Original Article

Immune Responses in Cutaneous Leishmaniasis: *in vitro* Thelper1/Thelper2 Cytokine Profiles Using Live Versus Killed *Leishmania major*

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Abstract

Background: Recovery from cutaneous leishmaniasis (CL) leads to protection against further lesion development. In contrast, vaccination using killed parasites does not induce enough protection; the reason(s) is not currently known but might be related to different immune response induced against live versus killed parasites. In this study, Th1/Th2 cytokine profiles of CL patients were evaluated against live versus killed *Leishmania major*.

Methods: In this study peripheral blood mononuclear cells (PBMC) of the volunteers with active CL lesion (CL), history of CL (HCL) and healthy volunteers were cultured and stimulated with live or killed *Leishmania major*, the supernatants were collected and levels of IFN-γ, IL-5 and IL-10 were titrated using ELISA method.

Results: The results showed that IFN-γ levels in CL patients ($p< 0.001$) and HCL volunteers ($p< 0.005$) are significantly higher when stimulated with live than stimulated with killed *L. major*. IFN-γ production in PBMC volunteers with CL and HCL stimulated with live or heat-killed *L. major* was significantly ($p< 0.001$) higher than in unstimulated ones. The level of IL-5 in CL patients ($p< 0.005$) and HCL volunteers ($p< 0.001$) are significantly lower when stimulated with live than killed *L. major*. There was no significant difference between the levels of IL-10 in PBMC stimulated with either live or killed *L. major*.

Conclusion: It is concluded that using live *Leishmania* induces a stronger Th1 type of immune response which justify using leishmanization as a control measure against CL.

Keywords: Cutaneous leishmaniasis; Live *Leishmania*; Heat-killed Leishmania; In vitro Immune response

Introduction

Leishmaniasis is a neglected tropical disease (1) caused by different *Leishmania* species and transmitted by the bite of various *Phlebotomus* species. The disease is endemic in 102 countries, it is estimated that 10th of the world population is at risk to contract one form of the disease, worldwide at least 12 million are affected and 1.5–2 million is the annual incidence. Over 90% of VL occurs in six countries of Bangladesh, Ethiopia, Brazil, India, Sudan, and South Sudan and 70–75% of CL cases occur in 10 countries including Afghanistan, Algeria, Brazil, Iran, Peru, Ethiopia, North Sudan, Costa Rica, Colombia and Syria. The burden of the disease (DAILYs) is reported to be 3.3 million (2, 3). Leishmaniasis is a major public health problem in some endemic foci, infection with *Leishmania* parasites depends upon the *Leishmania* species causes the disease and the host genetic background which governs the immune response. The outcome of exposure to Leishmania might be asymptomatic or might induce a range of clinical manifestations from a simple self-healing skin lesion to non-healing form of CL, diffuse CL, mucocutaneous leishmaniasis, a fatal systemic disease and post

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kala-azar dermal leishmaniasis (PKDL). Current available measures are not practically effective to control leishmaniasis; there is ample evidence to prove that in principle development of a vaccine against leishmaniasis is possible. Numerous *Leishmania* antigens have been introduced as potential vaccine candidate and a few of first-generation vaccines have been tested in human clinical trials, but so far no vaccine is available against any form of human leishmaniasis. Control measures are not effective and chemotherapy accompanies drawbacks and is not always effective. In experimental murine models of leishmaniasis, control of infection and induction of protection require generation of Th1 type of immune response represented by production of a high level of IFN-γ in the absence of Th2 response, in most of the mouse strains like C57BL/6 mice, infected with *Leishmania major* induce a self-healing lesion and upon healing the animals are protected against further lesion development, so protection accompanies development of Th1 response, whereas in susceptible BALB/c mice infected with *L. major* induces progressive lesions which eventually generalized and kills all the mice, the progressive disease occurs with development of a Th2 type of immune response with high levels of IL-4, IL-5 and IL-10 and no IFN-γ production (4, 5). In human leishmaniasis recovery from CL induces by natural infection or Leishmanization (LZ) usually accompanies with protection against subsequent lesion development. Leishmanization is an inoculation of live virulence *Leishmania* intradermally into susceptible individuals to induce artificial CL lesion, LZ showed to be an effective control measure against CL. The surrogate marker(s) of cure and protection in human leishmaniasis is not well known, but in some studies, a correlation between the generation of Th1 type of immune response and protection is shown (6-13). In this study, volunteers with active of CL lesion, volunteers with a history of CL and healthy volunteers were recruited. Blood samples were collected and stimulated with either live or killed *L. major* and Th1/Th2 cytokine profile was checked.

