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Humoral and *In Vivo* Cellular Immunity against the Raw Insect-Derived Recombinant *Leishmania infantum* Antigens KMPII, TRYP, LACK, and papLe22 in Dogs from an Endemic Area

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Abstract. *Leishmania infantum* causes visceral leishmaniasis, a severe zoonotic and systemic disease that is fatal if left untreated. Identification of the antigens involved in *Leishmania*-specific protective immune response is a research priority for the development of effective control measures. For this purpose, we evaluated, in 27 dogs from an enzootic zone, specific humoral and cellular immune response by delayed-type hypersensitivity (DTH) skin test both against total *L. infantum* antigen and the raw *Trichoplusia ni* insect-derived kinetoplastid membrane protein-11 (rKMPII), tryparedoxin peroxidase (rTRYP), *Leishmania* homologue of receptors for activated C kinase (rLACK), and 22-kDa potentially aggravating protein of *Leishmania* (rpapLe22) antigens from this parasite. rTRYP induced the highest number of positive DTH responses (55% of leishmanin skin test [LST]-positive dogs), showing that TRYP antigen is an important T cell immunogen, and it could be a promising vaccine candidate against this disease. When TRYP-DTH and KMPII-DTH tests were evaluated in parallel, 82% of LST-positive dogs were detected, suggesting that both antigens could be considered as components of a standardized DTH immunodiagnostic tool for dogs.

INTRODUCTION

Visceral leishmaniasis (VL), a severe parasitic disease that is usually fatal if left untreated (http://www.who.int/leishmaniasis/en/) has an incidence of more than 500,000 new human cases each year. Zoonotic VL is caused by *Leishmania infantum* (syn. *Leishmania chagasi*[1]) in both the Palearctic and the Neotropical ecozones. Dogs are the natural hosts and the main domestic reservoirs of the parasite.[2] In ecoregions around the Mediterranean basin, the prevalence of canine infection reaches 67–80% in highly enzootic areas[3,4] with at least 2.5 million dogs infected on the northern shore.[2] Control of parasite burden in dogs by vaccination or treatment reduces the infectivity to the vector and the incidence of the disease in humans.[5,6] Identification of the mechanisms and components involved in *Leishmania*-specific immune responses to improve diagnosis, prognosis, and vaccination is a research priority.

L. infantum-infected humans and dogs can develop several immune responses, ranging from predominantly specific cell-mediated immunity (CMI) to a predominantly humoral immune response, which has been associated with active disease.[7,8] The cellular mechanisms underlying the protective immune response against *L. infantum* seem to involve a T_h1-like response, including production of interferon (IFN)-γ by sensitized T cells to induce macrophage activation.[9,10] Measurement of IFN-γ production using cultured peripheral blood cells in response to *Leishmania* antigens is expensive, time consuming, and cumbersome. Delayed-type hypersensitivity (DTH) reaction to an intradermal injection of a suspension of inactivated promastigotes (leishmanin skin test, LST) is an easy and semiquantitative test to measure *Leishmania*-specific CMI *in vivo*.[8,11] This test has been extensively used in large-scale immunoepidemiological studies in Southwestern Europe as a marker of exposure to *Leishmania* in humans[12–14] and much less in dogs.[15,16] The LST result is usually negative in advances stages of clinical VL and canine leishmaniasis (CanL),[7,8,17] whereas a positive LST result is detected during subclinical infection,[15,16] in early stages of VL and CanL,[18,19] or after successful treatment.[20,21] Several vaccination studies conducted in dogs have shown that animals that converted to a positive LST result were protected against the disease,[6,22,23] like in humans.[24,25] Unfortunately, the development of this immunodiagnostic method has been hampered by the lack of a standardized product, and a test to evaluate *Leishmania*-specific CMI *in vivo* in humans or dogs is currently not available in clinical practice.

Recombinant proteins make it possible to identify specific B cell and T cell immunogens so that more accurate diagnostic and prognostic tools can be developed. Moreover, discrimination between potentially protective and potentially aggravating antigens would allow a more rational development of experimental vaccines. Baculovirus–insect larvae expression system has proven to be a valuable system for the production of large amounts of high-quality, yet inexpensive recombinant proteins.[26] Folding and processing of proteins related to eukaryotic systems improve with insect larvae expression strategy compared with prokaryotic systems. However, some processing protein pathways of insect cells, such as the protein glycosylation pathway, are not exactly equivalent to those of other animals, especially vertebrates.[27] A wide variety of recombinant proteins have been expressed in insect larvae to be used for serodiagnosis,[28,29] vaccination strategies, or production of reagents.

