

Evaluation of *In Vitro* Production of IFN- γ , IL-10, IL-12 and IL-13 by Blood Cells in Patients with Cutaneous Leishmaniasis Lesions

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ABSTRACT

This study investigated the *in vitro* production of interferon- γ , interleukin (IL)-10, IL-12, and IL-13, after antigenic stimulation of the cells (with *Leishmania* antigen and lipopolysaccharide) using whole blood from patients with cutaneous leishmaniasis lesions caused by *Leishmania tropica* and in normal volunteers with history of cutaneous leishmaniasis.

ELISA results showed that the mean production of interferon- γ by cells of whole blood in patients with lesions in response to *Leishmania* antigen was significantly lower than corresponding values in volunteers with history of cutaneous leishmaniasis ($P < 0.05$) and significantly higher levels of IL-10 production in patients with lesions were observed compared with cured volunteers of the disease ($P < 0.01$). A similar level of IL-12, including p40 subunit of IL-12, was detected in both groups tested in this study in response to stimulation of parasite antigen. The levels of the IL-13 after stimulation with *Leishmania* antigen were significantly more in patients compared with volunteers with history of cutaneous leishmaniasis ($P < 0.01$). There was no significant difference in the mean production of IFN- γ , IL-10, IL-12 and IL-13 by PHA or LPS stimulated cells from patients with lesions and volunteers with history of the disease, indicating that there was no qualitative defect in cytokine production in these patients.

In this study, we have detected the decreased production of interferon- γ by cells of patients with lesions of cutaneous leishmaniasis in response to parasite antigen and unbalanced production of regulatory cytokines such as IL-10 and IL-13 using the whole-blood stimulation assay technique. The required small volume of blood and the rapid set up time are the advantages in this assay technique. Using this assay for further immunodetection of cytokines may confirm its value for clinical investigation.

Keywords: Interferon-Gamma; Interleukin 5; Interleukin 10; Interleukin 12; Interleukin 13; *Leishmania tropica*; Leishmaniasis

INTRODUCTION

Leishmaniasis is a public health problem in several countries of the Indian sub continent, South Western Asia, Africa and Latin America. Infection in humans with the parasite causes a spectrum of clinical manifestations, ranging from healing cutaneous to mucosal and visceral leishmaniasis.^{1,2}

Clinical outcomes depend on the parasite species and the specific immune responses to *Leishmania* antigens. It is known that a single strain of *Leishmania* can give rise to more than one clinical forms of the disease.³ Localized cutaneous leishmaniasis (LCL) caused by *Leishmania major* (*L. major*) or *L. tropica* is common in different parts of Iran.⁴ Infection with these species may be subclinical or manifest as cutaneous lesions which may persist for months to years, or heal spontaneously within a year.⁵

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The disease is initiated by the bite of an infected female sandfly and the concurrent intradermal inoculation of the protozoan parasite. In experimental infection of inbred mice with *L. major*, activation of two distinct CD4⁺ T helper cell subsets, Th1 and Th2, have been described. Resistant mice such as C57BL/6 and C3H when infected with *L. major*, resolution of infection is associated with expansion of Th1 that produces interferon- γ (IFN- γ), IL-2 and IL-12. In contrast, other strains of mice (notably BALB/c) develop severe and uncontrolled lesions following infection with *L. major*. In this strain of mice progression of infection is associated with expansion of Th2 subset that secretes IL-4, IL-5, IL-10, and IL-13.^{6,7}

