original article

Elevated TGF-beta levels in drug-resistant visceral leishmaniasis

Miskelyemen A. Elmekki,[a,b] Mogahid M. Elhassan,[a,b] Hani A. Ozbak,[a] Moawia M. Mukhtar[c]

From the [a]Department of Medical Laboratory Technology College of Applied Medical Sciences Taibah University, Almadinah Almonawara, Saudi Arabia; [b]Department of Medical Microbiology and Parasitolgy, College of Medical Laboratory Sciences, Sudan University of Science and Technology, Khartoum, Sudan, [c]Institute of Endemic Diseases, University of Khartoum, Khartoum, Sudan

Correspondence: Dr. Miskelyemen Elmekki · Taibah University, Department of Medical Laboratory Technology College of Applied Medical Sciences, Almadinah Almonawwara, Saudi Arabia · T: 966 14 8618888 · miskatti@yahoo.com

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BACKGROUND: Poor and neglected populations in Africa are particularly affected with visceral leishmaniasis. The widespread emergence of resistance to pentavalent antimonials occurs globally and the unavailability of a vaccine in clinical use constitutes a major obstacle in disease control.
OBJECTIVE: To investigate the cytokine profile in human visceral leishmaniasis.
DESIGN: A cross-sectional laboratory-based study.
SETTING: Single center study carried out at the Institute of Endemic Diseases, University of Khartoum, Sudan.
PATIENTS AND METHODS: Soluble lysates of *L major* and *L donovani* were used to stimulate the lymphocytes of two groups of confirmed VL patients (group 1 [n=20] had respond to pentostam treatment and group 2 [n=5] were recorded as drug resistant after follow up) in a cellular proliferation assay and the levels of IFNγ, IL-10, TNFα and TGFβ were detected by cytokine ELISA.
MAIN OUTCOME MEASURES: Levels of IFNγ, TNFα, IL-10 and TGFβ.
RESULTS: A significant increase of IFNγ and TNFα levels were reported in stimulated cells of drug susceptible and drug resistant groups, but no significant difference in IL-10 production was observed between the different antigens or between the patients groups. TGFβ from stimulated lymphocytes was secreted in statistically significant amounts in patients reported as drug resistant in response to both *L major* and *L donovani* antigens ($P<.001$).
CONCLUSIONS: In VL patients, IFNγ and TNFα are extremely produced in response to in vitro re-stimulation which means that the parasitic infection, although virulent and chronic, does not render patients as immunocompromised. However, TGFβ is mostly associated with treatment failure.
LIMITATIONS: This study assessed secretory TGFβ. A study with a larger sample size to assess TGFβ gene expression and to follow its intracytoplasmic synthesis in drug resistant VL patients is recommended.

Leishmaniasis, a parasitic disease caused by different members of the genus *Leishmania*, is a vector-borne disease transmitted by *Phlebotomous* spp (sandflies). The disease has three different forms of clinical presentation, including visceral leishmaniasis (VL) (the most fatal), mucocutaneous leishmaniasis and the cutaneous form (CL), which is self healing in most cases. According to the WHO,[1] leishmaniasis is endemic in more than eighty countries of the tropical and subtropical zones.

Disease severity is largely affected by the host immune response regardless of the species or strain responsible for causing the disease. Gamma-interferon and nitric oxide production by T helper-1 cells, which dominate cell-mediated immunity, usually increases the host ability to resist disease while susceptibility and disease progression are mostly associated by the T helper-2 type of immune response, which is the dominant type in humoral immunity.[2-4] Thus, in both visceral and cutaneous forms of the disease, and regardless of the different clinical presentations in each, resistance as well as recovery are linked to Th1 type of response dominancy while case deterioration is linked to Th2 type of response dominancy.[5] In addition, suppression of cel-

lular immunity (Th1 response) that results in a negative leishmanin skin test (delayed type hypersensitivity reaction to leishmania antigen) is a major characteristic of visceral leishmaniasis.[6]

