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In Vitro Analysis of Visceral Leishmaniasis and Pulmonary Tuberculosis Patient's Cytokines Responses to Related and Unrelated Antigen Stimulation

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Abstract

Background: Leishmanias-tuberculosis co-infection cases have been documented repeatedly in eastern Africa, however, limited information is known about the co-infection immunological interaction. A case control study was conducted for evaluating *in-vitro* cytokines reactions in patients affected with visceral leishmaniasis and patients affected with pulmonary tuberculosis.

Methods: Thirty leishmaniasis patients and thirty tuberculosis patients in addition to ten healthy individual cytokine profiles were conducted after being stimulated by live Leishmania Promastigotes and BCG. A subset of Th-1 cytokines IFN-γ and TNF-α and Th-2 (IL-10) in addition to the inflammatory cytokine IL-15 were determined using ELISA in stimulated whole blood samples supernatants.

Results: IFN-γ and TNF-α (Th-1 cytokines) concentration were significantly higher in stimulated whole blood supernatants of VL patients in contrast with TB patients basically when stimulated by *L. donovani* antigen. In TB patient's whole blood samples, IL-10 was significantly produced mainly when stimulated with BCG. Compared by healthy controls, significant concentration of the above cytokines was identified in stimulated whole blood samples of both visceral leishmaniasis and pulmonary tuberculosis patients.

Outcomes: Using homologous antigen as a stimuli in visceral leishmaniasis patients, the expressions of Th-1 cytokines were higher compared to the non-stimulated. Those findings suggest a strong adaptive response. In the meantime, the Th-2 cytokine IL-10 expression in TB patients stimulated by homologous antigen was greater than the non-stimulated which indicates a strong inhibition of the protective Th-1 cytokines expression. Therefore, TB infection reoccurrence might induce a stronger infection because of weak immune response due to suppression of Th-2 main cytokine.

Keywords: Visceral leishmaniasis; Pulmonary tuberculosis; Co-infection; Cytokines

Introduction

Visceral leishmaniasis and pulmonary tuberculosis are major health problems in Sudan, which is known as one of the largest countries with a very high prevalence for both diseases in the eastern region of the Mediterranean [1,2]. Although different in etiology and transmission mechanisms, the two diseases pathogenesis show several similarities, both are macrophage pathogen and their cure is associated with induction of Th-1 immune response while susceptibility and persistence of the two diseases is mediated by Th-2 response.

VL or kala-azar is considered one of the most important disease from the public health aspect in Sudan, as it is known to be one of the disease main foci in the world [1]. It has been reported since the beginning of the twentieth century when Neave first described the disease in 1904 in Southern Sudan. As from then VL has been on the top of the health problems in Sudan mainly in the endemic areas in the eastern and central regions: north-east from the Atbara river, heading south to the Sudanese-Ethiopian border near the Sobat river untill Nasir and Malakal, and spreading westwards across the White Nile [3].

It was reported that at least 1000 cases of VL occur yearly in Gedarif State with an about 38/1000 persons/year [4]. At the beginning of the 20th century, the eastern region in Gedarif State, mainly in the Atbara River area, was heavily affected with the disease.

TB is a worldwide well known life threatening disease that is responsible of the world's one-third population death, in addition to its impact on HIV/AIDS disease as a common opportunistic infection [5] and considered a major health problem in Sudan [6,7]. The annual risk of TB infection is estimated at 1.8%, putting Sudan among the high TB prevalence countries in the Eastern Mediterranean Region, with an average of 80.6% of pulmonary cases and 19.4% of other TB forms.

Several clinical cases of associated VL-TB have been documented in Sudan [8,9] and other parts of the world. Nevertheless, little is known about the co-infection immunological interactions. Chaudhuri has reported that VL can reactivate a latent mycobacterial infection. Similarly, Montalban and Callejia [10] suggested that TB represents one of the immunosuppressive conditions which can lead to the

development of latent leishmanial infection to leishmaniasis. We conducted a case control study that was carried out to investigate cytokines responses *in vitro* in visceral leishmaniasis (VL) patients and pulmonary tuberculosis (TB) patients to homologous and heterologous antigens.

Materials and Methods

Ethical consideration

The study was authorized by the ethics committee of the Institute of Endemic Diseases, University of Khartoum. Participants were consented before participating in the study.

