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Research Article

Correlation between Immune Responses of Th1,Th2 Cytokines, Macroscopic and Microscopic Findings of Mice and its Spleen and also both Spleen's and Lymph Nodes' CD3+, CD4+, CD8+, CD25+ Pre-Challenge of New Leishmania Major Vaccine in Balb/c Mice

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Abstract

Objective: Cutaneous leishmaniasis is a disease that is common in human and animal reservoir hosts with wild animals as the second reservoir. This parasite is seen as amastigote in vertebrates and promastigote in insects. Leishmaniasis affects about 12 million people from 88 countries. This parasite (Leishmania) creates a complex disease that is known as leishmaniasis.

Method: There are two paths for T helper cells (Th), which divide into Th1 (T Helper1) leading to increasing protection against intracellular pathogens and to Th2, leading to the deterioration of the disease. Based on their role and results from previous author experiments, the decision was made to evaluate new Leishmania vaccine's effects on both cellular and humoral factors (Th1 & Th2 cytokines) ,macroscopic evaluation of Balb/c mice and macroscopic spleen amounts, and also, compared variation of both spleen white pulp size and lymph nodes'CD3+, CD4+, CD8+ and CD25+cells with together.

Results: Our results show that IL-12 and number of pulp spleen had significant differences (p=0.001). CD3+ of lymph node was P<0.063 and CD4+ of spleen (P=097) almost near to significant and notable. IL-4, IL-10, CD25+, mouse weight, spleen weight, percentage of spleen weight/mouse weight, IL-17,and IL-23 had not significant differences. Interferon gamma, mean of pulp size, CD3+ and CD8+ from spleen had P<0.15, which is debatable.

Keywords: Leishmania, Vaccine, Th1, Th2, IL-17, IL-23 Spleen, Lymph node, CD3+, CD4+, CD8+, CD25+

Introduction

Leishmania parasite has mechanisms which enable its amastigote stage to survive in the hostile environment of the phagolysosome. To date no vaccine strategy in humans has had satisfactory results [1]. Results of one study showed that NKT cells should be considered when treating active leishmania infection as well as in the development of vaccines. Also, NKT cells activated by L. major observed in various models of leishmania infection have been variable and often conflicting. Most of this is probably due to different infection models and leishmania strains applied [2]. Recent progresses in understanding of how the innate immune system recognizes microbial stimuli and regulates adaptive immunity is being applied to "systems vaccinology" in vaccine discovery [3]. In previous studies, we showed that: [1] The new vaccine formula was safe and not toxic (Article on leishmania safety), [2] It increased spleen white pulp size expansion in Balb/c (susceptible) and small white laboratory (resistant) mice [3]

It changed from negative to positive DTH [2,4] in 48 hours [4,5]. Meanwhile, the study also identified Teucrium Polium as a new adjuvant used in BCG vaccine and evaluated its effects after repeated exposure on spleen white pulp [5,6]. Other researchers reported that increasing IL-12 and decreasing IL-10 cause immune protection against leishmaniasis infection.

In addition, we found that: Balb/c female mice that produced high IL-12 had higher

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survival rate and male mice with high IL-10 had lower survival rate [7]. We observed that this new vaccine in some injection doses and groups could not only increase IL-12, IL-4 and CD4+ pre-challenge, but also IL-12 and Il-4 were highest and IL-10 and IFN-γ lowest in the control group with 100 per cent survival rate [8]. It had effects on spleen white pulp expansion, the number of spleen white pulp, L-23 and IL-17 [9]. We know today that interleukin-1-beta and TNF-α (pro-inflammatory cytokines) are different in people with chronic, acute and healed leishmaniasis and the healthy ones [10]. Regarding the above studies [5,7], and this project results confirmed one of our hypotheses, that "alcohol extract of Teucrium Polium could be a safe adjuvant" in the Balb/c mice. Our second hypothesis, "the crude cocktail antigen preparation plus alcoholic extract of Teucrium Polium and BCG (LBT) or plus BCG alone (LB) is not recommended for the provisional vaccine in spite of their potency in both cell mediated and humoral immunity" was also confirmed, since the live BCG could produce mycobacterial infection particularly in the immune compromised or immune deficient subjects. Observing our pre-challenge data, we decided to find the correlation between immune responses of Th1, Th2 cytokines, macroscopic, microscopic findings of mice and its spleen and also both spleen's and lymph nodes' CD3+, CD4+, CD8+, CD25+ pre-challenge of Leishmania vaccine in Balb/c mice.

