

Short Report

## Interferon Gamma Unresponsiveness Due to Down-Regulation of IFN- $\gamma$ R Expression in Experimental Cutaneous Leishmaniasis

Amina Kariminia<sup>\*1</sup>, Sussan K. Ardestani<sup>2</sup>, Nargess Al-Sadat Ardestani<sup>3</sup>, Houri Sepehry<sup>3</sup> and Haideh Darabi<sup>1</sup>

<sup>1</sup>Dept. of Immunology, the Pasteur Institute of Iran; <sup>2</sup>Laboratory of Immunology, International Center of Biophysics and Biochemistry, Tehran University; <sup>3</sup>Dept. of Physiology, Faculty of Science, Tehran University, Tehran, Iran

Received 12 July 2005; revised 17 July 2005; accepted 20 July 2005

### ABSTRACT

It is now well documented that interferon gamma (IFN- $\gamma$ ) is the indispensable cytokine for inducing protective immunity against experimental and human cutaneous leishmaniasis. The importance of IFN- $\gamma$  receptor (IFN- $\gamma$ R) has also been studied. In the present study, we made attempts to find out whether *L. major* infection is able to alter the expression of IFN- $\gamma$ R *in vivo*. In addition, we studied the responsiveness to IFN- $\gamma$  *ex vivo*. To do that, we assessed the expression of CD119 (IFN- $\gamma$ R $\alpha$ ) on CD45+ cells isolated from draining lymph nodes of infected and uninfected BALB/c and C57BL/6 mice by flow cytometry. The MFI (mean fluorescence intensities) of CD119 on uninfected BALB/c mice were  $192.8 \pm 18.4$  but the CD119 MFI of infected BALB/c mice were remarkably decreased ( $107.9 \pm 40.8$ ). CD119 MFI of uninfected and infected C57BL/6 mice were  $276.2 \pm 17.1$  and  $140.4 \pm 43.0$  respectively. Moreover, we measured the production of nitric oxide (NO) by these cells in the presence of IFN- $\gamma$  in order to study the function of IFN- $\gamma$ R. NO production by draining lymph nodes cells of infected C57BL/6 mice in response to recombinant murine IFN- $\gamma$  was significantly higher than the cells of infected BALB/c mice ( $37.5 \pm 0.6$  and  $11.6 \pm 0.5$   $\mu$ M respectively,  $p < 0.05$ ). Therefore, our results confirm the *in vitro* reports regarding the impairment of IFN- $\gamma$  responsiveness due to *Leishmania* infection. *Iran. Biomed. J. 10 (2): 105-109, 2006*

**Keywords:** Interferon gamma receptor (IFN- $\gamma$ R), *Leishmania major*, Mice

### INTRODUCTION

The presence of resistance and susceptible murine strains makes it possible to define and characterize the potential immunopathologic mechanism(s) underlying susceptibility to *Leishmania* infection. The development of T helper 2 (Th2) response defined by interleukin-4 (IL-4) production in susceptible and development of Th1 defined by interferon gamma (IFN- $\gamma$ ) production in resistant murine strains have long been demonstrated [reviewed in 1]. However, because of the discrepancies obtained from different reports, it is difficult to come up with a consensus conclusion [2]. It is now well documented that IFN- $\gamma$  is the indispensable cytokine for inducing

protective immunity against experimental and human cutaneous leishmaniasis [3, 4]. The role of IFN- $\gamma$  in parasite killing and induction of protective immunity has been reported in both human and murine cutaneous leishmaniasis [5, 6]. The importance of IFN- $\gamma$  receptor (IFN- $\gamma$ R) has also been studied. Intra-dermal injection of even very low numbers of infective *L. major* promastigotes in mice knocked-out for IFN- $\gamma$ R alpha chain gene (IFN- $\gamma$ R $\alpha$ -/-) with resistance background induced disseminated fatal disease despite mounting a Th1 response in these mice [7]. Recently, it has been found that U937 cells infected with *L. donovani* showed down-regulation of IFN- $\gamma$ R expression in addition to the impairment of IFN- $\gamma$  signaling [8]. Such alterations induced by *Leishmania* infection in

\*Corresponding Author; Fax: (+98-21) 6696 68857; E-mail: akariminia@institute.pasteur.ac.ir

macrophages, the main mammalian host cells where parasites persist and proliferate, have already been reported. The decrease of protein kinase C activity [9-10], down-regulation of B7 [11, 12] and major histo-compatibility class II antigens (MHC class II) expressions [13,14] are the examples of the influence of *Leishmania* infection on the host cells.