Case control study design

The study was conducted throughout the period from February 2011 to August 2012. Visceral leishmaniasis (VL) patients recruited for this study were mostly from the White Nile state and Gadarif state, age range from 3 years to 55 years, 19 males and 11 females with symptoms duration from one to six months. TB patients recruited were mostly from Khartoum state, age range from 17 years to 85 years, 26 males and 4 females, with symptoms duration of one month to 4 months. Healthy controls were also included in the study.

30 visceral leishmaniasis patients (diagnosed detection of agglutinating antibodies in patients` sera using DAT, and affirmed by demonstration of the parasite in an aspirate from lymph node isolated and cultured in NNN biphasic media, at the Institute of Endemic Diseases) and 30 tuberculosis patients (diagnosed by demonstration of acid fast bacilli *Mycobacterium tuberculosis* in sputum samples in Educational Khartoum Hospital laboratories) were randomly selected and referred to the study. Ten healthy controls were both Mantoux and DAT negative.

Preparation of antigens for stimulation

Leishmania parasite culture: Leishmania parasites were isolated according to Evans [11] using an adjusted biphasic NNN media consisting of solid and liquid phases.

Preparation of live parasite antigen: Two milliliters of live *L. donovani* (MHOM/SD/00/MW125) MON82, isolated from VL patient in stationary phase were centrifuged at 1200 round-per-minute for ten min. Then the pellet was washed three times using sterile phosphate buffer saline (PBS) before suspended in 1 ml of RPMI -1640. Ten µl from the suspended pellet was added to equal volume of 2% formalin, mixed and loaded into a haemocytometer under the 40X objective at light microscope. The number of parasites/ml was counted.

BCG preparation: Freeze-dried Bacillus Calmette-Guerin (BCG, manufactured by BB-NCIPD Ltd, Sofia, Bulgaria) tuberculosis vaccine was used as antigen for whole blood stimulation. The BCG was prepared in sterile environment by removing metal ring with a vial opener and adding 1 ml of diluent for BCG Vaccine (Saline) (BB-NCIPD Ltd, Sofia, Bulgaria). The prepared solution was kept in -20°C till used.

Whole blood stimulation

Dilution of the whole blood: One ml of heparinized venous blood from each patient from both groups and the healthy control was placed into a 15 ml centrifuge tubes (Corning, #25330, USA) and diluted by adding two ml of sterile RPMI-1640 medium (250 µl blood containing about 5×10^5 lymphocytes).

Stimulation: One ml/well of diluted whole blood were dispensed into a sterile flat-bottomed 24-well cell culture plates (Coaster # 3526, USA). Whole blood stimulation was done by adding 4×10^6 parasite/well of live *L. donovani*, BCG in a concentration of 5×10^5 viable unit to another well and the third well remained as a non-stimulated negative control for each sample. The culture plates were incubated at 37°C with 5% CO_2. After 48 h, the cells were harvested by centrifugation at 1200 rpm for 10 min, kept in -80°C till used for DNA extraction and detection by PCR. The supernatants were collected and frozen for cytokines measurement by ELISA.

Cytokines measurement by ELISA

Concentrations of Interferon gamma (IFN-γ), Tumor necrosis factor (TNF-α), Interleukin 10 (IL-10) and Interleukin 15 (IL-15) produced by both Leishmania-reactive cells and BCG-reactive cells were calculated in aliquots of cell-free supernatants using a sandwich ELISA (BD PotEIA TM ELISA SET B - BD Biosciences, USA, Catalog number: 550534). The ELISA procedure was done as directed by the manufacturer (BD Biosciences, USA). Hundred microliters of capture antibody diluted in coating buffer was added to each well of the 96-wells ELISA plates and incubated at 4°C overnight. After being washed three times using a washing buffer, 200 µl of assay diluents and blocking buffer was added to each well and kept for one hour at room temperature. After being washed three times, 100 µl of standard and samples (which were prepared as recommended by the manufacture) were added into each well and incubated for 2 h at room temperature. After washing five times, 100 µl of working conjugate (Detection antibody + Enzyme reagent) was added to each well and again incubated for 1 h at room temperature. After seven washes, 100 µl of substrate solution (Tetramethylbenzidine TMB and Hydrogen Peroxide H_2O_2) was added to each well. The reaction mixture was then kept in the dark for another 30 minutes at room temperature. Fifty microliters of a stop solution ($2NH_2SO_4$) was then added to each mixture. Lastly, the plate optical density was determined using micro-plate instrument (Thermo Labsystems, Finland) at 450 nm. The mean absorbance of each set of duplicate standards, controls and samples was calculated. The cytokines concentration of the unknown was determined from the standard curve and multiplied by the dilution factor.

