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Original Article

Evaluation of Cellular Immune Responses in Dogs Immunized with Alum-Precipitated Autoclaved *Leishmania major* along with BCG and Imiquimod

Mohammad Barati [1], Mehdi Mohebali [2], Ali Khamesipour [3], Fariborz Bahrami [1], Haiedeh Darabi [1], Vahid Khaze [1], Farhad Riazi-Rad [1], Gholamreza Habibi [4], Soheila Ajdary [1], *Mohammad Hossein Alimohammadian [1]

1. *Department of Immunology, Pasteur Institute of Iran, Tehran, Iran*
2. *Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran*
3. *Center for Research and Training in Skin Diseases and Leprosy, Tehran University of Medical Sciences, Tehran, Iran*
4. *Razi Vaccine & Serum Research Institute, Karaj, Iran*

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***Correspondence**
Email:
mhalimoham@gmail.com

Abstract

Background: We aimed to investigate the potential effects of BCG and imiquimod on improvement of current experimental *L. major* vaccine against dogs in an endemic area of Zoonotic visceral leishmaniasis (ZVL) in Iran.

Methods: During 2012 till 2014, seven mixed-breed shepherd dogs with no anti-*Leishmania* antibodies and no response to Leishmanin reagent were immunized with 2 doses of alum-precipitated autoclaved *L. major* (Alum-AML) while BCG and imiquimod (for skin pre-treatment) were used as adjuvants. The productions of a few characteristic cytokines of T-helper immune responses and the development of delayed-type hypersensitivity (DTH) of the immunized animals were then evaluated, up to 300 days. Blood samples were collected at 0, 30, 80 and 300 d post-vaccination and the concentrations of IFN-γ, IL10, IL-12 and TGF-β cytokines secreted from PBMCs at these time-points were quantified by ELISA. DTH was evaluated by Leishmanin skin test (LST).

Results: Although a similar LST conversion was observed at all time-points, the cytokine measurement results indicated significantly higher levels of IFN-γ at day 80 and elevated levels of IL-10 at days 80 and 300, post-vaccination. Moreover, a significantly higher IFN-γ/IL-10 ratio was observed at day 30 post-vaccination compared to the other time-points.

Conclusion: Although a Th1-like response could be observed at day 30 post-vaccination, the development of cytokine profiles was inclined toward mixed Th1 and Th2 responses at days 80 and 300 post-vaccination. This situation may indicate the requirement of an additional boosting by this Alum-AML formula, in order to induce long-lasting protection against ZVL.

Introduction

Zoonotic visceral leishmaniasis (ZVL), is caused by an intracellular protozoan which is endemic mainly in Indian subcontinent, north-east Africa, South America, the Mediterranean basin and the Middle-East, including Iran (1, 2). The disease is caused by *Leishmania* (*L.*) *infantum* in the Old World and *L. chagasi* in the New World through biting of different species of infected sandflies. *Leishmania* parasites survive and proliferate solely in macrophage lineages of the mammalian hosts and cause a wide range of clinical manifestations in humans, including the cutaneous and the visceral (also known as kala-azar) forms of the disease while the latter is fatal if left untreated (3-5). The global mortality rate of VL is estimated to be 20,000 to 40,000 deaths, annually (1, 6).

Dogs are the main reservoir hosts of *L. infantum* in ZVL and play an important role in transmission and spreading of the infection to humans (7). Four known endemic regions of *L. infantum* infection in Iran with respect to canine visceral leishmaniasis (CVL) are the following. The first region is located in rural areas of Meshkin-Shahr City of Ardabil Province in the north-west of Iran (7). The second area includes Ahar and Kalibar cities in East Azarbaijan Province (north-west of Iran) (8). The third region encompasses Kazeroun, Nourabad, Firouzabad and Darab cities, in Fars Province (9) while the fourth zone is in Boyer-Ahmad district, in Kohgiluyeh and Boyer-Ahmad Province (10). Although the clinical signs of CVL are variable, a considerable percentage of the dogs in the endemic areas are exposed to *L. infantum* (11).

