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Failure of a multi-subunit recombinant leishmanial vaccine (MML) to protect dogs from *Leishmania infantum* infection and to prevent disease progression in infected animals

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Abstract

We report results of a Phase III trial of the multi-subunit recombinant *Leishmania* polyprotein MML for the protection of dogs against infection by *Leishmania infantum*. The antigen, also known as Leish-111f, is the first antileishmanial human vaccine entered Phase I clinical testing. The study was performed in a leishmaniasis endemic area of southern Italy. Three groups of 15 *Leishmania*-free beagle dogs each, received 3 monthly injections with vaccines A (MML + MPL®-SE adjuvant), B (sterile saline = control) and C (MML + Adjuprime adjuvant), respectively, before transmission season 2002. The surviving dogs received a second three-dose vaccine course 1 year later. The dogs were naturally exposed to sandfly bites for 2.5 months in 2002, and for 5 months in 2003. Every 2 months post vaccination, dogs were examined by clinical and immunological evaluation, and by specific serology, microscopy, culture and PCR. A weak lymphoproliferative response to MML was seen in A and C groups throughout the study period. One year after the first vaccine course, the cumulative incidence of leishmanial infections was 40% in group A, 43% in group B and 36% in group C. Two-year post-vaccination (1 year after the second vaccine course) the cumulative incidence was 87% in group A (with three symptomatic cases), 100% in group B (with no symptomatic cases) and 100% in group C (with two symptomatic cases). The efficacy of the MML vaccine as an immunotherapeutic agent for the prevention of disease progression (subpatent infection → asymptomatic patent infection → symptomatic patent infection) was evaluated through follow-up of dogs found infected prior to the second vaccination. Among 15 infected animals, progression to a subsequent stage of infection was found in 5/6 dogs of group A, 3/6 of group B and 2/3 of group C. We conclude that vaccination with MML is not effective to prevent leishmaniasis infection and disease progression in dogs under field conditions.

Keywords: Recombinant polyprotein; *Leishmania*; Canine leishmaniasis; Vaccine

1. Introduction

Zoonotic visceral leishmaniasis (ZVL) is a severe sandfly borne disease caused by the protozoan parasite *Leishmania infantum* and widely distributed in temperate and subtropical countries of both the Old and New World [1]. The domestic reservoir of ZVL are dogs, which may suffer from a severe disease characterized by chronic evolution of viscero-cutaneous signs occurring in less than 50% of infected animals [2]. On the other hand, both asymptomatic and symptomatic dogs with detectable antibodies can be infectious to phlebotomine vectors [3,4].

Mass detection of seropositive dogs followed by culling and/or drug treatment, or the mass application of deltamethrin-impregnated collars, were shown to have an impact in reducing human and canine ZVL prevalence in endemic areas of the Old World [5–7], although the efficacy of eliminating seropositive canines has been debated in Brazil [8,9]. The above control measures are either not

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acceptable, expensive or not very effective. Mathematical models used to compare the effectiveness of various tools for controlling ZVL, suggest that a dog vaccine may be the most practical and effective method [10]. Therefore, the development of vaccines able to protect dogs from leishmanial infections and/or to prevent disease progression in infected animals, is highly desirable for the implementation of ZVL control programs as well as for the veterinary community.

A few Phase I/II vaccine trials have been performed in dogs, using killed *Leishmania* promastigotes, purified leishmanial fractions or recombinant DNA [11,12]. Recently, a fucose-mannose-ligand (FML) enriched fraction of *Leishmania donovani* entered a Phase III vaccine trial against symptomatic canine leishmaniasis, with about 80% clinical efficacy [13,14]. The same antigen conferred 90% protection from disease progression when used for the immunotherapy of asymptomatic animals [15].

