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Pre-challenge Evaluation of Immune Response in Serum Cytokines (Th1 and Th2) and T-cell Markers (CD4, CD8, CD3, and CD25) Following Administration of New Formulated Leishmania Vaccine in Balb/c Mice

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Abstract

Leishmaniasis is considered an endemic disease that is a major public health concern in Iran and elsewhere. It is suggested that an effective immune response against leishmaniasis is T cells-mediated immunity that provides immunity against *leishmania* infection. The formulation and production of an immunogenic, effective, and safe vaccine to control *leishmania* infection is a necessity. Due to the complexity of the biological behavior of the *leishmania* parasite and its host immune response, the formulation and production of a safe and a protective vaccine is a difficult but worthwhile endeavor to tackle health problems.

Methods: In this study, we evaluated pre-challenged immune responses related to the Th1 (IFN-gamma and IL-12) and Th2 (IL-4 and IL-10) cytokine profiles, and the CD4, CD8, CD3, and CD25 markers of T cells. This measurement was followed by vaccination accompanied by two one-week interval boosters with the *leishmania* major antigen preparations adjuvant with BCG or alcoholic extract of *Teucrium polium* plant or both at 100 and 200 micrograms of the crude antigen/0.1 ml per mouse. This experiment was performed on six groups of *leishmania*-susceptible Balb/c mice.

Results: The statistical analysis of the data related to the T cells or lymphoid cells with the different markers, including CD8, CD3, and CD25, indicated that there were no significant differences between seven groups of animals; however, the differences were significant when the CD4 T cells were considered. On comparing the cytokines levels in the antigen-injected groups and the control group, the results showed only significant differences in serum IL-12 levels. Conclusion: It was concluded that, as shown in previous studies and the present research, the vaccine could not only induce a protective immunity in Balb/c mice, but it also did not produce deleterious responses as shown through clinical monitoring and even resulted in a 100% survival rate of the experimental animals.

Keywords: New vaccine; Th1 and Th2 cytokines and CDs; pre-challenge; Balb/c

Introduction

There are three forms of leishmaniasis-namely cutaneous, visceral, and mucocutaneous. Leishmaniasis often occurs in areas where poverty, malnutrition, famine, and illiteracy are common. The disease is transmitted to vertebrate hosts by the bite of female phlebotomine sandflies [1]. Until now, an effective vaccine capable of controlling the *leishmania* infection by preventing cutaneous leishmaniasis has not been available, and it is speculated that one will be developed in the near future. Patients who have recovered from *L. major* infections develop high levels of immunity to the pathogen [2]. Leishmaniasis has a long history. Designs on pre-Colombian pottery and the existence of thousand-year-old skulls with evidence of leishmaniasis prove that the disease had been present in the Americas for a long time. It had also been present in Africa and India since at least the mid-18th century [3]. Today, there are an estimated 12 million cases of leishmaniasis worldwide with an estimated number of 1.5–2 million new cases occurring annually, which include 1–1.5 million cases of cutaneous and 500,000 of visceral leishmaniasis [1,4].

In vertebrate hosts, *leishmania* parasites survive and multiply as non-motile amastigotes, primarily in macrophages. The genus *Leishmania* comprises 30 species, of which around 20 are pathogenic for humans [5]. Sores can result in permanent scars and disfigurement. Treatment may reduce their severity. Medication can sometimes cure the disease; however, treatment is most effective when it is started before injuries enter the immune system. Visceral leishmaniasis often proves fatal within two years if it is not treated properly. For most species, humans are accidental hosts since leishmania is primarily a zoonotic disease [6]. Until now, a successful vaccination strategy against leishmaniasis has been limited to cutaneous leishmanization in which small doses of living virulent *L. major* promastigote are deliberately injected intradermally [7]. Since the mouse model is close to humans, it has been used for the study of both cutaneous and visceral leishmaniasis, but reflects the human cutaneous leishmaniasis rather than the visceral disease. Until now, several vaccine formulations have been prepared, but they were not found to be protective in primates [8,9]. That is why there is a need for new methods to assess the immune response of animals to vaccination that can better predict the response to virulent organism

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challenges in primates and humans. New methods would be useful for the detection of parasites as well as assessment by using pathological methods. The effective diagnosis of the progression of infection with my candidate vaccine will require an assessment of the genetic stability of the agent. The results obtained from the mouse model, although practical and informative, must be further confirmed in a primate model that more closely predicts pathogenesis and immunogenicity in humans [10]. The limitation of this unique alternative is that by default, the Th1/Th2 unique parameter of the mouse model is reflected on the leishmaniasis. In other words, compensating for the Th2 strategy of the parasite can be an effective alternative for the development of the vaccine against leishmaniasis instead of enhancing the Th1 response that occurs during the infection [11]. To date, two host systems have been classified for studying leishmania infection on the basis of host susceptibility and resistance. This observation extends to the murine *L. major* model-for example, C57BL/6 mice being uniformly resistant and BALB/c consistently susceptible, which reflect the immune potentiality toward *leishmania* infection [12]. Dendrite cells (DCs) are potent antigen-presenting cells and can induce T cell activation efficiently [13]. It has been also shown that DCs are the source of different cytokines such as IL-12, IL-10, and IFN-γ [14-16].

The incubation of *leishmania* promastigote with dendritic cells can induce early IL-12 production *in vitro*, which might have originated from the preexisting pool of IL-12 p70 that was secreted soon after the ligation of any microbial product [17], suggesting the role of DCs in the initiation of T cell immune response in *leishmania* infection. It is also reported that the uptake of *leishmania* amastigotes by skin-derived DCs induces IL-12 p70, and up-regulates co-stimulatory molecules and vaccinates against *L. major* infection. In marked contrast, *L. major* inhibits IL-12 production in macrophages [18,19]. IL-10 appears to constitute a major regulatory control in the outcome of infection, as well as the failure to produce IL-12 associated with the active form of the disease [20]. Our previous findings on the same newly formulated vaccine in two groups of mice (susceptible/Balb/c) and (resistance/conventional) showed that it produced positive DTH [21] and led to an increase in white pulp size [22], which was statistically significant and correlated with effective immune responses, and was dependent on antigen doses, types of adjuvants, and injection groups [23]. Also, in other studies, our new formulated provisional vaccine was fortified by using BCG and the alcoholic extract of the *Teucrium polium* plant as an adjuvant in Balb/c mice, and after that the vaccinated subjects were challenged with live promastigote that were harvested from the culture medium. In this experiment, Types 1 and 2 cytokines, spleen changes, and rate of survival were evaluated [24-26]. The findings indicated the safety, efficacy, and productivity of the new leishmania vaccine.

