

Cytokine Profile of *Leishmania Infantum* Fucose-Mannose Ligand in Vaccinated Dogs in the Northwest of Iran

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ABSTRACT

Background: Canine visceral leishmaniasis (CVL) caused by *Leishmania infantum* is endemic in the northwest and south of Iran. An appropriate vaccine can help to prevent and control visceral leishmaniasis in both humans and animals. Few studies have confirmed that the fucose-mannose ligand (FML) antigen of *Leishmania donovani* produced protective immunity in dogs against CVL. **Objective:** To evaluate the immune responses of vaccinated dogs against FML antigen of *L. infantum*. **Methods:** We isolated the FML antigen from native *L. infantum* and vaccinated the dogs with FML-saponin in an endemic area of VL in Iran to evaluate the immune responses of vaccinated dogs against this antigen. **Results:** Our results indicated a significant increase in the expression of IFN- γ , IL-10 and IL-13, but not IL-12A, gene transcripts in PBMCs of FML-saponin vaccinated dogs in comparison with controls. Our findings showed a significant difference in the ratio of IFN- γ /IL-10 mRNA expression in FML-saponin vaccinated dogs in comparison with two control groups. Moreover, a significant level of anti-FML antibodies was detected in serum of vaccinated dogs. **Conclusion:** These findings showed that FML-saponin stimulates both Th1 and Th2 immune responses with predominant Th1 and strong humoral immune responses to produce protective immunity against CVL.

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Keywords: Canine Leishmaniasis, Fucose Mannose Ligand, *Leishmania Infantum*, Vaccination

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INTRODUCTION

Canine visceral leishmaniasis (CVL) is endemic in the northwest and south of Iran. *Leishmania infantum* is the causative agent of CVL in Iran and the Mediterranean Basin (1). Sero-epidemiological surveys reported the prevalence of approximately 14.2% for CVL in endemic areas of Iran, however, higher prevalence was reported in molecular studies (2,3). Infected dogs are the main reservoirs of parasite, and these animals play an important role in persistence of infection in the environment and transmission of parasite to humans. Elimination of seropositive infected dogs being recommended as a preventive measure in some situations by the WHO, is usually invasive and is not acceptable for owners of dogs, especially when the dogs are asymptomatic (4). Thus, a suitable vaccine against CVL can help to prevent and control the disease in both humans and animals.

A vast variety of antigens have been used for vaccination of dogs against CVL. Among them, fucose-mannose ligand (FML) antigen, isolated from *Leishmania donovani* promastigotes, in association with saponin, as an adjuvant, is one of the most appropriate vaccine candidates producing protective immunity in dogs against CVL. FML was the first vaccine (Leishmune®) to be licensed against CVL in Brazil (5). Leishmune® has been reported to be safe (6), effective (7) and a transmission blocking vaccine against CVL (8).

Recent studies elucidated that Leishmune® increased the level of IFN γ , IL-4, IgG2 and number of CD8⁺ T cells, while it reduced the number of CD4⁺/CD25⁺ T cells in vaccinated dogs (5,9-11). Thus far, the FML antigen used for vaccination of dogs is *L. donovani* origin, however, few studies on *L. chagasi* are available. Here, we isolated the FML antigen from native *L. infantum* and vaccinated the dogs in an endemic area of CVL in Iran to evaluate the immune responses of vaccinated dogs against the FML antigen.

MATERIALS AND METHODS

Study Area and Animal Selection. The study was conducted in a CVL endemic area, Agh-Bulagh Village, in the south of Meshkin-Shahr city in the northwest of Iran. Meshkin-Shahr is the center of Meshkin-shahr County, in Ardabil Province, north-west of Iran where CVL and human visceral leishmaniasis (HVL) are endemic (1). Being in Alborz Mountain Range and near the high Sabalan Mountain, it enjoys a moderate mountainous climate. Sixty ownership dogs (≤ 2 years old) were selected from the village. All animal works were carried out in strict compliance with the approval of the Institutional Animal Ethics Committee of the Shiraz University of Medical Sciences, following the NIH guidelines for care and use of experimental animals. Consent forms were completed by the owners, and peripheral blood samples were collected with and without EDTA to obtain buffy coat and sera for PCR and DAT, respectively. From 60 dogs, 40 dogs, which were negative for *Leishmania*, by both PCR and DAT, were selected for the study.

Preparation of the FML Antigen. FML antigen of native *L. infantum* was prepared as originally described by Palatnik *et al.* (12). Briefly, native *L. infantum* (MCAN/IR/07/Moheb-gh) was mass cultivated in BHI medium. The promastigotes, in stationary phase, were harvested (washed by saline), and the pellet was mixed with cold

distilled water, and centrifuged twice, and the supernatant was collected. Supernatant was boiled for 15 min in 100°C, and centrifuged, and the supernatant was lyophilized for size exclusion chromatography by Bio Gel P-10 Gel (Biorad, U.S.A.). FML antigen was collected in void volume, and lyophilized and stored at -20°C until use. Antigenic fractions were detected by SDS-page and western blotting.

