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Reproducibility of the new Leishmania vaccine: Changes in T1, T2 and spleen white pulp responses after re- exposure to live promastigote of Leishmania major

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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 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 more experiments over our previous new formulation leishmania major vaccine which had satisfactory experiments results.

Method: For detail procedure refers to author's previous publications. Briefly, hundred and twenty Balb/cmice were randomly divided into four groups as LT, LB, LBT and control groups. LT, LB and LBT injected subcutaneously with the antigen and the same booster doses with two weeks intervals. The expansion rates of the spleen white pulp size was evaluated using a light microscope 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 IL12, lowest levels of IL10 and highest increase in the spleen white pulp size. Significant correlation was observed between IL12 and IL10 but not IFN gamma or IL4.

Conclusion: present study indicated that the LBT group which received crude cocktail leishmania antigen plus alcoholic extract of Teucrium polium and BCG as adjuvant showed satisfactory cytokines profile comparing to groups LT and LB,since highest levels of IL12 and lower levels of IL10 which could help the infected subjects to inhibit or eradication the intracellular leishmania amastigotes, and also was seen highest increase in the spleen white-pulp size which pointed to the synergistic effects of alcoholic extract of *T. polium* and BCG.

Keywords: L. major; Th 1; Th 2; Spleen; *Teukrium polium*; BCG; Adjuvant; Challenge

1. Introduction

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 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

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can be divided to cutaneous, visceral, and mucosal syndromes. Two million new human cases arise every year that at least 350 million of exposed people are leishmania parasite infection at risk (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). The parasite maybe protected from hostile extracellular environment (6)endocytos and presentation on MHC class-II molecules helping survival of the organisms . 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 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 (8) It killed vaccine is not recommended. Other vaccination strategies exploit the specific interaction of infectious pathogens with 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 These vaccine candidates are used in combination with a wide array of adjuvant (9) 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 may effectively mimic a natural infection. It is known to promote an adequate memory immune response in the majority of people infected with L.major (9). Other vaccination strategies exploit the specific interaction of infectious pathogens with DCs to target a leishmania antigen to the DCs compartment. These vaccines involve the expression of specific Leishmania peptides.More recently susceptibility and resistance to leishmania infection in the mouse model demonstrated which, associated with the emergence of a unique subset of T cells, namely the T regulatory cells (T reg) and with the levels of the cytokine IL-10 (10,11).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 skin lesions(12)Therefore T reg cells and IL-10 are important and integrated mediators or regulation 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 (13)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.(13) 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 (14)CD 40 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 self-healing phenotype (15). A report demonstrated a dual role of IL-4 in L.major infection where depending on the phase of response and the antigen-presenting cells, IL-4 promoted Th1 response (16). Administration of anti-IL-10 receptor antibody was shown to cure the leishmania infection (17, 18).Another report (19) suggested the role of IL-10 by using IL-10 gene deficient mice of both Balb/c and C57BL/6.These mice were resistant to leishmania donovani infection (19) and were producing more IL-12 and IFN- γ suggesting that IL-10 is the critical factor for disease progression. It has also been shown that co administration of IL-10 plasmid with low dose of L. major inoculums, known to induce protective Th1 phenotype, promoted the disease in Balb/c mice(20)further confirming the disease progressive role of IL-10(21).Induction of Il-10 increased by addition of IL-2 and the suppressive role of IL-10 in leishmaniasis are also demonstrated (M.Bodas and B.Saha, unpublished observations).IL-12,produced by macrophages, and IFN- γ produced by NK cells, cells, are the potential candidate cytokines based on their known ability to influence Th1 development in vitro in various systems(22,23,24,25). Notice above experiments and our previous studies on the same preliminarily vaccine about three injection groups, five injectiondoses, and two types mice (susceptible and resistance), and it's satisfactory results on skin test, or DTH measurement (26), expansion of spleen's white pulp size (27), and comparison DTH and expansion of spleen's white pulp size against to new vaccine (28),evaluation Th1(I-12,IFNg) and Th2(IL-,IL-10) cytokines post challenging(29) and assessment of effects on spleen white pulp size after re exposure with live L. major promastigote(30), The aim of this study was more evaluation effects on Th1 & Th2 cytokines and secondary lymphoid tissue (Spleen White pulp) over our previous new formulation leishmania major vaccine which has had satisfactory experiments results and correlated them with together. In this regard we decided accomplish more experiments over this new vaccine with the same methodology (26, 27, 28, 29, 30,). In thisregards we selected two previous study's successfully doses (100 & 200 μg/o.1ml), three injection groups : Leishmania plus BCG (LB), Leishmania plus new adjuvant (Teucrium Polium)[LT], leishmania plus BCG and Teucrium Polium (LBT),and one susceptible mice (Balb/c) and measure two type cytokines:Th1(IFN- γ, IL-12)and Th2 (IL-4,IL-10) and expansion of white pulp size after challenge with live leishmanial.