As delayed type hypersensitivity was found to be restored upon effective treatment,[7] immunosuppression was also shown to be quickly reversible following successful chemotherapy.[8] Moreover, intracellular killing of *leishmania* parasites by interferon gamma-activated macrophages is the main mechanism for control of visceral leishmaniasis by both adaptive and innate immune responses.[9] On the other hand, an early humoral response preceding the abnormalities of the cellular response has been detected through the elevation of antileishmanial specific antibodies of almost all antibody classes.[10,11] These antibodies may result in the acquisition of naturally protective immunity in endemic areas as large populations with asymptomatic or subclinical infections were found to have high levels of specific antileishmanial antibodies.[12]

PATIENTS AND METHODS

We conducted a cross-sectional laboratory-based study in visceral leishmaniasis patients referred for diagnosis to the laboratory of the Institute of Endemic Diseases from the Tropical Medicine Hospital, Suba University Hospital, Omdurman and Khartoum Teaching Hospitals.

Patients with a positive direct agglutination test (DAT) gave informed consent and were enrolled after confirmation of infection by parasitological methods. Smears from lymph node aspiration were prepared and stained with Gimsa and all the study subjects had positive results (presence of Leishman-Donovan (LD) bodies). Five of the 25 VL patients enrolled in this study were resistant to pentostam (sodium stibogluconate) according to follow-up records.

In smear-positive patients, 10 mL of blood were drawn into heparinized vacutainer tubes. The tubes were centrifuged at 2000 rpm for separation of plasma, which was removed to a new tube. The rest of the blood was diluted with an equal volume of complete media (RPMI 1640). In a 15-mL tube, 3 mL of Ficoll-Hypaque (Histopaque-1077, Sigma Aldrich) was added and the diluted blood was carefully layered on the Ficoll-Hypaque. The tubes were then centrifuged at 2000 rpm for 15 minutes. The lymphocytes layer in the interphase was collected and transferred to a new tube. Cells were washed twice with 10 mL of complete media. Cells were then pelleted and the pellets were resuspended in 2 mL of complete media to concentrate the cells.

Autochthonous isolates MHOM/SD/00/MW1 (zymodeme MON-74) of *L major*, were isolated from a Sudanese CL patient and MHOM/SD/00/MW81 (zymodeme MON-82) of *L donovani* isolated from a Sudanese VL patient and grown in the lab in stationary phase were used for protein preparation. Cultures were centrifuged, the pellets washed three times using sterile phosphate buffered saline (PBS), then freezed and thawed six times in liquid nitrogen and a water bath at 55°C. The protein concentration was then measured and adjusted to the desired concentration and stored frozen.

Into a sterile 24-well flat-bottomed plate, aliquots of cells (10^6 cells per mL of complete media) were placed together with one of the leishmania antigens (MW1 and MW81 lysates) each in a separate well in a final concentration of 100 µg/mL. For each sample, a well with cultured unstimulated cells was kept as a negative control and a well stimulated with phytohaemagglutinin mitogen (PHA) was kept as a positive control. Lymphocytes were cultured at 37°C in a humidified, 5% CO_2 incubator. After 72 hours, the wells were harvested by centrifugation at 6000 rpm for 3 minute to remove the cells. The supernatant was separated and stored at -20°C in small aliquots for later cytokine analysis.

Cytokines were determined by sandwich ELISA using a BD OptEIA ELISA Set for the human cytokines interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα), tumor necrosis factor beta (TGFβ) and interleukin 10 (IL-10). ELISA was performed according to manufacturer instructions.

Data was analyzed by statistical software package, Version 16 (SPSS Inc., Chicago, IL, USA). Descriptive data analysis was used to visualize differences within the data. Independent sample t-tests and one-way ANOVA were used for comparison of means.