In the present study, we evaluated and compared the serological and DTH response to crude total *L. infantum* antigen (CTLA) and four recombinant evolutionarily conserved antigens from this parasite: kinetoplastid membrane protein-11 (KMPII; formerly known as KMP-11), tryparedoxin peroxidase (TRYP; previously known as TSA), *Leishmania*

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homologue of receptors for activated C kinase (LACK), and 22-kDa potentially aggravating protein of *Leishmania* (papLe22). These antigens have been used elsewhere as targets for vaccines[30–32] and serodiagnosis of CanL[33,34]; however, their role in *L. infantum*-specific CMI in naturally infected dogs has not been evaluated. The main objective of this work was to determine whether these antigens produced in recombinant baculovirus-infected *Trichoplusia ni* larvae are T cell immunogens in dogs naturally infected with *L. infantum* and to assess their inclusion in a standardized DTH-based immunodiagnostic tool or their usefulness as possible vaccine candidates in the canine model.

MATERIALS AND METHODS

Production of recombinant KMPII, TRYP, LACK, and papLe22 proteins. Recombinant proteins were obtained in baculovirus-infected *T. ni* larvae as previously described.[34] Table 1 shows the Genbank accession numbers of the sequences used for primer design and the nucleotide sequences of forward and reverse primers for each *L. infantum* gene cloned. Recombinant bacmids carrying *KMPII*, *TRYP*, *LACK*, and *papLe22* genes and an additional bacmid with non-insert clones produced by the Bac-to-Bac system (Invitrogen, Carlsbad, CA) were used to transfect *Spodoptera frugiperda* Sf21 cells to obtain the recombinant baculoviruses and the wild-type baculovirus, respectively. Wild-type baculovirus was used to obtain the control raw protein extract (Ni) for both enzyme-linked immunosorbent assay (ELISA) and the DTH skin test. Fifth instar *T. ni* larvae were intracoelomically injected near the proleg with the recombinant baculovirus preparations. Inoculated larvae were kept at 28°C for 96 hours and then frozen immediately at –20°C. Total protein was solubilized from frozen larvae by homogenizing on ice using extraction buffer: phosphate buffered saline, pH 7.2, 0.01% Triton X-100, 25 mM dithiothreitol, and a protease inhibitor cocktail (Complete; Roche, Basel, Switzerland). After centrifugation, the supernatant was filtered, recentrifuged, and lyophilized overnight.

Total soluble protein (TSP) extracts were analyzed for production of recombinant proteins by sodium-dodecyl-sulphate polyacrilamide gel electrophoresis (SDS-PAGE), Western blot, and glycoprotein detection assays. Thirty micrograms of each TSP extract were electrophoretically separated by 15% SDS-PAGE and stained with Coomassie brilliant blue (Bio-Rad Laboratories Inc., Hercules, CA), and bands corresponding to *L. infantum* recombinant proteins were quantified using a Tina 2.0 image analyzer software package (Raytest, Straubenhardt, Germany).

Table 1

Genebank accession number and primer sequences of *L. infantum* recombinant antigens

Gene (accession number)	Direction	Primer
*KMPII (*X95627)	Forward	5′-ATGGCCACCACGTACG AGGAGTTTTCG-3′
	Reverse	5′-TTACTTGGATGGGTACT GCGCAGC-3′
TRYP (AF044679)	Forward	5′-CCAGCCATGTCCTGCGGT AACGCCAAG-3′
	Reverse	5′-AGGTTTACTGCTTGCTG AAGTATCCTTC-3′
LACK (U49695)	Forward	5′-ACCATGAACTACGAGGG TCACC-3′
	Reverse	5′-TTACTCGGCGTCGGAGAT GGACC-3′
papLe22 (AF123892)	Forward	5′-GGCCACTTCTCTCTTCT CCA-3′
	Reverse	5′-CTTGCCACATACACCAA TCG-3′

Western blotting was performed to detect recombinant antigens in protein larva extracts without further purification, as previously described.[34] Briefly, proteins were electrophoretically transferred onto an Immobilon nitrocelulose membrane (GE Healthcare, Fairfield, CT). The membrane was blocked overnight with phosphate-buffered saline 0.05% Tween 20 (PBST) and 4% dried skimmed milk and subsequently incubated at room temperature with a pool of six sera that were highly positive to CTLA-based ELISA for 1 hour. After washing with PBST (one time for 15 minutes and then two times for 5 minutes), protein A–horse radish peroxidase (HRP) (Pierce, Rockford, IL) was used as secondary antibody and incubated for 1 hour at room temperature, and the membrane was thoroughly washed as described above. Color was developed by enhanced chemiluminescence system (ECL), and chemiluminiscence was detected using Chemidoc (Bio-Rad) and analyzed using the Image laboratory Software, version 2.0.1 (Bio-Rad).