2. Material and methods

This 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. For detail procedure please refer to Latiynia A. and et al. (26, 27, 28, 29, & 30).Briefly, leishmania parasites and antigens from promastigotes of L.major (WHO) strain were kindly provided by the University of Medical Sciences of Tehran and they were grown in NNN medium (14 gr bactopeptone, 6gr Nacl, Rabbit blood 300ml and up to 1200ml H2O2) and in the second step they were grown 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 -70c until use. By the time the harvested parasite was diluted to a concentration of 5.92×10^{1}ºparasite per milliliter.One hundred and twenty young adult male and female Balb/c mice were obtained from Razi Vaccine and Serum Research Institute and randomly assigned to four standardpolycarbonate boxes of three antigen injected groups (LT, LB, LBT) and control which received no antigen injection. All groups were fed ad lib with the commercial mice chaw and kept in polycarbonate boxes in a well ventilated animal room located in the Tehran University of Medical Sciences, School of Medicine, Tehran, and Islamic Republic of Iran. The new formulated antigen was adjuvented with BCG at levels of 2x105 CFU live BCG /0.1 ml or 400 mg teucrium polium dissolved in 1 ml distilled water and 2.5 mg /0.1 alcoholic extract of Teucrium polium or both adjuvant. 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 adjuvant and group four was kept as control group. Groups LT, LB and LBT received subcutaneously antigen and then same doses as booster with two weeks intervals. A week after the last booster, all animals including were challenged with 3x105/0.1 ml live Leishmania major promastigotes. The animals survived, were euthanized, their spleen was removed, and 4-5 microns paraffin sections were prepared and stained with Harris Hematoxylin and Eosine method. All doses were injected intra dermal in tails in susceptible Balb/c mice both male and female in three injection groups: LT [Leishmania antigen doses (100, 200µg / 0.1ml) accompanied to teucrium polium as adjuvant], LB [Leishmania antigen doses (100, 200µg /0.1 ml) accompanied to BCG and teucrium polium as adjuvant], LBT [Leishmania antigen doses (100, 200µg / ml) accompanied to BCG and teucrium polium as adjuvant]. After first injection we have two same booster doses which its interval was one week. Past 20 days after third leishmania injection or second booster dose, mice had challenged with 300000 lived leishmania major. The protective response was evaluated by the challenge effects which notice almost daily for 70 days over all mice. Evaluation was contain: inducing lesion, and survival and another critical signals .After this time lived mice's serum used for cytokine levels and their level measured with Measurement of cytokine production by enzyme linked immunosorbent assay (ELISA). Levels of IL-4, IL-10, IL-12, and IFN-γ in the three injections groups and normal group were determined by sandwich ELISA, according to the recommendations of manufacturer. Mice serum Levels of IL-4, IL-10, IL-12 and IFN-γ in the subjects measured by ELISA, using an automated micro plate reader, set at 405 nm. The sensitivity limit was 20 piqogram /ml for IL- 4, IL-10, and IFN-γ. After that the animals survived of post challenging were euthanized using diethyl- ether, necropsied 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 Eosine method. The expansion rate of thespleen white pulp size was evaluated using a light microscope with eye- piece graticule. Data obtained from the experiment were analyzed using SPSS (SPSS Inc., Chicago, IL, USA). Means were compared by a 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) is significant. 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.