In this paper, we report results of a Phase III trial of the multi-subunit recombinant *Leishmania* polyprotein MML, also known as Leish-111f [16,17], for the protection of dogs against infection by *L. infantum*. This chimeric antigen was generated from three recombinant *Leishmania* antigens screened for their ability to elicit human and murine cellular immune responses. Recombinant TSA (=MAPS), obtained from an *Leishmania major* amastigote cDNA expression library, elicited strong T-cell immune responses in mice and conferred protective immunity against *L. major* when administered with IL-12. This antigen also stimulated proliferative responses in peripheral blood mononuclear cells (PBMC) from human leishmaniasis patients [18]. Recombinant LmSTI1 (=M15) was also selected from an *L. major* amastigote cDNA expression library. Both cellular and humoral responses against this antigen were shown in infected BALB/c mice and in human leishmaniasis patients. In particular, recombinant LmSTI1 was demonstrated to be capable of shifting these toward a Th1-type cellular response in mice with advanced *L. major* infection [19]. A mixture of TSA and LmSTI1 antigens, administered with IL-12 and alum, was found to protect from experimental cutaneous leishmaniasis in a non-human primate model [20]. Recombinant LeIF, originating from a *Leishmania braziliensis* expression library, was found to stimulate the production of IFN-γ and IL-2, but not IL-4 or IL-10, in PBMC from human leishmaniasis patients, and IL-12 in PBMC from both patients and uninfected individuals [21]. The ability of LeIF to influence an early Th1 cytokine profile by IL-12-dependent mechanisms was shown in a SCID mouse model [22]. Since LeIF confers only partial protection against *L. major* in BALB/c mice when used alone, it may have a potential role as a Th1-type adjuvant when used in combination with other leishmanial antigens. A candidate vaccine consisting of Leish-111f formulated in monophosphoryl lipid A stable emulsion (MPL® - SE) entered Phase I clinical testing in healthy volunteers in January 2003 [23].

2. Materials and methods

2.1. Study area and dogs

The study was performed in a rural setting of the Naples province, southern Italy. This area has long been under investigation due to a high incidence of human and canine ZVL. An average of about 40 human cases is reported annually from a cluster of villages and towns surrounding Vesuvius [24], whereas canine leishmaniasis seroprevalence averages 23% [6]. Adult females of the local phlebotomine vector, *Phlebotomus perniciosus*, are usually active from the end of May through to October. In the study area, this species was found to be naturally infected at high rates with *L. infantum* zymodemes known to cause disease in man and dog [25].

Forty-five beagle dogs (23 males) born in January 2002, purchased by a local dog breeder from a laboratory animal company located in a non-endemic area of northern Italy (Green Hill 2001, Montichiari, Brescia), were enrolled in the vaccine study. The dogs had received routine vaccinations against leptospirosis, distemper, adenovirosis-2, hepatitis, parainfluenza and parvovirus (CEPPiL, Merial, France), and were negative for anti-*Leishmania* antibodies by immunofluorescent antibody test (IFAT).

The first two doses of the vaccines under study were administered at the facilities of the laboratory animal company, while the third dose was given after the animals were moved to the study area in July 2002. Here, the dogs were placed in three contiguous open kennels and kept under constant veterinary care during the study period. The use of topical or environmental insecticides was avoided to allow natural exposure of dogs to sandfly bites. Tick control was affected by mechanical measures. The collection of biological samples from the dogs was performed in accordance with the national guidelines for animal welfare, under the supervision of the veterinary services of the Local Health Unit.

2.2. Vaccine and vaccination

Two vaccine preparations, differing in adjuvant composition, consisted of 45 μg/dose MML plus 50 μg/dose MPL®-SE (vaccine A), or 45 μg/dose MML plus 1 mg/dose Adjuprime (Pierce Chemical, IL, USA) (vaccine C), respectively, to give a final volume of 1 ml/dose. A third preparation consisted of 1 ml/dose sterile saline (vaccine control, B). The study was blinded, as the vaccine doses were prepared by Novartis Animal Vaccines Ltd. (Braintree, UK) following procedures reported by Skeiky et al. [17], and neither the veterinarian in charge nor the scientific staff were informed of the identity of the vaccine batches and compositions.

Dogs were randomized by sex and assigned to three groups of 15 animals each, to receive three subcutaneous injections with A, B and C vaccines, respectively, at 28-day intervals starting from 3rd June 2002. The surviving dogs (15 of group A, 14 of group B and 13 of group C) received a second three-dose vaccine course 1 year later, starting from 1st July 2003.

2.3. Follow-up and laboratory procedures

Approximately every 2-month post-vaccination, the following biological samples were obtained from each dog: (a) peripheral blood (PB) for specific serology, immunology and clinical evaluation; (b) bone marrow (BM) aspirate for microscopy and leishmanial DNA detection by polymerase chain reaction (PCR); (c) popliteal lymph node (LN) aspirate for parasite culture.

Detection of antileishmanial IgG antibodies was performed by IFAT and ELISA-K39 techniques. The in-house antigen for IFAT consisted of promastigotes of the WHO Reference Strain for *L. infantum* (MHOM/TN/80/IPT1), and the assay procedure followed the OIE protocol [26]. The threshold titre for positivity was set at 1:80. ELISA plates sensitized with the recombinant *Leishmania* antigen K39 were obtained from Heska Corp. (Fort Collins, CO, USA). The test procedure and cut-off (absorbance [A]: 0.400) were those reported by Scalone et al. [27].

