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Strength and medium-term impact of HisAK70 immunization in dogs: Vaccine safety and biomarkers of effectiveness for *ex vivo Leishmania infantum* infection

Abel Martínez-Rodrigo[a], Alicia Mas[a], Javier Fernández-Cotrina[b], Silvia Belinchón-Lorenzo[b], José A. Orden[a], Pablo Arias[c], Ricardo de la Fuente[a], Javier Carrión[a,*], Gustavo Domínguez-Bernal[a]

[a] INMIVET, Department of Animal Health, Faculty of Veterinary Science, Complutense University of Madrid, 28040 Madrid, Spain
[b] LeishmanCeres Laboratory (GLP Compliance Certificated), Parasitology Unit, Veterinary Faculty, University of Extremadura, Avenida de la Universidad s/n, 10003, Cáceres, Spain
[c] Hospital Central de la Defensa Gómez Ulla, 28047 Madrid, Spain

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ABSTRACT

HisAK70 candidates have successfully been tested in cutaneous (CL) and visceral leishmaniosis (VL) mouse models. Here, we analyse different biomarkers in dog trials after a heterologous immunization strategy with a HisAK70 candidate (plasmid DNA plus adoptive transfer of peripheral blood-derived dendritic cells (DCs) pulsed with the same pathoantigen and CpG ODN as an adjuvant) to explore the antileishmanial activity in an *ex vivo* canine co-culture system in the presence of *Leishmania infantum* parasites. In the canine model, the heterologous HisAK70 vaccine could decrease the infection index in the DC-T cell co-culture system by up to 54% after 30 days and reach almost 67% after 100 days post-immunization, respectively, compared to those obtained in the control group of dogs. The observed security and potential to fight *ex vivo L. infantum* infection highlight a HisAK70 heterologous immunization strategy as a promising alternative to evaluate its effectiveness against canine VL.

1. Introduction

Leishmania infantum is the causative agent of zoonotic visceral leishmaniosis (VL) in humans and other mammals. These infections occur in the drier parts of Latin America as well as in the Mediterranean countries of the Old World, with the domestic dog serving as the main reservoir host [1]. VL, also known as kala-azar, is fatal if left untreated in over 95% of cases. It is characterized by irregular bouts of fever, weight loss, enlargement of the spleen and liver, and anaemia. An estimated 50,000–90,000 new cases of VL occur worldwide each year (http://www.who.int/leishmaniasis/en/). In Spain, VL is a vector-borne zoonosis that is mainly transmitted by the bite of infected female phlebotomine sandfly species *Phlebotomus perniciosus* [2]. The latest human VL outbreak in Spain confirmed the endemic nature and the high prevalence of the disease in the Mediterranean area [3–5]. Although dogs are considered the main domestic reservoirs for *L. infantum*, other animals, including hares and rabbits, have been significant competent reservoirs of leishmaniosis in the latest outbreak in Spain [6–8]. Canine VL (canVL) is endemic and represents a public health problem of global concern not only in Mediterranean countries but also in other parts of Europe where the disease has spread [2].

A current review describes that the progression of *L. infantum* infection in dogs depends on the genetic and non-genetic features of the host when it comes into contact with the parasite. Most infected dogs resist the infection asymptomatically, while the rest are susceptible dogs that present clinical signs of canVL (cutaneous alterations, onychogryphosis, lymphadenomegaly, general muscular atrophy, and renal disease derived from immune-complex deposition). In addition to these signs, increased levels of specific antibodies and high parasite burdens, together with a depression of antileishmanial cellular immune response, are characteristics of the disease [9]. Particularly remarkable is the fact that developing methods of infection control in dogs has the benefit of restricting the risk of infection in humans [10,11]. Since most of the common drugs used in the treatment of canVL have multiple toxicities and are increasingly ineffective due to the development of parasite resistance, vaccination is probably the best way of controlling a vector-borne disease such as leishmaniosis. Vaccines against canVL have been trialled in Brazil (Leishmune® and Leish-Tec®; both focused

* Corresponding author.
E-mail address: javier.carrion@ucm.es (J. Carrión).

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on protection against *L. donovani* and *L. infantum*) and Europe (CaniLeish® and Letifend®, where *L. infantum* is the species responsible for this zoonosis) with mixed results [12–15]. A large, randomized, blinded phase III clinical trial conducted in Brazil showed that Leishmune® induced protection rates of 90% on canine populations. Unfortunately, after a short commercialization period, the Leishmune® prescription was discontinued in Brazil. On the other hand, the other two commercial vaccines available have a low protective efficacy of approximately 68–71% (CaniLeish® 68.4%; Leish-Tec® 71%). The LetiFend® vaccine has been recently licensed by the European Commission, and its efficacy for preventing canVL in non-infected dogs was shown to be 72% [14]. Clearly, more effective vaccines against the disease are required to decrease the incidence of canVL, as an effective way of reducing the transmission of *L. infantum* to humans.

Our research group reported that HisAK70 candidates, as DNA vaccines encoding seven *Leishmania* genes (H2A, H2B, H3, H4, A2, KMP11 and HSP70), generated effective cross-protective immunity in mice against VL and CL [16,17]. Recently, using an approach for vaccination that exploits an attenuated mutant of *Salmonella enterica* serovar Choleraesuis as a carrier to deliver a plasmid encoding the protein HisAK70, our group has described an alternative way to induce a resistant phenotype against murine VL [18]. In this article, taking another step towards achieving global control of VL, we explore different host biomarkers in dog trials after immunization with HisAK70 candidates using a heterologous immunization strategy (plasmid DNA plus adoptive transfer of peripheral blood-derived DCs pulsed with the same pathoantigen and CpG ODN as an adjuvant) against subsequent *ex vivo* parasite infection. It should be noted that we have optimized an innovative system to study the efficiency of the immune function conferred after heterologous vaccination in the canine model, based on the *ex vivo* interaction of DCs and autologous lymphocytes derived from the peripheral blood of dogs in the presence of the infective stage of *L. infantum* promastigotes.

2. Materials and methods

2.1. Ethics statement

This study was approved by the Local Ethical Committee of the Complutense University of Madrid and regional competent authorities (reference number PROEX 396/15). All procedures were conducted in accordance with law RD 53/2013 regarding animal experimentation in Spain and with the European Directive 2010/63/EU concerning the protection of animals used for scientific purposes. The dogs included in the study lived in indoor kennels properly equipped to avoid exposure to the outdoor sandflies. After the experiment was completed (140 days after immunization), all dogs were healthy and were offered for adoption.

