In the name of God

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University of Tehran

TH1, Th2 serum cytokines and spleen white pulp changes against preliminary L. major vaccine injection and challenge with live L.major promastigotes in Balb /c mice.

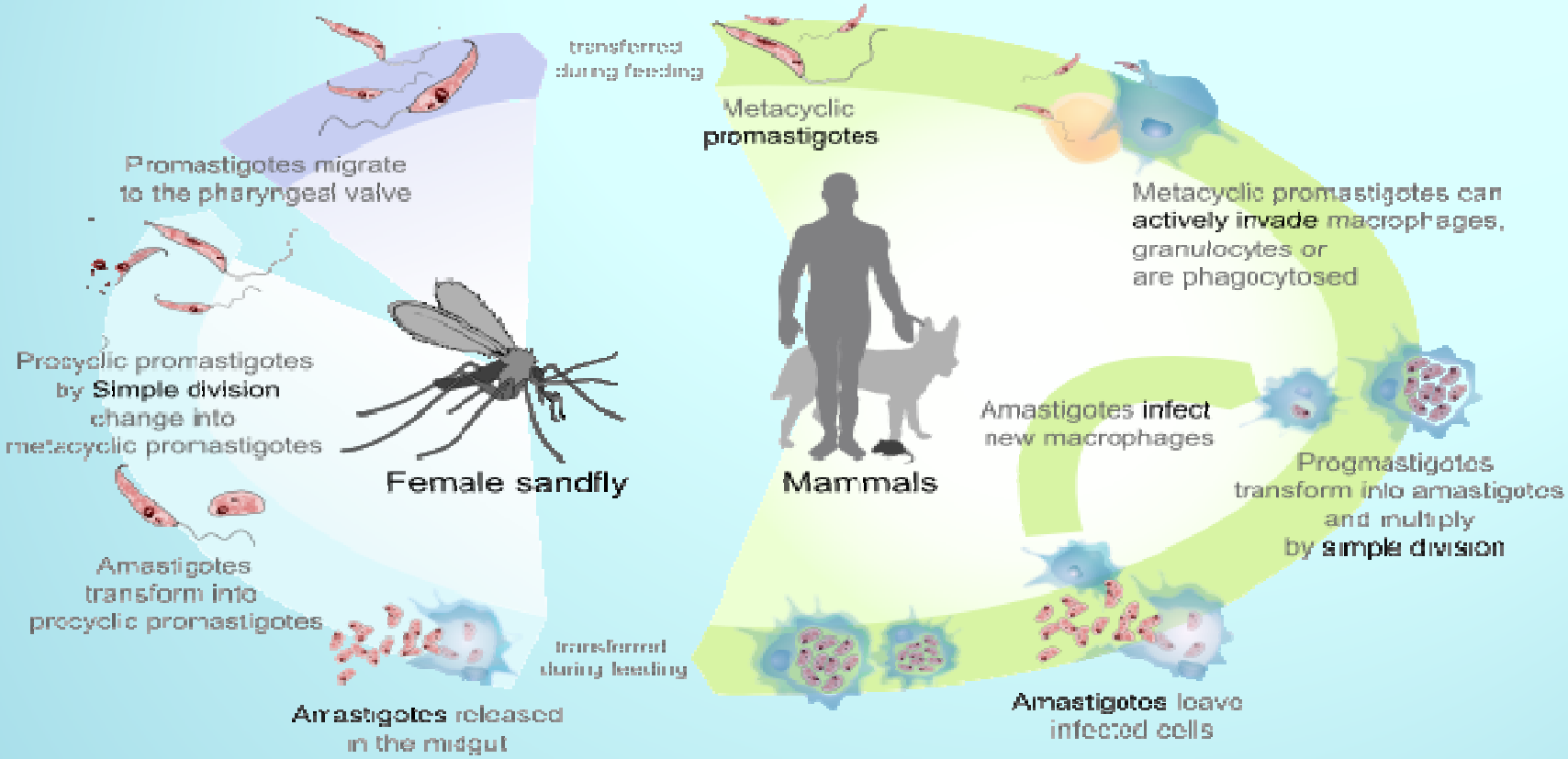
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Promastigotes of *Leishmania major*, 10×100, Giemsa stain.