In the present study, we made attempts to find out whether *L. major* infection is able to alter the expression of IFN- $\gamma$ R *in vivo*. In addition, we studied the responsiveness to IFN- $\gamma$  *ex vivo*. To do that, we assessed the expression of CD119 (IFN- $\gamma$ R $\alpha$ ) on CD45+ cells isolated from draining lymph nodes of infected and uninfected BALB/c and C57BL/6 mice. CD45 is the marker of pan leukocyte therefore the expression of IFN- $\gamma$ R was assessed on all types of cells in the draining lymph nodes. Moreover, we measured the production of nitric oxide (NO) by these cells in the presence of recombinant murine IFN- $\gamma$  in order to study the function of IFN- $\gamma$ R.

## MATERIALS AND METHODS

**Reagents.** Flow cytometry reagents including FITC-anti murine CD45mAb, Biotin-anti murine CD119 (IFN- $\gamma$ R  $\alpha$  chain), phycoerythrin (PE)-StrepAvidin and recombinant murine IFN- $\gamma$  were all purchased from PharMingen, USA. All other reagents used for cell culture and NO measurement were purchased from Sigma (USA).

**Parasites.** *Leishmania major* promastigotes, MHRM/IR/75/ER, were grown in Schneider's medium supplemented with 10% heat-inactivated fetal bovine serum, 292  $\mu$ g/ml L-glutamine, 4.5 mg/ml glucose, 100  $\mu$ g/ml streptomycin and 100 IU/ml penicillin at 23-25°C as previously described [15]. The parasites were kept in a virulent state by regular passage in susceptible BALB/c mice. Stationary phase promastigotes were harvested and centrifuged at 1811  $\times$ g at 4°C for 10 min. The pellet was washed three times in PBS (8 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.75 mM KH<sub>2</sub>PO<sub>4</sub>, 0.25 mM KCl, 137 nM NaCl).

**Mice and infection.** female BALB/c and C57BL/6 mice (4-6 weeks old) were obtained from the Animal Breeding Stock Facility of the Pasteur Institute of Iran (Karaj). The mice were divided into two groups (8 mice per group): the infected and non-infected. The infected groups of mice received  $2 \times 10^5$  infective stationary phase promastigotes in the hind footpad (s.c) and the non-infected groups

received only PBS. The draining lymph nodes (popliteal lymph nodes) were removed 4 weeks after challenge and total draining lymph node cells were isolated and washed three times with sterile PBS to be used for flow cytometry study and for NO production.

**Flow cytometry of CD119.** Based on manufacturer's instruction, one million mononuclear cells isolated from draining lymph nodes were washed three times with PBS and then fixed with paraformaldehyde. The cells were washed again 2X with PBS and 1X with staining buffer, (containing) PBS with 10% FBS. The cells were then incubated with proper amount of FITC-anti murine CD45 on ice for 20 min, washed, and stained for IFN- $\gamma$ R $\alpha$  chain with biotin-anti murine CD119 on ice for 20 min. At the end of incubation, the cells were washed again 3X with PBS. Cell acquisition and cytometry were then performed by FACScan (Becton Dickinson, USA) and the data analyses were done using LYSIS II software (Becton Dickinson, USA).

**Cell culture condition for NO production.** Two million of the draining lymph node cells were cultured in 1 ml of RPMI-1640 supplemented with 10% FBS, 20  $\mu$ M L-glutamine, 100  $\mu$ g/ml streptomycin and 100 IU/ml penicillin in the presence or absence of recombinant murine IFN- $\gamma$  (50 ng/ml). The optimum dose of IFN- $\gamma$  was already determined (data not shown). After incubation at 37°C for 24 h with 5% CO<sub>2</sub>, the supernatants were collected and kept frozen until used.

**Measurement of NO.** NO<sub>3</sub>/NO<sub>2</sub> was measured based on Greis method [16, 17]. Briefly, 100  $\mu$ l of vanadium chloride and 50  $\mu$ l of Greis reagent [1:1 (V/V) of 0.01% naphthylethylenediaminedihydrochloride in H<sub>2</sub>O + 2% sulphanilamine in 5% H<sub>3</sub>PO<sub>4</sub>] was added to 100  $\mu$ l of supernatant. The mixture was then incubated at 37°C for 40 min. The absorbances were read out at 540 nm.

