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# Th1, Th2 Serum Cytokines and Spleen White Pulp Changes Against Preliminary L. Major Vaccine Injection and Challenge With Live L. Major Promastigotes in Balb/C Mice

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## Abstract

**Introduction:** Human Leishmaniasis is distributed worldwide and is mainly in the tropics and subtropics, with a prevalence of 12 million cases and an approximately incidence of 0.5 million cases of visceral Leishmaniasis(VL) and 1.5 million cases of cutaneous Leishmaniasis (CL). *Leishmania* parasites are vector-born protozoan pathogens found in tropical and subtropical regions of both the old and new world. The disease in human can be divided into cutaneous, visceral, and mucosal syndromes. The aim of this study was to conduct further studies over our new formulation of *Leishmania* major vaccine which experimentally had satisfactory results.

**Method:** For the detail of the procedure it is referred to the author's previous publications. Briefly one hundred and twenty Balb/c mice were randomly divided into four groups as LT, LB, LBT and control groups. Groups LT, LB and LBT injected subcutaneously with the antigen and the same booster doses with a week interval. The expansion rates of the spleen white pulp size was evaluated and the levels of the serum TH1 (IFN-γ, IL-12) and TH2 (IL-4, IL-10) cytokines measured with the ELISA method.

**Results:** Comparing to the LT and LB groups, the LBT group had highest levels of serum IL-12, lowest levels of IL-10 and highest increase in the spleen white pulp size. Significant negative correlation was observed between IL-12 and IL-10 but not IFN-γ or IL-4.

**Conclusion:** The present study indicated that the LBT group which received crude cocktail *Leishmania* antigen plus alcoholic extract of *Teucrium polium* and BCG as adjuvants showed satisfactory cytokines profile comparing to groups LT and LB, since highest levels of IL-12 and lower levels of IL-10 could help the infected subjects to inhibit or eradicate the intracellular *Leishmania amastigotes*, and also highest increase in the spleen white pulp size which pointed to the synergistic effects of BCG and alcoholic extract of *T. polium*.

**Keywords:** *Leishmania* major vaccine; Th1& Th2 cytokines; Spleen; *Teucrium polium*; BCG

## Introduction

Human Leishmaniasis is distributed worldwide mainly in the topics and subtropics, with a prevalence of 12 million cases and an approximate incidence of 0.5 million cases of visceral Leishmaniasis (VL) and 1.5 million cases of cutaneous Leishmaniasis (CL) [1]. Leishmaniasis denotes the human disease caused by any species in the protozoan parasite genus *Leishmania*. The parasites exist as flagellated extracellular promastigotes in their vectors, phlebotomine sand flies. The disease in human can be divided into coetaneous, visceral, and mucosal syndromes. Two million new human cases arise every year that at least 350 million of exposed people are at risk of *Leishmania* parasite infection [2]. Whole-killed vaccine strategy is to identify pathogen peptides or other pathogen components that promote a Th1 response. For example, the parasite antigen Leif (*Leishmania* elongation initiation factor) induces IL-12 production from the antigen-presenting cells and promotes a skewing of the antigen specific recall response toward a Th1 response [3]. Therefore, certain peptide-based vaccines may be better at exclusively expanding a population of cells that are highly skewed toward a Th1 response [4,5]. These vaccine candidates are used in combination with a wide array of adjuvants [6]. Presumably they stimulate a predominant $CD4^+$ T cell response via endocytosis and presentation on MHC class-II molecules. It is possible that their limited efficacy may result from inadequate cross-presentation and little $CD8^+$ T cell activation. In addition different individual microbial antigens can induce DCs to promote either Th1 or Th2 responses [7]. It is probable that the immune response to a relatively complex antigenic mixture would have some aspects of both a Th1 and a Th2 response. Therefore a whole-killed vaccine may not able to target the appropriate population of T cells to consistently generate an effective Th1 response [3], for this reason the killed vaccine is not recommended. Other vaccination strategies exploit the specific interaction between infectious pathogens and DCs

