The Patterns of Interferon-Gamma and Interleukin-10 Production as a Potential Immunological Biomarker for the Outcome of Mycobacterium Tuberculosis Infection

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Abstract: Deficiencies in current tuberculosis (TB) immunodiagnostics pipeline demand new approaches to control TB. Because the balance in key pro- and anti-inflammatory cytokines production could determine Mycobacterium Tuberculosis (MTb) infection outcome, this study aimed to determine the patterns of MTb-specific antigen-stimulated Interferon-gamma (IFN-y) and Interleukin-10 (IL-10) production in different clinical forms of MTb infection and to evaluate their concomitant changes during anti-TB treatment (ATT). Overall, 84 BCG-vaccinated HIV-negative adults, consisting of 25 Healthy Community Controls (HCC), 27 Latent Tuberculosis Infection (LTBI) cases, and a cohort of 32 Acute Pulmonary Tuberculosis (APTB) patients were investigated for IFN-y and IL-10 responses at enrollment (base-line) and during ATT at 2-month (ATT1) and 6-month (ATT2). At enrollment, groups didn't differ significantly in age, gender, or CD4+ T counts but differed in the other socio-demographics, and hematological parameters, p<0.05. Base-line Sandwich ELISA – measured IFN-y responses were significantly higher in HCC (223.50±58.11pg/ml) compared with LTBI (128.82±41.81pg/ml) and APTB (47.82±22.05pg/ml), p<0.0001 in each case. During treatment, IFN-y levels increased significantly at ATT1 (125.37±16.09pg/ml) and ATT2 (203.35±23.24pg/ml), p<0.0001. Conversely, base-line IL-10 responses increased significantly in APTB (17.53±6.30pg/ml), compared with LTBI (10.71±2.39pg/ml) and HCC (7.49±2.02pg/ml), P<0.0001, but declined significantly at ATT1 (10.54±2.25pg/ml) and ATT2 (5.25±1.45pg/ml), P=<0.0001. Cytokines response combination ratio showed: 'High' HCC, 'Intermediate' LTBI, or 'Low' APTB ratio that increased during successful ATT; the two identified MDR-TB patients recorded fluctuating but constantly low ratio during ATT. These results demonstrate the immunocompetence of MTb-exposed adults, and that IFN-y and IL-10 cytokines cross-regulate, and strongly suggest a shift toward IFN-y-mediated pro-inflammatory host immune phenotype during effective control of MTb infection. The IFN-y/IL-10 response ratio is a novel potential immunological biomarker to assess if MTb infection is going to resolve, result in latency, progress to TB; or become drug-resistant.

Keywords: Adults, Mycobacterium Tuberculosis Infection, Immunodiagnostics, Cytokines Production Ratio, TB Control

1. Introduction

Tuberculosis (TB) caused by mycobacterium tuberculosis (MTb) bacillus is the oldest documented infectious disease. TB remains a major public problem worldwide, especially in developing countries, despite some advances in diagnostics and availability of chemotherapies to combat the disease [1]. The challenge of TB is worsening with the spread of multidrug-resistant (MDR) TB disease and the increased susceptibility of HIV-infected individuals to developing TB [2]. Globally, an estimated 3.3% of new TB cases and 20% of previously treated individuals had MDR-TB in 2014 [3]. TB, a predominantly inflammatory disease of the lungs, is the result of a complex interaction between the causative agent (MTb) and the host's innate and adaptive immune responses. In the vast majority of MTb-infected individuals, an effective cell-mediated immune response shortly after infection results in asymptomatic latent tuberculosis infection (LTBI), which may remain undiagnosed and could reactivate later in life to cause active (infectious) TB [4, 5]. It has been established that co-ordination/co-operation between the various cellular elements, especially the macrophages and CD4+T lymphocytes, which is dependent on the interplay of cytokines secreted by these cells, are critical for the control of MTb infection [4, 5, 6]. Those with cellular immune defects involving cytokine response dysregulation could be especially at risk for developing active TB. Accurate diagnosis of the clinical form of MTb infection is a cornerstone of TB control.

A century old sputum smear microscopy is the most widely used test in high TB-endemic countries and this test is inadequate to diagnose early TB disease and sputum smear-negative TB; it cannot differentiate between the clinical forms of pulmonary MTb infection.

The detection of MTb and discrimination between different states of MTb infection is possible by immunodiagnostic testing. One of the major limitations of the currently available immunodiagnostic methods for MTb infection is that neither the Tuberculin Skin Test (TST) nor Interferon Gamma Release Assays (IGRAs) are able to distinguish between individuals with LTBI and active TB infection [7, 8]. There is currently no standard test to distinguish between LTBI and active TB, a distinction critical for clinical management as the treatment of LTBI and active TB differs, especially in geographic regions where TB prevalence is high.

