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Primary vaccination with the LiESP/QA-21 vaccine (CaniLeish®) produces a cell-mediated immune response which is still present 1 year later☆

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ABSTRACT

Canine leishmaniasis, an important zoonotic disease of dogs, is the result of an ineffective and inappropriate immune response to infection with *Leishmania infantum*. It is widely accepted that the appropriate immune response is characterised by a T-helper (Th)1-dominated profile in an overall mixed Th1/Th2 response. The absence of a strong Th1 response is associated with progression to the clinical disease. Thus, there is a need for an effective vaccine that could modulate the immune response to a more appropriate profile against the parasite. In this study we measured the impact of the LiESP/QA-21 canine vaccine, recently launched commercially in Europe, on selected humoral and cellular immune markers for one year after a primary vaccination course. The humoral response to vaccination was characterised by a predominantly IgG2 profile. Vaccinated dogs developed long-lasting cell-mediated immune responses against *L. infantum*, specifically with a stronger ability of macrophages to reduce intracellular parasite burdens in co-culture with autologous lymphocytes compared to control dogs ($p = 0.0002$), which was correlated with induction of inducible nitric oxide synthase (iNOS) and production of nitric oxide (NO) derivatives. These results confirm that vaccination with LiESP/QA-21 is capable of inducing an appropriate Th1-dominated immune profile which persists for a full year.

1. Introduction

Canine leishmaniasis (CanL), a complex disease caused by an inappropriate immune response to infection with *Leishmania* spp. (*Leishmania infantum* in the Mediterranean basin) is a significant problem for the canine population and their owners in the world's endemic areas (Solano-Gallego et al., 2009). It is currently estimated that at least 2.5 million dogs are infected in southwestern Europe alone (Moreno and Alvar, 2002), with the endemic area spreading steadily northwards (Maroli et al., 2008). This provides a large reservoir of infection with the potential for zoonotic spread (Gramiccia and Gradoni, 2005).

The outcome of infection with *L. infantum* is highly variable in dogs, with some progressing rapidly to the clinical syndrome while others remain healthy (Solano-Gallego et al., 2001). Data from the visceral form of the disease in humans and from murine models of visceral leishmaniasis have shown that a mixed response with a dominant Th1 profile is required for protection (McMahon-Pratt and Alexander, 2004; Bhowmick and Ali, 2009; Sharma and Singh, 2009). This also seems to be the case in dogs where the response is always a complex balance between the Th1 and Th2 profiles (Strauss-Ayali et al., 2007), and it is now generally accepted that resistance to developing CanL is primarily dependent on whether or not a dog develops an appropriate Th1-dominated cell-mediated immune response against the parasite (Santos-Gomes et al., 2002; Carrillo and Moreno, 2009). The protective response is believed to be primarily mediated by the induction of inducible nitric oxide synthase (iNOS) in macrophages upon stimulation by Th1 cytokines such as IFN-γ. This results in a leishmanicidal oxidative burst with a simultaneous increased production of leishmanicidal nitric oxide (NO) and reduced production of the polyamines which are essential for the growth and survival of the parasite within the macrophage (Barbiéri, 2006; Zafra et al., 2008). It is also probable that further to its direct leishmanicidal effect, NO may play additional immunoregulatory roles (Bogdan et al., 2000). Despite the complexity of the situation, it is now widely recognised that the ideal protective immune response in the dog is mediated by a dominant CD4$^+$ Th1 influence in an overall mixed cellular response (Santos-Gomes et al., 2002; Carrillo and Moreno, 2009).

Therefore, there is a clear need to be able to reinforce a dog's immune response in order to increase the probability that it will correctly manage the infection and remain healthy. In addition, when the immune system correctly controls parasite replication, this in turn reduces the number of dogs infectious to sandflies. It is for this reason that several authors have expressed the opinion that an effective vaccine against CanL would be the best control strategy for both canine and human disease in areas of the world where transmission to humans is primarily zoonotic (Dye, 1996; Alvar et al., 2004).

This has been made possible for the first time in Europe by the relatively recent launch of the LiESP/QA-21 vaccine (CaniLeish®, Virbac, France). This vaccine is licensed for use with a primary vaccination course of three injections at three week intervals, followed by annual booster vaccinations. It is very important to be able to understand as much as possible about the mode of action of this vaccine on the immune response of the dog. Data demonstrating the onset of an appropriate Th1-dominated cellular immune response after vaccination with the LiESP/QA-21 vaccine has been published (Moreno et al., 2012). However, as with any vaccine, the key objective is the ability to produce an appropriate and specific memory response to infection months or even years later. Thus, it is essential to demonstrate that this response persists for a suitable period of time in order to determine an appropriate revaccination interval for the vaccine. Two other canine leishmaniosis vaccines (Leishmune®, Zoetis, and LeishTec®, Hertape Calier) are available, but only in Brazil (Otranto and

Dantas-Torres, 2013). Both of these also have a primary vaccination course consisting of three injections at three week intervals followed by annual booster vaccinations.