RESULTS

In both groups of patients there was a significant increase in interferon gamma (IFNγ) levels in response to the homologous visceral antigen compared with heterologous cutaneous antigen ($P<.001$) (**Figure 1**). However, there was no significant difference between the drug-susceptible and drug-resistant patients peripheral mononuclear cells (PMNC) in production of this cytokine. Interleukin-10 (IL-10) increased slightly when the lymphocytes were stimulated by PHA and *L major* lysate compared to non-stimulated cells and cells stimulated with *L donovani* antigen (**Figure 2**). Neither group of patients had a significant difference in IL-10 production.

Significant high levels of tumor necrosis factor alpha (TNFα) were detected in culture supernatants, with special reference to the cellular response to *L major* antigen compared to L donovani antigen ($P<.001$) (**Figure**

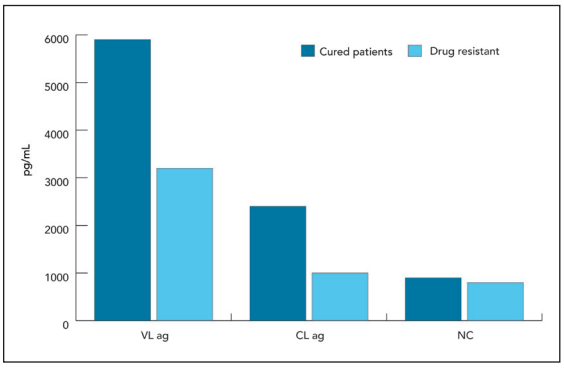

Figure 1. Levels of IFNγ produced by lymphoproliferative response of drug-sensitive and drug-resistant VL patients to *L major* (CL ag) antigen and *L donovani* (VL ag) antigen. (*P*<.001 homologous visceral antigen compared with heterologous cutaneous antigen); NC: non-stimulated cells (negative control).

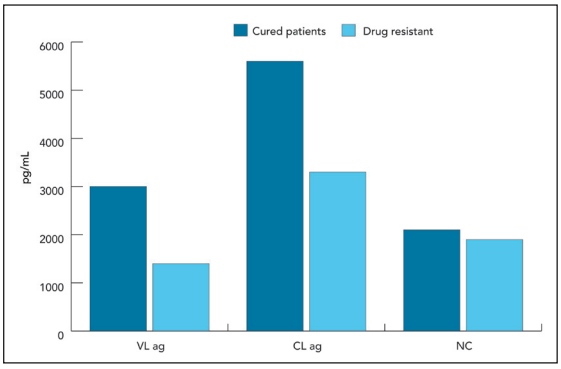

Figure 3. Levels of TNFα produced by lymphoproliferative response of drug-sensitive and drug-resistant VL patients to *L major* (CL ag) antigen and *L donovani* (VL ag) antigen (*P*<.001 CL ag vs VL ag). NC: non-stimulated cells (negative control).

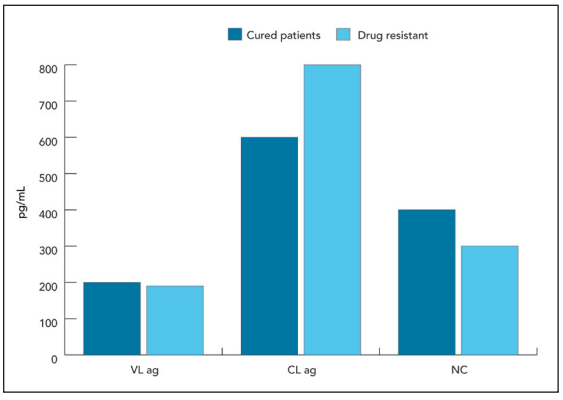

Figure 2. Levels of IL-10 produced by lymphoproliferative response of drug-sensitive and drug-resistant VL patients to *L major* (CL ag) antigen and *L donovani* (VL ag) antigen; NC: non-stimulated cells (negative control).