Statistical analysis

ELISA conventional results were statistically analyzed using SPSS computer software, the mean concentration and the median, SD, and P values were calculated by ANOVA.

Results

IFN-γ concentration was significantly high in supernatants of samples of VL patients as compared with TB patients and healthy controls (p values between the three groups=0.089) (Figure 1). A representative increase in IFN-γ was conducted in VL samples stimulated with live *Leishmania d. promastigote* compared with BCG stimulation (p values<0.00-0.05).

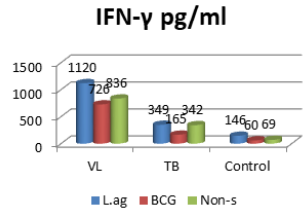

Figure 1: Comparison of IFN-γ concentrations in visceral leishmaniasis patients samples, pulmonary tuberculosis patients and healthy controls when stimulated with *L. donovani* antigen, BCG & non-stimulated.

The concentration of TNF-α in supernatants of VL patients was significantly greater compared to patients with TB and healthy controls (Figure 2). A significant increase of TNF-α was conducted in VL samples stimulated by life *Leishmania d. promastigote* ($p<0.05$).

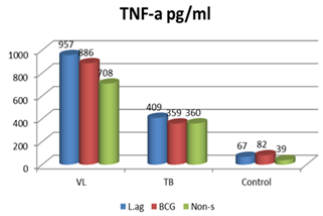

Figure 2: TNF-α concentrations in supernatants of visceral leishmaniasis patients, pulmonary tuberculosis patients and healthy controls stimulated with live *Leishmania d. promastigote*, BCG compared with non-stimulated samples.

IL-10 concentration was significantly greater in TB samples in comparison with VL and healthy controls (Figure 3). Significant IL-10 production was measured in samples of TB patients stimulated with BCG or *L. donovani* ($p<0.05$).

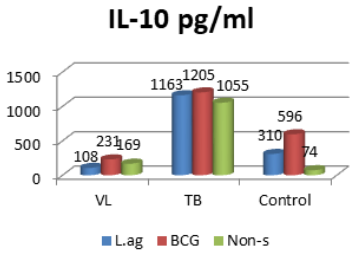

Figure 3: IL-10 concentrations in supernatants of visceral leishmaniasis patients, pulmonary tuberculosis patients and healthy controls when stimulated with live *Leishmania d. promastigote*, BCG and non-stimulated.

A significant IL-15 concentration was measured in supernatants of samples of VL and TB patients in both stimulated and non-stimulated cells (Figure 4).

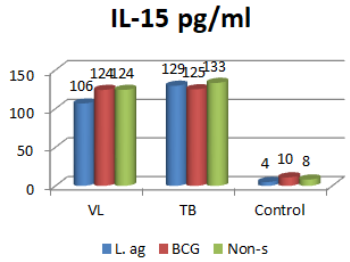

Figure 4: IL-15 concentrations in supernatants of visceral leishmaniasis patients, pulmonary tuberculosis patients and healthy controls when stimulated with live *Leishmania d. promastigote*, BCG and non-stimulated.

Discussion and Conclusion

In this study, IFN-γ was significantly produced by VL and TB patients' blood samples when stimulated with live *Leishmania d. promastigote* compared to BCG stimulation (Figure 5 and Figure 6). IFN-γ production was higher in VL patients in comparison with TB patients, which indicates a strong Th1 response in visceral leishmaniasis patients. This is similar to the findings reported by Nylen et al. [12] who reported high concentration of IFN-γ production. Interestingly, live Leishmania parasites induced IFN-γ, a known Th-1 cytokine–more efficiently than BCG which is used as vaccine against Tuberculosis. Neither live *Leishmania* nor BCG was able to induce significant IFN-γ in healthy controls.