Study Design and Vaccination of Dogs. The study was conducted in November 2010 and in a cold season when the sand flies are not active as vectors. The selected 40 seronegative and PCR negative dogs were divided into three groups. The first 20 dogs, as the test group, received FML+ saponin; the second group, 10 dogs as the control group, received saline injection, and the third group, 10 dogs as the second control group, received saponin injection alone. The animals in all three groups received three subcutaneous injections, every three weeks, on days 0, 21 and 42, respectively. Groin in the right leg was selected as injection site. The test group received 1 mL of saline containing 1.5 mg of FML and 0.5 mg of saponin in every injection, whereas the saline control group received 1 mL of saline alone, and the third group received 1 mL of saline containing 0.5 mg of saponin.

Sample Collection from the Vaccinated Dogs. Ten days after the last injection, totally 17 mL of peripheral blood sample was taken from each dog. Seven mL in heparin contained vacutainer tubes for culturing peripheral blood mononuclear cells (PBMCs). Serum samples were collected from 5 mL of clotted peripheral blood of dogs to detect anti-FML antibodies by FML-ELISA. To detect possible adventitious infection in vaccinated dogs, the remained 5 mL of peripheral blood samples was collected in EDTA for PCR assay.

kDNA Amplification by Nested PCR to Identify *Leishmania* Infected Dogs. A nested PCR was used, as described by Noyes *et al.* (13) using external primers CSB2XF (C/GA/GTA/GCAGAAAC/TCCCGTTCA) and CSB1XR (ATTTTTCG/CGA/TTTT/CGCAGAACG), and internal primers 13Z (ACTGGGGGTTGGTGTAATAATAG) and LiR (TCGCAGAACGCCCT), to amplify the kDNA of *Leishmania* on buffy coat samples of all 60 selected dogs and on buffy coat of 40 vaccine-inoculated dogs to identify possible adventitious infection in the animals.

Direct Agglutination Test (DAT). DAT was performed to detect anti-*Leishmania* antibodies in the sera of dogs as described by Mohebbi *et al.* (1,3).

Fucose-Mannose Ligand-ELISA (FML-ELISA). Serum samples from all 40 dogs were collected 10 days after the last injection for evaluation of anti-FML antibodies, by FML-ELISA. FML-ELISA was performed as described before (14).

Cultivation of PBMCs from Immunized Dogs. The PBMCs were separated from the blood samples of the 40 dogs by Ficoll (Biosera, Korea) based on the manufacturer's instruction. Cells were cultivated in RPMI media (2×10^6 cells/mL) supplemented with 10% of fetal calf serum (Gibco, U.S.A.) and 1% penicillin-streptomycin 100X (Biosera, Korea) in a humidified incubator at 37°C and 5% CO₂ for 12 hours. A four-well culture plate was used for each dog (2 mL media containing 4×10^6 cells/well). FML (100 µg/mL), concanavalin A (Con A) (4 µg/mL) and FML+ Con A were added to three of the wells, while one well remained untreated. The ConA co-cultures cells were used as a positive control for lymphocyte proliferation and cytokine expression, while FML-ConA co-cultures were used to evaluate possible effect of ConA on cytokines expression. The cells were harvested after 12 hours and washed with phosphate buffered saline (PBS) before RNA extraction.

RNA Extraction and cDNA Synthesis. Total RNA of cultivated PBMC was extracted by TRIzol reagent (Invitrogen, U.S.A.) based on the manufacturer's instruction. Briefly, harvested cells were washed two times with ice cold PBS and resuspend in 100 μ L of PBS. One mL of TRIzol was added to each tube and incubated on ice for further lysing. Then 250 μ L of chloroform was added to each tube and samples were centrifuged at 15000 \times g for 20 min, and RNA in the upper phase was pelleted by absolute isopropanol. The RNA pellet was washed by 75% ethanol and eluted in the DEPC-treated water. To prevent DNA contamination, 5 μ g of total extracted RNA was mixed by 2 units of DNase I (Invitrogen-Gibco, Paisley, U.K.) and incubated at 37°C for 30 min and then at 65°C for 10 min to inactivate DNase I. A kit was used for cDNA synthesis (Fermentas, Lithuania) and cDNA was prepared based on the manufacturer's instruction. Briefly, 5 μ g of total RNA in 10 μ L of DEPC treated water and 1 μ L of oligo dT and 1 μ L of Random Hexamer primer were added into 0.5 ml microtubes and incubated for 5 min at 70°C in Dri-Block (Techne, U.K.). Then 4 μ L of reaction buffer (5 X), 2 μ L of dNTP (10 mM) and 1 unit of RNase inhibitor were added to each microtube. The microtubes were incubated for 5 min at 37°C followed by 5 min at 25°C. Then 1 μ L of reverse transcriptase enzyme (5 units) was added to all microtubes, and samples were incubated at 42°C for 90 min in thermal cycler (Eppendorf, Germany). The samples were incubated at 70°C for 10 min to inactivate the reverse transcriptase enzyme, and the prepared cDNA were stored at -70°C until use.