Therefore, there is need to identify other laboratory parameters specific to TB which could be useful surrogate (host) markers of response during treatment of pulmonary TB. The lack of reliable biomarkers to indicate or predict the different clinical outcomes of MTb infection has been given as a key reason for the failure of development of new diagnostic and prognostic tools for MTb infection [9]. There are apparent deficiencies in the existing TB diagnostic and treatment pipelines. New approaches to control TB worldwide are needed.

The progression of LTBI to active pulmonary TB has been shown to be related to the patterns of the inflammatory cytokines secreted by the CD4+ T lymphocytes [5, 10]. Although a broad spectrum of cytokines may contribute to protection, the Type 1 response, dominated by the pro-inflammatory Interferon-gamma (INF-γ) secretion, is a principal mediator of protective immunity against MTb [6], and the Type 2 response, on the other hand, is characterized by, in particular, the anti-inflammatory cytokine, Interleukin-10 (IL-10) associated with reduced resistance and chronic progressive TB [11, 12]. Because differences in the pro- and anti- inflammatory cytokines could possibly determine the clinical outcome of MTb infection, it is useful to study cytokine responses during the different stages of infection.

While the pro-inflammatory IFN-γ level can serve as a marker for previous MTb antigen encounter, its value while measured alone at determining disease state has been observed to be limited [7, 13]. The IL-10 response, which has been linked with the ability of MTb evasion, and the protective INF-γ could be simultaneously measured to perform accurate immunodiagnostics of LTBI versus active TB disease.

Given the impact of mycobacterial exposure and its immunoregulatory consequences for host immunity, it is important to study the pattern of the production of these key MTb-specific cytokines in TB-endemic areas since most individuals will be exposed to tuberculous and environmental mycobacteria. For instance, a recent study of a cross-section of apparently healthy individuals in the Okada community, Nigeria, revealed a high proportion of the population has latent tuberculosis infection [14]. Nigeria, the most populous country in Africa, leads the continent in the number of TB cases, and is currently rated 4th globally among the 22 high TB-burden countries (HBCs) [15].

As part of TB infection control program, it was decided to conduct a prospective study of IFN-γ and its inhibitory IL-10 levels among adults, consisting of healthy community controls, apparently healthy individuals with latent tuberculosis infection, and a cohort of active pulmonary tuberculosis patients receiving anti-tuberculosis treatment— all resident in the TB-endemic southern Nigeria. The present study aimed to determine the patterns of MTb-specific antigen-induced INF-γ and IL-10 cytokine responses in relation to MTb infection outcome and to also assess the changes during anti-TB treatment. The knowledge and usefulness of cytokine immunoassays are limited in Nigeria, and no work has been reported from this area on the evaluation of concomitant changes in pro-and anti-inflammatory cytokine responses during MTb infection.

2. Materials and Methods

2.1. Study Participants and Data Collection

The study participants consisted of three diagnostic groups of adults in our community. Healthy Community Controls

(HCC) recruited from students and staff of Igbinedion University (IUO) & Igbinedion University Teaching Hospital (IUTH), Okada; asymptomatic individuals with latent tuberculosis Infection (LTBI) as indicated by positive (≥10mm) TST result and free of clinical symptoms and signs suggestive of pulmonary TB and who have been in close contact and lived with a patient with microbiologically-confirmed TB for longer than 1 month; and newly diagnosed active pulmonary tuberculosis (APTB) patients based on clinical, radiological, microbiological and pathological results and registered for anti-TB treatment (ATT) at the IUTH Chest Outpatient Clinic. The APTB patients were evaluated according to the Nigeria National Tuberculosis and Leprosy Control Program (NTLCP) guideline [16] and had not been commenced on anti-TB treatment at the time of enrollment. The chest radiographs were read by radiologists who were blinded to the status of the participants; and the enrolled HCC and LTBI subjects had normal X-ray results.

After giving written informed consent, each study volunteer answered a pre-designed semi-structured questionnaire to glean information on socio-demographic data (age, gender, occupation, marital status, contact address), history of coughing, fever and night sweats, risk factors for TB such as smoking habit, alcohol consumption pattern, results of prior tuberculin skin test (TST) and sputum smear examination for acid fast bacilli (AFB), BCG (bacillus Calmette -Guerin) vaccination history, chest radiographic findings; and history of TB in family or close relation. The information supplied by each participant were confirmed from subject's medical records at the Igbinedion University Teaching Hospital (IUTH) or at the Okada Health Centre. Data were analyzed unanimously.