**Parasite load.** The number of amastigotes were determined in 1,000 of cells isolated from the draining lymph node using Wright-Geimsa staining after methanol fixation.

**Statistical analysis.** Statistical significance between groups was analyzed by student's *t*-test using SPSS ver. 10. *p*<0.05 was considered significant.

## RESULTS

**Parasite load.** Number of amastigotes in 1000 total draining lymph node cells isolated from infected BALB/c and C57BL/6 mice were determined as follows: the cells from draining lymph nodes were isolated, washed, fixed with methanol and stained with Wright-Giemsa and investigated by light microscope. There was a significant difference between the resistance and susceptible mice since the number of amastigotes in 1,000 total draining lymph node cells of C57BL/6 was  $30 \pm 13$  and for BALB/c mice was  $160 \pm 67$  ( $p < 0.001$ ).

**CD119 expression.** The results of mean fluorescence intensities (MFI) of CD119 expression on CD45+ cells isolated from popliteal lymph nodes in different groups of mice clearly showed that *L. major* infection significantly down-regulated the expression of IFN- $\gamma$ R $\alpha$  chain (CD119) on CD45+ cells in both resistance and susceptible mice. The MFI of CD119 on uninfected BALB/c mice were  $192.8 \pm 18.4$  but the CD119 MFI of infected BALB/c mice were remarkably decreased ( $107.9 \pm 40.8$ ). The same down-regulation was demonstrated in C57BL/6 since the CD119 MFI of uninfected and infected mice were  $276.2 \pm 17.1$  and  $140.4 \pm 43.0$  respectively (Fig. 1 and Table 1). Nonetheless, the expression of CD119 in both infected and uninfected groups of C57BL/6 were significantly higher than the respective groups of BALB/c mice.

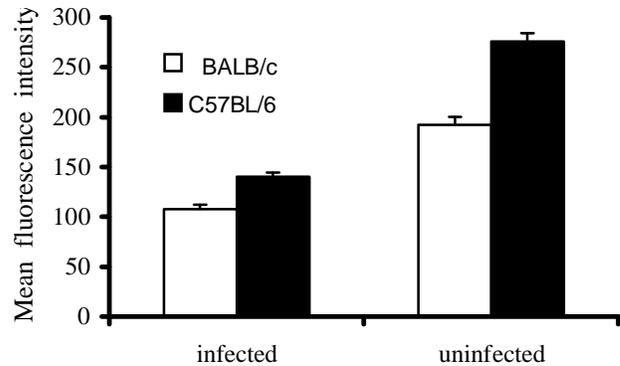
**Table 1.** Mean fluorescent intensity of IFN- $\gamma$ R (CD119) on CD45+ cells. The cells from draining lymph nodes of mice were isolated and stained with fluorochrom conjugated mAb to CD119-PE and CD45-FITC. The values show mean fluorescence intensity of CD119-PE.

	BALB/c	C57BL/6
Uninfected	$192.5 \pm 18.4^*$	$276.2 \pm 17.1^{**a}$
Infected	$107.9 \pm 40.8$	$140.4 \pm 43.0^b$

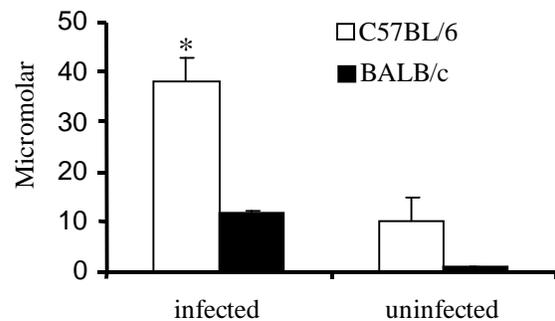
\*Significant difference between uninfected and infected BALB/c mice ( $p < 0.001$ ); \*\*Significant difference between uninfected and infected C57BL/6 mice ( $p < 0.001$ ). <sup>a</sup>Significant difference between uninfected of BALB/c and C57BL/6 mice ( $p < 0.001$ ). <sup>b</sup>Significant difference between infected of BALB/c and C57BL/6 mice ( $p < 0.001$ ).

