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Vaccination with LetiFend® reduces circulating immune complexes in dogs experimentally infected with *L. infantum*

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ABSTRACT

Domestic dogs constitute the main reservoir of *Leishmania infantum* and play a key role in transmission to humans. The main tool for controlling infection spread is a safe and effective vaccine, as successful immunization of dogs could significantly reduce the incidence of human visceral leishmaniosis (VL) and is the most cost-effective control strategy.

The factors that determine disease progression in canine leishmaniosis (CanL) remain poorly understood, though a previous study in naturally infected dogs has demonstrated a clear relationship between the presence of circulating immune complexes (CIC) in the blood and disease progression. Thus, the aim of this study was to compare CIC levels in serum samples from dogs vaccinated or unvaccinated with LetiFend®, a new vaccine containing recombinant Protein Q, and experimentally infected with *L. infantum*.

CIC were isolated from vaccinated or unvaccinated dogs after experimental infection with *L. infantum* and their levels measured by ELISA. Furthermore, reverse phase-liquid chromatography-mass spectrometry (RP-LC-MS/MS) analysis was used to investigate the protein composition of precipitated CIC.

At all the time points analyzed after infection, the amount of CIC was lower in the vaccinated group compared to the placebo group. Furthermore, there were differences in the protein composition of precipitated CIC between the vaccinated and unvaccinated groups.

In conclusion, administration of LetiFend® was able to reduce CIC elicited after experimental infection with *L. infantum* in a dog model in a process that may be related to complement system activation.

1. Introduction

Canine leishmaniosis is a systemic disease caused by *L. infantum*, which may lead to various pathologies affecting different organs and is often fatal if left untreated. The disease, which is endemic in the Mediterranean basin, Africa, Asia, and South America, is a zoonosis and dogs are the main infection reservoir for humans [1].

In CanL, progressive disease is associated with an increasing state of immunosuppression, attributed to the presence of immune regulatory cytokines [2,3] and exacerbated by a marked increase in parasite-specific antibody titers. As has been reported, the clinical manifestations of CanL are a consequence of the host immune response and linked to deposition of circulating immune complexes (CIC) in different tissues (mainly the kidney), causing lesions characterized by tubular and glomerular damage. Infected dogs, showing little or no humoral response, do not develop renal lesions [4,5]. Thus, the presence of immune complexes, formed by anti-*Leishmania* IgG and IgM and complement system fractions [6,7] correlates with an inability to resolve infections, and could be detrimental to the infected host due to the adverse influence on development of cell-mediated immune responses [8]. In this sense, a previous study has demonstrated a clear relationship between the presence of CIC and disease stage [9], and propose them as a promising biomarker of disease progression, which is

Abbreviations: BCA, bicinchoninic acid; CanL, canine leishmaniosis; CIC, circulating immune complexes; ELISA, enzyme linked immunosorbent assay; IFAT, immunofluorescence antibody testing; PEG, polyethylene glycol; SLA, soluble *Leishmania* antigen; RP-LC-MS/MS, reverse phase-liquid chromatography-mass spectrometry.

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of major value considering that a single parameter of cellular or humoral immune response is insufficient to clearly define disease severity. Integrated studies of multiple biomarkers are needed to better understand their role in the outcome of *L. infantum* infection. On the other hand, previous studies have postulated that the complement system is one of the first barriers for avoiding the infection of the parasite although the identification of the complement pathways and related proteins, as well other families of proteins, is not fully elucidated [10–12].

The role of dogs as the main vertebrate reservoir makes VL a good example of the importance of embracing a One Health approach for efficient canine and human disease surveillance and control [1,13]. To control *Leishmania* infection in dogs, insecticide (applied topically or via collars), drug therapy or vaccines are used [14]. Among them, vaccination appears to be one of the most appropriate tools for prevention and remains the most promising approach.