This study aimed to assess the ability of the LiESP/QA-21 vaccine to stimulate an appropriate, specific, Th1-dominated immune response against *L. infantum* in dogs for at least 1 year after the last administration of the vaccine.

2. Materials and methods

2.1. Ethics statement

The study was approved by the Ethical Committee of Harlan Laboratories Bioservice GmbH, Germany.

2.2. Summary of the study design

This study compared the general characteristics of the specific humoral and cellular immune responses against *L. infantum* between unvaccinated and vaccinated dogs during 1 year after a primary vaccination course.

2.3. Animals' characteristics

20 conventional Beagle dogs (10 males and 10 females) aged approximately 6 months old (between 5 months and 3 weeks, and 7 months old) on the day of the first vaccination were assigned to two groups (10 vaccinated and 10 control) according to their sex and litter of birth. There were 5 males and 5 females per group.

All animals had previously been vaccinated with conventional vaccinations against Distemper virus, Adenovirus, Parvovirus, Parainfluenza virus, *Leptospira* spp. and Rabies before the beginning of the study, and annual booster vaccinations for these valencies were given during the study.

They were housed in controlled indoor kennel conditions in a non-endemic country to avoid any possibility of exposure to *L. infantum* parasites, and dewormed with a combination product containing Febantel, Pyrantel embonate and Praziquantel (Drontal® Plus flavour, Bayer, Germany) 1 week prior to the date of the first administration of the LiESP/QA-21 vaccine and then again 7 months later.

2.4. Vaccine and vaccination protocol

The LiESP/QA-21 vaccine is authorised in the European Union under the trade name CaniLeish® (Virbac, France). It is composed of purified excreted-secreted proteins of *L. infantum* (LiESP), produced by means of a patented cell-free, serum-free culture system invented by the Institut de Recherche pour le Développement (Patent Application number FR 2 705 358, 1993, J Lemesre), and adjuvanted with QA-21, a highly purified fraction of the *Quilaja saponaria* saponin. The doses used in this study were formulated at 100 μgESP and 60 μg QA-21. This is consistent with the minimum accepted antigen levels in commercially available doses. Each dose was reconstituted immediately before use in 1 ml 0.9% NaCl solution.

Dogs in the vaccinated group ($n = 10$) were given one dose of the LiESP/QA-21 vaccine subcutaneously every 21 days for a total of three doses. Vaccinations were administered in the interscapular area. Dogs in the control group ($n = 10$) did not receive any vaccination. No additional booster vaccinations of LiESP/QA-21 were given to vaccinated dogs during the study.

2.5. Clinical follow-up

The general health of each animal was observed daily, and specific attention was also given to the injection site areas in the weeks following vaccination. The injection site area was clipped in both groups of dogs at the start of the study to allow for easier, unbiased visual assessment and palpation of any local reactions. In the absence of any suspected problems which would trigger an additional full clinical examination, the animals received a routine full clinical examination including assessment of bodyweight and rectal temperature every one to two weeks during the first three months of the study, and then monthly thereafter.

2.6. Analyses and schedule

2.6.1. Serology testing of the humoral immune response

ELISA testing was performed on the day of each vaccination (weeks 0, 3, 6) and also two weeks after the last vaccination (week 8) and then on weeks 12, 30, 42, 54 and finally on week 58 (1 year after the last vaccine administration) to dose the level of IgG1 and IgG2 antibodies to both LiESP and also specifically to Parasite Surface Antigen (PSA), which is a major antigenic component of LiESP. Blood was collected in uncoated tubes and the serum separated before performing the analyses.

On each occasion when ELISA testing was performed, Immunofluorescence testing (IFAT) was also performed on the same samples to dose the level of total anti-*Leishmania* IgG antibodies.

Briefly, the techniques were performed as follows:

2.6.1.1. ELISA. A NUNC Maxisorp plate was coated with either 0.1 μg ESP or 0.1 μg PSA per well in carbonate buffer (0.16% sodium carbonate with 0.29% sodium bicarbonate, pH 9.6) for 90 min at 35–37 °C. Non-specific sites were blocked with PBS-Tween (SIGMA, France) 0.5%-milk powder (Régilait, France) 5% for 90 min at 35–37 °C. Then serial three-fold dilutions of the serum to be tested, from 1/150 to 1/12,150, were made in PBS-milk powder 0.5% buffer and added to the plate. After 60 min of incubation at 35–37 °C any antibodies fixed to the ESP or PSA, respectively, were revealed with a specific peroxydase-conjugated polyclonal anti-IgG1 or anti-IgG2 secondary antibody (Bethyl Laboratories, Montgomery, USA) and ABTS colouration. The titre corresponded to the first dilution with an optical density at 405 nm inferior to 0.4. Dogs were considered as negative when the titre was inferior to 1/450.

