

IMMUNOLOGICAL ASPECTS

The effect of HIV coinfection, HAART and TB treatment on cytokine/chemokine responses to *Mycobacterium tuberculosis* (*Mtb*) antigens in active TB patients and latently *Mtb* infected individuals



Desta Kassa^{a, b, *}, Wilco de Jager^c, Gebremedhin Gebremichael^a, Yodit Alemayehu^a, Leonie Ran^b, Justin Fransen^b, Dawit Wolday^d, Tsehaynesh Messele^a, Belete Tegbaru^a, Tom H.M. Ottenhoff^e, Debbie van Baarle^{b, 1}

^a HIV/AIDS and Tuberculosis Research Directorate, Ethiopian Health and Nutrition Research Institute (EHNRI), Addis Ababa, Ethiopia

^b Department of Internal Medicine and Infectious Diseases, Laboratory of Transitional Immunology, Department of Immunology, University Medical Center Utrecht, Utrecht, The Netherlands

^c Department of Internal Medicine and Infectious Diseases, Department of Pediatric Immunology, Laboratory of Translational Immunology, University Medical Center Utrecht, Utrecht, The Netherlands

^d Medical Biotech Laboratory, Addis Ababa, Ethiopia

^e Department of Infectious Diseases, Leiden University Medical Center, Leiden, The Netherlands

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SUMMARY

Identification of *Mtb* specific induced cytokine/chemokine host biomarkers could assist in developing novel diagnostic, prognostic and therapeutic tools for TB.

Levels of IFN- γ , IL-2, IL-17, IL-10, IP-10 and MIP-1 α were measured in supernatants of whole blood stimulated with *Mtb* specific fusion protein ESAT-6/CFP-10 using xMAP technology. The study groups were HIV positive TB patients (HIV⁺TB⁺), HIV negative TB patients (HIV⁻TB⁺), HIV positive tuberculin skin test positive (TST⁺) (HIV⁺TST⁺), HIV negative TST⁺ (HIV⁻TST⁺), and HIV⁻TST⁻ individuals.

Compared to HIV⁻TST⁻, latent TB infection led to increased levels of IP-10, IFN- γ and IL-17, while levels of IL-2 and IP-10 were increased with active TB. Levels of IFN- γ , IL-17, MIP-1 α , and IL-10 were increased in HIV⁻TST⁺ individuals compared to HIV⁻TB⁺ patients. HIV coinfection decreased the level of IFN- γ , IL-17, IP-10 and IL-2. After six months (M6) of anti-TB treatment (ATT) in HIV⁻TB⁺ patients, IFN- γ , IL-10, and MIP-1 α levels normalized. After M6 and M18 of ATT plus HAART in HIV⁺TB⁺ patients, levels of MIP-1 α and IL-10 normalized, while this was not the case for IFN- γ , IL-2, IL-17, and IP-10 levels. In HIV⁺TST⁺ patients on HAART, levels of IFN- γ , IL-17, IL-10 and MIP-1 α normalized, while no change in the levels of IL-2 and IP-10 were observed.

In conclusion, the simultaneous measurement of IFN- γ , IL-17 and IP-10 may assist in diagnosing LTBI; IL-2 and IP-10 may assist in diagnosing active TB; while IFN- γ , IL-17, MIP-1 α , and IL-10 levels could help to discriminate LTBI and active TB. In addition, IL-10 and MIP-1 α levels could help to monitor responses to TB treatment and HAART.

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1. Introduction

Despite that nearly 20 million lives have been saved from tuberculosis (TB) associated deaths in the past 17 years, and mortality from TB has decreased by 41% since 1990, TB remains a major health problem particularly in developing countries where 90% of TB incidence and death occurs. In 2011, there were 8.8 million incident TB cases (13% co-infected with HIV) and 1.4 million deaths from TB worldwide [1].

* Corresponding author. Ethiopian Public Health Institute (EPHI), P.O. Box 1242, Addis Ababa, Ethiopia. Tel.: +251 0911 479212; fax: +251 0112 75 63 29.

E-mail addresses: dkassa2003@gmail.com (D. Kassa), wjager@umcutrecht.nl (W. de Jager), gereachem@yahoo.com (G. Gebremichael), yudanet@gmail.com (Y. Alemayehu), leonie.ran@roche.com (L. Ran), j.h.fransen@umcutrecht.nl (J. Fransen), dawwol@gmail.com (D. Wolday), tmessele@aslm.org (T. Messele), bt2362@columbia.edu (B. Tegbaru), T.H.M.Ottenhoff@lumc.nl (T.H.M. Ottenhoff), debbie.van.baarle@rivm.nl (D. van Baarle).

¹ Current address: Center for infectious disease control, National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands.

Due to the lack of an effective vaccine, control of TB largely depends on the diagnosis and treatment of active TB [2]. However, Acid Fast Bacilli (AFB) smear sputum microscopy, the gold standard for active TB diagnosis especially in resource limited settings where TB is endemic, has low sensitivity (50–70%) [3] especially in individuals coinfecting with HIV (~35%) [4] and children (<50%) [5]. In addition, *Mtb* sputum culture has limitations including the relatively higher costs per test, long time to get results (4–8 weeks) and requirement of higher expertise and laboratory infrastructure [3]. Moreover, the current recommended regimen for treatment of active TB is requiring a minimum of 6 months to complete and is often hampered by non-adherence and drug-related toxicity.

Nonetheless, treatment of asymptomatic latent TB infection (LTBI), which is recommended when the risk of reactivation is high, is a critical strategy to control TB [6]. The role of the century old Tuberculin Skin Test (TST) and the recently emerged IFN- γ release assay (IGRA) is important to diagnose LTBI [7]. However, TST and IGRA have low sensitivity and specificity especially in malnourished and HIV infected individuals [8]. The fact that TST and IGRAs are dependent on the detection of a single biomarker, IFN- γ [9], is the main reason for the lower performance of both TST as well as IGRA [10].

Based on the notion that *Mtb* infection is associated with a spectrum of overlapping clinical conditions which can be poorly separated, there is a need to identify additional biomarkers that correlate with the clinical stages of *Mtb* infection. This could accelerate the development of novel diagnostic and therapeutic tools for both latent and active TB. Furthermore, for better utilization of TB biomarkers in clinical practice, the effect of HIV infection and therapy on the biomarker profile also needs to be investigated [11–13].

The clinical outcome of *Mtb* infection is determined by a complex interplay of various cytokines intercellular signaling molecules that regulate the differentiation, proliferation, and activation of immune cells [14]; and chemokines (8–10 kDa cytokines that direct cell migration) [15]. The main cytokines shown to be important in the occurrence, progression and control of TB infection are the pro-inflammatory (INF- γ , TNF- α , IL-12, IL-2), inflammatory (IL-6, IL-17), and immunoregulatory cytokines (IL-10, TGF- β) [16]. A combination of IP-10, IL-2 and TNF- α [17], and IP-10 and IFN- γ [18], could be promising biomarkers for active TB diagnosis. IP-10 and MCP-2 were reported to be able to discriminate TB disease from latent infection [19].