**NO production.** In order to know whether down-regulation of IFN- $\gamma$ R could affect IFN- $\gamma$  responsiveness, the total draining lymph node cells were cultured in the presence or absence of recombinant murine IFN- $\gamma$  (50 ng/ml). The production of NO was consequently measured by standard Greis reaction. As shown in Figure 2 NO

production by draining lymph nodes cells of infected C57BL/6 mice in response to recombinant murine IFN- $\gamma$  was significantly higher than the cells of infected BALB/c mice ( $37.5 \pm 0.6$  and  $11.6 \pm 0.5$   $\mu$ M respectively,  $p < 0.05$ ). Interestingly, the level of NO production in both control groups including uninfected resistance and susceptible strains was significantly lower than the infected groups (under  $0.9$   $\mu$ M).



**Fig. 1.** The expression of CD119 (IFN- $\gamma$ R $\alpha$  chain) is shown on CD45+ cells isolated from draining lymph nodes of different groups of mice. The values show mean fluorescence intensity of the receptor determined by flow cytometry.



**Fig. 2.** NO<sub>3</sub>/NO<sub>2</sub> concentration was determined in the supernatants of draining lymph nodes cells based on Greis reaction. The details was presented in Materials and Method. \*significantly different from BALB/c mice.

## DISCUSSION

This is the first report showing the effect of *Leishmania* infection on IFN- $\gamma$ R expression *in vivo* in the animal model. We were able to demonstrate that *Leishmania* infection induces down-regulation of IFN- $\gamma$ R $\alpha$  chain (CD119) on the CD45+ cells of the draining lymph nodes. IFN- $\gamma$ R $\alpha$  chain is the

IFN- $\gamma$  binding subunit and the signal transducing subunit is the beta chain however the importance of alpha chain for IFN- $\gamma$  responsiveness was already studied. It has been shown that intradermal injection of even very low numbers of infective *L. major* promastigotes in mice knocked-out for IFN- $\gamma$ R $\alpha$ -/- gene with resistance background induced disseminated fatal disease despite mounting a Th1 response in these mice [7]. There is several reports showing that *Leishmania* infection alters the expression of molecules involved in developing protective immunity. B7 is the co-stimulatory molecules that transduces activation signals to T cells during M $\phi$ -T cells interaction. Its expression on M $\phi$  infected with *Leishmania* parasites is decreased compared to the uninfected cells [11, 12]. Down-regulation of MHC class II molecules expression on infected M $\phi$  is another example of the antigen presentation interference induced by *Leishmania* [13]. These changes make the infected macrophage a safe site for the parasite to persist and proliferate without activating M $\phi$  parasite killing mechanisms.

In order to see whether there is a correlation between parasite load and the expression of CD119, the number of amastigotes were determined in the total cells isolated from the draining lymph nodes. However, no correlation was observed between the parasite load and expression of CD119 in both strains of mice (data not shown). This would imply that the infection *per se* affects expression of IFN- $\gamma$ R. Therefore, it was necessary to investigate the function of IFN- $\gamma$ R in susceptible and resistance mice with or without *Leishmania* infection.

It is well documented that NO production is the major parasite killing mechanism in murine model of experimental leishmaniasis and IFN- $\gamma$  is the potent inducer of inducible NO Synthetase, the enzyme of NO synthesis, in macrophages [17]. However, upon *Leishmania* infection, the production of NO is down-regulated [18]. It is also demonstrated that such inhibition is not due to either the effect of secreted inhibitory cytokines such as IL-10 and IL-4 or decreased production of IFN- $\gamma$  since cultured macrophages isolated from infected BALB/c mice produced very low NO even in the excessive amount of IFN- $\gamma$  [18]. Therefore, the mechanism(s) involved in inhibition of NO production upon *Leishmania* infection has/have not been resolved. In the present study, we showed that the expression of IFN- $\gamma$ R $\alpha$  chain is down-regulated upon *Leishmania major* infection in resistance and susceptible mice. Nonetheless, it was necessary to