2.6.1.2. IFAT. The Fluoleish® kit (BVT, France) was used according to the manufacturer's instructions using serial dilutions of the serum from the 1/100th to 1/12,500th. The

titre in this test corresponded to the last dilution where at least 50% of the parasites displayed visible fluorescence.

For the graphical representation of the mean reciprocal titres (in $\log_{10}$) of all serological analyses, dogs with negative results were regarded as having a result of zero after log conversion of the reciprocal titres to allow these negative results to be represented on the charts.

2.6.2. Cellular immune response assays

The three cell-mediated immunity tests, [Lymphoblastic Transformation Test (LTT), IFN-γ Enzyme-Linked Immunospot Assay (ELISpot) and Canine Macrophage Leishmanicidal Assay (CMLA)] were performed as previously described, and as summarised below, at baseline (week 0), three weeks after the third vaccination (week 9) and thereafter on weeks 30 and 58 (1 year after the last vaccine dose).

2.6.2.1. LTT. This assay was used to reveal the ability of the specific memory T cells produced as a result of vaccination to proliferate after being exposed to Soluble Leishmania Antigens (SLA). It was performed in a manner similar to that previously described (Moreno et al., 1999; Carrillo et al., 2008; Moreno et al., 2012).

Briefly, heparinized blood samples were fractionated by centrifugation over lymphocyte separation medium. PBMCs obtained were incubated at a density of 10^6 cells/ml for 5 days (37 °C, 5% CO_2) in presence of either 10 μg/ml ConA, or 10 μg/ml SLA, or with medium alone. The cells were pulsed during the last 24 h with 10 μM 5-bromo-2-deoxyuridine (BrdU), which is incorporated into the DNA of proliferating cells. BrdU incorporation was determined with a specific ELISA system (GE Healthcare, Chalfont St. Giles, UK) that uses peroxydase-labelled anti-BrdU antibodies which are in turn detected by a substrate reaction using 3,3′5,5′-tetramethylbenzidine. Absorbance values at 450 nm correlate directly to the amount of DNA synthesis and thereby to the number of proliferating cells in culture. The results were expressed as the lymphoproliferation index, which is the ratio of the mean optical density obtained for the SLA stimulated samples compared to the mean optical density obtained for the non-stimulated samples. ConA was used as a positive control and the medium alone was used as a negative control.

2.6.2.2. ELISpot. This assay was used to determine the proportion of T cells that release IFN-γ after stimulation with SLA in order to quantify the level of stimulation of a specific Th1-polarity immune memory response. It was performed in a manner similar to that previously described (Holzmuller et al., 2005; Moreno et al., 2012). Heparinized blood samples were fractionated by centrifugation over lymphocyte separation medium. The PBMCs obtained were incubated at a density of 10^6 cells/ml for 3 days in multiscreen HTS filter plates (Millipore, Billerica, USA) previously coated with canine IFN-γ capture antibody (R&D System, Minneapolis, USA), in presence of 10 μg/ml ConA, or 10 μg/ml SLA antigens, or with medium alone, in a humidified 37 °C CO_2 incubator. The quantity of IFN-γ was revealed with a specific biotinylated antibody and incubation with Streptavidin-AP and the BCIP/NBT

Chromogen (R&D System, Minneapolis, USA). The number of specific spots was determined by an automated ELISpot reader. ConA was used as a positive control and the medium alone was used as a negative control. The data presented are the number of spots per 2×10^5 cells after stimulation with SLA minus the equivalent value obtained with the negative control using medium alone.

2.6.2.3. CMLA. This assay was used to determine the ability of monocyte-derived canine macrophages to kill *Leishmania* parasites in a co-culture system due to the stimulation of iNOS expression and the resulting production of NO derivatives when the macrophage is exposed to autologous lymphocytes derived from canine PBMC. It was performed in a manner similar to that previously described (Neogy et al., 1994; Vouldoukis et al., 1996, 2006; Moreno et al., 2012).

Briefly, monocytes separated from lymphocytes by adherence were cultured at a density of 2×10^5 cells per well at 37 °C and 5% CO_2 for 6 days in complete RPMI 1640 medium containing 25 mM Hepes.