Detection of serum IgG antibodies against MML antigen was performed by an ELISA technique. Briefly, 96-well plates were sensitized overnight with 2 μg/well MML in carbonate buffer. Sera were assayed at the dilution of 1:1000, and the anti-dog IgG peroxidase-conjugated antiserum (Sigma) was used at the 1:200 dilution. The cut-off value was set at the mean + 5S.D. of the A values of sera obtained from the 45 beagles before they were moved to the field. A pool of sera from dogs with canine leishmaniasis was used as positive control.

For the evaluation of cellular immune responses, a lymphocyte proliferation assay was performed using a whole-blood micro assay technique [28]. Briefly, duplicate cultures of PB diluted 1:15 in RPMI medium were cultivated at 37 °C in 24-well culture plates for 3 days in presence of concanavalin A (ConA) (1.5 or 3 μg/ml), and for 5 days in presence of MML (20 μg/ml) or *Leishmania* soluble antigen (LSA, 50 μg/ml). Twenty-four hours before harvesting, cells were pulsed with 5-bromo-2′-deoxyuridine (BrdU) (Boheringer/Roche, Mannheim, Germany) and BrdU incorporation was recognized using a specific monoclonal antibody. To enhance sensitivity of BrdU measurement by a peroxidase-conjugated second antibody, *p*-iodophenol-luminol was used as substrate and the resulting chemoluminescence was measured with the aid of a luminometer at 405 nm. Proliferative response was expressed as stimulation index (SI), i.e. the ratio of counts per second (cps) of stimulated cultures to the cps of untreated cultures.

BM aspirate material was partly smeared onto slides and stained with Giemsa's stain, and partly examined by nested-PCR assay, as follows. DNA was extracted from 350 μl of BM sample using the Easy-DNA™ Kit (Invitrogen, San Diego, CA, USA) and stored at −20 °C until use. The first PCR amplification was carried out in a 50 μl volume containing 10 μl BM DNA plus 40 μl PCR Master Mix (Promega) containing 50 pmol of the kinetoplastid-specific primers R221 (GGTTCCTTTCCTGATTTACG) and R332 (GGCCGGTAAAGGCCGAATAG) [29]. For the second amplification, 3 μl of the first PCR product were added to 22 μl of PCR Master Mix (Promega) containing 3 pmol of the *Leishmania*-specific primers R223 (TCCCATCGCAACCTCGGTT) and R333 (AAAGCGGGCGCGGTGCTG) [29]. The amplification products were analysed by 1.5% agarose gel and visualized under UV light. Positive samples yielded a predicted nested-PCR product of 358 bp.

LN aspirate material was cultured in Evans' Modified Tobie's medium at 22 °C. Cultures were examined for promastigote growth during 1 month.

Clinical assessment was performed by accurate inspection of dogs for the presence of seven signs attributable to *Leishmania* infection (dermatitis, skin ulcers, alopecia, ocular lesions, lymph adenopathy, onychogryphosis, weight loss) and by the evaluation of laboratory data (full blood count, total proteins and albumin/globulin ratio). Animals were scored for clinical and laboratory signs on a scale from 0 to 2/3, and the scores added up to give a clinical score.

2.4. Definition of infection

By the comparative analysis of serological, parasitological and clinical findings from longitudinal samples, the stage of *Leishmania* infection detected at each assessment was assigned to one of the following categories:

(i) *Subpatent infection*: detection of parasite DNA in BM samples, and/or low positive IFAT titre (1:80) followed by occasional conversion to negative in subsequent assessment(s); negative BM microscopy, ELISA-K39 and LN culture; clinical score ≤3;
(ii) *Asymptomatic patent infection*: steady detection of parasite DNA in BM sample; steady or increasing IFAT titre (≥1:160); positive LN culture; positive ELISA-K39 and/or positive BM smear; clinical score ≤3;
(iii) *Symptomatic patent infection*: laboratory findings as above; clinical score >3.

3. Results

3.1. Adverse events

The two MML vaccine preparations were well tolerated and no local or systemic adverse reactions were recorded during or in the period after vaccine injection.

There were no deaths in group A; one dog of group B, and five dogs of group C died in the period from September 2002 to October 2003. All deaths were attributed to acute haemorrhagic disease of probable infectious origin.