In humans, the healing of lesions in cutaneous leishmaniasis (CL) appears to be associated with resistance to experimental or natural reinfection,^{8,9} however the roles of immune cells and their various mediators in healing or progression of leishmaniasis remain unclear.¹⁰ Studies have shown that lymphocytes from patients with active Leishmaniasis produce mainly IFN- γ and some IL-4 in response to stimulation with the parasite antigen.¹¹ Cure following an infection is associated with the production of IFN- γ .¹² In mice, the inability to control Leishmaniasis has been correlated with the absence of INF- γ production by T-cells leading to failure to activate macrophages to kill intracellular *Leishmania* parasites.⁶ IFN- γ is regulated by other cytokines such IL-10 and IL-12. IL-10 inhibits the production of IFN- γ and thus IL-10 has been referred to as a cytokine that promotes the development of a Th2 response to *L. major* infection in mice.¹³ IL-12 has been shown to play a role in the optimal production of IFN- γ . Injection of recombinant IL-12 during the first week of infection with *L. major* in susceptible BALB/c mice resulted in the development of a Th1 response and allowed healing their lesions.⁹ Several observations indicated that IL-13 is involved in susceptibility to *L. major* infection.^{14,15} The use of IL-13 deficient mice and IL-13 transgenic mice indicates that IL-13 is important for the generation of the Th2 cells.¹⁴

This study was undertaken to assess the *in vitro* production of interferon- γ , interleukin (IL)-10, IL-12, and IL-13, after antigenic stimulation of the cells (with *Leishmania* antigen and lipopolysaccharide) using whole blood in patients with CL lesions caused by *Leishmania tropica* and to compare these values with those in volunteers with history of the disease.

MATERIALS AND METHODS

Patients and Control Group

Two groups of patients, from Bam area (South East of Iran) where localized CL caused by *Leishmania tropica* is endemic, took part in this study: Group one consisted of sixteen patients (nine women and seven men) with lesion(s) of CL. These patients were diagnosed by a combination of clinical and parasitological criteria. Lesion(s) in these patients lasted from six months to two years and during this time all these patients showed resistance to pentavalent antimonial drug therapy. As the lesions caused by *L. tropica* may persist up to two years, these patients were considered as patients with lesions of CL. A physical examination was performed on each patient by a dermatologist. The patients ranged in age from 10 to 42 years. Group two consisted of volunteers with history of CL and regarded as positive control group. These individuals who resided in endemic area and previously presented cutaneous lesion caused by *L. tropica* were cured following appropriate treatment with Glucantime. They ranged in age from 18 to 29 years and lesion(s) in these individuals had healed at least six months before blood sampling. The patients and control groups consented to take part in this study. Four to five milliliters of venous blood was drawn from each subject into sterile endotoxin-free blood collection tube containing 100 units sodium heparin. Each blood sample was processed within one hour after collection.

Antigens and Mitogen

Leishmania antigen and lipopolysaccharide (LPS, *Escherichia coli* 0111; B4, Boivin method, Difco Laboratories, Detroit, MI) were used as antigens. Based on cross reactivity antigens between *L. major* and *L. tropica*, *L. major* antigen was used and prepared by freeze-thawed, whole-cell sonicate of promastigotes in stationary phase.¹⁶ The prepared *Leishmania* antigen in PBS containing cocktail of Protease inhibitors (Sigma, St Louis, MO, USA) and LPS were stored in 250 μ l aliquots at -20°C. Undiluted aliquots were taken once a week and stored at 4°C. 10 μ l of aliquots obtained and added to the RPMI-diluted whole blood aliquots to obtain *Leishmania* antigen at final concentration of 50 μ g/ml and LPS at final concentration of 1 μ g/ml.¹⁷⁻¹⁹ Preliminary experiments revealed that the selected *Leishmania* antigen concentration was the most appropriate for IFN- γ , IL-10, IL-12 and IL-13 production in the whole blood assay. Phytohemagglutinin

(PHA, 5 µg/ml, Sigma Diagnostics) was used as a positive control for cell reactivity.^{19,20}

Whole Blood Stimulation System

Heparinized whole blood sample from each subject was diluted 1:5 with RPMI-1640 (Gibco Life Technologies, Paisly, UK). The diluted blood was cultured in one ml volume in endotoxin-free sterile tube in duplicate. The cells of diluted whole blood were either cultured alone as control or stimulated with *Leishmania* antigen (50µg/ml) or LPS (1 µg/ml)¹⁷⁻¹⁹ or PHA (5 µg/ml).^{19,20} The tubes were incubated in a humidified air atmosphere at 37°C with 5% CO₂ for 72 hours. Culture supernatant fluids were collected at 3 days post stimulation for all antigen treated and PHA treated cells and control and were then frozen at -20°C until assayed for cytokines. These fluids were coded and laboratory personnel were blinded throughout the experimental procedures.