Quantitative Real-Time PCR to Evaluate Dog Cytokines Profile after Vaccination with FML-Saponin. Quantitative real-time PCR was used to evaluate the expression of IL-10, IL-12A, IL-13, IFN γ in vaccinated along with controls dogs. Beta-actin housekeeping gene was used as an indicator for other target genes expression levels. The primers used to identify *Canis familiaris* IL-10, IL-12A, IL-13, IFN γ and beta-actin gene transcripts were: IL-10 forward, (5-AGGCTGCGACGCTGTCACC-3) and reverse, (5-TGCGCTCTTCACCTGCTCCA-3); IL-12A forward, (5-TCACAAAGGAT AAAACCAGCACAG-3) and reverse, (5-GCACAGGACCGTCATAAAAGAGG-3); IL-13 forward, (5-GCGGCAGGGCAGATTTCC-3) and reverse, (5-AGTGGTCA GGTCTGGGTCTAC-3); IFN γ forward, (5-GTGTTCTTCTGGCTGTAAGTCTC-3) and reverse, (5-TTGTCACCTCTCCTCTCCATTTTC-3); β -actin forward, (5-TATGTTTAATACCGCCTATTCCAG-3) and reverse, (5-CCTTCCCAACCCTG TTAGAC-3). The primers were selected on different exons for each cytokine. Each PCR reaction was performed in a final volume of 20 μ L containing 0.5 μ g of the cDNA product, 150 nM of each primer, and 1X reaction mixture consisting of FastStart DNA polymerase, dNTPs, reaction buffer and SYBR green I provided by supplier (ABI, U.S.A.).

Thermal cycling to amplify all genes was performed with initial heating to 95°C for 10 min (denaturation step), followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 56°C for 20 s and extension at 60°C for 60 s, respectively. Fluorescence acquisition was measured in the final step of each extension step. Accuracy of the quantitative real-time PCR amplification was verified by performing melting curve analysis after each run. The efficiency of real-time PCR was determined using logarithmic dilutions of one of the cDNA samples in reactions with different primer pairs, and standard curves were plotted for these samples. The efficiency of reactions was calculated by the equation: Efficiency=10^(-1/slope).

Statistical Analysis. The levels of IFN- γ , IL-10, IL-12A, and IL-13 gene transcripts were evaluated between different groups of dogs using $2^{-\Delta Ct}$ method combined with Pfaffl model (15). To calculate the target gene expression in each condition, the following equation was applied;

$$\text{Expression target gene/ACTB (housekeeping gene)} = \frac{(1 + \text{efficiency of target gene})^{-Ct}}{(1 + \text{efficiency of ACTB})^{-Ct}}$$

Upper calculation was executed by Microsoft Excel 2007 software. Gene expression difference between groups was revealed by nonparametric Mann-Whitney and Spearman correlation nonparametric tests, using SPSS software v. 15.0. In all statistical analyses, $p < 0.05$ was considered significant.

RESULTS

Antigenic pattern of the FML antigen of native *L. infantum*.

Antigenic fractions relevant to glycoprotein antigens were detected by SDS-page and western blot (Fig. 1). Western blotting of native *L. infantum* FML-Ag showed 36 kDa band similar to FML-Ag of *L. donovani* (12). Furthermore, the stronger bands were observed in approximately 22 and 25 kDa zones, respectively.

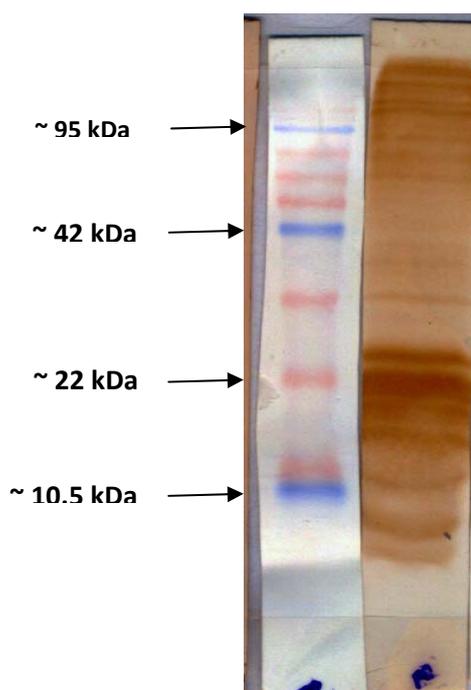


Figure 1. Western blot pattern of FML antigen of native *L. infantum*.