2.2. Dogs

Sixteen healthy beagles (12–24 months old) of both genders (8 males and 8 females) were purchased from the Jesús Usón Minimally Invasive Surgery Centre (JUMISC, Cáceres, Spain), bred under vector-free conditions, maintained in quarantine before vaccination, and housed under vector-free conditions at the Animal Service Facilities of the Gómez Ulla Military Hospital under constant veterinary supervision. They had direct contact with pen mates and neighbouring pens and received daily exercise in the facility corridors. There was environmental enrichment in the form of several toys. After quarantine, all dogs received routine vaccinations, had negative anti-*Leishmania* antibody tests and were *Leishmania*-negative by bone marrow PCR. Dogs were allocated into three groups: (1) **HisAK70** vaccinated group (n = 8 dogs), (2) vector-**VAX** immunized group (n = 4 dogs) and (3) non-vaccinated **Control** group (n = 4 dogs).

Table 1
Synthetic peptides used for immunizations.

Protein	Peptide	Amino acids Sequences
H2A [23]	**H2A$_{1\text{-}20}$**	MATPRSAKKSARKSGSKSAK
	H2A$_{106\text{-}132}$	HSGVVPNISKAMAKKKGGKKGKATPSA
H2B [24]	**H2B$_{1\text{-}30}$**	MASSRSAPRKASNPHKSHRKPKRTWNVYVG
	H2B$_{30\text{-}50}$	RSLKAINAQMSMSHRTMKIV
H3 [25]	**H3**	MSRTKETARAKRTITSKKSKKAPSAASGVK
H4 [24]	**H4**	MAKGKRSADAKGSQRRQKKVLRDNIRGITR
A2 *	**A2N26**	EPHKAAVDVGPLSVGPQS
	A2rep	VGPLSVGPQSVGPLSVGPQSVDVSPVS
Kmp11 [26]	**K**	LDRLDEEFNRKMQEQNAKFF

*Designed from the sequence of the complete A2 protein from GenBank: AY255809.1 Ver tb [Genbank: GQ290460].
*Designed after *in silico* analysis from the complete sequence of A2 protein from GenBank: AY255809.1.
[23–26].

2.3. Parasites, vaccine schedule and vaccine safety

L. infantum parasites were isolated from a naturally infected dog (strain code: M/CAN/ES/96/BCN150), were typed at the reference WHO laboratory of Barcelona University (Spain) as *L. infantum* zymodeme MON-1, which is responsible for most cases of VL in humans and dogs [19,20], and were maintained as previously described [21]. Soluble *Leishmania* antigen (SLA) was prepared from stationary cultures of promastigotes as previously described [22]. DNA vaccines pVAX-HisAK70 (HisAK70) and the empty vector pVAX (VAX) were previously constructed and described [17]. They were purified using the EndoFree plasmid Giga kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The endotoxin-free DNA plasmids were resuspended in sterile saline solution and stored at −20 °C until the day of immunization. The HisAK70 polyprotein cocktail contains nine immunogenic peptides (six from nucleosomal histones: H2A, H2B, H3, H4 -**His**-; two representing repetitions of the pathoantigen A2, -**A**-; one from Kmp11, -**K**-) together with one protein (Hsp70 GenBank: CAA59793.1, -**70**-). The amino acid sequences of the peptides used are shown in Table 1. These peptides and the Hsp70 protein were purchased from GenScript (Piscataway, NJ, USA). The vaccination schedule consisted of three subcutaneous (s.c.) doses, given as follows: HisAK70 and VAX-Vector groups of dogs received two doses of the plasmid DNA (300 µg of pVAX-HisAK70 or pVAX, respectively) on days -42 and -21 resuspended in 1 ml of PBS. The control group received PBS alone. On day 0, peripheral blood mononuclear cells were collected in pool from each group (15 ml from each animal) and subsequently differentiated into DCs as described below (Section 2.5). DCs were seeded at a cell density of 1×10^6 cell/ml and pulsed overnight with 20 µg/ml of the complete polyprotein cocktail, containing 2 µg of each peptide along with 2 µg of the Hsp70 protein plus 5 µg/ml of CpG (ODN 2395) for the HisAK70-vaccinated group. Subsequently, the HisAK70 group of dogs was s.c. immunized with 1×10^6 HisAK70-CpG-pulsed DCs. Similarly, the VAX group of dogs was s.c. immunized with 1×10^6 CpG (ODN 2395) pulsed DCs. In parallel, the control group was inoculated with 1×10^6 un-pulsed DCs using the same procedure.

Dogs were monitored weekly during the immunization period to evaluate possible adverse reactions (vaccine safety) upon vaccination as previously described [14]. Complete clinical examinations, including local erythema, pain, oedema and inflammation as well as body weight, temperature measurements and complete evaluation of the general status of animals, were carried out. Blood samples were collected at days 0 and 30 after the vaccination protocol to assess haematological and biochemical evaluation (Laboratorio de análisis veterinario Arturo Soria, S.L.).

2.4. Antigen-specific antibody responses

Peripheral blood samples from each dog were collected at different time points during the experiment as follows: before immunization, during vaccination period (days -42, -21 and 0) and after vaccination (days 30 and 100). Standard endpoint ELISA was performed as previously described [18] to determine both anti-SLA and anti-polyprotein cocktail HisAK70 antibodies (Abs). Briefly, standard 96-well plates were coated overnight at 4 °C with 100 µl of SLA (8 µg/ml) or HisAK70 peptides separately (10 µg/ml of four histone peptides, 10 µg/ml of A2 peptides, 10 µg/ml of Kmp11, or 10 µg/ml of Hsp70) diluted in PBS. Positive and negative control sera were obtained from parasite-free and *Leishmania* naturally infected dogs. Serial dilutions of the sera were carried out to determine the titre, which is defined as the inverse of the highest serum dilution with an absorbance three times higher than the negative control. As secondary Abs, peroxidase-labelled goat anti-dog IgG (Southern Biotech) was used (dilution 1/6000). The enzyme-labelled complexes were detected by reaction with TMB substrate. The reaction was stopped with 50 µl of 1 M sulfuric acid, and the optical density was read at 450 nm using a spectrophotometer.