Enzyme-Linked Immunosorbent Assay for Interferon-γ, IL-10, IL-12, and IL-13

The frozen culture supernatant fluids were thawed at room temperature and cytokines levels were measured with commercial assay kits for IFN-γ, IL-10, IL-12 and IL-13 supplied by Biosource Europe S.A. (Nivelles, Belgium) according to the manufacturer's instructions. The absorbance of each well was read at 492 nm and cytokine concentrations in the samples were calculated with a standard curve generated from recombinant cytokines. Cytokine values were expressed as picograms/milliliter (pg/ml).

Statistical Analysis

Statistical analysis was performed using SPSS, version 11. The results were presented as the mean ±

standard deviation (S.D.). Student's *t* test was used to analyze differences in variables between cases and cured volunteers. Pearson's correlation coefficient (*r*) and the significance (*P*) were calculated to determine whether correlations existed between the different response parameters. *P*<0.05 was considered significant.

RESULTS

Data on IFN-γ production by cells of whole blood after stimulation with *Leishmania* antigen and LPS are presented in Table 1. The mean production of IFN-γ by cells of patients with lesions was significantly lower than corresponding values in volunteers with history of CL (mean±SD, 132.3±83.8 vs. 566.6±266.1 pg/ml, *P*<0.01). The mean production of IFN-γ in response to PHA was similar in both groups (883.9±323.1 vs. 1060±89.6, *P*=0.08) (Table 1 and Figure 1).

Regarding the production of IL-10, it was observed that significant levels of IL-10 were produced in response to *Leishmania* antigen in patients with lesions compared with volunteers with history of the disease (mean, 73.14±43.7 vs. 15.0±6.8, *P*<0.01). Stimulation of the cells of these patients with LPS resulted in the elevated level of IL-10 in the culture supernatants compared with volunteers with history of CL (87.1±61.2 vs. 56.2±17.1, *P*=0.1), (Table 1 and Figure 1).

The levels of the secretion of IL-13 after stimulation with *Leishmania* antigen in patients with lesions were significantly higher compared with the value in volunteers with history of the disease (138.4±60.6 vs. 47.0±20.6 pg/ml), *P*< 0.005).

Table 1. *In vitro* cytokine production (mean ± SD) of stimulated whole blood cells with either *Leishmania* antigen or lipopolysaccharide (LPS) or mitogen (PHA) from cases with lesions of cutaneous leishmaniasis (group 1) and volunteers with history of the disease (group 2).

Groups	Cytokines	Mean Production of Cytokine (pg/ml) Induced by <i>Leishmania</i> Antigen	Mean Production of Cytokine (pg/ml) Induced by LPS	Mean Production of Cytokine (pg/ml) Induced by PHA
Group 1 (Cases)	IFN-γ	132.3±83.8	208.6±93.3	883.9±323.1
	IL-10	73.14±43.7	87.1±61.2	402.1±323.3
	IL-12	22.6±16.5	123.4±74.4	240.7±60.6
	IL-13	138.4±60.6	75.1±16.5	1025.1±750.7
Group 2 (Cured Volunteers)	IFN-γ	566.6±266	185.0±123.8	1060±89.6
	IL-10	15.0±6.8	56.2±17.1	323.3±173.7
	IL-12	16.0±9.3	178.3±86.5	341.6±155.3
	IL-13	47.0±20.6	70.0±25.3	1293.3±732.6