The aim of the present study is to further elucidate the effect of the new adjuvanted vaccine on the immune responses of Balb/c mice, pre challenge and post vaccination. In the present experimental design, we evaluated Th1 cytokines (IFN-gamma and IL-12) and Th2 cytokines (IL-4 and IL-10), and the markers belonging to T cell subtypes, including CD4, CD8, CD3, and CD25 markers, which are considered to be mostly involved in immune response against leishmania parasites and occur in the vaccinated animals.

Material and Method

Culture and isolation of *Leishmania* parasites

Leishmania parasites and antigens from the promastigote of the *L. major* (WHO) strain were provided by the Tehran University of Medical Sciences, and they were grown in NNN medium (14 g bacto peptone, 6 g NaCl, 300 ml rabbit blood, and up to 1200 mL H_2O_2) and in the second step were grown in RPMI 1640 culture, both FCS 5% and 10%. The harvested parasites were washed three times with normal saline solution (0.9%) or phosphate buffer saline (PBS). The parasites were counted in a Neubar chamber and then kept at 70°C till use. After parasite accumulation in one flask, it was diluted to a concentration of 1.87×10^{10}.

For details of the procedure, please refer to the previous studies by Latifynia et al. [21–26]. In time, the harvested parasites were diluted to a concentration of 5.92×10^{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 formulation and preparation of the vaccine. The protein content of each dose was estimated by the Lowry method [27]. 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, and 1 ml H_2O for injection).The amount of BCG for each injection dose was 2×10^5 CFU/0.1 ml. To prepare the Teucrium polium adjuvant, 400 mg of alcoholic extract of Teucrium polium [28-30] was dissolved in 1 ml of 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 and 200 µg/ml of antigens supplemented with adjuvants were prepared.

Experimental design

Forty young adult female and male Balb/c mice were obtained from the Pasture and Serum Research Institute, and they were randomly assigned to four standard polycarbonate boxes of four treatment groups. All the groups were fed ad lib with commercial mice kept in the polycarbonate boxes in a well-ventilated animal room located in the University of Medical Sciences of Tehran, School of Medicine, Tehran, and Islamic Republic of Iran. The experimental design consisted of six antigen-injected groups (LT 100, LT 200, LB 100, LT 200, LBT 100, and LBT 200) and a control group that received no antigen injection. Group LT received 100 or 200 µg/0.1 ml of the crude cocktail antigen preparation plus alcoholic extract of *Teucrium polium* as adjuvant, Group LB received 100 or 200 µg/0.1 ml of the crude cocktail antigen plus BCG as adjuvant, Group LBT received 100 or 200 µg/0.1 ml of the crude cocktail antigen preparation plus the 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. The control group did not receive antigen injection. All the six groups received a booster a week later. All the mice were scarified 25 days after the booster injection.

ELISA method

To evaluate cytokine levels in the sera of the animals, at most 2 ml of blood sample was taken from each mouse, and its serum was separated by using the routine standard method. The levels of IL-4, IL-10, IL-12, and IFN-γ in the six injection groups and the control group of mice 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 by using an automated micro plate reader set at 450 nm. The sensitivity limit was 20 pg/ml for IL-4, IL-10, and IFN-γ.

Spleen cell isolation and flow cytometry

Splenic cells and lymphocytes were obtained from Balb/c mice by the collagenase method. Following this, 10^7 cells were treated with 4 mg/ml proteinase-free collagenase (Sigma-Aldrich, #C6079) for 20 min at 37°C in saline solution at pH 7.5 under gentle agitation, followed by the neutralization of collagenase with an equal volume of complete RPMI media. The cells were centrifuged at 8006 g, re-suspended in saline solution containing 1% BSA, and passed through a 100 µm filter mesh before analysis. Negatively sorted CD4 T cells were obtained at higher than 90% purity according to FACS analysis by cell passage through mouse CD4 subset column kit #MCD4C according to the manufacturer's instructions (R&D Systems Minneapolis, MN). DNCD3 T-cells were isolated either by depletion of CD4 and CD8 T-cells using tandem CD4 and CD8 mouse column kits (#MCD4C and #MCD8C 1000, R&D Systems), or by FACSAria cell sorter (BD, San Jose, CA) at 98% purity. For purification of DN, DP, and SP4 T-cell subsets, single-cell suspensions were triple-stained with CD3 Ab-FITC, CD4 Ab-PE, and CD8 Ab-PerCP conjugates (BD Phar Mingen, CA) and then FACS-sorted in three simultaneous windows in a FACS Aria instrument. In some experiments, the TCRcd/NK cell depletion of FACS-sorted DNCD3 splenocytes was carried out by the incubation of cells with 2 µg/10^6 cells of anti-mouse TCRcd Ab-PE (clone #GL3, BD Phar Mingen, San Jose, CA) and 2 µg/10^6 cells of anti-asialo-GM1 Ab-PE conjugates (clone #SH34, ATCC), followed by incubation with anti-PE Abs coupled with magnetic beads and passage through MACS paramagnetic columns according to the manufacturer's instructions (Miltenyi Biotech Inc., Auburn, CA) [28].

Data analysis and ethics

The data obtained from the experiments were analyzed by using SPSS (SPSS Inc., Chicago, IL, USA). The means were compared by the standard analysis of variance/simple factorial tests, and by one-and two-way student Newman Keuls methods. The correlation coefficient analysis was determined by a Pearson average two-tailed test of significance. The study was done in compliance with the Helsinki Declaration, and the protocol was approved by the research deputy of the Tehran University of Medical Sciences, Tehran, Iran.