Serological evidence of HIV infection, non-BCG vaccination, pregnancy, chronic cardiovascular or metabolic diseases, immunosuppressive medication, age less than 18years, and incomplete data records constituted exclusion criteria.

Clinical examination of qualified participants at enrollment included measurement of body mass index (BMI) for evaluation of body weight, early-morning sputum smear staining for AFB by Ziehl-Neelsen technique, and BCG vaccination was confirmed by presence of BCG scar (s).

Ethics: The study was conducted with the prior approval of the Ethics Committee of the Igbinedion University & Igbinedion University Teaching Hospital, Okada, Nigeria.

2.2. Tuberculin Skin Testing

Tuberculin skin tests were performed by one of us (Immunologist) on all the participants at the time of initial assessment using 5-TU (Tuberculin Unit) of protein purified derivative (PPD) RT23 (Statens Serum Institute, Copenhangen, Denmark). The largest transverse diameter of the palpable hardened area was measured 48-72hrs with a plastic millimeter ruler and the cut off for a positive result in a subject was diameter ≥10mm. All LTBI and APTB cases were positive in the PPD skin test as per their definition in this study.

2.3. Anti-tuberculosis Treatment

Free anti-tuberculosis treatment was provided to all the enrolled APTB patients in accordance with the NTLCP guideline and consisted of a standard regime of Isoniazide, rifampicin, pyrazinamide and ethambutol (2HRZE4H3R3). Treatment was supervised once weekly by Direct Observation Therapy Short-course (DOTS) program [17]. Treatment was evaluated at 2- month (ATT1) and 6- month (ATT2) of standard anti-TB therapy.

2.4. Blood Collection and Processing

Peripheral blood samples from Healthy Community Controls, LTBI and APTB cases were taken at enrollment (baseline) by venipuncture from a vein in the pre-cubital fossa following strict aseptic measures. For the determination of the hematological parameters, 2.5ml of whole-blood was drawn and transferred into ethylenediamine tetraacetic acid (EDTA) and analyzed in the Hematology Analyzer (Sysmex ICX-21N), an automated differential hematology analyzer, for the estimation of hemoglobin (Hb) concentration, white blood cell (WBC), lymphocyte and monocyte levels. Total CD4+T cell counts were determined using Becton Dickinson FACS Flow Auto Counter (Kaplan Scientific Inc., Japan); and 2ml of the blood was used for erythrocyte sedimentation rate (ESR) determination by the Westergren tube method. For the cytokine assay, 5ml of baseline whole blood sample was allowed to clot for 2hrs at RT^o before centrifugation at 5000rpm for 10min; sera were collected and stored at -20°C until they were tested for cytokines by enzyme- linked immunosorbent assay (ELISA). Repeat blood samples were also taken from the APTB patients after 2-month (ATT1) and 6- month (ATT2) of TB treatment and blood samples were stored frozen until tested for cytokines assay by ELISA.

2.5. Quantitative ELISA for INF-ɣ and IL-10

The stored sera for ELISA were allowed to thaw out at RT^o and assayed in batches for either INF-ɣ or IL-10 cytokine following the manufacturer's instructions on the respective commercial Human Cytokine ELISA Kit (Elabscience Biotechnology Inc.).

The ELISA kit uses Sandwich-ELISA as the method. Briefly, 100µl/well of the re-constituted cytokine Kit standard and serum sample were dispensed into appropriately-labelled high affinity 96-well plastic micro plates pre-coated with the corresponding specific monoclonal antibodies. The plates were incubated for 90mins at 37°C and the supernatant removed. Next, 100µl/well of the diluted biotinylated anti-cytokine antibody was added and the plates were incubated for 1hr at 37°C and then washed x3times with the Kit wash buffer. After this step, 100µl/well of avidin-horse radish peroxidase (HRP) was added. The plates were incubated x30mins at 37°C and washed again x5 times with the Kit wash buffer. Then, 90µl/well Substrate Reagent solution was added and incubated x15 mins at 37°C. Next, 50µl/well Stop solution was added and absorbance was read immediately at 450nm. The concentration of the cytokine in

the analyzed serum was calculated by comparing the optical density (OD) of the sample wells to the Standard curve. The OD value is proportional to the concentration of the corresponding human cytokine.