In 2016, LetiFend® was licensed for veterinary use in Europe (https://www.ema.europa.eu/en). The active ingredient of LetiFend® is Protein Q, whose efficacy was assessed in a large-scale dog population of different breeds and ages over a 24-month field study [15]. Results demonstrated that vaccination clearly reduces clinical signs related to the progression of disease. Considering that a previous study has established a clear relationship between the disease severity and CIC levels, the purpose of this study was to investigate how LetiFend® reduces disease progression and controls disease development in experimental *L. infantum* infection.

2. Materials and methods

2.1. Animals, vaccination and infection

All study procedures were performed in accordance with Spanish and European legislation for protection of animals used for experimentation and other scientific purposes (European Directive 2010/63/UE, Spanish Law RD 53/2013, FELASA Guidelines and Humane Endpoints Guidance Document of the OECD (ENV/JM/MONO(2000)7). The study design and study protocol were approved by the Ethics Committee on Animal and Human Experimentation (CEEAH) at the Universidad Autónoma de Barcelona [16].

Furthermore, this study was conducted according to Good Laboratory Practice (GLP) requirements of European Directive 2004/10/EC and Spanish Royal Decree 1369/2000.

Forty-four beagle dogs (22 males and 22 females; aged 5.4–5.9 months) were acclimatized in a randomized, double-blinded, placebo controlled laboratory study and raised under parasite-free conditions in an animal facility (Vivotecnia Research, S.L., Madrid, Spain). All dogs were considered eligible for vaccination on day 0 and randomly allocated into one of two groups (vaccinated or placebo), each with 22 animals (11 males and 11 females). Those included in the vaccinated group received a single subcutaneous dose of LetiFend® vaccine (50 μg of active substance, Protein Q) whereas the placebo group received a control product, containing only excipient ingredients (sodium chloride, arginine hydrochloride and boric acid).

On day 28, all dogs were challenged intravenously with a single dose (0.5 mL) containing 500,000 infectious promastigotes of the *L. infantum* M/CAN/ES/96/BCN 150 (zymodeme MON-1) strain.

For preparation of the challenge, *L. infantum* amastigotes from infected hamster liver and spleen homogenates were cultured *in vitro* to differentiate them into the infectious promastigote form. Parasites were grown at 26 °C in Schneider's medium (Sigma-Aldrich) supplemented with 20% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich), 20 mM l-glutamine, 200 U/mL penicillin and 100 μg/mL streptomycin at pH 7.4. Differentiated parasites were isolated at the end of the logarithmic phase (onset of stationary phase) and the infectious inoculum was prepared and administered intravenously through the cephalic vein.

2.2. Serum sample collection

Blood samples were collected from all dogs before administration of the vaccine or placebo, and at 90, 240, 273, 330 and 363 days post-infection (dpi) by jugular puncture, using a IV indwelling cannula (20G Introcan®, B Braun, Melsungen, Germany) coupled to a luer adapter. Afterwards, blood samples were kept at room temperature for approximately 30 min and then centrifuged for 10 min at 1200 xg at 4 °C. After centrifugation, a minimum volume of 3 mL of serum per animal was obtained and stored at −80 °C until further use.

2.3. CIC isolation

Serum samples were processed for CIC isolation after modifying and adapting a previously reported method [17]. In short, samples were centrifuged and the upper layer collected and mixed with equal volume of 7% polyethylene glycol (PEG) (Sigma-Aldrich, St Louis, MO, USA) and kept overnight. Afterwards, PEG-precipitated CIC were pelleted by centrifugation, reconstituted in 0.01 M PBS and stored at −80 °C for further use. The protein content of PEG-precipitated CIC was estimated by a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

2.4. L. infantum culture and antigen preparation

L. infantum (MCAN/ES/1996/BCN/150/MON-1) parasites were grown at 26 °C in Schneider's medium (Sigma-Aldrich) supplemented with 20% heat-inactivated FBS (Sigma-Aldrich), 20 mM l-glutamine, 200 U/mL penicillin, 100 μg/mL streptomycin, and 50 μg/mL gentamicin at pH 7.4.

Soluble *Leishmania* antigen (SLA) was prepared from stationary-phase promastigotes of *L. infantum*, as reported previously [18].