Result

In continuing the pre challenge experiments, we compared the immune responses of the Th1 cytokine profile (IL-12, INF-γ) and Th2 (IL4, IL10) with cluster determinants (CD4+, CD8+, CD3+, and CD25+) that were induced by the new vaccine of *L. major*, which was injected in each of these three injection groups (LB, LT, LBT) that received 100 or 200 µg/0.1ml protein with only one booster dose. Our results were as follows:

IL-12: The ANOVA test showed that the means square of IL-12 compared between the injection groups and the others (INF-γ, IL4, IL10, CD4+, CD8+, CD3+, and CD25+) was significant (P<0.001), and between the injection and the control groups, Th1 cytokine (IL-12) had significant differences (P=0.001) too (Table 1). The highest amount of IL-12 was related to normal, which was not vaccinated (3896 pg/ml), but among the three injection groups and two injection doses, LB 200 µg/ml (3688.3 pg/ml) had the highest level of IL-12 and the lowest amount belonged to the LBT injection group of dose 100 µg/ml (2353 pg/ml) (Table 3). The amounts of Il-12 were as follows: Normal>LB 200>LBT 200>LT 200>LT100>LB 100>LBT 100 (Figures 1-3). Considering IL-12 and multiple comparisons of IL-12 with Tukey's (HSD) test washonestly significant different (HSD) test and 95% confidence interval showed that the mean difference was significant at the 0.05 level (Table 3). Pearson correlation with two-tailed test revealed that virtually none of INF-γ, IL-10, CD3+, CD8+, and CD25+ was significantly associated with IL-12, but IL-4 had more correlation. IL-12 and correlation with IL-10 and INF-γ, although low, were inversely related, and when IL-

		Sum of Squares	df	Mean Square	F	Sig
Interleukin 12	Between Groups	12232030.067	2	6116015.033	8.081	.001
	Within Groups	26488453.472	35	756812.956		
	Total	38720483.539	37			
Interferon gamma	Between Groups	18.571	2	9.286	2.341	.110
	Within Groups	146.765	37	3.967		
	Total	165.336	39			
Interleukin 4	Between Groups	.034	2	.017	.694	.506
	Within Groups	.915	37	.025		
	Total	.950	39			
Interleukin 10	Between Groups	2.719	2	1.360	.541	.587
	Within Groups	92.978	37	2.513		
	Total	95.697	39			
Cluster Determinant 3	Between Groups	593.168	2	296.584	2.686	.117
	Within Groups	1104.295	10	110.430		
	Total	1697.463	12			
Cluster Determinant 8	Between Groups	18.508	2	9.254	2.196	.158
	Within Groups	46.362	11	4.215		
	Total	64.869	13			
Cluster Determinant 4	Between Groups	552.505	2	276.253	2.910	.097
	Within Groups	1044.183	11	94.926		
	Total	1596.689	13			
Cluster Determint 25	Between Groups	1.270	2	.635	.901	.434
	Within Groups	7.755	11	.705		
	Total	9.025	13			

Table 1: The results of analysis variance (ANOVAs) for serum level IL-12, IL-4, INF-γ, IL-10, and spleen cell determinants: CD3+, CD8+, CD4+, and CD25+ between two injection doses(100 and 200 µg/ml) no considering to three injection groups (LB, LT and LBT) and two injection doses (100 and 200 µg/0.1 ml) (P<0.01).

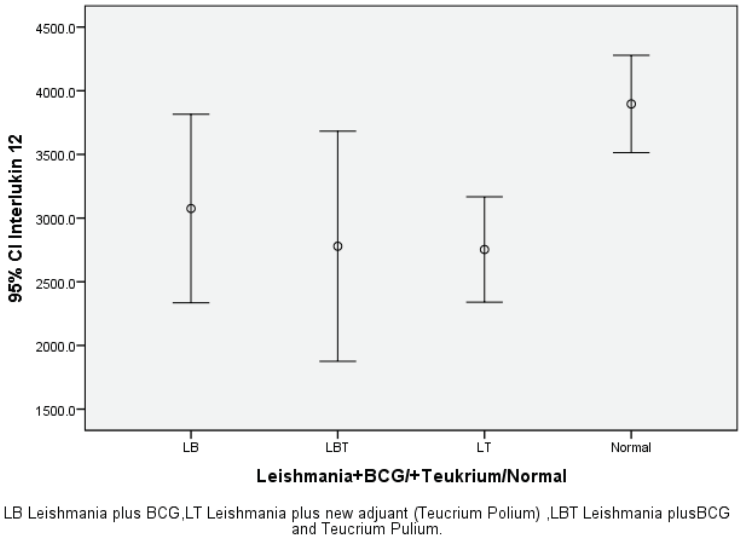


Figure 1: Mean of IL-12 for three injections groups: LB, LT, and LBT with normal group in serum of Balb/c mice after vaccination with new *leishmania* vaccine.

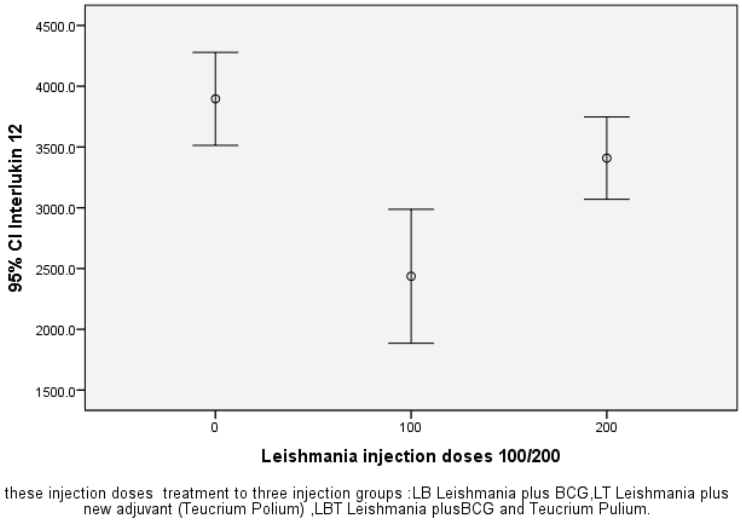


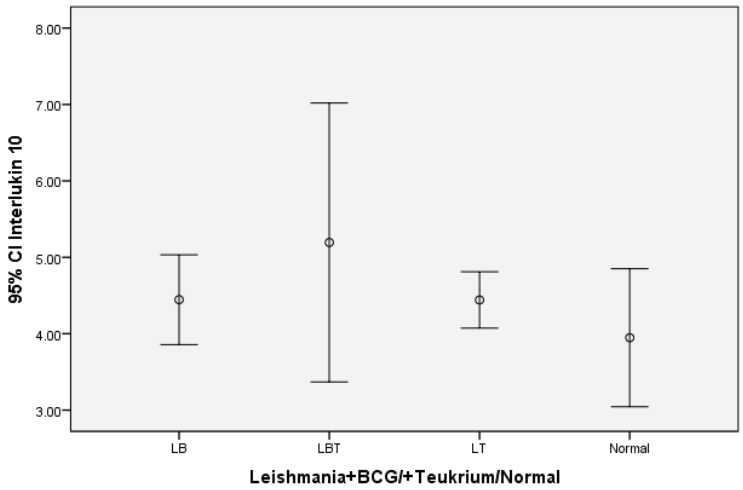
Figure 2: Mean of IL 12 for two injections dose: 100 and 200 microgram/0.1 ml with normal group 0.00 microgram/0.1 ml in serum of Balb/c mice after vaccination with new *leishmania* vaccine.