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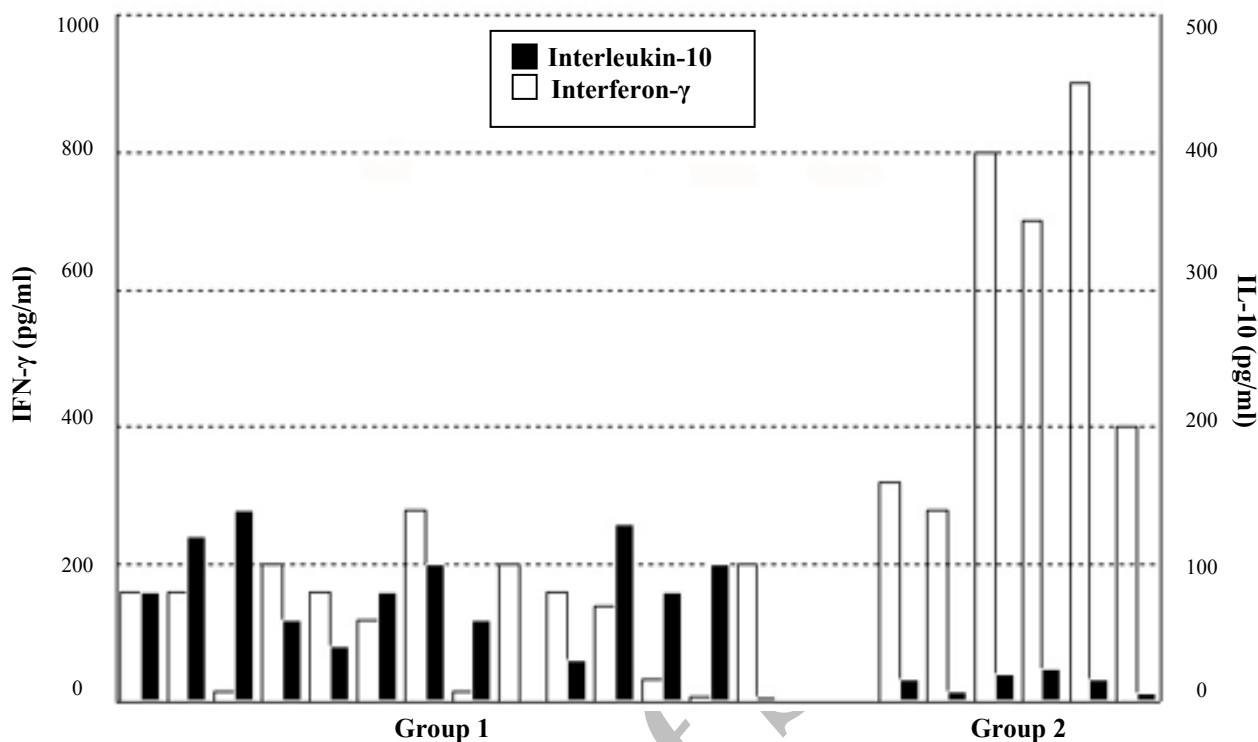


Figure 1. Cytokines (IFN- γ and IL-10) production by *Leishmania* antigen stimulated whole blood cells from patients with lesion (s) of cutaneous leishmaniasis (group 1) and volunteers with history of the disease (group 2).