Resistance to CVL is associated with induction of parasite-specific cytotoxic T cells and secretion of IFN-γ, IL-2 and TNF-α by PBMCs which lead to activation of the macrophages and killing of the intracellular parasites (12). The level of IFN-γ and TNF-α mRNA is higher in the lymph nodes (LNs) of the asymptomatic dogs compared to the symptomatic ones naturally infected with *L. chagasi* as well as the non-infected dogs. Moreover, the asymptomatic dogs with higher levels of these cytokines are suggested to have the ability to control the parasite proliferation and burden in their LNs while in the symptomatic dogs, higher levels of tissue parasitism are shown to be present. Such profiles indicate that these cytokines play a role in protection against the infection. IL-12 is a main cytokine of Th1 response and is considered necessary for control of the parasite inside the host (13, 14). Meanwhile, the expressions of IL-10 and TGF-β along with higher loads of the parasite have been reported in the symptomatic dogs, indicating their role in the exacerbation of the disease (15). Taken together, the clinical manifestation of naturally-infected dogs is believed to depend upon a balance between the expression of protective cytokines such as IFN-γ and the expression of disease-promoting cytokines such as IL-10 and TGF-β in the dog's LNs (15). Various lines of evidence have shown that imiquimod is a strong immune-modifying agent. Imiquimod enhances both Th1 and Th2 responses via activation of Toll-like receptors (TLR) on dendritic cells. Upon binding of imiquimod to TLR7 and TLR8 in humans and TLR7 in mice, the dendritic cells are activated and produce cytokines such as interferon, TNF-α and IL-12 (16-18).

In the present study, dogs with no response to *Leishmania* antigens were selected and injected twice with alum-precipitated autoclaved *L. major* (Alum-ALM) in a main ZVL endemic area in north-west of Iran. During the trial, blood samples were collected over 300 days and productions of certain indicative cytokines as well as the development of delayed-type hypersensitivity (DTH) was evaluated.

Materials and Methods

Ethical considerations

All procedures on the animals including blood sample collection, vaccination and skin testing were carried out according to Ethics Committee of Islamic Republic of Iran's Ministry of Health and Medical Education (2013 directives). The approval for the experiments was confirmed by Ethical Committee of National Institute of Health Research (Ethical Committee of Tehran University of Medical Sciences; No. 240/M/559) in accordance with Helsinki Declaration and guidelines. The animals were physically examined and healthy dogs were recruited with their owners' written consents.

Study location, animals selection and vaccination procedure

This study was carried out in villages located in vicinity of Meshkin-Shahr City in Ardabil Province (north-west of Iran, approximately 680 km west of Tehran) in 2012 till 2014. Estimated annual incidence for human VL in this area has been evaluated as 300-600 (1) and incidence rate of canine VL has been estimated as 18% (19). This region is a well-known ZVL endemic area where ownership mixed breed shepherd dogs are the main reservoirs of *L. infantum*.

Blood samples were collected from each dog and examined for anti-*Leishmania* antibodies using direct agglutination test (DAT). Leishmanin skin test (LST; using Leishmanin produced under GMP guidelines at Pasteur Institute of Iran) was performed on dogs with negative DAT and then dogs with no LST reaction were selected for the vaccination. Dogs were injected intradermally (i.d.) in the left hind leg with Alum-ALM experimental vaccine, containing 200 µg of *L. major* protein absorbed in 1,400 µg alum, produced under GMP guidelines at Razi Vaccine and Serum Research Institute (Hesarak, Iran), mixed with Bacillus Calmette–Guérin (BCG) vaccine (~ 2×10^6 CFU), immediately before use. Following application of a 125-mg load of imiquimod cream (5%, Aldara, 3M, Canada) on a shaved area of the hind-leg and robbing it on a surface of 1-2 cm^2 for 5 min, the prepared vaccine was immediately injected. Three weeks later, both groups received a booster injection as above.