No evidences of N-glycosylation in KMPII, TRYP, LACK, and papLe22 proteins exist, but glycosylation prediction analysis (http://www.oppf.ox.ac.uk/opal/OPAL.php) detected the possible presence of several O-glycosilation sites in both TRYP ($N = 19$) and papLe22 ($N = 5$) proteins. For glycosylation detection assays, 200 μg of TSP per lane were resolved in a 15% SDS-PAGE, and glycosylated proteins were stained using the GLYCOPRODetection Kit (Sigma-Aldrich, St. Louis, MO) following the manufacturer's instructions.

Dogs. The study was conducted in June 2008 in Mallorca, Spain, a highly enzootic area for *L. infantum*.[4] Twenty-seven dogs of different breeds (14 females and 13 males) from the Animal Pound of Palma de Mallorca were included in the study. Their ages ranged from 6 months to 7.5 years, with a mean ± standard deviation (SD) of 3 ± 1.8 years. Before sampling, all dogs were examined to detect clinical signs compatible with CanL. The dogs were then sedated using 10 $\mu g.kg^{-1}$ of medetomidine (Domtor; Pfizer, New York, NY) intramuscularly to take blood samples and perform DTH tests. Dogs were considered infected by *Leishmania* when they had a positive result to serology testing with CTLA and/or LST and/or real-time polymerase chain reaction (qPCR) in blood samples.[16,35] All the procedures were performed with the approval of the Animal Pound of Palma de Mallorca Ethics Committee.

Blood samples. Blood was collected by jugular venipuncture before DTH tests were performed. Sera were obtained after centrifugation of blood samples at 2,000 × *g* for 20 minutes. Serum and blood clot samples were stored at –20°C until use.

Real-time PCR amplification of *L. infantum* DNA in blood samples. *L. infantum* DNA was specifically detected and quantified by Taqman qPCR (Applied Biosystems, Foster City, CA) performed by the Servei Veterinari de Genètica Molecular, Universitat Autònoma de Barcelona, as described elsewhere.[36] Briefly, DNA was extracted from blood clots, and qPCR was performed, targeting conserved DNA regions of the kinetoplast minicircle DNA from *L. infantum*. The eukaryotic 18S rRNA Pre-Development TaqMan Assay reagents (Applied Biosystems) were used as an internal reference.

L. infantum DNA load in each sample was determined by relative quantification using the $2^{-\Delta\Delta Ct}$ method.[37] Results were expressed as *x*-fold more DNA copies than the calibrator sample (dog M7).

Recombinant and crude total *L. infantum* antigen-based ELISA. ELISA to detect serum antibodies against rKMPII, rTRYP, rpapLe22, and rLACK antigens and against CTLA was performed as previously described.[34,38] Microtiter plates were coated with raw protein larva extracts containing the recombinant proteins (1.3 µg per well for rKMPII, 5.0 µg for rTRYP, 24.4 µg for rLACK, and 10.0 µg for rpapLe22), with the control Ni extract at the same concentrations or with CTLA (2 µg per well). Protein A-HRP was used as secondary antibody. Each serum sample was tested under equal conditions against both protein larva extracts containing each recombinant antigen and control Ni extract in the same plate. A known positive serum used as calibrator (~1 optical densities [OD]) was included in all plates, and plates with interassay variations ≥ 10% were ruled out. The ELISA cut-off values (mean + 3 SD for 76 dogs from non-enzootic areas) were 0.180 OD for CTLA, 0.100 OD for rKMPII, 0.082 OD for rTRYP, 0.060 OD for rpapLe22, and 0.005 OD for rLACK.

Results were expressed as OD. For ELISAs using recombinant antigens, absorbances were corrected by subtracting the absorbance achieved by the serum on the control antigen Ni extract from that achieved on the protein larva extract containing specific recombinant antigen.