3. Results

Our results showed that: When compared three injection groups of leishmanial antigen with control group, the spleen white pulp size increased in the groups LBT (highest expansion) and LB but had not increasing in LT group.IL-12: Highest amounts of IL-12 (2305.5 pg/ml) related to the LBT group and lowest of IL-12(1032and 1037pg/ml) was related to the LT and LB groups which are almost equal. IL-12 was higher in female (2091pg/ml) than male mice (611.08 pg/ml) (table1).Correlation was significant on 0.05 level with two tailed analysis ($p<0.005$) Correlation between three injections and normal groups, considering IL-12 and Multiple Comparisons of IL-12 with Tukey (HSD) and95% Confidence interval show 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 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).

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 promastigote

Group	Sex	IFN-γ min(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]	451.20	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 2 A statistically comparison between the SWPS expansion rates of the groups LT, LB and LBT an control challenged with live L. major promastigotes.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	639993.436	3	213331.145	7.362	0.001
Within Groups	608538.611	21	28978.029		
Total	1248532.047	24			

Table3 ANOVA test show that means square of IL-12 between groups and compare to other Th1, Th2 cytokines is significant.

	ANOVA	Sum of Squares	df	Mean Square	F	Sig.
IFNγ Levels	Between Groups	98.548	3	32.849	.812	.497
	Within Groups	1295.035	32	40.470		
	Total	1393.583	35			
IL12 Levels	Between Groups	1.696E7	3	5653365.438	4.651	.008
	Within Groups	3.890E7	32	1215595.185		
	Total	5.586E7	35			
IL10 Levels	Between Groups	263.395	3	87.798	1.553	.220
	Within Groups	1809.174	32	56.537		
	Total	2072.569	35			
IL4 Levels	Between Groups	487.502	3	162.501	1.207	.323
	Within Groups	4308.083	32	134.628		
	Total	4795.586	35			

Table 4 Correlation between IL-12 and IL-10, IL-4, IFN-γ on Doses 100 and 200 µg / 0.1ml combined with together. Pearson Correlation with 2 – tailed test IL-4 , IL-10, IL-12 , IFN- γ & SWPs expansion

Correlations		SWPS (micrometer)	IL12 Levels	Il-10 levels	Il-4 levels	IFN-γ levels
SWPS (micrometer)	Pearson Correlation	1	.582**	-.351	-.021	-.173
	Sig. (2-tailed)		.002	.085	.919	.409
	N	25	25	25	25	25
IL12 levels	Pearson Correlation	.582**	1	-.342*		
	Sig. (2-tailed)	.002		.041	UD	UD
	N	25	36	36		
IL10 levels	Pearson Correlation	-.351	-.342*	1		
	Sig. (2-tailed)	.085	-.041		UD	UD
	N	25	36	36		
IL4 levels	Pearson Correlation	-.021			1	UD
	Sig. (2-tailed)	.919				
	N	25	UD	UD	36	
IFN--γ levels	Pearson Correlation	-.137				1
	Sig. (2-tailed)	.409				
	N	25	UD	UD	UD	36

**Correlation is significant at the 0.05 level (2-tailed). ** is significant, UD is Undetermined

Show that IL-2 and IL-10 have reversed with together and when IL-12 is increased Il-10 decreased. Correlation is significant at the 0.05 levels (2-tailed) and also correlation between IL-12, IL10, Il-4, IFN-γ and SWPs expansion at doses 100 and 200 µg / 0.1ml Pearson Correlation with 2 - tailed test show that IL-12 and SWPs expansion is direct and when IL-12 is increased SWPs expansion is increased also.

In this 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, IL12 increased also. (This relation is good and Pearson Coefficient is 0.582, which is very strong, and its Coefficients of Determination is 0.34, which relatively is strong. Correlation 2-tailed is significant (P< 0.05) and this suggests that in 99.8% of cases this conclusion is true and significant (table4). 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 level, but the LT group was higher than the LBT group and also lower than LB group. IL-10 in male (25.27 pg/ml) was higher than female mice (23.67 pg/ml) (table 1, 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 showed that IL-2 and IL-10 had inverse relationship which any time IL-12 increased Il-10 decreased and Vice versa (Table3). In this regard correlation between levels of IL-10 expression and increasing of SWPs was Inverse linear (negative), also, which show when the white pulp increased; IL10 decreased, and vice versa. But this relation is weak and Pearson Coefficient is 0.351, which is moderate, and its Coefficients of Determination is 0.12. Correlation 2-tailed is 9 not significant (P< 0.085) and this suggests although 0.085 is greater than critical number 0.05,but this difference was very small and only .03 larger than of .05,and near to significant(table 4).