The detection range for the INF-ɣ and IL-10 were 15.63 to 1000pg/ml and 7.81 to 500pg/ml respectively. Sensitivity was 9.38pg/ml for INF-ɣ and 4.69pg/ml for IL-10. Indeterminate ELISA results due to inadequate cytokine responses after stimulation with the positive controls were excluded from the study data because the test could not be repeated since test had deployed.

The investigator who performed the cytokine ELISA was blinded to the identity of the coded sample.

2.6. Cytokine Intra-Assay and Inter-Plate Quality Controls

For assay precision, the reliability and accuracy of individual ELISA kits in measuring INF-ɣ or IL-10 was assessed using the quality controls provided with each Kit. The levels of each of the measured cytokines fell within the manufacturer's expected range. As inter-plate quality controls, we used five aliquots from the serum of one patient; one aliquot of the inter-plate control was included in each plate with each run. For each of the cytokines measured, the variation of the inter-plate control did not generally exceed 10%.

2.7. Statistical Analysis

Demographic, clinical and laboratory raw data from each study participant were collated with the aid of Microsoft Access Database (Microsoft Corp., Redmond, Australia). Results are given as means ± Standard deviation, frequencies or percentages. Data analysis were performed using Graph-Pad Prism 400 (Graph-pad Software 5.0 Inc., Carnegie, Australia). Differences in cytokine levels among groups were evaluated by the Kruska-Wallis and Mann-Whitney U tests. One-Way ANOVA was used to analyze the changes over time in cytokine levels in the APTB patients. A p-value of ≤0.05 was considered as statistically significant.

2.8. Limitations of Study

There are some limitations to the present study. This is a study with limited population size. The specific cellular sources of IFN-ɣ and IL-10 cytokines in the sera of our subjects were not identified.

3. Results

An initial total of 88 volunteers who met the study criteria were recruited. Four participants were excluded from the final results: two 18 year-old male recruits who did not return for the TST reading, two APTB patients (one male and one female) who had indeterminate INF-ɣ and IL-10 test results respectively in our cytokine ELISA. Overall, a final total of 84 individuals were enrolled and included in the study analysis. The enrolled participants consisted of 25 healthy community controls (HCC), 27 latent tuberculosis infection (LTBI) cases and 32 newly diagnosed active pulmonary tuberculosis (ATB) patients.

Baseline characteristics of Participants

The demographic and clinical characteristics of the subjects at enrollment are reported in Table 1. There were no significant differences among the groups regarding age or gender. At the point of enrollment, the untreated APTB patients had significantly higher body temperature than in LTBI and healthy controls and had symptoms of pulmonary tuberculosis, such as chronic cough, persistent fever, night sweats and unexplained weight loss. The body mass index (BMI) in APTB patients (18.9 ± 0.8 kg/m^2) was significantly lower than that in LTBI (22.3 ± 0.5 kg/m^2, p<0.05) cases and in healthy controls (22.5 ± 0.3kg/m^2, p<0.05). Cavitary TB lesions of varying grades were recorded in all the APTB patients. The mean purified protein derivative (PPD) response diameter in the TST was statistically higher in the APTB (16.5 ± 4.7 mm) and LTBI (13.5 ± 5.5mm) cases compared to healthy controls (7.2 ± 1.3mm, P < 0.0001). All the subjects have BCG scar (s). Risk factors for TB, such as smoking (53.1%) and alcohol ingestion (43.8%) were observed more frequently among the APTB patients. Hematological parameters showed anemia (as defined by hemoglobin (Hb) level) in APTB compared with LTBI and HCC groups; raised erythrocyte sedimentation rate (ESR), significant leukocytosis and lymphopenia among APTB patients. The CD4+T counts in all the three groups were within normal range as for the Nigerian adult population (853.2 ± 297.6 cells/μl) [18] and showed no statistical difference (P= 0.3679) between the groups (Table 1). All of the enrolled subjects were serologically negative for HIV infection.

Table 1. Baseline Characteristics of the Study Participants (N=84).

Parameter	HCC (n=25)	LTBI (n=27)	APTB (n=32)
Age (range), years	27 (18-56)	32 (20-55)	25 (18-54)
Male/female	14/11	12/15	17/15
Mean TB symptoms Duration ±SD (wk.)	0 (0.0)	0 (0.0)	13±2.2
BMI (Range) (kg/m²)	22.5±0.3	22.3±0.5	18.9±0.8
Mean TST Induration ±SD (mm)	7.2 (±1.3)	13.5±5.5	16.5±4.7
BCG-vaccinated (%)	25 (100.0)	27 (100.0)	32 (100.0)
Smoking (%)	3 (12.0)	2 (7.4)	17 (53.1)
Alcoholism (%)	2 (8.0)	3 (11.1)	14 (43.8)