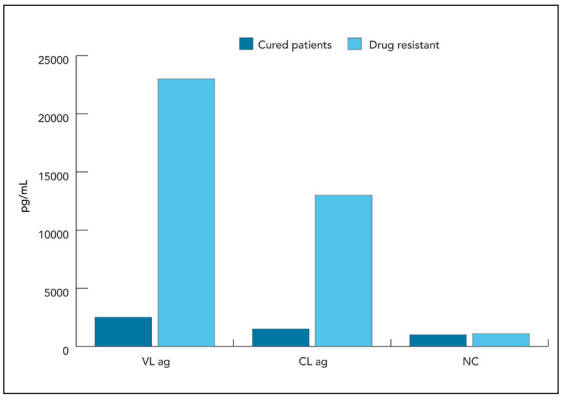

Figure 4. Levels of TGFβ produced by lymphoproliferative response of drug-sensitive and drug-resistant VL patients to *L major* (CL ag) antigen and *L donovani* (VL ag) antigen (*P*<.001 CL ag vs VL Ag). NC: non-stimulated cells (negative control).

3). No significant difference was observed between the two groups of patients. Transforming growth factor beta (TGFβ) from stimulated patients lymphocytes was secreted in statistically significant amounts in patients reported as drug resistant. (*P*<.001) (**Figure 4**). This was true for both *L major* and *L donovani* antigens.

DISCUSSION

The high levels of IFNγ detected in response to leishmania antigens, with more specific stronger response to *L donovani* antigen, are consistent with a previous study, which reported that high levels of IFNγ are secreted at the initial stages of exposure to the parasites as observed in seroconverted or sub-clinically infected individuals in an endemic area.[13] Another study that examined the cells of cutaneous leishmaniasis patients recorded that both CD8+ cells and CD4+ cells are sources of biologically active IFNγ when the cells of patients are stimulated in vitro with *L major* antigen compared with control cells.[14]

Similarly, considerable amounts of TNFα were detected in the cells of patients included in this study, which is consistent with a previous finding by Pirmez, et al.[15] Another study found high TNFα levels in the serum of active VL patients compared to patients with cryptic leishmanial infection (asymptomatic, self-healing subclinical infection, and posttreatment VL cases) and normal volunteers.[16] Moreover, VL is an extremely rare example of opportunistic infection in patients treated with TNFα antagonists. A few cases have been described for patients developing VL under biological therapy.[17] Another study found that levels of circulating TNFα, assessed by ELISA, were higher in patients than in healthy controls, and declined significantly with improvement in clinical and laboratory parameters after successful treatment, but these plasma levels when

evaluated by cytotoxicity assay were not well defined, a fact that could be linked to the presence of factor(s) that can affect both the release and activity of TNFα.[18]

We detected low and insignificant levels of IL-10. The production of IL-10 during *L donovani* infection, and the role of IL-10 in the regulation of immune responsiveness during visceral leishmaniasis is well documented.[19] The results of our study are not consistent with previous investigations on Brazilian patients, which showed that IL-10 production from *L chagasi* antigen-stimulated PBMC cultures of acute VL patients was significantly higher than in cured individuals, whereas asymptomatic leishmanin skin test (LST)-positive individuals did not produce IL-10.[20] A previous study proved that the high parasite load in VL is strongly correlated with a high level of IL-10, implicating IL-10 as a marker of disease severity that can be assayed for diagnosis as well as prognosis of both VL and post-kala-azar dermal leishmaniasis (PKDL),[21] Investigators in 2011 nominated IL-10 as an approach to therapy in human VL following the fact that IL-10 neutralization promoted parasite killing in and complete clearance in the majority of VL patients.[22] Moreover, the splenic cells secreted increased levels of both TNFα and IFNγ under IL-10-neutralizing conditions.[22] Lymphocytes are the known source of IL-10 and previous data implicated CD25-Foxp3 T cells as the source of IL-10 in the pathogenesis of human VL.[23]