Briefly, 2×10^8 promastigotes were washed three times in cold sterile PBS. After six freeze–thaw cycles followed by ultrasonication (Labsonic® M, Sartorious, Goettingen, Germany) with five cycles of 30 s at 38 MHz, the suspension was centrifuged at 8000 xg for 30 min at 4 °C and the supernatant containing the SLA was collected. The protein concentration was estimated by the Bradford method (Bio-Rad, Hercules, CA, USA), and 500 μL aliquots were stored at −80 °C.

2.5. CIC levels

An enzyme-linked immunosorbent assay (ELISA) was used to measure PEG-precipitated CIC. In summary, microtiter immunoassay plates (NUNC, Roskilde, Denmark) were coated with *L. infantum* SLA at 10 μg/mL dissolved in 0.01 M PBS overnight at 4 °C. Microplates were washed four times with PBS containing 0.1% Tween 20 (PBS-T) and blocked with 200 μL/well of PBS containing 0.1% Tween 20 and 1% BSA (Sigma-Aldrich) at room temperature (RT). After washing, microplates were incubated with a 1/100 dilution of precipitated CIC for 1 h at RT. Microplates were then washed again and incubated with 1/10,000 HRP-conjugated anti-dog IgG (Bethyl Laboratories, Montgomery, TX, USA) at RT for 1 h. After washing, microplates were developed with a solution of Sigma Fast o-phenylene diamine dihydrochloride (OPD) and H_2O_2 in phosphate-citrate buffer. After 30 min, the reaction was stopped and microplates read in an ELISA microplate spectrophotometer (Bio-Rad).

All samples were tested in duplicate and the mean value was recorded; mean OD + 2 SD of the placebo group was considered as the cutoff value. Any sample exhibiting absorbance above the cutoff value was considered to be positive.

2.6. Determination of L. infantum antibody titer by indirect immunofluorescence

An immunofluorescence antibody testing [19] was performed to determine the level of total anti-*Leishmania* IgG antibodies as reported elsewhere [20], on serial dilutions from 1/40 to 1/640. Seropositivity was defined by a cut-off $\geq$ 1/80.

2.7. Proteomic analysis

The protein composition of PEG-precipitated CIC from serum samples collected from vaccinated and placebo groups was assessed by reverse phase-liquid chromatography-mass spectrometry (RP-LC-MS/MS) analysis (Dynamic Exclusion Mode).

First, PEG-precipitated CIC were quantified by SDS-PAGE electrophoresis and digested *in situ* with sequencing grade trypsin (Promega, Madison, WI, USA) as previously reported with minor modifications [21]. After digestion, samples were dried down and desalted onto C18 OMIX Pipette tips (Agilent Technologies, Santa Clara, CA, USA) for mass spectrometric analysis.

Desalted and digested PEG-precipitated CIC samples were analyzed by RP-LC-MS/MS in an Easy-nLC II system coupled to an ion trap LTQ-Orbitrap-Velos-Pro hybrid mass spectrometer (Thermo Scientific, Waltham, MA, USA) located in the protein chemistry facility of the Center of Molecular Biology "Severo Ochoa" (CBMSO, Madrid, Spain). Peptides were eluted using a 90 min gradient from 5 to 40% solvent (0.1% formic acid, 80% acetonitrile in water). Orbitrap resolution was set at 30,000 FWHM (Full Width at Half Maximum) at m/z 400.

Peptides were identified from raw data using the PEAKS Studio 8.5 search engine (Bioinformatics Solutions Inc., Waterloo, ON, Canada). A database search was performed against uniprot-*canis lupus.fasta* and uniprot-*leishmania infantum*.fasta (decoy-fusion database). The following constraints were used for searches: tryptic cleavage after Arg and Lys; up to two missed cleavage sites; tolerances of 10 ppm for precursor ions and 0.6 Da for MS/MS fragment ions and searches were performed enabling optional Met oxidation and Cys carbamidomethylation. The false discovery rate (FDR) for peptide spectrum matches (PSM) was limited to 0.01. Only those proteins with at least two different peptides discovered from LC/MS/MS analyses were considered reliably identified.