Detection of anti-FML antibodies in vaccinated dogs.

FML-ELISA was performed to evaluate anti-FML antibodies in serum samples of FML-saponin vaccinated dogs and control groups. Significant level of anti-FML

antibodies was detected in the sera of all of the 20 FML-saponin injected dogs, but not in the sera of saline or saponin injected control groups (Fig. 2).

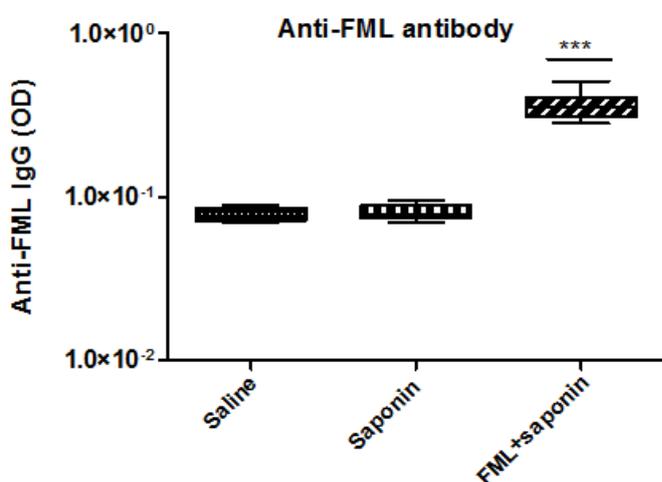


Figure 2. Levels of anti-FML antibodies in vaccinated dogs. Anti-FML IgG in serum samples of FML-saponin injected dogs and control groups. Rate of antibody in saline injected dogs (0.0796 ± 0.006802) was equal with saponin injected dogs (0.0801 ± 0.008212). Rate of produced anti-FML IgG in FML-saponin injected dogs (0.3596 ± 0.05627) was significant in comparison with control groups ($p < 0.0001$). Median of each column was showed by solid line. In box plots max and min of each column were showed by error bar. 75% and 25% percentile were showed in box. Data were analyzed by t-test and p value less than 0.05 considered as statistically significant and represented by (*) as p value less than 0.05 and (**) as p value less than 0.01 and (***) as p value less than 0.001 in the graph.

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IFN- γ mRNA expression.

Analysis of real-time PCR data showed that IFN- γ mRNA expression was significantly up-regulated in FML-saponin injected dogs in comparison to other two groups of animals ($p < 0.0001$). Moreover, the levels of expression of IFN- γ were significantly elevated in ConA and FML-ConA treated groups in comparison to those in the control group (Fig. 3).

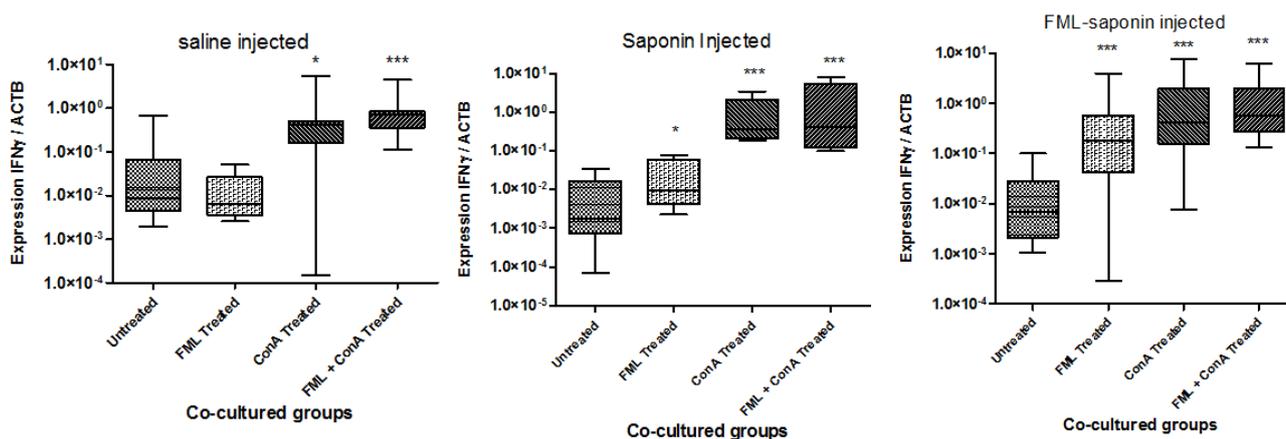


Figure 3. Levels of IFN- γ mRNA in PBMCs co-culture of vaccinated dogs. Median of each column was showed by solid line. In box plots max and min of each column were showed by error bar. 75% and 25% percentile were showed in box. Data were analyzed by Mann Whitney t-test and p value less than 0.05 considered as statistically significant and represented by (*) as p value less than 0.05 and (**) as p value less than 0.01 and (***) as p value less than 0.001 in the graph.

