Leucocytes and Th-associated Cytokine Profile of HIV-Leishmaniasis Co-Infected Persons Attending Abuja Teaching Hospital, Nigeria

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ABSTRACT

Objective: T-helper cells (Th)-1& -2 cytokines homeostasis control or predict clinical outcome of infected persons, especially those with HIV/AIDS. This case-control study evaluated the leucocytes differentials, TNF-alpha, interleukin (IL)-2 and -10 levels among HIV-infected persons with serological evidence of leishmaniasis attending University of Abuja Teaching Hospital, Nigeria

Materials and Methods: Blood samples from 28 HIV infected persons who had Leishmania donovani rK39 and Immunoglobulin-G (IgG) positive (group 1), 30 age- & -sex matched HIV infected persons without Leishmanias antibodies (group 2) and 30 apparently healthy persons without HIV and Leishmania antibodies (group 3). Full blood counts, TNF alpha, IL-2 and -10 levels were analyzed using automated hematology analyzer and ELISA, respectively. Structured questionnaires were used to collate biodata and clinical presentations of participants.

Results: Ten (35.7%) participants in group 1 were on ART, 15 (50%) in group 2 were on ART, while group 3 were ART naïve. There were significantly higher values in basophil (4.4±2.5%) and eosinophil counts (12.9±3.8%) in HIV/leishmania coinfected persons (p<0.005). However, other white cells subpopulation was significantly lower in HIV/leishmania co-infected participants (p<0.05). There was significantly reduced CD4+ T cell counts ([19±26 versus 348±63 versus 605±116 cells/mm3], TNF-alpha ([36.82±8.21 versus 64.67±12.54 versus 254.98±65.59 pg/mL]) and IL-2 levels ([1.24±20.91 versus 507.6±84.42 versus 486.62±167.87 pg/mL]) among HIV/Leishmania co-infected participants compared to group 2 and group 3 participants, respectively. However, higher IL-10 level (80.35±14.57 pg/mL) was found in HIV/Leishmania co-infected participants as opposed to the HIV monoinfected (62.2±10.43 pg/mL) and apparently healthy persons (23.97±4.88 pg/mL) (p<0.001).

Conclusion: Eosinophil, basophil counts and serum IL-10 level were high in HIV/Leishmania coinfected persons, demonstrating parasite-induced hypersensitivity and immunosuppression.

Keywords: Cellular immunity, cytokines, leishmaniasis, proinflammation, HIV coinfection

Introduction

Human immunodeficiency virus (HIV)/Leishmania coinfection poses a major threat to people living with HIV (PLWHV) and vice versa [1]. HIV and leishmaniasis are restricted within the confinement of mutual reinforcement [2]. In PLWHV, leishmaniasis induces and hastens acquired immune deficiency syndrome (AIDS) onset and limits lifespan [3]. Furthermore, HIV infection escalates the risk of clinical visceral leishmaniasis (VL) (a severe form of leishmaniasis) more than 100 times [4]. HIV/Leishmaniasis coinfection increases disease burden by inhibiting T-helper-cell-mediated immune responses [1, 4]. In addition, there is a fundamental change in the cytokine network signaling pattern [1, 4].

Leishmaniasis could be cutaneous (CL), mucocutaneous, or VL depending on the etiologic species and the site or tissue/organ affected. CL poses an enormous health challenge in most global regions, especially in the third-world countries [5]. Leishmaniasis is considered endemic in at least 88 countries [6]. In sub-Saharan Africa, PLWHV are afflicted more frequently with VL as the third most common infection [7]. As a result of the VL affecting the destitute and indigent patients, underreporting of the presented cases in several affected regions is usually due to the absence of diagnostic equipment to investigate the coinfection and inefficient reporting systems [1, 7].

Importantly, individuals living with VL/HIV coinfection are regarded as superspreaders of VL infection, which presents a major challenge to the efforts of eradicating leishmaniasis [1]. Based
on these reasons, it was proposed that Leishmania organisms are critical in the pathogenesis of HIV-1 infection. In addition, it was observed that L. donovani amastigotes enhance HIV replication via tumor necrosis factor alpha (TNF-α) production and activates CD4+ T cells with the aid of its lipophosphoglycan [8]. Putting together, it has been proven that antileishmanial therapy is efficacious in the inhibition of HIV replication [9]. However, the balances of pro- and anti-inflammatory cytokines are needed to prevent immunopathological disorders [9].