2.5. Isolation of lymphocytes and isolation, differentiation and characterization of canine monocyte-derived dendritic cells

Fifteen-millilitre aliquots of blood from each dog were collected in heparinized tubes and combined into a pooled sample per group. Canine dendritic cells (DCs) were obtained following the standard protocol used for human peripheral blood mononuclear cells with some modifications [27]. Briefly, canine peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats by passage over a density gradient medium (Histopaque 1077, Merck). After separation, PBMCs were washed by centrifugation at 500 *g* for 10 min and resuspended in PBS pH 7.2 and 2% of inactivated foetal bovine serum (FBS, F7524, Sigma Aldrich). The washing step was repeated 3 times, and the cells were resuspended in PBS pH 7.2, 2 mM EDTA and 0.5% bovine serum albumin (BSA, PROLABO). Monocytes were isolated by CD14-positive selection (Miltenyi, 130050-201) and resuspended in completed medium (CM) consisting of Roswell Park Memorial Institute (RPMI) 1640 (R0883) medium supplemented with 10% FBS, 2 mM L-glutamine (K 0282), 100 U/ml of penicillin, 100 mg/ml of streptomycin (A 2213) and 10 mM HEPES (L 1613). CD14-negative cells were collected and frozen in CM containing 40% FBS and 10% dimethyl sulfoxide (DMSO) and stored at −80 °C until the infection assay was performed. CD14-positive monocytes (6×10^6 cells per ml) were seeded in 25 ml flasks and differentiated into canine monocyte-derived dendritic cells (DCs) by the addition of 33 ng/ml recombinant human Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) and 50 ng/ml recombinant canine IL-4 (both from Kingfisher Biotech, Saint Paul, MN) for a period of 7 days at 37 °C, 5% CO_2. At day 3, the medium was replaced with the same concentration of freshly added growth factors.

To determine the phenotype of canine monocytes and DCs, both cells were double stained with αCD14 (mouse anti-human CD14:RPE, TÜK4, Bio-Rad) in combination with αMHC-II (rat anti-dog MHC class II, YKIX334.2, Bio-Rad), and αCD86 (mouse anti-human CD86:RPE, BU63, Bio-Rad) in combination with αCD40 (mouse anti-human CD40:FITC, LOB7/6, Bio-Rad). CD14-negative cells were stained with αCD3 (rat anti-canine CD3, CA17.2A12, Bio-Rad) to characterize them as T-lymphocytes ($CD14^-CD3^+$). Fluorescence intensities were quantified using a FACScan flow cytometer (BD Biosciences, CAI, UCM, Madrid, Spain), and data were processed with FlowJo software (Tree Star, Ashland, OR, USA).

2.6. Antigen-specific cytokine secretion

After seven days of differentiation as described above, DCs were incubated on ice for 20 min for detachment. DCs (5×10^5 cells/ml) were cultured overnight in 24-well plates either pulsed or not with SLA (6 µg/ml). Subsequently, DCs were incubated in the presence of autologous lymphocytes at a 1:5 ratio (DCs: $CD14^-$ $CD3^+$). Supernatants were collected after 24 h (TNF-α) and 72 h (IFN-γ and IL-10), and cytokine levels were measured by commercial ELISA kits following the manufacturer's instructions (DuoSet® ELISA by Development System R &DTM, UK).

2.7. Killing activity ex vivo assay

DCs (10^5 cells/ml) were seeded into 24-well plates with polylysine-treated circular coverslips (13 mm diameter, VWR) and cultured overnight using 500 ml of complete medium per well. On the following day, stationary-phase *L. infantum* promastigotes were added at a 5:1 ratio (parasite:DC). After 4 h of incubation at 37 °C, extracellular parasites were removed by washing, and cells were incubated for 24 h and 72 h in the presence of autologous lymphocytes at a 1:5 ratio (DC:$CD14^-CD3^+$) for each group. After Giemsa staining, cells were mounted with Coverquick 3000, and 300 cells were counted using an Olympus BX41 microscope. The percentage of infected cells and the mean number of amastigotes per infected cell were evaluated. The infection index was calculated by the multiplication of both parameters to account for the overall parasite load, as previously described [4].

2.8. Statistical analysis

The statistical analyses were performed using Statgraphics version 18 and SigmaPlot version 11 software packages. Analyses of the killing activity *ex vivo* assays were conducted using one-way ANOVA with the multiple range tests to determine which means (from Control, Vector, and HisAK70 groups) were significantly different. Significant differences compared to the control group are indicated by * (p-value is $\leq$ 0.05), whereas significant differences between HisAK70 and the rest of the groups (control and vector) are depicted by ** (p-value is $\leq$ 0.05). Antigen-specific cytokine secretion data were assessed for normality, and subsequent statistical analyses were performed by one-way ANOVA followed by a Bonferroni test. Means were defined as significantly different when p-value $\leq$ 0.05.

3. Results

3.1. Safety evaluation

Vaccine safety was established by direct observation of dogs immediately after immunization, every single day one week after each immunization and weekly during the procedures. Haematological and biochemical parameters remained normal during the experiment in all groups of dogs. The dogs were under veterinary supervision during the vaccination period. None of the animals (vaccine and controls) showed any local reactions or systemic clinical signs that could be attributed to the immunization.

3.2. Specific antibody responses in dogs

To evaluate the humoral response induced by HisAK70 heterologous vaccination, blood serum samples were obtained on days -42, -21, 0, 30 and 100. ELISA assays were performed using both SLA and HisAK70 polyprotein as described above. Notably, all tested dogs (vaccinated and control) showed negative (data not shown) anti-SLA or anti-HisAK70 antibody titres (total IgG and specific IgG1 and IgG2 antibodies) after vaccination, thus confirming the low humoral immunogenicity of the HisAK70 candidate [17].