After 6 days of culture, monocyte-derived macrophages were infected with stationary growth phase *L. infantum* (MCAN/82/GR/LEM 497) promastigotes at a ratio of 1:5 for 5 h; then the cells were washed and fresh medium was added for 24 h. This point was then considered as time zero. The cells were checked to ensure that greater than 55% were infected. The infected cells (t0) were washed and then 2×10^5 macrophages were incubated in each well alone or in the presence of 10^5 autologous lymphocytes for 72 h in complete medium containing additionally 10 mM HEPES and 5×10^{-5} M 2-mercaptoethanol. After 72 h of co-culture, the lymphocytes were then removed by several gentle washings, the cell free supernatants were conserved for analysis and the macrophages were fixed in order to evaluate the leishmanial killing. One part of the fixed macrophages was stained with Giemsa and the leishmanicidal activity was determined microscopically by counting in triplicate the number of intact parasites per 100 cells in the macrophages co-cultured with the lymphocytes and contrasting this with the number of intact parasites per 100 cells in the macrophages cultured with the medium alone (no T cells). The difference between these results was expressed as the CMLA index using the following formula: CMLA index = 100 − (mean number of amastigotes per macrophage multiplied by the percentage of infected cells when co-cultured with lymphocytes)/(mean number of amastigotes per macrophage multiplied by the percentage of infected cells when cultured without lymphocytes) × 100.

The other part of the fixed macrophages was used to evaluate the % of inducible nitric oxide synthase (iNOS) expression by immunolabelling with NOS specific antibodies, as described previously (Vouldoukis et al., 2006). Briefly, the cells were incubated with rabbit polyclonal antibodies directed against NOS (Santa Cruz Biotechnology, USA) at a dilution of 1:100 in PBS for 1 h at 4 °C, followed by 3 washes in PBS, then the binding of the antibody was revealed by use of a labelled anti-rabbit IgG in an immunofluorescence assay to determine the percentage of iNOS positive macrophages.

The production of NO_2 (involved in the NO cascade) was determined in the culture supernatants using the modified Griess reference technique (Green et al., 1982). When evaluating this leishmanicidal activity test, a result was considered as successful, when the % inhibition of the parasitic index (CMLA) was associated with the activation of the NO pathway and directly correlated with a significant increase of iNOS expression and the production of NO derivatives.

3. Statistical analyses

All statistical tests were performed using the SAS v9.1 software, and for all analyses the significance threshold was set at $p = 0.05$.

Assessments for bodyweight, rectal temperature, LTT, ELISpot, CMLA, NO_2 and iNOS were performed by use of linear mixed-effects models including group, time and their interaction as fixed effects the dog as a random effect.

Where significant differences were found in the linear mixed-effects models, Wilcoxon two-sample tests were also used to assess the differences between groups at each time point.

4. Results

4.1. Growth and rectal temperatures

During the study period both groups of dogs grew normally with no significant differences between them. Some dogs in each group (5 vaccinated and 5 control dogs) presented single-day rectal temperatures slightly above the normal range. This was not associated with any clinical signs and was unrelated to the administration of the vaccine in the vaccinated group. These episodes were attributed to excitement and handling of the animals.

4.2. Clinical condition

Minor clinical signs were observed in both groups before the commencement of the vaccination course including conjunctivitis and mild swelling of the cervical lymph nodes. During the one-year course of the study, some minor clinical signs such as conjunctivitis, skin irritation at sites clipped for blood sampling and minor wounds were noted in nine of the vaccinated dogs and all the unvaccinated control dogs. None of these were related to vaccination. No adverse events (local or systemic) were reported after any vaccination.

4.3. Serology testing of the humoral immune response

At the start of the study, all animals were negative for antibodies against *L. infantum* by all the tests used. No control dogs were positive by any serological test at any point during the study.

After vaccination, all LiESP/QA-21 vaccinated dogs developed a strong IgG2 response to both ESP (range 1/1350–1/12,150) and, in particular, to PSA (range 1/450–1/4050). Six months after the vaccine course (week 30) all vaccinated dogs still had detectable IgG2 titres to
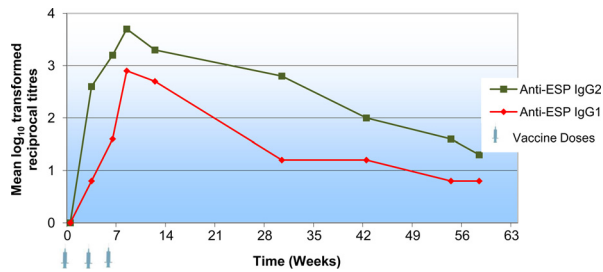
Fig. 1. Progression in log-transformed anti-ESP IgG1 and IgG2 titres during primary vaccination and the subsequent year. The data presented here are the means of the log-transformed reciprocal titres in the vaccinated group ($n = 10$). The titre is taken to be the first dilution with an optical density of less than 0.4 measured at 405 nm. The sera were tested using an ESP-coated ELISA. The ESP used was identical in profile to the antigen of the vaccine. Dogs with a negative result were regarded as having a result of zero after log conversion of the reciprocal titres to allow this to be represented on the charts.