Proinflammatory cytokines secreted by Th1 cells are instrumental in regulating the proliferation and differentiation of T-lymphocytes in defense against HIV/Leishmania coinfection. Despite the role of these cytokines, it is vital that these populations are balanced with their anti-inflammatory counterparts so as to avoid unpleasant immunopathological defects.

These defects that could be due to unregulated Th1 cell activity and excessive role of pro-inflammatory cytokines can have a negative impact on the survival of T cell clones. The T cells that survive are dysfunctional and lack the ability to increase in number as well as secrete specific interferon-gamma targeted at antigenic stimuli. Previous research has shown that CD4+ T lymphocytes with diverse lymphokine patterns are more efficient compared with humoral B cells in reducing the burden of Leishmania infection. In contrast, the anti-inflammatory cytokines (IL-4 and IL-10) when secreted by Th2 cells in discriminate proportions can promote the survival of parasites within cells and increase disease burden [1, 10].

A scientific study currently reported the existence of a dominant cytokine profile secreted by Th1 cells and possess high protective role for pathogens compared with Th2 cytokines in HIV infection. It has been hypothesized that a shift from Th1 to Th2 cytokines depletes CD4 population and enhances viral replication in AIDS [9, 11]. In order to determine the expression profile of Th1 and Th2 cytokines in the case of HIV/Leishmania coinfection, this study aims to investigate the plasma levels of both pro- (IL-2, interferon-gamma) and anti- (IL-10) inflammatory cytokines as well as white cell differentials of persons coinfected by the HIV and leishmaniasis attending the study area.

Materials and Methods

Study Area

This hospital-based study was conducted at Abuja in the Federal Capital Territory (FCT), Abuja, Nigeria. Blood samples were collected at the antenatal clinics and analyzed at the immunology laboratory. Gwagwalada is about 45 km away from the FCT. It is one of the six area council headquarters of the FCT. The town lies in the downstream of River Usuma and is located between 8°55’ and 9°00’N and longitudinal 7°00’ and 7°05’E.

The centrality of this town in relation to other area councils’ headquarters makes it influential and important in various socioeconomic activities. The climate condition of this town is not far-fetched from that of the tropics having several climatic elements in common, especially the wet and dry season characteristics. The temperature of the area ranges from 30°C to 37°C yearly, with the highest temperature experienced in the month of March and mean total rainfall of approximately 1650 mm per annum. The area council is an industrial zone of the FCT that stands out as the second most cosmopolitan city of the FCT after the capital city with 10 political wards and consists of more than 26 Federal organizations. These have brought about the inflow of people into the council. About 60% of Gwagwalada residents live in rural settlements and are predominately farmers.

Sample Collection and Preparation

5-ml whole blood samples were collected aseptically. 2-ml ethylenediaminetetraacetic acid-preserved blood samples were used for CD4+ cell counts and full blood count, while 3-ml lithium-heparin-preserved blood was used to harvest plasma for cytokine measurement using enzyme immunoassay. Samples were collected between April 7, 2015 and October 10, 2015. Blood samples were analyzed within 1 h of collection.

Laboratory Analytical Procedures

White Cell Count and Differentials

The Sysmex™ XS-1000i five-part automated hematology analyzer was used for the total white cell count, monocyte, basophils, eosinophils, and lymphocyte differentials using the direct current detection method with coincidence correction. The automatic discriminators separated the cell populations based on complex algorithms. The intensity of the electronic pulse from each analyzed cell was proportional to the cell volume.