DTH tests using leishmanin and recombinant *L. infantum* proteins. Leishmanin reagent consisted of a suspension of 3×10^8 inactivated *L. infantum* (MHOM/FR/78/LEM75) promastigotes per milliliter in 0.4% phenol-saline solution (provided by Instituto de Salud Carlos III). Working concentrations of recombinant protein extracts for use in DTH reactions were set up in a pilot study (data not shown). Recombinant DTH test reagents were set at 4 µg·mL^{-1} of specific *Leishmania* antigen diluted in pyrogen-free saline solution. The negative control, Ni reagent, was set at 730 µg·mL^{-1} of raw larva extract in pyrogen-free saline solution, equivalent to the maximum concentration of raw larva extract used in specific *Leishmania* antigen preparations (rTRYP reagent). A volume of 0.1 mL of each reagent was intradermally inoculated. The six injections were distributed 3 cm apart on both sides of the groin skin. The DTH response was assessed by measuring the size of the induration and erythematous area (mean of two perpendicular diameters) produced against each reagent at 48 and 72 hours post-injection. A DTH response against leishmanin reagent or against each of the recombinant antigens > 5 mm was considered positive.[8] Dogs with a positive DTH response to Ni reagent were not included in the analysis.

Data analysis. All statistical tests were performed using SPSS version 15.0 (SPSS Inc, Chicago, IL). Data were analyzed by means of non-parametric tests. A P value < 0.05 was considered significant.

RESULTS

Detection of *L. infantum* recombinant proteins in *T. ni* larvae. The recombinant KMPII, papLe22, TRYP, and LACK proteins were successfully produced in the inoculated *T. ni* larvae (Figure 1). Specific bands corresponding to rKMPII and rLACK in *T. ni* extracts were visible using Coomassie brilliant blue staining (Figure 1A) and Western blotting (Figure 1B). Because of bands of Ni antigen having the same size as rTRYP and rpapLe22 in Coomassie blue-stained gels, expression of these antigens was confirmed by Western blotting (Figure 1B). rKMPII, rpapLe22, rTRYP, and rLACK were identified as the expected single band with an electrophoretic mobility of around 11, 20, 22, and 34 kDa, respectively. In the case of rLACK, the band with a higher molecular weight (37 kDa) could be because of alternative post-raductional modifications of the antigen conserving the common epitopes. Because no bands appear in the negative control, it is very unlikely a non-specific immunoreactivity with seemingly unrelated proteins. In the case of rpapLe22, a proteolytic degradation of the sample should be suspected, because a band of lower molecular weight (18 kDa) is detected.

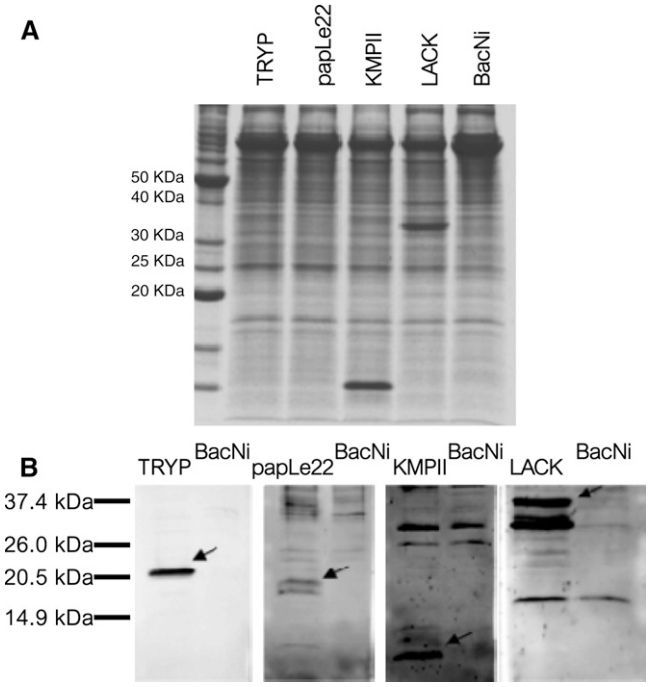

FIGURE 1. Detection of the recombinant proteins rKMPII, rpapLe22, rTRYP, and rLACK in *T. ni* larvae. Total protein extracts were analyzed by (**A**) Coomassie brilliant blue staining of SDS-PAGE gels and (**B**) Western blotting using a pool of canine sera from total *Leishmania* antigen-positive samples. Arrows indicate the position of the recombinant proteins.