IL-4: Highest IL-4 (25.52 pg/ml) related to the LT and lowest IL-4 (17.52 pg/ml) to the LBT and LB groups. These two groups were equal together and normal group (Fig. 3). IL-4 in male (23.99 pg/ml) was higher than female mice (21.7 pg/ml). IL-4 was not significant at the 0.05 level (2-tailed) (Table 1, 2). Correlation between injection groups and Th1 cytokines (IL-12, IFN-) and Th2 cytokines (IL-4, IL-10) and combined doses (100 and 200 µg/0.1 ml) with ANOVA test showed that means square of IL-4 between groups compared 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 show when the white pulp increased, IL4 decreased. On the contrary, SWPs decreased, when the IL4 increase. This relation was weak and Pearson Coefficient -0.021, which was moderate, and its Coefficients of Determination was 0.001.Correlation 2-tailedwas not significant (P=0.919) but very larger than critical number (0.05) which was very large (table4). IFNin the LB group had highest level (35.4 pg /ml) and27.2 pg/ml related to the LT and alsolowest concentration belonged to the LBT group (19.39 pg/ml) .IFN in male (32 pg/ml) washigher than female mice (26.23 pg/ml) (table1). IFN- was not significant with 0.05 level (2-tailed) (Table2). Correlation between injection groups and Th1 cytokines (IL-12, IFN-) and Th2cytokines (IL-4,IL-10) with combined doses (100 and 200 µg/0.1 ml) showed that three injectionand normal groups', considering IL-12 and Multiple Comparisons of IL-12 with Tukey HSDwith95% Confidence by mean difference was significant at the 0.05 level. ANOVA test also showedthat means square of IFNbetween groups and compared to the other Th1, Th2 cytokines wasnot 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 Ɣ decreased. On the contrary, when SWPs was decreased, IFN Ɣ increase. Pearson Coefficients for this relation was weak (-0.173) and Coefficient of determination was 0.03, which showed week relation. Correlation of 2-tailedwas not significant (P=0.409) and this was very larger than critical number (0.05), this difference was very large and finding and correlation is not significant (Table 4).

4. Discussion

There was significant difference between LBT and LT groups (P<0.002), LBT and LB(0.03) , also,LBT 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) 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 the experiment indicate that compare to control group, the higher survival rate was seen in LT group which received the antigen plus alcoholic extract of T.polium challenged by live L.major promastigote. 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. In addition this preliminary vaccine also could induce cell mediated immunity which it seems to be a protective response as shown in previous studies. (41). LB group has the highest level of IFN- (35.4 pg/ml), (27.2 pg/ml) related to the LT whether lowest amount belong to the LBT group (19.39 pg/ml). Male mice had higher IFN (32 pg/mL) than female (26.23 pg/ml). IFN-is not significant at the 0.05 level (2-tailed) (table 1) .IL-12 and Multiple Comparisons of IL-12 with Tukey HSD and 95% Confidence Interval, show that the mean difference is significant at the 0.05 level (Table 2). ANOVA test show that means square of IFNbetween groups and compared to another cytokines is not significant(table 3) (44). The resistance to leishmaniaconferred by T-helper type-1 (Th1) cells while the susceptibility is conferred by Th2