Material and Methods

This study was conducted in accordance with the Helsinki Declaration. The protocol is supported by both the research fellows of the School of Medicine and the Deputy Director of Research of Tehran University of Medical Sciences. Tehran, Iran. For detail procedures please refer to Latiynia, et al. [8,9]. In brief, Balb/c mice (n=40) were obtained which were three months old.

Culture and isolation of leishmania parasites

Leishmania parasites and promastigote antigens from the L. major, WHO strain prepared from the Pasteur Institute. It was cultured and grown in NNN medium supplemented with 5–10% fetal calf serum. The harvested parasites were washed three times with normal saline (0.9 per cent) or PBS. The parasites were counted using a Neubar chamber and then kept at 80°C until use. The collected parasites then reached the concentration of 5.92×10^{10} and were divided into five tubes of equal volume and the vaccine was prepared. Detailed procedures have already been described [8,9].

Vaccine injection

Just before the injection, the BCG vaccine 0.01 mg (2×10^5 CFU/0.1ml) was added to each bottle containing promastigote. Based on previous studies, 100 mg/0.1 ml or 200 mg/0.1 ml of leishmania protein was selected at each dose of the temporary vaccine to formulate and prepare the vaccine. The protein content of each dose was estimated by the Lowry method [11]. To prepare Teucrium adjuvant Palm, 400 mg of Teucrium Polium alcoholic extract in 1 ml distilled water 2.5 mg/0.1 ml was used for each of the doses of the leishmania antigens given above, and two injection doses containing 100 µg/0.1 ml antigens or 200 µg/0.1 ml containing adjuvants [8,9].

Vaccination

All doses of vaccine antigens of Leishmania and the bold reminder to the tail of mice were intra-dermally injected. Dosages (100, 200 µg protein) were used as follow: Group LB (100,200) received 100, 200 µg/0.1 ml antigen combined with BCG, Group LT (100, 200) received 100, 200 µg/ 0.1 ml antigen combined with BCG and alcoholic extract of Teucrium Polium, Group LBT (100, 200) received 100, 200 µg/0.1 ml antigen combined with BCG and alcoholic extract of Teucrium Polium. The control group did not receive any injection. All groups were injected subcutaneously with vaccine preparation at the tail and one week later using similar dose as booster dose. Twenty-five days after booster injection, all animals, plus the control group, were weighed and blood sampling was performed and they spleens' and lymphoid nodes' removed.

Spleen cell isolation and Hematoxylin and Eosin staining

All spleens were cut in equal length and width and fixed in 10 per cent formaldehyde buffer solution. The formalin fixed tissues include spleen was processed in a tissue processor. Paraffin blocks were made and tissue sections of 4 to 5 microns were prepared and stained with Haris Hematoxylin and Eosin. The fixed spleen was stained in paraffin blocks and stained in a tissue processor (tissue sections 5–6µm thick) with hematoxylin and eosin. The number and diameter of the white pulp of the spleen (lymphoid spleen follicles) were examined using a microscope light with the fragment of the eye. The diameter of the SWP sections was measured and compared with that in the other groups as well as the control group.

Cytokine assay

ELISA and sandwich methods were used to evaluate the ILs-4, 10, 12, 17, 23 and IFN-γ in animal serum and their serum levels were measured using an auto-reader at 405 nm.

Spleens and lymph nodes' cell isolation and flow cytometry

Splenic cells and lymph nodes' lymphocytes were obtained from of Balb/c mice for the collagenase method [8,12].

Statistical Analysis

Data were obtained using statistical software (SPSS Inc., Chicago, IL, USA). The means were analyzed using standard variance analysis / simple factorial test with two-way Student–Newman–Keuls method. The correlation coefficient was determined using a Pearson bivariate test.