Leishmaniasis life cycle



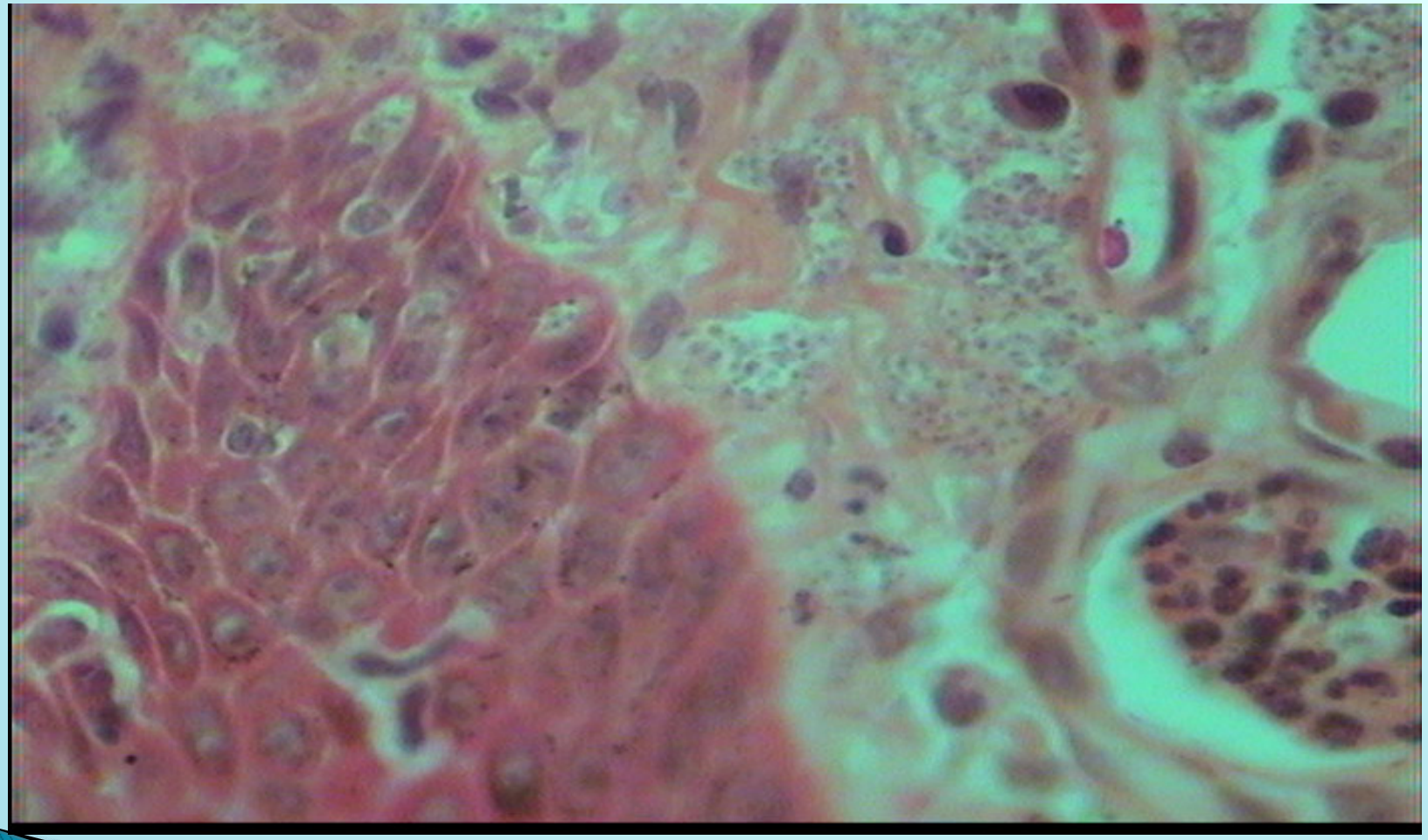
Cutaneous Leishmaniasis Sore(human)



Cutaneous Leishmaniasis Sore(dog)



Amastigotes of *Leishmania major within macrophges*, 4×100, H&E staining method(cutaneous lesion).