ESP. At the end of the study, 5 vaccinated dogs still had detectable IgG2 titres against ESP, and 1 had a detectable titre against PSA (see also Figs. 1 and 2).

All vaccinated dogs also developed an IgG1 response to ESP by week 8 (range 1/450–1/4050). By week 30, only 4 vaccinated dogs still had detectable IgG1 titres against ESP.

Only one vaccinated dog developed a detectable IgG1 anti-PSA response (maximum titre 1/1350), which was evident only from week 8 to week 12 (see also Figs. 1 and 2).

All vaccinated dogs were also positive by IFAT on week 8 and week 12 (maximum titre 1/500) and one vaccinated dog still had a titre of 1/200 on week 30. All dogs were negative by IFAT (<1/200) on week 42 (see Fig. 3).

4.4. Cellular immune response assays

4.4.1. LTT

The PBMCs of all animals in both vaccinated and control groups were able to respond effectively to the non-specific positive control stimulation with ConA (no difference between groups, data not shown, $p = 0.235$). A comparison of the result after SLA stimulation at each time point is shown in Fig. 4. There was a significant difference between
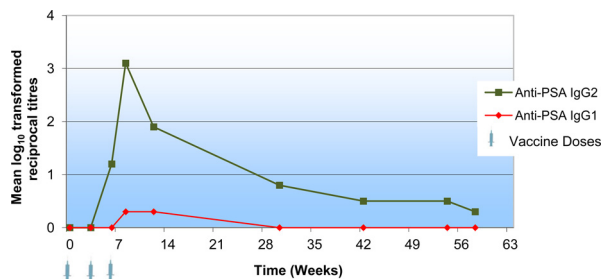

Fig. 2. Progression in log-transformed anti-PSA IgG1 and IgG2 titres during primary vaccination and the subsequent year. The data presented here are the means of the log-transformed reciprocal titres in the vaccinated group ($n = 10$). The titre is taken to be the first dilution with an optical density of less than 0.4 measured at 405 nm. The sera were tested using a PSA-coated ELISA. PSA is a dominant antigen in ESP and therefore a key antigen in the vaccine. Dogs with a negative result were regarded as having a result of zero after log conversion of the reciprocal titres to allow this to be represented on the charts.
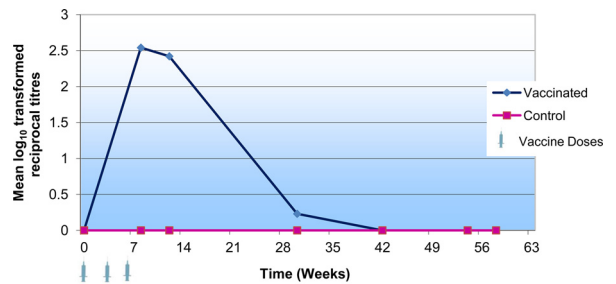
Fig. 3. Progression in log-transformed anti-*L. infantum* IgG titres during one year after primary vaccination. The data presented here are the means of the log-transformed reciprocal titres in both vaccinated ($n = 10$) and control ($n = 10$) dogs. The titre is taken to be the last dilution where at least 50% of the parasites display visible fluorescence. The sera were tested using commercially available IFAT kit. Dogs with a negative result were regarded as having a result of zero after log conversion of the reciprocal titres to allow this to be represented on the charts.

the groups over the course of the study in a linear mixed effect model ($p = 0.0025$). The p-values of the comparison between groups at each time point are displayed in Fig. 4 where these are <0.05.

4.4.2. ELISpot

The PBMCs of all animals in both vaccinated and control groups were able to respond effectively to the non-specific positive control stimulation with ConA (no difference between groups, data not shown, $p = 0.235$). A comparison of the result after SLA stimulation at each time point is shown in Fig. 5. There was a significant difference over time as assessed by a linear mixed effects model ($p = 0.0214$). The p-values of the comparison between groups at each time point are displayed in Fig. 5 where these are <0.05.

