1 and T2) and tick infestation (T3) into sterile

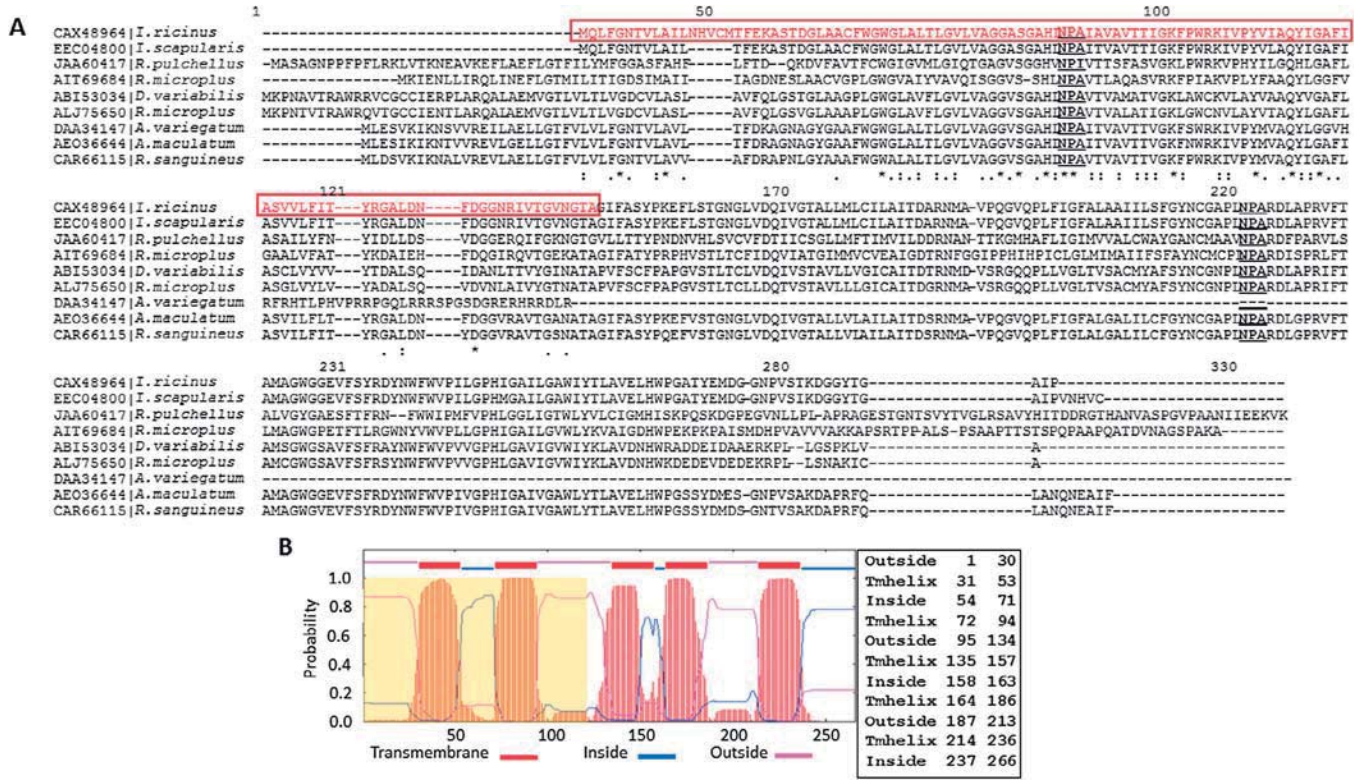

Fig. 1. Characterization of tick AQP protein sequences. (A) Amino acid sequence alignment of tick AQPs. Protein accession numbers and tick species are shown for each sequence. The two NPA motifs (Asn-Pro-Ala) are shown in bold, underlined letters. The conserved region selected for CoAQP is shown in the square. An "*" (asterisk) indicates positions which have a single, fully conserved residue. A ":" (colon) indicates conservation between groups of strongly similar properties – scoring >0.5 in the Gonnet PAM 250 matrix. A "." (period) indicates conservation between groups of weakly similar properties – scoring =<0.5 in the Gonnet PAM 250 matrix. (B) Prediction of the structure of *I. ricinus* AQP protein topology using the TMHMM server. The values indicate the amino acids forming part of each region. The region included into the CoAQP is highlighted. Abbreviation: Tmhelix, transmembrane helix.

tubes and maintained at 4 °C until arrival at the laboratory. Serum was then separated by centrifugation and stored at −20 °C. An indirect ELISA test was performed to detect IgG antibodies against IrAQP or CoAQP proteins in serum samples from vaccinated and control rabbits collected at T1-T3 as described previously [25,27]. High absorption capacity polystyrene microtiter plates were coated with 50 μl (0.02 μg/ml solution of purified recombinant proteins) per well in carbonate-bicarbonate buffer (Sigma-Aldrich). After an overnight incubation at 4 °C, coated plates were blocked with 200 μl/well of blocking solution (5% skim milk in PBS). Serum samples or PBS as negative control were diluted (1:10, v/v) in blocking solution and 50 μl/well were added into duplicate wells of the antigen-coated plates. After an overnight incubation at 4 °C, the plates were washed three times with a washing solution (PBS containing 0.05% Tween 20). A goat anti-rabbit IgG-peroxidase conjugate (Sigma-Aldrich) was added (diluted 1:500 in blocking solution) and incubated at room temperature (RT) for 1 h. After three washes with washing solution, 200 μl/well of substrate solution (Fast OPD, Sigma-Aldrich) was added. Finally, the reaction was stopped with 50 μl/well of 3 N H_2SO_4 and the optical density (OD) was measured in a spectrophotometer at 450 nm. Antibody titers in vaccinated and control rabbits were expressed as the OD_{450nm} ($OD_{rabbit\ sera}$–$OD_{PBS\ control}$) and compared between vaccinated and control groups by ANOVA test ($p = 0.05$). A correlation analysis was conducted in Microsoft Excel (version 12.0) between the antibody titers (OD_{450nm}) at time of tick infestations (T3) and larval molting (No. nymphs/No. replete larvae) in individual vaccinated and control rabbits to compare the effect of vaccination on tick larval molting with antibody titers after feeding on vaccinated and control rabbits.

2.8. Analysis of rabbit IgG antibody response by Western blot

Proteins were extracted from the *I. ricinus* embryo-derived tick cell line IRE/CTVM20 [28] and unfed larvae [23] using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Valencia, CA, USA) according to manufacturer instructions. For Western blot analysis, 10 μg of purified recombinant proteins and 20 μg of total proteins from tick cells and larvae were separated by electrophoresis in an SDS-12% polyacrylamide gel (Life Science, Hercules, CA, USA) and transferred to a nitrocellulose membrane. The membrane was blocked with 5% BSA (Sigma-Aldrich) for 2 h at RT, washed four times with TBS (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5% Tween 20). Pooled sera collected at T3 from IrAQP and CoAQP vaccinated rabbits were used as primary antibodies. Primary antibodies were used at a 1:500 dilution in TBS, and the membrane was incubated overnight at 4 °C and washed four times with TBS. The membrane was then incubated with an anti-rabbit IgG-horseradish peroxidase (HRP) conjugate (Sigma-Aldrich) diluted 1:1000 in TBS with 3% BSA. The membrane was washed five times with TBS and finally developed with TMB (3,3′, 5,5′- tetramethylbenzidine) stabilized substrate for HRP (Promega, Madrid, Spain) according to the manufacturer recommendations.

3. Results and discussion

The objective of this study was to evaluate the protective capacity of the IrAQP and CoAQP antigens for the control of *I. ricinus* larvae infestations in vaccinated rabbits. Larvae were selected for infestation because in this tick species, larvae are the

first developmental stage to infest hosts and acquire infection, which then transmit pathogens that are transovarially transmitted or after molting to nymphs.

The alignment of the amino acid sequences of different tick AQPs showed a conserved region containing a NPA (Asn-Pro-Ala) motif in the transmembrane loop B (Fig. 1A and B). The highly conserved NPA motifs are the most important structural domains that play a crucial role in water-selective permeation in AQP water channels [29]. The conserved region selected for CoAQP also included sequences from two predicted extracellular regions (Fig. 1B). Recombinant IrAQP and CoAQP proteins showed the expected molecular weight of 35 and 15 KDa, respectively when produced in *E. coli* (Fig. 2A) and after purification to >95% of total cell proteins by Ni affinity chromatography (Fig. 2B).

Sera from vaccinated rabbits recognized recombinant proteins and the native IrAQP in protein extracts from *I. ricinus* tick cell line IRE/CTVM20 and unfed larvae (Fig. 2C). These results showed that the IgG antibody response in vaccinated rabbits was directed against AQP. The IgG antibody response against IrAQp and CoAQP in vaccinated rabbits increased after immunization, with the highest increase after the second immunization on T2 (Fig. 3A and B). *I. ricinus* larval mortality significantly increased in 6.25 and 5.17 fold in rabbits vaccinated with IrAQP and CoAQP, respectively when compared to controls (Table 1). The effect on larval mortality was not significant in rabbits vaccinated with CoAQP probably due to animal-to-animal variations in both control and vaccinated groups (Table 1). Nevertheless, the larval molting to nymphs was significantly reduced in IrAQP and CoAQP vaccinated groups by 13% and 73%, respectively when compared to controls (Table 1). Furthermore, a correlation between vaccination and tick phenotype was conducted as one of the proposed correlates of protection in tick vaccine trials [17]. As in previous tick vaccine experiments [25–27], a negative correlation was obtained between the effect of vaccination on larval molting and antibody titers in vaccinated and control rabbits at T3 (Fig. 3C and D), showing a correlation between the effect of vaccination (antibody titers) and vaccine efficacy on tick phenotype. These results provided an additional support for the effect of vaccination with IrAQP and CoAQP on *I. ricinus* larvae molting to nymphs, which is a critical step in the completion of tick life cycle. The IrAQP and CoAQP vaccines showed an overal efficacy of 32% and 80%, respectively on the control of *I. ricinus* larvae by considering the cumulative effect on reducing tick survival and molting. Tick vaccines are not designed to prevent tick infestations but to reduce tick populations by affecting feeding, reproduction and development of ticks feeding on immunized animals and ingesting antigen-specific antibodies that interact with and affect protein function [17]. Therefore, vaccine efficacy is determined by considering the effect on different tick developmental stages [17].

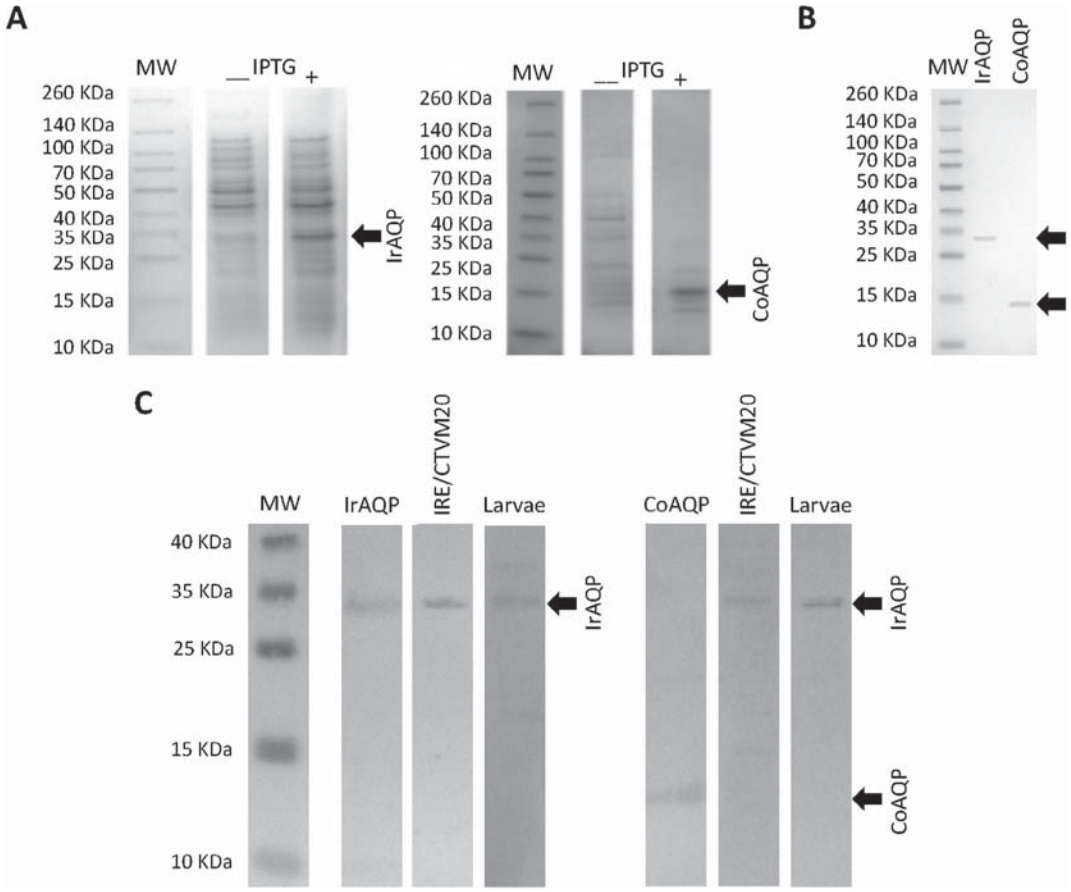

Fig. 2. Production and characterization of recombinant tick AQPs. (A, B) Production of the recombinant IrAQP and CoAQP in *E.coli*. Samples were taken (A) before and after induction with IPTG and (B) after purification to >95% of total cell proteins by Ni affinity chromatography. Ten μg proteins were loaded per well in an SDS-12% polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue. The position of the recombinant proteins is indicated with arrows. (C) Proteins were extracted from *I. ricinus* tick cell line IRE/CTVM20 and unfed larvae and used for Western blot analysis. Ten μg of purified recombinant proteins and 20 μg of total proteins from tick cells and larvae were separated by electrophoresis in an SDS-12% polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was incubated with pooled sera collected at T3 from IrAQP and CoAQP vaccinated rabbits. The position of the recombinant or native AQPs is indicated with arrows. Abbreviation: MW, molecular weight markers (Spectra multicolor broad range protein ladder; Thermo Scientific).