Isolation and culture of peripheral blood mononuclear cells (PBMCs)

Seven eligible dogs were chosen for the cytokine assays. At 4 different time points (before vaccination (i.e. day 0) and at days 30, 80 and 300 post-vaccination), blood samples were collected from the dogs and PBMCs were isolated by density-gradient centrifugation, using Histopaque 1077 (Sigma, Germany). Viable cells (2×10^6) were then cultured using 24-well microplates containing RPMI, supplemented with 2 mM L-glutamine, 10% heat-inactivated FBS, 100 U/ml penicillin and 100 µg/ml streptomycin in a final volume of 1 ml per well. The cells were re-stimulated with 30 µl of freeze-thawed *L. infantum* (1.5×10^8/ml) or equal volumes of phytohemagglutinin P (PHA, 50 mg/ml, Difco Laboratories, Detroit, MI, USA) as a mitogen (i.e. the positive control) or the medium (i.e. the negative control). The supernatants of the cultures were collected after 72 h and stored at -80 ºC until use.

Cytokine assessment

The concentrations of canine IFN-γ, IL10, IL-12 (p40 subunit) and TGF-β were quantified by sandwich ELISA technique using commercial kits (R&D Systems, MN, USA), according to the manufacturer's instructions. The experiments were done in duplicate.

DTH measurement

The TDR/WHO reference Leishmanin, produced from *L. major* (MRHO/IR/75/ER) under guidelines of GMP at Pasteur Institute of Iran (20), was used to evaluate DTH by skin testing. Briefly, 0.1 ml of Leishmanin (6 ×

10^6/ml, 55 µg/ml) was injected intradermally into the inner surface of the skin in forearm of the animal. After 48-72 h, the diameter of indurations was measured using a millimeter graduated ruler. The mean of the two diameters at 90° angle was used to define each reaction, based on procedure (21). An average induration ≥ 5 mm in diameter was considered as a positive reaction.

Statistics

All the data analyses were performed using GraphPad Prism ver. 6.01 for Windows (GraphPad Software Inc., La Jolla, CA, USA). One-way ANOVA followed by Tukey's multiple comparisons test was used to compare the differences between the groups. $P \leq 0.05$ was considered as significant.

Results

Cytokine responses

The concentrations of 4 main cytokines of T-helper immune responses, namely IFN-γ, IL-10, IL-12 and TGF-β were measured in the supernatant of canine PBMCs cultures.