to target a *Leishmania* antigen to the DCs compartment. These vaccines involve the expression of specific *Leishmania* peptides as part of other attenuated organisms, such as *salmonella* or BCG [6]. In this system, DC response is determined by the DC interaction and maturation pathway evoked by the" carrier organism, and the *Leishmania*- specific T cell response is determined by the parasite-specific protein or peptide that has been placed into the attenuated through genetic deletions which in turn it may effectively mimic a natural infection. The peptide is known to promote an adequate memory immune response in the majority of people infected with L. major [6]. More recently susceptibility and resistance to *Leishmania* infection in the mouse model has been demonstrated and it is associated with the emergence of a unique subset of T cells, namely the T regulatory cells ($T_{reg}$) and also with the levels of the cytokine IL-10 [8,9]. $T_{reg}$ cells ($CD4^+CD25^+$ ) suppress the activity of effectors T cell populations ($CD4^+CD25^-$) specific for self-antigens as well as foreign invaders such as *Leishmania* parasites. IL-10 is a potent inhibitor of IFN-γ production and has been shown to be a key cytokine that favors the persistence of the parasites in the skin lesions [10]. Therefore $T_{reg}$ cells and IL-10 are important and integrated mediators or regulators of resistance/susceptibility to Leishmaniasis. The protection induced by lpg2- parasites is basically not associated with enhanced IFN-γ production in response to *Leishmanial* antigens but clearly with a dramatic suppression of IL-4 and IL-10 responses to the same antigen. The use of the more susceptible animal models seems appropriate because they mirror more closely the immunological status of the individuals in the human population who are ultimately the targets of the vaccine, i.e., the susceptible and not the resistant or self-healing individuals [11]. It has been reported that endogenous IL-12 is required to eliminate *Leishmania* growth in IFN-γ gene knockout mice whereas IL-12 knockout macrophage are lacking *Leishmania* preventive phenotypes [12]. CD40 ligation induces IL-12 which in turn activates the T cell to produce IFN-γ and Leishmaniacidal function. Priming of susceptible BALB/c mice with exogenous rIL-12 during *Leishmania* infection also promises protection and gives a self-healing phenotype [13]. A report demonstrated a dual role of IL-4 in L. major infection where it is depending on the phase of response and the antigen-presenting cells since IL-4 promoted Th1 response [14]. Administration of anti-IL-10 receptor antibody was shown to cure the *Leishmania* infection [15,16]. Another report [17] suggested the role of IL-10 by using IL-10 gene deficient mice of both Balb/c mice and C57BL/6 mice. These mice were resistant to *Leishmania donovani* infection [17] and they were producing more IL-12 and IFN-γ, suggesting that IL-10 is the critical factor for disease progression. Also, it has been shown that co- administration of IL-10 plasmid with low dose of L. major inoculums, known to inhibit protective TH1 phenotype, and promoted the disease in Balb/c mice [18]; further confirming the disease progressive role of IL-10 [19]. Induction of IL-10 by addition of IL-2 and the suppressive role of IL-10 in Leishmaniasis has also been demonstrated (M. Bodas and B. Saha, unpublished observations). IL-12 produced by macrophages, and IFN-γ produced by NK cells, are the potential candidate cytokines based on their known ability to influence Th1 development in vitro in various systems [20-23]. Regard to above experimental findings and our previous studies on the presented preliminarily and experimentally tested vaccine conducted on two types of mice (susceptible and resistance), and it's satisfactory results on skin testing or DTH response in three injection groups and five injection doses [24], expansion of spleen's white pulp size [25], and comparing DTH and expansion of spleen's white pulp size against the new vaccine [26], we continue to evaluate further the new antigen furmula upon Th1 (IL-12, IFN-γ) and Th2(IL-4, IL-10) serum cytokines levels and effects on spleen white pulp size after re exposure to live *Leishmania* major post challenging [27]. The aim of the present to evaluate the effects of the new formulation *Leishmania* major antigen on Th1 & Th2 cytokines and secondary lymphoid tissue (Spleen White pulp) with the same methodology used in our previous studies [24-28]. The two previous studied successfully doses (100 µg/o.1 ml and 200 µg/o.1 ml), three injection groups: the *Leishmania* antigen plus BCG (LB group), the *Leishmania* antigen plus the new adjuvant namely *Teucrium polium* (LTgroup), *Leishmania* plus BCG and *Teucrium Polium* (LBTgroup), a susceptible mice (Balb/c), measurement of two types of cytokines: Th1 (IFN-γ, IL-12)and Th2 (IL-4, IL-10) and expansion of white pulp size after challenge with live *Leishmania* were considered in the present murine model experimental study.

## Material and Methods

For detail of the procedure please visit the previous studies by Latifynia et al. [24-28]. Briefly, *Leishmania* parasite promastigotes of the L. major strain (WHO strain) were kindly provided by the University of Medical Sciences of Tehran and they were grown in NNN medium (14 g bactopeptone, 6 g Nacl, Rabbit blood 300 ml and up to 1200 ml $H_2O$) and in the second step they were cultivated in RPMI 1640 culture medium supplemented with 5-10% fetal calf serum. Harvested parasites were washed three times with normal saline solution (0.9%) or phosphate buffer saline (PBS). The parasite were counted in a Neubar chamber and then kept at -70°C until use. By the time the harvested parasite was diluted to a concentration of $5.92\times10^{10}$ parasites per milliliter. Based on the previous studies, 100 µg/0.1 ml or 200 µg/0.1 ml *Leishmania* protein per dose of the provisional vaccine was selected for the vaccine formulation and preparation. The content of protein in each dose was estimated by the Lowry method [29]. The vaccine was stored at 4°C until injection. BCG Vaccine (Mycobacterium bovis, Bacillus Calmette Gurine, BCG Strain Pasteur Institute of Iran, Frozen-dried BCG Vaccine Pasteur France. 1173 P2 secondary seed lot C. batch No.179.Feb.1995) was suspended and diluted in the SSI solution (125 mg $MgSO_4$, 125 mg $K_2PO_4$, 1 mg L-aspargine, 12.5 mg iron ammonium citrate, 18.4 mg 85% glycerol, 0.5 mg citric acid, $H_2O$ 1ml for injection). Amount of BCG for each injection dose was $2\times10^5$ CFU/0.1 ml. To prepare Teucrium polium adjuvant, 400 mg of alcoholic extract of *Teucrium polium* [30] was dissolved in 1 ml distilled water; 2.5 mg/0.1 ml was used for each of the injection dosages of the antigen (100-200 µg/0.1 ml). The adjuvants were added to the *leishmania* antigen solutions mentioned previously, and two injection doses containing 100 µg/ml or 200 µg/ml antigen supplemented with adjuvants were prepared.