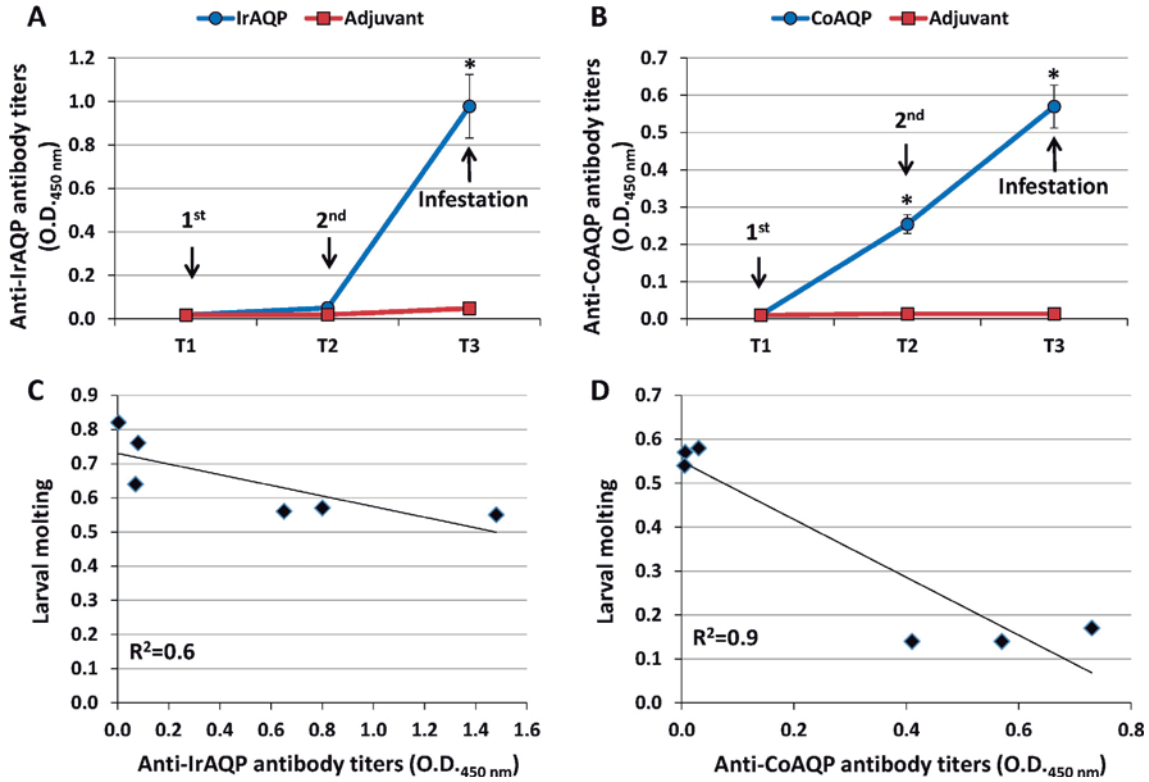

Fig. 3. Effect of vaccination in rabbits infested with *I. ricinus* larvae. (A, B) Antibody titers were determined by ELISA in vaccinated and control rabbits against the recombinant (A) IrAQP or (B) CoAQP protein used for vaccination. Serum samples were collected before 1st (T1) and 2nd (T2) immunizations and tick infestation (T3) with *I. ricinus* larvae. Antibody titers in vaccinated rabbits were expressed as the average ±S.D. OD_{450nm} ($OD_{rabbit\ sera}$-$OD_{PBS\ control}$) and compared between vaccinated and control groups by ANOVA test ($^{*}P < 0.05$). (C, D) Antibody titers negatively correlated with vaccine effect on *I. ricinus* larval molting in rabbits vaccinated with (C) IrAQP or (D) CoAQP. The correlation analysis was conducted using Microsoft Excel (version 12.0) to compare the vaccine effects on tick biology after feeding on vaccinated and control rabbits with antibody titers at time of tick infestation (T3). The linear correlation coefficients (R^2) are shown (N = 6).

Table 1
Effect of rabbit vaccination with IrAQP and ConsAQP on *I. ricinus* infestations.

Parameter	Rabbit number	Experiment 1		Experiment 2	
		Ticks fed on			
		Control rabbits	IrAQP vaccinated rabbits	Control rabbits	ConsAQP vaccinated rabbits
Larval mortality (No. dead/Total No. larvae)	Rabbit 1	0.05	0.26	0.11	0.18
	Rabbit 2	0.07	0.25	0.04	0.11
	Rabbit 3	0.01	0.25	0.03	0.63
	Ave ± S.D.	(0.04 ± 0.03)	(0.25 ± 0.01)**	(0.06 ± 0.04)	(0.31 ± 0.28)
DT, % reduction in the number of larvae[a]			DT = 22%		DT = 27%
Larval feeding (weight/larvae) (mg)	Rabbit 1	0.41	0.44	0.43	0.52
	Rabbit 2	0.41	0.46	0.45	0.53
	Rabbit 3	0.40	0.46	0.43	0.53
	Ave ± S.D.	(0.41 ± 0.00)	(0.45 ± 0.01)*	(0.44 ± 0.01)	(0.53 ± 0.01)**
DW, % reduction in larval weight[b]			DW = 0%		DW = 0%
Larval molting (No. nymphs/No. replete larvae)	Rabbit 1	0.84	0.64	0.57	0.14
	Rabbit 2	0.80	0.82	0.54	0.17
	Rabbit 3	0.91	0.76	0.58	0.14
	Ave ± S.D.	(0.85 ± 0.05)	(0.74 ± 0.09)*	(0.56 ± 0.02)	(0.15 ± 0.02)**
DM, % reduction in larval molting[c]			DM = 13%		DM = 73%
E, vaccine efficacy[d]			E = 32%		E = 80%

Results are shown for each infested rabbit (N = 3) with average (Ave) ± S.D. in parenthesis. Data was analyzed statistically to compare results between ticks fed on vaccinated and control rabbits by Student's *t*-test ($^{*}p < 0.05$, $^{**}p < 0.005$).

[a] DT = 100 − [(No. dead larvae in vaccinated rabbits × 100)/No. dead larvae in control rabbits].

[b] DW = 100 − [(larval weight in vaccinated rabbits × 100)/larval weight in control rabbits].

[c] DM = 100 − [(larval molting in vaccinated rabbits × 100)/larval molting in control rabbits].

[d] E = 100 × [1 − (DT × DM)].

A previous experiment that was conducted in cattle vaccinated with recombinant *R. microplus* AQP1 showed the effect of the vaccine on the reduction of cattle tick infestations and weight with a 68%-75% overall vaccine efficacy (E) [19]. *R. microplus* is a one host tick, and therefore in this experiment data was not obtained on the effect of the vaccine of tick molting. Nevertheless, the effect of the *R. microplus* AQP1 vaccine on the decrease in the number of female cattle ticks completing feeding could be due at least in part to the

effect on tick molting reported here in *I. ricinus*. In our study, we did not see a decrease in larval weight after feeding on vaccinated rabbits (Table 1), which in fact was slightly (1.1–1.2 fold) higher in ticks fed on vaccinated rabbits when compared to controls (Table 1). Although this increase in larval weight may not be physiologically relevant, it evidenced a difference in the results of both trials. One possible explanation to this discrepancy is the fact that in our experiment data was obtained in tick larvae and not adult females, together with possible differences between tick species and hosts in the response to vaccination with AQP antigens. The mechanism responsible for the weight increase in ticks fed on vaccinated rabbits may be related to the effect of antibodies to vaccine antigens on AQP function to concentrate blood components for efficient digestion during tick feeding [11,12,30], resulting in reduced water elimination and tick weight gain. Therefore, considering the AQP function during tick feeding and other physiological processes [12–16], and the protective mechanisms of other tick vaccines [17], it is possible to hypothesize that the effect of AQP-based vaccines is based on the interactions between anti-AQP antibodies and tick AQP that affect protein function resulting in reduced tick fitness.

4. Conclusions

The results of this study confirmed the efficacy of the tick AQP antigens for the control of tick infestations by showing the effect of IrAQP and CoAQP vaccination on *I. ricinus* tick larvae in rabbits. Furthermore, the efficacy of the vaccine containing the AQP conserved region included in the CoAQP antigen was higher than that of the IrAQP vaccine, therefore suggesting the possibility of using this antigen for the control of different tick species. *I. ricinus* is one of the tick species responsible for the growing prevalence of tick-borne diseases in companion animals in Europe [31]. The effect of the AQP-based vaccines on *I. ricinus* larvae infestation and molting could result in the reduction of tick infestations in vaccinated animals, and supports that CoAQP might be a candidate protective antigen for the control of different tick species feeding on the same host.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Bacterial membranes enhance the immunogenicity and protective capacity of the surface exposed tick Subolesin-*Anaplasma marginale* MSP1a chimeric antigen

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Original article

Bacterial membranes enhance the immunogenicity and protective capacity of the surface exposed tick Subolesin-*Anaplasma marginale* MSP1a chimeric antigen

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ABSTRACT

Ticks are vectors of diseases that affect humans and animals worldwide. Tick vaccines have been proposed as a cost-effective and environmentally sound alternative for tick control. Recently, the *Rhipicephalus microplus* Subolesin (SUB)-*Anaplasma marginale* MSP1a chimeric antigen was produced in *Escherichia coli* as membrane-bound and exposed protein and used to protect vaccinated cattle against tick infestations. In this research, lipidomics and proteomics characterization of the *E. coli* membrane-bound SUB-MSP1a antigen showed the presence of components with potential adjuvant effect. Furthermore, vaccination with membrane-free SUB-MSP1a and bacterial membranes containing SUB-MSP1a showed that bacterial membranes enhance the immunogenicity of the SUB-MSP1a antigen in animal models. *R. microplus* female ticks were capillary-fed with sera from pigs orally immunized with membrane-free SUB, membrane bound SUB-MSP1a and saline control. Ticks ingested antibodies added to the blood meal and the effect of these antibodies on reduction of tick weight was shown for membrane bound SUB-MSP1a but not SUB when compared to control. Using the simple and cost-effective process developed for the purification of membrane-bound SUB-MSP1a, endotoxin levels were within limits accepted for recombinant vaccines. These results provide further support for the development of tick vaccines using *E. coli* membranes exposing chimeric antigens such as SUB-MSP1a.

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1. Introduction

Diseases caused by arthropod-borne pathogens greatly impact human and animal health. Ticks are considered to be second to mosquitoes as vectors of pathogens to humans and the most important vectors of pathogens that cause disease in cattle (de la Fuente et al., 2007). Control of tick infestations has been based primarily on the use of chemical acaricides that has resulted in selection of acaricide-resistant ticks and environmental pollution (de la Fuente et al., 2007). Vaccination with the *Rhipicephalus microplus* BM85/BM95 gut antigen emerged as an alternative for tick control that has shown the advantage of being cost-effective while reducing acaricide applications and the drawbacks associated with their use (de la Fuente et al., 2007; Willadsen, 2006).

Subolesin (SUB), the ortholog of insect and vertebrate Akirins (AKR) is an evolutionary conserved protein that was recently discovered in *Ixodes scapularis* as a tick protective antigen (Almazán et al., 2003; de la Fuente et al., 2011). SUB is involved in tick innate immune response and in other molecular pathways including those required for feeding, reproduction and pathogen infection and multiplication (de la Fuente et al., 2011). Vaccination with recombinant SUB/AKR showed a reduction in tick, mosquito, sand fly and poultry red mite infestations by reducing ectoparasite numbers, weight and/or oviposition and tick infection with different pathogens such as *Anaplasma phagocytophilum*, *Anaplasma marginale*, *Babesia bigemina* and *Borrelia burgdorferi* (de la Fuente et al., 2013). Recently, vaccination with recombinant *Aedes albopictus* AKR reduced *Plasmodium berghei* infection in mosquitoes fed on immunized mice when compared to controls (da Costa et al., 2014). These results suggested that SUB/AKR is a good candidate antigen for the development of vaccines for the control of multiple arthropod vectors and vector-borne diseases (de la Fuente et al., 2011, 2013; da Costa et al., 2014; Moreno-Cid et al., 2013).

Proteins exposed on the cell membrane such as major surface proteins are good targets for vaccine development. Recently, recombinant chimeras comprising tick proteins such as BM95 immunogenic peptides and SUB fused to the N-terminal region of the *A. marginale* Major Surface Protein 1a (MSP1a) (BM95-MSP1a and SUB-MSP1a chimeras, respectively) were produced in *Escherichia coli* as membrane-bound and exposed proteins (Canales et al., 2008; Almazán et al., 2012). Furthermore, the use of bacterial membranes containing the membrane-bound BM95-MSP1a or SUB-MSP1a proteins as vaccines resulted in the control of *R. microplus* and *R annulatus* infestations in cattle (Almazán et al., 2012; Canales et al., 2009). This system provides a novel, simple and cost-effective approach for the production of tick protective antigens by surface displaying antigenic protein chimera on the *E. coli* membrane and demonstrated the possibility of using recombinant bacterial membrane fractions in vaccine preparations to protect cattle against tick infestations (Almazán et al., 2012; Canales et al., 2009, 2010).