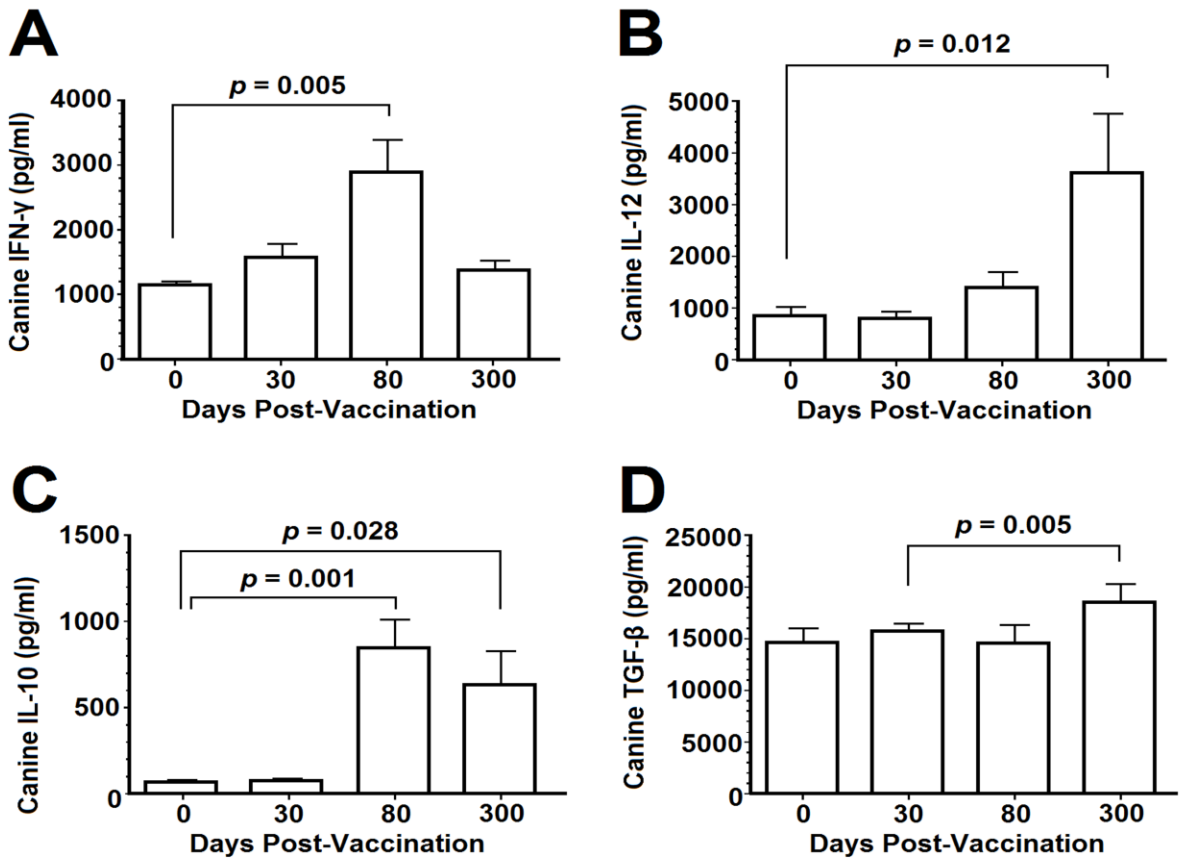

Fig. 1: Canine cytokines released from the PBMCs. This figure indicates the concentrations of the cytokines released from the collected PBMCs, cultured for 72 h in presence of freeze-thawed *L. infantum*, quantified by ELISA at each time-point. (A) IFN-γ concentrations secreted by the cell cultures. (B) IL-12 concentrations secreted by the cell cultures. (C) IL-10 concentrations secreted by the cell cultures. (D) TGF-β concentrations secreted by the cell cultures. Means with SEM are shown (n=7)

As shown in Fig. 1A, the level of secreted IFN-γ increased after day 30 post-vaccination (mean: 1,566.7±214.6 pg/ml), followed by a significant increase (*P*=0.005) at day 80 (mean: 2,888.6 ±519.3 pg/ml), compared to day 0 and then decreased at day 300 post-vaccination (1,408.3±123.7 pg/ml).

IL-12 production increased after day 30 (mean: 807.5 ± 122.6 pg/ml) to 1,405.4±297.9 and 3,622.4±1124 pg/ml at days 80 and 300 post-vaccination with a significant difference (*P*=0.012) between the levels of IL-12 at day 300 and day 0, as shown in Fig. 1B. Similarly, the levels of IL-10 production induced in PBMCs culture increased after day 30 to significantly higher (*P*=0.001) quantities at days 80 and remained significantly high (*P*=0.028) at day 300 post-vaccination (mean: 844.0 ± 164.8 and 637.5±194.9 pg/ml, respectively), compared to day 0. The levels of TGF-β produced by canine PBMCs culture were comparatively similar at days 0, 30 and 80 post-vaccination (Fig. 1D). However, the production of TGF-β was significantly higher (*P*=0.005) at day 300, compared to the first-month post-vaccination.

Meanwhile, when the ratios of secreted IFN-γ/IL-10 for the last three time-points were compared, with the progress of time, the ratio decreased significantly. As shown in Fig. 2, the IFN-γ/IL-10 ratio was the highest at day 30 (23.2 ± 2.38), compared to day 80 (7.99 ± 2.22) and day 300 (3.52 ± 0.595) post-vaccination (*P*=0.004, *P*≤0.0001, respectively).