3.2. Exposure to sandfly bites and tick infestation

The duration of dog's exposure to bites of *P. perniciosus* sandflies was estimated through routine entomological

data obtained from sandfly collecting stations sited in villages of continental southern Italy in years 2002 and 2003 (Maroli, personal communication). In the transmission season 2002, the vector was present at low density from June through September, due to unfavourable climatic conditions. In 2003, high densities of *P. perniciosus* were recorded from the end of May through to October, with two peaks in July and September, respectively. Hence, we assume that the dogs were exposed to sandfly bites for about 2.5 months in 2002, and for about 5 months in 2003.

Despite mechanical control measures, in July 2002 and 2003 all dogs showed heavy infestations with the common dog tick, *Rhipicephalus sanguineus*, which required single-dose ivermectin (Ivomec, Merial, France) treatment, as well as chemoprophylactic control of *Ehrlichia* infections with 100 mg/kg doxycycline (Bassado, Pharmacia Italia) for 10 days. Despite drug treatment, all dogs developed specific antibodies against *Ehrlichia canis* during the study period.

3.3. Antibody response of dogs to MML antigen

Sera samples collected before each vaccine dose and 35 days after the last vaccine dose in 2002, and before the second vaccination in 2003, were examined for anti-MML IgG antibodies. The mean A value for all dog sera before the 2002 vaccination was 0.046 (S.D.: 0.055), whereas the A value of positive control serum was 1.622. After the first vaccine dose, 2/15 dogs of group A and none of groups B and C were positive to the antigen. After the second vaccine dose, 13/15 of group A, none of group B and 5/15 of group C were positive. After the third vaccine dose, 15/15 of group A, 1/15 of group B (a weak response) and 8/15 of group C had antibodies against MML.

One year after the first vaccine course, 15/15 of group A, 13/14 of group B and 8/14 of group C were positive to MML antigen. The high prevalence of MML reactors in the B control group indicated that responses to vaccine antigen overlapped with responses to MML epitopes from natural *Leishmania* infections occurred in the 2002 season (Fig. 1).

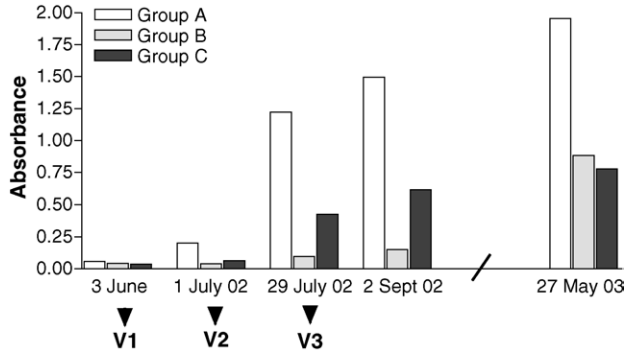

Fig. 1. Mean levels of anti-MML antibodies measured by ELISA in dogs that received three doses (V) of MML vaccines (groups A and C) or saline (group B) and were exposed to natural *Leishmania* transmission in 2002.

3.4. Lymphoproliferative responses

Lymphoproliferative responses against ConA, MML and LSA were determined on eight consecutive PB samples obtained from each dog in the period August 2002–May 2004.

As shown in Fig. 2, a weak proliferative response to MML was seen in A and C groups in October 2002, after the first vaccine course. A more robust response to MML (but never exceeding the ConA response) appeared again in these groups from April to June 2003, concomitantly with specific responses to LSA antigen. From January 2004, also dogs from group B responded to MML antigen, suggesting a general response of dogs to natural *Leishmania* infections. Notably, there was a significant progressive decrease in the non-specific response to ConA in all animals, starting from