IL-10 mRNA expression.

Expression of IL-10 mRNA was significantly ($p=0.0001$) elevated in FML-saponin vaccinated dogs in comparison with the rest of groups (Fig. 4).

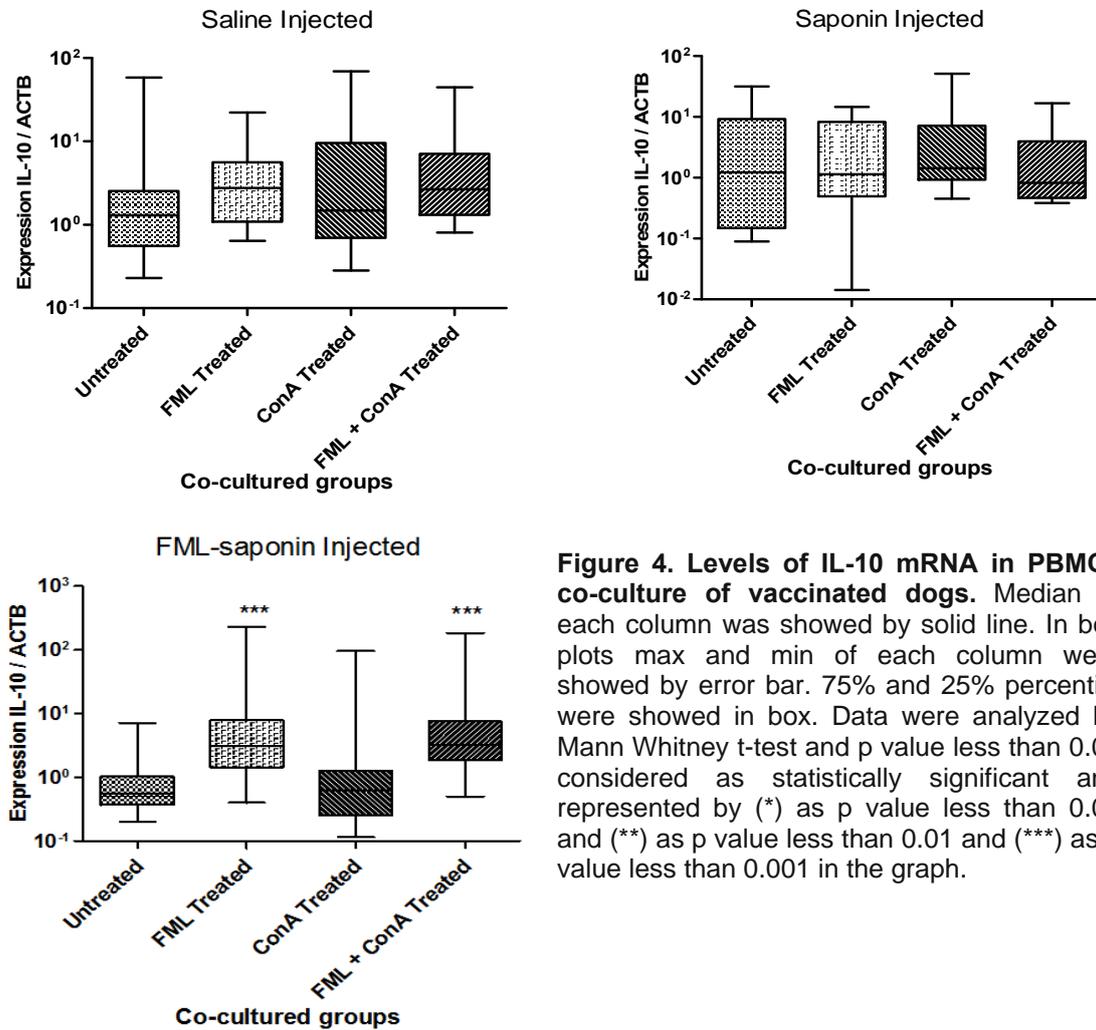


Figure 4. Levels of IL-10 mRNA in PBMCs co-culture of vaccinated dogs. Median of each column was showed by solid line. In box plots max and min of each column were showed by error bar. 75% and 25% percentile were showed in box. Data were analyzed by Mann Whitney t-test and p value less than 0.05 considered as statistically significant and represented by (*) as p value less than 0.05 and (**) as p value less than 0.01 and (***) as p value less than 0.001 in the graph.

IL-12A mRNA expression.

There were no significant differences in expression of IL-12A between FML-saponin vaccinated dogs and other groups. However, in saponin and FML-saponin injected dogs, the differences in expression of IL-12A were significant in ConA treated and FML-ConA treated groups compared to the untreated group (Fig. 5).