One hundred and twenty young adult female and male Balb/c mice were obtained from The Razi Vaccine and Serum Research Institute and randomly assigned to four standard polycarbonate boxes of four treatment groups. All groups were fed ad lib with the commercial mice chaw and kept in the polycarbonate boxes in a well-ventilated animal room located in the University of Medical Sciences of Tehran, School of Medicine, Tehran, Islamic Republic of Iran. The experimental design consisted of three antigen injected groups (LT, LB and LBT) and a control group which received no antigen injection. The new formulated antigen was adjuvented with BCG at levels of $2\times10^5$ CFU live BCG/0.1 ml or 2.5 µg/0.1 ml of alcoholic extract of *Teucrium polium* or both. Group LT received 100-200 µg/0.1 ml of the crude cocktail antigen preparation plus alcoholic extract of *Teucrium polium* as adjuvant, Group LB received 100-200 µg/0.1 ml of the crude cocktail

antigen preparation plus BCG as adjuvant, group LBT received 100-200 µg/0.1 ml of the crude cocktail antigen preparation plus alcoholic extract of *Teucrium polium* and BCG as adjuvants. Groups LT, LB and LBT were injected subcutaneously with the antigen preparations at the base of the tail and they were boosted two times using the same doses with a week interval. A week after the last booster, all animals including the control group was challenged with $3\times10^5$/0.1 ml live L. major promastigotes.

To evaluate cytokines levels in the sera of the animals survived post challenging, a blood sample of each animal was taken and they were euthanized using diethyl-ether, necropsies and spleen was removed and fixed in 10% buffered formaldehyde solution. The fixed spleen tissues were processed in a tissue processor, paraffin blocks were made and 4-5 microns tissue sections were prepared and stained with Harris Hematoxyline and Eosin method. The expansion rates of the spleen white pulp size were evaluated using a light microscope with eye-piece graticule.

The levels of IL-4, IL-10, IL-12, and IFN-γ in the three injection groups and control group were determined by the sandwich ELISA method according to the recommendations of the manufacturers. Mice serum levels of IL-4, IL-10, IL-12 and IFN-γ in the subjects were measured using an automated micro plate reader set at 405 nm. The sensitivity limit was 20 pg/ml for IL-4, IL-10, and IFN-γ. Data obtained from the experiment were analyzed using SPSS (SPSS Inc., Chicago, IL, USA). Means were compared by standard analysis of variance/simple factorial tests, and by one and two way, student Newman-Keuls methods. Correlation coefficient analysis was determined on a Pearson average two tailed test of significance. The study was done in compliance with the Helsinki Declaration, and the protocol was approved by research deputy of Tehran University of Medical Sciences, Tehran, Iran.

## Results

Our results showed that when comparing three *Leishmanial* antigen injected groups with control group, an increase in the spleen white pulp size was noticed in the groups LBT and LB but not in LT group. The highest expansion was evident in the LBT group.

| Group | Sex | IFN-γmin(pg/ml) | IL-12 min(pg/ml) | IL-4 min(pg/ml) | IL-10 min (pg/ml) | SWPs min(µm) |
|---|---|---|---|---|---|---|
| LT | F | 31.2(23.1-39.1) | 710(326-1864) | 17.9(13.9-21.8) | 23.5(18-26.5) | 573.8(308-657.5) |
| | M | 33.8(27.5-45.6) | 2293(668-4000) | 18.4(20.8-34.1) | 16.2(13.6-18) | 503.2(392.7-8.6.97) |
| | T | 33.03(23.1-45.6)[a] | 1185(326-4000)[a] | 21.1(13.9-34.1)[a] | 21.1(13.6-26.5)[a] | 524.16(308-806.96) |
| LB | F | 30.2(26.5-34.9) | 1364(307-3032) | 17.5(14.2-19.9) | 21.5(17.2-27) | 620.5(565.72-699.2) |
| | M | 30.5 | 55 | 19.9 | 27 | 588 |
| | T | 30.2(26.5-34.9)[a] | 1102(55-3032)[a] | 17.6(14.2-19.9)[a] | 22.7(17.2-27)[a] | 614(565.72-699.2)[a] |
| LBT | F | 25.5(12.6-34.4) | 3098(1828-4094)[b] | 18.4(17.1-18.9) | 18.8(13.6-26) | 914.3(623.4-1016.5)[b] |
| | M | ND | ND | ND | ND | ND |
| | T | 25.5(12.6-34.4) | 3098(1828-4094)[b] | 18.4(17.1-18.9) | 18.8(13.6-26) | 914.3(623.4-1016.5)[b] |
| Control | F | 26.7(23.8-29.4) | 2709(864-3686) | 19.2(14.2-27.5)[a] | 17.8(14.7-22.7) | 504.1(460.9-672.9) |
| | M | ND | ND | ND | ND | ND |
| | T | 26.7(23.8-29.4) | 2709(864-3686) | 19.2(14.2-27.5)[a] | 451.2 | 504.1(460.9-672.9) |