Diagnosis

- *L. major* should be considered chronic lesions in endemic area
- similar lesions :paracoccidiodomycosis, histoplasmosis, sporotrichosis, lobomycosis, lupus vulgaris, *Mycobacterium ulcerans*, syphilis, cutaneous sarcoidosis, and leprosy should all be considered as well
- Identify amastigotes in a Wright-Giemsa-stained touch preparation or through isolation of the parasites in cultures.

Materials and Methods

- :
- One hundred and twenty young adult female and male Balb/c mice
- Culture and Isolation of Leishmania Parasites:
- Leishmania promastigotes of L. major (WHO) strain were provided by the Tehran University of Medical Sciences
- were grown in NNN medium and RPMI 1640 supplemented with 5% - 10%FCS
- Harvested parasites were washed three times with normal saline solution (0.9%) or PBS .
- counted in a Neubar chamber and kept at -70°c until use. accumulated parasites diluted to a concentration of 5.92×10^{10}

Materials and Methods

- *Vaccine Preparation*

- suspended 5.92×10^{10} parasite was divided into 5 batches
- procedure compsed of: freeze and thaw, autoclave, or inactivation at 56°C
- At least antigen processed
- each vaccine was adminstered at 100 or 200 µg/0.1 mL *Leishmania protein* per dose
- content of protein in each dose was estimated by the Lowry method

Materials and Methods

- *Vaccine Preparation*

- The content of protein in each dose was estimated by the Lowry method
- two injection doses (100, 200 µg/mL) were prepared
- The vaccine was stored at 4°C until use
- Just before injection, 2×10^5 CFU live BCG /0.1 ml or 400 mg/0.1ml alcoholic extract of Teucrium polium dissolved in 1 ml distilled water and 2.5 mg/0.1 ml of used for each injection dosage solution (100, 200 µg/ml) .

Materials and Methods

- *Injection Groups*
- LT received 100-200 µg /0.1 ml of the crude cocktail antigen plus alcoholic extract of Teucrium polium as adjuvant
- LB received 100-200 µg /0.1 ml of the crude cocktail antigen preparation plus BCG as adjuvant
- LBT received 100-200 µg/0.1 ml of the same antigen preparation plus alcoholic extract of Teucrium polium and BCG as adjuvant
- Control had not any received antigen.
- Groups LT, LB and LBT were injected subcutaneously and were boosted two times with a weeks interval.
- *Challenge*
- A week after the last booster, all animals including were challenged with 3×10^5/0.1 ml live Leishmania major promastigotes.
- *Protectivity*
- The protective response was evaluated for 70 days. Evaluation parameters was composed of : lesion induction , and rates of survival .


LT-100
3-30-6149


LT.100
86-03-30-6149

Materials and Met animals survived hods

- *Cytokine assay*
- The serum of the survived animals was used for cytokine levels
- Levels of IL-4, IL-10, IL-12, and IFN-γ in the three injection groups and normal group were determined by sandwich ELISA and an automated micro plate reader, set at 405 nm, according to the recommendations of manufacturer
- The sensitivity limit was 20 picogram /ml for IL-4, IL-10, and IFN-γ.

Materials and Methods

- *White Pulp Size Assay*
- 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 the spleen white pulp size was evaluated using a light microscope with eye-piece graticule.

- *Statistical Analysis*
- 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.

Results

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).
- The ANOVA test between injection groups, Th1 cytokines (IL-12, IFN-γ) , Th2 cytokines (IL-4, IL-10)in 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-12 and IL-10 had inverse relationship and any time IL-12 increased IL-10 decreased also and Vice versa (Table 3).