show that such down-regulation of receptor expression could affect the production of NO. In order to do that, we used *ex vivo* cell culture to assess the IFN- $\gamma$ R down stream signaling. Consequently, we isolated the cells from the draining lymph nodes. Total draining lymph node cells were washed and cultured in the presence of recombinant murine IFN- $\gamma$ . The supernatants were harvested 24 hrs after incubation and NO<sub>3</sub>/NO<sub>2</sub> production was measured based on Griess method. It was demonstrated that NO production was decreased in the infected mice. Nonetheless, NO production by infected BALB/c mice was significantly lower than C57BL/6 mice. This finding clearly showed that even in the presence of excessive amount of rmIFN- $\gamma$  the cells remain unresponsive to the main inducer of NO. It should be noted that we can not ruled out the effect of inhibitory cytokine(s) produced during incubation time. Similar results were obtained when the infection was induced by freshly isolated amastigotes (data not shown).

Our findings are in consonance with the *in vitro* studies regarding the effect of *Leishmania* infection on IFN- $\gamma$ R expression [8]. It has been shown that the peripheral blood monocytes of individuals with active visceral leishmaniasis (VL) show reduced expression of IFN- $\gamma$ R1 [19]. Furthermore, it has been reported that anti-leishmanial drugs may up-regulate IFN- $\gamma$ R1 on human macrophage cell line (THP1) [19]. However, the role of the anti-*Leishmania* drugs on IFN- $\gamma$ R expression needs to be deeply explored.

Recently, IFN- $\gamma$ R gene polymorphism has been shown to be associated with different auto immune diseases [20-22]. Similar study has been reported on human VL. Longitudinal studies in Sudan show ethnic differences in incidence and clinical phenotypes associated with *Leishmania donovani*. IFN $\gamma$ R1 was linked ( $P = 0.031$ ) and associated ( $P = 0.007$ ) to post kalaazar disseminated leishmaniasis but not VL or VL *per se* [23]. It would be of clinical interest to study the cytokine and cytokine receptor gene polymorphism in different clinical forms of cutaneous leishmaniasis.

## REFERENCES

1. Gumy, A., Louis, J. and Launois, P. (2004) The murine model of infection with *Leishmania major* and its importance for the deciphering of mechanisms underlying differences in Th cell differentiation in mice from different genetic