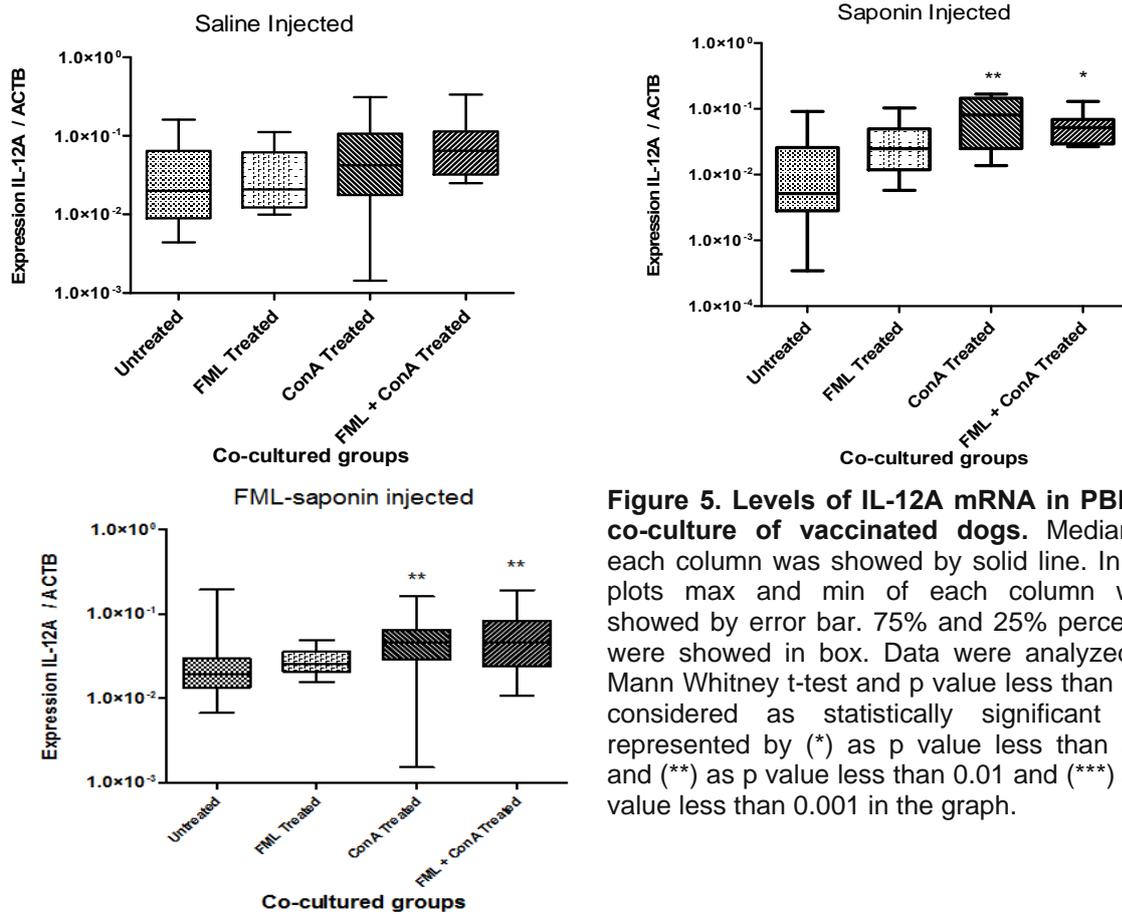


Figure 5. Levels of IL-12A mRNA in PBMCs co-culture of vaccinated dogs. Median of each column was showed by solid line. In box plots max and min of each column were showed by error bar. 75% and 25% percentile were showed in box. Data were analyzed by Mann Whitney t-test and p value less than 0.05 considered as statistically significant and represented by (*) as p value less than 0.05 and (**) as p value less than 0.01 and (***) as p value less than 0.001 in the graph.

IL-13 mRNA expression.

While the differences in expression of IL-13 in control groups were insignificant, these differences in saponin injected (p=0.0379) and FML-saponin vaccinated dogs were significant (p<0.0001) (Fig. 6).