cells.Th1 cells secret IL12 and IFN- γ where as 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. 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 (42).Most inbred mouse strains (e.g., C57BL/6, CBA/J, C3H, B10D2) are resistant to infection with leishmania major .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 mice 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)Many investigators have observed that several leishmanial antigens against a Th1 response is developed during the infection are not necessarily protective antigen (40). 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 cell type I (Th1) and type 2 (Th2) cells, during the disease process. Upon infection with L.major mice of the resistant phenotype clearly develop a dominant a Th1 phenotype of immune response to the parasite's antigens. By contrast Balb/c mice develop a typical Th2 response. Based on these results, we can say, our findings about this preliminary vaccine that showed most expression of IL-12 in survival LBT group 70 days Post, and conversely, lowest expression of IL-10 in survival LBT group 70 days Post challenging, and also increasing IFN γ expression in almost all 3 injection groups, and finally IL4 slightly increased only in LT group. Our results almost confirmed other experiments results which mentioned above. In several systems have been used to correlate resistance/susceptibility with Th1/Th2 response but perhaps the most compelling one is that involving mice genetically deficient in either interferon-γ (IFN- γ) or interleukin 4(IL-4),the phenotypic 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 susceptibly factor (21). Recently it has been shown that IL-10 plays a suppressive or regulatory role in autoimmune diseases (24), host versus graft rejection (25), and parasitic infection (26, 27). Administration of antiIL-10 antibodies during L.major infection reduced the susceptibility of IL-4 receptor α gene deficient mice (28). It has been shown that IL-10 dictates the susceptibility to L.donovani infection (29, 30) and is required for higher parasite persistence in both resistant C57BL/6 and susceptible BALB/c mice (31, 32). There is arguments that for vaccine development against leishmaniasis,the use of Immunological mediators of polarized Th1-specific immune response to parasite antigens as readouts for antigen discovery and selection seems to be redundant and 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 in combination with them ,modulates a strong Th1 response. Thus preventing the emergence of disease favoring antigen – specific Th2 clones. (8). Several vaccination strategies for both cutaneous and visceral leishmaniasis are being developed .Most of the vaccines target the host DCs with adjutants, 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 (18,19). The adjuvant stimulate DCs through various PRR, in particular TLR, provide pathogen signals recognized by the DCs can leading to maturation Th1 for responding antigen –specific T cells. Other adjuvant strategies being developed include the administration of Th1 promoting cytokinesvaccines with BCG as an adjuvant promote predominantly a cell mediated type immuneHowever, the efficacy of these vaccines is not clear (20).In our previous research, spleen white pulp statue showed that, lowest increasing size in the resistant mice and injection group I(one) that received 100 μg of the antigen, pre challenge. The same result was found in; mice type 1 and group I that injected with 200 μg of antigen. Correlation of DTH 24h and 48h showed when SLT (24 hrs) increasing, SLT (48hrs) increased too. Correlation of DTH at 24 hrs post inoculation (PI) and 48 hrs 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 received 100 or 200 μg of antigen with a single booster either in mice type 1 or 2(13). The injection of purified Leishmania subunit proteins, conferred protection in the mouse model of leishmanasis against subsequent challenges, but such vaccines seems to require continuous boosters and presence of immune adjuvant. Understanding how antigen dose influences the development of Th1and Th2cells 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 first studies include two types of mice: susceptible (type 1) and resistant (type 2), and five injection doses of antigen (100,200,300,400,500 μg /0.1ml) and three injection groups: group I (Leismania plus thesame dose as booster) , group II (Leismania plus BCG), and group III (Leismania plus BCG plus the same dose as booster). Its results indicated that the DTH responses and spleen white pulp statue had difference significantly when mice type 1 and 2. Comparing with 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 animals that had received 100 or 200 μg of the antigen with a single booster either in mice type 1 or 2. One of the previous studies shows that CD4+ Th1and 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 gamma and IL-12by Th1response that lead to parasite killing, and probably in future challenge subject is immune. Scott et al suggested that low antigen doses may preferably promote a CD4+ Th2response in vivo, whereas high doses may favor Th1cells develop (13). Antigen doses could affect T helper cell development and our results provide additional insight that doses of antigen might influence the efficacy of vaccines and immunotherapy. 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 immune response is induces that subsequently antibody production. Upon antigenic challenge, these primaryfollicles develop into characteristic secondary follicles containing germinal centers, where rapidly dividing B cells (centroblasts) and leading to formation of plasma cells are surrounded by dense clusters of concentrically arranged lymphocytes (45). In one study results 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 invarious 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 (46). 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” (47). 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 (di-hydro- folate- 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 (48).