Materials and Methods

Ethical considerations and Study population

The study was carried out in leishmaniasis clinic of Center for Research and Training in Skin Diseases and Leprosy, Tehran University of Medical Sciences (CRTSDL, TUMS) during March 2017 to March 2019. The study proposal was approved by the Ethical Committee of Tehran University of Medical Sciences. The study groups are as follow: (i) 16 parasitologically proven patients with active CL lesion(s), (ii) 25 volunteers with history of CL, (iii) 15 healthy volunteers from non-endemic areas with no history of leishmaniasis. The lesion of every CL patient and volunteers with history of CL were parasitologically confirmed by direct smear, and/or culture, PCR was used to identify the *Leishmania* species.

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from a heparinized blood sample which was collected from each volunteer by using Ficoll- Hypaque density centrifugation.

Parasites and Antigens

Leishmania major (MRHO/IR/75/ER) was isolated from the spleen of an infected BALB/c mouse, the parasites were cultured in NNN media and sub-cultured in complete RPMI media supplemented with 10% FCS and penicillin/ streptavidin. The promastigotes were harvested at stationary phase, washed 3 times, and resuspended in PBS and then divided into two parts; one part was used as it was to stimulate PBMCs (5×105 live promastigotes/well), and the second part was killed by boiling for 5 minutes and checked microscopically to assure that the parasites are killed, the killed *Leishmania* was used to stimulate PBMCs (the same number as live promastigote, 5×105/well).

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Blood sampling collection

Heparinized blood samples (10mL) were collected from every volunteer, blood samples were diluted 1:1 using RPMI. The diluted blood samples were overlaid gradually with Ficoll-Hypaque (30–40% of blood volume) in 50ml disposable centrifuge tubes. Isolated PBMC was washed 3 times using PBS and resuspended in RPMI containing 10% heat- inactivated fetal calf serum, 100U/ml penicillin and 100µ/ml/ml streptomycin.

Peripheral blood mononuclear cell culture

The cell number was adjusted to a final concentration of 1×106PBMC/ml, the cells were cultured in triplicates in U-bottom 96 well culture plates (2×105 cells in 200µl volume/well) for 72h at 37 °C in 5% CO2, the PBMCs were stimulated with either PHA(5µg/ml), live parasites (5×105/well) or heat-killed *L. major* (5×105/well), or culture media alone with no stimulation as a negative control. After 72 hours of incubation, 150µl of the supernatants were carefully collected from each well and the triplicates were pooled and kept at -80 °C until used.

Cytokine assay

The level of IL-5, IL-10, and IFN-γ were titrated in culture supernatants using ELISA method (Biosciences, USA) according to the manufacture's recommendation. Briefly, the plates were coated with anti-IFN-γ/IL-5/IL-10 mAb in PBS, pH 7.4, and incubated at 4 °C overnight. After blocking the wells using buffer containing PBS plus 0.05% (v/v) Tween 20, supernatants were added to each well. Biotin-labeled mAb in incubation buffer was added to each well and as enzyme streptavidin-HRP was used. The reaction was developed using 3, 3′, 5, 5′-tetramethylbenzidine (TMB) substrate and stopped with 0.5 M H2SO4 solution. The plates were washed after each step of incubation using PBS +0.05% (v/v) Tween20. The plates were read at 450nm using a reader (BioTek, USA). The mean optical densities (ODs) of triplicate cultures were compared with the standard curves prepared using recombinant IL-5, IL-10, and IFN-γ. The cytokine levels represent the differences between the ODs of test and background wells. The results are expressed in pg/ml (mean +SD) of triplicates experiments (11, 12).