Proteins were clustered in a heat map if they exhibited a similar expression trend across the samples. Hierarchic clustering was generated using a neighbor joining algorithm with a Euclidean distance similarity measurement of the log2 ratios of the abundance of each sample relative to the average abundance.

2.8. Statistical analysis

Nonparametric Student's t-tests (Mann-Whitney) were performed with GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). Data were expressed as mean ± standard error of the mean (SEM); differences were considered statistically significant when $p < 0.05$.

Correlation coefficient (r value) and statistical significance of pairwise comparisons between PEG-precipitated CIC levels and IFAT titer or total protein content was determined using the Pearsońs correlation.

3. Results

3.1. Measurement of PEG-precipitated CIC

PEG-precipitated CIC levels measured by ELISA are shown in Fig. 1. CIC were not detected either on day 0 or 90 dpi for both groups analyzed (vaccinated and placebo). However, CIC were detected in both groups at 240, 273, 330, and 363 dpi with the vaccinated group showing lower levels of CIC compared to the placebo group at all time points. Statistically significant differences were observed ($p < 0.05$) at 240 and 273 dpi.

3.2. Protein content on PEG-precipitated CIC

PEG-precipitated CIC protein content is shown in Fig. 2A. Protein content was higher in the vaccinated group compared to the placebo group at 240, 273, 330, and 363 dpi, though significantly different only on day 240.

Fig. 2B shows a significant positive correlation between protein concentration and level of PEG-precipitated CIC in the vaccinated group ($r = 0.916$, $p = 0.029$) and also in the placebo group ($r = 0.910$, $p = 0.0318$).

3.3. IFAT analysis

Anti-*L. infantum* IgG levels determined by IFAT (Fig. 3A) ranged from negative to 1/640. Vaccinated dogs showed the lowest antibody titers at 240, 273, 330, and 363 dpi. Statistically significant differences were observed at these time points between the vaccinated group and placebo ($p < 0.05$).

Fig. 3B shows a significant positive correlation between IFAT titers and levels of PEG-precipitated CIC in the vaccinated group ($r = 0.979$; $p = 0.0006$) and placebo group ($r = 0.997$; $p < 0.0001$).

3.4. Proteomic analysis

Relative protein abundance is shown in Fig. 4. The heat map shows results from vaccinated and placebo groups according to protein expression pattern.

Relative intensity is shown in Fig. 5 (vaccinated and placebo) for those proteins with a fold-increase considered to be biologically significant (>1.5). Differences in protein concentration between groups (vaccinated vs. placebo) were observed. Twelve proteins related to the complement system were more abundant in the vaccinated dogs than in the placebo group. Some of them belong to the membrane attack complex (C7 and C9), proteins involved in the

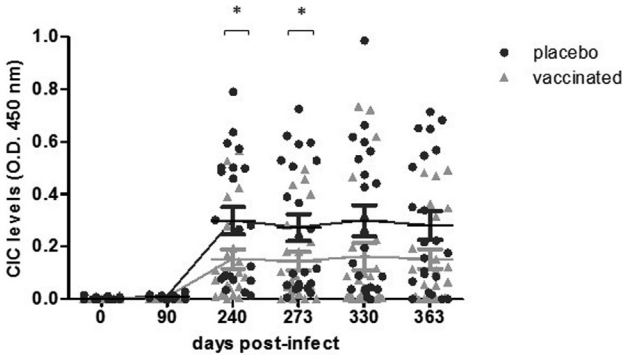

Fig. 1. Levels of PEG-precipitated CIC. CIC quantification was performed in PEG-precipitated serum samples of the vaccinated and placebo groups. Data show values of individual animals and mean ± SEM. Comparison between both groups was determined by nonparametric Student's *t*-test (Mann-Whitney) for each time point. Statistical significance is indicated (*$p < 0.05$).