Flow Cytometry Assay for Lymphocyte Population

Based on the manufacturer’s instructions, the CD4+ cell counts in the whole blood were analyzed using a Partec™ CyFlow Analyzer (Sysmex, Norderstedt, Germany) Model SL3. This device used the principle of light-scattering property (based on dissimilarity in cell size or granularity)
and the fluorescence of cells following staining with monoclonal antibodies to markers on the cell surface bound to fluorescent dyes. Flow cytometry data were analyzed using FlowJo v.7.6.5 software (BD, Oregon, USA). Cell populations of interest were then gated after identification. The generated percentages were multiplied by the total number of lymphocytes in the hemogram to derive absolute values for circulating lymphocytes. Absolute CD4+ cell counts were subsequently analyzed using a single-platform technique. Values for apparently healthy participants (group 3) were used as a reference.

**Enzyme-Linked Immunosorbent Assay for Anti-Leishmania IgG Antibody**

Indirect ELISA was carried out according to the method described by kit manufacturer (product code: KA3295) (Abnova®, Walnut, CA, USA). The *Leishmania* ELISA test is a three-incubation process, whereby the first incubation involved the coating of the wells with *Leishmania* spp antigens. During this step, all antibodies that are reactive with the *L. donovani* antigens bind to the wells. Next, the wells were washed to remove the test sample and other non-IgG antibodies. At this point, enzyme conjugate was added. During this second incubation, the enzyme conjugate specifically bound to IgG antibodies present.

Before the third incubation step, three more washings were done. Then, a chromogen (tetramethylbenzidine or TMB) was added. With the presence of enzyme conjugate and the peroxidase causing the consumption of peroxide, the chromogen changed to a blue color. The blue color turned to a bright yellow color after the addition of the stop solution, which ended the reaction. The ELISA plate reader was used for the optical densities (ODs) of every well, and results were calculated from the ODs.

**Detection of Leishmania Donovani rK39 Antibodies by Immunochromatography**

This test was conducted using the Inbios® Kalazar Detect™ Rapid Test (USA). The Kalazar Detect™ Test for VL is a qualitative, membrane-based immunoassay for the detection of antibodies to *Visceral Leishmaniasis* in human serum. The membrane was pre-coated with rK39 on the test line region and chicken anti-protein A on the control line region. During testing, the serum sample reacted with the dye conjugate (protein A-colloidal gold conjugate) which has been pre-coated in the test device. The mixture then migrates upward on the membrane chromatographically and reacted with recombinant VL antigen on the membrane and generates a red line. The presence of this red line indicated a positive result, while its absence indicated a negative result. Regardless of the presence of antibody to rK39, as the mixture continues to migrate across the membrane to the immobilized chicken anti-protein A region, a red line at the control line region will always appear. The presence of this red line served as verification for sufficient sample volume and proper flow and as a control for the reagents. In addition, external control sera with known *L. donovani* result were run alongside the samples. This test has sensitivity of 89.8% and specificity of 100%.

**Enzyme-Linked Immunosorbent Assay for Cytokines**

ELISA was carried out according to the method described by kit manufacturer (Abcam®, Cambridge, UK). Accordingly, IL-2 (product code: ab174444), IL-10 (product code: ab100549), and TNF-α (product code: ab181421) were investigated.

The simple-step ELISA® employs an affinity-tag-labeled capture antibody and a reporter-conjugated detector antibody, which immunocapture the sample analyte in solution. This entire complex (capture antibody/analyte/detector antibody) is, in turn, immobilized via immunoadsorption of an antitag antibody coating the well. To perform the assay, samples or standards were added to the wells, followed by the antibody mix. After incubation, the wells were washed to remove unbound material. TMB development solution was added, and during incubation, the reaction was catalyzed by Horseradish Peroxidase (HRP), generating blue coloration. This reaction was then stopped by addition of Stop Solution completing any color change from blue to yellow. Signal is generated proportionally to the amount of bound analyte, and the intensity was measured at 450 nm.

**Statistical Analysis**

SPSS (version 24, IBM SPSS Corp.; Armonk, NY, USA) was used to analyze data, which were expressed as mean±SD, and statistical significance was considered for p<0.05. One-way analysis of variance was used to compare categorical variables. P values <0.05 at a confidence interval of 95% were considered statistically significant.