Besides that measuring of multiple pro- and anti-inflammatory cytokine/chemokines specific to TB will give insight into the pathogenesis mechanism of *Mtb* infection, it may also provide the opportunity to identify candidate immunologic biomarkers for TB disease and infection. Therefore, we measured the secretion of six cytokines [T helper cell (Th) type 1 (Th1) (INF- γ , IL-12), IL-17 and IL-10], and two chemokines (IP-10, MIP-1 α) after 7 day culture of whole blood stimulated with Early Secreted Antigenic Target-6/ Culture Filtrate Protein-10 (ESAT-6/CFP-10) antigen. Five clinical groups namely HIV⁺TB⁺, HIV⁺TB⁻, HIV⁺TST⁺, HIV⁺TST⁻ and HIV⁻TST⁻ were included in this study.

2. Materials and methods

2.1. Study population and samples

This observational cohort study was performed at St Peter Specialized Referral TB Hospital, Akaki and Kaliti Health centers, in Addis Ababa, Ethiopia from April 2007–February 2011. Adults (age 18–69 years) of both sexes who were naïve to antiretroviral therapy (ART) and TB treatment were enrolled after informed and written consent was sought.

Diagnosis of active TB was based on both clinical and bacteriological evidence. At least two sputum smears were required to be microscopy positive for Acid Fast Bacilli (AFB) by Ziehl-Neelsen method [20]. Except for TB patients, TST test was performed for all participants by intradermally injecting 2 TU (tuberculin unit) of PPD RT23 (Statens Serum Institut, Copenhagen, Denmark) by Mantoux method. A diameter of skin induration was measured by a trained nurse after 48–72 h. The cut-off for TST positivity was ≥ 10 mm in HIV un-infected, and ≥ 5 mm in HIV-infected individuals [20].

A total of 79 participants in five clinical groups were included in this study: 1) HIV positive TB patients (HIV⁺TB⁺, $n = 26$) of whom 14 were on anti-TB treatment (ATT) and were followed-over a six month (M6) period, and 12 were on ATT plus HAART and were followed for 6 and 18 months; 2) HIV negative TB patients (HIV⁻TB⁺, $n = 14$), all were on ATT and followed for 6 month; 3) HIV positive TST positive individuals (HIV⁺TST⁺, $n = 19$), all were on HAART and followed for 6 and 18 months; 4) HIV negative TST positive individuals (HIV⁻TST⁺, $n = 10$), and 5) Healthy Controls without HIV infection, *Mtb* infection and without signs of active TB (HIV⁻TST⁻, $n = 10$). HAART and ATT were provided to the patients free of charge and according to the national guidelines [20].

At enrollment and during the follow-up visit each participant was interviewed using a standard questionnaire and detailed clinical, anthropometric and demographic data were recorded by a clinician. A total of 20 ml heparinized venous blood was collected and transported to the Ethiopian Health and Nutrition Research Institute (EHNRI) for laboratory tests. All laboratory tests were performed by automated machines by adhering to the manufacturer's manual. Quantification of absolute counts of **CD4⁺ T cells** was performed using a FACScan (Becton Dickinson, San Jose, USA). **Plasma HIV RNA** load was determined using the NucliSens EasyQ NASBA diagnostic 2007/1 assay (Organon, Teknika) which quantifies HIV-1 with a linear dynamic range from 50 to 3,000,000 copies/ml.

2.2. Whole blood stimulation in vitro

We adopted a previously established whole blood assay (WBA) and determined cytokines in the supernatants [21]. Whole blood obtained from each participant before and after treatment was diluted 1 in 5 with complete Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma, cat R0883) supplemented with 10% Fetal Calf Serum (FCS) (Invitrogen, cat 10106169), and 1% Penicillin/Streptomycin (P/S). Then 100ul of blood/well was transferred in triplicate in round-bottom microtiter plates (Nunc Surface; NUNC; Roskilde, Denmark) which has been coated with ESAT-6/CFP-10, and Culture Medium (RPMI 1640) as negative Control. The final concentration of ESAT-6/CFP-10 was 10 μ g/ml. Culture plates were incubated in a 5% CO₂ incubator at 37 °C for 7 days, and supernatants were collected and stored at -80° for further analysis. The ESAT-6/CFP-10 fusion protein was produced at the department of Infectious Diseases, Leiden University Medical Center [22].

2.3. Measurement of cytokine/chemokine biomarkers

The level of 4 cytokines (IFN- γ , IL-2, IL-17, IL-10) and two chemokines (IP-10, MIP-1 α) was measured in the 7th day whole blood culture supernatant using xMAP technology (Luminex, Austin TX, USA) using a biorad FlexMap3D system (biorad USA). Acquisition was performed using xPonent 4.2 software (Luminex) and data analysis was performed using BioPlex Manager 6.1.1 (biorad) at the multiplex core facility of the University Medical Center, Utrecht (UMCU), The Netherlands, as described previously [23].

2.4. Data analysis

Initially, the value of each cytokine/chemokine measured after no stimulation (RPMI medium) was subtracted from that of ESAT-6/CFP-10 induced cytokine/chemokines. After background subtraction, negative values were converted to zero. The value of the cytokine induced after no stimuli (RPMI medium) by each clinical group at baseline are shown in the [Supplementary Table 3](#).

One-way analysis of variance to test if all the means of a specific cytokine/chemokine induced by each clinical groups were equal (null hypothesis), showed the F statistic was significant for all the cytokine/chemokines, indicating that at least some of the means must differ from one other. Hence, we examined the pair-wise difference between the groups without adjusting for multiple comparisons. Thus, nonparametric statistical methods (Wilcoxon signed rank test for paired data, Mann–Whitney U tests for unpaired data, and Kruskal–Wallis tests for data from different groups) were performed to compare differences within and between groups. Spearman's rank correlation analysis was performed between cytokines/chemokines within each patient group. All data analysis was done using Intercooled STATA version 11.0 (College Station, Texas, USA). A p-value of <0.05 was considered significant.

3. Results

3.1. Study population characteristics at baseline

The demographic, clinical and laboratory characteristics of the study populations are shown in [Table 1](#). A total of 79 participants were included among which 26 HIV⁺TB⁺, 14 HIV[−]TB⁺, 19 HIV⁺TST⁺, 10 HIV[−]TST⁺ and 10 Controls (HIV[−]TST[−]). Mean age of the HIV infected groups (HIV⁺TB⁺ and HIV⁺TST⁺) was significantly higher than the HIV negative groups (HIV[−]TB⁺, HIV[−]TST⁺, HIV[−]TST[−]) (p < 0.05 for all).