Glycosylation assay did not show any positive band in the recombinant protein larvae extracts.

Clinical status and *L. infantum* infection rates. Clinical signs compatible with CanL, parasite load, specific antibody reactivity, and DTH test results against CTLA and the recombinant proteins in the 27 dogs from Mallorca are listed in Table 2.

Five of twenty-seven dogs (18%) included in the study presented clinical signs compatible with CanL. Two showed generalized lymphadenomegaly, two presented periauricular cutaneous lesions, and one showed classic clinical signs of CanL, namely severe emaciation and generalized exfoliative dermatitis. These five dogs were the only ones that showed specific antibodies against CTLA. In addition, the dog that manifested more severe clinical signs (M19) was the one with the highest *L. infantum*-specific antibody levels (3.0 OD).

TABLE 2

Clinical signs, specific antibody reactivity, DTH test results against total *L. infantum* antigen, Ni reagent, and recombinant *Leishmania* proteins rKMPII, rTRYP, rLACK, and rpapLe22, and qPCR in the blood of 27 dogs in Mallorca, Spain

		ELISA*					DTH†						qPCR
ID	Signs	CTLA	KMP11	TRYP	LACK	papLe22	LST	Ni	KMPII + Ni	TRYP + Ni	LACK + Ni	papLe + Ni	Blood‡
M1	–	0.017	0.000	0.000	0.000	0.000	**5.5**§	0.0	0.0	**5.5**	0.0	0.0	**131.2**
M2	–	0.063	0.000	0.000	0.000	0.000	0.0	8.5	7.5	9.5	4.0	0.0	0.0
M3	–	0.035	0.000	0.000	0.000	0.000	0.0	6.5	0.0	0.0	0.0	0.0	**34.7**
M4	–	0.045	0.000	0.000	0.000	0.000	**13.0**§	0.0	0.0	**5.5**	0.0	0.0	**1.0**
M5	–	0.036	0.000	0.000	0.000	0.000	**8.0**	8.5	14.0	7.5	6.0	6.5	0.0
M6	–	0.039	0.000	0.000	0.000	0.000	**7.5**	5.0	10.5	15.5	11.5	12.5	**1.0**
M7	+	**0.260**	0.000	0.000	0.000	0.000	**10.5**§	0.0	0.0	0.0	0.0	0.0	**1.0**
M8	–	0.133	0.000	0.000	0.000	0.000	**8.5**	5.0	8.5	15.0	0.0	0.0	**22.8**
M9	–	0.08	0.000	0.000	0.000	0.000	0.0	0.0	0.0	0.0	0.0	0.0	**3.5**
M10	–	0.015	0.000	0.000	0.000	0.000	0.0	0.0	0.0	**5.5**	0.0	0.0	0.0
M11	–	0.031	0.000	0.000	0.000	0.000	0.0	0.0	0.0	0.0	0.0	0.0	0.0
M12	+	**0.330**	0.000	0.000	0.000	0.000	0.0	0.0	0.0	0.0	0.0	0.0	0.0
M13	–	0.037	0.000	0.000	0.000	0.000	0.0	0.0	0.0	**6.0**	0.0	**6.0**	**14.0**
M14	–	0.028	0.000	0.000	0.000	0.000	**6.5**§	0.0	**12.0**	0.0	0.0	**7.0**	**1.9**
M15	–	0.141	0.000	0.000	0.000	0.000	**14.0**§	0.0	0.0	**12.5**	**6.0**	0.0	**2.2**
M16	+	**1.860**	0.000	0.000	0.000	**0.369**	**6.5**§	0.0	**6.0**	0.0	0.0	0.0	0.0
M17	–	0.043	0.000	0.000	0.000	0.000	**8.0**	6.5	8.5	17.5	12.0	6.0	**7.0**
M18	–	0.013	0.000	0.000	0.000	0.000	0.0	0.0	0.0	0.0	**5.5**	**7.5**	0.0
M19	+	**3.000**	**1.737**	0.000	0.000	**0.255**	**5.5**§	0.0	0.0	0.0	0.0	0.0	**45.7**
M20	–	0.047	0.000	0.000	0.000	0.000	**5.5**§	0.0	**8.0**	0.0	0.0	0.0	0.0
M21	–	0.057	0.000	0.000	0.000	0.000	**8.0**	5.0	0.0	0.0	0.0	13.0	0.0
M22	–	0.079	0.000	0.000	0.000	0.000	**9.0**	5.0	0.0	13.0	15.0	0.0	0.0
M23	–	0.061	0.000	0.000	0.000	0.000	**16.5**	5.0	0.0	0.0	6.5	8.0	0.0
M24	+	**0.330**	0.000	0.000	0.000	0.000	**8.0**§	0.0	0.0	**8.5**	0.0	0.0	0.0
M25	–	0.032	0.000	0.000	0.000	0.000	0.0	0.0	0.0	0.0	0.0	0.0	0.0
M26	–	0.024	0.000	0.000	0.000	0.000	**5.5**§	0.0	0.0	**7.0**	0.0	0.0	0.0
M27	–	0.037	0.000	0.000	0.000	0.000	**10.0**§	0.0	**15.0**	**6.0**	0.0	0.0	**13.7**
n	**5**	**5**	**1**	**0**	**0**	**2**	**18**		**4**	**8**	**2**	**3**	**13**