In the present study, significantly high levels of TGFβ were detected, especially in patients with treatment failure. These results were consistent with previous reports that showed TGFβ production in visceral leishmaniasis.[24,25] A previous study from India was consistent with out results in that they found that the retention and maintenance of residual IL-10 and TGF-beta in some sodium antimony gluconate-treated individuals and the elevation of IL-10 and TGF-beta in PKDL, a sequel to kala-azar, probably reflects the role of these cytokines in reactivation of the disease in the form of PKDL. Contrastingly, ambisome treatment of VL resulted in negligible TGF-beta levels and absolute elimination of IL-10, reflecting the better therapeutic activity of ambisome and its probable role in the recent decline in PKDL occurrences.[26] Moreover, elucidation of immune responses in Indian PKDL patients revealed a spectral pattern of disease progression where disease severity could be correlated inversely with lymphoproliferation and directly with TGF-beta, IL-10, and antibody production.[26]

In conclusion, in VL patients, IFNγ and TNFα are produced in high levels in response to in vitro re-stimulation, which means that the parasitic infection, although virulent and chronic, does not render patients as immunocompromised. Insignificant production of IL-10 was reported. TGFβ is mostly associated with the pathology of the disease since significant increases are associated with treatment failure.

Conflict of interest

Authors declare that there is no conflict of interests to report related to this article.