Results

ANOVA results showed that IL-12 and (Mean Number Spleen White Pulp) MNSWP had significant differences (P=0.001), CD4+ in spleen = 0.097 and CD3+ in lymph nodes P=0.063 were near to significant P=0.05 (Table 1). The following results were obtained (Tables 2-4) and related to their scores:

IL-12: The lowest level related to injection group LBT and injection dose 100, while the highest level was seen in LB group with dose 200 µg/0.1ml.

IL-4: The highest level related to injection group LT and injection dose 100 and the lowest to groups LB and LBT and dose 200 µg/0.1ml.

		Sum of Squares	df	Mean Square	F	Sig.
Interlukin 12	Between Groups	12232030.067	2	6116015.033	8.081	.001***
	Within Groups	26488453.472	35	756812.956		
	Total	38720483.539	37			
Interlukin 4	Between Groups	.034	2	.017	.694	.506
	Within Groups	.915	37	.025		
	Total	.950	39			
Interferon gamma	Between Groups	18.571	2	9.286	2.341	.110
	Within Groups	146.765	37	3.967		
	Total	165.336	39			
Interlukin 10	Between Groups	2.719	2	1.360	.541	.587
	Within Groups	92.978	37	2.513		
	Total	95.697	39			
Interlukin17	Between Groups	604.110	2	302.055	1.100	.344
	Within Groups	10161.667	37	274.640		
	Total	10765.777	39			
Interlukin 23	Between Groups	1177.172	2	588.586	1.365	.268
	Within Groups	15950.937	37	431.106		
	Total	17128.109	39			
Cluster Determinant 3(S)	Between Groups	593.168	2	296.584	2.686	.117
	Within Groups	1104.295	10	110.429		
	Total	1697.463	12			
Cluster Determinant 8(S)	Between Groups	18.508	2	9.254	2.196	.158
	Within Groups	46.362	11	4.215		
	Total	64.869	13			
Cluster Determinant 4(S)	Between Groups	552.505	2	276.253	2.910	.097**
	Within Groups	1044.183	11	94.926		
	Total	1596.689	13			
Cluster Determint 25(S)	Between Groups	1.270	2	.635	.901	.434
	Within Groups	7.755	11	.705		
	Total	9.025	13			
Cluster Determinant 3(L)	Between Groups	351.616	2	175.808	3.085	.063**
	Within Groups	1481.751	26	56.990		
	Total	1833.367	28			
Cluster Determinant 8(L)	Between Groups	141.777	2	70.888	1.846	.182
	Within Groups	844.887	22	38.404		
	Total	986.664	24			
Cluster Determinant 4(L)	Between Groups	299.420	2	149.710	1.064	.362
	Within Groups	3237.299	23	140.752		
	Total	3536.719	25			
Cluster DeterminantCD25 (L)	Between Groups	18.205	2	9.102	.753	.483
	Within Groups	253.744	21	12.083		
	Total	271.949	23			
Number of pulp Spleen	Between Groups	562.416	2	281.208	7.802	.001***
	Within Groups	1333.559	37	36.042		
	Total	1895.975	39			
Mean of pulp Spleen size	Between Groups	240199.287	2	120099.644	1.984	.152
	Within Groups	2240235.660	37	60546.910		
	Total	2480434.948	39			

**statistical differences☺.05<P<0.1, ***statistical differences P=0.001

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

IL-10: The highest level related to injection group LBT and injection dose 200 and the lowest to groups LB and LT and dose 100μg/0.1ml.

IFN-γ: The highest level related to injection groups LB and LT and injection dose 100 and the lowest to group LBT and dose 200μg/0.1ml.