At baseline, HIV⁺TB⁺ patients had higher HIV RNA (copies/ml) than the HIV⁺TST⁺ patients; and lower body mass index (BMI) (Kg/m²) and CD4⁺ T-cell counts (cells/μl) than HIV⁺TST⁺, HIV[−]TST⁺ and Controls, (p < 0.05 for all). BMI and hemoglobin (Hgb) were lower in HIV⁺TB⁺ and HIV[−]TB⁺ patients compared to Controls (p < 0.05). The majority of HIV⁺TST⁺ patients (68%) were on D4T/3TC/NVP antiretroviral regimen and the majority of HIV⁺TB⁺ patients (57%) were on D4T/3TC/EVZ.

3.2. Comparison of cytokine/chemokine responses to Mtb antigen at baseline

Comparison of the concentration of each cytokine in response to ESAT-6/CFP-10 stimulation among the five clinical groups at

baseline is shown in [Figure 1](#) and [Table S2](#). Compared to HIV[−]TST[−] controls, HIV[−]TST⁺ individuals had elevated IFN-γ (p = 0.04), IL-17 (p = 0.0007) and IP-10 (p = 0.03) levels, while HIV[−]TB⁺ patients had elevated IL-2 (p = 0.03) and IP-10 (p = 0.02), but lower IL-10 (p = 0.0001) levels. Interestingly, the chemoattractants showed similar expression during TB disease and infection, where IP-10 levels were elevated and MIP-1α level were decreased in both HIV[−]TB⁺ and HIV[−]TST⁺ compared to HIV[−]TST[−] (p < 0.05 for all) ([Figures 1 and 2](#)). We found significantly elevated IFN-γ (p = 0.005), IL-17 (p = 0.0001), MIP-1α (p = 0.009), and IL-10 (p = 0.0005) in HIV[−]TST⁺ individuals compared to HIV[−]TB⁺ patients ([Figures 1 and 2](#)).

3.3. The effect of HIV coinfection on Mtb specific immune response

We next studied the effect of HIV coinfection on the cytokine/chemokine response to ESAT-6/CFP-10 ([Figures 1 and 3](#)). Interestingly, HIV infection lead to lower secretion of Th1-derived cytokines (IFN-γ, IL-2), IL-17 and IP-10 in active TB patients (HIV⁺TB⁺ vs. HIV[−]TB⁺, p = 0.02, 0.02, 0.0001, 0.003, respectively) as well as in LTBI individuals (HIV⁺TST⁺ vs. HIV[−]TST⁺; p = 0.002, 0.0002, 0.07, 0.01, respectively). The secretion of all cytokines (IFN-γ, IL-17, IL-10, IL-2, IP-10 and MIP-1α) was significantly lower in HIV⁺TB⁺ patients compared to HIV⁺TST⁺ patients (p < 0.05 for all).

3.4. Comparison of cytokine/chemokine levels using heat map analysis

Hierarchical cluster analysis of all of the HIV negative participants (TST⁺HIV[−], TB⁺HIV[−], TST[−]HIV[−]) displays a distinct pattern of cytokine/chemokine production in each group in response towards ESAT-6/CFP-10. Increased levels of IFN-γ, IL-17, IL-2 and IP-10 are observed in TST⁺HIV[−] participants, whereas IL-2 and IP10 is increased in TB⁺HIV[−] participants; and IL-10 and MIP-a in healthy controls (TST[−]HIV[−]) ([Figure 2A](#)).

Furthermore, cluster analysis of all of the HIV positive participants (TB⁺HIV⁺ and HIV⁺TST⁺), revealed decreased levels of IFN-γ, IL-17, and IL-2 in TB⁺HIV⁺ group ([Figure 2B](#)).

3.5. The effect of anti-TB treatment (ATT) and HAART on the cytokine/chemokine responses

Analysis of the cellular immune responses after therapy provides novel opportunities to understand the complex pathogenesis mechanism of Mtb infection as well as identify biomarkers for monitoring treatment effect. Therefore, we measured the dynamics

Table 1
Baseline demographic and clinical characteristics of the 5 clinical groups (n = 79).

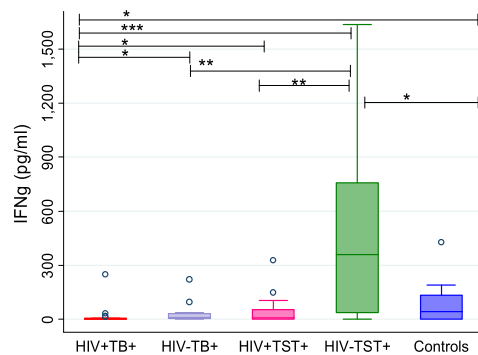
	HIV ⁺ TB ⁺ (n = 26)	HIV [−] TB ⁺ (n = 14)	HIV ⁺ TST ⁺ (n = 19)	HIV [−] TST ⁺ (n = 10)	HIV [−] TST [−] (n = 10)
Age, years	32.7 ± 7.2	25.3 ± 8.1 [*]	34.6 ± 8.0 [†]	26.6 ± 7.9 ^{*,‡}	27.5 ± 8.2
Male, n (%)	14 (50.0)	8 (57.14)	8 (42.1)	6 (60)	4 (40)
BCG scar frequency, n (%), 27/76 (35.5%)	4/23 (17.4%)	5/14 (35.7%)	7/19 (36.9%)	5/10 (50%)	6/10 (60%)
WHO stage, n (%)					
I + II	1 (4.2)	NA	7 (38.9)		
III + IV	23 (95.8)		11 (61.1)	NA	NA
BMI (kg/m ²)	18.9 ± 3.3	17.7 ± 2.8	21.6 ± 3.8 ^{*,†}	21.0 ± 2.5 ^{*,‡}	21.0 ± 2.2 ^{*,†}
Hgb (g/dl)	12.4 ± 3.7	14.2 ± 2.3	12.7 ± 2.3	16.5 ± 2.0 ^{*,‡}	17.7 ± 1.0 ^{*,†}
CD4 ⁺ count/μl	163 ± 110.7	445 ± 236.3 [*]	166.5 ± 95.5 [†]	901.7 ± 316.7 ^{*,‡}	727.8 ± 315.3 ^{*,†}
HIV RNA (copies/ml)	93,015.0 ± 172,824.4 [*]	67,592.3 ± 140,772.5	NA	NA	NA

Data are means ± standard deviations (SD); n (%): Number of patients; BMI: Body mass index; Hgb: Hemoglobin; WHO: World Health Organization; BCG, Bacillus Calmette–Guérin; NA: not applicable.