3.3. Antigen-specific cytokine secretion

To analyse the ability of the HisAK70 vaccine in promoting the

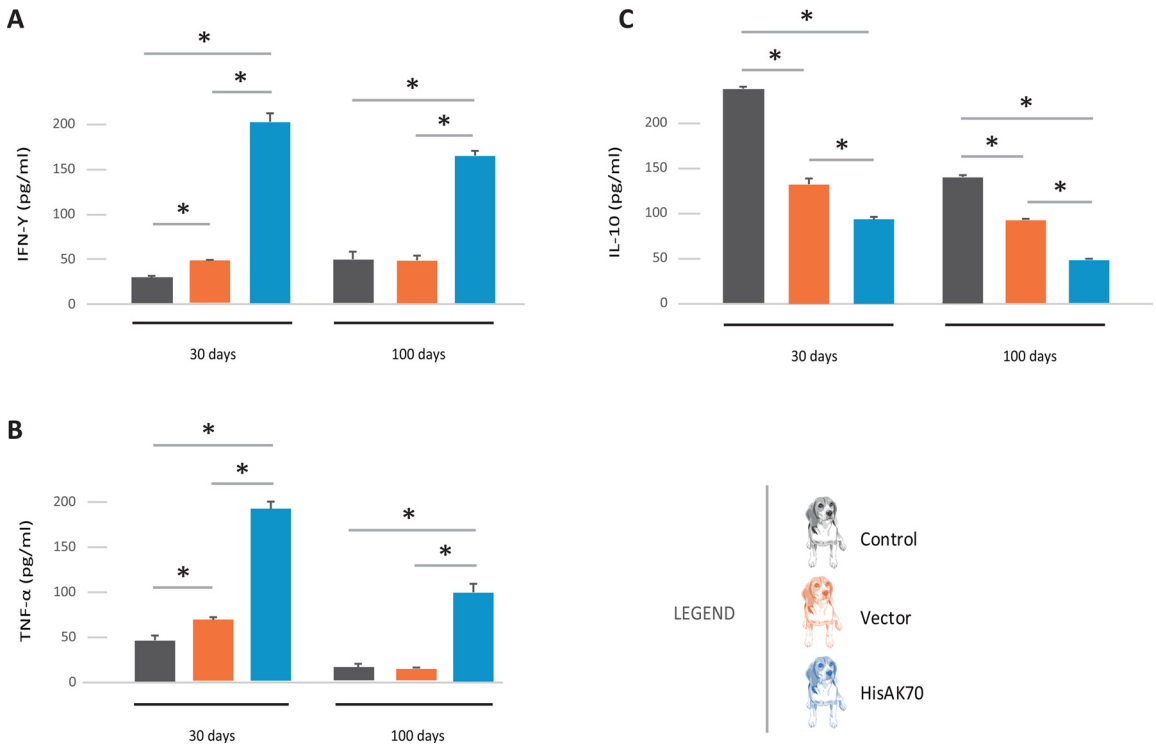

Fig. 1. Cytokine production by the co-culture immune system of DCs and T-lymphocytes. A pool of canine DCs from the non-immunized control (PBS), vector (VAX) and HisAK70 groups was pulsed for 24 h with 6 μg/ml of *L. infantum* SLA followed by co-culture with autologous $CD3^+$ T-cells. The supernatants were collected after 72 h for IFN-γ (A) and IL-10 (B) and after 24 h for TNF-α (C). Cytokine levels were determined by ELISA. Significant differences between groups are labelled as * (p-value ≤ 0.05).

cellular immune response, cytokine production was evaluated. Large quantities of IFN-γ and TNF-α were secreted from co-cultured cells (DCs:lymphocytes) from dogs that received heterologous immunization with HisAK70, compared to those found in the vector and control groups, both at 30 and 100 days after immunization (Fig. 1A–C). Nevertheless, co-cultured cells from HisAK70-vaccinated dogs, when stimulated with SLA, released lower levels of parasite-specific IL-10 than those obtained from the control group. Furthermore, HisAK70 vaccinated dogs showed significantly increased ratios of IFN-γ / IL-10 (mean ± SE at 30 days: 1.99 ± 0.36; and at 100 days: 3.31 ± 0.29) and TNF-α / IL-10 (mean ± SE at 30 days: 1.87 ± 0.17; and at 100 days: 2.00 ± 0.27), reaching the highest ratios at day 100 after immunization. In contrast, vector (IFN-γ / IL-10 mean ± SE at 30 days: 0.032 ± 0.04; and at 100 days: 0.48 ± 0.15; TNF-α / IL-10 mean ± SE at 30 days: 0.47 ± 0.04; and at 100 days: 0.13 ± 0.04) and control group (IFN-γ / IL-10 mean ± SE at 30 days: 0.12 ± 0.02; and at 100 days: 0.32 ± 0.16; TNF-α / IL-10 mean ± SE at 30 days: 0.19 ± 0.05; and at 100 days: 0.10 ± 0.06) showed significantly lower values. Taken together, these data indicate that although the heterologous regimen of immunization with HisAK70 did not induce antigen-specific antibody responses, it did promote a strong antigen-specific Th1 cellular response at 30 and 100 days post-immunization, as evidenced by the IFN-γ / IL-10 and TNF-α / IL-10 ratios.

3.4. Assessment of ex vivo L. infantum infection

Having verified that heterologous HisAK70 immunization promoted a lasting antigen-specific Th1 cellular response, we assessed whether this response could protect (understanding this term as killing activity in an *ex vivo* model) against *L. infantum* infection. On the basis of both the percentage of infected DCs and the mean of the number of amastigotes per infected DC, we were able to identify dogs from both the HisAK70 vaccinated and vector (Tables 2 and 3) groups as they were potentially less susceptible to *ex vivo* infection. Furthermore, the optical

Table 2
Anti-leishmanial assay after 30 days post-immunization. The infection index value was calculated for each time after infection by multiplying the mean percentage of infected cells by the mean number of amastigotes per infected cell. The resulting values are expressed in arbitrary units. One representative experiment out of three is shown. The mean ± standard error of the mean is shown. Significant differences compared to the control group are indicated by * (p-value is ≤ 0.05), whereas significant differences between HisAK70 and the rest of the groups (control and vector) are indicated by ** (p-value is ≤ 0.05).

30 days post-vaccination

		Infected Cells (%)	Amastigotes per infected cell	Infection Index
CONTROL	24h	78.53 ± 2.05	3.07 ± 0.16	241.00 ± 16.55
	72h	80.37 ± 1.87	4.04 ± 0.23	324.50 ± 16.63
VECTOR	24h	69.34 ± 2.18 *	2.83 ± 0.19	197.50 ± 16.55
	72h	73.13 ± 2.13 *	3.45 ± 0.24	251.50 ± 16.63
HisAK70	24h	60.08 ± 2.67 *	2.71 ± 0.16	165.50 ± 16.55 *
	72h	55.66 ± 2.21 **	2.71 ± 0.23	150.00 ± 16.63 **

Media ± Error Std.