F: Female, M: Male, T: Total, SWPs: Spleen

White Pulp size min :( Min-Max)/2

[a-c]Means within a column with no common superscripts are significantly different (P<0.005)

**Table 1**: Th1 and Th2 serum cytokines levels and spleen white pulp sizes (SWPs) in the control and vaccinated groups of Balb/c mice after challenging with live L.major promastigot

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 639993.4 | 3 | 213331.1 | 7.362 | 0.001 |
| Within Groups | 608538.6 | 21 | 28978.03 | | |
| Total | 1248532 | 24 | | | |

**Table 2**: A statistically comparison between the SWPS expansion rates of the groups LT, LB and LBT and control challenged with live L.major promastigotes.

**IL-12:** Highest levels of IL-12 (2305.5 pg/ml) related to the LBT group and lowest levels of IL-12 (1032 pg/ml and 1037 pg/ml) were related to the LT and LB groups which in this respect they are almost equal. IL-12 was higher in the female (2091 pg/ml) than male mice (611.08 pg/ml) (Table 1). Correlation was significant on 0.05 level with two tailed analysis ($p$<0.005) Correlation between three injected groups and normal group, regarding IL-12 and Multiple Comparisons of IL-12 with Tukey (HSD) and 95% confidence interval shows that the mean difference is significant with 0.05 level ($p$<0.005) (Table 2). The ANOVA test between injection groups, Th1 cytokines (IL-12, IFN-γ), Th2 cytokines (IL-4, IL-10) over both doses of 100 μg/0.1 ml and 200 μg/0.1 ml showed that means square of IL-12 between groups and compared to other Th1, Th2 cytokines is significant ($p$<0.005). Pearson Correlation with the 2-tailed test shows that IL-2 and IL-10 had inverse relationship and any time IL-12 increased IL-10 decreased also and vice versa (Table 3). The study showed that correlation between level of IL-12 expression and increasing of SWPs is straight linear (positive) which show when the white pulp increased, IL-12 increased also. This relation is good and Pearson Coefficient is 0.582, which is very strong, and their coefficient of determination was 0.34, which relatively is strong. The 2-tailed test of correlation is significant ($P$<0.05) and this suggests that in 99.8% of cases this conclusion is true and significant.

| ANOVA | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| IFNγ Levels | Between Groups | 98.548 | 3 | 32.849 | 0.812 | 0.497 |
| | Within Groups | 1295.035 | 32 | 40.47 | | |
| | Total | 1393.583 | 35 | | | |
| IL12 Levels | Between Groups | 1.70E+07 | 3 | 5653365 | 4.651 | 0.008 |
| | Within Groups | 3.89E+07 | 32 | 1215595 | | |
| | Total | 5.59E+07 | 35 | | | |
| IL10 Levels | Between Groups | 263.395 | 3 | 87.798 | 1.553 | 0.22 |
| | Within Groups | 1809.174 | 32 | 56.537 | | |
| | Total | 2072.569 | 35 | | | |
| IL4 Levels | Between Groups | 487.502 | 3 | 162.501 | 1.207 | 0.323 |
| | Within Groups | 4308.083 | 32 | 134.628 | | |
| | Total | 4795.586 | 35 | | | |

**Table 3**: ANOVA test show that means square of IL-12 between groups and compare to other Th1, Th2 cytokines is significant.

**IL-10:** Highest level of IL-10 (27.2 pg/ml) related to LB and lowest concentration belonged to LBT group (19.39 pg/ml).This is almost equal to the normal group's levels, but in the LT group was higher than the LBT group and also lower than LB group. IL-10 in the male mice (25.27 pg/ml) was higher than female mice (23.67 pg/ml) (Tables 1 and 2). Correlation between IL-10 and IL-12 at doses 100- 200 μg/0.1 ml was significant ($p$<0.005) and Pearson Correlation with the 2-tailed test of correlation showed that IL-2 and IL-10 had inverse relationship, any time IL-12 increased IL-10 decreased and vice versa (Table 3). In this regard correlation between level of IL-10 expression and increasing of SWPs was inverse linear (negative), also, it was shown that when the white pulp increased, IL-10 decreased, and vise versa. But this relation is weak and Pearson Coefficient is -0.351, which is moderate, and its Coefficients of Determination is 0.12. The 2-tailed is not significant ($P$<0.085) and this suggests that although 0.085 is greater than critical number 0.05, but this difference was very small and only 0.03 larger than of 0.05, which is near to significant.