### Results

**HIV/Leishmania-Coinfected Participants Showed Preponderance of Female Gender**

The evaluation of the preponderance of female gender on the basis of the ratio of all the participant groups (1–3), indicated that the female-to-male ratio (19:9) was mainly maximum in participants coinfected with HIV and leishmaniasis. The mean±SD age of participants was 26.6±4.9, 28.2±5.8, and 26.9±5.1 years for groups 1, 2, and 3, respectively. Only 10 (35.7%) participants in group 1 were on Antiretroviral Therapy (ART) and 15 (50%) in group 2 were on ART, while participants in group 3 were ART naïve (Table 1).

**HIV/Leishmania-Coinfected Participants Indicated Varied Leukocyte Profile Concentrations in Whole Blood**

Serum levels of neutrophil (p=0.04) and lymphocytes (p=0.001) were significantly decreased in *Leishmania*/HIV-coinfected participants compared with the HIV-monoinfected participants. In contrast, the serum levels of basophils (p=0.001), eosinophils (p=0.001), and monocytes (p=0.001) were significantly increased in the *Leishmania*/HIV-coinfected group of participants as opposed to the HIV-monoinfected group of participants (Table 2).

**CD4+ T Cell Population Decline in Response to HIV/Leishmania**

CD4+ T cell population significantly (p=0.001) decreased in participants with HIV/Leishmania coinfection compared with their HIV-monoinfected counterparts. All HIV seropositive participants with or with anti-Leishmania IgG had their CD4+ T lymphocyte below the reference lower limit of 500 cells/mm³ (Table 3).

| Table 1. Demographic of participants with and without anti-Leishmania IgG |
|-----------------------|-------------------------|-------------------------|-------------------------|
| Variable              | HIV Persons with anti-Leishmania IgG seropositive (n=28) | HIV Persons with anti-Leishmania IgG seronegative (n=30) | Persons without HIV and anti-Leishmania IgG seronegative (n=30) |
| Age (years) Mean±SD   | 26.6±4.9                | 28.2±5.8                | 26.9±5.1                |
| Male-to-female ratio   | 9:19                    | 9:21                    | 9:21                    |
| Number of participants on ART | 9                      | 15                      | 0                      |

SD: standard deviation; HIV: human immunodeficiency virus
HIV-monoinfected counterparts. In contrast, significantly increased serum levels of anti-inflammatory cytokine [IL-10 (p=0.001)] were observed in the Leishmania/HIV-coinfected participants as opposed to the HIV-monoinfected participants (Table 3).

Discussion

Leishmania/HIV's capability to influence host cellular immunity and coexist in the lymphoid tissues indicates their ability to mutually communicate by mutually re-enforcing their replication when coexisting in the same host cells [11]. To the best of our knowledge, this is one of a few studies that demonstrate, for the first time, the influence of the interaction of these pathogens on cytokines and leukocytes, which are orchestrators of the host defense system and play vital functions in modulating the host immune response against Leishmania/HIV coinfection. Comparative analysis of leukocyte and cytokine profiles from co- and monoinfected participants highlighted significant variations in immune responses displayed against coinfection, confirming the ability of Leishmania and HIV to mutually interact at the immunological level.

Participants coinfected with Leishmania and HIV responded with an overall decrease in pro-inflammatory cytokine (IL-2 and TNF-α) and the increase in anti-inflammatory cytokine (IL-10) release. Low levels of these proinflammatory cytokines have been reported in Indian patients with active leishmaniasis and elevated levels observed in those treated for leishmaniasis [12]. IL-2 and TNF-α have been implicated in protective immunity toward parasitic and viral infections [13]. TNF-α, a vital mediator of both innate and adaptive inflammatory responses [14], is observed to possess a crucial role in the formation and maintenance of granuloma against visceral Leishmania. This antiparasitic function of TNF-α is mediated by the activation of infected macrophages for the killing of intracellular Leishmania amastigote [15]. IL-2, which is produced by activated CD4+ T cells and was formally referred to as “T-cell growth factor,” has been thought to induce the proliferation and differentiation of Th2 cells (B-cells, natural killer cells, monocyte/macrophages, oligodendrocytes, and lymphocyte activated killer cells) and aid in the release of IgG1 and IgE-producing cells, which is vital for the resolution of leishmaniasis [16], suggesting that this cytokine may be crucial for Leishmania clearance. Hence, decreased concentrations of IL-2 observed in the coinfected versus the monoinfected cohort, apart from the already diminished levels of TNF-α, may be indicative of a nonfavorable, poor prognosis for Leishmania.