However, important issues still need to be addressed before *E. coli* membranes with surface-exposed antigens such as the SUB-MSP1a chimera could be used in commercial vaccine formulations. These issues include the characterization of the role of *E. coli* membranes in enhancing the immunogenicity of membrane-bound SUB-MSP1a chimeric antigen and the potential problems associated with the content of bacterial endotoxins in the SUB-MSP1a antigen preparation. In this study, we addressed these issues by characterizing the composition and immunogenicity of the membrane-bound SUB-MSP1a chimeric antigen. The results showed that the purified membrane-bound SUB-MSP1a chimeric antigen is more immunogenic than the membrane-free SUB-MSP1a and SUB antigens with endotoxin levels acceptable for vaccine formulations, therefore providing further support for the development of tick vaccines using *E. coli* membranes exposing chimeric antigens such as SUB-MSP1a.

2. Materials and methods

2.1. Production of recombinant SUB-MSP1a chimeric antigen and SUB in E. coli

Unless otherwise indicated, all reagents used in this work were purchased either from Sigma–Aldrich (St. Louis, MO, USA) or VWR International Eurolab S.L. (Mollet del Vallés, Barcelona, Spain). For the production of the membrane-bound *R. microplus* SUB-MSP1a chimera, recombinant *E. coli* JM109 cells transformed with the pMBXAF3 expression vector were used (Almazán et al., 2012). In this construct, as for the BM95-MSP1a chimera (Canales et al., 2008), the inserted SUB coding region is fused to MSP1a and is under the control of the inducible tac promoter (Almazán et al., 2012). Recombinant *E. coli* were propagated in 1 l flasks containing 250 ml Luria–Bertani (LB) broth supplemented with 10 g tryptone l^{-1}, 5 g yeast extract l^{-1}, 10 g NaCl l^{-1}, 50 μg/ml ampicillin and 0.4% glucose (Laboratorios CONDA S.A., Madrid, Spain) for 2 h at 37 °C and 200 rpm and then for 5.5 h after addition of 0.5 mM final concentration of isopropyl-β-D-thiogalactopyranoside (IPTG) for induction of recombinant protein production as previously described (Canales et al., 2008). Cell growth was monitored by measuring OD at 600 nm. Protein concentration was determined using bicinchoninic acid (BCA). The cells were harvested by centrifugation at 10,000 × *g* for 15 min at 4 °C and then 1 g of cell pellet was resuspended in 5 ml of disruption buffer (100 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM PMSF, 5 mM $MgCl_2 \cdot 6H_2O$ and 0.1% (v/v) Triton X-100) and disrupted using a cell sonicator (Model MS73; Bandelin Sonopuls, Berlin, Germany). After disruption, the insoluble and soluble protein fractions containing the membrane-bound and membrane-free SUB-MSP1a, respectively were collected by centrifugation at 21,500 × *g* for 15 min at 4 °C and stored at −20 °C until used for characterization and vaccine formulations. Recombinant SUB was produced in *E. coli* and purified by Ni affinity chromatography to >95% purity as previously described (Almazán et al., 2010).

2.2. Polyacrylamide gel electrophoresis and western blot for the characterization of recombinant SUB-MSP1a

The recombinant SUB-MSP1a was analyzed by SDS-PAGE and western blot. Ten micrograms of total proteins were loaded onto a 12% SDS-polyacrylamide gel (Criterion XT precast gels, Bio-Rad, Hercules, CA, USA) and either stained with Coomassie Brilliant Blue or transferred to a nitrocellulose membrane for western blot analysis. The percent of total cell proteins corresponding to recombinant SUB-MSP1a was determined in the stained SDS-polyacrilamide gel by densitometric analysis using ImageJ 1.44p (National institute of Health, USA).

For western blot analysis, proteins in the gel were transferred to a nitrocellulose membrane during 1 h at 12 V in a Mini-Genie Electroblotter semi-dry transfer unit (Idea Scientific, Corvallis, OR, USA). The membrane was blocked with 5% skim milk for 1 h at room temperature, washed three times in TBS and probed with rabbit antibodies. Serum from rabbits immunized with recombinant *R. microplus* SUB (Almazán et al., 2010) was diluted 1:500 in 3% BSA in TBS and the membrane was incubated with the diluted sera for 1 h at room temperature, and washed three times with TBS. The membrane was then incubated with an anti-rabbit horseradish peroxidase (HRP) conjugate (Sigma, St. Louis, MO, USA) diluted 1:1000 in TBS. The membrane was washed three times with TBS and finally developed with TMB stabilized substrate for HRP (Promega) for 20 min. Recombinant *A. phagocytophilum* superoxide dismutase

(SOD; Uniprot accession number Q2GKX4) protein was produced in *E. coli*, purified similar to SUB by Ni affinity chromatography (Almazán et al., 2010) and used as control to confirm the specificity of anti-SUB antibodies.

2.3. Lipidomics and proteomics profiles of the E. coli membrane-bound SUB-MSP1a chimera

2.3.1. Lipidomics

Lipid extraction was carried out from 100 μg of membrane-bound SUB-MSP1a using the Lipid Extraction (chloroform free) kit (Cell Biolabs, Inc., San Diego, CA, USA). After extraction, lipid samples were evaporated to dryness, redissolved in 100 μl of acetonitrile (AcN) and introduced by direct infusion at a flow rate of 5 μl/min onto a LTQ mass spectrometer (Thermo Scientific, San Jose, CA, USA). Data were collected in the positive ion mode (ES+) during 5 min with a capillary temperature of 270 °C and capillary voltage of 25 V. The range of acquisition was from m/z 150–2000. The total ion count was 10^7 ions and mass values for lipids were obtained from an average of 1000 μscans. All procedures were performed using two independent replicates. The masses of detected ions expressed as $[M+H]^+$ of the lipid compounds obtained for each sample with a maximum tolerance ± 1 Da are indicated in Supplementary Table 1. Theses masses where searched against the lipidomics gateway (http://www.lipidmaps.org/) to obtain the lipid categories, classes and subclasses of the detected lipid ions (Supplementary Table 1).

Supplementary table related to this article can be found, in the online version, at doi:10.1016/j.ttbdis.2015.07.010

2.3.2. Lipopolysaccharides

Lipopolysaccharides (LPS) were extracted from 2 mg of membrane-bound SUB-MSP1a using the LPS extraction kit (iNtRON Biotechnology, South Korea) following the manufacturer's instructions. The LPS were diluted to approximately 250 μg/ml with sample buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 12.5 mM EDTA, 0.02% bromophenol blue) and 20 μl of diluted samples were loaded onto a 12% SDS-polyacrylamide gel (PAGEgel™ Inc., San Diego, CA, USA) and stained with Pro-Q Emerald 300 Lipopolysaccharide Gel Stain kit (Invitrogen-Life Technologies Inc., Grand Island, NY, USA) following manufacturer's instructions. The LPS from *E. coli* serotype 055:B5 were used as standard.

2.3.3. Proteomics

Hundred μg of bacterial membranes containing SUB-MSP1a were resuspended in urea buffer (8 M urea, 25 mM ammonium bicarbonate, pH 8), reduced with 10 mM DTT for 1 h at 37 °C and then alkylated with 50 mM iodoacetamide for 45 min at room temperature in darkness. The mixture was diluted 4-fold to reduce urea concentration and, after the addition of trypsin (Sequencing grade, Promega, Madison, WI, USA) (1:20 protease to protein ratio), was incubated at 37 °C overnight. Digestion was stopped by the addition of trifluoroacetic acid (TFA) to a final concentration of 1% and the peptides were finally desalted onto OMIX Pipette tips C18 (Agilent Technologies, Santa Clara, CA, USA), dried-down and stored at −20 °C until mass spectrometry analysis. The desalted protein digest was resuspended in 8 μl of 0.1% formic acid and analyzed by RP-LC-MS/MS in an Agilent 1100 system coupled to a linear ion trap LTQ-Velos mass spectrometer (Thermo Scientific). The peptides were separated by reverse phase chromatography using a 0.18 mm × 150 mm Bio-Basic C18 RP column (Thermo Scientific), operating at 1.8 μl/min. Peptides were eluted using a 240 min gradient from 5% to 40% solvent B (Solvent A: 0.1% formic acid in water, solvent B: 0.1% formic acid, 80% acetonitrile in water). ESI ionization was done using a microspray "metal needle kit" (Thermo Scientific) interface. Peptides were detected in survey scans from 400 to 1600 amu (1 μscan), followed by fifteen data dependent MS/MS scans (Top 15), using an isolation width of 2 u (in mass-to-charge ratio units), normalized collision energy of 35%, and dynamic exclusion applied during 30 s periods. Peptide identification from raw data was carried out using the SEQUEST algorithm (Proteome Discoverer 1.4, Thermo Scientific). Database search was performed against uniprot-*E. coli*.fasta database complemented with the sequences of *R. microplus* SUB and *A. marginale* MSP1a proteins (uniprot accession numbers DQ159964 and AAG29248, respectively). The following constraints were used for the searches: tryptic cleavage after Arg and Lys, up to two missed cleavage sites, and tolerances of 1 Da for precursor ions and 0.8 Da for MS/MS fragment ions and the searches were performed allowing optional Met oxidation and Cys carbamidomethylation. Search against decoy database (integrated decoy approach) were also performed. All procedures were performed using two independent replicates. A false discovery rate (FDR) < 0.01 was considered as condition for successful peptide assignments and at least 2 peptides per protein in one of the samples analyzed was the condition for successful protein identification (Supplementary Table 2). Peptide spectrum matches (PSMs) were used to characterize relative protein abundance. The biological process of the identified *E. coli* proteins was assigned using the Blast2GO software (version 3; http://www.blast2go.org/).

Supplementary table related to this article can be found, in the online version, at doi:10.1016/j.ttbdis.2015.07.010

2.4. Live-cell immunofluorescence assay (IFA)

A live-cell IFA was used to detect surface expression of recombinant SUB-MSP1a fusion protein as previously reported (Canales et al., 2008), using rabbit preimmune serum and polyclonal antibodies against *R. microplus* SUB (Almazán et al., 2010). Controls included *E. coli* expressing recombinant SUB (Almazán et al., 2010). The *E. coli* cells were grown and induced as described above. One milliliter culture (approximately 3×10^8 cells) were collected by centrifugation (5000 × *g* for 5 min) and washed with 1 ml PBS. Intact cells were resuspended in 100 μl of rabbit preimmune or immune serum and incubated for 30 min at room temperature. After incubation with primary antibodies, cells were collected by centrifugation, washed with PBS, resuspended in 100 μl goat anti-rabbit IgG labeled with fluorescein (KPL, Inc., Gaithersburg, MD, USA) (1/100 final dilution in 3% goat serum (Sigma)/PBS) and incubated for 30 min at room temperature. Cell–antibody complexes were finally collected by centrifugation, washed with PBS, resuspended in 100 μl in 3% goat serum/PBS and 20 μl were smeared onto a glass slide and air dried. The smears were briefly fixed in methanol, rinsed in PBS, and cover slips were mounted by using Mowiol/glycerol/1,4-diazabicyclo-(2,2,2)-octane (DAPCO, Sigma) as the mounting medium. The smears were then examined by epifluorescence microscopy (Eclipse 50i, Nikon Instruments Inc., Melville, NY, USA).

2.5. Vaccine formulations

The membrane-bound insoluble protein fraction and the membrane-free soluble protein fraction containing over 50% of total proteins corresponding to the SUB-MSP1a chimera were resuspended in PBS, pH 7.4. To prepare cell membrane-free SUB-MSP1a for immunization in mice, 250 μg of the membrane-bound SUB-MSP1a were loaded onto a preparative 12% SDS-polyacrylamide gel, separated by SDS-PAGE and stained with Coomassie Brilliant Blue. The band corresponding to the SUB-MSP1a chimera was cut off the gel, crushed using a sterile razor blade and incubated in 1 ml 150 mM Tris–HCl, pH 7.4, containing 0.1% SDS, with gentle agitation overnight at 4 °C. After

centrifugation for 15 min at 4 °C, the membrane-free SUB-MSP1a protein was collected in the supernatant and dialyzed against PBS, pH 7.4. Both membrane-bound and membrane-free SUB-MSP1a proteins were adjuvated in Montanide ISA 50 V2 (Seppic, Paris, France) at a concentration of 125 μg SUB-MSP1a ml^{-1} (Almazán et al., 2012).

2.6. Endotoxin quantitation

The endotoxin levels in the membrane-bound SUB-MSP1a protein fraction were determined by standard kinetic chromogenic Limulus Amebocyte Lysate (LAL) assay (Thermo Scientific, Hudson, NH, USA) following the manufacturer's instructions. The reaction was stopped by the addition of 25% acetic acid and the absorbance was measured at 405 nm.

2.7. Immunization in mice and rabbits

Three groups of five female 5 weeks old Balb/c mice per group were each immunized three times at weeks 0, 3 and 6 with 0.2 ml doses (25 μg) injected subcutaneously in the dorsum near the base of the tail using a 1-ml tuberculin syringe and a 27.5-G needle. Mice were immunized with membrane-bound SUB-MSP1a, gel-extracted membrane-free SUB-MSP1a or adjuvant/saline alone as control. Blood was collected at weeks 0, 6 and at the end of the experiment (week 8) and used for serum preparation. Two groups of three rabbits each were immunized once with 0.4 ml doses (50 μg) as previously described for mice but using recombinant membrane-bound SUB-MSP1a, soluble membrane-free SUB-MSP1a or adjuvant/saline alone as control. Blood was collected 4 weeks after immunization and used for serum preparation. Mice and rabbits were housed at the Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Portugal (Permit No. 023357).