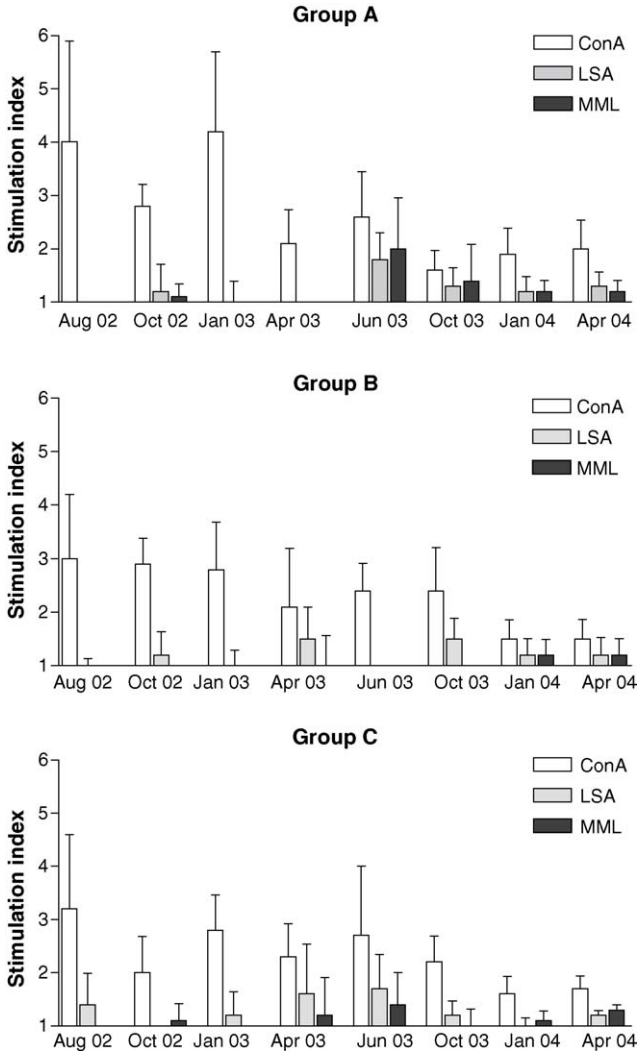

Fig. 2. Lymphoproliferative responses to ConA, MML and LSA, in dogs vaccinated with MML (A and C) or saline (B) and exposed to natural *Leishmania* transmission in 2002 and 2003. Bars represent the mean (±S.D.) stimulation index of whole blood cultures from each dog group.

Table 1
Serological and parasitological positive findings in dogs vaccinated with MML antigen (A and C) or saline (B) and exposed to natural *Leishmania* transmission in 2002 and 2003

Dog group (no.)	May 2003				
	IFAT	ELISA-K39	BM n-PCR	BM smear	LN culture
A (15)	4 (27%)	2 (13%)	6 (40%)	2 (13%)	2 (13%)
B (14)	1 (7%)	1 (7%)	6 (43%)	0	0
C (14)	1 (7%)	1 (7%)	5 (36%)	1 (7%)	1 (7%)
Dog group (no.)	May 2004				
	IFAT	ELISA-K39	BM n-PCR	BM smear	LN culture
A (15)	11 (73%)	6 (40%)	13 (87%)	6 (40%)	7 (47%)
B (14)	8 (57%)	4 (29%)	14 (100%)	4 (29%)	6 (40%)
C (10)	6 (60%)	3 (30%)	10 (100%)	2 (20%)	3 (30%)

BM: bone marrow; LN: popliteal lymph node.

April 2003 ($p = 0.0001$), indicating a generalized decrease in cellular immune reactivity.

3.5. Incidence of leishmanial infections

Results of serological and parasitological data on samples obtained through May 2003 and 2004, respectively, were used to estimate the cumulative incidence of *Leishmania* infections after the 2002 transmission season – to evaluate the efficacy of the first vaccination and after the 2003 transmission season – to evaluate the combined efficacy of the two vaccinations (Table 1).

Through May 2003, leishmanial infections were detected in 6/15 dogs of group A (40%), 4 of which being subpatent and 2 asymptomatic patent infections. In group B, 6/14 dogs showed subpatent infection only (43%). In group C, 5/14 animals were found positive (36%), 4 with subpatent and 1 with asymptomatic patent infections. No dogs from the three groups developed clinical signs of canine leishmaniasis.

Through May 2004, 13/15 dogs of group A were found infected (87%), 6 with subpatent, 4 with asymptomatic patent and 3 with symptomatic patent infections. 14/14 dogs of group B were detected positive (100%), 8 of them with subpatent and 6 with asymptomatic patent infections. No symptomatic cases have occurred in this group. In group C there were 10/10 infected dogs (100%), 7 with subpatent, 1 with asymptomatic patent and 2 with symptomatic patent infections.

None of the 11 dogs that developed asymptomatic patent leishmaniasis showed spontaneous conversion to a subpatent condition, suggesting the progressive nature of the disease in these animals. Similarly, none of the five dogs with symptomatic disease showed spontaneous resolution of clinical signs. On the other hand, animals with subpatent infections showed, by definition, occasional conversion to negative in one or more assessments.

Cumulative incidence rates and the relative proportion of animals found at different infection stages did not differ significantly between the three groups for any of the periods considered.

3.6. Effect of the second vaccination on disease progression

The efficacy of the MML vaccine as an immunotherapeutic agents for the prevention of disease progression, was evaluated through follow-up of dogs found infected prior to the second vaccination performed in July–August 2003. Immunotherapeutic efficacy was defined as the ability of the vaccines to control the following progression 'subpatent infection → asymptomatic patent infection → symptomatic patent infection', in whatever infection stage the animals were found at time of re-vaccination.