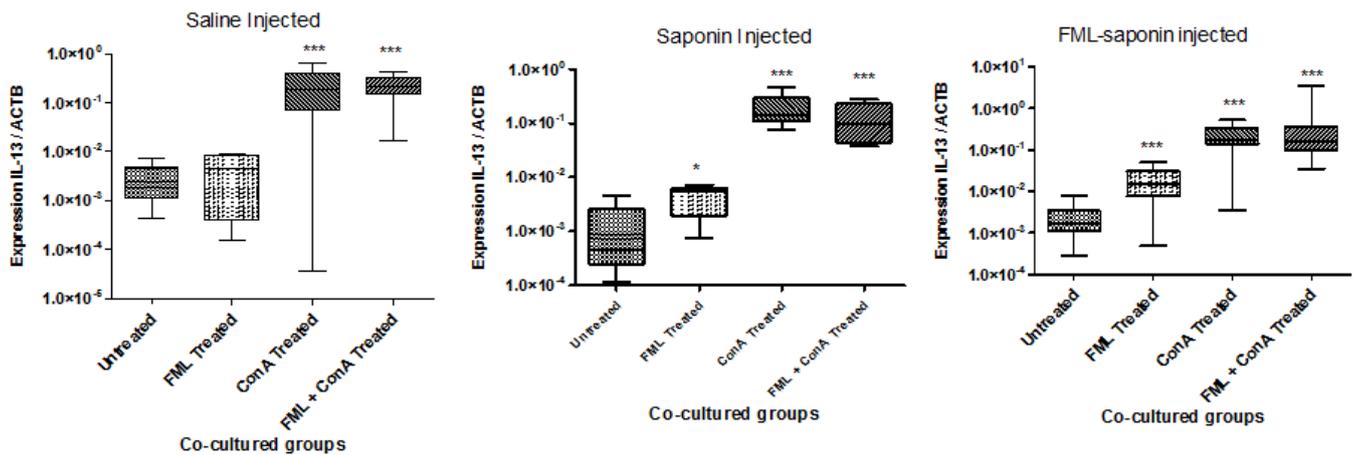


Figure 6. Levels of IL-13 mRNA in PBMCs co-culture of vaccinated dogs. Median of each column was showed by solid line. In box plots max and min of each column were showed by error bar. 75% and 25% percentile were showed in box. Data were analyzed by Mann Whitney t-test and p value less than 0.05 considered as statistically significant and represented by (*) as p value less than 0.05 and (**) as p value less than 0.01 and (***) as p value less than 0.001 in the graph.

IFN- γ /IL-10 mRNA expression ratio.

IFN- γ /IL-10 expression ratio was calculated to evaluate Th1/Th2 responses in vaccinated dogs. Analysis of results demonstrated a significant difference in ratio of IFN- γ /IL-10 expression in FML-saponin vaccinated dogs ($p=0.0275$), while such differences were not found in saline and saponin injected dogs (Fig. 7).

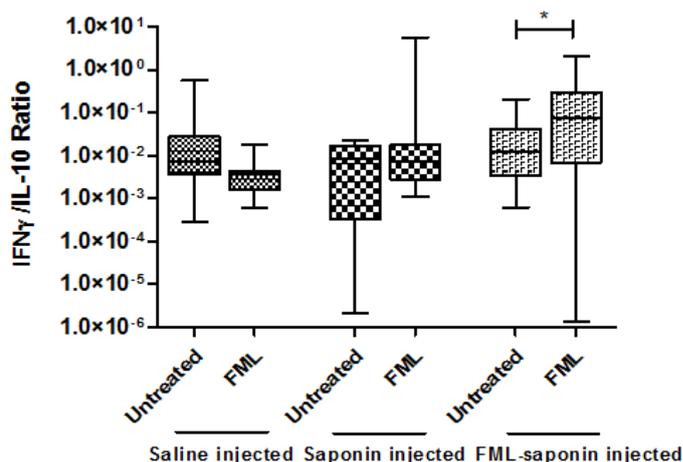


Figure 7. IFN- γ /IL-10 expression ratio in PBMCs co-culture of vaccinated dogs. Median of each column was showed by solid line. In box plots max and min of each column were showed by error bar. 75% and 25% percentile were showed in box. Data were analyzed by Mann Whitney t-test and p value less than 0.05 considered as statistically significant and represented by (*) as p value less than 0.05 in the graph.

DISCUSSION

Many vaccine candidate antigens have been evaluated for vaccination of dogs against canine visceral leishmaniasis. Among these, satisfactory results have been reported for FML-saponin in phase III trials, and a vaccine against CVL (Leishmune®) has been licensed and is commercially available in Brazil (16). In the current study, we prepared the FML antigen from native *L. infantum* in Iran and vaccinated the dogs in an endemic area of CVL to evaluate the immune responses of vaccinated dogs against the FML antigen.

Our results demonstrated a significant increase in expression of IFN γ , IL-10 and IL-13 gene transcripts in PBMCs co-cultured from FML-saponin injected dogs in comparison to control group. However, such differences were not seen in expression of IL-12A. We also found a significant difference in ratio of IFN- γ /IL-10 mRNA expression in FML-saponin injected dogs in comparison with control group. Levels of expression of IFN- γ and IL-4 in dogs after vaccination with FML antigen have been evaluated in few studies. Araujo *et al.* (10) demonstrated mixed Th1/Th2 responses to Leishmune® in PBMC culture of vaccinated dogs with increase in IL-4+ T-cells and predominant increase in IFN- γ + T-cells. Significant increase in the IFN- γ + /IL-4+ T-cells ratio verified a Th1 response in vaccinated dogs in their study. These findings are in concordance with our results. Increased level of IFN- γ in Leishmune® vaccinated dogs was also reported by de Lima *et al.* (11).