2.8. Oral immunization in pigs

Ten 2–3 month-old domestic piglets were randomly separated into two groups of five animals each and orally immunized at weeks 0 and 4 with 5 ml PBS, pH 7.4 containing 5 mg recombinant *R. microplus* SUB (Almazán et al., 2010), 5 mg membrane-bound SUB-MSP1a. Adjuvant was not included in the antigen preparation. Control animals ($N = 3$) were treated orally with PBS alone. Blood was collected for serum preparation before immunization at weeks 0 and 4 and at the end of the experiment at week 8. Animal handling procedures and sampling frequency were designed to reduce stress and health risks for subjects, according to European (86/609) and Spanish legislation (R.D. 223/1988, R.D. 1021/2005).

2.9. Determination of antibody titers by ELISA

Purified recombinant SUB (Almazán et al., 2010) (0.1 mg/well) was used to coat ELISA plates overnight at 4 °C. Sera from mice, rabbits and pigs collected before immunization (preimmune sera), at the time of 2nd/3rd immunizations and 2–4 weeks after the last immunization were serially diluted from 1:10 to 1:1000 in PBST (PBS/0.5% Tween 20, pH 7.2) and 10% fetal bovine serum (Sigma). The plates were incubated with the diluted sera for 1 h at 37 °C and then incubated with 1:10,000 rabbit anti-mouse, goat anti-rabbit or rabbit anti-pig IgG–HRP conjugates (Sigma) for 1 h at 37 °C. The plates were washed with PBST, the color reaction was developed with SigmaFast OPD (Sigma) following the manufacturer's recommendations and the OD at 450 nm was determined. Antibody titers were considered positive when they yielded an $OD_{450\,nm}$ value at least twice as high as the preimmune serum. Antibody titers were expressed as the $OD_{450\,nm}$ ($OD_{mouse\ sera} - OD_{PBS\ control}$) at the highest serum dilution (1:100 or 1:1000) and compared between immunized and control groups by Student's *t*-test with unequal variance ($P = 0.05$).

2.10. Tick capillary feeding

R. microplus partially engorged adult female ticks collected from cattle were used for capillary feeding using sera from orally immunized pigs as previously reported (Antunes et al., 2014). Briefly, collected ticks were cleaned, weighed and fixed on expandable polystyrene plates (19 cm × 10 cm) with double-sided adhesive tape (3M, St. Paul, MN, USA). Female ticks were discarded if they had damaged mouthparts or their weight did not lie between 20 and 60 mg. Citrated cattle blood was used to fill microhematocrit capillary tubes (75 mm × Ø1.5 mm) that were placed over the ticks' mouthparts. Tubes were replaced every 2–3 h as described previously (Antunes et al., 2014). Female ticks were divided into experimental groups of 15 individuals each and fed for 28 h with blood collected from cattle and supplemented with and equal volume of pooled sera from pigs orally immunized for 4 weeks with SUB-MSP1a, SUB or PBS alone as described before. After feeding, ticks were detached from the double-sided tape and weighed again to determine tick weight increase during feeding. Weight increase during feeding (mg/tick) were compared between ticks fed with blood supplemented with sera from orally immunized pigs and ticks fed with blood supplemented with control pig sera by Student's *t* test with unequal variance ($P = 0.05$). The protocol was approved by the Committee on bioethics and animal welfare, Universidad Autónoma de Tamaulipas, Mexico (permit CBBA-14-10-2).

3. Results and discussion

3.1. Production of the recombinant membrane-bound SUB-MSP1a chimera

The expression of the membrane-bound SUB-MSP1a chimera was characterized in *E. coli* grown in 250 ml working volume at bench-top scale. The yield in terms of biomass production and SUB-MSP1a protein levels were similar to that previously reported for BM95-MSP1a (Canales et al., 2008) (Fig. 1A). Cells grew on 1% glucose as sole carbon source exponentially at the maximum growth rate of 2.3 h^{-1}, reaching an optical density of $0.8 \pm 0.4 OD_{600\,nm}$ before induction with IPTG for expression of the recombinant protein.

A kinetics study was conducted to determine the optimal induction time for SUB-MSP1a production (Fig. 1B). The SUB–MSP1a chimera started to accumulate after 1 h of IPTG induction and its concentration reached the maximum of 9.5% of total cell proteins 5.5 h after induction (Fig. 1B). A simple process previously reported for the production of bacterial membranes containing the chimeric BM95-MSP1a antigen (Canales et al., 2008) was used for SUB-MSP1a recovery. This process involved cell disruption and phase separation by centrifugation, yielding an enriched membrane fraction with a total recovery of 90% of the recombinant protein. The purity of the membrane-bound SUB-MSP1a antigen was higher than 50% in the final fraction, as demonstrated by SDS-PAGE (Fig. 1C) and western blot (Fig. 1D) using antibodies specific for tick SUB (Fig. 1E). The overall operating time in the downstream process was 79% less when compared to intracellular SUB production in *E. coli* (Almazán et al., 2010) (Table 1). The productivity, calculated as the total amount of recombinant protein obtained per culture volume and unit time, was compared between SUB-MSP1a (186.7 mg/lh) and SUB (1.7 mg/lh) production processes showing 91% higher productivity for SUB-MSP1a.

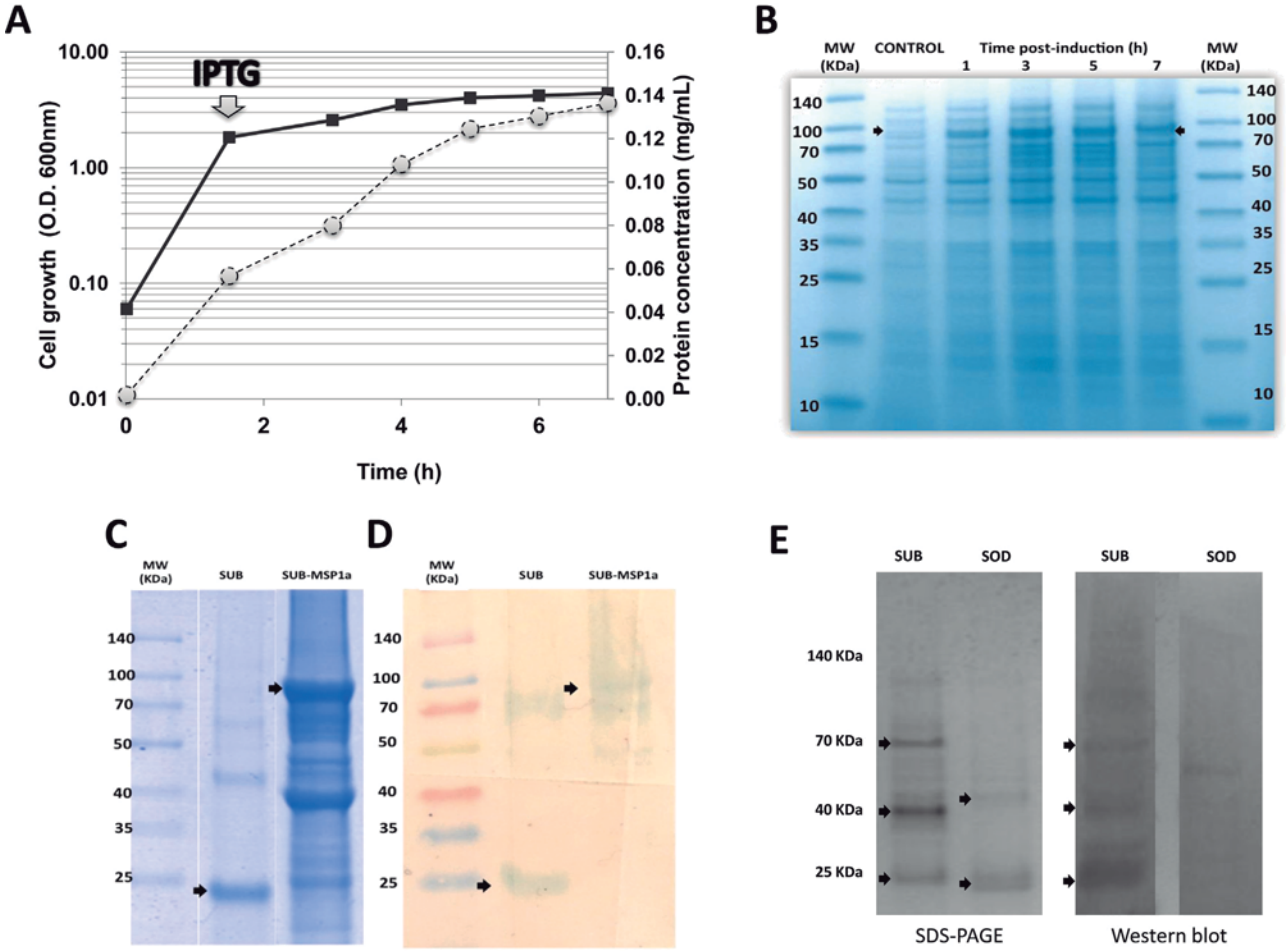

Fig. 1. Production of recombinant SUB-MSP1a chimera in *E. coli*. (A) Cell growth and total protein content were monitored by measuring $OD_{600\,nm}$ (solid line) and protein concentration (dashed line), respectively in *E. coli* transformed with the expression vector pMBXAF3 during multiplication in 250 ml. The time point of the addition of IPTG for expression induction is indicated. (B) Kinetics of recombinant SUB-MSP1a production in *E. coli* transformed with the expression vector pMBXAF3 and grown in 250 ml flasks. Samples were taken at different time points after induction with IPTG and 10 μg total proteins were loaded per well in a 12% SDS-polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue. Control *E. coli* transformed with the vector alone and induced for 7 h were included as control. The position of the recombinant SUB-MSP1a protein is indicated with arrows. (C) Coomassie Brilliant Blue stained 12% SDS-polyacrylamide gel with 10 μg recombinant purified SUB and SUB-MSP1a (arrows). (D) Western-blot analysis of the gel in (C) using rabbit antibodies against recombinant SUB. The position of the recombinant antigens is indicated with arrows. Higher and lower molecular weight bands may correspond to protein polymers or degradation products, respectively. (E) Western-blot analysis of 10 μg recombinant SUB and SOD proteins with His tags and purified by Ni affinity chromatography using rabbit antibodies against recombinant SUB to show the specificity of anti-SUB antibodies. The position of the recombinant proteins, including multimers, is indicated with arrows. Molecular weight markers (MW; Color Burst electrophoresis markers, Thermo Scientific) are indicated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The protein recovery, calculated as protein concentration in the purified final fraction divided by total protein concentration, was also higher for SUB-MSP1a (90% recovery) than for SUB (30% recovery).

Table 1
Estimated time per unit operation for the production and recovery of recombinant antigens in *E. coli*.

Process operation	Estimated time per operation (h)	
	Intracellular SUB	Membrane-bound SUB-MSP1a
Seed	12	12
Multiplication	7	7
Cell harvesting I	0.25	0.5
Homogenization I	1	1
Cell disruption	0.5	0.5
Cell harvesting II	0.25	0.5
Homogenization II	1	1
Microfiltration	1	NR
Chromatography	1	NR
Dialysis	12	NR
Total time	35	21

Abbreviation: NR, not required for antigen production.

These results reinforced the advantages and cost-effectiveness of the production of surface-exposed membrane-bound chimeric antigens (Almazán et al., 2012; Canales et al., 2010).

3.2. The SUB-MSP1a chimera is localized on the membrane of recombinant E. coli

The specificity of rabbit antibodies against tick SUB was demonstrated in western blot analysis of purified SUB and SUB-MSP1a proteins (Fig. 1D and E). As previously shown for the BM95-MSP1a chimeric antigen (Canales et al., 2008), the SUB-MSP1a chimera but not SUB was localized on the membrane of recombinant *E. coli* (Fig. 2), thus confirming membrane exposure of the SUB-MSP1a antigen.