Results

- *SWP & IL-12 correlation*

- 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
- 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)

Results

- 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.
- 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-12 and IL-10 had inverse relationship which any time IL-12 increased Il-10 decreased and Vice versa (Table3).

Results

- *SWP & IL-10 correlation*

- correlation between level 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,
- its Coefficients of Determination is 0.12. Correlation 2-tailed is 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 0.3 larger than ofo.5,which is near to significant(table 4).

Results

- 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 and normal group also .
- 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-γ and Th2 cytokines (IL-4, IL-10) and combined doses (100 and 200 µg/0.1 ml) and ANOVA test showed that means square of IL-4 between groups compared other Th1, Th2 cytokines was not significant (Table 3).

Results

- *SWP & IL-4 correlation*

- Correlation between level of IL-4 expression and increasing of SWPs is Inverse linear (negative) which show when the white pulp increased, IL-4 decreased. On the contrary, SWPs decreased, when the IL-4 increase. This relation was weak,
- Pearson Coefficient -0.021, which was moderate, and its Coefficients of Determination was 0.001. Correlation was not significant (P=0.919) but greater than critical number (0.05) (table4).

Results

- IFN-γ:

- The LB group had highest level (35.4 pg /ml) and27.2 pg/ml of IFN-γ related to the LT and also lowest concentration belonged to the LBT group (19.39 pg/ml) .IFN-γ in male (32 pg/ml) was higher than female mice (26.23 pg/ml) (table1). IFN- γ was not significant with 0.05 level (2-tailed) (Table2).

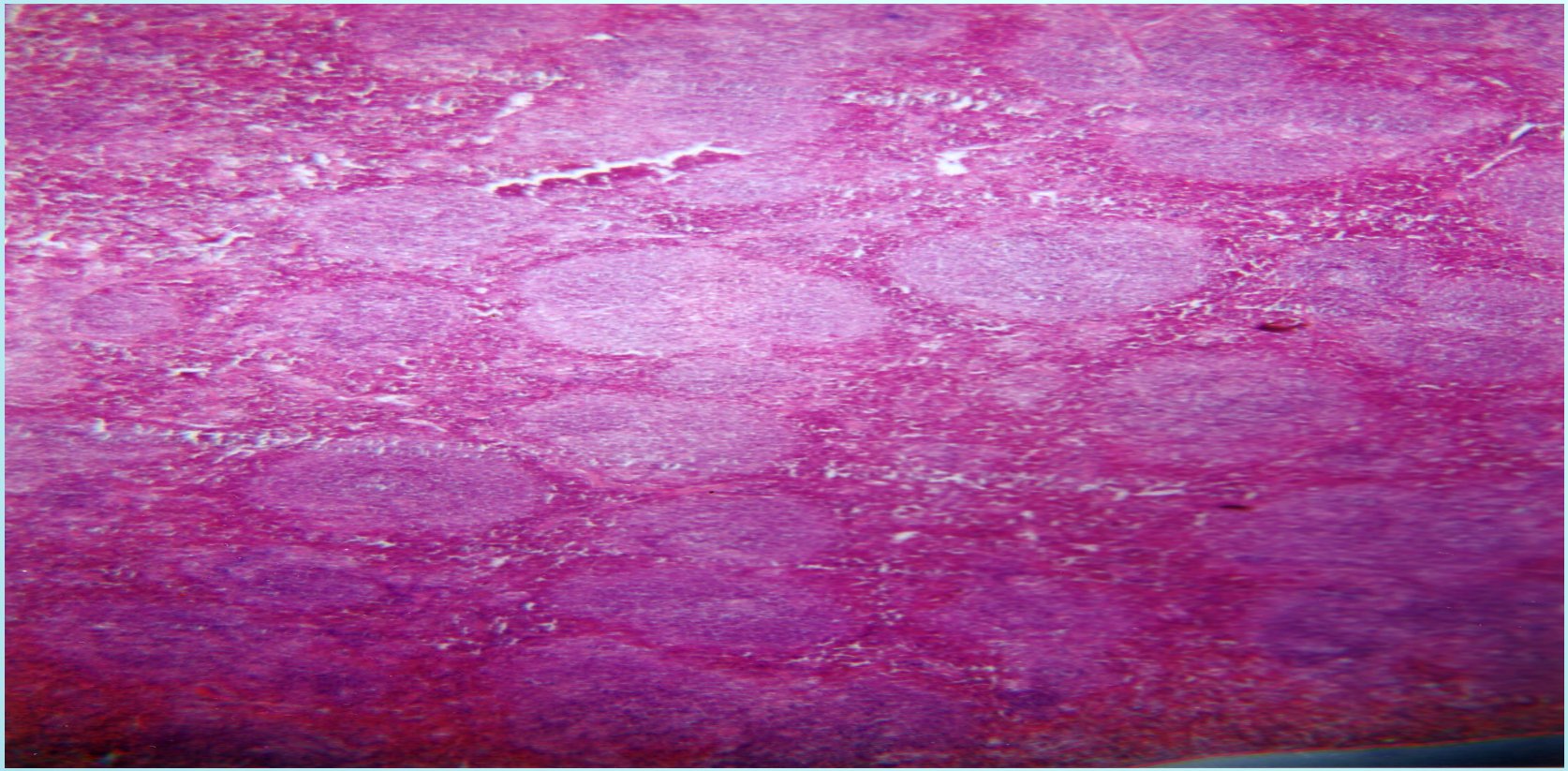
- Correlation between injection groups and Th1 cytokines (IL-12, IFN-γ) and Th2 cytokines (IL-4, IL-10) with combined doses (100 and 200 µg/0.1 ml) showed that three injection and normal groups', considering IL-12 and Multiple Comparisons of IL-12 with Tukey HSD with 95% Confidence by mean difference was significant at the 0.05 level. ANOVA test also showed that means square of IFN-γ between groups and compared to the other Th1, Th2 cytokines was not significant (Table 3).