12 increased, IL-10 decreased subsequently, and vice versa, when IL-10 increased, IL-12 decreased (Table 2).

INF-γ: The ANOVA test showed that the means square of INF-γ between the injection groups in comparison to the others (IL-12, IL4, IL10 and CD4+, CD8+, CD3+, and CD25+) showed no significant statistical differences (P=0.110), but their differences were very near to significant (Table 1). The amounts of INF-γ were as follows: LT 100>LBT 100 and LB 200=LBT 200>LT 200=Normal group. The highest amount was related to the injection dose of 100 μg/0.1 ml and the injection group LT (Tables 2 and 3; Figure 2). The highest level of IFN-γ (27.84 piqogram/ml) was related to LT 100 μg/0.1 ml and the lowest IFN-γ to the normal group (without vaccine injection) (6.1 pg/ml<200 μg/0.1 ml [Table 2]). Pearson correlation was not significant at the 0.05 level with a two-tailed analysis (P<0.05) (Table 1). The correlation between two injection doses and normal groups with regard to IFN–γ and multiple comparison of IFN-γ with Tukey's HSD test and 95% CI showed no significant differences (P<0.005).Between two injection doses and control amounts were the following: 0 and 200 μg/0.1 ml (P=0.997), 100 and 200 μg/0.1 ml (P=0.125), 0 and 100 μg/0.1 ml (P=0.342), but 100 and 200 μg/0.1 ml was near to significant (Table 3).

IL-4: The ANOVA test showed that the means square of IL-4 between the injection groups in comparison to the others (IL-12, INF-γ, IL10 and CD4+, CD8+, CD3+, and CD25+) was not statistically significant (p=0.506) (Table 1). The amounts of IL-4 were as follows: LBT 100 μg/0.1 ml (2.84 pg/ml)>LT 100> LT 200>LB 100=LB 200 (1.96)>LBT 200 (1.6)>Normal (1.29) (Table 3; Figure 3).

The highest amount of IL-4 (2.84 pg/ml) was related to LBT 100 μg/0.1 ml and the lowest concentration was related to the normal group (1.29 pg/ml). The injection groups LB 100 and 200 μg/0.1 ml were equal (2.43 and 2. 46 pg/ml) and LB 200 was 1.6 pg/ml



LB Leishmania plus BCG,LT Leishmania plus new adjuant (Teucrium Polium) ,LBT Leishmania plusBCG and Teucrium Pulium.

Figure 3: Mean of IL-12 for three injections groups: LB, LT, and LBT with normal group in serum of Balb/c mice after vaccination with new *leishmania* vaccine.

		injection doses 100/200	IL- 12	IL- 4	IFN-γ	IL- 10	CD 3+	CD 8+	CD4+	CD25+
Leishmania injection doses 100/200	Pearson Correlation	1	0.059	-0.06	-0.123	0.157	-0.024	0.49	-0.202	0.357
	Sig. (2-tailed)		0.726	0.712	0.449	0.334	0.938	0.075	0.488	0.21
	N	40	38	40	40	40	13	14	14	14
Interlukin 12	Pearson Correlation	0.059	1	0.225	-0.133	-0.065	0.311	0.241	0.156	0.255
	Sig. (2-tailed)	0.726		0.174	0.425	0.7	0.3	0.407	0.593	0.379
	N	38	38	38	38	38	13	14	14	14
Interlukin 4	Pearson Correlation	-0.06	0.225	1	0.015	0.056	0.114	0.056	0.227	0.102
	Sig. (2-tailed)	0.712	0.174		0.927	0.73	0.711	0.849	0.435	0.73
	N	40	38	40	40	40	13	14	14	14
Interferon gamma	Pearson Correlation	-0.123	-0.133	0.015	1	-0.004	-0.303	-0.096	-0.293	0.169
	Sig. (2-tailed)	0.449	0.425	0.927		0.979	0.314	0.745	0.309	0.564
	N	40	38	40	40	40	13	14	14	14
Interlukin 10	Pearson Correlation	0.157	-0.065	0.056	-0.004	1	-0.224	-0.031	-0.249	0.042
	Sig. (2-tailed)	0.334	0.7	0.73	0.979		0.462	0.916	0.391	0.886
	N	40	38	40	40	40	13	14	14	14
Cluster Determinant 3	Pearson Correlation	-0.024	0.311	0.114	-0.303	-0.224	1	0.367	.958**	0.07
	Sig. (2-tailed)	0.938	0.3	0.711	0.314	0.462		0.218	0	0.82
	N	13	13	13	13	13	13	13	13	13
Cluster Determinant 8	Pearson Correlation	0.49	0.241	0.056	-0.096	-0.031	0.367	1	0.223	0.204
	Sig. (2-tailed)	0.075	0.407	0.849	0.745	0.916	0.218		0.444	0.485
	N	14	14	14	14	14	13	14	14	14
Cluster Determinant 4	Pearson Correlation	-0.202	0.156	0.227	-0.293	-0.249	.958**	0.223	1	-0.073
	Sig. (2-tailed)	0.488	0.593	0.435	0.309	0.391	0	0.444		0.804
	N	14	14	14	14	14	13	14	14	14
Cluster Determant 25	Pearson Correlation	0.357	0.255	0.102	0.169	0.042	0.07	0.204	-0.073	1
	Sig. (2-tailed)	0.21	0.379	0.73	0.564	0.886	0.82	0.485	0.804	
	N	14	14	14	14	14	13	14	14	14

Table 2: Correlations: The results of Correlations of serum level IL-12, IL-4, INF-γ, IL-10, and spleen cell determinants: CD3+, CD8+, CD4+, and CD25+ between two injection doses(100 and 200 µg/ml) no considering to three injection groups (LB, LT and LBT) (P<0.05)

(Table 3; Graphs 1 and 2). Pearson correlation with a two-tailed test of IL-4 with IL-12 was 0.225 and not significant (p=0.174), but near to it. This means that both IL-12 and IL-4 can increase or decrease. In this context, there was no observed significant correlation between IL-4 and two injection doses (100, 200 µg/0.1 ml) with another measured cytokines and the other Th1 and Th2 measured cytokines and Cluster determinants (CDs) (Table 2).The results of multiple comparisons, Tukey's HSD test, and 95% CI between the two injection doses of 100 and 200 µg/0.1 ml were not significant at the 0.05 level (P<0.05).