3.3. Bacterial membranes increase the immunogenicity of the recombinant SUB-MSP1a chimeric antigen

The protection elicited by tick vaccines is mediated by antibodies against the vaccine antigen that are ingested by ticks during feeding on vaccinated hosts and result in antibody–antigen

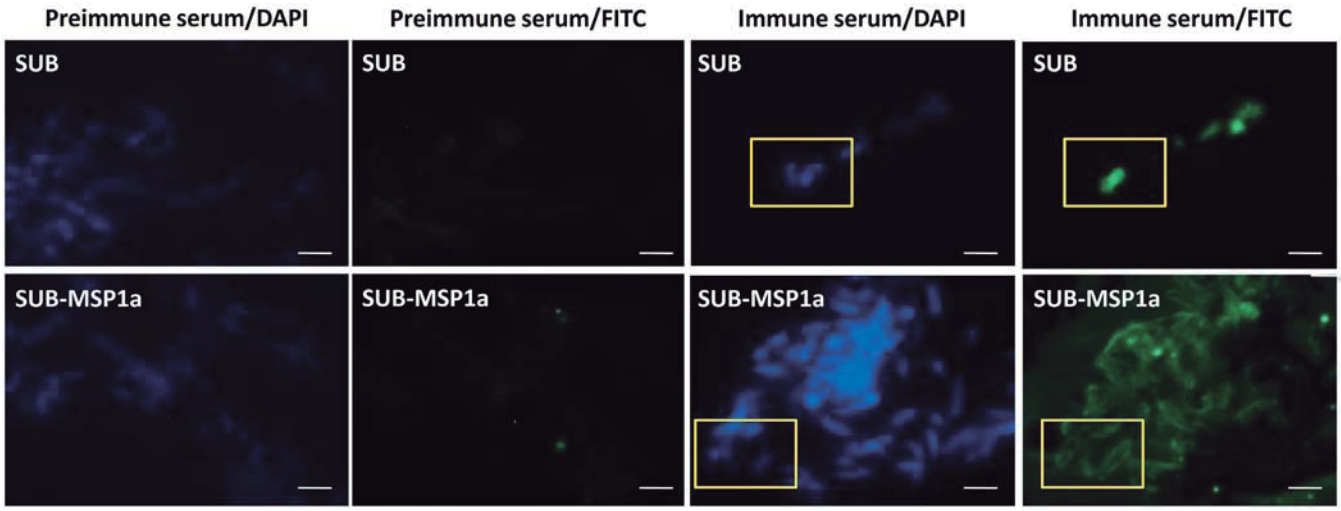

Fig. 2. Cellular localization of recombinant SUB-MSP1a. Surface display of the SUB-MSP1a fusion protein on *E. coli*. Live-cell immunofluorescence assay of recombinant *E. coli* expressing SUB and the SUB-MSP1a fusion protein and reacted with preimmune serum/DAPI, preimmune serum/FITC, anti-SUB immune serum/DAPI and anti-SUB immune serum/FITC primary antisera, followed by a secondary reaction with fluorescein-labeled affinity purified goat antibody to rabbit IgG. Bacterial nucleic acids are stained with DAPI in blue and SUB proteins are stained in green. Representative images are framed in yellow. Bars, 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

interactions that reduce tick numbers, feeding and/or oviposition (Elvin and Kemp, 1994; Popara et al., 2013). Consequently, antibody titers in vaccinated hosts positively correlate with vaccine efficacy (Willadsen, 2006; Moreno-Cid et al., 2013; Merino et al., 2011). These facts support the importance of increasing the immunogenicity of tick vaccine antigens.

One possibility to increase antigen immunogenicity that was targeted with membrane-bound tick proteins fused to MSP1a such as SUB-MSP1a is the use of the adjuvant effect of some components present in the membrane of microorganisms (Rodríguez et al., 2001; da Silva et al., 2008; Macmillan et al., 2008; Yokoi et al., 2009). *E. coli* surface-exposed SUB-MSP1a includes bacterial membranes containing the recombinant antigen together with components with adjuvant effect (Supplementary Tables 1 and 2). However, despite the protective effect elicited by these antigens on the control of cattle tick infestations (Almazán et al., 2012; Canales et al., 2009), the effect of bacterial membranes on antigen immunogenicity has not been characterized.

Herein, three experiments were conducted to characterize the effect of bacterial membranes on SUB-MSP1a antigen immunogenicity. In the first experiment, mice were immunized with membrane-bound and gel-extracted membrane-free SUB-MSP1a antigens to compare the antibody titers. Gel-extracted membrane-free SUB-MSP1a antigens were used in this experiment because the recovery of the soluble membrane-free SUB-MSP1a fraction was very low (less than 1% of the recombinant protein). The results showed that antibody titers against tick SUB after immunization were significantly higher in mice immunized with the membrane-bound antigen when compared to animals immunized with the membrane-free antigen or controls (Fig. 3A). However, because of the possibility that gel-extracted membrane-free SUB-MSP1a antigens reduced the immune response in vaccinated animals due to antigen purification procedure, a second experiment was conducted in rabbits. In this experiment, rabbits were immunized with membrane-bound SUB-MSP1a and soluble membrane-free SUB-MSP1a to compare the antibody titers. Preliminary results showed that after a single immunization, antibody titers against tick SUB were significantly higher in rabbits immunized with the membrane-bound antigen when compared to animals immunized with the soluble membrane-free antigen or controls (Fig. 3B). This experiment confirmed that membrane-bound SUB-MSP1a antigen was more immunogenic than the membrane-free antigen independently of the extraction procedure use to purify the membrane-free SUB-MSP1a antigen.

3.4. Antibodies in pigs orally immunized with SUB-MSP1a reduce tick weight

The first two experiments demonstrated the effect of bacterial membranes in increasing the immunogenicity of the SUB-MSP1a antigen. Then, a third experiment was conducted to further characterize the immunogenicity of the membrane-bound SUB-MSP1a antigen in relation with its potential protective capacity against tick infestations. The rationale for this experiment was to compare the orally induced immunogenicity and potential protective capacity in the absence of adjuvant of the membrane-bound SUB-MSP1a antigen in comparison with tick SUB, which has been shown to be effective against infestations by various tick species in different hosts (de la Fuente et al., 2011, 2013). In this experiment experiment, pigs were orally immunized with recombinant SUB or membrane-bound SUB-MSP1a antigens in the absence of adjuvant. This model has been used before to characterize the efficacy of oral vaccines on tuberculosis control and demonstrated the activation of adaptive antibody response after oral immunization (Garrido et al., 2011). Furthermore, orally administered tick vaccine formulations may be relevant for the immunization of wildlife reservoirs against tick infestations to prevent tick-borne pathogens from crossing the interspecies barrier and cause diseases in humans and domestic animals (Gortazar et al., 2014). The results showed that the membrane-bound SUB-MSP1a antigen induced an antibody response in pigs that was significantly higher when compared to SUB-immunized and control pigs while the antibody response with the *E. coli* membrane-free recombinant SUB only differed from controls after the second immunization (Fig. 4A). Furthermore, the antibody titers rose after the first immunization and lasted for at least 8 weeks in pigs orally immunized with the membrane-bound SUB-MSP1a antigen (Fig. 4A).

Tick capillary feeding was then used to characterize the potential protective capacity of the antibodies raised against recombinant membrane-free SUB and membrane-bound SUB-MSP1a in orally immunized pigs. Capillary feeding allows obtaining preliminary information on the efficacy of candidate tick protective

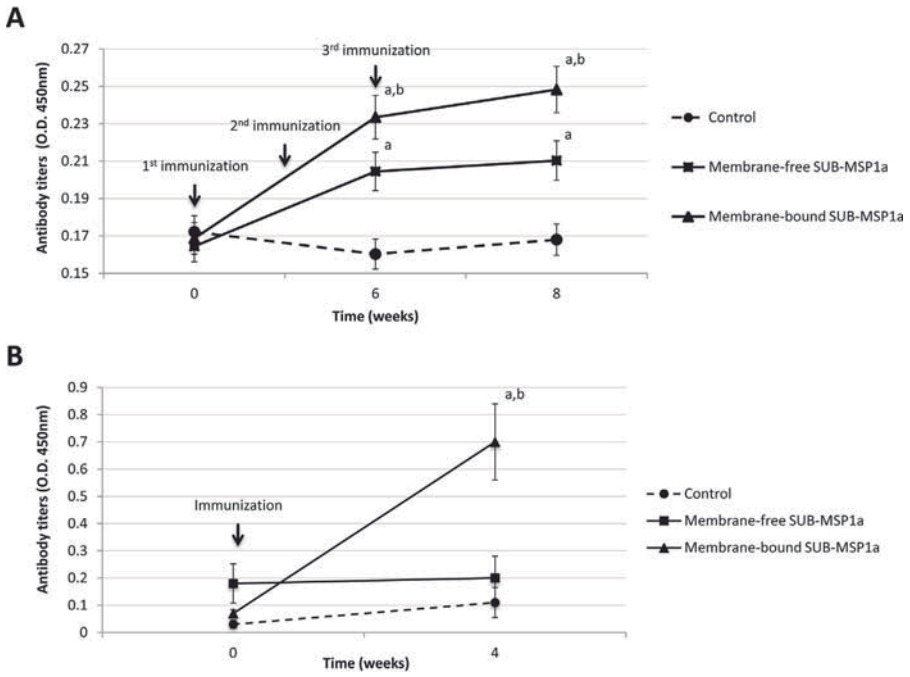

Fig. 3. Immunogenicity of recombinant SUB-MSP1a chimeric antigen. (A) Balb/c mice ($N = 5$ per group) were immunized with membrane-bound SUB-MSP1a, membrane-free SUB-MSP1a or adjuvant/saline alone as control. Antibody titers were determined in mice at different time points and expressed as the $OD_{450\,nm}$ ($OD_{mouse\ sera} - OD_{PBS\ control}$) at the highest serum dilution (1:1000) and compared between immunized and control groups ($^{a}P \leq 0.01$) and between both immunized groups ($^{b}P \leq 0.01$) by Student's *t*-test with unequal variance. Immunization times are indicated with arrows. (B) Rabbits ($N = 3$ per group) were immunized once with recombinant membrane-bound SUB-MSP1a, soluble membrane-free SUB-MSP1a or adjuvant/saline alone as control. Antibody titers were determined in rabbits 4 weeks after immunization and expressed as the $OD_{450\,nm}$ ($OD_{mouse\ sera} - OD_{PBS\ control}$) at the serum dilution (1:100) and compared between immunized and control groups ($^{a}P \leq 0.05$) and between both immunized groups ($^{b}P \leq 0.05$) by Student's *t*-test with unequal variance. Immunization time is indicated with an arrow.

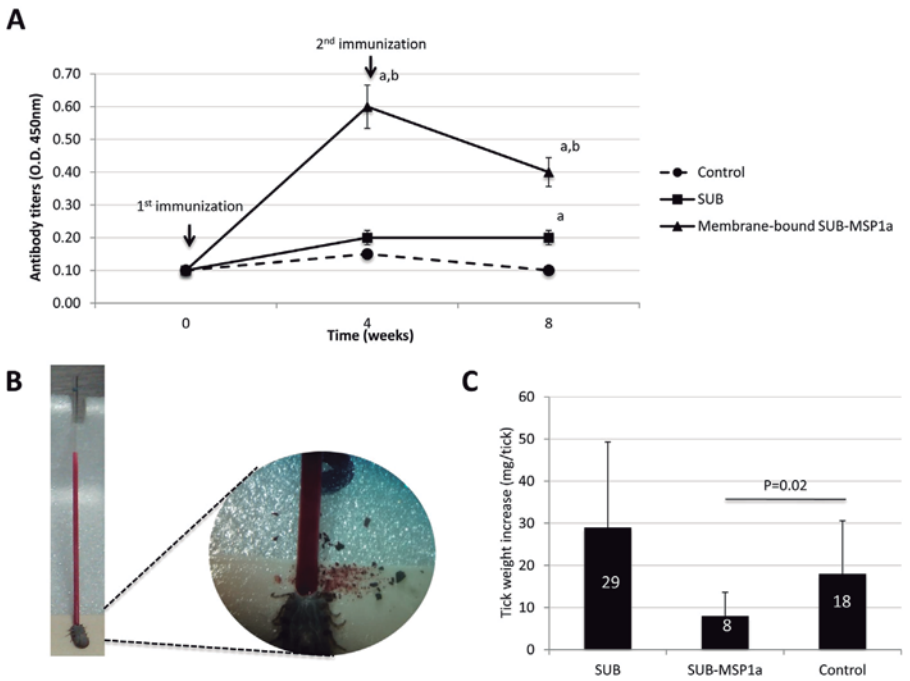

Fig. 4. Effect of antibodies from orally immunized pigs on tick weight. (A) Iberian piglets ($N = 3$–5 per group) were orally immunized with recombinant SUB, membrane-bound SUB-MSP1a or adjuvant/saline alone as control. Antibody titers were determined in pigs at different time points and expressed as the $OD_{450\,nm}$ ($OD_{mouse\ sera} - OD_{PBS\ control}$) at the highest serum dilution (1:1000) and compared between immunized and control groups ($^{a}P \leq 0.01$) and between both immunized groups ($^{b}P \leq 0.01$) by Student's *t*-test with unequal variance. Immunization times are indicated with arrows. (B) Representative images of *R. microplus* female tick capillary feeding. (C) Ticks ($N = 15$ per group) capillary-fed on cattle blood supplemented with sera from pigs orally immunized with SUB, SUB-MSP1a or PBS (Control) were weighed before and after capillary feeding, the tick weight increase calculated as final minus initial tick weight, expressed as Ave + S.D. (mg/tick) and compared between the control group and the other groups by Student's *t*-test. Only significant differences are shown.