4.4.3. CMLA

No significant leishmanicidal activity was detected for any dog at week 0, and there was no statistical difference between groups for any of the three parameters tested in this assay at week 0.
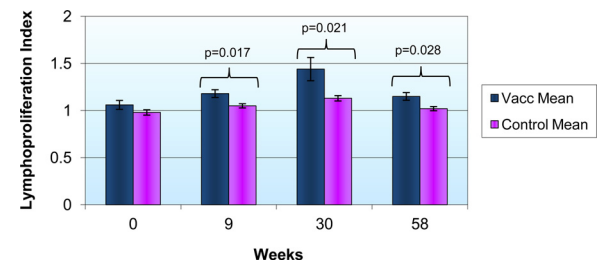

Fig. 4. Lymphoproliferation index before and for 1 year after the completion of the primary vaccination course. This assay detects the ability of the specific T cells produced as a result of vaccination to proliferate after being exposed to Soluble *Leishmania* Antigens (SLA). The lymphoproliferation index is the ratio of the mean optical density obtained for the SLA stimulated samples compared to the mean optical density obtained for the non-stimulated samples using a BrdU specific ELISA system. There were 10 dogs per group. There was a significant difference between the groups over the course of the study in a linear mixed effect model ($p = 0.0025$). The p-values for the difference between groups at specific time points as assessed using a Wilcoxon two-sample test are noted where these are <0.05.
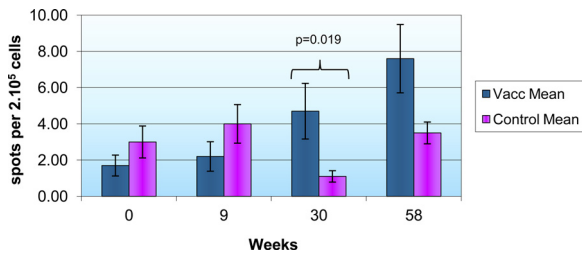
Fig. 5. ELISpot detection of IFN-γ secreting lymphocytes before and for 1 year after the completion of the primary vaccination course. This assay detects the ability of lymphocytes to secrete IFN-γ after specific stimulation with Soluble *Leishmania* Antigens (SLA) by detecting spots (which represent a clone of cells secreting IFN-γ) using specific biotinylated antibodies and an automated ELISpot reader. The data presented here are the number of spots per 2×10^5 cells after stimulation with SLA minus the equivalent value obtained with the negative control using medium alone. There were 10 dogs per group. There was a significant difference over time as assessed by a linear mixed effects model ($p = 0.0214$). The p-values for the difference between groups at specific time points as assessed using a Wilcoxon two-sample test are noted where these are <0.05.

In the control group the results for all three parameters rose slightly by week 9, but no dog reached the threshold in any parameter throughout the study.

In the vaccinated group, a significant rise was seen in all three parameters by week 9, and all dogs remained above the threshold for all three parameters for the remainder of the study. There was a highly significant difference between the groups over time ($p < 0.0001$) for all three parameters. Between week 9 and the end of the study the results for the vaccinated dogs remained significantly higher than those for the control dogs in all three parameters at every time point (see Fig. 6a–c, where * signifies $p = 0.0002$).

5. Discussion

The results of this study were broadly in line with those of a previous study published by our group, in which we assessed the same parameters during the initial vaccination phase up until 3 weeks after the third vaccination (Moreno et al., 2012).

Once again it has been confirmed that the antibody profile induced by the vaccine is predominantly IgG2. However, the current study demonstrates that the IgG2 titres are not only higher but also more persistent than the IgG1 titres. Although a correlation between antibodies of the IgG1 subtype and a Th2 response, and antibodies of the IgG2 subtype and a Th1 response has been demonstrated in mice (Heinzel et al., 1989) the IgG1/IgG2 ratio has not been correlated with protection or resistance in dogs (Day, 2007a). Nevertheless, a previous study using a prototype LiESP vaccine formulated with MDP adjuvant found that the IgG2 induced by vaccination was functionally active in that it was leishmanicidal to both amastigotes and promastigotes, and could also reduce the infectivity of pre-treated amastigotes to canine macrophages *in vitro* (Bourdoiseau et al., 2009). Even if the main determinant of resistance to canine leishmaniasis is an effective Th1-dominated cell-mediated response, IgG2, which is effective in opsonisation and complement fixation, may still be
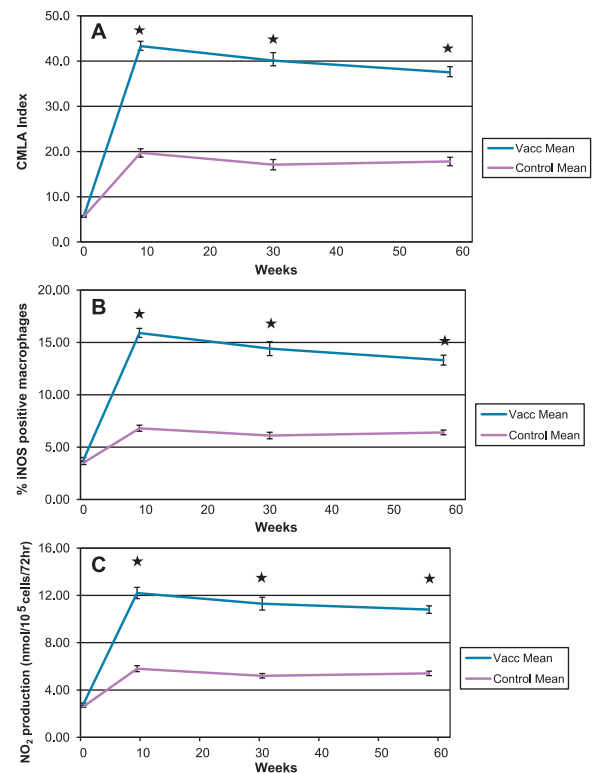