**IL-4:** Highest IL-4 levels (25.52 pg/ml) related to the LT and lowest IL-4 (17.52 pg/ml) related to the LBT and LB groups. These LBT and LB groups and also normal group relatively had equal levels of IL-4 (Figure 1). IL-4 in the male mice (23.99 pg/ml) was higher than female mice (21.7 pg/ml). IL-4 was not significant at the 0.05 level (the 2-tailed test of coefficient) (Tables 1 and 2). Correlation between injection groups and TH1 cytokines (IL-12, IFN-γ) and Th2 cytokines (IL-4, IL-10) and combined doses (100 μg/0.1 ml and 200 μg/0.1 ml) with ANOVA test showed that means square of IL-4 between groups compared to other Th1, Th2 cytokines was not significant (Table 3). Correlation between level of IL-4 expression and increasing of SWPs is inverse linear (negative) which showed when the white pulp increased, IL-4 decreased. On the contrary, SWPs decreased, when the IL-4 increased. This relation was weak and Pearson Coefficient -0.021, which was moderate, and its Coefficients of Determination was 0.001. The 2-tailed test of correlation was not significant ($P$=0.919) but greater than the critical number (0.05) which was very great by itself.

**IFN-γ:** The LB group had highest levels of IFN gamma (35.4 pg/ml) and 27.2 pg/ml related to the LT group and also lowest concentration belonged to the LBT group (19.39 pg/ml). The serum levels of IFN-γ in male (32 pg/ml) was higher than female mice (26.23 pg/ml) (Table1). The level of IFN-γ was not significant with 0.05 levels (Table 2). Correlation between injected groups and Th1 cytokines (IL-12, IFN-γ) and Th2 cytokines (IL-4, IL-10) with combined doses (100 μg/0.1 ml and 200 μg/0.1 ml) showed that in the three injected and normal groups', considering IL-12 and Multiple Comparisons of IL-12 with Tukey HSD with 95% Confidence by mean difference was significant at the 0.05 level. ANOVA test also showed that means square of IFN-γ between groups and compared to the other Th1, Th2

cytokines was not significant (Table 3). Correlation between level of IFN-γ expression and increasing of SWPs is Inverse linear (negative) which show when the SWPs increased, IFN-γ decreased. On the contrary, when SWPs was decreased, IFN-γ increases. Pearson Coefficients for this relation was weak (-0.173) and Coefficient of determination was 0.03, which showed week relation. 2-tailed test of correlation was not significant ($P$=0.409) and this was very larger than critical number (0.05). This difference was very great and correlation is not significant.

## Discussion

The resistance to *Leishmania* conferred by T-helper type-1 (Th1) cells while the susceptibility is resulted from Th2 cells responses. Th1 cells secret IL-12 and IFN-γ whereas Th2 cells secret IL-4, IL-5 and IL-10. It has been shown that IFN-γ activates macrophages to express iNOS2, the enzyme catalyzing the formation of nitric oxide; nitric oxide kills the intracellular amastigotes [31]. In contrast, Th2 immune response limits the action of Th1 functions via IL-10 and IL-4, which deactivate macrophages helping intracellular parasite growth and disease progression [32,33] Most inbred mouse strains (e.g., C57BL/6, CBA/J, C3H, B10D2) are resistant to the infection with *Leishmania* major. Upon intradermal/sub-cutaneous injection with L. major, these animals develop a small lesion that subsides within 6-8 weeks. By contrast, BALB/c mice are highly susceptible to infection with these organisms [34,35].

Many investigators have observed that several *Leishmanial* antigens inducing a Th1 response is developed during the infection are not necessarily protective antigens [36]. Administration of anti-IL-10 antibodies during L. major infection further reduced the susceptibility of IL-4 receptor α gene deficient mice [37]. It has been shown that IL-10 dictates the susceptibility to *L. donovani* infection [38,39] and it is required for higher parasite persistence in both resistant C57BL/6 mice and susceptible BALB/c mice [15,40]. The prepared antigen in this study also could induce cell mediated immunity which it seems to be a protective response as shown in previous investigations. There were significant differences between LBT group and LT group ($P$<0.002) and the differences were significant as well when LBT group compared to LB (0.03) and control groups (0.05).It seems that, when BCG and alcoholic extract of *T. polium* were used together they show a remarkable synergistic effects (Table 2). Spleen is a lymphoid organ of the secondary lymphatic system that contains two types of tissues, red pulp and white pulp. The white pulp is the place where occurring immune response which leading to subsequently antibody production. Upon antigenic challenge, these primary follicles develop into characteristic secondary follicles containing germinal centers, where rapidly dividing B cells (centroblasts) and plasma cells are surrounded by dense clusters of concentrically arranged lymphocytes [41]. The largest white pulp size were seen in the female Balb/c LBT group, and smallest white pulp size were seen in the LT group of male Balb/c (Table 3). The data obtain from this experiment indicate that compare to control group, the higher survival rate was seen in LT group which received the antigen preparation plus the alcoholic extract of *T. polium* followed by live L. major challenge. Comparing, four groups of LT, LB, LBT and control, the lower survival rate was seen in control group challenged by live L. major promastigots. Higher white pulp size in the LBT group indicated induction of humoral immunity which it could not protect the animals against *Leishmania* infection or progression. In addition this antigen preparation also could induce cell mediated immunity which it seems to be a protective response as shown in previous studies [42]. Antigen doses could affect T helper cell development and our results provide additional insight in that, the doses of antigen might influence the efficacy of vaccines and immunotherapy.