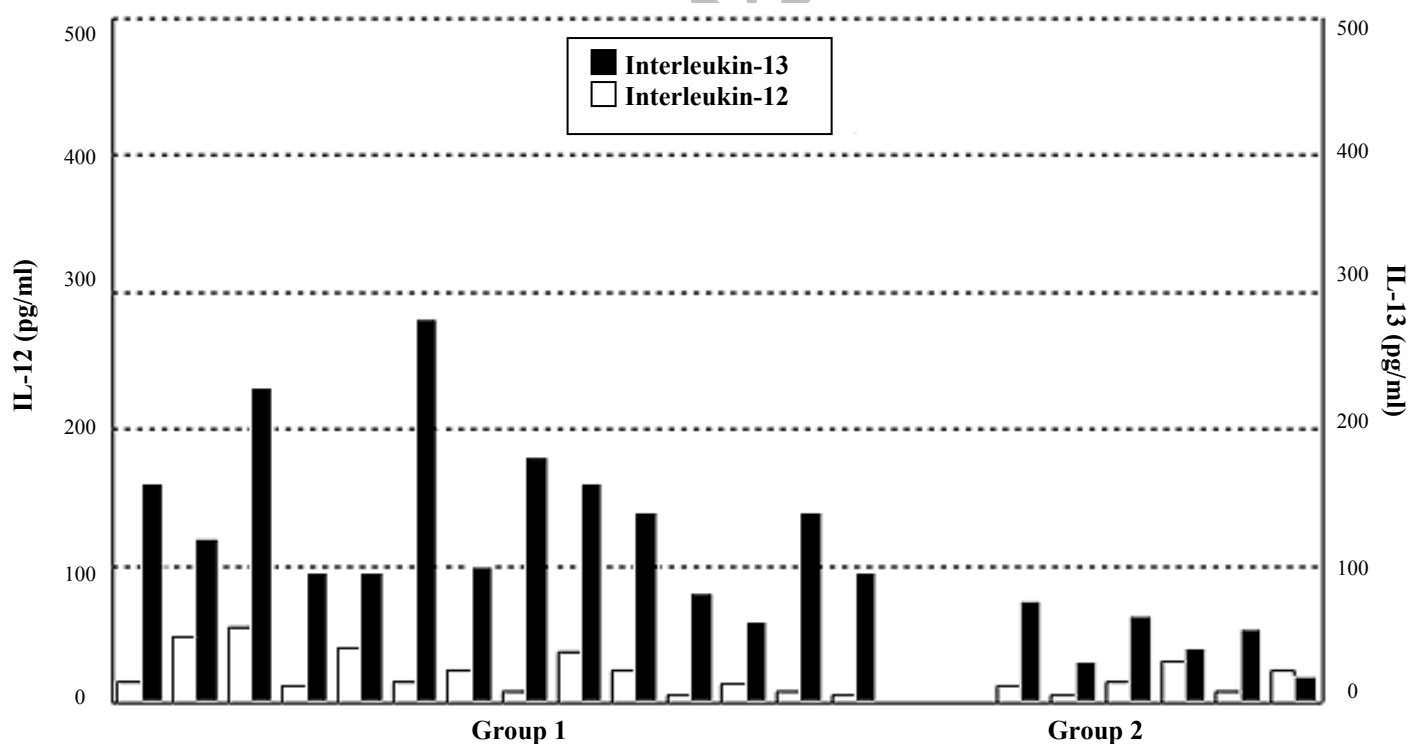


Figure 2. Cytokines (IL-12 and IL-13) production by *Leishmania* antigen stimulated whole blood cells from patients with lesion (s) of cutaneous leishmaniasis (group 1) and volunteers with history of the disease (group 2).

LPS induced similar IL-13 production in the culture fluids of patients and cured volunteers (75.1 ± 16.5 vs. 70.0 ± 25.3 , $P=0.6$). IL-12, including p40 subunit of IL-12, was released in lower level in both groups tested in this study in response to stimulation with parasite antigen (mean: 22.6 ± 16.5 in patients with lesions vs. 16.0 ± 9.3 in cured volunteers, $P=0.3$). LPS induced similar IL-12 production in cells from patients and cured volunteers (123.4 ± 74.4 vs. 178.3 ± 86.5 , $P=0.2$) (Table 1 and Figure 2).

Overall, there was no significant difference in the mean production of IFN- γ , IL-10, IL-12 and IL-13 by either PHA or LPS stimulated cells from patients with lesions and volunteers with history of the disease. Mean production of IFN- γ in these patients was not correlated with IL-12 production but a significant correlation was observed between IFN- γ and IL-10 production in culture supernatants after stimulation with *Leishmania* antigen. ($n=20$, $r=0.53$, $P<0.05$). A significant correlation was also observed between IL-13 and IL-10 production in culture supernatant fluids from these patients ($n=20$, $r=0.46$, $P<0.05$).

DISCUSSION

During early invasion of *Leishmania* parasite, T cells and the cytokines that they release play a critical role in determining the nature of the immune response and the outcome of the infection.⁷ Therefore in both human and experimental leishmaniasis, identification of the pattern of cytokine production for a better understanding of disease progression is of great importance.