Statistical analysis

Shapiro-Wilk test was used to evaluate the normality of numerical variables. The Quantitative variables were expressed as mean ±SD (standard deviation), and qualitative variables as a percentage. Chi-square test and Fisher's exact test was used to examine the relation between qualitative variables. Pair T-test /Wilcoxon signed-rank test was used to the intergroup comparison. One-way ANOVA with a followed post-hoc test (Bonferroni) was used to multiple comparisons between the groups. P-value less than 0.05 was considered statistically significant. The SPSS Version 16 (SPSS Inc., Chicago, IL, USA) software was used for all statistical analyses.

Results

The background information of the volunteers including gender, age and, cutaneous leishmaniasis status are presented in Table 1. In healthy volunteers, the mean +SD of IFN-γ production in PBMC stimulated with live or heat-killed parasites versus unstimulated were (408.5+198.2), (369.5+199.07) and (179.7+88.1), respectively. There was a significant difference in IFN-γ production in PBMC of healthy volunteers stimulated with live *L. major* ($p< 0.001$), heat-killed *L. major* ($p< 0.005$) *vs.* unstimulated, there was no significant difference in IFN-γ production in PBMC of healthy volunteers stimulated with heat-killed *L. major* or live *L. major* (Fig. 1A), In active lesion of CL volunteers the mean +SD of IFN-γ production in stimulated with live or heat-killed *L. major vs.* unstimulated were (1,953.2+1,032.8), (1,157.0+678.3) and (220+182), respectively. IFN-γ production in PBMC volunteers with active lesion stimulated with live or

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heat-killed *L. major* was significantly ($p< 0.001$) higher than with non-stimulated (Fig. 1a), IFN-γ production in PBMC of volunteers with active lesion stimulated with live *L. major* was significantly ($p< 0.001$) higher than stimulated with heat-killed *L. major* (Fig. 1a). In volunteers with history of CL, the mean +SD of IFN-γ production in PBMC stimulated with live *L. major*, heat-killed *L. major* and unstimulated were (2337.4+879), (1729.4+597.1), and (209+107), respectively. IFN-γ production in PBMC stimulated with live or heat-killed *L. major* was significantly ($p< 0.001$) higher than unstimulated PBMC, there was a significantly higher IFN-γ ($p< 0.005$) production in PBMC of history of CL volunteers stimulated with live *L. major* in comparison with PBMC stimulated with heat-killed *L. major* (Fig. 1a). In healthy volunteers, the mean +SD of IL-5 production in stimulated with live or killed *L. major* and unstimulated were (157.3+53.9), (191.2+ 86.6) and (160.3+36.8), respectively. There was no significant difference in IL-5 production in PBMC of healthy volunteers stimulated with live or killed *L. major* and unstimulated PBMC, There was no significant difference in IL-5 production in PBMC of healthy volunteers stimulated with live *L. major* or heat-killed *L. major* (Fig. 1b). In PBMC collected from volunteers with active CL lesion, the mean +SD of IL-5 production in PBMC stimulated with live, heat-killed *L. major* or unstimulated were (187.8+69.1), (272.6+113.5) and (198.7+97.5), respectively. There was no significant difference in IL-5 production in PBMC of active lesion CL volunteers stimulated with live *L. major* compared with unstimulated PBMC, but there was a significant difference ($p< 0.05$) in IL-5 production in PBMC of volunteers with active CL lesion stimulated with killed *L. major* compared with unstimulated PBMC. IL-5 production in PBMC of volunteers with active lesion stimulated with live *L. major* was significantly ($p< 0.005$) lower than PBMC stimulated with heat-killed *L. major* (Fig. 1b). In volunteers with a history of CL, the mean +SD of IL-5 production in PBMC stimulated with live, heat-killed *L. major* and unstimulated were (133.4+50.4), (196.7+68.8) and (148.6+ 675), respectively. There was no significant difference in IL-5 production in PBMC of volunteers with a history of CL stimulated with live *L. major* in comparison with unstimulated but there was a significant difference ($p= 0.005$) in IL-5 production in PBMC of volunteers with history of CL stimulated with heat-killed *L. major* in comparison with unstimulated (Fig. 1b). IL-5 production in PBMC of volunteers with history of CL stimulated with live *L. major* was significantly ($p< 0.001$) lower than that of PBMC stimulated with heat-killed *L. major* (Fig. 1B). In healthy volunteers, the mean+SD of IL-10 production in PBMC of volunteers stimulated with live *L. major*, heat-killed *L. major* and unstimulated were (272.2+180.2), (263.4+ 236.4) and (212.3+160.2), respectively. There was no significant difference in IL-10 production in PBMC of healthy volunteers stimulated with live *L. major*, heat-killed *L. major* and unstimulated PBMC, there was no significant difference in IL-10 production of PBMC of healthy volunteers stimulated by heat-killed *L. major* and PBMC stimulated with live *L. major* (Fig. 1c). In volunteers with active CL lesion, the mean +SD of IL-10 production in PBMC stimulated with live *L. major*, heat-killed *L. major* and unstimulated were (451.1+ 291.1), (464.4+264.6) and (286.6+28.1), respectively. There was no significant difference in IL-10 production in PBMC of volunteers with active CL lesion stimulated with live *L. major*, and unstimulated but there was a significant difference ($p< 0.05$) in IL-10 production in PBMC of volunteers with active CL lesion stimulated with heat-killed *L. major* or unstimulated PBMC, there was no significant difference in IL-10 production in PBMC of volunteers collected from active CL lesion stimulated with live *L. major* or killed *L. major* (Fig. 1c). In volunteers with history of CL, the (mean +SD) of IL-10 production in PBMC stimulated with live *L. major*, heat-killed *L. major* and un-