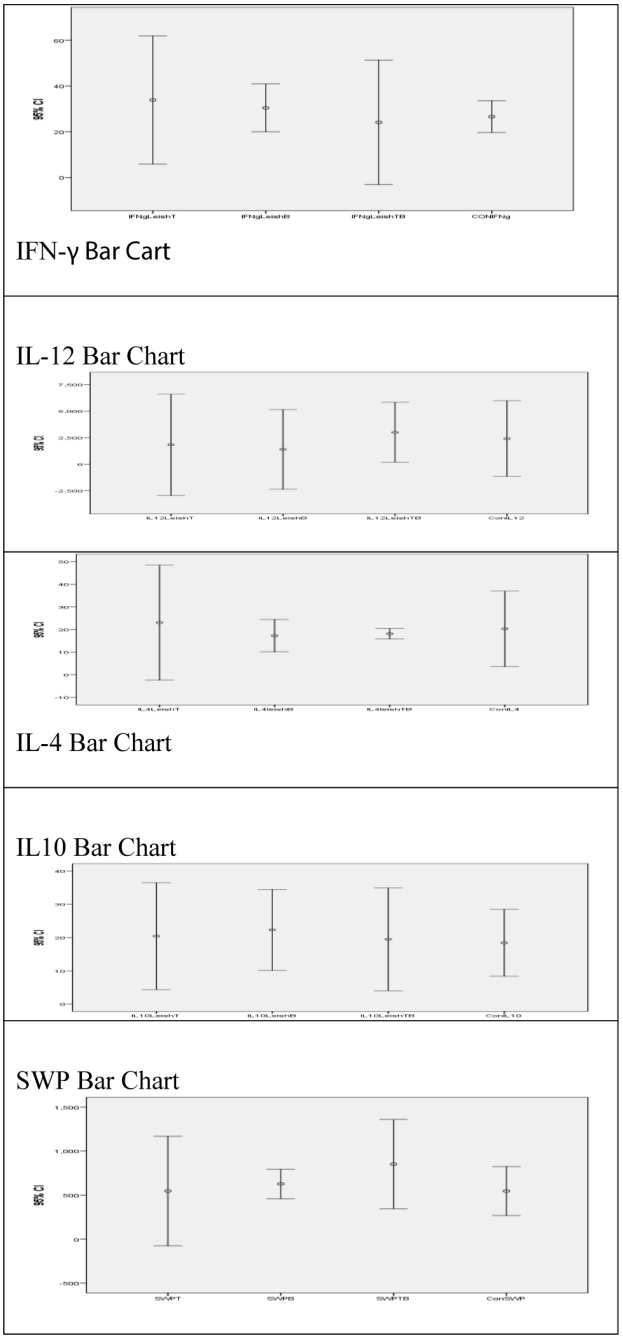


**Figure 1:** Bar Chart related to IFN- γ, IL-12, IL-4, IL-10 that compared *Leishmania* vaccine+ *Teucrium*, *Leishmania* vaccine +BCG and *Leishmania* vaccine +*Teucrium*+ BCG with controland SWP and consideration: confidence interval 95%.

The LB group had the highest level of IFN-γ (35.4 pg/ml), and the lowest levels belonged to the LBT group (19.39 pg/ml) however the