Several versions of a GalCer and other glycolipids have been synthetically generated and vary in terms of cytokine responses, is favor more of a Th-1 or Th-2 response (49, 50) 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 (51). 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 (52-27). 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 leishmanial antigen or BCG after vaccination even though such response had a huge variability (16–68%conversion rate) in these studies (53). An important finding in our previous study is that, lower expansion of WPS and highest increasing in DTH was seen in groups received 100 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 gamma and IL12by Th1 response that lead to parasite inhibition which in turn it confer immunity to future challenge (12).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 for SLT and low SWPs increasing, while low DTH and high WPS was seen in group II, III that received 400 μg/0.1ml and 500 μg/0.1ml antigen. This finding leads us to the point of high and low dose concepts that proposed by Uzonna et al in 2004. In present research also, highest SWPs was related to LBT group and lowest SWPs was also to group however, LB group had SWPs greater than LT group and less than LBL 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 complex of immune responses needed for protection (13). Regard to all of the Th1& Th2 cytokines and SWPs expansion results in this study which new vaccine could had induce Th1 pattern expression (IFN γ,IL-12), and prevent of Th2 (IL-10,IL-4) pattern induction ,and effect on spleen and SWPs which had adverse or direct effect with same injection doses and diverse injection groups(author previous experiments 9, 12,13,41&44). In present study Pearson Correlation with 2 - tailed test show that IL-2 and IL-10 have reversed with together and when IL-12 is increased Il-10 decreased. Correlation is significant at the 0.05 levels (2-tailed) and also correlation between IL-12, IL10, Il-4, IFN-γ and SWPs expansion at doses 100 and 200 μg / 0.1ml Pearson Correlation with 2 - tailed test show that IL-12 and SWPs expansion is direct and when IL-12 is increased SWPs expansion is increased also. Correlation is significant at the 0.05 level (2-tailed).(table 4). In this study we also conducted a similar study after a few years for the reproducibility (53),of the newly prepared vaccine and verification of its previous tests, which fortunately got very good results that showed that this new vaccine with the same initial formula is not only reproducible and the results, they are similar to each other, but our experiments also confirmed the correctness of our previous experiments. We showed once again that our vaccine against re-exposure to live promastigote not only affected and activated the immune system, but also in some groups, it was able to protect the mice. It also protected against live parasites and they were alive until we killed them ourselves for cytokines and spleen pathology tests. In our another studies, 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 (54), also IL23 and IL17 expression measured, after injection new leishmania vaccine and after re-exposure with live leishmania major (6).We with our past and present experiments about new formulation vaccine confirmed other scientist findings Regard to our findings: we know now;1) preliminary vaccine could have been induced protective effect after vaccination and against re exposure with live leishmania promastigote in Blab/c mice,2)because of Il-10 expression has lowest level in LBT group and second LT , down regulation for IL-10 and up regulation IL-12 for LBT group was seen post challenging,3)IL-4 expression influence on regulatory of this preliminary vaccine protective effects, although, LT group had highest IL-4 expression,5)this new vaccine could induce Th1 pattern which could survive a number of mice after 70 days and probably it could be more,6)This new vaccine induce Th1 pattern and increasing SWPs and prevent of induce Th2 pattern in LBT group,7) LT group has lowest level of SWPs and IL-12 less than LBT group, IL-10 less than LB group but more than LBT,8) LT group had most level of IL-4 and IFN-γ. While we are still don't know to yet; whether SWPs is Place to create and induce humoral antibody; so if it increase SWPs in order to induce humoral and induction of antibody, so does the production of type II cytokines. Our concluded is, if LBT group did not produce antibodies,their cytokine production would be fine, and also, if LT group did not produce antibodies, their cytokine production would be better than LBT. Because of most survived mice are in LT group, if they have had acceptable level of Th1 cytokines expression, it is the best group for vaccination.BCG has very side effect and these side effects are very severe in MSMD patients. Most important suggestions is: According to the satisfactory results of the studies conducted on the new cutaneous leishmania vaccine in the published articles, this article and three other articles under print that will be published soon, we suggest that the animal clinical trial (phase 1, II, III) is terminated. So that we can take firm steps to implement the human clinical trial.

5. Conclusion

Because of most survived mice are in LT group, if they have had acceptable level of Th1 cytokines expression, it is the best group for vaccination.BCG has very side effect and these side effects are very severe in MSMD patients. Most important suggestions is: According to the satisfactory results of the studies conducted on the new cutaneous leishmania vaccine in the published articles, this article and three other articles under print that will be published soon, we suggest that the animal clinical trial (phase 1, II, III) is terminated. So that we can take firm steps to implement the human clinical trial.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare that there is no conflict of interest

Statement of ethical approval

The present research work does not contain any studies performed on animals/humans subjects by any of the authors.

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