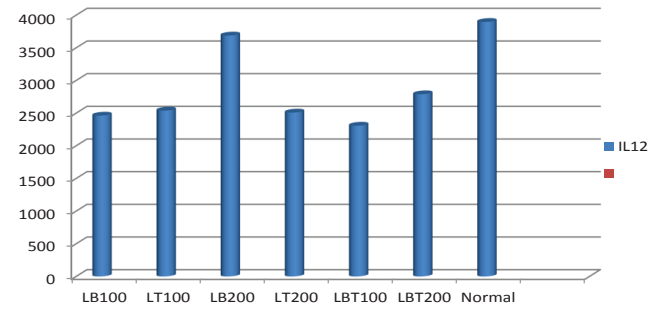
GROUP	Dosages Of Antigen µg/0.1 ml	Number of Balb/C mice	IL12pg/ml $\bar{x}$ Min.-max.	IL-4 pg/ml $\bar{x}$ Min.-max.	IFN-γ pg/ml $\bar{x}$ Min.-max	IL-10 pg/ml $\bar{x}$ Min.-max.	CD3+ $\bar{x}$ Min.-max.	CD8+ $\bar{x}$ Min.-max.	CD4+ $\bar{x}$ Min.-max.	CD25+ $\bar{x}$ Min.-max.	Survival rates (percent)
LB	100	6	2462 3941-65.5	1.96 1.15-1.81	12.2 10.2-5.2	4.73 7.2-3.95	47.7 46.6-48.8	15.6 14.9-16.3	35.5 30-41	4.19 3.48-4.9	100%
LT	100	6	2963.3 3211.6-1898	2.43 5-1.06	27.84 70.4-7.6	4.22 5.2-3.66	48.5 44.6-51.5	13.9 13.8-14	32.1 30.2-34	5.84 5.81-5.9	100%
LBT	100	6	2353 3896.3-1004	2.84 4.9-1.2	11.35 17.4-5.8	4.77 7.8-3.51	57 57.9-56.1	19.35 18.7-20	37.05 36.5-37.6	6.61 6.41-6.8	100%
LB	200	6	3688.3 4303-2651.7	1.93 2.7-1.1	14.94 19.8-5.8	4.15 4.6-3.75	57.97 46.2-78.9	16.33 13.2-18.5	37.13 32.3-46.4	5.18 4.3-6.3	100%
LT	200	6	3010 2196-3859	2.46 5.6-1.1	7.4 10.4-4.9	4.65 5.7-4	62.83 47.1-84.2	16.43 13.9-19.9	41.2 31-63.9	5.75 5.34-6.2	100%
LBT	200	5	3486 3862-3079	1.6 3-1.1	9.9 16.4-5.6	5.7 12.6-3.8	72.83 47.1-86.4	12.27 14.1-18.9	55.57 31.5-69.1	5.57 5.41-5.7	100%
Control	0	5	3896 4262-3548	1.29 1.5-1.2	7.48 8.4-6.1	3.94 4.9-3.02	62 51.3-72.7	15.23 13.4-16.2	47.2 37.6-58.1	5.39 5.0-5.7	100%

Mean within column with no common superscripts are significantly different (P<0.005)

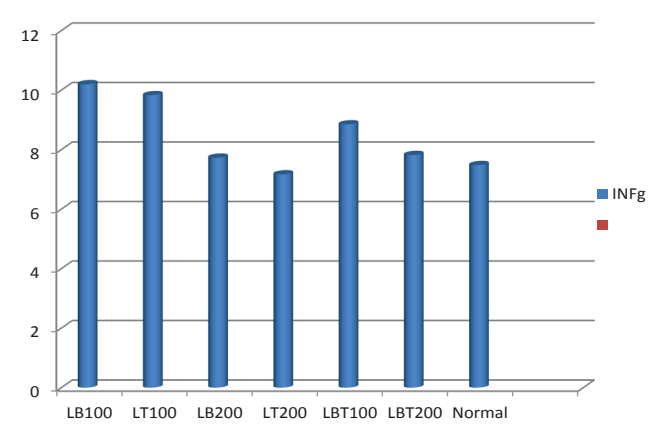
IL-12: Interleukin-12; IL-4: Interleukin-4; IFN-γ: Interferon-gamma; IL-10: Interleukin-10;

CD3+: Cluster Determinant 3+; CD8+: Cluster Determinant8+; CD25+: Cluster Determinant CD25+

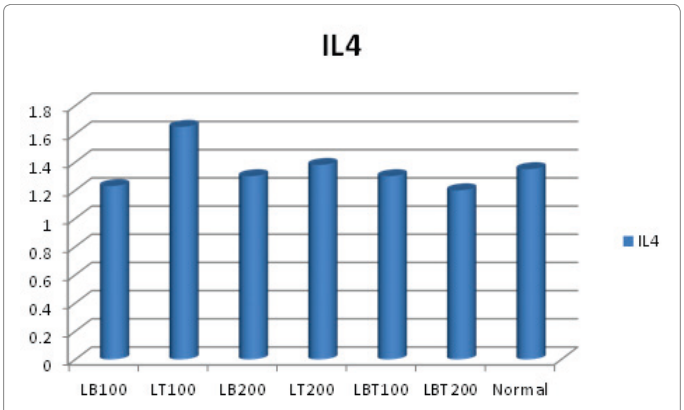
Table 3: Effects of provisional Leishmania Vaccine on spleen parameters, IL-12, IL-4, IFN-γ, IL-10, CD3+, CD8+, CD25+and Survival Rates of the Female Balb/C Mice.



Graph 1: The effects of the crude cocktail L. major antigen preparation on IL-1, production, in two injection doses (100, 200 µg/0.1 ml) and three injection groups (LT, LB and LBT) compared to control group.



Graph 2 : The effects of the crude cocktail L. major antigen preparation on INF-γ production, in two injection doses (100, 200 µg/0.1 ml) and three injection groups (LT, LB and LBT) compared to control group.



Graph 3: The effects of the crude cocktail L. major antigen preparation on IL-4 production, in two injection doses (100, 200 µg/0.1 ml) and three injection groups (LT, LB and LBT) compared to control group.

IL-10: The ANOVA test showed that the means square of Th2 cytokine (IL-10) between the injection groups and in comparison to others (IL-12, INF-γ, IL4 and CD4+, CD8+, CD3+, and CD25+) showed no significant statistical differences (p=0.506) (Table 1). The amounts of IL-4 were as follows: Normal group (1.29 pg)<LBT 200 µg/0.1 ml (1.6 pg)<LB 100=LB 200 (1.96 pg)<LT 100=LBT 200 (2.46)<LBT 100 (2.84) (Table 3; Figures 4 and 5). The highest level of IL-10 was related to LBT 100 µg/0.1 ml (5.7 pg/ml), and the lowest concentration was related to the control group (1.29 pg/ml). The results also showed that the mean of IL10 for the two injection doses 100, 200 µg/0.1 ml and 0.00 µg/0.1 ml (control), while considering the group injection in the serum of Balb/c mice after vaccination with the new leishmania vaccine with 95% CI, showed that the doses of 100 and 200 µg/0.1 ml had no significant differences with each other, but had significant differences with the 0.00 injection dose (see Graph 3; Figures 4 and 6).