Parameter	HCC (n=25)	LTBI (n=27)	APTB (n=32)
Hb Level ±SD (g/dl)	13.2±0.2	13.3±0.4	11.8±0.6
Mean ESR±SD (mm/hr.)	17.5±2.6	18.2±2.2	28.3±6.3
WBC (range)(10³/μl)	4.3-4.7	4.7-4.8	5.7-5.9
Lymphocyte (range) (%)	38.2-41.1	39.5-42.5	31.2-33.5
Monocyte (range) (%)	5.9-7.2	6.8-8.5	10.2-11.9
CD4+T counts ±SD cells/μl	851±235.2	885±228.9	895±322.6

HCC-Healthy Community Control; LTBI-Latent Tuberculosis Infection; APTB-Active Pulmonary Tuberculosis; TB-Tuberculosis; SD-Standard Deviation; BMI-Body Mass Index; TST-Tuberculin Skin Test; BCG-Bacillus Calmette-Guerin; Hb- Hemoglobin, ESR-Erythrocyte Sedimentation Rate; WBC- White Blood Cell.

Outcome of Anti-tuberculosis Treatment

Clinically all the TB patients had mild disease. After the completion of the 6-month anti-TB therapy, 30 (93.75%) of the APTB patients were cured on the basis of conversion to negative sputum microscopy results. They all showed improved clinical appearance and reduction of the lesion areas in chest x-ray. Treated APTB patients had significant weight gain (BMI: 22.4 ±0.3 kg/m^2, p<0.01) and a decreased ESR (17.5±2.1mm/hour, P<0.03). Also, other blood parameters approached values close to healthy control subjects at 6 -month.

Two of the APTB patients showed persistently positive sputum AFB microscopy results and were identified to have treatment failure, however, they had gained weight compared to when they were in the active TB phase at enrollment. They were accordingly given follow-up treatment.

Pattern of serum cytokine levels in participants

The cytokine levels were calculated by comparing the optical density of the sample wells with the generated standard curve for each assay run. The serum IFN-ɣ response levels in the healthy community controls (HCC), latent tuberculosis infection (LTBI) cases and active pulmonary tuberculosis (APTB) patients at enrollment and during the course of anti-tuberculosis treatment are shown in Figure 1 and Figure 2 respectively. The baseline IFN-ɣ levels were HCC (223.50±58.11pg/ml), LTBI (128.82±41.81pg/ml) and APTB (47.82± 22.05pg/ml), p<0.0001 in each case; the correlation coefficient for HCC versus APTB=0.03917). The IFN-ɣ production increased at 2-month, ATT1 (125.37 ±16.09pg/ml), and at the end of 6-month therapy, ATT2 (203.35±23.24pg/ml), P<0.0001. IFN-ɣ production during treatment correlated inversely with TB disease activity, P< 0.05 in each case (Figure 2).

The Interleukin-10 (IL-10) levels in the different groups at baseline and during the course of anti-TB therapy are shown in Figure 3 and Figure 4 respectively. In contrast to IFN-ɣ, Interleukin-10 responses at baseline showed it was slightly but significantly increased at enrollment point for APTB (17.53±6.30pg/ml), compared to LTBI (10.71±2.39pg/ml) and HCC (7.49±2.02pg/ml, p<0.0001, correlation coefficient=0.008379). In the course of treatment, the IL-10 level decreased in APTB patients at 2-month, ATT1 (10.54±2.25pg/ml) and at 6- month, ATT2 (5.25±1.45pg/ml), compared to baseline level (17.53±6.30pg/ml, p =<0.0001, p=<0.0001 respectively; correlation coefficient between

ATTO and ATT2= 0.0108306). Significant difference was found in APTB patients between the 2- and 6-month post-therapy, (p<0.05).
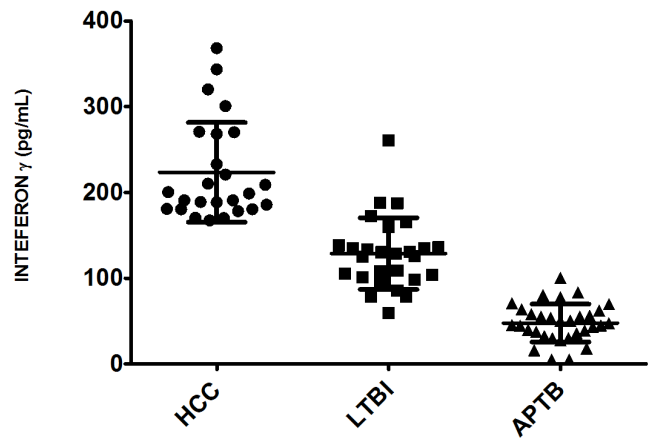