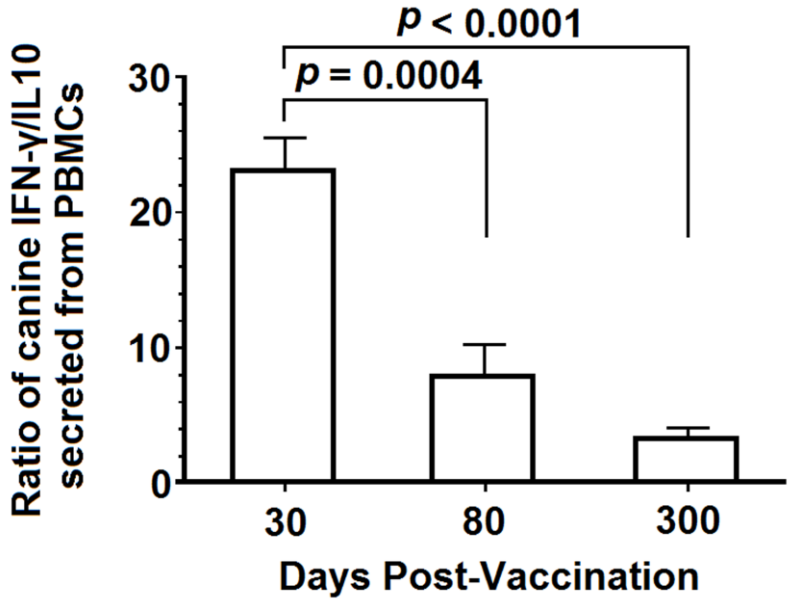

Fig. 2: The ratios of the secreted IFN-γ over IL-10 from PBMCs collected at indicated time-points and cultured for 72 h in presence of freeze-thawed *L. infantum*, quantified by ELISA. Means with SEM are shown (n=7)

The prevalence of LST positivity

LST-positive responses were detected in 66.7% of the vaccinated dogs at days 30 and 80 post-vaccination and 60% of the dogs at day 300 post-vaccination. Moreover, similar magnitudes (mean diameters of the indurations) of LST responses in the responder dogs were detectable at days 30, 80 and 300 post-vaccination, without any significant differences among them (Fig. 3).

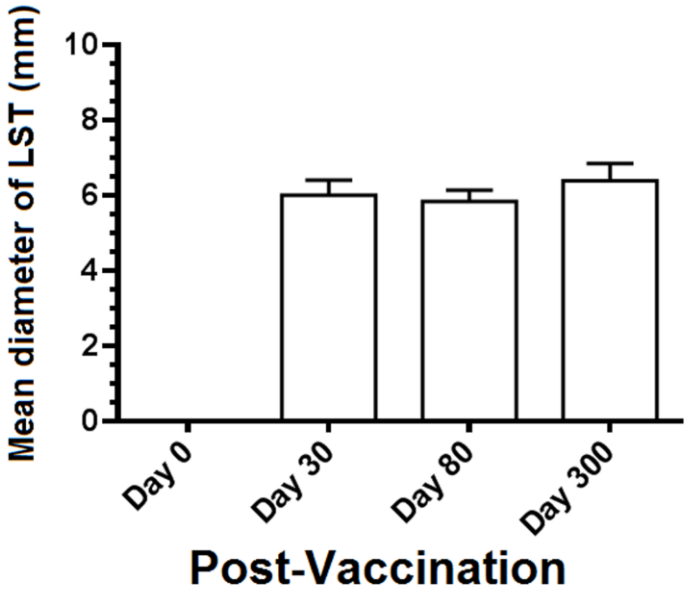

Fig. 3: DTH prevalence over 300 days. The figure shows mean diameter of indurations following LST at indicated time points. Means with SEM are shown (n = 7)

Discussion

So far, attempts to develop a vaccine against canine leishmaniases have not been fully successful and have resulted in different immunological outcomes. Using LBSap vaccine (composed of *L. braziliensis* promastigote proteins plus saponin as an adjuvant), prominent Th1 responses have been reported with high production of IFN-γ and IL-12 and lower levels of TGF-β (22). The administration of LiESP/QA-21 vaccine, composed of a purified-secretion of *L. infantum* (LiESP) plus QA-21 adjuvant (authorized in the European Union under the trade name of CaniLeish), has resulted in induction of *Leishmania*-specific T cells with a dominant Th1 response, as manifested by high levels of IFN-γ production upon stimulation with soluble *Leishmania* antigen (23).