CTLA = crude total *L. infantum* antigen; LST = leishmanin skin test.

*ELISA results are expressed as optical densities (OD). OD results above the cut-off values were considered positive (numbers in bold). The cut-off values (mean + 3 SD for 76 dogs from non-endemic area) were established at 0.180 OD for CTLA, 0.100 OD for rKMPII ELISA, 0.082 OD for rTRYP ELISA, 0.060 OD for rpapLe22 ELISA, and 0.005 OD for rLACK ELISA.

†Mean millimeter of the largest perpendicular diameter of induration and eritamatous area and its perpendicular one at 48 and 72 hours post-injection. DTH responses > 5 mm were considered positive (numbers in bold).

‡Results expressed as *x*-fold more DNA copies than the calibrator sample (dog M7).

§Dogs with a positive LST result and no reaction to Ni reagent. Ni reagent is raw non-infected larva extract in pyrogen-free saline solution.

The remaining dogs (82%) showed neither clinical signs compatible with CanL nor specific antibodies against CTLA.

Eighteen dogs (67%), including four dogs with clinical signs, developed a positive LST reaction at 48 and/or 72 hours post-injection. The intensity of the reaction recorded in dogs with a positive LST response (median in millimeters; interquartile interval) was 8 mm (6.5–9.8).

L. infantum DNA was detected in the blood samples of 13 dogs (48%). The amount of *Leishmania* DNA was generally low, with a median of 7.0-fold increase over M7 (range = 1.00–131.2). Although only 2 of these 13 dogs showed antibodies against CTLA, 10 of them developed a positive LST reaction.

Combining the results of the three techniques (CTLA serology, LST, and qPCR), we estimated the rate of *L. infantum* infection in this group of dogs to be 82% (22/27).

Specific humoral response against recombinant *L. infantum* antigens. Two dogs (M16 and M19) showed positive anti-rpapLe22 antibody levels. One of these dogs (M19) also showed antibodies against rKMPII. Anti-rTRYP or anti-rLACK antibodies were not detected in any dog.

DTH response against recombinant *L. infantum* antigens. In the 11 LST-positive Ni-negative dogs, the highest percentage of DTH responses was detected against rTRYP (6/11; 55%). Lower percentages of positive DTH reactions were detected against rKMPII (4/11; 36% of the LST-positive dogs). Finally, only one dog showed a DTH-positive response against rLACK, and one showed a DTH-positive result against rpapLe22 (1/11; 9% of LST-positive dogs, respectively). The remaining seven LST-positive dogs showed a DTH-positive reaction against raw antigen Ni at 48 and/or 72 hours post-injection.

The intensity of reaction recorded in dogs with a positive DTH response (median; interquartile interval) was 10.0 mm (7.5–12.8) against rKMPII and 6.0 mm (5.5–7.4) against rTRYP.

We evaluated the potential of the recombinant antigens for detecting dogs with a positive LST reaction. rTRYP produced the highest number of DTH-positive reactions among dogs with positive LST (6/11), but its sensitivity in relation to LST was low: 0.55 (95% confidence interval [CI] = 0.22–0.84). Thus, we combined in parallel the results of rTRYP with the results obtained with the second most recognized antigen (rKMPII) to improve sensitivity. The combination of the rTRYP and rKMPII DTH results revealed a sensitivity of 0.82 (CI = 0.49–0.95), a specificity of 0.71 (CI = 0.31–0.93), and an accuracy of 0.78 (CI = 0.59–0.97) in relation to LST. This combination showed a significant relationship with the LST test (Fisher exact test, $P = 0.049$).