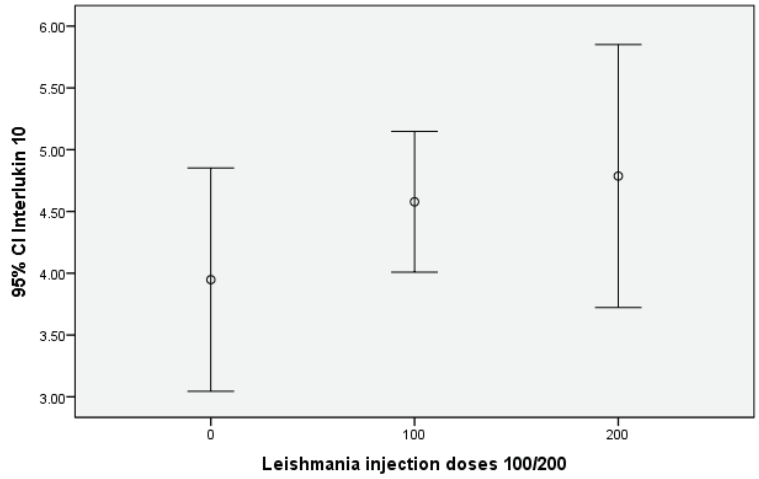
Cluster determinant 3 (CD3+): The ANOVA test showed that the means square of Th2 cytokine (CD3+) between the injection groups and compared to the others (IL-12, INF-γ, IL4, IL-10, CD4+, CD8+, and CD25+) was not significant, but very near to it (P=0.117) (Table 1).The amounts of CD3+ were as follows: LBT 200 µg/0.1 ml (72.83 pg/ml)>LBT 100 (62.83) ≥ Normal (62)>LT 20 (57.97) ≥ LB 200 (57.0)>LT 100(48.5) ≥ LB 100 (47.7) (Table 3). The highest level was related to LBT 200 and the lowest was related to LB 100 and LT 100. Pearson correlation with a two-tailed test of CD3+ with CD4+ was significant (0.958), with CD8+ (0.367), IL-12 (0.311), and IFN-γ (0.303) were relatively high but not significant, and with

doses of 100 and 200 µg/0.1 ml (0.024), IL-4 (0.114), 10 (0.224) were low and not significant (Table 2).

Cluster Determinant 8 (CD8+): The ANOVA test showed that the means square of Th2 cytokine (CD8+) between injection groups and compared to others (IL-12, INF-γ, IL4 , IL-10 , CD4+, CD3+, and CD25+) had no significant statistical differences but were very near to it (P=0.158) (Table 1). The amounts of CD3+were as follows: LB 200 µg/0.1 ml (12.27 pg)<LT 100 (13.9)<control (15.23)<LB 100 (15.6)<LB 200=LT 200 (16.43)<LBT100 (19.35) (Table 3; Figure 5). Among the injection groups, CD8+ did not show statistical differences (P=0.158), which was not significant (Table 2), but very near to it. The highest amounts were related to LB 200 and the lowest was related to LBT 100 (Table 3). Pearson correlation with a two-tailed test on CD8+ with doses of 100 and 200 µg/0.1 ml (0.490) and 0.075 significance revealed relatively high results but not significant ones.

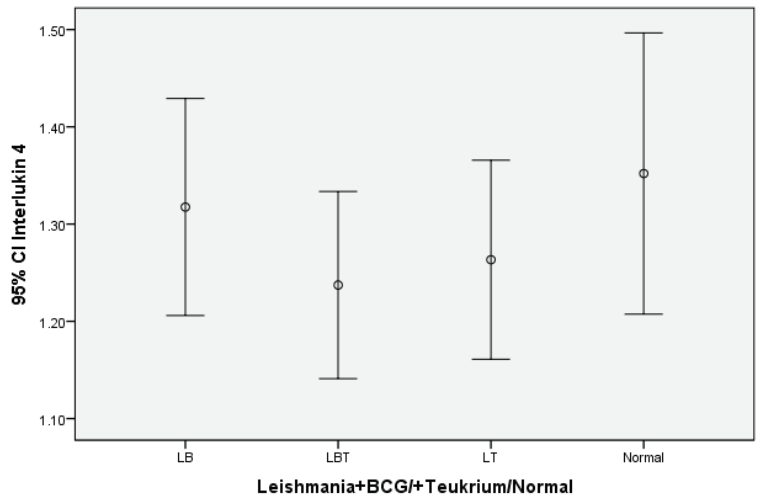
Cluster Determinant 4 (CD4+): The ANOVA test showed that the means square of Th2 cytokine (CD8+) between the injection groups and compared to the others (IL-12, INF-γ, IL4 ,IL-10 , CD8+, CD3+, and CD25+) had significant statistical differences, and were very near to it (P=0.097) (Table 1). The amounts of CD3+ were as follows: LT 100 µg/0.1 ml (32.1 pg)<LB 100 (35.5)<LBT 100=LB 200<LT 200 (41.2) <control (47.2) <LBT 200 (55.57). The highest level was related to LBT200 and the lowest was related to LT100 (Table 3). Pearson correlation with a two-tailed test of CD4+ with CD3+(0.958) and 0.000 significance, with doses of 100 and 200 µg/0.1 ml (-0.202), IFN-γ (-0.293), and IL-10 (-0.249) were relatively dependent, but negative, while IL-12 (0.156), IL-4 (0.227), and CD8+ (0.223) were relatively dependent (Table 2).

Cluster Determinant 25 (CD25+): The ANOVA test showed that the means square of Th2 cytokine (CD8+) between the injection groups



these injection doses treatment to three injection groups :LB Leishmania plus BCG,LT Leishmania plus new adjuvant (Teucrium Polium) ,LBT Leishmania plusBCG and Teucrium Pulium.

Figure 4: Mean of Interleukin 10 for two injections dose: 100 and 200 microgram/0.1 ml with normal group 0.00 microgram/0.1 ml in serum of Balb/c mice after vaccination with new *leishmania* vaccine.



LB Leishmania plus BCG,LT Leishmania plus new adjuant (Teucrium Polium) ,LBT Leishmania plusBCG and Teucrium Pulium.