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REFERENCES

1. WHO expert committee report, Control of the leishmaniasis. Technical report series 1991; 793.
2. Peter E. Kima and Lynn Soong, Interferon Gamma in Leishmaniasis Front Immunol 2013; 4: 156
3. Alexander, J., Satoskar, A.R., and Russel, D.G. Leishmania species: models of intracellular parasitism . J. Cell Science 1999; 112, 2993-3002
4. Solbach, W., and Laskay, T . The host response to Leishmania infection. Adv. Immunol 2007; 4, 275-317.
5. Castellano LR, Filho DC, Argiro L, Dessein H, Prata A, Dessein A, Rodrigues V. Dichotomy of the T cell response to Leishmania antigens in patients suffering from cutaneous leishmaniasis; absence or scarcity of Th1 activity is associated with severe infections. Th1/Th2 immune responses are associated with active cutaneous leishmaniasis and clinical cure is associated with strong interferon-gamma production. Hum Immunol 2009; 70(6):383-90
6. Gidwani K., Rai M., Chakravarty J., Boelaert M., and Sundar S. Evaluation of Leishmanin Skin Test in Indian Visceral Leishmaniasis. Am J Trop Med Hyg 2009 80 (4): 566-567
7. Reiner, S.L., and Locksley, R.M. The regulation of immunity to Leishmania major. Annu Rev Immunol 1995; 13, 151-77.
8. Cenini P., Berhe N., Hailu A., McGinnes K. and Frommel D. Mononuclear Cell Subpopulations and Cytokine Levels in Human Visceral Leishmaniasis before and after Chemotherapy J Infect Dis 1993; 168(4): 986-993
9. Ribeiro-de-Jesus, A., Almeida, R.P., Lessa, H., Bacellar, O., and Carvalho, E.M. Cytokine profile and pathology in human leishmaniasis. Braz J Med Biol Res 1998; 31: 143-8.
10. da Matta VL, Hoshino-Shimizu S, Dietze R, Corbett CE. Detection of specific antibody isotypes and subtypes before and after treatment of American visceral leishmaniasis. J Clin Lab Anal 2000; 14 : 5-12.
11. Saha S., Mondal S., Banerjee A., Ghose J., Bhowmick S. and Ali N. Immune responses in kala-azar, Indian J Med Res, 2006, 245-266
12. Lakhal-Naouar I., Boussoffara T., Meddeb-Garnaoui A., Ben Achour-Chenik Y., Louzir H., and Chenik M. Cellular and Humoral Responses to Leishmania major Virulence Factors in Healed Cutaneous Leishmaniasis and Mediterranean Visceral Leishmaniasis Patients Clin Vaccine Immunol. 2009; 16(6): 956–958.
13. Bacellar, O., Barral-Netto, M., Badaro, R., and Carvalho, E.M. Gamma interferon production by lymphocytes from children infected with L. chagasi. Braz J Med Biol Res. 1991. 24, 791-5.
14. Nateghi Rostami M1, Keshavarz H, Edalat R, Sarrafnejad A, Shahrestani T, Mahboudi F, Khamesipour A. CD8+ T cells as a source of IFN-γ production in human cutaneous leishmaniasis. PLoS Negl Trop Dis. 2010; 12;4(10):e845.
15. Pirmez, C., M. Yamamura, K. Uyemura, M. Paes-Oliveira, F.Conceicao-Silva, and R. L. Modlin. Cytokine patterns in the pathogenesis of human leishmaniasis. J. Clin. Invest. 1993. 91:1390-1395.
16. Barral-Netto M1, Badaró R, Barral A, Almeida RP, Santos SB, Badaró F, Pedral-Sampaio D, Carvalho EM, Falcoff E, Falcoff R. Tumor necrosis factor (cachectin) in human visceral leishmaniasis. J Infect Dis. 1991;163(4):853-7.
17. De Leonardis F1, Govoni M, Lo Monaco A, Trotta F Visceral leishmaniasis and anti-TNF-alpha therapy: case report and review of the literature. Clin Exp Rheumatol. 2009; 27(3):503-6.
18. Salomão R1, Castelo Filho A, de Medeiros IM, Sicolo MA. Plasma levels of tumor necrosis factor-alpha in patients with visceral leishmaniasis (Kala-azar). Association with activity of the disease and clinical remission following antimonial therapy. Rev Inst Med Trop Sao Paulo. 1996 ;38(2):113-8.
19. Ghalib H W, Piuvezam M R, Skeiky Y A, Siddig M, Hashim F A, el-Hassan A M, Russo D M and Reed S G Interleukin 10 production correlates with pathology in human Leishmania donovani infections. J Clin Invest. 1993;92(1):324–329.
20. Holaday, B.J., Pompeu, M.M., Evans, T., Braga, D.N., Texeira, M.J., Sousa, Ade, Q., et al. Correlates of Leishmania-specific immunity in the clinical spectrum of infection with Leishmania chagasi. J Infect Dis. 1993. 167, 411-7.
21. Verma S, Kumar R, Katara GK, Singh LC, Negi NS, Ramesh V, Salotra P. Quantification of parasite load in clinical samples of leishmaniasis patients: IL-10 level correlates with parasite load in visceral leishmaniasis. PLoS One. 2010; 5(4): 10107.
22. Gautam S1, Kumar R, Maurya R, Nylén S, Ansari N, Rai M, Sundar S, Sacks D. IL-10 neutralization promotes parasite clearance in splenic aspirate cells from patients with visceral leishmaniasis. J Infect Dis. 2011 204(7):1134-7.
23. Nylén S., Maurya R., Eidsmo L., Das Manandhar K., Sundar S. and Sacks D. Splenic accumulation of IL-10 mRNA in T cells distinct from CD4+CD25+ (Foxp3) regulatory T cells in human visceral leishmaniasis. JEM 2007; 204 (4) : 805-817
24. Nascimento ET, Gantt KR, Pontes N, Bacelar O, Luz VE,Wilson ME,. TGF- in acute visceral leishmaniasis. Am J Trop Med Hyg; 2002; 67 : 262.
25. Gantt KR, Schultz-Cherry S, Rodriguez N, Jeronimo SM, Nascimento ET, Goldman TL, Activation of TGF beta by Leishmania chagasi: importance for parasite survival in macrophages. J Immunol; 2003. 170 : 2613-20.
26. Saha S1, Mondal S, Ravindran R, Bhowmick S, Modak D, Mallick S, Rahman M, Kar S, Goswami R, Guha SK, Pramanik N, Saha B, Ali N. IL-10- and TGF-beta-mediated susceptibility in kala-azar and post-kala-azar dermal leishmaniasis: the significance of amphotericin B in the control of Leishmania donovani infection in India. J Immunol. 2007; 15;179(8):5592-603.