Results

- *SWP &* IFN-γ *correlation*

- Correlation between level of IFN-γ expression and increasing of SWPs is Inverse linear (negative) which show when the SWPs increased, IFN ϒ decreased. On the contrary, when SWPs was decreased, IFN ϒ increase. Pearson Coefficients for this relation was weak (-0.173) and Coefficient of determination was 0.03, which showed week relation.
- Correlation of 2-tailed was not significant (P=0.409) and this was very greater than critical number (0.05). This difference was great and the correlation is not significant (Table 4).

Spleen White Pulp size-Normal Balb/c × 3.5



Group LB. dose100 Balb/c ×3.5

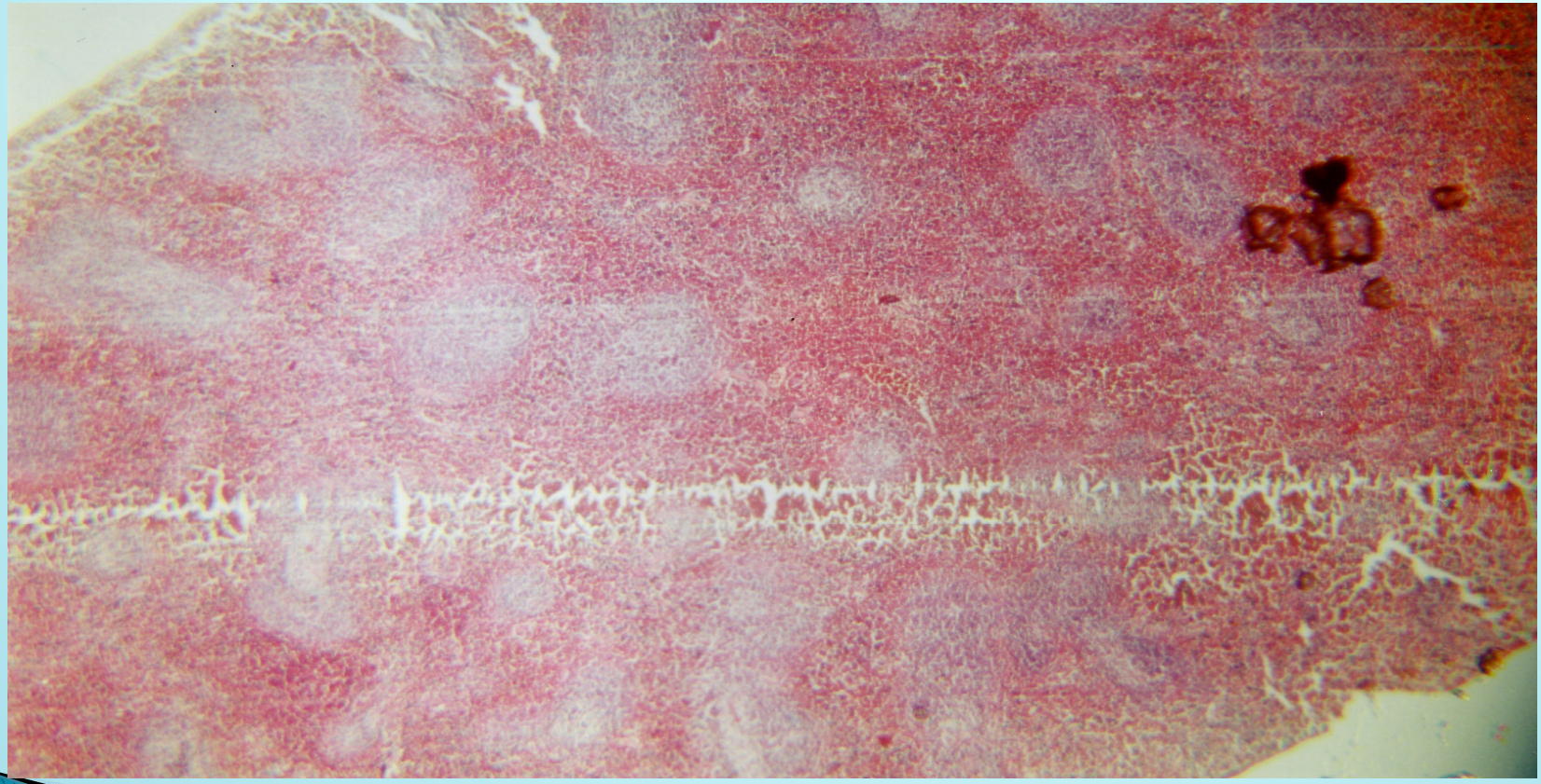


Table 1 : Th1 and Th2 serum cytokines levels and spleen white pulp sizes (SWPs) in the control and vaccinated groups of Balb/c mice after challenging with live L.major promastigot