Fig. 6. CMLA assay: inhibition of the macrophage parasitic index, iNOS activity and production of NO derivatives.

Error bars denote the standard error of the means. Data are presented for week 0 (immediately before starting the primary vaccination course), week 9 (three weeks after the completion of the primary vaccination course), and then weeks 30 and 58 (which is one year after completion of the primary vaccination course). There were 10 dogs per group.

Panel A is a comparison of the ability of the dogs' macrophages to inhibit parasite multiplication after interaction with autologous lymphocytes. The CMLA index is obtained by comparing the number of intact parasites in artificially infected monocyte-derived macrophages when they are co-cultured with autologous lymphocytes for three days (beginning one day after the infection of the macrophages) in contrast to those with medium alone. This panel demonstrates the increase in the leishmanicidal capacity of the dogs' macrophages as a result of vaccination.

Panel B is a comparison of the rate of expression of iNOS in the dogs' macrophages after 3 days of exposure to autologous lymphocytes. The expression of iNOS is measured by immunolabelling with NOS specific antibodies.

Panel C is a comparison of the rate of production of NO derivatives from the dogs' macrophages during co-culture with autologous lymphocytes, and is expressed in nmol NO_2 per 10^5 cells per 72 h. NO_2 is determined in the culture supernatants using the modified Griess technique, providing a correlation with the production of the short-lived NO radical.

When these three measurements are consistent, this provides evidence of an increased NO-mediated pathway of parasite killing as a result of vaccination. There was a highly significant difference between the groups over time ($p < 0.0001$) for all three parameters. Between week 9 and the end of the study the results for the vaccinated dogs remained significantly higher than those for the control dogs in all three parameters at every time point (* signifies $p = 0.0002$).

able to play a complementary role by targeting individual amastigotes released from infected macrophages (Day, 2007a).

The fact that the IFAT results were positive, and in some cases at more than 4 times the laboratory threshold for a short time after vaccination, confirms that caution

is required in the interpretation of such results in dogs vaccinated under field conditions. Until the advent of vaccination in Europe, standard guidelines suggested that IFAT titres of at least 4 times the laboratory cut-off level were indicative of the disease, and that levels between the threshold and 4 times the threshold raised a suspicion of the disease (Solano-Gallego et al., 2009). IFAT is a non-specific test that detects IgG antibody fixation to whole parasites. As some of the component proteins of the LiESP are found in both surface and excreted forms, it was not unexpected to obtain such a result.

In light of the clear consensus that a Th1-dominated profile within a mixed Th1/Th2 response is the most desirable outcome for protection in the dog (Baneth et al., 2008), the most important data in this paper are those relating to cellular immunity. In this study, we chose to assess some key markers of the appropriate Th1 response. The choice of markers studied was based on the knowledge available at the time, and also on the availability of validated assays as required in a study conducted with a view to vaccine registration. Consequently, not all possible markers could be assessed. For example, it would have been interesting to look at the IL-10/IFN-γ ratio as IL-10 is a key marker of the Th2 profile, and some studies have suggested that in the presence of very high levels of IL-10, even significant IFN-γ production may not be effective (Alexander and Bryson, 2005). The lack of a validated assay at the time of the study precluded this analysis. Likewise, it appears that IFN-γ from stimulated PBMCs is insufficient alone, and other co-factors such as TNF-α are also required for an effective response in dogs, mice and human beings (Nacy et al., 1991; Vouldoukis et al., 1995; Carrillo and Moreno, 2009). However, the fact that we also included the CMLA assays in this study adequately compensates for any apparent lack. In the CMLA assay, the functional interaction between the T lymphocytes and the macrophages is assessed. Therefore, if there were a dominant inhibitory IL-10 effect blocking the ability of IFN-γ to stimulate the macrophages' leishmanicidal activity, or an inability to produce the TNF-α cofactor, this effect would have been seen in the CMLA results. Indeed it has been previously demonstrated that IL-10 inhibition of *Leishmania* killing correlates with a decrease in NO generation and down-regulated leishmanicidal activity in macrophages (Vouldoukis et al., 1997). The positive results obtained in our CMLA assay therefore clearly confirm that the dominant effect of macrophage activators such as IFN-γ is not abolished by a lack of TNF-α or an excess of IL-10, and confirm that the overall response is indeed Th1-dominated.