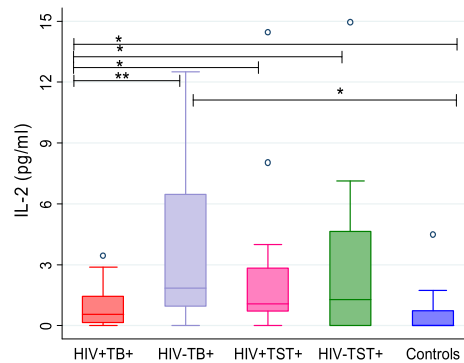
^{*} Comparison with HIV⁺TB⁺, p < 0.05.

[†] Comparison with HIV[−]TB⁺, p < 0.05.

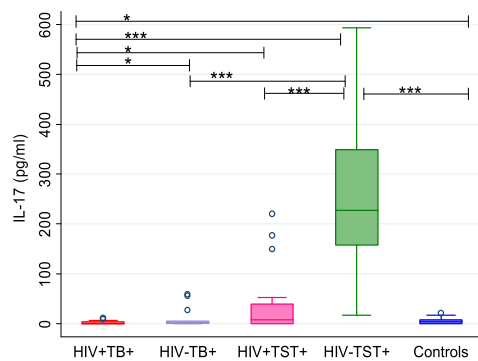
[‡] Comparison with HIV[−]TST⁺, p < 0.05.

IFN- γ 

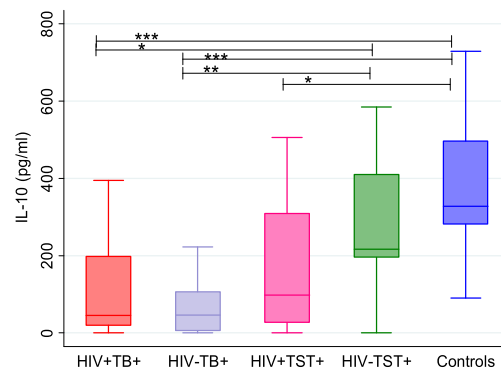
IL-2



IL-17



IL-10



IP-10

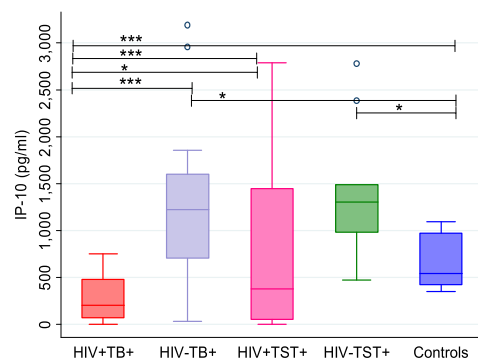
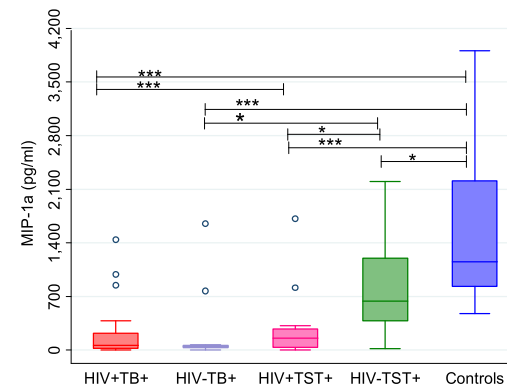
MIP-1 α 

Figure 1. Comparison of cytokine/chemokine concentration among five clinical groups at baseline: HIV + TB+, (n = 24); HIV – TB+ (n = 14); HIV + TST+ (n = 17), n=; HIV – TST+ (n = 10), and Controls (HIV + TST–, n = 10). Y-axis represents cytokine/chemokine concentrations and X-axis represents the study groups. The concentration of Th1 (IL-2, IFN- γ), Th17 (IL-17), cytokines, and chemokines (MIP-1 α , IP-10) (pg/ml) was measured by Luminex assay from culture supernatants of whole blood stimulated with ESAT-6/CFP-10. In the figure, horizontal lines in the boxes show median values, boxes boundaries representing 25th and 75th percentiles and whiskers represent the highest and lowest values. The responses were compared using a Mann–Whitney U test. P-values of those with significant difference are shown on the horizontal lines. *: p < 0.05; **: p < 0.01, ***: p < 0.001.

of the cytokine response to ESAT-6/CFP-10 in patients who were on ATT and/or HAART (Figures 3 and 4).

In the HIV-TB+ patients by M6 of ATT, there was a significant increase in IFN- γ (p = 0.005), IL-10 (p = 0.003), and MIP-1 α (p = 0.05), but a decrease in IL-2 (p = 0.003) and IP-10 (p = 0.02) relative to baseline values, and all reached normal control values (p > 0.45 for all) (Figure 3). However, in the HIV+TB+ patients by M6

of ATT without HAART, we found no significant change in the level of Th1 derived cytokine (IFN- γ , IL-2), IL-17, and IP-10. However, the level of both IL-10 and MIP-1 α increased and reached normal control values (Figure 3).

In HIV+TB+ patients by M6 and M18 on combined ATT and HAART, the levels of Th1 derived cytokines (IFN- γ , IL-2), IL-17, and IP-10 remained impaired, while there was an increase in MIP-1 α