Group	sex	IFN-γmin(pg/ml)	IL-12 min(pg/ml)	IL-4 min(pg/ml)	IL-10 min(pg/ml)	SWPs min(µm)
LT	F M T	31.2(23.1-39.1) 33.8(27.5-45.6) 33.03(23.1-45.6)a	710(326-1864) 21185(326-4000) a 293(668-4000)	17.9(13.9-21.8) 21.1(13.9-34.1) a 18.4(20.8-34.1)	23.5(18-26.5) 16.2(13.6-18) 21.1(13.6-26.5) a	573.8(308-657.5 503.2(392.7-8.6.97) 524.16(308-806.96)b
LB	F M T	30.2(26.5-34.9) 30.5 30.2(26.5-34.9)a	1364(307-3032) 55 1102(55-3032)a	17.5(14.2-19.9) 19.9 17.6(14.2-19.9)b	21.5(17.2-27) 27 22.7(17.2-27)a	620.5(565.72-699.2) 588 614(565.72-699.2)b
LBT	F M T	25.5(12.6-34.4) ND 25.5(12.634.4)b	3098(1828-4094) b ND 3098(1828-4094) b	18.4(17.1-18.9) ND 18.4(17.1-18.9)b	18.8(13.6-26) ND 18.8(13.6-26)a	914.3(623.4-1016.5) ND 914.3(623.4-1016.5) a
Control	F M T	26.7(23.8-29.4) ND 504.1(460.9-672.9) b	2709(864-3686) ND 2709(864-3686)c	19.2(14.2-27.5)a ND 26.7(23.8-29.4)b	17.8(14.7-22.7) ND 19.2(14.2-27.5)a	504.1(460.9-672.9) ND 17.8(14.7-22.7)a