	N	Minimum	Maximum	Sum	Mean		Std. Deviation	Variance
	Statistic	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic	Statistic
Leishmania injection doses 100/200	40	0	200	5200	130.00	10.860	68.687	4717.949
mous weight	40	11.109	24.879	819.516	20.48790	.365305	2.310393	5.338
SPleen weigth	40	.038	.980	7.776	.19440	.039843	.251990	.063
percent spleen weight/ mous weight	40	34	74	2130	53.25	1.206	7.628	58.192
Number of pulp Spleen	40	25	54	1459	36.48	1.102	6.972	48.615
Mean of pulp Spleen size	40	1180.0	2234.0	73561.3	1839.033	39.8751	252.1922	63600.896
Interlukin 12	38	65.5	4303.0	114459.6	3012.095	165.9502	1022.9856	1046499.555
Interferon gamma	40	4.9	14.3	326.2	8.156	.3256	2.0590	4.239
Interlukin 4	40	1.06	1.81	51.34	1.2835	.02467	.15605	.024
Interlukin 10	40	3.02	12.60	183.52	4.5880	.24768	1.56645	2.454
Interlukin17	40	.00	69.80	668.15	16.7038	2.62700	16.61462	276.046
Interlukin 23	40	13.5	138.6	895.8	22.396	3.3135	20.9567	439.182
Cluster Determinant 3	13	44.6	85.0	703.6	54.123	3.2987	11.8935	141.455
Cluster Determinant 8	14	13.2	20.0	223.9	15.993	.5970	2.2338	4.990
Cluster Determinant 4	14	30.0	66.1	538.6	38.471	2.9619	11.0825	122.822
Cluster Determint 25	14	3.48	6.80	78.34	5.5957	.22268	.83319	.694
Cluster Determinant 3	29	70.6	99.4	2624.2	90.490	1.5026	8.0918	65.477
Cluster Determinant 8	25	8.0	29.3	423.0	16.921	1.2824	6.4118	41.111
Cluster Determinant 4	26	29.1	66.4	1108.5	42.635	2.3326	11.8941	141.469
Cluster DeterminantCD25 of lymph node	24	5.04	16.80	209.79	8.7412	.70190	3.43858	11.824
Valid N (listwise)	4							

Table 2: Descriptive Statistics: The results of Correlations of serum level IL-12, IL-4, INF-γ, IL-10, Il-17,IL-23 ,balb/c mice and it's spleen macroscopic findings and both spleen cell determinants: CD3+, CD8+, CD4+, CD25+ and lymph nodes cell determinants: CD3+, CD8+, CD4+, CD25+ between two injection doses(100 & 200µg/ml) no considering to three injection groups (LB,LT&LBT) (P<0.05)

IL-17: The highest level related to injection group LT and injection dose 100 and the lowest to group LBT and dose 200µg/0.1ml.

IL-23: The highest level related to injection group LT and injection dose 100 and the lowest to groups LBT and LB and dose 200µg/0.1ml.

MSWPS: The highest level related to injection group LB and injection dose 200 and the lowest to group LT and dose 100µg/0.1ml.

MNSWP: The highest level related to injection group LB and injection dose 200 and the lowest to group LT and dose 100µg/0.1ml.

Multiplication of MSWP × MNSW: The highest level related to injection group LB and injection dose 200 and the lowest to group LT and dose 100µg/0.1ml.

CD3+ (spleen): The highest level related to injection group LBT and injection dose 200 and the lowest to group LB and dose 100µg/0.1ml.

CD8+ (spleen): The highest level related to injection group LB and injection dose 100 and the lowest to group LT and dose 200µg/0.1ml.

CD4+ (spleen): The highest level related to injection group LBT and injection dose 200 and the lowest to group LB and dose 100µg/0.1ml.

CD25+ (spleen): The highest level related to injection group LBT and injection dose 200 and the lowest to group LB and dose 100µg/0.1ml.

CD3+ (Lymph nodes): The highest level related to injection group LT and injection dose 200 and the lowest to group LB and dose 100µg/0.1ml.

CD8+ (Lymph nodes): The highest level related to injection group LB and injection dose 100 and the lowest to group LT and dose 200µg/0.1ml.

CD4+ (Lymph nodes): The highest level related to injection group LB and injection dose 100 and the lowest to group LT and dose 100µg/0.1ml.

CD25+ (Lymph nodes): The highest level related to injection group LBT and injection dose 100 and the lowest to group LT and dose 200µg/0.1ml.