Of the 17 dogs found infected before re-vaccination, 15 could be evaluated through May 2004. From the initial condition and at three time-points shown in Table 2, several dogs

Table 2
Effect of re-vaccination with MML antigen (A and C) or saline (B) on disease progression in dogs naturally infected with *Leishmania*

Group (no.)	Before re-vaccination (May 2003)			October 2003			February 2004			May 2004		
	SP	AP	S	SP	AP	S	SP	AP	S	SP	AP	S
A (6)	4	2	0	4	1	1	1	3	2	1	2	3
B (6)	6	0	0	6	0	0	5	1	0	3	3	0
C (3)[a]	2	1	0	2	0	1	2	0	1	1	0	2

SP: subpatent infection; AP: asymptomatic patent infection; S: symptomatic patent infection.
[a] Further two dogs found infected before re-vaccination were lost at follow-up.

showed progression to a subsequent stage of infection: 5/6 in group A, 3/6 in group B and 2/3 in group C.

4. Discussion

At the end of the 2-year study, 37/39 dogs that could be evaluated showed a leishmanial infection (95%), confirming the extremely high *L. infantum* infection pressure in the study site. Results have demonstrated that the MML antigen, at the dose employed and in combination with MPL-SE or Adjuprime as adjuvants, failed to confer protection on the dogs, neither from natural *Leishmania* infection nor from disease progression. It is noteworthy that the only animals scored symptomatic by the end of the study were from the MML-vaccinated groups. These findings are somewhat unexpected, since previous small-scale studies on dogs performed in the same study area, using a mixture of 15 μg each/dose of recombinant TSA, LmSTI1 and LeIF, plus Adjuprime as adjuvant, had provided encouraging results (Gradoni et al., unpublished). In a first study, none of three naive beagles vaccinated with the antigen cocktail showed evidence of infection as compared to 7/7 control beagles that became positive by serology and BM culture within 14 months from natural exposure to sandflies. When two of the vaccinated dogs were re-exposed to a second sandfly season, they acquired clinical leishmaniasis within 2–6 months, but vaccine re-treatment induced rapid improvement of the animals' clinical conditions. In a second study, none of 7 young stray dogs vaccinated with the antigen mixture showed a patent infection, in contrast with 5/12 unvaccinated animals which became infected within 12 months after natural *L. infantum* exposure. The contrasting results obtained in the present study as opposed to the previous investigations may reflect differences in the presentation of the immunogenic epitopes between the mixture of the individual recombinant antigens and the chimeric trifusion protein, although the immune responses to the individual components appear to have been maintained in the latter case, as shown in a Balb/c mouse model of *L. major* [17].

The low ability of MML to elicit cellular immune responses in dogs was evidenced by the scarce lymphocyte proliferative responses to the antigen detected in the period following the first vaccination, despite a high level of anti-MML IgG produced by the animals. After a high proportion of vaccinated dogs have been infected by *Leishmania*, the levels of proliferative response to MML increased in parallel with those to LSA, and this occurred also in unvaccinated animals. Furthermore, the length of the periods devoid of an infection or with a subpatent infection did not correlate with the response levels to MML.

Preliminary results of an on-going analysis of cytokines expressed longitudinally by the dogs enrolled in the present study (data not shown) indicate that animals with a long-lasting subpatent condition and those with rapidly progressing disease, both exhibit elevated levels of IL-4 and IL-10 cytokines expressed by their unstimulated or antigen-stimulated PBMC, which strengthens the evidence for the absence of a polarized Th1/Th2 response to natural or experimental *Leishmania* infection in canines [11,12,30,31].

The outcome of present study also stresses the dramatic differences that may exist between Phase I/II trials with canine vaccines following single-dose experimental challenge with *L. infantum* amastigotes or promastigotes and Phase III trials in vaccinated dogs naturally exposed to parasite transmission in endemic areas. From data available on natural *Leishmania* infection rates of phlebotomine vectors and sandfly biting rates on dogs in rural settings of the Mediterranean area, it can be inferred that, under optimal vector conditions, canines may receive up to one infectious bite/hour/night during warm months [32]. Although it was demonstrated that a single infection of dogs can lead to a long prepatent phase characterized by low expression of cytokines, including those associated with a Th2 phenotype [31], the overlap of several and prolonged immunogenic stimuli may result in a variable immunological background. Furthermore, under field conditions, other pathogenic agents may contribute to an increased immunological susceptibility to *Leishmania*. During the present trial, all dogs showed evidence of *Ehrlichia* co-infection, and it has been recently demonstrated that this pathogen can downregulate canine MHC class II receptors and, hence, impair cellular immune responses [33]. In this respect, it is worth mentioning that a few months after field exposure, all dogs included in the present study showed a significantly decreased non-specific lymphoproliferative response to ConA mitogen, as was previously reported from another study in beagle dogs [34].