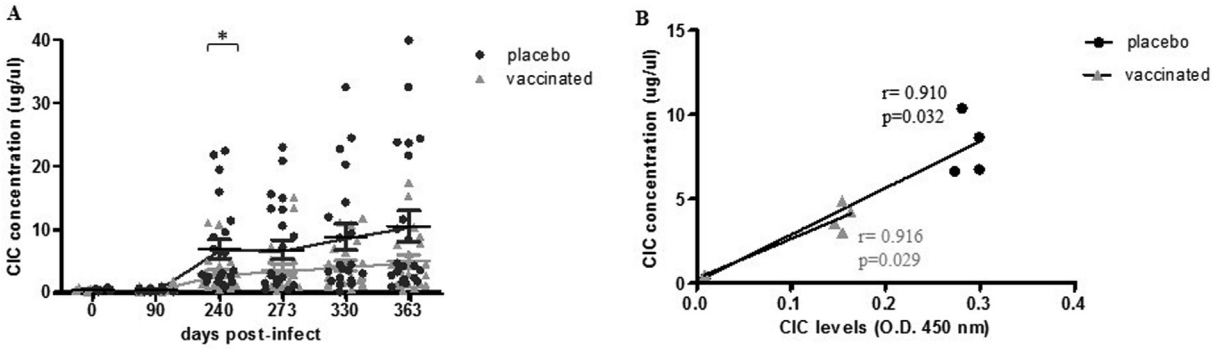

Fig. 2. PEG-precipitated CIC protein content. A. CIC protein content in PEG-precipitated serum samples of vaccinated and placebo groups, determined by BCA protein assay kit. Data show individual values and mean ± SEM. Comparison between both groups was determined by nonparametric Student's *t*-test (Mann-Whitney) for each time point. Statistical significance is indicated (*p < 0.05). B. Correlation between protein content (μg/μl) and levels (O.D. 450 nm) of PEG-precipitated CIC. For both groups (vaccinated and placebo), data are represented as the mean of the individual values for each time point (0, 90, 240, 273, 330 and 363 days post-infection). Pearson's correlation for vaccinated groups (r = 0.916; p = 0.029) and placebo group (r = 0.910; p = 0.032).

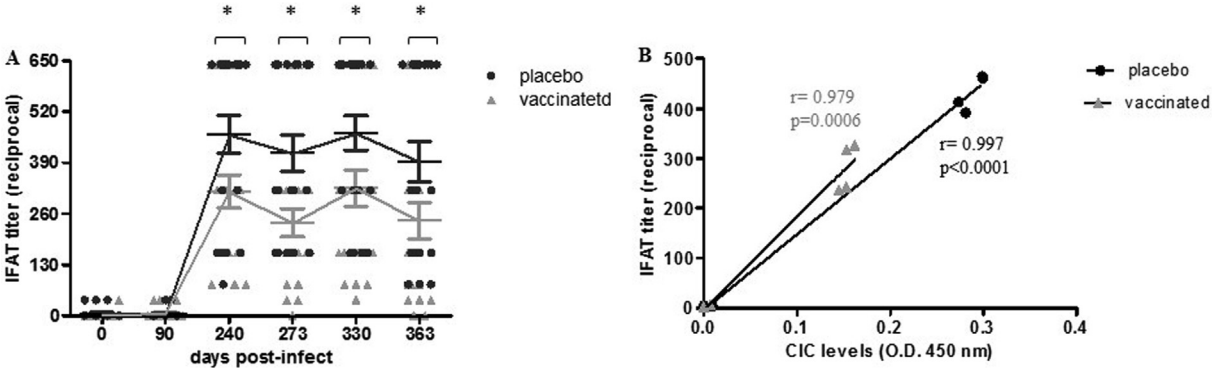

Fig. 3. IFAT analysis (A) and correlation between IFAT and PEG-precipitated CIC levels (B). A. IFAT titers/animal (reciprocal representation) of vaccinated and placebo groups and mean ± SEM values. Comparison between both groups was determined by nonparametric Student's *t*-test (Man-Whitney) for each time point. Statistical significance is indicated (*p < 0.05). B. Correlation between IFAT titer and levels (O.D. 450 nm) of PEG-precipitated CIC. For both groups (vaccinated and placebo), data are represented as the mean of the individual values for each time point (0, 90, 240, 273, 330 and 363 days post-infection). Pearson's correlation for vaccinated groups (r = 0.979; p = 0.0006) and placebo group (r = 0.997; p < 0.0001).

complement activation process (C1r, C1q A chain, C1s, Component 4 binding protein beta and Complement factor P (properdin)) and regulatory factors that act in different steps of the activation pathways. In addition, five proteins belonging to the serpin family were also more abundant in vaccinated dogs (G1, F1, C1, A1 and A5).