- backgrounds. *Int. J. Parasitol.* 34 (4): 433-444.
- Sacks, D. and Noben-Trauth, N. (2002) The immunology of susceptibility and resistance to *Leishmania major* in mice. *Natl. Rev. Immunol.* 2 (11): 845-858.
  - Rosas, L.E., Keiser, T., Pyles, R., Durbin, J. and Satoskar, A.R. (2003) Development of protective immunity against cutaneous leishmaniasis is dependent on STAT1-mediated IFN signaling pathway. *Eur. J. Immunol.* 33 (7): 1799-1805.
  - Chang, K.P. and McGwire, B.S. (2002) Molecular determinants and regulation of *Leishmania* virulence. *Kinetoplastid Biol. Dis.* 1 (1): 1.
  - Rosenthal, E. and Marty, P. (2003) Recent understanding in the treatment of visceral leishmaniasis. *J. Postgrad. Med.* 49 (1): 61-68. Review.
  - Louis, J.A., Gumy, A., Voigt, H., Launois, P. and Rocken, M. (2003) The use of the murine model of infection with *Leishmania major* to reveal the antagonistic effects that IL-4 can exert on T helper cell development and demonstrate that these opposite effects depend upon the nature of the cells targeted for IL-4 signaling. *Pathol. Biol. (Paris)* 51 (2): 71-73. Review.
  - Swihart, K., Fruth, U., Messmer, N., Hug, K., Behin, R., Huang, S., Del Giudice, G., Aguet, R. and Louis, J.A. (1995) Mice from a genetically resistant background lacking the interferon gamma receptor are susceptible to infection with *Leishmania major* but mount a polarized T helper cell 1-type CD4+ T cell response. *J. Exp. Med.* 181 (3): 961-971.
  - Ray, M., Gam, A.A., Boykins, R.A. and Kenney, R.T. (2000) Inhibition of interferon-gamma signaling by *Leishmania donovani*. *J. Infect. Dis.* 181 (3): 1121-1128.
  - Holm, A., Tejle, K., Magnusson, K.E., Descoteaux, A. and Rasmusson, B. (2001) *Leishmania donovani* lipophosphoglycan causes periphagosomal actin accumulation: correlation with impaired translocation of PKC $\alpha$  and defective phagosome maturation. *Cell. Microbiol.* 3 (7): 439-447.
  - Pingel, S., Wang, Z.E. and Locksley, R.M. (1998) Distribution of protein kinase C isoforms after infection of macrophages with *Leishmania major*. *Infect. Immun.* 66 (4): 1795-1799.
  - Compton, H.L. and Farrell, J.P. (2002) CD28 costimulation and parasite dose combine to influence the susceptibility of BALB/c mice to infection with *Leishmania major*. *J. Immunol.* 168 (3): 1302-1308.
  - Mbow, M.L., DeKrey, G.K., and Titus, R.G., (2001) *Leishmania major* induces differential expression of costimulatory molecules on mouse epidermal cells. *Eur. J. Immunol.* 31 (5): 1400-1409.
  - Fruth, U., Solioz, N. and Louis, J.A. (1993) *Leishmania major* interferes with antigen presentation by infected macrophages. *J. Immunol.* 150 (5): 1857-1864.
  - Kwan, W.C., McMaster, W.R., Wong, N. and Reiner, N.E., (1992) Inhibition of expression of major histocompatibility complex class II molecules in macrophages infected with *Leishmania donovani* occurs at the level of gene transcription via a cyclic AMP-independent mechanism. *Infect. Immun.* 60 (5): 2115-2120.
  - Alimohammadian, M.H., Darabi, H., Kariminia, A., Rivier, D., Bovay, P., Mauel, J., Ajdary, S. and Kharazmi, A. (2002) Adjuvant Effect of *Leishmania major* Promastigotes on the Immune Response of Mice to Ovalbumin. *Iran. Biomed. J.* 16 (4): 123-128.
  - Cabrera, M., Rodriguez, O., Monsalve, I., Tovar, R. and Hagel, I. (2003) Variations in the serum levels of soluble CD23, nitric oxide and IgE across the spectrum of American cutaneous leishmaniasis. *Acta Tropica.* 88 (2): 145-151.
  - Brandonisio, O., Panaro, M.A., Sisto, M., Acquafredda, A., Fumarola, L., Leogrande, D. and Mitolo, V., (2001) Nitric oxide production by *Leishmania*-infected macrophages and modulation by cytokines and prostaglandins. *Parasitologia* 1: 1-6. Review.
  - Bogdan, C., Rollinghoff, M. and Diefenbach, A. (2000) The role of nitric oxide in innate immunity. *Immunol. Rev.* 173: 17-26.
  - Dasgupta, B., Roychoudhury, K., Ganguly, S., Kumar, S.P., Vimal, S., Das, P. and Roy, S. (2003) Antileishmanial drugs cause up-regulation of interferon-gamma receptor 1, not only in the monocytes of visceral leishmaniasis cases but also in cultured THP1 cells. *Ann. Trop. Med. Parasitol.* 97 (3): 245-257.
  - Schrijver, H.M., Hooper-van Veen, T., Van Belzen, M.J., Crusius, J.B., Pena, A.S., Barkhof, F., Polman, C.H., and Uitdehaag, B.M. (2004) Polymorphisms in the genes encoding interferon-gamma and interferon-gamma receptors in multiple sclerosis. *Eur. J. Immunogenet.* 31 (3): 133-140.
  - Fraser, D.A., Loos, B.G., Boman, U., Van Winkelhoff, A.J., Van der Velden, U., Schenck, Z. and Dembic, Z. (2003) Polymorphisms in an interferon-gamma receptor-1 gene marker and susceptibility to periodontitis. *Acta Odontol. Scand.* 61 (5): 297-302.
  - Tanaka, Y., Nakashima, H., Hisano, C., Kohsaka, T., Nemoto, Y., Nihiro, H., Otsuka, T., Otsuka, T., Imamura, T. and Niho, Y. (1999) Association of the interferon-gamma receptor variant (Val14Met) with systemic lupus erythematosus. *Immunogenetics* 49 (4): 266-271.
  - Mohamed, H.S., Ibrahim, M.E., Miller, E.N., Peacock, C.S., Khalil, E.A., Cordell, H.J., Howson, J.M., El Hassan, A.M., Bereir, R.E. and Blackwell, J.M. (2003) Genetic susceptibility to visceral leishmaniasis in The Sudan: linkage and association with IL4 and IFNGR1. *Genes Immun.* 4 (5): 351-355.