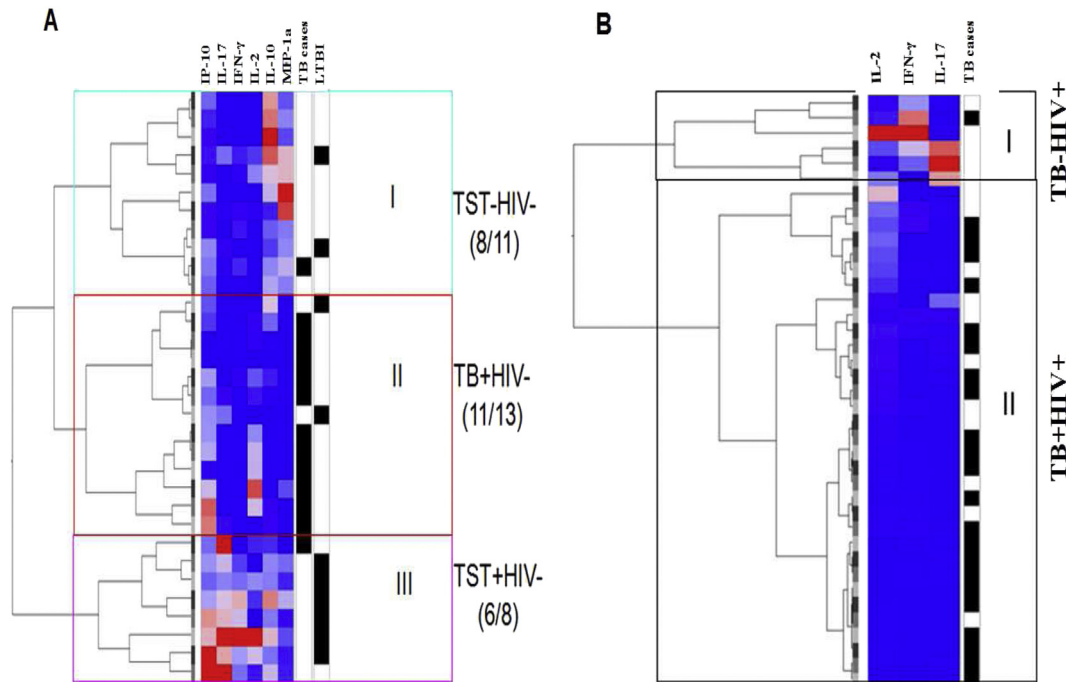


Figure 2. Heat map analysis comparing cytokine levels after stimulating with ESAT-6/CFP-10. A) HIV negative participants: Black box under “TB cases” column = Active TB patients (TB⁺HIV⁻); Black box under “LTBI” column = tuberculin skin test (TST) positive individuals (HIV⁻TST⁺); white box under both “TB cases” and “LTBI” = controls (TST⁻HIV⁻). **B) HIV positive participants:** Black box under “TB cases” column = HIV positive TB patients (TB⁺HIV⁺), and white box = HIV positive non-TB individuals (TB⁻HIV⁺). The individual values are represented as colors. Data was normalized for each parameter individually displaying, highest level in red and lowest level in blue. Symbols I, II and III, indicates clustering of each study participant in response to individual cytokine levels. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

($p = 0.05, 0.02$) and IL-10 ($p = 0.05, 0.003$) which reached normal values by M18 (Figure 4).

In HIV⁺TST⁺ patients by M6 and M18 of HAART, there was an increase in IL-10 ($p = 0.02, 0.008$) and MIP-1 α ($p = 0.06, 0.0007$) levels, and a decrease in IL-2 ($p = 0.03, 0.32$) and IL-17 ($p = 0.03, 0.01$) levels and all reached normal values by M18. Although there was no significant change in IFN- γ by M6, it sharply increased by M18 ($p = 0.006$).

Of special interest, this study showed a distinctive expression pattern of the chemoattractants (MIP-1 α and IP-10) and IL-10 in response to therapy (Figures 3 and 4). Whereas the concentration of both IL-10 and MIP-1 α normalized in all the patients by M6 of ATT, ATT plus HAART, and HAART, respectively; there was no significant change in the level of IP-10 in HIV⁺TB⁺ and HIV⁺TST⁺ patients on standard therapy, while it reached normal values in the HIV⁻TB⁺ patients by M6 of ATT.

3.6. The effect of anti-TB treatment (ATT) and HAART on IFN- γ , IL-17, and IL-2/IL-10 ratios overtime

We analyzed the dynamics of IFN- γ /IL-10, IL-17/IL-10 and IL-2/IL-10 ratios over time in response to ATT and/or HAART. Interestingly, we found a completely different expression in the ratio of the cytokines in HIV⁺TB⁺ versus HIV⁻TB⁺ patients by M6 of ATT alone. While there was a sharp increase in IFN- γ /IL-10 but a decrease in IL-17/IL-10 and IL-2/IL-10 ratio in HIV⁻TB⁺ patients (Figure 5D), the reverse was observed in the HIV⁺TB⁺ patients (Figure 5C).

However, there was an increase in the IFN- γ /IL-10, IL-17/IL-10 and IL-2/IL-10 ratio in the HIV⁺TB⁺ patients by M6 of ATT and HAART (Figure 5A), as also found in HIV⁺TB⁺ patients by M6 of ATT without HAART (Figure 5C), which could be associated with the occurrence of Immune Reconstitution Inflammatory Syndrome (IRIS).

In the HIV⁺ TST⁺ patients on HAART (Figure 5B), we found a decrease in IL-17/IL-10 and IL-2/IL-10 by M6 and M18 (a shift to Th2), but a sharp increase in IFN- γ /IL-10 by M18 (a shift to Th1).

The results suggested that IL-7/IL-10 and IL-2/IL-10 ratios could be used to monitor TB treatment and HAART responses, except in HIV⁺ TB⁺ patients. IFN- γ /IL-10 ratio behaves differently relative to the IL-17/IL-10 and IL-2/IL-10 ratio in all the clinical groups, which indicates less value of IFN- γ /IL-10 to monitor therapy responses

4. Discussion

In this study, the levels of four cytokines (IFN- γ , IL-12, IL-10, IL-17) and two chemokines (MIP-1 α , IP-10) in a 7 day culture supernatants of whole blood stimulated with ESAT-6/CFP-10 antigen were compared in five clinical groups HIV⁺TB⁺, HIV⁻TB⁺, HIV⁺TST⁺, HIV⁻TST⁺ and controls (HIV⁻TST⁺) before and after treatment. As a result, candidate cytokine/chemokine biomarkers that may serve as diagnostic and prognostic markers for TB were identified.

We use a 7 day culture system as compared to the more conventional *in vitro* overnight stimulation assays like the antigen-induced proliferation and Interferon secretion, due to the reasons that, the whole blood assays, as compared to the standard assays using PBMC, are relatively simple, require less manipulation and can be used with small quantities of blood, and thus could be used in endemic field situation.

4.1. Distinct expression of cytokines in LTBI individuals

The increased expression of IFN- γ , IP-10 and IL-17 cytokines in LTBI individuals (HIV⁻TST⁺) relative to healthy controls (Figures 1 and 2), indicates that immune cells producing these cytokines are abundantly present in most LTBI individuals [7]. Our results suggest