level of the cytokine in the LT group was 27.2 pg/ml which lowers than LB group but higher than LBT group. Male mice had higher level of IFN-γ (32 pg/mL) than female (26.23 pg/ml). Comparing to treatment groups, serum concentration of IFN-γ is not significant at the 0.05 level (Table 1). Multiple Comparisons of IL-12 levels in the injected groups and control group using Tukey HSD with 95% Confidence Interval show that the mean difference is significant at the 0.05 level (Table 2). ANOVA test shows that means square of IFN-γ between groups and compared to the other Th1, Th2 cytokines is not significant (Table 3) [43]. The findings may indicate the beneficial effects of the vaccine preparations in IL-12 induction which favors Th1 responses against *Leishmania* infections. As shown experimentally, several inbred mouse strains (e.g., C57BL/6, CBA/J, C3H, and B10D2) are resistant to *Leishmania* major infection. Upon intra dermal /sub-cutaneous injection with L. major, these animals develop a small lesion that subsides within 6-8 weeks. By contrast, BALB/c mice are highly susceptible to infection with these organisms [4,5]. These animals fail to control the infection and develop extensive lesions. The parasites metastasize to the internal viscera (primarily liver, spleen, and bone marrow), an event that may lead to the animal's death [7]. one of the most striking concepts arising from these studies is the clear association of resistance and susceptibility with the emergence of the two phenotypically distinct subsets of $CD4^+$ T cells, namely T helper cells type 1 (Th1) and type 2 (TH2), during the disease process. Upon infection with L. major mice of the resistant phenotype clearly develop a dominant Th1 phenotype of immune response to the parasite's antigens. By contrast Balb/c mice develop a typical Th2 response. Based on the results presented in this studies, it could be suggested that higher expression and induction of IL-12 in survived LBT group 70 days post challenging and also an increased in IFN-γ expression in almost all three injected groups and conversely, lowest expression of IL-10 in survival LBT group and finally a small increase in IL-4 only in the LT group post challenging can confirm above mentioned experimental results. In several systems to correlate resistance/susceptibility, Th1/Th2 responses have been used but perhaps the most compelling one is that involving mice genetically deficient in either Interferon-γ or Interleukin-4, the phenotypes that surrogates of Th1 and Th2 $CD4^+$ T cell responses, respectively [7]. The results of other researches finally shifted focus from IL-4 to IL-10 as a susceptibility factor [19]. Recently it has been shown that IL-10 plays a suppressive or regulatory role in autoimmune diseases [24], host versus graft rejection [23], and parasitic infection [24,25]. There is an argument related to vaccine development against Leishmaniasis, in that the use of the immunological mediators enable to polarize Th1-specific immune response to parasite antigens as an approach for antigen discovery and selection, however it seems to be redundant and may be irrelevant. Rather potent Th2-inducing antigens that are expressed or secreted by the parasites after infection (particularly during the initiation of the infection), seem to be more appropriate target molecules for vaccine development, as long as they are administered with an adjuvant that, in combination they could modulate a strong Th1 response, thus preventing the emergence of disease favoring antigen-specific Th2 clones [3]. Several vaccination strategies for both coetaneous and visceral Leishmaniasis are being developed. Most of the vaccines target the host DCs with adjuvants, such as Bacillus Calmette Guerin (BCG), *C. parvum*, or more recently, with non-methylated CpG oligo deoxy nucleotides, which mimic nucleotide sequences common to bacterial DNA [16,17]. The adjuvant stimulate DCs through various PRR, in particular the TLR, and provide pathogen signals recognized by the DC population to promote maturation of a shifted Th1 from the responding antigen-specific T cells. Other adjuvant strategies being developed include the administration of Th1 promoting cytokines, such as IL-12, within the vaccine. In clinical trials of both humans and dogs, whole killed vaccines with BCG as an adjuvant promote predominantly a cell mediated-type immune response. However, the efficacy of these vaccines is not clear [18]. In our previous research, measurement of white pulp statue showed that, the lowest white pulp size was seen in the resistant mice, group I that had received 100 µg of the antigen, pre challenge. The same result was found in group I, resistant mice injected with 200 µg of antigen. However when the types of mice were not considered, the lowest increase in SWPF size was shown in group I that had received 200 µg of the antigen, pre challenge. Correlation of DTH 24h and 48h showed that the results of Skin leishmanin test (SLT) at 24h and 48h increased following skin testing. Correlation of DTH at 24h post inoculation (PI) and 48h PI with percentage of SWPF size expansion is reversed and linear (-0.0797), that is near to (-1.0). There is a correlation between groups injected with antigen plus BCG and PPD skin test reversed and linear (-0.0797), that is near to (-1.0) susceptible (type 1) and resistant (type 2). The higher DTH responses and lower spleen white pulp size were noticed in animals that had received 100 µg or 200 µg of antigen with a single booster either in mice type 1 or 2 [11]. The injection of purified *Leishmania* subunit proteins, conferred protection in the mouse model of leishmaniasis against subsequent challenges, but such vaccines seems to require continuous boosters and presence of immune adjuvant. Understanding how antigen dose influences the development of Th1 and Th2 cells is important for designing vaccines and until the time of being, experiments that have addressed this issue have had conflicting results. The last approach is what we call cocktail based vaccination which has been already used in various pathologic conditions and in animal models. Our studies include two types of mice: susceptible (type 1) and resistant (type 2), and five injection doses of antigen (100 µg/0.1 ml, 200 µg/0.1 ml, 300 µg/0.1 ml, 400 µg/0.1 ml or 500 µg/0.1 ml) and three injection groups: group I (*Leismania* antigen plus the same dose as booster), group II (*Leismania* antigen plus BCG), and group III (*Leismania* antigen plus BCG plus the same dose as booster). Its results indicated that the DTH responses and spleen white pulp statue differed significantly when mice type 1 and 2 were considered. Comparing groups I, II and III results showed a statistically significant difference among groups in an antigen dependent manner. The higher DTH responses and lower spleen white pulp size were noticed in the animals that had received 100 µgs or 200 µgs of the antigen with a single booster either in mice type 1 or 2. Previous studies show that $CD4^+$ Th1 and Th2 regulate infection development. When L. major causes a single cutaneous lesion, or undergo spontaneous cure, subject is resistant and probably infection is inhibited in macrophage *via* innate immunity and production of interferon-γ and IL-12 by Th1 response that lead to parasite killing, and probably in the post challenging, the subject is immune. Scott et al. suggested that low antigen doses may preferably promote a $CD4^+$ Th2 response *in vivo*, whereas high doses may favor Th1 cells develop [11].