DISCUSSION

In the present study, we used DTH tests to investigate the role of four evolutionarily conserved *L. infantum* proteins proposed as vaccine candidates—KMPII, TRYP, LACK and papLe22—as T cell immunogens in dogs living in an enzootic area. To our knowledge, only one recently published study has used

a recombinant *Leishmania* protein as a DTH test reagent (the recombinant cysteine proteinase rLdccys1) to detect CMI *in vivo* in naturally infected dogs, with very promising results.[39] Our study is the first to use recombinant proteins expressed in baculovirus-infected larvae for DTH testing. One limitation of other expression platforms is the high cost associated with large-scale production. As an alternative, the use of lepidoptera larvae has been reported as a suitable method for inexpensive production of large amounts of recombinant proteins. Moreover, previous studies[34,40–42] show that crude protein extracts without further purification can be used as immunodiagnostic reagents, because the recombinant proteins preserve their antigenic activities. However, some interference of insect proteins, such as cross-reactions, cannot be ruled out, especially in dogs with a positive DTH response against Ni proteins. Therefore, if Ni-positive dogs are excluded, DTH tests with raw insect-derived recombinant proteins without further purification can be used as a low-cost tool for rapid screening of potential DTH or vaccine antigen candidates. Future studies using purified recombinant *L. infantum* proteins will be conducted by our group.

The dogs included in the study were from Mallorca, a highly *L. infantum*-enzootic area.[16,43] By combining the detection of anti–*Leishmania*-specific humoral (CTLA ELISA) and cellular (LST reaction) immune responses with the detection of parasite DNA in blood (qPCR), we estimated the rate of *L. infantum* infection in the study dogs to be 82% (22/27). This high rate of infection is similar to that of previous reports in this area (77%).[16] Although 18% of dogs presented clinical leishmaniasis, 67% developed a positive reaction to LST. A positive LST result indicates previous *Leishmania* exposure[44] and is generally thought to reflect durable and protective CMI.[6,8,15,22] In agreement with previous studies, our results show that most dogs living in a *Leishmania*-enzootic area display a resistant immunological profile against *L. infantum* infection[15,16] and that clinical disease represents only a small part of the real infection by the parasite.[45]

To avoid false-positive DTH reactions against recombinant antigens because of possible cross-reactions with insect proteins, dogs with a positive DTH response against Ni reagent were not included in the analysis of results. rTRYP induced the highest percentage of positive DTH responses in the 11 LST-positive dogs (6/11; 55%). TRYP has shown immunogenicity in dogs[46] and protection against cutaneous or mucosal leishmaniasis in mice and non-human primates,[47] and it is a component of the trifusion Leish-111f vaccine, now being assayed in humans (http://clinicaltrials.gov/show/NCT00121862). Supporting its value as a vaccine candidate against VL, TRYP has proven highly immunogenic in a DNA/modified vaccinia virus ankara (MVA) vaccine strategy against CanL.[32] Furthermore, the DTH response against this antigen in vaccinated dogs correlated with high production of *Leishmania*-specific IFN-γ. These results indicate that TRYP could be a potent T cell immunogen in dogs and that it might be involved in protective *L. infantum*-specific CMI, at least in one-half of naturally infected dogs. However, it was not possible to verify the potential glycosilation sites detected by prediction analysis of neither rTRYP nor rpapLe22 antigens with the detection method used in this work. If these predicted glycosylated sites are real and located in immunodominant regions of native TRYP antigen, then the results obtained in the present study might be underestimating the true percentage of dogs that recognized this protein.

The present results show that only 4 of 11 LST-positive dogs (36%) developed a positive DTH skin reaction against rKMPII, indicating that, compared with rTRYP, this antigen is a weak inducer of CMI in naturally *L. infantum*-infected dogs. Accordingly, the antigenic and T cell immunogenic potential of KMPII studied during experimental canine *L. infantum* infection showed that, although this antigen induced a T_h1 cytokine expression pattern, the production of IFN-γ was only moderate.[48] In humans, KMPII has been shown to stimulate T cells from patients who have recovered from *L. donovani* infection[49] and to induce production of both IFN-γ and interleukin (IL)-4,[50] whereas both IFN-γ and IL-10 were found during subclinical *L. infantum* infection.[51] Thus, KMPII is not playing a T_h1 role but a mixed T_h1/T_h2 role in these individuals.