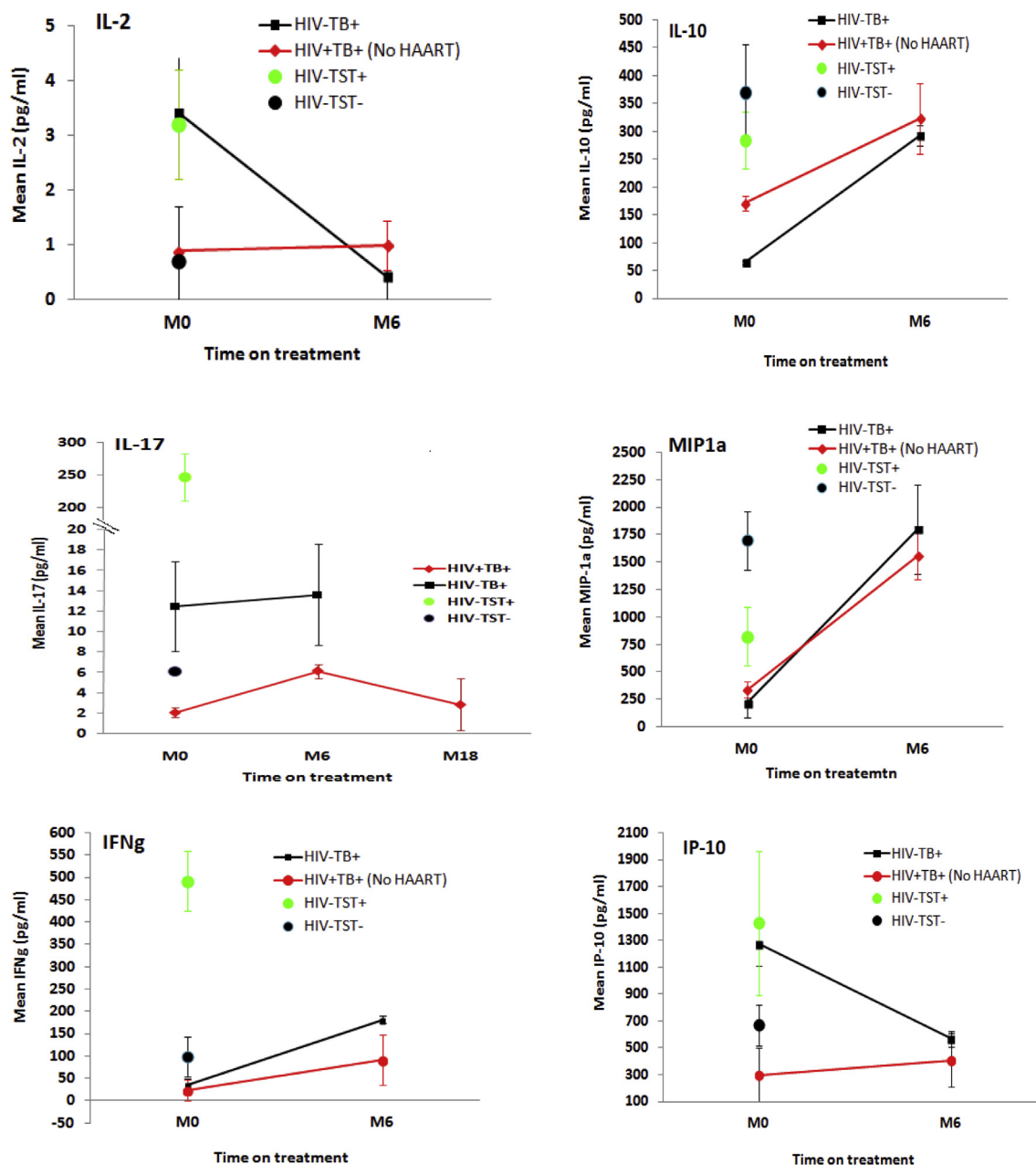


Figure 3. Change in cytokine/chemokine concentrations during TB treatment. HIV negative TB patients on TB treatment (HIV – TB+, $n = 14$) (black line); HIV positive TB patients on TB treatment with no HAART (HIV + TB+, $n = 12$) (red line), HIV – TST+ individuals ($n = 10$) (green dot), and HIV-TST-controls ($n = 10$) (green dot). The level of cytokine/chemokines measured in 7th day culture supernatants of whole blood stimulated with ESAT-6/CFP-10 at baseline (M0), and by six month (M6) and M18 of HAART and/or anti-TB treatment. Months at which cytokines were measured are shown on X-axis, and the cytokine values expressed in mean and standard deviation are shown on Y axis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

that simultaneous measurement of these cytokine/chemokines could increase the accuracy to predict and diagnose LTBI. To support this, previous studies indicated the value of IP-10, IFN- γ and IL-2 [24], and IP-10 and MCP-2 [19], to diagnose LTBI. Even more, since IP-10 is produced by a variety of cells (neutrophils, monocytes, endothelial cells and fibroblasts) which are less affected by HIV infection [25], detection of IP-10 could improve the sensitivity to diagnose LTBI in populations with HIV infection [26,27].

4.2. Distinct expression of cytokines in active TB patients

In agreement with other reports [27] active TB patients (HIV-TB+) in this study showed elevated expression of IL-2 and IP-

10 (Figures 1 and 2), which suggest that combined assessment of these cytokines may assist in the prediction and diagnosis of active TB. Indeed, others showed that combined detection of IP-10, IFN- γ and MIP-1 β [27]; IFN- γ , IP-10 with MIG [27]; IP-10, IL-2 with TNF- α [17], and IP-10 with IFN- γ [18], in response to *Mtb* specific antigens, improved diagnostic performance for active pulmonary TB. However, the secretion of IFN- γ , IL-2 and IP-10 at baseline was suppressed in active TB patients coinfecting with HIV (Figures 1 and 3), which could be due to a reduction in the number and functionality of *Mtb* and HIV specific T cells and macrophages due to infection with *Mtb*, HIV or TB/HIV [28]. Thus, the value of IFN- γ , IL-2 and IP-10 to diagnose active TB could be compromised during HIV co-infection.