Figure 5: Mean of IL-4 for three injections groups: LB, LT, and LBT with normal group in serum of Balb/c mice after vaccination with new *leishmania* vaccine.

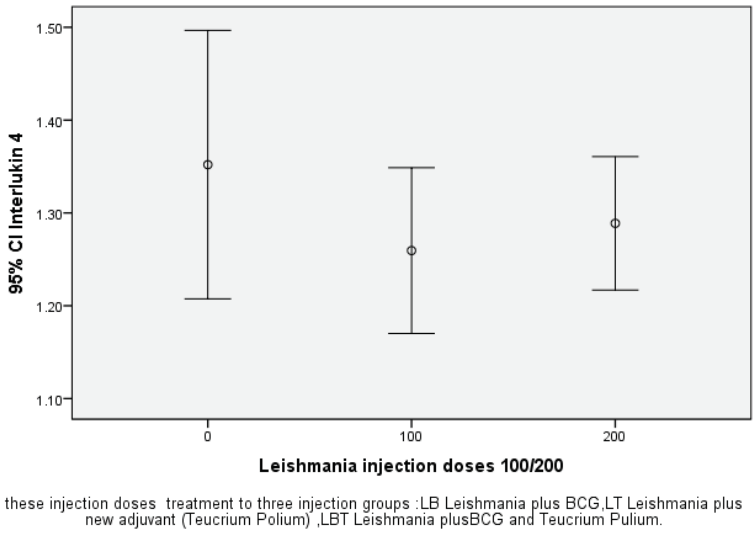
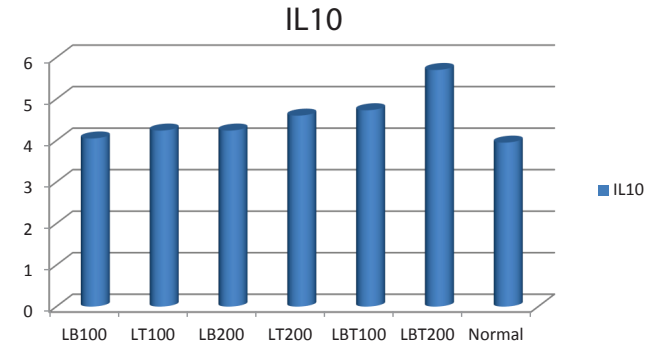


Figure 6: Mean of Interleukin 4 for two injections dose: 100 and 200 microgram/0.1 ml with normal group 0.00 microgram/0.1 ml in serum of Balb/c mice after vaccination with new *leishmania* vaccine.

and as compared to the others (IL-12, INF-γ, IL4, IL-10, CD8+, CD3+, and CD25+) had significant statistical differences , but were very near to it (P=0.097) (Table 1). The amounts of CD3+ were as follows: LB 100 µg/0.1 ml (4.19 pg)<LB 200 (5.18)<control (5.39)<LB T 200 (5.57)<LT 100 (5.75)<LT 200 (5.84)<LBT 100 (6.61). The highest level was related to LBT 100 and the lowest was related to LB 100 (Table 3). Pearson correlation with a two-tailed test of CD4+ with CD3+(0.958), and 0.000 significance, with doses of 100 and 200 µg/0.1 ml (-0.202), IFN-γ (-0.293), and IL-10 (-0.249)were relatively dependent, but negative, while IL-12 (0.156), IL-4 (0.227), and CD8+ (0.223) were relatively dependent (Table 2).

Discussion

In this study, we prepared the *L. major* antigen as a *leishmania* vaccine for the third time. According to this topic, we measured the safety, toxicity, and reproducibility in different conditions, and confirmed the authors' previous results with the same experiments as well as additional ones. Our present results showed that without considering the injection doses, IL-12 had significant differences (P=0.001) and CD4+ had nearly significant differences (P=0.097) among all the groups. INF–γ (P=0.110), CD3+ (P=0.117), and CD8+ (P=0.158) had no significant differences, but were approximately near to it. IL-4 (P=0.506) and IL-10 (P=587) showed no significant differences among all the groups (Table 1). In our previous post challenge experiments, IL-12 was found to be significant among all the groups (P=0.008), and IL-4 and Il-10 had no significant differences (P=0.323 and P=0.22) (26). The present Th1 and Th2 cytokine results confirmed our previous findings (21, 23, 24, and 26). This new vaccine increases DTH pre-challenge and leads to an increase of IL-12 in the post-challenge phase. As per the post-challenge results, we did not know whether the challenge with live *leishmania* promastigote increased IL-12 or if the increase was due to our preliminary vaccine. But we were satisfied to see that the new vaccine induce to production significant immune response which was significant and resistant-in mice against live promastigote with high survival rates have had with high and significantly IL-12 between all of the groups. An important finding for us was that the injections of the new vaccine pre-challenge directly increased IL-12 with different adjuvants, and the two injection doses of 100 and 200 µg/ml showed significant differences between all the injection groups (LB, LT, and LBT) (Table 1). This is an important point for the new vaccine, because not only did Il-12 and IFN-γ increase, but also IL-4 and 10 decreased compared to normal, which suggests that this harmless new vaccine provides an immune defense against intracellular pathogens in Balb/c mice. Now, if our study was reversed, interferon gamma and interleukin 12 would decrease and IL-4 and IL-10 would increase; we had seen this as it was a breach of the vaccine, which had shifted antibodies after exposure to intracellular pathogens (L.) and ultimately fatal visceral *leishmaniasis* that led to failure of the vaccine. The reason for this is that in this case, the animal model has produced humoral immunity and antibody. But fortunately, this new vaccine has no significant difference in IL-4 production, and 10 injections were observed for any of the two injection doses and three injection groups (Table 1; Graphs 1-4; Figures 1-4) (Figures 7 and 8). In the first study, we measured the delayed type hypersensitivity (DTH), resistant, and susceptible mice before the challenge. We came up with different results for different injection groups, and observed that the new vaccine could not only stimulate the delayed immune system, but it could also be sensitized 48 h after injecting the new antigen, and it turned from negative to positive and the expansion of spleen white pulp size was seen in resistant and



Graph 4: The effects of the crude cocktail L. major antigen preparation on IL-10 production, in two injection dose (100, 200 µg/0.1 ml) and three injection groups (LT, LB and LBT) compared to control group.