Results indicated in Table 3 show that:

LB group: Had the highest level of IL-12 (3075pg/ml), but it was less than the control group's level. The highest IFN-g (20.05 pg/ml), MSWPS (1785.5 pg/ml), MNSWP (38.6 pg/ml), multiplication of MSWPS × MNSWP (68920.3micron) also belonged to this group. It had the highest levels of CD3+ in spleen and CD4+ in lymph nodes. On the other hand, the lowest amounts of IL-23 (18.33 pg/ml), IL-17 (13.05 pg/ml) and IL-4 (1.95 pg/ml) which was equal to that of LBT and IL-10 (4.44 pg/ml) equal to LT and also the lowest levels of CD4+ (36.12), CD3+ (52.84), CD25 (5.0) in spleen, both CD3+ (86.85)and CD25 (8.56) in lymph nodes, were in this group.

LT group: The highest levels of IL-4 (2.45pg/ml), IL-17 (16.74 pg/ml), IL-23 (23.899 pg/ml) and CD3+ (96.35) in lymph nodes belong to this group. However, it had the lowest levels of IL-10 (4.44 pg/ml) which was equal to that of LB, MSWPS

Group	Number of Balb/C mice	Meant of Serum IL12 (pg/ml) $\bar{x}$ Min.-max.	Meant of Serum IL4(pg/ml) $\bar{x}$ Min.-max.	Meant of Serum IL10 (pg/ml) $\bar{x}$ Min.-max.	Meant of Serum IFN-γ (pg/ml) $\bar{x}$ Min.-max.	Meant of Serum IL17 (pg/ml) $\bar{x}$ Min.-max.	Meant of Serum IL23 (pg/ml) $\bar{x}$ Min.-max.	MSWPS (micron) $\bar{x}$ Min.-max.	MN-SWP $\bar{x}$ Min.-max.	multiplication of MNSWP & MSWPS	CD3+ $\bar{x}$ Min.-max. Spleen	CD8+ $\bar{x}$ Min.-max. Spleen	CD4+ $\bar{x}$ Min.-max. Spleen	CD25+ $\bar{x}$ Min.-max. Spleen	CD3+ $\bar{x}$ Min.-max. Lymph nodes	CD8+ $\bar{x}$ Min.-max. Lymph nodes	CD4+ $\bar{x}$ Min.-max. Lymph nodes	CD25+ $\bar{x}$ Min.-max. Lymph nodes	Survival rates (percent)
LB	12	3075	1.95	4.44	20.05	13.05	18.33	1785.50	38.6	68920.3	52.84	15.97	36.12	5.00	86.85	17.78	44.21	8.56	100%
LT	12	2986.7	2.45	4.44	17.62	16.74	23.89	1697.52	34.5	58564.4	55.67	15.17	36.65	5.80	96.35	12.23	37.25	7.58	100%
LBT	12	2919.5	1.95	5.24	10.63	16.38	18.43	1777.53	37.5	66657.4	64.92	15.81	46.31	6.01	92.8	16.17	41.84	9.36	100%
Control	6	3896	1.29	3.94	7.48	24.56	16.96	1897.22	35.0	66402.7	62	15.23	47.2	5.39	81.82	21.82	49.72	9.67	100%
Dose 100µg/0.1ml	18	2592.8	2.41	4.57	17.10	18.11	22.48	2022	32.8	66321.6	51.07	16.28	34.83	5.55	92.22	17.7	42.87	9.31	100%
Dose 200µg/0.1ml	18	3394.8	1.99	4.83	15.08	12.66	17.58	1800.98	40.8	73480	64.55	10.01	44.63	5.50	99.44	14.44	39.28	7.72	100%

Table 3: Effects of provisional Leishmania Vaccine on spleen parameters, IL-12, IL-4, IFN-γ, IL-10, and both CD3+, CD8+, CD4+ in spleen and CD3+,CD8+.CD4+,CD25+ in lymph nodes and Survival Rates of the Female Balb/C Mice.