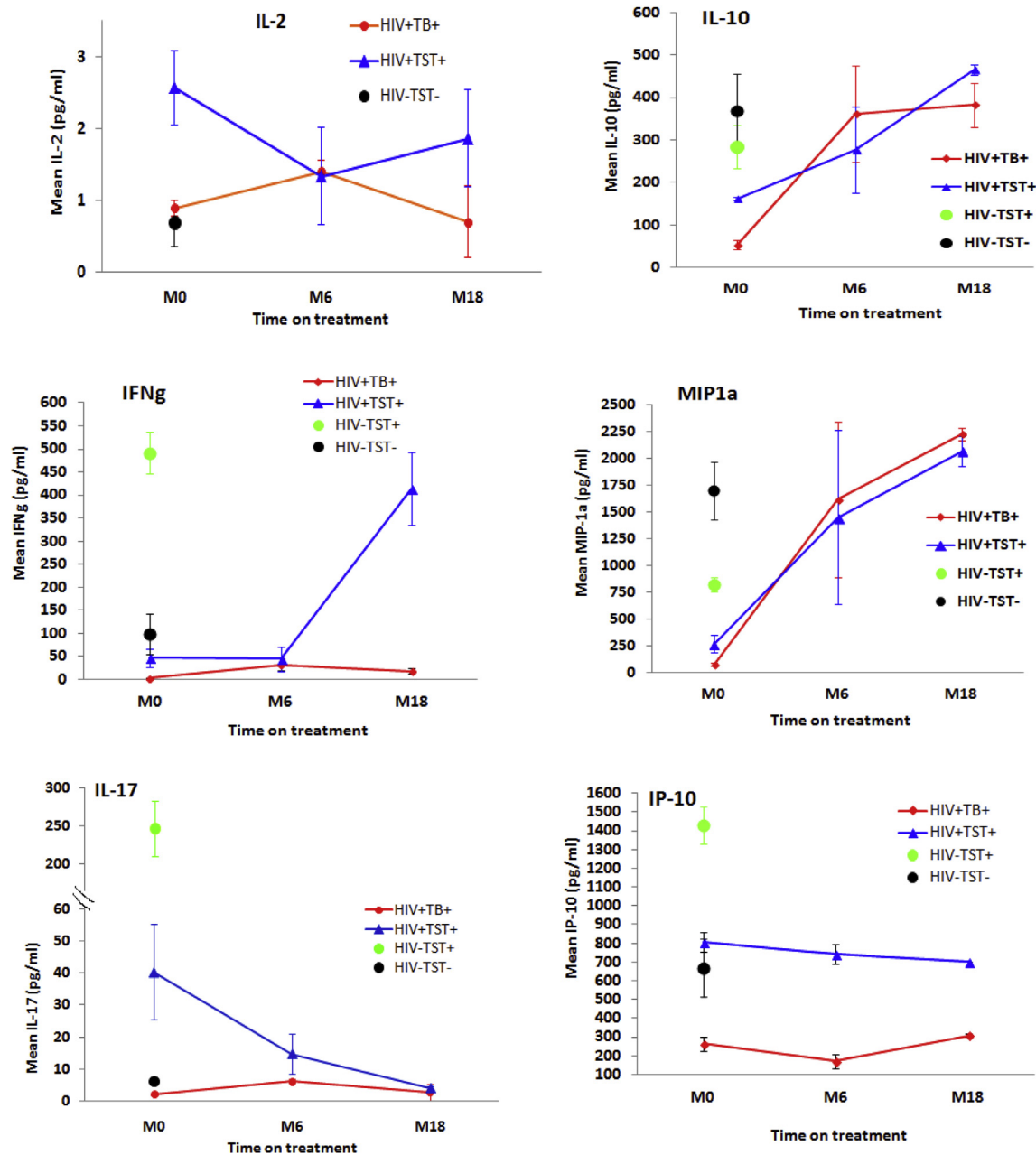


Figure 4. Change in cytokine/chemokine concentrations during HAART and/or TB treatment. HIV positive TB patients on HAART and TB treatment (HIV + TB+, n = 12) (red line); HIV infected TST positive groups on HAART (HIV + TST+, n = 17) (blue line); HIV – TST+ individuals (n = 10) (blue triangle) and HIV-TST-controls (n = 10) (black dot). The level of cytokine/chemokines was measured in 7th day culture supernatants of whole blood stimulated with ESAT-6/CFP-10 at baseline (M0), and by six-month (M6) and M18 of HAART and/or anti-TB treatment. Months at which cytokines were measured are shown on X-axis, and the cytokine values expressed in mean and standard deviation are shown on Y-axis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4.3. Distinct expression of cytokines between LTBI individuals and active TB patients

Identification of stage specific cellular biomarkers which can accurately discriminate LTBI from active TB could play a significant role in control of TB [8]. In this study, HIV⁺ TST⁺ individuals showed elevated IFN- γ , IL-17, IL-10 and MIP-1 α response to ESAT-6/CFP-10 antigen stimulation compared to HIV-TB⁺ patients, which suggests that simultaneous analysis of these cytokines can discriminate LTBI from active TB, which is in agreement to other studies [29,30]. However, IP-10 appears to be less relevant to distinguish LTBI from active TB in this study as reported by others [31].

The distinctive expression of cytokine/chemokines in LTBI and active TB groups in the present study also indicates the role of these cytokine in the pathogenesis of *Mtb* infection. Whereas increased secretion of Th1 (IFN- γ), IL-17, and IP-10 cytokines in HIV⁺ TST⁺ individuals may indicate the protective role of the cellular immune response against *Mtb* infection; the increased production of IL-10 in HIV⁺ TST⁺ may indicate the positive effect of IL-10 to counter-act immunopathology [16]. Moreover, whereas increased production of IP-10 in HIV-TB⁺ patients may show ongoing pro-inflammatory response during active TB, the lower production of IFN- γ in HIV-TB⁺ patients may confirm the defective Th1 response accompanying active TB disease [32].