microscopy data revealed that all dogs from the HisAK70 vaccinated group had a significantly lower infection index at 30 and 100 days post-vaccination (mean ± SE: 150.00 ± 16.63 and 49.50 ± 11.53, respectively) compared to dogs from the vector (mean ± SE: 251.50 ± 16.63 and 117.50 ± 11.53, respectively) and control groups (mean ± SE: 324.50 ± 16.63 and 147.00 ± 11.53, respectively) at 72 h post-infection. The infection index was calculated by the multiplication of both parameters to account for the overall parasite load, as previously described. It should be noted that 72 h, when parasites have transformed into their intracellular amastigote stage, was also sufficient time for suitable interaction between DCs (characterized by flow cytometry as $CD14^+$ MHC^+/-, $CD40^+$ and $CD86^+$ as described elsewhere [28] and autologous lymphocytes ($CD14^-CD3^+$

Table 3
Anti-leishmanial assay after 100 days postimmunization. The infection index value was calculated for each time after infection by multiplying the mean percentage of infected cells by the mean number of amastigotes per infected cell. The resulting values are expressed in arbitrary units. One representative experiment out of three is shown. The mean ± standard error of the mean is shown. Significant differences compared to the control group are indicated by * (*p*-value is ≤ 0.05), whereas significant differences between HisAK70 and the rest of the groups (control and vector) are indicated by ** (*p*-value is ≤ 0.05).

100 days post-vaccination				
		Infected Cells (%)	Amastigotes per infected cell	Infection Index
CONTROL	24h	65.68 ± 2.25	2.94 ± 0.14	193.5 ± 28.83
	72h	60.14 ± 2.22	2.44 ± 0.10	147.0 ± 11.53
VECTOR	24h	74.03 ± 2.15 *	2.02 ± 0.15 *	149.5 ± 28.83
	72h	50.51 ± 2.16 *	2.35 ± 0.11	117.5 ± 11.53 *
HisAK70	24h	32.93 ± 2.15 **	1.88 ± 0.15 *	62.5 ± 28.83 *
	72h	31.74 ± 2.36 **	1.56 ± 0.15 **	49.5 ± 11.53 **

Media ± Error Std.

cells) in this canine *ex vivo* model. Finally, our data were expressed as the percentage of reduction of the infection index as a vaccine effectiveness marker against VL (Fig. 2A and B) at 30 and 100 days post-immunization. Thus, the heterologous HisAK70 immunization was able to propagate a potent percentage of reduction of the infection index, which markedly increased after 30 (means: 31.60% and 53.41% at 24 and 72 h, respectively) to 100 days after immunization (means: 67.47% and 66.63% at 24 and 72 h, respectively).

4. Discussion

In recent years, a plethora of new vaccine candidates and different strategies have been developed to achieve a gold standard for immunization against human and canine leishmaniosis. The existence of multiple and different ongoing strategies illustrates both the necessity and the complexity of the development of a vaccine against leishmaniosis. The most evolved vaccines depend on the combination of different strategies to generate a long-lasting immunity [29]. The fine-tuning of the host-parasite interaction hampers the development of efficient vaccines.

Previous investigations from our group in experimental murine models have demonstrated the features of the HisAK70 vaccine against different forms of leishmaniosis. We have already described the use of a HisAK70 DNA vaccine encoding seven *Leishmania* genes (H2A, H2B, H3, H4, A2, KMP11 and HSP70) for the immunization of mice to assess the induction of a resistant phenotype against VL and CL caused by *L. infantum* and *L. major* [17]. These parasite antigens play an essential role in the infectivity stage and other relevant biological features of the protozoan *Leishmania* [16]. Recently, we evaluated the use of a novel approach that exploits an attenuated mutant of *Salmonella enterica* serovar Choleraesuis as a carrier to deliver a plasmid encoding the HisAK70 polyprotein in the well-described murine model of VL [18]. These results suggest that immunization with HisAK70 promotes the acquisition of an immune-resistant phenotype against murine VL using different vaccine strategies [16–18], and encouraged us to extrapolate from research in mice to dogs as a definitive host model of VL. Thus, in the present study, we performed an initial approach in a canine *in vivo* model to determine the safety and capacity of heterologous immunization with HisAK70 in generating an adequate immunological response. Finally, we designed an *ex vivo* efficacy trial to analyse the immunogenicity properties and the vaccine potential of HisAK70 against an infection with *L. infantum*.

Given that successful vaccination against different intracellular pathogens is mostly related to the induction of antigen-specific Th1 responses, with the aim of enhancing the immunogenicity of previous DNA vaccine strategies, our rationale behind the use of DCs remains in their pivotal role in the induction of adaptive immunity-priming naïve T-cells and the consequent orchestration of immune response upon vaccination. Indeed, recent studies have shown the potential of DCs loaded with parasite antigens as a vaccine against numerous infectious diseases [30–32]. The increasing knowledge of DCs biology furthers hopeful perspectives for the development of vaccination strategies in different fields. To exploit the full potential of DCs as tools for vaccination, in the present study, once accurately differentiated *ex vivo* from peripheral blood mononuclear cells, canine DCs were loaded with HisAK70 polyprotein cocktail (2 µg of each peptide plus 5 µg/ml of

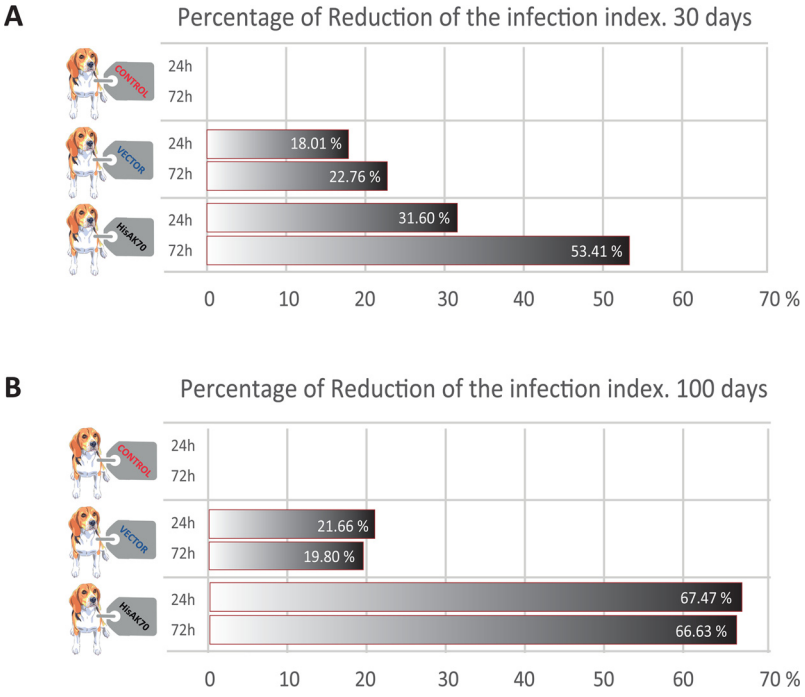

Fig. 2. Schematic representation of vaccine effectiveness expressed as reduction of the infection index at 30 (A) and 100 (B) days post-immunization.