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stimulated PBMC were (359.2+120.4), (365.3+ 198.6) and (241+98), respectively. There was no significant difference in IL-10 production in PBMC of volunteers history of CL stimulated with heat-killed *L. major* or live *L. major* with unstimulated, there was no significant difference in IL-10 production in PBMC of volunteers with a history of CL stimulated with live *L. major* or killed *L. major* (Fig. 1c). There was a significant difference ($p< 0.001$) between the IFN-γ levels in supernatants of PBMC collected from volunteers with active CL lesion and PBMC of healthy volunteers stimulated with heat-killed or live *L. major* (Fig. 1a). There was a significant ($p< 0.001$) difference between the IFN-γ levels in supernatants of PBMC of volunteers with history of CL and PBMC of healthy volunteers stimulated with heat-killed or live *L. major* (Fig. 1a). There was a significant difference between the IFN-γ levels in supernatant of PBMC of volunteers with history of CL and volunteers with active CL lesion stimulated with heat-killed *L. major* ($p< 0.005$) and but there was no significant difference between the IFN-γ levels in supernatant of PBMC of volunteers with history of CL and volunteers with active CL lesion stimulated with live *L. major* (Fig. 1a). There was a significant difference between the IL-5 levels in supernatants of PBMC of volunteers with active lesion and PBMC of healthy volunteers stimulated with heat-killed *L. major* ($p< 0.005$) but there was no significant difference between the IL-5 levels in supernatants of PBMC of volunteers with active lesion and that of healthy volunteers stimulated with live *L. major* (Fig. 1b). There was a significant ($P< 0.005$) difference between the IL-10 levels in supernatants of PBMC of volunteers with active lesion and that of healthy volunteers stimulated with live *or* heat-killed *L. major*. But there was no significant difference between the IL-10 levels in supernatants of PBMC of volunteers with a history of CL lesion and that of volunteers with active lesion stimulated with live or heat-killed *L. major*. There was no significant difference between the IL-10 levels in supernatants of PBMC of volunteers with active lesion and that of volunteers with history of CL stimulated with live *L. major* or heat-killed *L. major* (Fig. 1c).

Table 1. Characteristic of the volunteers

	HCL Cases	Active lesion	Healthy
Number of volunteers	25	16	15
Mean of Age (Year)	32	33.6	45
Gender (M/F)	16.9	13.3	13.2
Number of lesion	30	51	0
Position of the lesion			
Upper limb	11	12	-
Lower limb	2	2	-
Trunk	1	1	-
Face	4	2	-
Mean duration of the lesion (months)	5.4	2	-