Figure 1. *Distribution of Interferon-ɣ Levels at Base-line in the Serum of Healthy Community Controls (HCC) (N=25), Latent Tuberculosis Infection (LTBI) cases (n=27) and active Pulmonary Tuberculosis (APTB) Patients (n=32).*

Each value represents one individual. Data is expressed in picogram per milliliter (pg. /ml),
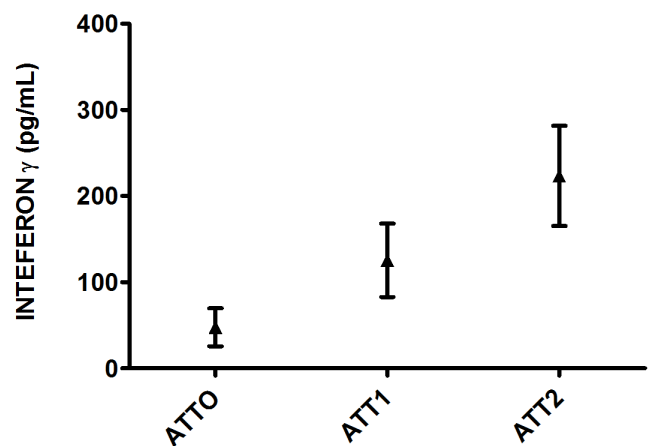Mean value ± standard deviation for each group is indicated, p < 0.0001.

Figure 2. *Changes in Serum Interferon-ɣ Level of Active Pulmonary TB Patients (n=32) during Anti-TB Treatment.*

Time Point: ATTO-Base-line, ATT1--2-month, ATT2—6-month.
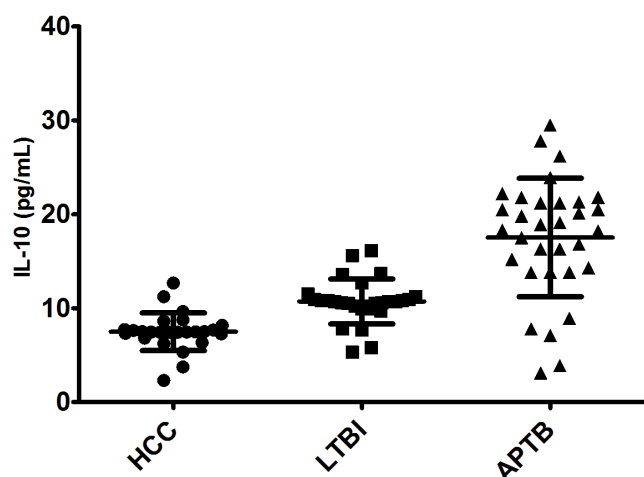Mean value± Standard deviation for each group at different time points is indicated, p<0.0001.

Figure 3. *Distribution of Interleukin-10 Levels at Base-line in the Serum of Healthy Community Controls (HCC) (n=25), Latent Tuberculosis Infection (LTBI) cases (n=27) and Active Pulmonary Tuberculosis (APTB) patients (n=32).*

Each value represents one individual. Data is expressed in picogram per milliliter (pg. /ml).
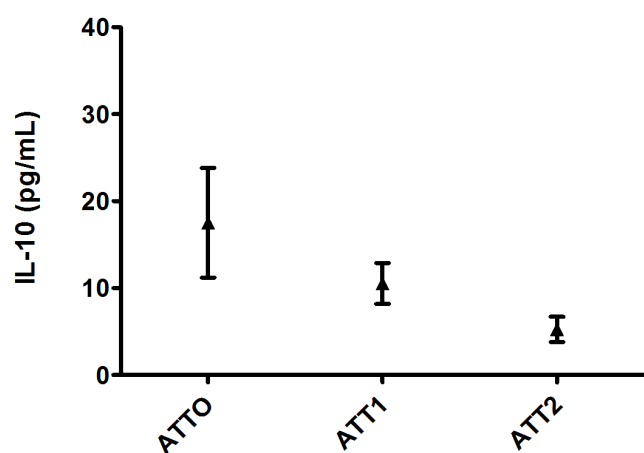Mean value ± Standard deviation for each group is indicated, p < 0.0001.

Figure 4. *Changes in Serum Interleukin-10 Levels of Active Pulmonary TB Patients (n=32) during Anti-TB Treatment.*

Time Point: ATTO-Base-line, ATT1--2-month, ATT2—6-months.
Mean value± Standard deviation for each group at different time points is indicated, p<0.0001.