Our results showed a general up-regulation of anti-FML immune responses in vaccinated dogs with predominant Th1 immune responses. Many studies have been conducted on symptomatic and asymptomatic dogs as well as vaccinated dogs to

explore the role of Th1 and Th2 responses in developing immunity to CVL. Results of such studies confirmed that Th1 mediated immune responses contributed to resistance, and Th2 mediated immune responses contributed to susceptibility to infection. However, co-existence of mixed Th1/Th2 immune responses against *Leishmania* infections in symptomatic and asymptomatic animals has been reported in quite a few studies (17,18). In one study on cytokine profile in spleen cells of dogs naturally infected by *L. chagasi*, Lage *et al.* demonstrated an increased level of both Th1 and Th2 immune responses in both symptomatic and resistant asymptomatic dogs with differences in proportion of produced related cytokines (19).

Evaluation of immune responses in asymptomatic dogs, as an animal model with immune competent and resistant to clinical disease, can be useful. In one study on cytokine expression levels in asymptomatic naturally infected dogs by Manna *et al.* (18), high level of IFN- γ , IL-2 and IL-18 cytokines expression at the beginning of infection, and appearance of IL-4 and IL-10 cytokines in addition to IFN- γ , IL-2 and IL-18, six month later, was confirmed. IL-12 was the only undetected cytokine in asymptomatic dogs at the beginning, 6 and 24 months after infection in animals. In the same study, six months after infection, high levels of IL-2, IL-4 and IL-10 and low level of IFN- γ were found in symptomatic dogs. Interestingly, significant level of IL-12 was found only in symptomatic dogs six months after infection, representing IL-12 as a marker of active disease (18).

In our study, contrary to our expectation, the level of IL-12A mRNA expression in vaccinated dogs was not significant. IL-12 has a heterodimer structure, combined of a light chain (known as p35 or IL-12 α) and a heavy chain (known as p40 or IL-12 β) that encoding by separate genes (20,21). Recent studies demonstrated that expression of the IL-12 p35 controls IL-12 production (22,23). Babik *et al.* (24) showed that the p35 gene was highly regulated and controlled in translation and transcription. Accordingly, higher level of IL-12 in vaccinated dogs might have been seen in our study if we measured the IL-12 in secreted protein form. Our results showed a significant increase in the expression of IL-10 mRNA in vaccinated dogs. Direct correlation has been found between the increased level of IL-10, clinical signs and high parasite load in CVL (19). Contrary to these findings, Quinnell *et al.* (25) showed that the level of IL-10 and IL-13 in infected dogs was not significant and suggested that IL-10 might not play an immunosuppressive role in dogs as it does in the human VL.

The Leishmune® vaccine causes an increase in CD8+ total lymphocytes population in the blood (9,26) and a decrease in CD4+/CD25+ T cells in vaccinated dogs (11). These cells might be the source of IL-10 in our study. We have detected a significant level of IL-13 in vaccinated dogs in our study. Sanchez-Robert *et al.* (27) maintain that absence of IL-4 and IL-13 in the first stage of *Leishmania* infection in asymptomatic dogs can create protection against replication of parasite. Our results are inconsistent with the study of Quinnell *et al.* showing no enhancement in IL-13 in infected dogs (25). Considering the protective effect of FML-saponin vaccine and the results of our study as well as in view of the above-mentioned studies, increased levels of IL-10 and IL-13 in vaccinated dogs could not be solely considered an immunosuppressive response. Our findings demonstrated a significant level of anti-FML antibodies in serum of vaccinated dogs. This in turn shows a strong humoral response to FML antigen in FML-saponin vaccinated dogs. This finding is in line with findings reported in other studies (7,11,28). Bourdoiseau *et al.* (29) reported enhanced level of IgG2 anti-*Leishmania* excretory-secretory antigen (anti-LiESAp) in serum of vaccinated dogs, and showed that vaccine-

induced antibody in serum of vaccinated dogs reduced capacity of promastigotes to infect canine macrophages *in vitro* and had an inhibitory effect on the viability and proliferation of parasites. In our study, the level of total IgG was measured in vaccinated dogs, while subclass of IgG might be important in immune responses of dogs against the vaccinated antigen. Increased IgG2 anti-FML antibody has been detected in dogs vaccinated with FML-vaccine (10,30), while enhanced total IgG along with enhanced IgG1 was detected in serum of dogs with active CVL (30).

In conclusion, FML-saponin stimulates Th1 and Th2 associated with predominant Th1 immune responses as well as strong humoral immune responses to produce protective responses against CVL. Considering the diversity of cytokines functions in various conditions and in different animal models, further studies on natural reservoirs of VL are needed to explore the details of immune responses induced by the FML-saponin vaccine.

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