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 and control challenged with live L.major promastigotes.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	639993.436	3	213331.145	7.362	.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.
IFN-γ Levels	Between Groups	98.548	3	32.849	.812	.497
	Within Groups	1295.035	32	40.470		
	Total	1393.583	35			
IL-12 Levels	Between Groups	1.696E7	3	5653365.43812	.651	.008
	Within Groups	3.890E7	32	15595.185		
	Total	5.586E7	35			
IL-10 Levels	Between Groups	263.395 1809.174	3	87.798	1.553	.220
		2072.569	32	56.537		
	Within Groups		35			
	Total					
IL-10 Levels	Between Groups	487.502 4308.083	3	162.501	1.207	.323
		4795.586	32	134.628		
	Within Groups		35			
	Total					

Table-4: Correlation between IL-12 and IL-10, IL-4, IFNγ on Doses 100 and 200 µg / 0.1ml combined with together. 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). ** is significant, UD is Undetermined,

	Correlations	SWPS(micrometer)	IL12 Levels	Il-10 levels	Il-4 levels	IFN-γ levelsg/ml)
SWPs min(µm)	Pearson Correlation Sig. (2-tailed) N	1 25	.582** .002 25	-.351 .085 25	-.021 .919 25	-.173 .409 25 25
IL12min(pg/ml)	Pearson Correlation Sig. (2-tailed) N	.582** .002 25	1 36	-.342* .041 36	UD	UD
IL10min(pg/ml)	Pearson Correlation Sig. (2-tailed) N	.351 .085 25	-.342* .041 36	1 36	UD	UD
IL4min(pg/ml)	Pearson Correlation Sig. (2-tailed) N	-.021 .919 25	UD	UD	1 36	UD
IFNγmin(pg/ml)	Pearson Correlation Sig. (2-tailed) N	-.173 .409 25	UD	UD	UD	 36

Discussion

- **Higher** survival rate was seen in LT group which received the antigen preparation plus the alcoholic extract of T.polium followed by live L.major challenge.
- Comparing, four groups of LT, LB, LBT and control, the lower survival rate was seen in control group challenged by live L.major promastigots.
- Higher white pulp size in the LBT group indicated induction of humoral immunity which it may not protect the animals against leishmania infection or progression.
- this antigen preparation also could induce cell mediated immunity which it seems to be a protective response as shown in previous studies. (41)

Discussion(continued)

- LB group has the highest level of IFN-γ (35.4 pg/ml), whether (27.2 pg/ml) related to the LT is lowest which belong to the LBT group (19.39 pg/ml).
- Male mice had higher IFNγ (32 pg/mL) than female (26.23 pg/ml).

- The resistance to leishmania conferred 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).

- Ldp23 is a powerful and selective activator of Th1 cytokines by mouse cells (41).

Discussion(continued)

- 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).
- 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.

Discussionc(continued)

- **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 challenging, 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 little increasing 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).