However, an increase in the level of IL-10 (an anti-inflammatory cytokine) and reduction in cell-mediated immunity are seen in Leishmania infection. IL-10 dampens the production of many proinflammatory cytokines (including TNF-α, IL-1, IL-6, and IL-12), it also decreases the expression of MHC-II and other costimulatory molecules on the surface of macrophages, and these inhibit macrophage-mediated activation of CD4+ helper T cells, leading to a reduction in both adaptive and innate immune responses [17].

The immunosuppressive role of IL-10 in human VL leads to a drastic decrease in the accumulation of monocyte-derived macrophages, which is controlled by the migration inhibition factor. Furthermore, IL-10 is observed to encourage the Th1 cell dysfunction, which enhances intracellular infection for interferon-gamma production, deactivating parasitized tissue macrophages and downregulating antigen presentation by dendritic cells [17].

IL-10 has also been implicated in inhibiting the leishmanicidal roles of macrophages by decreasing the synthesis and release of proinflammatory molecules (reactive nitrogen intermediates by macrophages, interferon-gamma by Th1 natural killer cells, and IL-12-mediated activation of macrophages). These are suppressive roles of IL-10, which is released by alternatively activated macrophages and interferon-gamma coproduc-

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### Table 2. Comparison of white cell count and differentials of participants with and without anti-Leishmania IgG

| Parameter                        | Value (mean±SD) | Value (mean±SD) | Value (mean±SD) | F    | p     |
|----------------------------------|-----------------|-----------------|-----------------|------|-------|
|                                  | HIV persons     | HIV persons     | Persons without |     |       |
|                                  | with            | with            | HIV and         |      |       |
|                                  | anti-Leishmania | anti-Leishmania | anti-Leishmania |      |       |
|                                  | IgG seropositive| IgG seronegative| IgG seronegative|      |       |
| WBC 10³L⁻¹                       | 3.7±1.2         | 4.2±1.9         | 5.9±2.1         | 12.23| 0.0001|
| Neutrophils (%)                  | 58.9±11.6       | 61.6±10.4       | 64.1±13.2       | 5.834| 0.040 |
| Lymphocytes (%)                  | 22.5±9.8        | 29.9±10.6       | 30.7±9.9        | 9.994| 0.001 |
| Basophils (%)                    | 4.4±2.5         | 3.1±1.2         | 1.4±0.8         | 11.01| 0.001 |
| Eosinophils (%)                  | 12.9±3.8        | 4.3±2.6         | 3.2±2.3         | 62.63| 0.001 |
| Monocytes (%)                    | 1.3±1.5         | 1.1±0.2         | 0.6±0.1         | 13.97| 0.001 |

WBC: white blood cells; SD: standard deviation; HIV: human immunodeficiency virus

### Table 3. Comparison of cytokines and CD4+ T cell count and differentials of participants with and without anti-Leishmania IgG

| Parameter                        | Value (mean±SD) | Value (mean±SD) | Value (mean±SD) | F    | p     |
|----------------------------------|-----------------|-----------------|-----------------|------|-------|
|                                  | HIV persons     | HIV persons     | Persons without |     |       |
|                                  | with            | with            | HIV and         |      |       |
|                                  | anti-Leishmania | anti-Leishmania | anti-Leishmania |      |       |
|                                  | IgG seropositive| IgG seronegative| IgG seronegative|      |       |
| CD4+ T cell count (cells/mm³)    | 119±26          | 348±63          | 605±116         | 278.0| 0.001 |
| IL-2 (pg/mL)                     | 142.1±20.91     | 507.6±84.42     | 486.6±167.87    | 291.0| 0.001 |
| IL-10 (pg/mL)                    | 80.35±14.57     | 62.2±10.43      | 23.97±4.88      | 211.0| 0.001 |
| TNF-α (pg/mL)                    | 36.82±8.21      | 64.67±12.54     | 254.98±65.59    | 269.0| 0.001 |

SD: standard deviation; HIV: human immunodeficiency virus; IL: interleukin; TNF-α: tumor necrosis factor alpha.
cing CD4+ T cells (type I regulatory T cells) at moderate-to-high levels owing to the low-to-zero levels of pro-inflammatory cytokines and the decreased number of multifunctional CD4+ T cells [17]. During the initial phases, infection justifies why it is associated with decreased immunity against Leishmania infection. Because of the suppressive role of IL-10, the parasite burden continues to build up even when interferon-gamma levels are elevated [18].