In the CMLA assays it is not simply the final reduction of the macrophage parasite load that is demonstrated, but also a correlation between this and the currently understood mode of action of an effective immune response – a leishmanicidal oxidative burst due to Th1 cytokine-mediated induction of iNOS leading to production of NO (Vouldoukis et al., 1996; Noël et al., 2004; Holzmuller et al., 2005; Perez et al., 2006; Wanasen and Soong, 2008). This greatly reinforces confidence in the results relating to the leishmanicidal effects. While it would also have been interesting to assess the presence of alternatively activated macrophages, permissive to the parasite development (Vouldoukis et al., 1997), it was not possible in this

study, and in any case the CMLA results reflect the global picture created by the ensemble of all the macrophage types present.

In a recent review (Reis et al., 2010) the authors concluded that, amongst others, *Leishmania*-specific IgG isotype levels, IFN-γ expression, the proliferative response to leishmanial antigen and the leishmanicidal activity in macrophages were valuable, reliable and measurable biomarkers when determining the immunogenicity of vaccines against canine leishmaniasis. However, despite also considering IL-10 in their review, there did not appear to be sufficient evidence in dogs for them to propose this marker for canine studies in their conclusions. The choice of parameters in this study therefore remains in line with the current literature.

As noted in our previous study, which assessed the onset of immunity (Moreno et al., 2012), there was a slight but non-significant rise in the CMLA responses in the control group in this study. In the previous work, we hypothesised that this may simply be due to the age of the dogs and the natural maturation of the cell-mediated arm of the adaptive immune response, which is slower than that of the humoral response (Day, 2007b). The fact that in the current study the initial rise, which was noted during the period of maturation of the dogs, levelled off subsequently and did not change further lends additional support to this hypothesis. Regardless of this slight rise in the control group, the differences between groups were marked and highly significant throughout the full period of the study after the onset of immunity in the vaccinated group.

It is accepted that no single marker is sufficient to be a direct correlate of protection or susceptibility to canine leishmaniasis as the effective immune response operates as a complex network of regulatory and counter-regulatory interactions involving multiple cytokines and cell types (Day, 2007a; Resende et al., 2013). However, it is possible to consider multiple parameters together and to obtain a general view of the overall direction of the immune response. The concept of a dynamic spectrum in the immune responses of dogs, where the overall balance of the various factors present in the mixed Th1/Th2 response will determine the final outcome was recently proposed (Reis et al., 2010). Accepting this model, the current study clearly demonstrated the ability of vaccination with LiESP/QA-21 to induce an appropriate immune response and to maintain this for a full year. While no specific assessment was made using cell-surface markers for the presence of *Leishmania*-specific memory T cells, it is clear that an effective response obtained one year after vaccination in animals that had never been exposed to the infectious agent will be due to the presence of such cells.

Despite the obvious limitations of an *in vitro* analysis of immune profile parameters in the face of the complexity of the immune response induced *in vivo*, the results obtained in this study strongly support the expectation of an effective response *in vivo*, and the ability of vaccinated dogs to control their parasite burdens when exposed to virulent parasites. It would be necessary to confirm the effectiveness of this immune profile using a virulent challenge to allow any conclusion to be drawn regarding its correlation with clinical protection. In addition, it must be

recognised that this study was performed in a relatively small, homogenous group of beagles, and so it is possible that in a wider and more varied population the range of responses could be slightly different. Nevertheless, for a study such as this it is important to have groups which are as similar and standardised as possible to eliminate the interference of variables other than the effects of the vaccine on the canine immune response.

6. Conclusion

The results presented here confirm that vaccination with LiESP/QA-21 is capable of stimulating an appropriate Th1-dominated cell-mediated immune response that is still present 1 year after the last injection of a primary vaccine course. This supports an expectation of *in vivo* efficacy that must be assessed by virulent challenge in vaccinated dogs.

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Conflict of interest

Several of the authors are employees of Virbac and played a direct role in the study design, the data collection, analysis and interpretation, the decision to publish and the preparation of the manuscript.

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