Only one LST-positive dog showed a DTH-positive response against rLACK and another one against rpapLe22 in the present study. These results indicate that a specific CMI against LACK or papLe22 is not common in clinically healthy, naturally *L. infantum*-infected dogs. In this sense, LACK does not induce lymphoproliferation in patients cured of VL,[52] and papLe22 elicits production of IL-10 but not IFN-γ.[53] Both antigens play a prominent role in the pathogenesis of *Leishmania* infection in patients with different clinical presentations.[53,54] However, vaccine strategies, such as DNA or prime-boost vaccinations, which are able to induce a strong T_h1 immune response against these antigens, have proven able to obtain protection against *L. infantum*, probably by promoting a redirection of their T_h2 immune response to a T_h1 profile.[30,55]

The lack of a standardized antigen is the main drawback of the LST. Leishmanin reagent is an amalgam of antigens, some of which could be activating a strong T_h1 immune response, such as TRYP, and others that could be inducing a T_h2 or weak T_h1 immune response, such as KMPII, LACK, or papLe22. Screening *Leishmania* antigens using DTH tests makes it possible to identify the antigens that may be related to host protection against the parasite. Recombinant *Leishmania* antigens could be used in the future as a standardized tool for diagnosis of subclinically *L. infantum*-infected dogs, in clinical trials with vaccines, and even to predict the outcome of disease and treatment. This approach has been applied in the diagnosis of bovine tuberculosis[56] and to evaluate clinical trials with vaccine against malaria[57] or hepatitis B[58] in humans. In the present study, a high percentage of dogs reacting to leishmanin reagent also showed a positive DTH response against rTRYP and/or rKMPII (82% of LST-positive dogs). Because of the different MHC-II repertory in dogs,[59] a cocktail of different antigens would provide better coverage for diagnosis of *L. infantum*. The estimated sensitivity, specificity, and accuracy of the combination of these two antigens used in parallel compared with LST were 82%, 71%, and 78%, respectively. Our results suggest that a multiantigenic DTH test including rTRYP and rKMPII could constitute a useful diagnostic tool for the detection of dogs subclinically infected with *L. infantum*.

Only dogs showing clinical signs (18%) showed specific antibodies against CTLA. In two of them, seroreactivity was found against rKMPII and/or rpapLe22. KMPII, TRYP, LACK, and papLe22 antigens have proven to be B cell immunogens in humans and dogs with clinical leishmaniasis,[34,60–62] but their seroprevalence in the study group was very low. It is important to highlight that most of the dogs in the present study did not manifest clinical signs and that overall CTLA seroprevalence was low. These findings are consistent with the association

between negative to medium levels of specific antibodies and subclinical *L. infantum* infection in dogs.[63]

Interestingly, four of five dogs with clinical leishmaniasis developed a positive LST reaction. This result raises the question of the existence of a defined polarized dichotomy between LST(+) protection and LST(−) susceptibility in CanL. A broad spectrum of immunological profiles coexists in clinically healthy canine populations living in *Leishmania*-enzootic areas.[15,16,64] Our results support the fact that such a wide range also exists in dogs with clinical leishmaniasis[63]: from dogs with self-limiting disease[65] or even dogs at the early stages of disease that develop specific CMI[19] to dogs with severe disease, a suppressed T cell response, and an exaggerated humoral response.[8,66] A positive LST result in a *Leishmania*-infected dog probably reflects control of parasites, even in the presence of clinical signs, although several factors can break the balance and severe disease can develop.[45] Longitudinal studies will be required to confirm the long-term protective capability of the observed LST responses.

In conclusion, our results indicate that the raw insect-derived recombinant antigens KMPII, TRYP, LACK, and papLe22 present T cell epitopes responsible for the induction of a DTH response in dogs living in a *L. infantum*-enzootic area. Whereas TRYP showed the highest DTH-positive percentage in clinically healthy dogs, KMPII, LACK, and papLe22 proved to be weak T cell immunogens. Thus, our results suggest that TRYP could be a promising vaccine candidate or a component of a standardized DTH test, particularly when combined with KMPII. Further studies are needed to confirm the diagnostic, prognostic, and vaccination potential of this approach.

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