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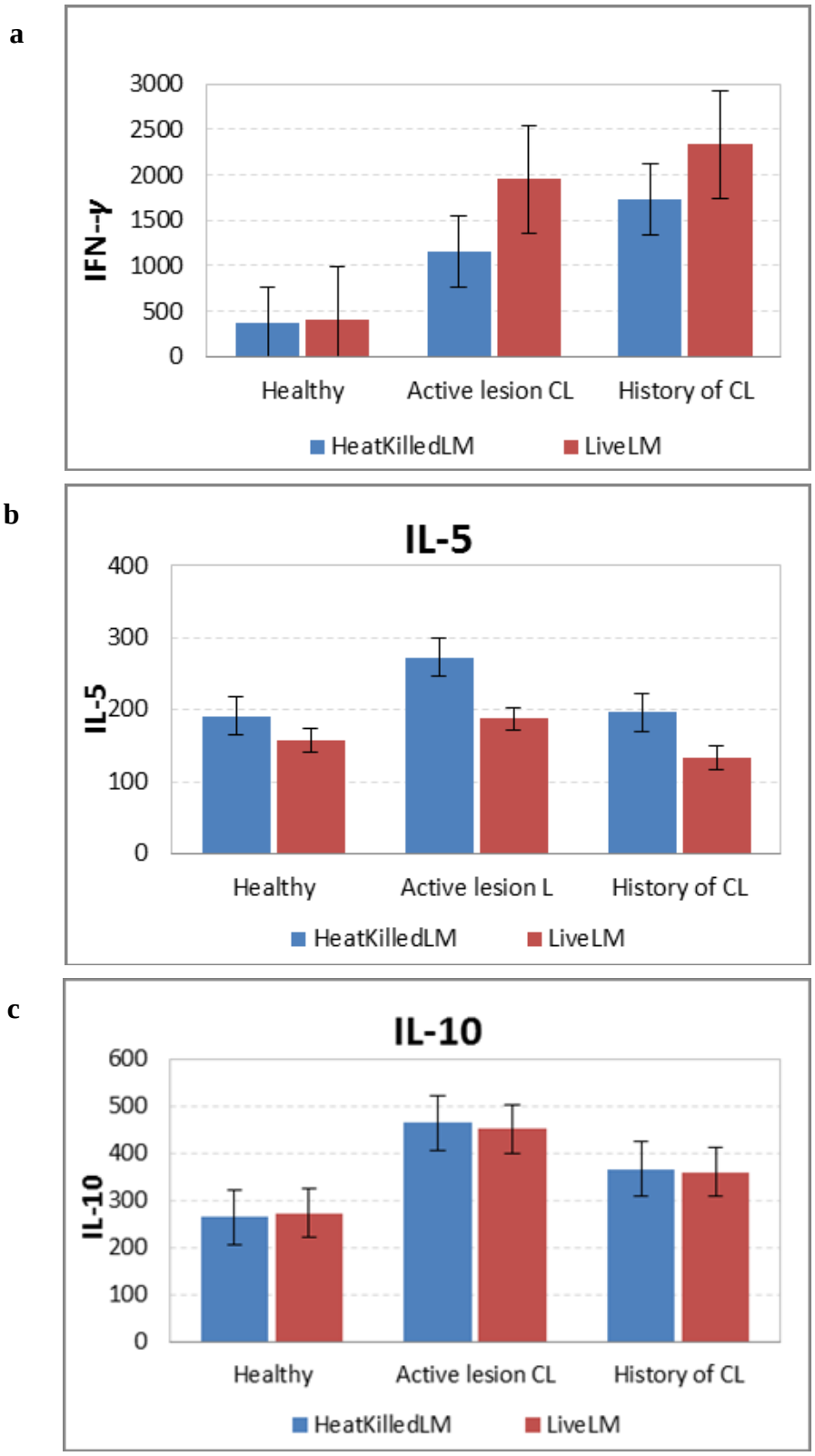

Fig. 1. Cytokine levels (pg/ml) in culture supernatants of peripheral blood mononuclear cell (PBMC) of different volunteers (healthy, active cutaneous leishmaniasis(CL) lesion, History of CL) stimulated with Live *L. major* or Killed *L. major*, (a) IFN-γ level in culture, (b) IL-5 level in culture, (c) IL-10 level in culture