CpG-ODN 2395) and subsequently used (1×10^6 DCs per dog) for s.c. immunization of the HisAK70 group of dogs. This strategy could be optimal for the development of an efficient memory cell response against a *Leishmania* infection. Thus, we designed a heterologous immunization strategy with a HisAK70 candidate consisting of two immunizations with HisAK70 plasmid DNA followed by an adoptive transfer of peripheral blood-derived DCs pulsed with the same polyprotein (HisAK70) suitably formulated with adjuvant CpG ODN 2395 (C-class CpG ODNs) that stimulate Th1 responses [33]. The third immunization with HisAK70 plus CpG-ODN 2395-pulsed DCs aims to optimize and consolidate the antigen-specific immune response generated after the first two genetic immunizations with HisAK70 in dogs.

Interestingly, our findings showed that 30 and 100 days after heterologous HisAK70 vaccination, there were no anti-SLA- or anti-HisAK70 polyprotein IgG specific antibodies generated, presenting values similar to those found in control dogs, thus confirming no crossed reactivity with SLA and also the low humoral immunogenicity of the HisAK70 candidate [17], as described for other DNA vaccines [34,35]. This is a key feature after HisAK70 immunization when the immune response appeared to be skewed towards an immune balance based primarily on the antigen-specific cell host response [16]. This fact can be considered an advantage conferred by the HisAK70 vaccine in the context of infection with *L. infantum*, since the production of high levels of anti-SLA antibodies is not efficient against the intracellular pathogen and constitutes a marker of canine susceptibility to disease evolution [36]. Indeed, these data could be extended in future experiments as an alluring perspective for affronting the new challenges related to the goal of distinguishing between naturally infected and vaccinated dogs by serological tests. On the other hand, with the aim of evaluating the predominant immune phenotype induced by the HisAK70 heterologous vaccination, we evaluated the specific production of cytokines as a marker of the cellular immune response, analysed by different *ex vivo* trials.

Several studies have already published the use of stimulated whole blood, serum, or even different host tissues to determine immunological parameters that allow the analysis of possible immune responses elicited after immunization with any vaccine candidate or infection [37–39]. Moreover, most *in vitro* studies previously described involve co-culture systems with lymphocytes where macrophages are the main antigen presenting cell [40]. Considering that DCs have been implicated in playing a central role in initiating a specific T-cell immune response, leading to a resistant immune phenotype in humans, mice and dogs [18,37,41–43], together with the importance of memory T cells in establishing an effective long-term immunity [29,44–46,43], prompts us to explore the possibility of designing a co-culture system where the players for the immune synapse are DCs and autologous lymphocytes ($CD14^-CD3^+$) to assess the predominant immune phenotype induced by the HisAK70 heterologous vaccination. Thus, assays were carefully planned and developed from the isolation and differentiation of the autologous DCs, which were then pulsed with SLA and subsequently co-cultured in the presence of autologous lymphocytes. This technique was intended to favour the interaction between DCs that process SLA and autologous T-lymphocytes, whose potential effector functionality and memory after immunization allowed us to show differences between the vaccinated and control dogs. Although the heterologous regimen of immunization with HisAK70 did not induce antigen-specific antibody responses, it did promote a strong antigen-specific effector Th1 cellular response at 30 days post-immunization, as evidenced by IFN-γ / IL-10 and TNF-α / IL-10 ratios, which were statistically significant compared to the control group and maintained as memory cell responses after 100 days post-immunization. Indeed, in this system, heterologous HisAK70 immunization resulted in an *ex vivo* effective percentage of reduction of the *Leishmania* infection index, which markedly increased after 30–100 days after vaccination.

The above findings indicated not only that it is feasible to establish an evaluation system based on the *ex vivo* interaction of *Leishmania* Ag pulsed DCs and T lymphocytes as a tool to determine the immunogenicity and the effector effect induced by vaccination strategies in dogs, but also reflect the acquisition of an improved resistant phenotype in HisAK70-vaccinated dogs compared to control dogs. The observed security and potential to fight *ex vivo L. infantum* infection highlight a HisAK70 heterologous immunization strategy as a promising alternative to evaluate its effectiveness against canine VL.

5. Conclusions

The purpose of this study was to achieve and implement in dogs the generation of an *L. infantum*-resistant immune phenotype through two genetic immunizations and memory consolidation by a boost based on an adoptive transfer of DCs pulsed with the HisAK70 vaccine candidate in the form of a protein cocktail. In the canine model, the heterologous HisAK70 strategy could decrease the infection index in DCs after 30 and 100 days post-immunization by increasing the immune ratios IFN-γ/IL-10 and TNF-α/IL-10 to those obtained in the control groups in the absence of a specific humoral response. These results strongly encourage further investigations to explore the potential clinical application of the HisAK70 vaccine against canVL. Furthermore, a functional high-throughput screen of candidate vaccines could be carried out to test their ability to activate the acquired memory cell response *ex vivo* since such assays could be predictive of the preclinical immunogenicity of *in vivo* behaviours.

Conflict of interest

The authors declare they have no competing interests.