4. Discussion

CanL is a multi-systemic disease that presents diverse clinicpathologic abnormalities and clinical outcomes. Furthermore, there appears to be significant individual variation following infection with the *L. infantum* parasite with only some dogs developing clinical disease [2]. CanL may potentially involve any organ, tissue or body fluid and is often manifested by nonspecific clinical signs mostly associated with the presence of immune complexes [5]. These complexes are formed as a consequence of *Leishmania* infection by the formation of huge aggregates of parasite proteins and anti-*Leishmania* IgG and IgM antibodies and, to a lesser extent, complement components [6,7,22]. In this sense, their deposition in specific organs has been defined as one of the main causes responsible for tissue damage and is related to renal failure and eventually death [23,24].

In a preliminary study, CIC were isolated and quantified using serum samples from animals suffering from clinical CanL at different stages [9]. The results reported a statistically significant correlation between CIC levels and pathology stage in dogs infected with *L. infantum*. In addition, a positive correlation between IFAT titer and CIC levels was also confirmed.

In this study, the capacity of LetiFend® to reduce CIC levels was analyzed in beagles experimentally infected with *L. infantum*. Data revealed that vaccinated dogs present significantly lower CIC levels than the placebo group, and these levels are related to a lower parasitic load, as confirmed by the positive correlation obtained (data not shown). Moreover, as a consequence of vaccination with LetiFend®, changes in the protein composition of CIC were detected, including a significant increase in complement system proteins in vaccinated dogs. We hypothesize that the higher amount of these proteins could be related to activation of this major player in innate immunity, and massive destruction of extracellular parasites [11,25,26]. In fact, a reduction in parasite load has also been previously observed in animals vaccinated with LetiFend® [15]. Most initial studies on the role of the complement system in parasite infections (such as leishmaniosis) have approached this system as an innate host component to control infection by inducing parasite killing. The complement system comprises more than thirty plasma and membrane-associated proteins that interact in a cascade by means of sequential activation of several proenzymes that catalyze activation of other enzymes [27]. As complement is present in tissue fluids and the blood, it encounters parasites immediately upon introduction into the host [28–31].

Taking into account the results above reported, in addition to the capacity of LetiFend® to induce an early and statistically