The results obtained from a study show that NKT cells should be considered both when treating active *Leishmania* infection as well as in the development of vaccines. The reported effects of L. major-activated NKT cells observed in various models of *Leishmania* infection have been variable and often conflicting which, most of this is probably due to both different infection models and *Leishmania* strains applied [43]. Recent progress in understanding how the innate immune system recognizes microbial stimuli and regulates adaptive immunity is being applied to vaccine discovery in what is termed

"systems vaccinology" [44]. In the laboratory, *Leishmania* strains additional chromosome copies have been observed in laboratory *Leishmania* strains when attempting to construct gene knockouts or after induction of drug resistance. The generation of null mutants by homologous recombination has proven unsuccessful for the dhfr-ts gene (dihydrofolate reductase-thymidylate synthase) in L. major. Aneuploidy is relatively frequent in eukaryotic pathogens and may be a mechanism to adapt to the host environment and prevailing drug pressure. In *candida*, *Leishmania*, and cancer, aneuploidy is often associated with drug pressure: whether it would also occur in the absence of drugs should be tested [45]. Several versions of a GalCer and other glycolipids have been synthetically generated and vary in terms of cytokine responses, is favor more of a Th1 or Th2 response [46,47] Multi copy genes are often preferred to enhance sensitivity, and thus are beneficial for detection, but due to potential variations and instability in copy number of the same gene both between and within species [48]. Systems vaccinology is one shoot out systems biology for which tools of a number of high-through put technologies including DNA microarrays, RNA-seq, protein arrays, deep sequencing, and mass spectrometry along with sophisticated computational tools have been originally developed [25,49]. The role of IL-10 in promoting pathology of VL has long been demonstrated in human studies. It must be noted that in several of the heat killed *Leishmania* vaccines, BCG was a common adjuvant and the immune reaction caused by BCG compounded the LST-based interpretation significantly. A meta-analysis further confirmed that LST conversion maybe associated with an immune response that can provide some protection by its ability to distinguish as a population of responders to *Leishmania-l* antigen or BCG after vaccination even though such response had a huge variability (16-68% conversion rate) in these studies [50]. Higher white pulp size in the LBT group indicates induction of humoral immunity which could not protect the animals against *Leishmania* infection. In addition this antigen could induce cell mediated immunity which it seems to be a protective response [28]. An important finding in our previous study is that, lower expansion of WPSF and highest increasing in DTH was seen in groups received 100 μg and 200 μg antigen. These findings may indicate that in the resistant animal and human subjects the infection will be probably resolved in the macrophage *via* innate immunity and production of interferon-γ and IL-12 by Th1 response that lead to parasite inhibition which in turn it confer immunity to future challenge [10]. In author previous published results show that group I that received 100 μg/0.1 ml and 200 μg/0.1 ml antigen had high DTH in SLT and low SWPs increasing, while low DTH and high WPS was seen in group II, III that received 400 μg/0.1 ml and 500 μg/0.1 ml antigen which lead to and to confirm high dose and low dose concepts [26].

In the present research also, highest SWPs was related to LBT group and lowest SWPs was also to LT group however, LB group had SWPs greater than LT group and lesser than LBT group. *Leishmania* vaccine development has proven to be a difficult and challenging task, which is mostly hampered by inadequate knowledge of parasite pathogenesis and the complexity of immune responses needed for protection [11]. Regard to all of the Th1& Th2 cytokines and SWPs expansion results appeared in this study, the new provisional vaccine could induce Th1 pattern cytokines expression (IFN-γ, IL-12), prevent Th2 type cytokines induction (IL-10, IL-4),and its effects on spleen white pulp size in the studied groups received different preparation of the antigen [6,10,11,39,51]. In this study and new vaccine experiments confirmed other scientist findings. Regard to the results obtained in the present study: we know now

- The preliminary vaccine could induce a protective response after vaccination and could protect animal following re exposure to live *Leishmania*, promastigote in Blab/c mice.
- Because of low levels of IL-10 production in particular in the LBT group and in the LT group, down regulation for IL-10 and up regulation IL-12 was seen in the LBT group post challenging.
- IL-4 expression may influence and regulate the preliminary vaccine protective effects, in the case, LT group had highest IL-4 expression comparing to other treated groups.
- This new vaccine could induce Th1 pattern which could help a number of mice to be survived 70 days or more post challenging.
- This new vaccine induces Th1 cytokine pattern, causes SWPs expansion and prevents induction of Th2 cytokines in the LBT group.
- LT group has the lowest level of SWP expansion, moderate inducer of IL-12 production (the serum levels of IL-12 is less than LBT group), a weak inducer of IL-10 (the level of serum IL-10 in LT group is less than LB group but more than LBT).
- LT group had highest levels of IL-4 and IFN-γ.
- In the present experiment, the LT group showed more survival ratio comparing to other groups.

It is concluded that, the groups which capable to produce higher ratios of Th1/Th2 responses characterized by higher IL-12 and lower IL-10 production, supposed to be able to prevent or contain the *Leishmania* infection following vaccination with the new provisional vaccine. The LBT which received the *Leishmania* antigen adjuvented with the BCG and the alcoholic extract of *Tocrium polium* plant seems to the vaccinated group that induces a satisfactory cytokines profile required for anti-*Leishmanial* immune response. However as seen in the Mendelian Susceptibility Mycobacterium Disease (MSMD) patients, in the immune deficient or immune compromised individuals, the vaccine containing BCG as adjuvant could produce mycobacterial infection, for this reason such vaccine absolutely is not recommended, instead in such a condition the *Leishmania* antigen plus the alcoholic extract of *T. polium* may be a safer candidate. Finally, additional experimental researches should be conducted before phase 1 clinical trial, including determination of IL-17 and IL-23 levels and measurement of anti *Leishmanial* antibodies (IgM an IgG classes) in the vaccinated groups.

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