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References

[1] P.D. Ready, Epidemiology of visceral leishmaniasis, Clin. Epidemiol. 6 (2014) 147–154.
[2] I. Martin-Martin, M. Jimenez, E. Gonzalez, C. Eguiluz, R. Molina, Natural transmission of Leishmania infantum through experimentally infected Phlebotomus perniciosus highlights the virulence of Leishmania parasites circulating in the human visceral leishmaniasis outbreak in Madrid, Spain, Vet. Res. 46 (2015) 138.
[3] M. Aguado, P. Espinosa, A. Romero-Mate, J.C. Tardio, S. Cordoba, J. Borbujo, Outbreak of cutaneous Leishmaniasis in Fuenlabrada, Madrid, Actas Dermosifiliogr. 104 (2013) 334–342.
[4] G. Dominguez-Bernal, M. Jimenez, R. Molina, L. Ordonez-Gutierrez, A. Martinez-Rodrigo, A. Mas, M.T. Cutuli, J. Carrion, Characterisation of the ex vivo virulence of Leishmania infantum isolates from Phlebotomus perniciosus from an outbreak of human leishmaniosis in Madrid, Spain, Parasit. Vectors 7 (2014) 499.
[5] P. Roth-Damas, M. Sempere-Manuel, A. Mialaret-Lahiguera, C. Fernandez-Garcia, J.J. Gil-Tomas, J. Colomina-Rodriguez, V. Palop-Larrea, Community Outbreak of Cutaneous Leishmaniasis in La Ribera Region of Valencia, spain: Public Health Measures], Enferm Infecc Microbiol Clin, 2016.
[6] M. Jimenez, E. Gonzalez, I. Martin-Martin, S. Hernandez, R. Molina, Could wild rabbits (Oryctolagus cuniculus) be reservoirs for Leishmania infantum in the focus of Madrid, Spain? Vet. Parasitol. 202 (2014) 296–300.
[7] G. Miro, C. Ruperez, R. Checa, R. Galvez, L. Hernandez, M. Garcia, I. Canorea, V. Marino, A. Montoya, Current status of L. infantum infection in stray cats in the Madrid region (Spain): implications for the recent outbreak of human leishmaniosis? Parasit. Vectors 7 (2014) 112.
[8] R. Molina, M.I. Jimenez, I. Cruz, A. Iriso, I. Martin-Martin, O. Sevillano, S. Melero, J. Bernal, The hare (Lepus granatensis) as potential sylvatic reservoir of Leishmania infantum in Spain, Vet. Parasitol. 190 (2012) 268–271.
[9] C. Maia, L. Campino, Biomarkers associated with leishmania infantum exposure,