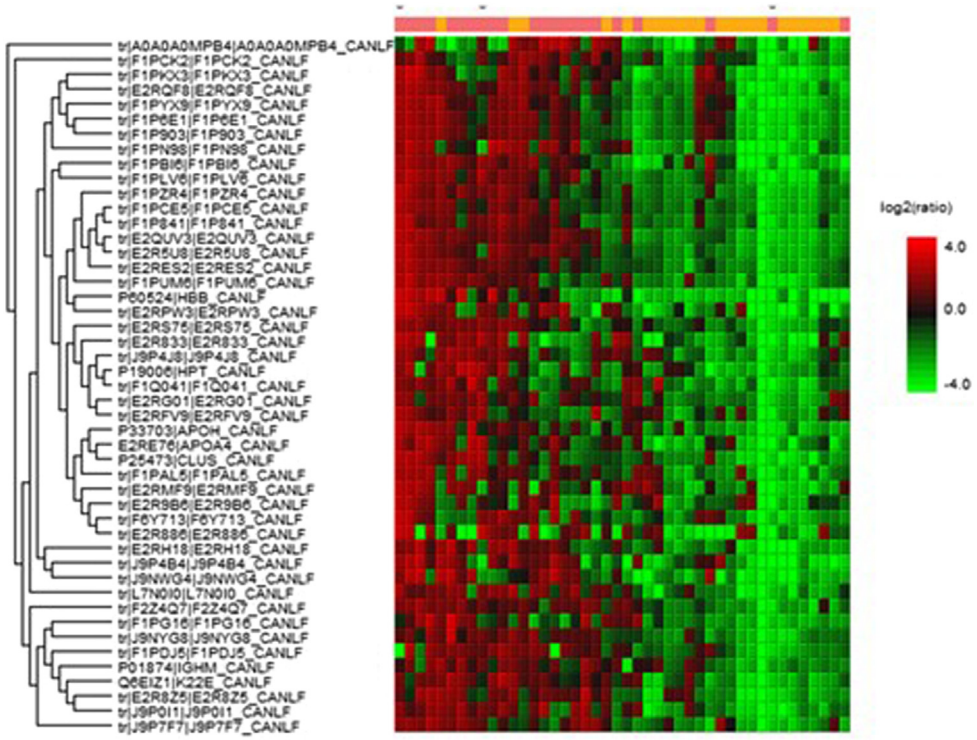

Fig. 4. Characterization by RP-LC-MS/MS of PEG-precipitated CIC. Protein composition in PEG-precipitated serum samples of the vaccinated and placebo groups (day 240 post infection) was assessed by RP-LC-MS/MS analysis (Dynamic Exclusion Mode) in a LTQ ORBITRAP VELOS PRO. The relative protein abundance is represented as a heat map. The proteins (indicated on the left) are clustered by similar expression trend across the samples (orange to placebo; red to vaccinated) and showed that vaccinated and placebo samples are grouped taking into account the protein expression pattern.

significant increase in IgG2 against Protein Q, as has been previously described [11,27,28], vaccination would lead to a decrease in levels of circulating immune complexes**,** a lower parasitic load and active maintenance of the complement system. These factors would correlate with the results observed in different vaccination studies [32–34] and in the field trial [15] which revealed decreased lesions and clinical signs, less disease severity and lower parasitic load.

In addition to the complement system proteins, an increase in the serpin family was detected in the vaccinated group. Serpins are a superfamily of serine protease inhibitors that modulate a wide variety of proteolytic cascades thus controlling many physiologic and pathologic reactions including immune-regulation processes. Previous studies have suggested that almost all organisms express serpins, and that these proteins play critical roles in host-pathogen interactions and regulation of inflammatory responses. In this sense, it has been reported that serpins are a defense strategy derived from the host to provide protection against parasitic diseases by various pathways, including direct inhibition of pathogen proteases, inhibition of pathogen binding, and enhancement of host immune cell functions [35–38]. This may suggest that the presence of higher amounts of proteins from the serpin family in vaccinated dogs compared to the placebo group would be useful to enhance the anti-parasitic protection mechanisms.

It has been reported that in experimental canine infection models used in vaccine studies, although high doses of parasites are administered intravenously, some dogs still do not develop clinical signs over the study period [39–42]. In this lab trial the infection was clearly established, as shown by the IFAT titer results in addition to parasite load and anti-SLA response (data not shown). However, over the one-year infection period in this trial, only minor clinical signs were observed. For this reason, it was not possible to establish a significant relationship between CIC levels and clinical signs, which would require a longer period of time to achieve greater disease severity.

In summary, the vaccination of dogs with LetiFend® seems to increase the amount of proteins from the complement system and the serpin family, resulted in a reduction in CIC levels. This may be involved in the mechanism by which the vaccine controls *L. infantum* infection in dogs, reducing parasite load as evidenced in a previous field trial [15]. Despite these results, the mechanism of action remains unclear and additional studies related to the mechanisms by which the antibodies induced by LetiFend® vaccination are able to reduce CIC levels and maintain activation of the complement system need to be performed. In this sense, the immunologic and genomic approaches will be applied to new studies (currently ongoing) that should enable an understanding of how LetiFend® functions to reduce the risk of pathologies associated with CanL.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