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References

[1] Desjeux P. The increase in risk factors for leishmaniasis worldwide. Trans R Soc Trop Med Hyg 2001;95:239–43.
[2] Lanotte G, Rioux JA, Perières J, Vollhardt Y. Ecologie des leishmanioses dans le sud de la France. 10. Les formes evolutives de la leishmaniose viscèrale canine. Ann Parasitol Hum Comp 1979;54:277–95.
[3] Molina R, Amela C, Nieto J, San-Andrés M, Gonzàles F, Castillo JA, et al. Infectivity of dogs naturally infected with *Leishmania infantum* to colonized *Phlebotomus perniciosus*. Trans R Soc Trop Med Hyg 1994;88:491–3.
[4] Courtenay O, Quinnell RJ, Garcez LM, Shaw JJ, Dye C. Infectiousness in a cohort of brazilian dogs: why culling fails to control visceral leishmaniasis in areas of high transmission. J Infect Dis 2002;186:1314–20.

[5] Ashford RW. New strategies for control forum: reservoir control. In: Hart DT, editor. Leishmaniasis: the current status and new strategies for control. New York: Plenum Press; 1989. p. 827–31.
[6] Maroli M, Mizzoni V, Siragusa C, D'Orazi A, Gradoni L. Evidence for an impact on the incidence of canine leishmaniasis by the mass use of deltamethrin-impregnated dog collars in southern Italy. Med Vet Entomol 2001;15:358–63.
[7] Mazloumi Gavgani AS, Hodjati MH, Mohite H, Davies CR. Effect of insecticide-impregnated dog collars on incidence of zoonotic visceral leishmaniasis in Iranian children: a matched-cluster randomised trial. Lancet 2002;360:374–9.
[8] Dietze R, Barros GB, Teixeira L, Harris J, Michelson K, Falqueto A, et al. Effect of eliminating seropositive canines on the transmission of visceral leishmaniasis in Brazil. Clin Infect Dis 1997;25:1240–2.
[9] Moreira Jr ED, Mendes De Souza VM, Sreenivasan M, Nascimento EG, Pontes De Carvalho L. Assessment of an optimized dog-culling program in the dynamics of canine *Leishmania* transmission. Vet Parasitol 2004;122:245–52.
[10] Dye C. The logic of visceral leishmaniasis control. Am J Trop Med Hyg 1996;55:125–30.
[11] Gradoni L. An update on antileishmanial vaccine candidates and prospects for a canine *Leishmania* vaccine. Vet Parasitol 2001;100:87–103.
[12] Ramiro MJ, Zarate JJ, Hanke T, Rodriguez D, Rodriguez JR, Esteban M, et al. Protection in dogs against visceral leishmaniasis caused by *Leishmania infantum* is achieved by immunization with a heterologous prime-boost regime using DNA and vaccinia recombinant vectors expressing LACK. Vaccine 2003;21:2474–84.
[13] da Silva VO, Borja-Cabrera GP, Correia Pontes NN, de Souza EP, Luz KG, Palatnik M, et al. A phase III trial of efficacy of the FML-vaccine against canine kala-azar in an endemic area of Brazil (Sao Goncalo do Amaranto, RN). Vaccine 2001;19:1082–92.
[14] Borja-Cabrera GP, Correia Pontes NN, da Silva VO, Paraguai de Souza E, Santos WR, Gomes EM, et al. Long lasting protection against canine kala-azar using the FML-QuilA saponin vaccine in an endemic area of Brazil (Sao Goncalo do Amarante, RN). Vaccine 2002;20:3277–84.
[15] Borja-Cabrera GP, Cruz Mendes A, Paraguai de Souza E, Hashimoto Okada LY, de A, Trivellato FA, et al. Effective immunotherapy against canine visceral leishmaniasis with the FML-vaccine. Vaccine 2004;22:2234–43.
[16] Coler RN, Skeiky YA, Bernards K, Greeson K, Carter D, Cornellison CD, et al. Immunization with a polyprotein vaccine consisting of the T-cell antigens thiol-specific antioxidant, *Leishmania major* stress-inducible protein 1, and *Leishmania* elongation initiation factor protects against leishmaniasis. Infect Immun 2002;70:4215–25.
[17] Skeiky YAW, Coler RN, Brannon M, Stromberg E, Greeson K, Crane RT, et al. Protective efficacy of a tandemly linked, multi-subunit recombinant leishmanial vaccine (Leish-111f) formulated in MPL adjuvant. Vaccine 2002;20:3292–303.