On a further examination of the results, it was observed that two APTB patients have consistently low IFN-y responses: ATTO (3.51±0.43pg/ml), ATT1 (5.20±1.24pg/ml), ATT2 (4.82±1.5pg/ml) but high IL-10 responses: ATTO (28.55±1.52pg/ml), ATT1 (14.11±1.10pg/ml), ATT2 (22.32±1.80pg/ml). Coincidentally, these two APTB patients also failed to attain curative treatment at 6-month.

Interferon-gamma to Interleukin-10 Ratio

Since the balance between pro-inflammatory and anti-inflammatory cytokines is important in clinical outcome in several human diseases, we performed cytokines combinational analysis by calculating the ratio of INF-y versus IL-10 (INF-y/ IL-10) responses at baseline and during the course of anti-TB treatment. The baseline IFN-y/IL-10 ratios recorded were: 29.83, 12.03 and 2.73 for the HCC,

LTBI and APTB groups respectively, which significantly differed, (p<0.05). The cytokines ratio progressively increased during the course of anti-TB treatment: ATTO (2.73), ATT1 (11.89) and ATT2 (42.54). The differences in the IFN-y/IL-10 ratio between the groups and at the different treatment time points were statistically significant, (p<0.05). The two APTB patients with unresolved TB after standard anti-TB treatment were also observed to have consistently low IFN-y/IL-10 ratio at the different time points: ATTO (0.12), ATT1 (0.37) and ATT2 (0.22).

4. Discussion

Inflammatory cytokines are important mediators in the regulation of the immune response to mycobacterial infection. This study investigated the production pattern of the principal pro-inflammatory cytokine, interferon-gamma (IFN-ʏ), in relation to its regulatory interleukin-10 (IL-10) in the serum of healthy community controls (HCC), latent tuberculosis infection (LTBI) cases, and in a cohort of active pulmonary tuberculosis (APTB) patients before and during standard anti-tuberculosis treatment.

This is a study with limited population size, a limitation shared by many other studies in this area (20-25). Nonetheless, the differences in the INF-y and IL-10 responses were sufficiently large enough to enable the detection of significant differences between the diagnostic groups and the time-points during treatment.

This study has shown that the immune status of active pulmonary TB patients and LTBI cases included in this study was not compromised, and that the healthy control subjects were not naive to MTb-specific antigens. The MTb antigen-stimulated secretion of INF-y and IL-10 cytokines by the healthy controls and LTBI cases could have arisen for several reasons, including vaccination with BCG, exposure to environmental bacteria, and infection with MTb in our TB-endemic setting.

Although there were inter- individual variation in the INF-y and IL-10 cytokine responses, generally, significantly higher INF-y level was recorded among the healthy controls compared with LTBI cases and the APTB patients, whereas the corresponding IL-10 response was higher among the APTB patients compared with LTBI and healthy controls--all at the point of enrollment. There was dose effect in the course of TB treatment, since the levels of IFN-ʏ were lower at 2-month compared to 6-month post-therapy, which showed lower IFN-ʏ level than the LTBI group and similar to the levels observed in the healthy control subjects at baseline. In contrast to IFN-ʏ production, this work found a negative correlation between IL-10 level and MTb curative treatment, and the levels normalized at the end of effective treatment. These results have shown that, during MTb infection, low INF-y response correlates with high IL-10 production, which suggests that IFN-ʏ and IL-10 cytokines cross – regulate during MTb infection.

The striking depression of IFN-ʏ production by the APTB patients at enrollment, in response to specific MTb

stimulation as observed in the present study, is in agreement with some previous reports from TB endemic areas in Africa [26, 27] and Indonesia [28]. This was different from a study in South Africa where over a 6-week anti-tuberculous treatment period, the plasma levels of IFN-ɣ were observed to decrease while the level of tumor necrosis factor alpha (TNF-α) increased [29]. Another study, in Turkey, a high TB-prevalence country, has reported that both IFN-ɣ and IL-10 levels were high in APTB patients and that the levels increased in their TB patients after 2 – and 6-month of therapy and concluded that anti-TB treatment affected the IFN-ɣ and IL-10 production in a similar way [22].

The rise in IFN-ɣ level in APTB patients during treatment as observed in the present work and supported by reports of some other studies [26-28] is consistent with the observation of the disappearance of specific immunosuppression and the recruitment of CD4+T lymphocytes from sequestered lesions in the lungs into the peripheral compartment after the death of mycobacteria caused by chemotherapy as previously reported [6, 11]. However, these possibilities were not measured in this study, future work will need to determine the frequencies of such responsive cells.