Two different vaccines namely Leishvaccine (consisted of a strain of *L. amazonensis* antigenic preparation, using BCG as an adjuvant) and Leishmune (consisted of purified *L. donovani* fucose mannose ligand, FML, using saponin as an adjuvant) have been evaluated recently. While the injection of Leishvaccine has been reported to induce a mixed cytokines to profile like higher levels of both IFN-γ and IL-4, the inoculation of Leishmune has led to the induction of a dominant Th1 type response and increased levels of IFN-γ and NO (24). Recently, the induction of partial protection in form of production of higher levels of IgG2, IFN-γ and TNF-α, but low levels of IL-10, has been reported in outbreed dogs immunized with a live vaccine based on recombinant *L. tarentolae*, expressing *L. donovani*A2 antigen along with cysteine proteinase genes (25). With respect to promising adjuvants in this line of research, imiquimod functions as a potential adjuvant for vaccines when applied topically (16). The application of imiquimod as an adjuvant has also been reported in an investigation using BALB/c mice in which autoclaved *L. major* has been used as a vaccine. The results of this study have suggested that the topical use of imiquimod cream enhances Th1 responses in a susceptible mouse model (26). Moreover, results obtained by nano-adjuvents such as polymethyl methacrylate

(PMMA) nanoparticles have recently been shown to be effective to promote cellular responses and reduced parasite burden in *L. major*-infected BALB/c mice (27).

The secretion of cytokines by the hosts during leishmaniases can play a pivotal role in the inflicted pathology. The development of Th1 response accompanies with cure and protection against re-infection whereas the induction of Th2 response leads to the exacerbation of the disease (28). In this regard, *L. major* crude antigens have been used in past ZVL studies under similar situations and this species has also been used as a vaccine in *L. infantum* studies (29, 30). The results of the current study generated during 10 months after the vaccination indicated that the level of IFN-γ production gradually increased after day 30 till day 80 post-vaccination when it was significantly higher than day 0. However, the secretion of IFN-γ declined afterward to its early levels at day 300. Even though after a month following the vaccination the level of IL-12 production had not changed, it gradually increased to its highest levels at day 300 post-vaccination which was almost 4 times higher than day 0. Since both IFN-γ and IL-12 are the main cytokines of Th1 response, the vaccination with 2 doses of Alum-ALM mixed with BCG and application of imiquimod cream prior to the injection, tend to shift the dogs' immune system toward a Th1 response.

Higher levels of intracellular IFN-γ in dogs immunized with Leishimmune and Leishvaccine have been reported (24). However, instead of 3 doses of Leishimmune or Leishvaccine, here we only used two doses of a first-generation vaccine. Our results also showed consistency with an earlier report in which LiesP/QA-21 was injected into conventional Beagle dogs and Th1 response was elicited (23).

The lower production of IFN-γ at day 300, along with significantly higher amounts of IL-10 production at days 80 and 300, as well as increased level of TGF-β at day 300 post-vaccination altogether point toward a Th2 response at the later time-points after the vaccination. The reason for the lower level of IFN-γ at day 300 may be attributed to the significantly increased production of IL-10 at the period between days 80 and 300 and increased levels of TGF-β around the end of this study period. At the same time, the ratio of IFN-γ/IL-10 was significantly decreased over this period in a stepwise manner. Moreover, LST conversion from negative to positive skin test was observed in the vaccinated dogs which indicate the development of cell-mediated immune responses against *Leishmania* antigens in majority of the vaccinated dogs. This was also in agreement with another report in which a whole parasite preparation was used as a vaccine in Beagle dogs and the results had shown strong cell proliferation after the first and the second injections (31).

Although there are several studies on canine cytokine profiles, to our knowledge there is no report on cytokine profiles of the vaccinated dogs in a ZVL endemic area. Our results showed that administration of two doses of Alum-ALM mixed with BCG at animal skin pretreated with imiquimod as adjuvants could induce a Th1 immune response at first month after the vaccination gradually shifted to a mixed Th1 and Th2 responses after day 80, up to 10 months post-vaccination. The tendency of the vaccinated dogs toward a Th2 response over the long time may be attributed to the requirement of a second booster injection (*i.e.* a third dose), in order to extend the protection period against *L. infantum*.

Conclusion

The results obtained here showed the development of a Th1-like response at day 30 post-vaccination. However, in animals immunized at days 80 and 300 post-vaccination, the development of the cytokine profiles inclined toward mixed Th1 and Th2 profiles. Hence, boosting the target animals with another Al-

um-ALM vaccine and adjuvants may enhance the long-term protection.

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Conflict of interest

The authors declare that there is no conflict of interest.

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