In addition to the varying levels of the cytokine profile, this study revealed a higher secretion of IL-10, which was associated with increased viremia and depleted CD4+ T cell population in Leishmania/HIV-coinfected participants compared to those of the HIV-monoinfected group. This corroborated with a recent study, which reported CD4+ T cell count as low as 49 cells/mm3 in HIV/Leishmania asymptomatic immune responders compared with the nonresponders [19]. The marked decrease in the number of CD4+ T cells is associated with the reduced leishmanicidal capacity of macrophages and the replication and uncontrolled systemic spread of the parasite throughout the body [20], which increases the risk of progression to VL by 100-2320 times based on the mutual interaction between HIV and Leishmania [21].

Based on the leukocyte profile, HIV/Leishmania-coinfected participants had significantly lower lymphocyte and neutrophil counts with significantly higher monocyte, basophil, and eosinophil counts, as observed in this study. These observations were in line with a study conducted in Brazil [22], which demonstrated significantly lower levels of lymphocyte and hemoglobin counts at diagnosis and considerably higher levels of eosinophil count in the initial hematologic evaluation in over 50% of coinfected participants. Similar observations were observed in the liver of genetically susceptible mice infected with experimental VL [19].

The primary cells of mice that are infected by Leishmania donovani amastigotes are the liver-resident tissue macrophages (Kupffer cells). These cells are observed to produce cytokine sand chemokines, which recruit monocytes and neutrophils to the infection site during the initial days of infection, thus further amplifying the production of more chemokines [23]. Neutrophils (primary antimicrobial cells) control infections by phagocytizing and killing invading pathogens. Although some of these pathogens are killed by the lysosomal effect of the neutrophil, others, which are either obligate or facultative intracellular organism, become resistant to the lysosomal enzymes and replicate in these cells, which become apoptotic. These apoptotic neutrophils (without parasitidal effect) either control Leishmania growth by undergoing depletion to reduce the load of this parasite and delaying the onset of leishmaniasis [24] or act as Trojan horses with delayed apoptosis by the parasite for onward transmission, infection, and replications of these parasites in monocytes and macrophages that phagocytize the infected apoptotic neutrophils.

Another exciting outcome observed in this study is the decrease in neutrophil count. Leishmania infection interferes with several signaling pathways in immune cells [25]. Monocytes have been observed to possess the antiparasitic function based on their plasticity and ability to differentiate into potent antigen-presenting or regulatory cells [26]. The recruitment of neutrophils, monocytes, and then the T cells into the Kupffer cells is crucial for the formation of granuloma around these cells, which is required for controlling the growth of Leishmania/HIV coinfection [27].

The cellular immune-mediated interactions between Leishmania donovani and HIV seem to determine the extent of immunological response that occur in the coinfected biological system as well as the intensity of the onset of the accompanying infection. Similar circumstances have been observed with other Leishmania coinfections, revealing that the inherent consequences that emerge from several pathogen-host relations need to resolve when designing Leishmania vaccine trials. Proper consideration of parasite interplays should be adopted when determining the best strategy for the treatment of Leishmania/HIV coinfections to achieve immune stability without inflicting harm during the clinical course of the infected individual.

In conclusion, Eosinophil, basophil counts, and serum IL-10 levels were high in HIV/Leishmania-coinfected persons, suggesting parasite-induced hypersensitivity and proinflammatory reaction; this could lead to severe forms of leishmaniasis, if prompt and appropriate therapy is not instituted.

Ethics Committee Approval: Ethics committee approval was received for this study from the Ethics Committee of the University of Abuja Teaching Hospital (approval number: UATH/HREC/PR/002/058).

Informed Consent: Informed consent was obtained from patients who participated in this study.

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