In experimental leishmaniasis, cure is related to the predominance of a Th1 response, since this leads to the production of IFN- γ and activation of parasite infected macrophages. Disease progression is associated with the development of Th2 response, as the consequence IL-4, IL-10 and IL-13 are generated leading to inactivation of infected macrophages.^{6,7,8}

In this study, by using whole-blood cell stimulation assay, we found the cells of CL patients with lesion(s) exhibited low IFN- γ production after *Leishmania* antigen stimulation (Table 1 and Figure 1). A previous study, which investigated the IFN- γ gene expression in peripheral blood mononuclear cells (PBMC) from cases with non-healing CL in response to *in vitro* stimulation of recombinant *Leishmania* antigen, reported low level of expression of mRNA for this cytokine in the PBMC.²¹ Thus, one possibility is that the diminished IFN- γ production in these patients might be due to down regulation at the transcriptional level. In experimental CL, it has been

suggested that the healing of lesions depends on the early presence of macrophages, the activation of these cells by type 1 interferon and, later, by low amounts of IFN- γ probably produced by NK cells, consequently, the Th1 cells amplify the killing of the parasite when they release more IFN- γ .²²

In a typical Th1 and Th2 responses, besides IFN- γ , other cytokines such as IL-10, IL-12 and IL-13 have been shown to be important factors in the regulation of immune responses. The pattern of production of these cytokines in this study is presented in figures 1 and 2. The concentration of IL-10 production in the culture supernatants from patients was higher compared with those from cured individuals ($P<0.01$). The role of IL-10 in experimental murine leishmaniasis as a suppressive cytokine has been shown by several experiments,^{11,13,23} BALB/c mice which are deficient in IL-10 production were more resistant to *Leishmania* infection.²³

In this study, we investigated whether low IFN- γ release which is seen in these patients, is associated with reduced IL-12 production. Our study showed decreased production of IL-12 in these patients. A previous report in experimental *L. major* infection in susceptible BALB/c mice indicated low production of IL-12 in the chronic phase of infection, suggesting that a rise in the production of biologically active IL-12 is required for persistent production of IFN- γ .²⁴

The level of IL-13 showed a significant increase in patients with lesion(s) when compared with cured volunteers ($P<0.005$), (Table 1). A study in experimental murine leishmaniasis has demonstrated a disease exacerbate role for IL-13 as well as IL-4 in the mice which were deficient in either IL-13 or IL-4 gene respectively. Thus, deficient mice were more resistant than wild-type controls against infection with *L. mexicana*. The mice which were deficient in both IL-4 and IL-13 genes IL-4(-/-) were significantly more resistant to parasite growth than their counterparts.²⁵ In human the role of IL-13 has been demonstrated by Bourreau and his colleagues who investigated the profile of cytokine expression in CL lesions caused by *L. guyanensis* by semi quantitative RT-PCR analysis and reported a Th2 cytokine expression pattern as showed by IL-4 and IL-13 mRNA expression in the lesions. The predominant cytokine in most lesions was IL-13.²⁶ Bosque and his colleagues who investigated the mechanisms of susceptibility and resistance to *L. viannia panamensis* infection demonstrated that macrophages from individuals from recurrent disease were more permissive *in vitro* to the entry of parasites than macrophages from subclinically infected individuals. In the

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presence of added recombinant IFN- γ , there was no difference in the permissiveness to the infection of macrophages from the clinically different groups, this indicates the difference is not intrinsic to the host cell and suggests that factors such as cytokines or accessory molecules may be involved in the susceptible phenotype.²⁷

In conclusion, in this study, we have detected the decreased production of IFN- γ by cells of patients with lesions of CL in response to parasite antigen and unbalanced production of regulatory cytokines such as IL-10 and IL-13 using the whole-blood stimulation assay technique. The required small volume of blood and the rapid set up time are the advantages in this assay technique. Using this assay for further immunodetection of cytokines may confirm its value for clinical investigation and special diagnostic applications.

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