Group	Number of Balb/C mice	Meant of Serum IL12 (pg/ml)_ $\bar{x}$ Min.-max.	Meant of Serum IL4(pg/ml)_ $\bar{x}$ Min.-max.	Meant of Serum IL10 (pg/ml) $\bar{x}$ Min.-max.	Meant of Serum IFN-γ (pg/ml) $\bar{x}$ Min.-max.	Meant of Serum IL17 (pg/ml) $\bar{x}$ Min.-max.	Meant of Serum IL23 (pg/ml)_ $\bar{x}$ Min.-max.	MSWPS (micron) $\bar{x}$ Min.-max.	MNSWP $\bar{x}$ Min.-max.	Multiplication of MNSWP & MSWPS	CD3+_ $\bar{x}$ Min.-max. Spleen	CD8+ $\bar{x}$ Min.-max. Spleen	CD4+ $\bar{x}$ Min.-max. Spleen	CD25+_ $\bar{x}$ Min.-max. Spleen	CD3+_ $\bar{x}$ Min.-max. Lymph nodes	CD8+ $\bar{x}$ Min.-max. Lymph nodes	CD4+ $\bar{x}$ Min.-max. Lymph nodes	CD25+_ $\bar{x}$ Min.-max. Lymph nodes	Survival rates (percent)
LB	12	--	+	+	+++ ++	----	++	--	++	++	--	++	----	+	+	-	-	---	100%
LT	12	---	++++	+	++++	--	+++++	----	-	--	-	-	---	++++	++++	-----	-----	-----	100%
LBT	12	----	+	++++	+	---	++	---	+	+	++	+	-	+++ ++	+++	---	---	-	100%
Control	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100%
Dose 100µg/0.1ml	18	-----	+++	++	+++	_	++++	+	--	-	---	+++	-----	++	++	--	--	--	100%
Dose 200µg/0.1ml	18	-	++	+++	++	-----	+	-	+++	+++	+	--	--	++	+++ ++	----	----	----	100%

Table 4: Results of core about the effects of provisional Leishmania Vaccine on spleen parameters, IL-12, IL-4, IFN-γ, IL-10, and both CD3+, CD8+, CD4+ in spleen and CD3+,CD8+.CD4+,CD25+ in lymph nodes and Survival Rates of the Female Balb/C Mice.

(1697.52micron), MNSWP (34.5) and multiplication of MSWPS × MNSWP (58564.4micron). Also, LT group had the lowest CD8+ (15.17) in spleen, CD8+ (12.23), CD4+ (37.25) and CD25+ (7.58) in lymph nodes.

LBT group: This group had the highest levels of CD3+ in spleen, IL-10 (5.24 pg/ml) which was more than that of the control group, the lowest IL-4 (1.95 pg/ml) which was equal to that of LBT, spleen's CD25+ and lymph nodes' CD25+. On the other hand, the lowest levels of IL-12, (2919.5 pg/ml), IL-4 equal to that of LB (1.95 pg/ml) and IFN-g (10.63 pg/ml) were in this group.

Dose 100 µg/0.1ml: This injection dose had the highest levels of IL-4 (2.41), IL-17 (18.11), MSWPS (2022 micron), multiplication of MSWPS × MNSWP (79869.0micron), spleen's CD25+ (5.55), and lymph nodes' CD8+ (17.7), CD4+ (42.87) and CD25+ (9.31). The lowest levels of IL-12 (2592.8 pg/ml), IL-10 (4.75 pg/ml), MNSWP (32.8), spleen's CD3+ (51.07), CD4+ (34.83), CD8+ (912.23), CD4+ (37.25) and CD25+ (7.58) in lymph nodes were in this injection dose.

Dose 200 µg/0.1ml: This injection dose had the highest IL-12 (3394.8 pg/ml), IL-10 (4.83 pg/ml), Spleen's CD3+ (64.55), CD4+ (44.63) and lymph nodes' CD+ (99.44). The lowest levels of IL-4 (1.99 pg/ml), IFN-g (15.08 pg/ml), IL-17 (12.66 pg/ml), IL-23 (17.58 pg/ml), MSWPS (1800.96micron), MNSWP (40.8), MSWPS × MNSWP (73480micron), CD8+ (10.01), spleen's CD25+ (5.50), and lymph nodes' CD8+ (14.44), CD4+ (39.28) and CD25+ (7.72) were in this injection dose.