Discussion(continued)

- **Most of** the vaccines target the host DCs with adjutants, such as Bacillus Calmette Guerin (BCG),, or, more recently ,with non methylated CpG oligo deoxy nucleotides, which mimic nucleotide sequences common to bacterial DNA (18,19).
- Other adjuvant strategies being developed include the administration of Th1 promoting cytokines, such as IL-12, within the vaccine.
- In our previous research white pulp statue showed the lowest increasing size in the resistant mice and injection
- group I 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).

Discussion(continued)

- **Our previous results** indicated that the DTH responses and spleen white pulp statue differed 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. Previous studies show that CD4+ Th1 and Th2 regulate infection development. When L. major causes a single cutaneous lesion, or undergo spontaneous cure, subject is resistant and probably infection is inhibited in macrophage via innate immunity and production of interferon gamma and IL12 by Th1 response 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+ Th2 response in vivo, where as high doses may favor Th1 cells develop (13).

Discussion(continued)

- **The higher** DTH responses and lower spleen white pulp size were noticed in animals that had 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 Th1 and Th2 cells is important for designing vaccines and until the time of being, experiments that have addressed this issue have had conflicting results.
- Our previous 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 the same dose booster) group, group II (Leismania plus BCG), and group III (Leismania plus BCG plus the same dose booster).

Discussion(continued)

- **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 induce that subsequently antibody production. Upon antigenic challenge, these primary follicles develop into characteristic secondary follicles containing germinal centers, where rapidly dividing B cells (centroblasts) and plasma cells are surrounded by dense clusters of concentrically arranged lymphocytes(45).
- NKT cells should be considered both when treating active Leishmania infection as well as in the development of vaccines.
- 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).

Discussion(continued)

- **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).*

Discussion(continued)

- **An important** finding in our previous study is that, lower expansion of SWPs 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 IL12 by 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 SWPs was seen in group II, III that received 400 µg/0.1ml and 500 µg/0.1ml antigen which lead to high dose and low dose concepts that proposed by Uzonna *et al in 2004.*

Discussion(continued)

- **In present research** also, highest SWPs was related to LBT group and lowest SWPs was also to LT group however, LB group had SWPs greater than LT group and 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)

Discussion(continued)

- The present confirmed other scientist findings.
- Regard to our results following suggestion could be presented at this stage of my study:
- 1- preliminary vaccine used in this study could have been induced protective effect after vaccination and against re exposure with live leishmania, in Blab/c mice.
- 2-IL-10 expression has lowest level in LBT group and, down regulation for IL10 and up regulation of IL12 for LBT group were seen post challenging.
- 3-IL-4 expression influence could regulate the preliminary Vaccine protective effects, in this regard the LT group had highest IL-4 expression.
- 4- This new vaccine could induce Th1 pattern which could increase survival rates in a number of mice after 70 days.
- 5-This new vaccine induce Th1 pattern and could increase SWPs and prevent induction of Th2 cytokines pattern in LBT group.
- 6- LT group has lowest level of SWPs and reasonable level of IL12,but, the level is lower than LBT group, IL-10 less in LT group is less than LB group but more than LBT.
- 7- LT group had reasonable levels of serum IL-4 and IFN-γ

Discussion(continued)

- **And while we do not know**:
- whether SWPs is the site to create and induce humoral antibody,
- The serum levels of leishmania antibody in the LBT group .whether LBT is a good inducer of antibody induce and cytokines, and alsowhether the LT group is a bad inducer of antibody and a good inducer of protective cytokines.
- Since most of the survived mice are in LT group, it can be imagined that the group received antigen plus alcoholic extract of T.polium produced a protective cytokines profiles or network which contained the organism at the site of enry .
- And we know that the BCG could have a side effect especially in MSMD patients.

suggestions

- Most important suggestions are;
- 1- Additional studies should be conducted on animal models towards final process of phase I clinical trial of this preliminary leishmania major vaccine in the future.Furthermore, we are planning to measure IL23 and IL17 expression, after adminstration of new leishmania vaccine folllowed by challenging with live leishmania major in the future.
- 2- In this regard the rate of antibody production (IgM, IgG) will be conducted .

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Thank you of your attention