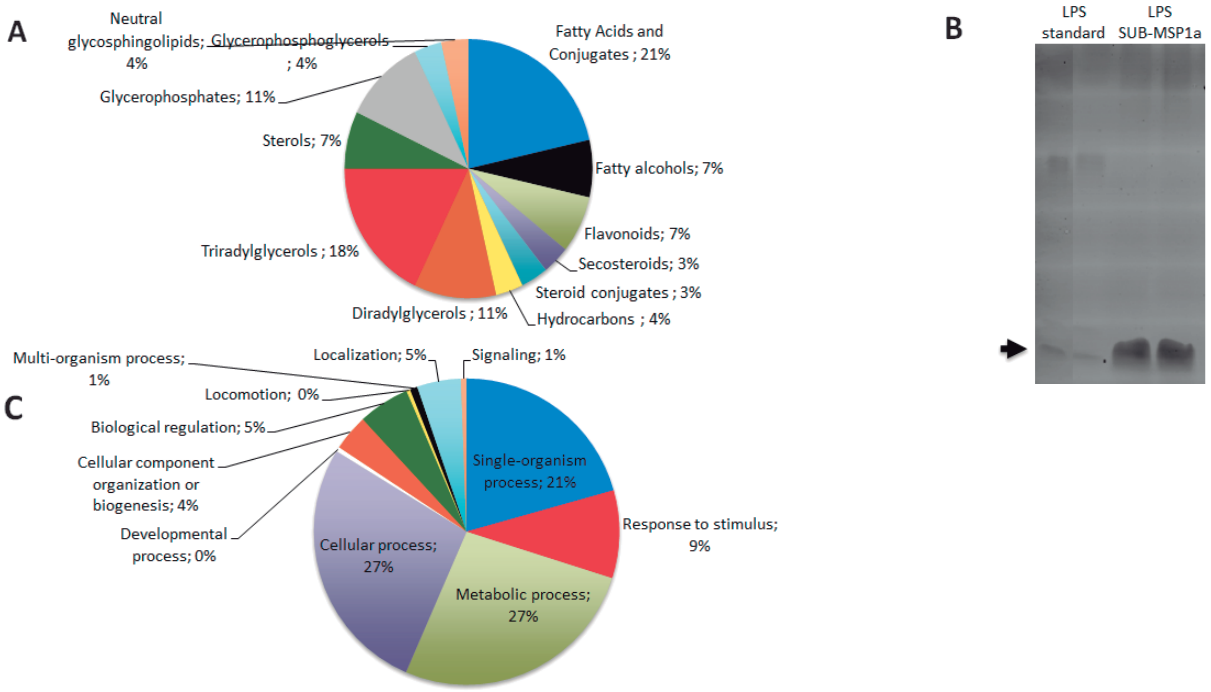

Fig. 5. Lipidomics and proteomics analysis of the membrane-bound SUB-MSP1a antigen preparation. (A) Main lipid classes identified by lipidomics analysis of the membrane-bound SUB-MSP1a antigen. (B) LPS profile of the membrane-bound SUB-MSP1a antigen. Two independent replicates of the isolated LPS were separated on a 12% SDS-polyacrylamide gel and stained with Pro-Q Emerald 300. The LPS from *E. coli* serotype 055:B5 were used as standard. The LPS core is indicated with an arrow. (C) Biological process assignments to *E. coli* proteins identified by proteomics analysis in the membrane-bound SUB-MSP1a antigen.

antigens (Antunes et al., 2014; Lew-Tabor et al., 2014; Gonsioroski et al., 2012). In these experiments, *R. microplus* female ticks were fed using capillaries containing blood supplemented with sera from orally immunized pigs (Fig. 4B). The results showed a significant reduction in the weight of ticks fed on blood supplemented with sera from pigs orally immunized with membrane bound SUB-MSP1a but not membrane-free SUB when compared to controls (Fig. 4C). These results correlated with the anti-SUB antibody titers obtained in orally immunized pigs after 4 weeks (Fig. 4A) and showed an effect of these antibodies on tick feeding. These results constitute the first evidence of the protective capacity of the membrane-bound SUB-MSP1a antigen administered orally to immunize hosts and supported the use of membrane-bound antigens in vaccine formulations.

3.5. The membrane-bound SUB-MSP1a chimeric antigen contains bacterial components with potential adjuvant effect

These results showed the effect of bacterial membranes on increasing the immunogenicity and protective capacity of the membrane-bound SUB-MSP1a antigen. To provide a preliminary characterization of the bacterial membrane components with adjuvant effect, the membrane-bound SUB-MSP1a antigen was subjected to lipidomics and proteomics analyses.

The lipidomics analysis of the membrane-bound SUB-MSP1a antigen resulted in the identification of 26 lipids that were classified into 12 main classes (Fig. 5A and Supplementary Table 1). The immunostimulatory activity of lipids associated with bacterial membranes has been recognized for several decades and exploited in a large variety of different adjuvant preparations (Andersen et al., 2009). Among the predominant lipid components in the membrane-bound SUB-MSP1a antigen, fatty acids and conjugates (21%), fatty alcohols (7%) and sterols (7%) (Fig. 5A) have been shown to act as adjuvant or have potent immunoregulatory effects (Bomford, 1981; Bashir et al., 2013). Additionally, the ability of bacterial LPS to function as an adjuvant and enhance the immune response to antigens has been well established (Hoogerhout et al., 1995) and this adjuvant activity resides in its lipid A moiety (Han et al., 2014). Therefore, the presence of LPS was also demonstrated in the membrane-bound SUB-MSP1a antigen (Fig. 5B).

The proteomics analysis identified 339 *E. coli* proteins in the membrane-bound SUB-MSP1a antigen preparation (Supplementary Table 2). As expected, the most abundant proteins were *A. marginale* MSP1a (193 ± 27 PSMs) and *R. microplus* SUB (186 ± 19 PSMs) (Supplementary Table 2). The identified *E. coli* proteins were annotated into 11 biological processes, including cellular, single-organism and metabolic process, response to stimulus, and cellular component organization or biogenesis that contain proteins involved in the stimulation of the immune response (Wells and Malkovsky, 2000; Tsukamoto et al., 2011) (Fig. 5C).

In summary, these results showed the presence in the membrane-bound SUB-MSP1a antigen of several molecules with potential stimulatory or regulatory effects on immune responses that likely contributed to its adjuvant effect to enhance the immunogenicity of this antigen preparation when compared to membrane-free antigens.

3.6. Endotoxin content in the membrane-bound SUB-MSP1a antigen is within the limits acceptable for recombinant vaccines

Considering the adjuvant effect of bacterial components in the membrane-bound SUB-MSP1a vaccine formulation, a balance should be reached between this positive effect and the detrimental effect of endotoxins associated with the membrane fraction that may raise safety issues. To address this issue, herein we used the LAL assay to determine the endotoxin content in the purified membrane-bound SUB-MSP1a preparation. The LAL assay has been used since the early 1970s for the detection of endotoxins and provides a reliable and validated tool for these studies (Brito and Singh, 2010).

Several approaches have been used to obtain endotoxin-free antigens produced in *E. coli* (Canales et al., 2010), including endotoxin removal using non-ionic detergents (Aida and Pabst, 1990). The process developed to recover the *E. coli* membrane fraction containing the exposed SUB-MSP1a antigen contains 0.1% Triton X-100 (Almazán et al., 2012). The residual endotoxin levels obtained in the crude fraction after cell centrifugation was equal to 173 ± 20 endotoxin units (EU)/mg. However, after cell disruption and treatment with Triton X-100, endotoxin levels decreased to 85 ± 5 EU/mg. Therefore, in the final vaccine formulation for cattle containing 0.1 mg antigen per dose (Almazán et al., 2012), endotoxin levels were equivalent to 8.5 ± 0.5 EU/dose. This endotoxin level is lower than the maximum recommended level of 20 EU/dose for recombinant vaccines (Brito and Singh, 2010).

These results showed that the use of 0.1% Triton X-100 was successful in reducing endotoxins to acceptable levels for vaccine formulations based on the membrane-bound SUB-MSP1a using a simple a cost-effective production system (Canales et al., 2010).

4. Conclusions

The results of this study provided further support for the development of tick vaccines using *E. coli* membranes exposing chimeric antigens such as SUB-MSP1a composed of tick polypeptides fused to MSP1a. The production of these membrane-bound antigens is simple and cost-effective (Canales et al., 2010) and results in antigen preparations with increased immunogenicity and protective capacity in immunized animals with endotoxin levels within the limits acceptable for recombinant vaccine formulations.

Conflict of interests

J.A. Moreno-Cid and M. Canales are currently working for biotech companies but their contribution to this research was while they worked at IREC. The other authors declare that there is no conflict of interests regarding the publication of this paper.

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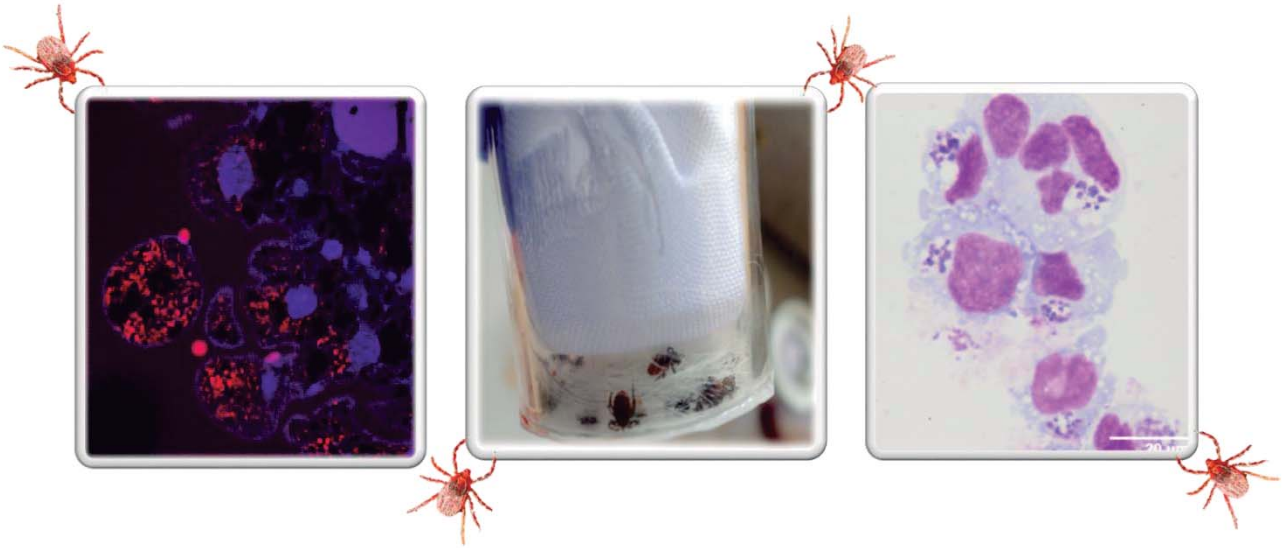
CHAPTER IV.
General discussion



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Review

Targeting a global health problem: Vaccine design and challenges for the control of tick-borne diseases

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ABSTRACT

It has been over twenty years since the first vaccines for the control of tick infestations became commercially available. These vaccines proved their efficacy and the potential of this approach for the control of tick-borne diseases (TBDs), which represent a growing burden for human and animal health worldwide. In all these years, research in this area has produced new tick-derived and pathogen-derived candidate protective antigens. However, the potential of vaccines for the control of TBDs has been underestimated due to major challenges to reduce tick infestations, pathogen infection, multiplication and transmission, tick attachment and feeding time and/or host pathogen infection. Nevertheless, vaccines constitute the most safe and effective intervention for the control of TBDs in humans, domestic and wild animals.

Contents

1. Introduction

Vector-borne diseases (VBDs) represent a growing burden for human and animal health worldwide [1–3]. Ticks (Acari: Ixodida) are obligate hematophagous arthropod ectoparasites that are second to mosquitoes as vectors of pathogens causing diseases in humans and the first cause of VBDs in farm animals [4]. Among the most prevalent tick-borne diseases (TBDs), Lyme disease caused by some species of the *Borrelia burgdorferi* sensu lato (s.l.) complex and anaplasmosis caused by *Anaplasma* spp. constitute a growing burden for humans, companion and farm animals worldwide [4–10] (Table 1).

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Table 1
Characterization of selected TBDs caused by TBPs with different transmission cycles.

TBP	TBD	TBD-affected hosts[a]	Main tick vector species	Tick cycle[b]	TBP transmission time at tick bite	Transovarial transmission
Bacteria						
B. burgdorferi s.l.	Lyme disease	H/C	*Ixodes* spp.	3	16–72 h	No
Anaplasma phagocytophilum	Human granulocytic anaplasmosis, tick-borne fever	H/F	*Ixodes* spp.	3	24–48 h	No
Anaplasma marginale	Bovine anaplasmosis	F	*Rhipicephalus* spp., *Dermacentor* spp.	1, 3	24–48 h	No
Anaplasma platys	Canine cyclic thrombocytopenia	C	*Rhipicephalus sanguineus*	3	16–72 h	No
Rickettsia rickettsii	Rocky Mountain spotted fever	H	*Dermacentor* spp., *Amblyomma* spp., *R. sanguineus*	3	10 h	Yes
Ehrlichia ruminantium	Heartwater	F	*Amblyomma* spp.	3	48–96 h	No
Protozoans						
Babesia divergens, Babesia microti	Human babesiosis	H	*Ixodes* spp.	3	48–72 h	Yes, No
B. divergens, Babesia bovis, Babesia bigemina	Bovine babesiosis	F	*Ixodes* spp., *Rhipicephalus* spp.	1, 3	48–216 h	No
Theileria annulata	Tropical theileriosis	F	*Hyalomma* spp., *Rhipicephalus appendiculatus*	3	48 h	No
Babesia canis, Babesia vogeli	Canine babesiosis	C	*R. sanguineus, Dermacentor* spp., *Haemaphysalis leachi*	3	48 h	Yes
Viruses						
Tick-borne encephalitis virus	Tick-borne encephalitis	H	*Ixodes* spp.	3	Immediate	Yes
Crimean-Congo hemorrhagic fever virus	Crimean-Congo hemorrhagic fever	H/F	*Hyalomma* spp.	3	Immediate	Yes
Louping ill virus	Louping ill	F	*Ixodes ricinus*	3	Immediate	No

Data compiled from de la Fuente et al. [4] and Schorderet-Weber et al. [10].
[a] TBD-affected hosts: H (human), C (companion animal), F (farm animal).
[b] The number of hosts involved in tick life cycle.