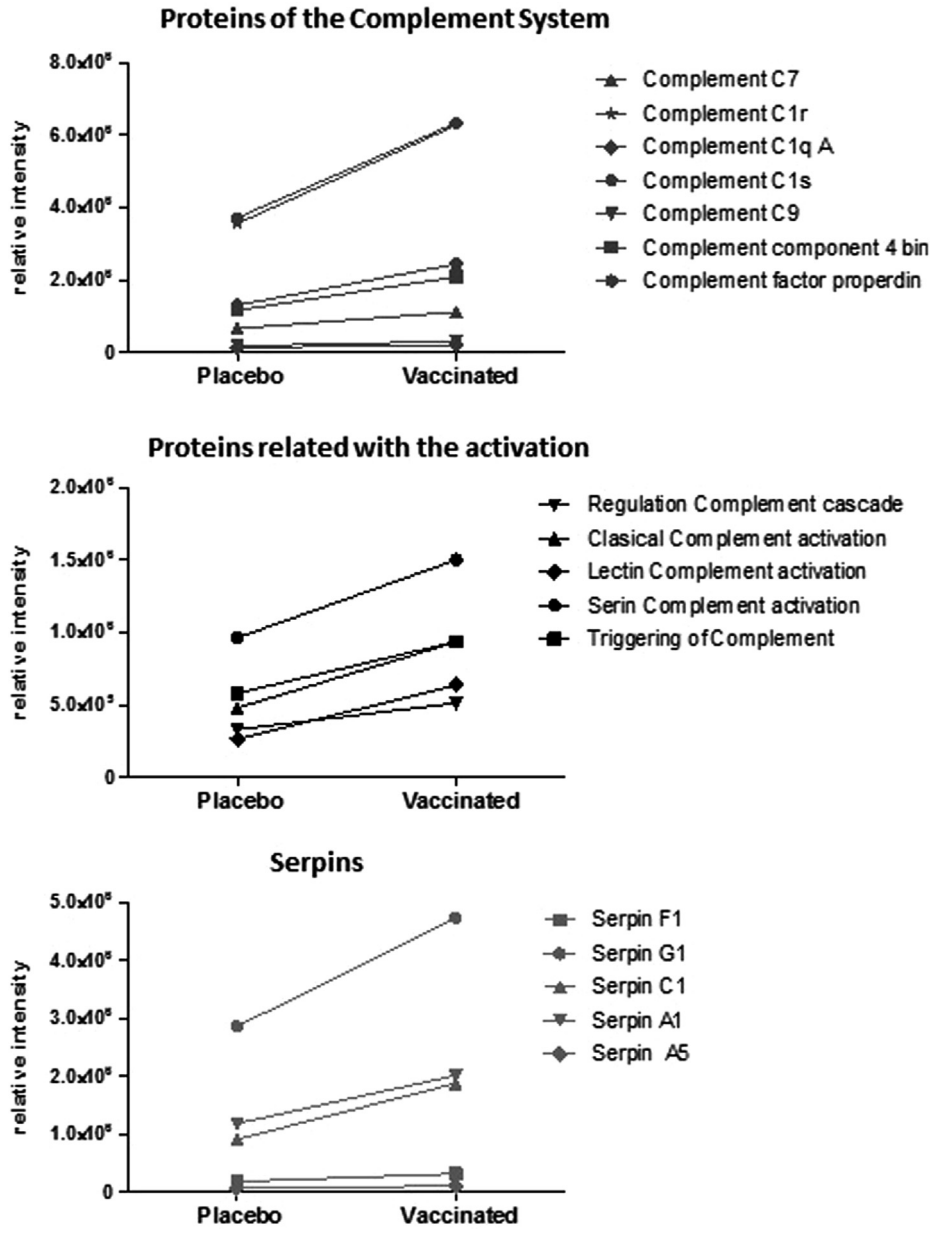

Fig. 5. Proteins profile in PEG-precipitated CIC characterized by RP-LC-MS/MS. The most abundant proteins found by RP-LC-MS/MS analysis in PEG-precipitated serum samples of the vaccinated and placebo groups (day 240 post-infection) are represented as relative intensity. Biological significance was defined by the fold-increase using cutoff of 1.5.

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