[18] Webb JR, Campos-Neto A, Ovendale PJ, Martin TI, Stromberg EJ, Badaro R, et al. Human and murine immune responses to a novel *Leishmania major* recombinant protein encoded by members of a multicopy gene family. Infect Immun 1998;66:3279–89.
[19] Webb JR, Kaufmann D, Campos-Neto A, Reed SG. Molecular cloning of a novel protein antigen of *Leishmania major* that elicts a potent immune response in experimental murine leishmaniasis. J Immunol 1996;157:5034–41.
[20] Campos-Neto A, Porrozzi R, Greeson K, Coler RN, Webb JR, Seiky YA, et al. Protection against cutaneous leishmaniasis induced by recombinant antigens in murine and nonhuman primate models of the human disease. Infect Immun 2001;69:4103–8.
[21] Skeiky YAW, Guderian JA, Benson DR, Bacelar O, Carvalho EM, Kubin M, et al. A recombinant *Leishmania* antigen that stimulates human peripheral blood mononuclear cells to express a Th1-type cytokine profile and to produce interleukin 12. J Exp Med 1995;181:1527–37.
[22] Skeiky YAW, Kennedy M, Kaufman D, Borges MM, Guderian JA, Scholler JK, et al. LeIF: a recombinant *Leishmania* protein that induces an IL-12-mediated Th1 cytokine profile. J Immunol 1998;161:6171–9.
[23] Reed SG, Campos-Neto A. Vaccines for parasitic and bacterial diseases. Curr Opin Immunol 2003;15:456–60.
[24] Gradoni L, Pizzuti R, Scalone A, Russo M, Gramiccia M, di Martino L, et al. Recrudescence of visceral leishmaniasis unrelated to HIV infection in the Campania region of Italy. Trans R Soc Trop Med Hyg 1996;90:234–5.
[25] Maroli M, Gramiccia M, Gradoni L, Troiani M, Ascione R. Natural infection of *Phlebotomus perniciosus* with MON 72 zymodeme of *Leishmania infantum* in the Campania region of Italy. Acta Trop 1994;57:333–5.
[26] Gradoni L, Gramiccia M. Leishmaniosis. In: Manual of standards for diagnostic tests and vaccines. Paris: Office International des Epizooties; 2000. p. 803–12.
[27] Scalone A, De Luna R, Oliva G, Baldi L, Satta G, Vesco G, et al. Evaluation of the *Leishmania* recombinant K39 antigen as a diagnostic marker for canine leishmaniasis and validation of a standardized enzyme-linked immunosorbent assay. Vet Parasitol 2002;104:275–85.
[28] Smedman L, Joki A, Perlmann P, Troye-Blomberg M. Whole-blood microassay for immunodetection of antigen specific cell mediated immunity using bromodeoxyuridine incorporation. J Clin Lab Immunol 1991;34:79–85.
[29] van Eys GJ, Schoone GJ, Kroon NC, Ebeling SB. Sequence analysis of small subunit ribosomal RNA genes and its use for detection and identification of *Leishmania* parasites. Mol Biochem Parasitol 1992;51:133–42.
[30] Quinnell RJ, Courtenay O, Shaw MA, Day MJ, Garcez LM, Dye C, et al. Tissue cytokine responses in canine visceral leishmaniasis. J Infect Dis 2001;183:1421–4.
[31] Santos-Gomes GM, Rosa R, Leandro C, Cortes S, Romao P, Silveira H. Cytokine expression during the outcome of canine experimental infection by *Leishmania infantum*. Vet Immunol Immunopathol 2002;88:21–30.
[32] Gradoni L. The diagnosis of canine leishmaniasis. In: Killick-Kendrick R, editor. Canine Leishmaniasis: moving towards a solution. Boxmeer: Intervet International; 2002. p. 7–14.
[33] Harrus S, Waner T, Friedmann-Morvinski D, Fishman Z, Bark H, Harmelin A. Down-regulation of MHC class II receptors of DH82 cells, following infection with *Ehrlichia canis*. Vet Immunol Immunopathol 2003;96:239–43.
[34] De Luna R, Vuotto ML, Ielpo MTL, Ambrosio L, Piantedosi D, Moscatiello V, et al. Early suppression of lymphoproliferative response in dogs with natural infection by *Leishmania infantum*. Vet Immunol Immunopathol 1999;70:95–103.