Several approaches have been implemented for reducing the risk of TBDs. These approaches include the use of chemical acaricides, which have been only partially successful and often accompanied by serious drawbacks including the selection of acaricide-resistant ticks and contamination of the environment and animal products with residues, the use of botanical acaricides and repellents, entomopathogenic fungi and the education about recommended practices to reduce exposure to ticks and available options for the management of drug resistance [11,12]. Additionally, integrated control programs that include habitat management and the genetic selection of hosts with higher resistance to ticks have been also proposed to reduce the use of acaricides for the control of tick infestations [11,12]. Recent developments have suggested the possibilities of combining chemicals with repellency and parasiticidal activity to reduce the risk of TBDs [10]. This approach intends to prevent both vector infestations and pathogen transmission [10]. However, major difficulties such as long-lasting effect and safety for human and animal use encourage the development of vaccines, which could induce a long-lasting protective immune response against vector infestation and pathogen infection and transmission [11].

Vaccines constitute one of the greatest advances in science with a substantial impact on improving human and animal health. Vaccines for the control of TBDs have been controversial due among other limitations to the impossibility of preventing tick infestations and consequently the possibility of pathogen transmission. For those involved in the development of vaccines for the control of tick infestations and TBDs, these limitations constitute a challenge that has been approached by developing new platforms for the identification and characterization of candidate tick-derived and pathogen-derived protective antigens [13,14]. As discussed in this paper, recent results support that vaccines are indeed the most effective and environmentally sound approach for the prevention and control of TBDs.

2. Current status of the vaccines for the control of TBDs

The first vaccines for the control of cattle tick infestations became commercially available in the early 1990s [15]. These vaccines contained the *Rhipicephalus microplus* BM86 or BM95 recombinant antigens and their use demonstrated that vaccines could constitute an effective component of the integrated programs for the control of TBDs [11,15,16]. These vaccines were not designed to prevent tick infestations, but to reduce tick populations and the prevalence of tick-borne pathogens (TBPs) by affecting feeding, reproduction and development of ticks feeding on immunized animals and ingesting with the blood meal antigen-specific antibodies that interact with and affect protein function [11,15,16]. Most of the data available on vaccine efficacy against tick infestations under field conditions have been obtained in cattle [11,15,16], but results are also available in other farm animals such as sheep and camels, companion animals such as dogs, and natural wild tick hosts such as deer [7,11]. Vaccines based on tick-derived antigens have also shown to have an effect on reducing pathogen infection and transmission with the possibility of targeting multiple tick species and other arthropod vectors [11,17]. Finally, the use of pathogen-derived antigens has proven effective for reducing the risk of Lyme disease under certain conditions in both humans [18] and natural reservoir hosts [19,20], but currently due to safety issues, commercial vaccines are not available for this disease [18]. Among TBDs for which vaccines are currently available, tick-borne encephalitis (TBE) caused by TBE virus (TBEV) is one of the most widespread in Europe [21,22]. An inactivated virus vaccine also provides partial protection against Louping ill virus (LIV) and Spanish goat encephalitis virus (SGEV) in sheep [23]. For other tick-borne viruses such as Crimean-Congo hemorrhagic fever virus (CCHFV) causing the CCHF, the efficacy of vaccines based on inactivated virus has not been clearly demonstrated [22].

The combination of tick-derived and pathogen-derived antigens to improve vaccine efficacy for the control of TBDs is still a pending challenge. Mice immunization with a combination of *I. scapularis* salivary protein Salp15 and *B. burgdorferi* s.l. outer surface proteins OspA/OspC provided better protection from *B. burgdorferi* s.l. infection than either antigen alone [24]. A vaccine combining the tick vector Salp15 protein used by the pathogen for infection of the vertebrate host together with the pathogen-derived OspC protein that binds to Salp15 during *Borrelia* transmission enhances vaccine protection against Lyme disease [24]. Although limited by the results available for vaccines using pathogen-derived antigens, these results encourage the combination of tick-derived and pathogen-derived antigens to target vector infestation and pathogen infection and transmission [11].

3. An approximation to the evaluation of the risk of TBDs and vaccine efficacy

Modifying the method used by Kilpatrick et al. [25] for mosquito-borne West Nile virus risk assessment, we propose that the risk (probability) that a tick species will infest a human, companion, farm or wild animal with transmission of a TBP could be estimated as Risk (R) = $A \times F_m \times P \times C_v \times T \times S$, where A is the abundance of the tick species in the study area, F_m is the fraction of blood meals taken from natural hosts for TBP, P is the TBP infection prevalence in the tick vector species, C_v is an index of tick vector competence corresponding to the fraction of TBP-infected ticks that will transmit pathogen after bite, T is the attachment success and feeding time of a tick vector species, and S is the vertebrate host (human, companion, farm or wild animal) susceptibility to TBP.

Considering these parameters for R assessment, the efficacy (E) of a vaccine to decrease the risk for TBDs can be estimated as $E = E_1 \times E_2 \times E_3 \times E_4 \times E_5$, where E_1 is related to A and F_m and represents the reduction in tick feeding (number of tick larvae, nymphs and adult females completing feeding), oviposition (number of laid eggs) and fertility (number of hatched larvae) calculated as previously discussed in de la Fuente and Contreras [11] and Aguirre Ade et al. [26] for one-host (tick species completing life cycle feeding on a single host) and multiple-host (tick species in which each developmental stage feeds on a different host) ticks, respectively, E_2 is related to P and represents the reduction in TBP infection and multiplication in the tick vector, E_3 is related to C_v and represents the reduction in TBP transmission by the tick vector, E_4 is related to T and represents the reduction in tick attachment and feeding time, and E_5 is related to S and represents the reduction in host pathogen infection.

Therefore, it is possible to implement research strategies for the identification of candidate tick-derived and pathogen-derived protective antigens by targeting different E components. For example, if the objective is to identify protective antigens with E1, then research should focus on proteins with relevant biological function in tick feeding, reproduction, development, and subversion of host immunity [14]. These proteins would have a key role in biological processes controlling tick feeding, oviposition and fertility, and could be identified by using different approaches [14].

Vaccines represent about one-quarter of the research on methods for the control of TBDs [27]. Nevertheless, recent results have shown the identification of tick protective antigens with different activities and vaccine E (Table 2). The vaccines based on these antigens reduce tick infestations (E_1), TBP infection and multiplication in the tick (E_2), TBP transmission (E_3), tick attachment and feeding time (E_4), and/or host TBP infection (E_5). Furthermore, indirect evidence support that vaccination with some antigens may reduce tick feeding time in vaccinated hosts, which affects pathogen transmission (e.g. [28]). Additionally, vaccine formulations with *B. burgdorferi* s.l. pathogen-derived antigens have provided evidence for reduction of host TBP infection (E_5) (Table 2).

4. Vaccines: Possibilities and challenges for the control of TBDs

Several factors need to be considered when designing effective vaccines for the control of TBDs. The proposed formulae to calculate R and vaccine E for TBDs help identifying these factors. The transmission cycles for TBDs identify different possible vaccine targets (Fig. 1), but differences in TBD-affected hosts, tick vector species, tick life cycle, TBP transmission cycle, and TBP transmission time after tick bite should be considered (Table 1). For TBDs in which TBPs are transmitted by one-host ticks, it is possible to focus on a single host for vaccine administration and efficacy. However, for multiple-host tick vector species, it may be necessary to consider multiple hosts for vaccination. As recently shown for tick Aquaporin antigens, the immune response to vaccination may differ between different hosts (e.g. [29]), and therefore should be considered during vaccine design and efficacy trials. Vaccines targeting wild reservoir hosts are more effective for TBPs that are only acquired by ticks feeding on infected hosts than for those transmitted transovarially from one tick generation to the next. TBP transmission time after tick bite is an important factor to consider for the design of vaccines to control TBDs. For TBPs requiring several hours of tick feeding for transmission, which is the case for most bacterial and protozoan pathogens, then the possibility of blocking pathogen transmission in vaccinated hosts in much easier than for tick-borne viruses that are transmitted immediately after tick bite (Table 1). In the case of viruses, vaccines affecting tick attachment and feeding time (E_4), and/or reduction in host TBP infection (E_5) are the only option to protect hosts from these TBDs. In addition to these considerations, which are valid for all TBD-affected hosts, vaccines targeting wild reservoir hosts, domestic animals or humans have different considerations.

4.1. Vaccines for wild reservoir hosts

Vaccines could be designed to target wild hosts for TBPs and tick vectors. Red deer (*Cervus elaphus*) and white-tailed deer (*Odocoileus virginianus*) are wild hosts for different tick vector species and TBPs playing a role in the maintenance and dispersal of TBDs such as Lyme disease [18]. Vaccination of these hosts with tick-derived BM86 or Subolesin (SUB) antigens in controlled pen and field trials showed reduction in tick infestations [30], which could reduce tick populations under field conditions [31]. Results of field trials using oral vaccines with *B. burgdorferi* s.l. outer surface proteins have shown that is possible to reduce tick infection prevalence by vaccinating wild white-footed mice (*Peromyscus leucopus*), reservoir hosts involved in maintenance and spread of Lyme disease [19,20]. In principle, other tick antigens affecting tick infestations (E_1) and tick pathogen infection (E_2) could be also used for vaccination of vertebrate hosts supporting the circulation of tick vectors and TBPs (Table 2). However, the challenge is to produce effective oral vaccine formulations by combining tick-derived antigens alone or in combination with pathogen-derived antigens to reduce E_1 and E_2 with administration methods to specifically and effectively target wild vertebrate host species. If effective, these vaccines would reduce the risk of TBDs by reducing both tick populations and infection prevalence.

4.2. Vaccines for domestic animals

The experience with the use of BM86/BM95 vaccines for the control of cattle tick infestations supports that this is a cost-

Table 2
Examples of vaccine antigens with different protective efficacy.

Vaccine antigens	Vaccinated hosts	Targeted tick species	TBP	Vaccine efficacy
Rhipicephalus spp. BM86/BM95	cattle, camel, dog, deer	*Rhipicephalus microplus, Rhipicephalus annulatus, Rhipicephalus decoloratus, Rhipicephalus sanguineus, Hyalomma dromedarii*	NE	E_1
Tick spp. Subolesin and Subolesin/Akirin chimeras	cattle, sheep, deer, rabbit, mouse	*R. microplus, R. annulatus, Ixodes scapularis, Ixodes ricinus, Hyalomma* spp., *Haemaphysalis* spp., *Amblyomma americanum, Dermacentor variabilis, Dermacentor reticulatus*	*Anaplasma phagocytophilum, Anaplasma marginale, Borrelia burgdorferi* s.l., *Babesia bigemina*	E_1, E_2, E_3
R. microplus Metalloprotease	cattle	*R. microplus*	NE	E_1
R. microplus Ribosomal protein P0	cattle	*R. microplus*	NE	E_1
I. ricinus, *R. microplus* Ferritin 2	cattle, rabbit	*R. microplus. R. annulatus, I. ricinus*	NE	E_1
I. ricinus, *R. microplus* Aquaporin	cattle, rabbit	*R. microplus, I. ricinus*	NE	E_1
R. microplus Silk	cattle	*R. microplus*	*Anaplasma marginale*	E_1, E_2
R. microplus TROSPA	cattle	*R. microplus*	*Babesia bigemina*	E_2
R. appendiculatus Serpins (RAS)	cattle	*R. appendiculatus*	*Theileria parva*	E_1, E_3
R. appendiculatus cement protein 64P	guinea pig, hamster, rabbit, mouse	*R. appendiculatus, I. ricinus*	Tick-borne encephalitis virus	E_1, E_2, E_3, E_4
I. scapularis salivary protein (Salp) Salp15	mouse	NE	*Borrelia burgdorferi* s.l.	E_5
I. scapularis Salp25D	mouse	NE	*Borrelia burgdorferi* s.l.	E_2
I. scapularis TROSPA	mouse	NE	*Borrelia burgdorferi* s.l.	E_2, E_3
I. scapularis tick Histamine release factor (tHRF)	mouse	*I. scapularis*	*Borrelia burgdorferi* s.l.	E_1, E_2
I. scapularis tick Salivary lectin pathway inhibitor (TSPI)	rabbit	*I. scapularis*	*Borrelia burgdorferi* s.l.	E_1, E_2
B. burgdorferi s.l. outer surface proteins (OspA/OspC)	mouse, hamster, dog, monkey, wild white-footed mouse, human	NE	*Borrelia burgdorferi* s.l.	E_2, E_3, E_5
B. burgdorferi s.l. BBI36/BBI39	rodent	NE	*Borrelia burgdorferi* s.l.	E_2, E_3, E_5

Results revised by de la Fuente and Kocan [16], Merino et al. [17], de la Fuente and Contreras [11], de la Fuente et al. [7] or recently reported by Šmit and Postma [18], Richer et al. [20], Gomes-Solecki [19], Contreras and de la Fuente [29,49], Singh et al. [50]. Abbreviation: NE, No effect reported on tick infestations or pathogen infection and transmission.

effective and environmentally sound approach to reduce tick populations and the subsequent reduction in the prevalence of some TBDs [11,15,16]. Several tick antigens have been effective in vaccine formulations to reduce tick infestations (E_1) in domestic animals (Table 2). Vaccines have been effective in reducing tick populations for one-host tick species such as *Rhipicephalus* spp. infecting cattle and acting as vectors of the highly economically relevant TBDs, bovine anaplasmosis and bovine babesiosis [11] (Table 2). However, the challenge is to produce cost-effective vaccine formulations with long lasting protective immune response, easy to administer and with high efficacy on reducing tick infestations (E_1) and/or attachment and feeding time (E_4). For bovine anaplasmosis, which is also transmitted by biting insects and blood-contaminated fomites [5], vaccines affecting tick pathogen infection (E_2), TBP transmission (E_3), and host TBP infection (E_5) in addition to the reduction of tick infestations (E_1) would be more effective for disease control.