infection, and disease in dogs, Front. Cell. Infect. Microbiol. 8 (2018) 302.
[10] M.S. Duthie, A. Lison, O. Courtenay, Advances toward diagnostic tools for managing zoonotic visceral leishmaniasis, Trends Parasitol. (2018).
[11] C.B. Palatnik-de-Sousa, I. Silva-Antunes, A. Morgado Ade, I. Menz, M. Palatnik, C. Lavor, Decrease of the incidence of human and canine visceral leishmaniasis after dog vaccination with Leishmune in Brazilian endemic areas, Vaccine 27 (2009) 3505–3512.
[12] G. Bongiorno, R. Paparcone, V.F. Manzillo, G. Oliva, A.M. Cuisinier, L. Gradoni, Vaccination with LiESP/QA-21 (CaniLeish) reduces the intensity of infection in Phlebotomus perniciosus fed on Leishmania infantum infected dogs-A preliminary xenodiagnosis study, Vet. Parasitol. (2013).
[13] G.P. Borja-Cabrera, F.N. Santos, F.B. Santos, F.A. Trivellato, J.K. Kawasaki, A.C. Costa, T. Castro, F.S. Nogueira, M.A. Moreira, M.C. Luvizotto, M. Palatnik, C.B. Palatnik-de-Sousa, Immunotherapy with the saponin enriched-Leishmune vaccine versus immunochemotherapy in dogs with natural canine visceral leishmaniasis, Vaccine 28 (2010) 597–603.
[14] J. Fernandez Cotrina, V. Iniesta, I. Monroy, V. Baz, C. Hugnet, F. Maranon, M. Fabra, L.C. Gomez-Nieto, C. Alonso, A large-scale field randomized trial demonstrates safety and efficacy of the vaccine LetiFend(R) against canine leishmaniosis, Vaccine 36 (2018) 1972–1982.
[15] L. Gradoni, Canine Leishmania vaccines: still a long way to go, Vet. Parasitol. 208 (2015) 94–100.
[16] G. Dominguez-Bernal, P. Horcajo, J.A. Orden, R. De La Fuente, A. Herrero-Gil, L. Ordonez-Gutierrez, J. Carrion, Mitigating an undesirable immune response of inherent susceptibility to cutaneous leishmaniosis in a mouse model: the role of the pathoantigenic HISA70 DNA vaccine, Vet. Res. 43 (2012) 59.
[17] G. Dominguez-Bernal, P. Horcajo, J.A. Orden, J.A. Ruiz-Santa-Quiteria, R. De La Fuente, L. Ordonez-Gutierrez, A. Martinez-Rodrigo, A. Mas, J. Carrion, HisAK70: progress towards a vaccine against different forms of leishmaniosis, Parasit. Vectors 8 (2015) 629.
[18] G. Dominguez-Bernal, A. Martinez-Rodrigo, A. Mas, M.M. Blanco, J.A. Orden, R. De La Fuente, J. Carrion, Alternative strategy for visceral leishmaniosis control: HisAK70-Salmonella Choleraesuis-pulsed dendritic cells, Comp. Immunol. Microbiol. Infect. Dis. 54 (2017) 13–19.
[19] J. Alvar, C. Canavate, R. Molina, J. Moreno, J. Nieto, Canine leishmaniasis, Adv. Parasitol. 57 (2004) 1–88.
[20] J. Martin-Sanchez, M. Gramiccia, T. Di Muccio, A. Ludovisi, F. Morillas-Marquez, Isoenzymatic polymorphism of Leishmania infantum in southern Spain, Trans. R. Soc. Trop. Med. Hyg. 98 (2004) 228–232.
[21] J. Carrion, M.A. Abengozar, M. Fernandez-Reyes, C. Sanchez-Martin, E. Rial, G. Dominguez-Bernal, M.M. Gonzalez-Barroso, UCP2 deficiency helps to restrict the pathogenesis of experimental cutaneous and visceral leishmaniosis in mice, PLoS Negl. Trop. Dis. 7 (2013) e2077.
[22] P. Scott, E. Pearce, P. Natovitz, A. Sher, Vaccination against cutaneous leishmaniasis in a murine model. I. Induction of protective immunity with a soluble extract of promastigotes, J. Immunol. 139 (1987) 221–227.
[23] M. Soto, J.M. Requena, L. Quijada, M. Garcia, F. Guzman, M.E. Patarroyo, C. Alonso, Mapping of the linear antigenic determinants from the Leishmania infantum histone H2A recognized by sera from dogs with leishmaniasis, Immunol. Lett. 48 (1995) 209–214.
[24] M. Soto, J.M. Requena, L. Quijada, M.J. Perez, C.G. Nieto, F. Guzman, M.E. Patarroyo, C. Alonso, Antigenicity of the Leishmania infantum histones H2B and H4 during canine viscerocutaneous leishmaniasis, Clin. Exp. Immunol. 115 (1999) 342–349.
[25] M. Soto, J.M. Requena, L. Quijada, L.C. Gomez, F. Guzman, M.E. Patarroyo, C. Alonso, Characterization of the antigenic determinants of the Leishmania infantum histone H3 recognized by antibodies elicited during canine visceral leishmaniasis, Clin. Exp. Immunol. 106 (1996) 454–461.
[26] M. Agallou, M. Margaroni, E. Karagouni, Cellular vaccination with bone marrow-derived dendritic cells pulsed with a peptide of Leishmania infantum KMP-11 and CpG oligonucleotides induces protection in a murine model of visceral leishmaniasis, Vaccine 29 (2011) 5053–5064.
[27] P. Crauwels, R. Bohn, M. Thomas, S. Gottwalt, F. Jackel, S. Kramer, E. Bank, S. Tenzer, P. Walther, M. Bastian, G. van Zandbergen, Apoptotic-like Leishmania exploit the host's autophagy machinery to reduce T-cell-mediated parasite elimination, Autophagy 11 (2015) 285–297.
[28] M.E. Ricklin Gutzwiller, H.R. Moulin, A. Zurbriggen, P. Roosje, A. Summerfield, Comparative analysis of canine monocyte- and bone-marrow-derived dendritic cells, Vet. Res. 41 (2010) 40.
[29] S. Iborra, J.C. Solana, J.M. Requena, M. Soto, Vaccine candidates against leishmania under current research, Expert Rev. Vaccines 17 (2018) 323–334.
[30] J. Carrion, A. Nieto, M. Soto, C. Alonso, Adoptive transfer of dendritic cells pulsed with Leishmania infantum nucleosomal histones confers protection against cutaneous leishmaniosis in BALB/c mice, Microbes Infect. 9 (2007) 735–743.
[31] M. Fajardo-Moser, S. Berzel, H. Moll, Mechanisms of dendritic cell-based vaccination against infection, Int. J. Med. Microbiol. 298 (2008) 11–20.
[32] C.R. Falcon, F.A. Carranza, P. Aoki, C.C. Motran, L. Cervi, Adoptive transfer of dendritic cells pulsed with Fasciola hepatica antigens and lipopolysaccharides confers protection against fasciolosis in mice, J. Infect. Dis. 205 (2012) 506–514.
[33] J.D. Marshall, K. Fearon, C. Abbate, S. Subramanian, P. Yee, J. Gregorio, R.L. Coffman, G. Van Nest, Identification of a novel CpG DNA class and motif that optimally stimulate B cell and plasmacytoid dendritic cell functions, J. Leukoc. Biol. 73 (2003) 781–792.
[34] S.S.A.A. Hasson, J.K.Z. Al-Busaidi, T.A. Sallam, The past, current and future trends in DNA vaccine immunisations, Asian Pac. J. Trop. Biomed. 5 (2015) 344–353.
[35] A. Kumar, M. Samant, DNA vaccine against visceral leishmaniasis: a promising approach for prevention and control, Parasite Immunol. 38 (2016) 273–281.
[36] L. Lima, L.A. Carneiro, M.B. Campos, T. Vasconcelos Dos Santos, P.K. Ramos, M.D. Laurenti, C.E.C. Teixeira, F.T. Silveira, Further evidence associating IgG1, but not IgG2, with susceptibility to canine visceral leishmaniasis caused by Leishmania (L.) infantum chagasi-infection, Parasite 24 (2017) 37.
[37] S. Hosein, D.P. Blake, L. Solano-Gallego, Insights on adaptive and innate immunity in canine leishmaniosis, Parasitology 144 (2017) 95–115 W.H.O http://www.who.int/leishmaniasis/en/.
[38] A. Rodriguez-Cortes, E. Carrillo, S. Martorell, F. Todoli, A. Ojeda, A. Martinez-Florez, A. Urniza, J. Moreno, J. Alberola, Compartmentalized immune response in leishmaniasis: changing patterns throughout the disease, PLoS One 11 (2016) e0155224.
[39] L. Solano-Gallego, S. Montserrrat-Sangra, L. Ordeix, P. Martinez-Orellana, Leishmania infantum-specific production of IFN-gamma and IL-10 in stimulated blood from dogs with clinical leishmaniosis, Parasit. Vectors 9 (2016) 317.
[40] K.F. Viana, J.A. Fiuza, S. Gannavaram, R. Dey, A. Selvapandiyan, D.C. Bartholomeu, D. da Silveira-Lemos, L.L. Bueno, W.O. Dutra, R.T. Fujiwara, H.L. Nakhasi, R.C. Giunchetti, Application of rapid in vitro co-culture system of macrophages and T-cell subsets to assess the immunogenicity of dogs vaccinated with live attenuated Leishmania donovani centrin deleted parasites (LdCen-/-), Parasit. Vectors 9 (2016) 250.
[41] R. Freitas-Silva, M.C. Brelaz-de-Castro, A.M. Rezende, V.R. Pereira, Targeting dendritic cells as a good alternative to combat leishmania spp, Front. Immunol. 5 (2014) 604.
[42] S. Iborra, M. Martinez-Lopez, F.J. Cueto, R. Conde-Garrosa, C. Del Fresno, H.M. Izquierdo, C.L. Abram, D. Mori, Y. Campos-Martin, R.M. Reguera, B. Kemp, S. Yamasaki, M.J. Robinson, M. Soto, C.A. Lowell, D. Sancho, Leishmania uses mincle to target an inhibitory ITAM signaling pathway in dendritic cells that dampens adaptive immunity to infection, Immunity 45 (2016) 788–801.
[43] E. von Stebut, S. Tenzer, Cutaneous leishmaniasis: distinct functions of dendritic cells and macrophages in the interaction of the host immune system with Leishmania major, Int. J. Med. Microbiol. (2017).
[44] N.D. Glennie, P. Scott, Memory T cells in cutaneous leishmaniasis, Cell. Immunol. 309 (2016) 50–54.
[45] N.D. Glennie, S.W. Volk, P. Scott, Skin-resident CD4+ T cells protect against Leishmania major by recruiting and activating inflammatory monocytes, PLoS Pathog. 13 (2017) e1006349.
[46] P. Scott, F.O. Novais, Cutaneous leishmaniasis: immune responses in protection and pathogenesis, Nat. Rev. Immunol. 16 (2016) 581–592.