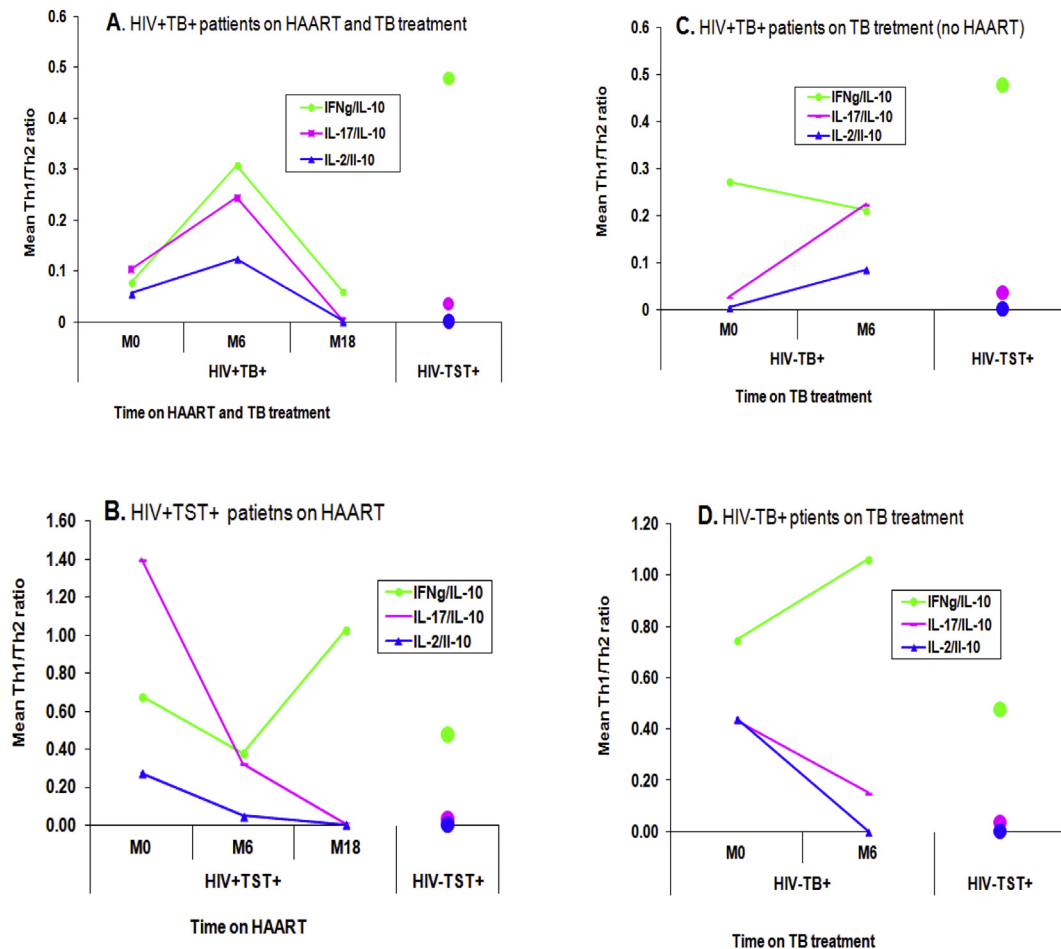


Figure 5. Dynamic changes in the cytokine ratio during treatment. A. HIV + TB+ (n = 11) on HAART and TB treatment; B. HIV + TST+ (n = 18) on HAART; C. HIV + TB+ (n = 10) on TB treatment with no HAART; and HIV + TB+ (n = 10) on TB treatment. The cytokine ratio was derived by dividing the concentrations (pg/ml) of Th1 cytokines (IFN- γ and IL-17) by that of IL-10 for each donor and then calculating the mean level in each group. Colored lines represent different cytokine ratios: IFN- γ /IL-10 ratio, (green line); IL-17/IL-10 ratio, (pink line); IL-2/IL-10 ratio, (Blue line). Y-axis represents mean values of the cytokine ratios in 7th day culture supernatants of whole blood stimulated with ESAT-6/CFP-10 at baseline (M0), and by six-month (M6) and M18 of HAART and/or TB treatment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4.4. The effect of ATT on cytokine responses in HIV – TB+ patients

IL-10, IFN- γ , IL-2, MIP-1 α , and IP-10 all increased to normal values in the HIV+TB+ patients by six month of ATT (Figure 4). Similarly a recent study done in Ethiopia showed that anti TB treatment significantly improves the plasma level of Th1 cytokines and level of chemokines in HIV negative TB patients [33]. Thus, our results suggests a role for IL-10, IFN- γ , IL-2, MIP-1 α , and IP-10 in the pathogenesis of TB on hand, and their value to monitor TB treatment. Others also showed the value of plasma IP-10 [34] and IFN- γ [35], and IP-10 and IFN- γ in response to ESAT-6 and CFP-10 stimulation [36] to monitor TB treatment in HIV negative TB patients.

4.5. The effect of ATT and/or HAART on cytokine responses in HIV + TB+ patients

In the HIV+TB+ patients by six month of ATT without HAART, except that MIP-1 α and IL-10 were normalized, there was no significant change in the restoration of IFN- γ , IL-2, IL-17 and IP-10 cytokines (Figure 4). Similarly a recent study done in Ethiopia, showed no restoration of the plasma level of Th1 cytokines in HIV positive individuals after anti TB treatment [33]. Our results

support the scenario of early HAART initiation in TB/HIV patients [1] which could boost optimal *Mtb* specific immune restoration. Nonetheless, we found persistently lower and weaker Th1 derived cytokines in the HIV + TB+ patients on ATT and HAART, which is similar to other reports [37]. This could be due to inefficient therapy, lack of treatment compliance [34], and exhaustion of the immune system to produce these Th1 derived cytokines [9].

However, there was a sharp increase in Th1/IL-10 ratio in the HIV+TB+ patients by six month of ATT and HAART (Figure 5), which is similar to previous reports [37]. This might reflect the occurrence of IRIS which can developed within a month to even years after commencing HAART [38]. Our results suggest, therefore, the need for diagnosis and management of IRIS in TB/HIV patients initiated HAART in settings like in Ethiopia where TB and HIV are endemic and access to ART is expanding [39].

4.6. The effect of HAART on cytokine/chemokine responses in HIV + TST+ patients

Chronic immune activation, inflammation, and immune dysfunction which cause non-AIDS pathologies including lymphoid fibrosis, cardiovascular diseases, lipodystrophy, and osteoporosis persist despite potent ART. Understanding the dynamics of antigen-

specific immune responses in HIV patients on HAART, may assist to identify biomarkers for ART monitoring, as well as to develop effective therapeutic strategies for the non-AIDS comorbidities and for HIV cure [40,41]. The progressive reduction in the pro-inflammatory cytokines (IL-2, IL-17, IL-2/IL-10 and IL-17/IL-10), but progressive increase in the anti-inflammatory cytokine (IL-10), and the chemoattractant (MIP-1 α) in HIV + TST+ patients (Figure 5), indicates the value of these cytokines to predict HIV disease progression, and also to monitor HAART outcomes in HIV patients. It seems that the Th1 and Th2 response in HIV patients on HAART behave in opposite direction. Others also showed a progressive increase in IL-10 [42]; IL-12 and IFN- γ [43]; but a decrease in IP-10 [44] in HIV patients on HAART. Our data indicate that HAART benefits HIV patients not only by inhibiting virus replication but also by adjusting pro- and anti-inflammatory cytokine production. The dynamics of cytokine/chemokines in response to therapy might have long-term implication for progression or regression of the immunological health of HIV patients [43], which strongly suggests the need to improve our understanding of when, where and how modulation of immune activation will be beneficial for HIV patients on HAART.

Of special interest, the level of IL-10 and MIP-1 α normalized in HIV + TB+, HIV-TB+ and HIV + TST+ groups on standard therapy (Figure 4), which could indicate the value of both IL-10 and MIP-1 α to predict HIV and TB disease progression and to monitor ATT and/or HAART outcomes.

In summary, we showed distinct cytokine/chemokine production in response to ESAT-6/CFP-10 in LTBI and active TB groups. Simultaneous measurement of IFN- γ , IL-17 and IP-10 may assist to diagnose LTBI, while IL-2 and IP-10 may assist to diagnose active TB. Likewise, combined measurement of IFN- γ , IL-17, MIP-1 α , and IL-10 may assist to discriminate LTBI from active TB. HAART plus ATT did not restore IFN- γ , IL-17, IP-10 and IL-2 response in HIV+TB+ patients. However, HAART adjusts pro- and anti inflammatory cytokine/chemokine production in HIV+TST+ patients except IP-10. Combined measurement of IL-2, IFN- γ and IP-10 for HIV-TB+ patients; and IL-10 and MIP-1 α for HIV-TB+, HIV+TB+ and HIV+TST+ patients, can be useful surrogate biomarkers to monitor therapeutic responses and disease progression. However, further studies should be performed to validate the diagnostic and prognostic value of these cytokine/chemokine biomarkers by including latent *Mtb* antigens as stimulants; and larger groups of LTBI individuals, extra pulmonary TB patients and children where the need of better diagnostic methods is enormous.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.tube.2015.05.015>.

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