4.3. Vaccines for humans

Vaccines designed to prevent TBDs in humans need to show high efficacy in reducing tick attachment and feeding time (E_4) and/or host TBP infection (E_5) (Table 2). The results using inactivated TBEV and recombinant *B. burgdorferi* s.l. outer surface proteins support the efficacy of vaccines with E_5 activity for the control of TBE and Lyme disease, respectively [18,21,22]. However, the combination of tick-derived and pathogen-derived antigens may be a more effective approach as shown for *B. burgdorferi* s.l. infection in mice [24]. The challenge is to produce safe vaccines with long-lasting protection and effective against multiple tick vector species and TBPs. These vaccines will likely contain a combination of protective antigens derived from both ticks and TBPs.

The α-Gal (Galα1-3 Galβ1-(3)4GlcNAc-R) syndrome is an emerging TBD affecting individuals mainly in the United States, Europe and Australia. The sensitization mediated by the IgE response to tick-produced proteins with α-Gal modifications could result in anaphylactic reactions to tick bite and/or red meat consumption [32–34]. The factors triggering the α-Gal syndrome are not known, but recent results supported a patient-specific IgE antibody response to tick proteins likely to be responsible for the anaphylaxis to tick bite [34]. Although still at its infancy, tick sialome (from the Greek sialos = saliva) proteins with α-Gal modifications and recognized by sera from patients with α-Gal syndrome but not healthy individuals exposed to tick bites may be selected as candidate protective antigens for the treatment and prevention of tick-borne anaphylactic reactions and other TBDs [34,35]. Vaccines with high efficacy on affecting tick attachment and feeding time (E_4) would also contribute to reducing the risk for tick-borne anaphylactic reactions.

5. Conclusions and future directions

Complete tick eradication by using acaricides does not appear to be a realistic goal for several reasons including (i) the possible elimination of enzootic stability for TBPs, which would result in

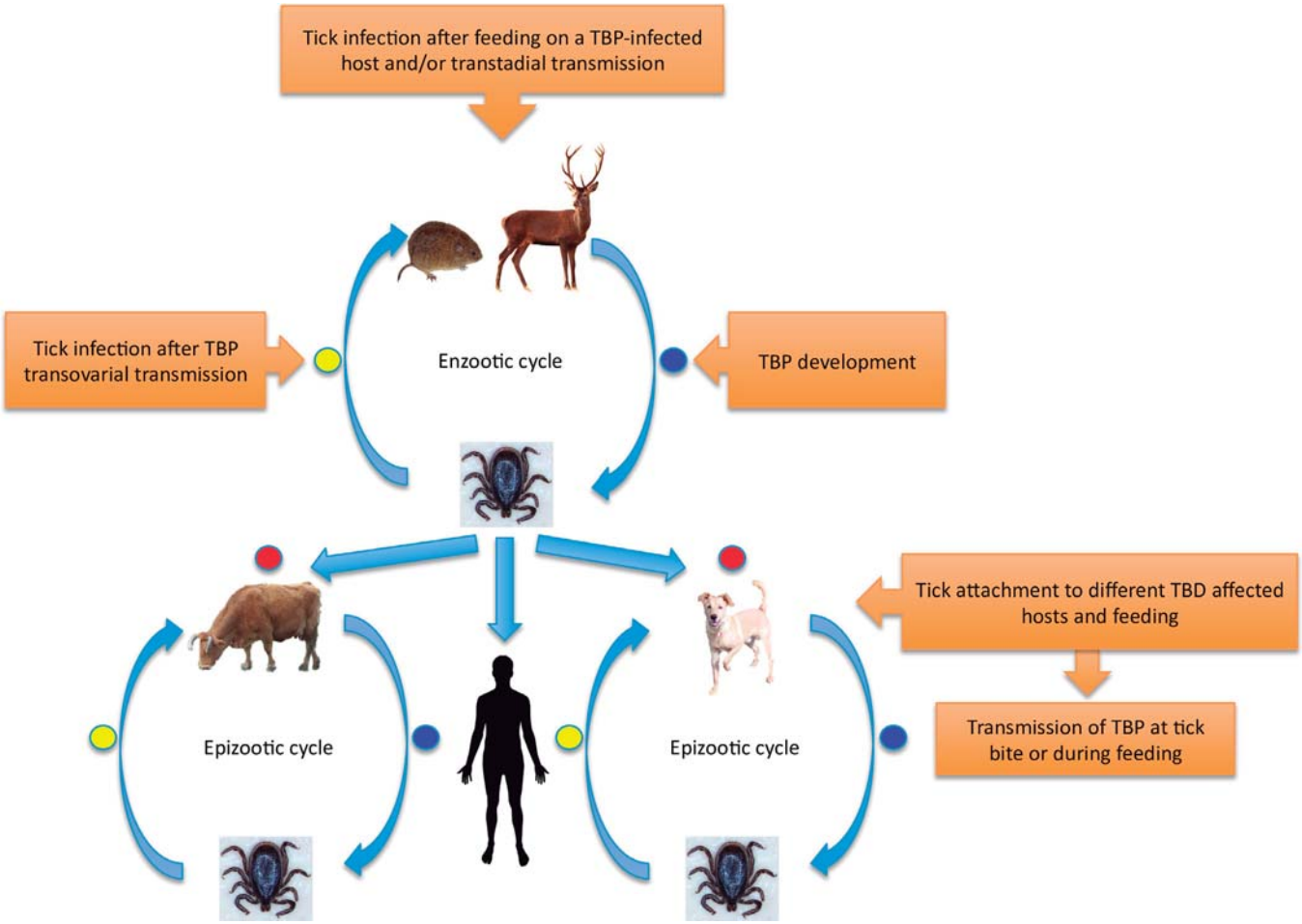

Fig. 1. Transmission cycles for TBPs. Ticks transmit a wide variety of pathogens, including bacteria, viruses, protozoa and helminthes [45]. Ticks acquire pathogens while feeding on infected hosts, and these pathogens can be transmitted from an infected tick to a susceptible vertebrate host, to another tick during co-feeding or to the next generation transovarially [46]. Ticks, vertebrate hosts and pathogens form robust networks in nature that are very resilient to specific host extinction and improve pathogen circulation [47,48]. Within these networks, specific pathogens and ticks are tightly associated to wild vertebrate hosts that play a prominent role in maintaining enzootic cycles [47,48]. Remarkably, companion and farm animals, which support epizootic cycles, play a predominant role in these tick-host-pathogen networks by increasing pathogen circulation[46]. The continuous exploitation of environmental resources and the increase in outdoor activities expose humans to tick vectors normally present in the field, but humans are only accidental hosts for ticks and TBPs.

highly susceptible populations for TBDs, (ii) the difficulty in sustaining it during conditions favorable for tick population expansion, and (iii) the effect on the reduction in biodiversity and the arsenal of bioactive compounds that might be isolated from ticks [3,27,36]. However, the control of tick populations and transmitted pathogens is essential to reduce the burden of TBDs for human and animal health.

Vaccines constitute the most safe and effective intervention for the control of TBDs. Vaccines producing a long-lasting immunity could prevent or reduce tick infestations and pathogen infection and transmission by combining tick-derived antigens with different functions alone or in combination with pathogen-derived antigens, and providing vaccine efficacy to reduce tick infestations (E_1), TBP infection and multiplication in the tick (E_2), TBP transmission (E_3), tick attachment and feeding time (E_4), and/or host TBP infection (E_5). New protective antigens will be identified by using latest omics technologies and focusing on biological processes involved in tick-host, tick-pathogen and host-pathogen interactions [11,13,14,37]. The sequencing and assembling of the first tick genome corresponding to the Lyme disease vector, *Ixodes scapularis* [38], together with the integration of transcriptomics, proteomics and metabolomics datasets [39] would facilitate the identification of effective protective antigens [40]. These vaccines should be designed considering the tick vector species, TBPs and TBD-affected hosts, and could be used alone or in combination with other control measures for the control of TBDs in humans, domestic and wild animals. Other control measures include new effective and safe formulations with repellency and parasiticidal activity [10], the application of novel technologies such as clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein-9 nuclease (Cas9) (CRISPR-Cas9) for genome editing to develop disease resistance in animals [41,42], the development of paratransgenic ticks with lower vector competence using recombinant bacteria from the tick microbiota [43], and autocidal tick control by knocking-down biologically relevant molecules for tick infestations and pathogen infection multiplication and transmission [28,44].

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Conflict of interest

The authors declare that there are no conflicts of interest.

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CONCLUSIONS

CONCLUSIONS

1. The application of vaccinomics allows the characterization and identification of candidate tick-derived and pathogen-derived protective antigens for the control of tick infestations and pathogen infection. Candidate protective antigens identified by vaccinomics are associated with different protective mechanisms.

2. The study of the *Anaplasma phagocytophilum* MSP4 and HSP70 membrane proteins shows that are involved in pathogen interaction with host cells, and HSP70 is directly implicated in pathogen infection. *A. phagocytophilum* MSP4 and HSP70 antigens are only partially protective against pathogen infection in sheep, but may constitute candidate protective antigens for the development of vaccines against tick borne diseases when used in combination with other antigens.

3. The application of vaccinomics was effective for the identification and characterization of the candidate tick protective antigens, lipocalin and lectin pathway inhibitor, for the control of *Ixodes* vector infestations and *A. phagocytophilum* infection. These proteins are involved in tick evasion of host defense response and pathogen infection and transmission, but targeting different immune response pathways.

4. The recombinant Subolesin-Akirin chimeric antigen Q38 could be used to develop vaccines targeting different tick species for the control of tick infestations while reducing the risk of pathogen transmission to humans and animals.

5. Recombinant Aquaporin-based vaccines reduce *Ixodes ricinus* infestations and molting. The results support that vaccination with CoAQP could protect against different tick species feeding on the same host.

6. The results of vaccination with the SUB-MSP1a chimera supports that *E. coli* membrane-bound antigens increase immunogenicity with acceptable endotoxin levels in cost-effective recombinant vaccine formulations to improve vaccine efficacy.

7. Vaccines could be the most safe and effective intervention for the control of tick-borne diseases. However, continuous challenges to reduce tick infestations, pathogen infection, multiplication and transmission, tick attachment and feeding time and/or host pathogen infection are required. The application and integration of novel technologies would facilitate the identification of effective protective antigens.

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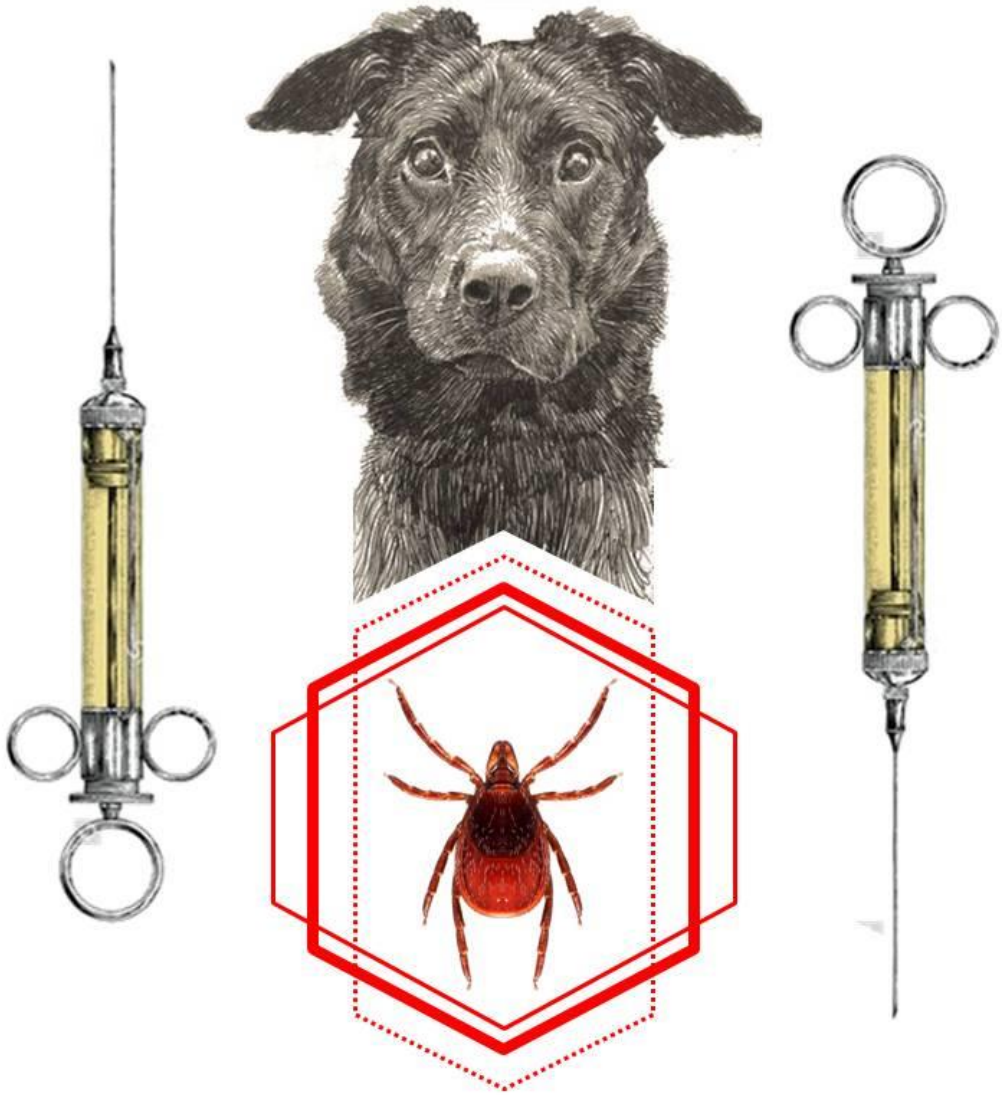

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