In contrast to IFN-ɣ production, IL-10 level was increased during active TB phase, suggesting that inflammatory cytokine production was not impaired in the naive APTB patients. This is in agreement with the observation in some other studies, which reported significantly elevated concentrations of IL-10 secreted by TB patients [21, 30, 22, 11, 31]. In the present study, IL-10 production was observed to correlate with TB disease activity and normalized during successful treatment.

Interestingly, it was discovered that 2 (6.25%) of the APTB patients had TB treatment failure and were possibly harboring the multidrug-resistant TB variant, despite the total (100%) BCG-coverage of the studied population. This observation corresponds with the global rating of Nigeria as one of the highest TB-burden countries [15] with high MDR-TB prevalence rate [2], and the observations that the BCG, which is up to date the only available TB vaccine has variable protective efficacy in different parts of the world and can only limit pediatric MTb infection but is not effective in preventing adult pulmonary TB [32].

On further examination, it was observed that high levels of discriminatory accuracy between the different clinical manifestations of MTb infection could be achieved by combinational analysis of the response data for the two cytokines. This distinction also manifests in the two APTB patients with treatment failure, who recorded low INF-y/IL-10 ratio at baseline and fluctuating but consistently low ratios at the different time points during their unsuccessful TB treatment. The persistently elevated IL-10 responses noted in the APTB patients and, particularly, in the two with unresolved TB correlates with the previously reported association of high IL-10 production in pulmonary TB patients with MDR-TB [24], and in those with possible TB recurrence [33]. Taken together, these observations indicate that an altered balance between the pro-(INF-y) and anti-(IL-

10) inflammatory cytokine is implicated in MTb infection progression and possibly resistance, rather than elevation or decrease of just one of the cytokines.

These results strongly suggest that the IFN-ɣ/IL-10 ratio may be a bio-marker signature to determine MTb infection progression and treatment outcome. This potentially represents a significant advance because current immunodiagnostic tests (i.e., TST and IGRAs) are unable to make the distinction. From a clinical perspective, the ability to discriminate between LTBI and active pulmonary TB based on a blood test alone, which can provide a result within two days, will be an important advantage, because this would allow clinicians to make timely management decisions, rather than having to wait for culture results, which would take 6-8weeks to be interpretable [34]. However, longer follow-up studies are needed to substantiate the possible permanence of the discordant patterns of these cytokines in TB- susceptible individuals, and to evaluate longitudinally the IFN-ɣ/IL-10 ratio in the course of latent tuberculosis infection progression to active pulmonary tuberculosis.

5. Conclusion

This prospective study of BCG-vaccinated adults in a TB-endemic setting has shown that the immune status of active pulmonary TB patients and subjects with latent tuberculosis infection was not compromised and that the healthy control subjects were not naive to MTb antigens. This work revealed the occurrence of a significant proportion (6.25%) of multidrug-resistant (MDR) TB variant among APTB patients, who recorded consistently low INF-ɣ/ IL-10 ratio; they are currently undergoing follow-up treatment.

This work has shown that, generally, the suppression of IFN-ɣ production in active pulmonary TB patients correlated with increased level of IL-10, and that both normalized during successful anti-tuberculosis treatment. Moreover, the patterns of the differences in the INF-y and IL-10 responses between the APTB and LTBI or healthy controls, the persistently high IL-10 but low INF-y level recorded by APTB patients with unresolved TB after regular treatment; and the corresponding low IFN-ɣ/IL-10 ratios at baseline that increased during anti-TB therapy—all strongly suggest a shift toward MTb-specific antigen-induced IFN-y-mediated pro-inflammatory host immune phenotype during effective control of MTb infection. Taken together, these results have demonstrated that these two key inflammatory cytokines cross-regulate during MTb infection and suggest that the impairment of this pathway could be related to MTb infection progression. Further studies are, however, necessary to identify the specific cellular sources of IFN-ɣ and IL-10 cytokines in response to MTb infection.

It is believed that the IFN-ɣ/IL-10 ratio may be a useful surrogate endpoint than a single cytokine determinant alone to assess if MTb infection is going to resolve, result in latency, progress to disease; or become drug- resistant.

Future evaluation and independent consideration of other potential cytokine biomarkers in different populations at

different geographical locations against clear clinical endpoints are required before the candidate IFN-γ/IL-10 response ratio biomarker can be recommended for routine clinical practice.

Conflicts of Interest Statement

The authors declare that they have no conflicting interests.

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