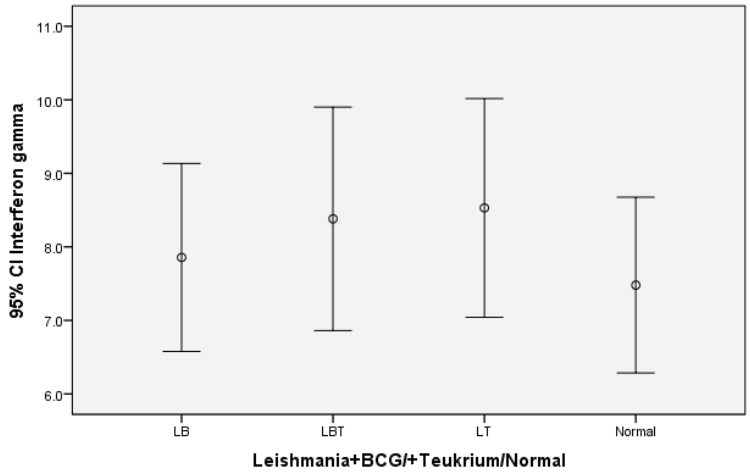


Figure 7: Mean of interferon gamma for three injections groups: LB, LT, and LBT with normal group in serum of Balb/c mice after vaccination with new *leishmania* vaccine.

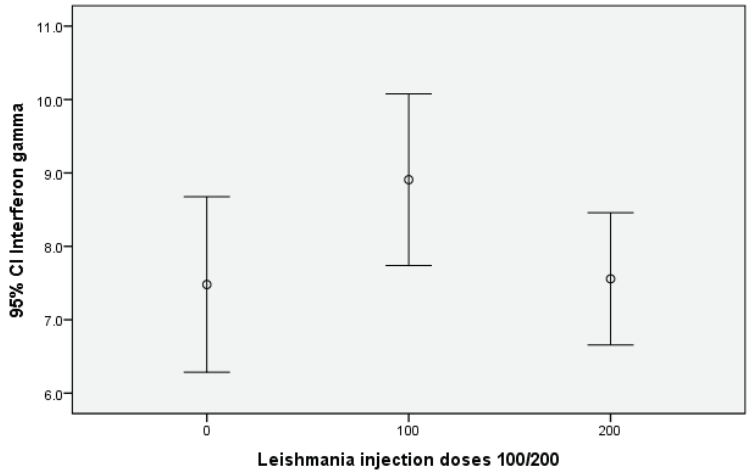


Figure 8: Mean of interferon gamma for two injections dose: 100 and 200 microgram/0.1 ml with normal group 0.00 microgram /0.1 ml in serum of Balb/c mice after vaccination with new *leishmania* vaccine.

susceptible mice [31-33]. For the second time, the different injection doses and injection groups showed significant differences again for the Th1 and Th2 cytokine profiles, and the expansion of spleen white pulp size in susceptible mice (Balb/c) against the same new vaccine post challenge with live promastigote [34-36].With regard to these results, this new vaccine was found to have the ability to stimulate a delayed immune system as well as produce positive DTH 48 h after vaccination [31,33] ,and the post-challenge increases in IL-12 and IL-10 were significant in some groups [34,36-41]. We had also observed in one of our previous studies that pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6), in non-healing, active *leishmaniasis* patients, and antioxidant levels were higher than in the other three patient and control groups [42]. Lipophosphoglycan *leishmania* could suppress TNF-α, IL-1β, and NO production by lipopolysaccharide-stimulated or PMA-stimulated macrophages. TNF-α induces mononuclear phagocytes and neutrophils to produce reactive oxygen intermediates (ROIs) [42], which led us to guess that increased amounts of Th1 cytokines such as IL-12 and IFN-γ cause a high DTH response. In contrast, Th2 cytokine (e.g. IL-4 and IL-10) levels enhanced a low DTH response in cutaneous leishmaniasis [21].

IL-4 and IL-10 act together in the presence of *leishmania* antigens [43]. When *L. major* causes a single cutaneous lesion or undergoes a spontaneous cure, the subject is resistant. In this case, probably, the infection was inhibited in the macrophage *via* innate immunity and production of IFN-γ and IL-12 by a Th1 response that led to the elimination of the parasite. In a future challenge, the subject would most likely be immune [23]. Scott et al. have suggested that low antigen doses may preferably promote a CD4+ Th2 response *in vivo*, whereas high doses may favor the development of Th1 cells [44]. The results of our present study also confirmed that CD4+ cells had significant differences depending on the two-dose injections and the three-group injections in

the pre-challenge phase of the new *leishmania* vaccine. The most visible disparity with our previous study's results, though not significantly, was the increase of IL-6 in the non-healing group, irrespective of whether IL-6 and TNF-α increased in the healing and active groups [42]. But an important and interesting point noticed about *in vivo* immune response (Balb/C) was that the one situation which was ready for innate immune response against intracellular bacteria and parasites pertained to high IL-12 and IFN-γ, and low IL-4, 10. When intracellular parasites enter the neutrophil, macrophage, or dendritic cells, immediately with the help of IL-12 and IFN-γ loop, the levels of IL-12 and IFN-γ increase, and if the immune system can be successful in this step then leishmaniasis or other intracellular diseases can be limited, and a recovery occurs, and if these cytokines have genetic or any other defects in producing humoral cytokines, including IL-4 and IL-10, then the infection progresses, leading to systemic leishmaniasis. But we still didn't know that, in our previous studies, after challenge when, IL-12 and interferon-gamma gamma increased after exposure, this effect related to pre-exposure vaccination or post challenging with live promastigote [43-44]. All previous articles, as well as the present study, expected that a successful vaccine would increase IL-12 and interferon-gamma. According to the results of all the studies, we answered some of many our questions:

1. We now know that DTH caused by injecting the new vaccine in the first study was re-established with live promastigote [21,23].
2. It subsequently led to a significant increase in IL-12, but significantly decreased IL-10 in some of the groups (see Figures 3 and 4; Table 1).
3. This new vaccine significantly increased IL-12 and decreased IL-10 in the pre-challenge phase, but significantly showed increase in some groups in the post-challenge scenario with live promastigote. This led to an inverse relationship between increased IL-12 and decreased IL-10, and in this study, CD 4+ also increased (Table 1).

Our results suggested that increased IL-12 suppressed and prevented the inducement of IL-10, and vice versa, IL-12 was suppressed and prevented by IL 10 [24,26].

With regard to our findings, we can propose that, first, the level of Th1 cytokines is high and Th2 is low pertaining to *leishmania*, but leishmaniasis disrupts its balance and causes its decrease. A successful vaccine must retain this feature, and not allow a decline in Th1 and an increase in Th2. Fortunately, this new vaccine was able to demonstrate this in our present experiments, and our research will continue to study this.

Acknowledgment

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