Discussion

The most important finding for us is that the injections of new vaccine pre-challenge increased IL-12 with different adjuvants, two injection doses of 100 and 200 µg/0.1ml, with significant differences between all injection groups (LB, LT and LBT) (Table 1). This is an important point for the new vaccine, because of not only IL-12 and IFN-γ increased but also IL-4 a decreased (compared to normal). So, we can conclude that this harmless new vaccine provides immune defense against intracellular pathogens in Balb/c.

If our results were reversed," interferon gamma and interleukin 12 decreased and interleukin 4 and interleukin 10 increased", we concluded that absolutely antibody would be produced after vaccination and after re-exposure to intracellular pathogens (Leishmania) that led to fatal visceral leishmaniasis, and surely the vaccine has been ineffective. But fortunately, this new vaccine has not led to significant IL-4 and production in any of the two injection doses and three injection groups (Table 1). We are pleased to confirm latest study on this new vaccine of authors that measured delayed type hypersensitivity (DTH) for 24 and 48 hours and was able to turn the negative skin test to positive, also [4,5].

In studies we used two types of resistant and susceptible mice because of evaluated other influencing factors. Resistant and susceptible mice before challenge showed different results in different injection groups and turned the negative skin test to positive 48 hours after new antigen injection [4,5]. This new vaccine could expand the size of spleen white pulp in resistant and susceptible mice [4,14]. Our next experiments showed that different injection doses and injection groups had significant difference of Th1 and Th2 cytokine profile and expansion of spleen white pulp size in susceptible mice (Balb/c) against same new vaccine post challenging with live promastigote that was confirmed by our another reports [1,6,7,16,17].

Our observations on the results of injection groups showed that although IL-12 is highest in LB group, but, LT group is higher than LBT. IL-4 is highest in LT group. Early secretion of IL-4 has a regulatory role toward Th1 and this is a point mentioned as protectivity for vaccines [17]. LT group has the lowest IL-10 and high IFN-γ and highest level of IL-17. Furthermore, highest IL-23 belonged to LT group with lowest levels of increasing of spleen white pulp size, number of spleen's pulp and total amount of spleen pulp size. In recent studies reported that IL-17 has an important role in protecting against leishmania [18,19] which is important to limit decision and conclusion of our results. Latest studies show that Il-23 and 17 can affect protectivity against leishmaniasis with the increasing of IFN-γ and nitric oxide [20]. In LT group, spleen's CD3+, CD4 and CD8+ decreased and CD25+ which have regulatory role in increasing of immune response , whereas in lymph nodes as secondary immune tissue CD3+ increased but CD4+, CD8+, and CD25+ sharply reduced. These cells can play a role in survival of Leishmania parasites [21]. Previous studies suggested that CD4+, CD25+ Treg cells are found in cutaneous lesions [22]. and increased of intra-lesional FoxP3 and IL-10 cells could help disease progression in both murine and human Leishmania infection [23]. Considering all of our results and another previous studies we select temporary LT group and secondary LB as the best injection groups for vaccination studies.

According to our observations on the results of injection doses, dose 100 µg/0.1ml had the lowest IL-12 and IL-10 and the highest IL-4 and IFN-γ, the lowest IL-17 and the highest IL-23 which in most cases leads to Th1 immune response. The lowest level of total amount of spleen pulp size also belonged to dose 100 µg/0.1ml. Lowest CD3+ and CD4+ and highest CD8+ were observed in dose 100 µg/0.1ml, but CD25+ was not different between 100 and 200 µg/0.1ml. We can select temporary dose 100 µg/0.1ml group firstly and secondary dose 200 µg/0.1ml for future vaccination choice and further researches. What can be deduced is that the vaccine has so far been well-matched to global standards and we are in the final investigation to complete animal model studies that are re-exposure to the live parasite on the new Leishmania vaccine, and we will report its final results soon.

Conclusion

It seems that best injection group is LT and best injection dose is 100 µg/0.1ml. But we must consider and compare results of both post-challenging with live promastigote and post-vaccination together before arriving at the absolutely best injection group and injection dose.

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