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Discussion

Recovery from leishmaniasis induced by natural infection or leishmanization leads to the development of lifelong protection against further disease development. Leishmanization is the most effective control measure against CL (14). Experiences with vaccination using killed *Leishmania* vaccines showed limited protection. It seems that the quantity/quality of immune response which generated in vaccines receiving killed *Leishmania* vaccine apparently is not similar to the immune response generated against live *Leishmania*. Although the reason of the different immune response against live *vs.* killed *Leishmania* is not well known but one reason might be that likely live *Leishmania* is presented by both endogenous and exogenous antigen presentation pathways but killed *Leishmania* is only presented by the exogenous pathway. Studies on murine model of leishmaniasis indicated that parasite persistence plays a crucial role in the induction of a strong and long-term immunity, another factor such as differences in the nature of the early inflammatory and/or immune responses to live *vs.* killed parasites might be important, animals inoculated with live *Leishmania* induced more IFN-γ and less IL-4 than those which were injected with killed *Leishmania* (it was shown that infection with live parasites, (virulent or avirulent) leads to extensive activation and IFN-γ production by both CD4+ and CD8+ T cells while injection with killed parasites only lead to activation of CD4+ T cells. This activation of CD8+ T cells may play a role in the regulation of the early immune response to live *vs.* killed parasites. Live *Leishmania* promastigotes showed to directly activate primary NK cells to produce IFN-γ in the absence of IL-12 and professional antigen presenting cells while Killed parasites unable to do so, this means that live *Leishmania* activates NK cells by direct mechanism but killed *Leishmania* do not activate NK cells. In another study, C57BL/6 mice which were clinically cured from *L. major* lesion, skin-resident CD4+ T cells were found in the skin far from the site of the primary infection and were able to enhance protection against a later challenge by producing IFN-γ and recruiting circulating T cells to the skin. It was shown that live and dead parasites can differ in their ability to induce cellular responses in healthy donors as defined by IFN-γ production and cell proliferation (15-25). Historically, leishmanization showed to be highly efficacious control measure against CL, in contrary, the results of several clinical trials using different *Leishmania* species killed by various ways showed that killed *Leishmania* does not induce immune response strong enough to protect against natural infection. In this study, the Th1/Th2 profile of volunteers with a history of CL and volunteers with active cutaneous leishmaniasis lesions were evaluated against live *L. major* in comparison with killed *L. major*. IFN-γ production in PBMC of volunteers with active lesion ($p< 0.001$) and a history of CL ($p< 0.005$) stimulated with live *L. major* was significantly higher than stimulated with heat-killed *L. major*. IL-5 production in PBMC of volunteers with active lesion ($p< 0.005$) and volunteers with history of CL ($p< 0.001$) stimulated with live *L. major* was significantly lower than PBMC stimulated with heat-killed *L. major*. There was no significant difference in IL-10 production in PBMC of volunteers with active CL lesion stimulated with live *L. major* and killed *L. major*. IL-5 and IL-10 in the supernatant of PBMC collected from volunteers with active lesion were significantly ($p< 0.005$) higher than healthy volunteers stimulated by live or killed *L. major*. The level of IFN-γ in volunteers with a history of CL was higher than active lesion but not significantly, and the level of IFN-γ in volunteers with active lesions was significantly higher ($p< 0.001$) than healthy volunteers, this means that Th1 response which is necessary for protection against leishmaniasis is induced upon recovery. IL-5 level in volunteers with active lesion(s) was more than vol-

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unteers with a history of CL. The results of this study showed that the intensity of Th1 type of immune response is significantly higher in PBMC stimulated with live *Leishmania* than stimulated with killed *Leishmania*, the current results showed why natural infection/l eishmanization induces protection but vaccination with killed *Leishmania* does not induce enough protection. This study is in agreement with previous studies carried out in which it was shown that responses to live and dead promastigotes are different in the induction of cytokines and response to live in which response to live parasites was parasites produced significantly more IFN-γ than the group of animals injected with killed stronger than dead ones (20). In another study, it was shown that the group of mice inoculated with live *Leishmania* parasites produced significantly more IFN-γ than the group of animals injected with killed parasites (25).

Conclusion

It is concluded that using live *Leishmania* parasites in vitro induces a significantly stronger Th1 type of immune response than killed *Leishmania* which justifies using live *Leishmania* (leishmanization) as a control measure to protect against cutaneous leishmaniasis.

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