Similarly, VL patients produced significantly higher concentration of TNF-α than TB patients. This is similar to the findings of Barral-Netto et al. [13] who reported high TNF-α concentration of active visceral leishmaniasis patients serums, whereas it was very low in normal volunteers' sera. TB patients produced higher TNF-α than healthy controls in agreement with the results reported by de Andrade et al. [14] who reported that there were significant differences in the levels of TNF-alpha between the tuberculosis and control groups.

In this study, high concentration of IL-10 was produced by TB patients compared to VL patients and to healthy controls, and it was the highest in whole blood samples stimulated with BCG of VL, TB and healthy control.

This can be contributed to the fact that all groups were previously vaccinated by BCG, as immunization by BCG induces the secretion of IL-10. Similar findings were reported by Hanekom [15] who showed that stimulation of whole blood with BCG lead to a consistent pattern of cytokine re-production: significant numbers of infants vaccinated by BCG produce either large amounts of the effector cytokine IFN-γ, or large amounts of the regulatory cytokine IL-10, but never both. IL-10 existence can lead to a stability shift from a pro-inflammatory and efficient immune response to a regulatory and dysfunctional immune response incapable of controlling disease development.

Recently IL-15 was discovered with the ability to stimulate the production activity of Th1 and/or Th2 lymphocytes [16]. Quite a few reports have shown that it increases protective immune response towards intracellular pathogens [17]. Remarkably, IL-15 concentrations in both VL and TB patients were significantly high compared to healthy controls. This is similar to the findings of Milano

et al. who reported significant increase of IL-15 in serum blood levels in acute phase of VL patients compared with healed ones; and to the findings of Abebe et al. that there was a progressive and significant increase in the expression levels of IL-15 during slowly progressive primary tuberculosis.

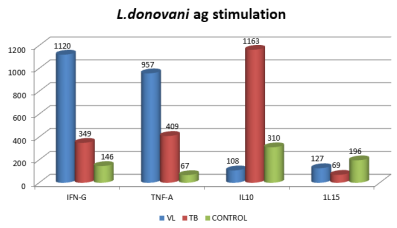

Figure 5: Cytokines production of VL, TB and healthy controls following *L. donovani* stimulation. VL patients` samples produced a significantly higher IFN-γ and TNF-α production in response to *L. donovani* stimulation. In contrast, TB patients` samples produced a significantly higher concentration of IL-10 when stimulated by BCG. There were no differences in IL-15 production by either VL, TB or healthy controls in response to *L. donovani* stimulation.

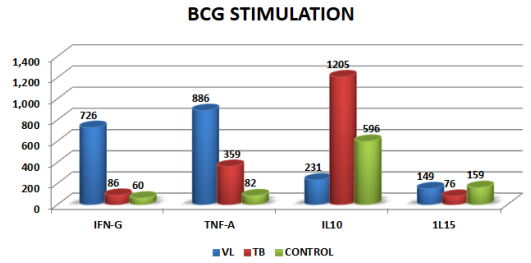

Figure 6: Stimulation of VL, TB and healthy controls samples with BCG resulted in significant production of IFN-γ and TNF-α by VL samples while induced a significant IL-10 production by TB patients' samples. No significant IL-15 was produced by either VL, TB or healthy controls samples.

When comparing stimulated (to homologous antigen) and non-stimultaed visceral leishmaniasis patients, the Th-1 cytokines expressions in the stimulated were higher compared to the non-stimulated, which could suggest a strong adaptive response. This discovery was similar to Kemp et al. [18] who observed that nonspecific Leishmania antigens like PPD presented a positive stimulation in the PBMCs of cured VL patients.

On the other hand, the Th-2 cytokine IL-10 expression was higher when stimulated with homologous antigen in TB patients than the non-stimulated that led to strong suppression the protective Th-1 cytokines expression. This discovery suggests that a re-occurring TB infection may generate a poor protective immune response which can result in more persistent infection. This finding is similar to Huygen et al. [19] who reported that, when PBMC from TB patients stimulated *in vitro* with PPD, release lesser levels of IFN-γ and IL-2, and to Vilcek et al. [20] who reported reduced IFN-γ [21].

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