Identification of cytokine signatures in HIV-infected individuals with and without *Mycobacterium tuberculosis* co-infection

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Abstract. Individuals with human immunodeficiency virus (HIV) infection are susceptible to immune system dysregulation, particularly during co-infection with *Mycobacterium tuberculosis* (MTB). Although there is an association between cytokine profiles and HIV-MTB co-infection, little is known about the cytokine-related host immune response mechanism to HIV-MTB co-infection. Therefore, the present study aimed to analyze expression of cytokines IL-17A, IFN-γ, TNF, IL-2, IL-10, IL-6 and IL-4 in individuals with HIV-MTB co-infection. A total of 30 patients with HIV and 40 with HIV-MTB co-infection were recruited into the present study, including those with active (A) (n=19) and latent (L)TB (n=21). HIV infection status was established based on national HIV guideline (Pedoman Nasional Pelayanan Kedokteran Tatalaksana HIV). ATB was confirmed using a positive acid-fast bacillus staining and culture of sputum; LTB status was established using IFN-γ release assay. Furthermore, the levels of cytokines IL-17A, IFN-γ, TNF, IL-10, IL-6, IL-4 and IL-2 were measured using flow cytometric bead array and CD4 cell count was performed by PIMA™ CD4 assay. IFN-γ, TNF, IL-10, IL-6 and IL-2 were able to significantly differentiate patients with HIV-ATB from those with HIV-LTB. Furthermore, in the patient subgroup with CD4 count <350 cells/μl, IFN-γ, IL-10 and IL-6 were able to differentiate between patients with HIV-ATB and HIV alone, as well as between patients with HIV-ATB and HIV-LTB. Based on these findings, the cytokine profiles are likely to be distinct between individuals with HIV infection with A- and LTB. Furthermore, the expression of CD4-positive T cells may influence the immune response in the body under HIV-MTB co-infection.

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Introduction

Human immunodeficiency virus (HIV) and *Mycobacterium tuberculosis* (MTB) are key pathogens contributing to patient morbidity and mortality in developing countries, particularly in Southeast Asia (1-3). TB is one of the leading causes of mortality among patients with HIV infection, accounting for ~187,000 global fatalities in 2021 (4). TB-associated mortality is 3-fold higher in patients with HIV compared with that in HIV-negative individuals. In particular, this risk is more pronounced when untreated latent TB (LTB) reactivates into clinically active TB (ATB) (5). The mandatory Bacillus Calmette-Guérin (BCG) vaccination program for children aged 0-2 months is a national program for vaccination against TB implemented by The Ministry of Health of the Republic of Indonesia (6). However, the capacity of T cells to expand and differentiate in response to BCG stimulation tends to wane with increasing age, increasing risk of TB infection later in adulthood (7).

The balance between cytokines produced by T helper (Th)1, 2 and 17 lymphocytes serves an essential role in regulating response of the immune system to specific pathogens. HIV and MTB function synergistically in causing an imbalance in cytokine production, leading to a dysregulated immune system. This disruption is key to understanding immunological dynamics of HIV/MTB co-infection (8-11).

The increased expression of pro-inflammatory cytokines produced by Th1 cells, including IFN-γ, TNF and IL-2, coupled with IL-17A produced by Th17 cells, serves an important role in the defense against MTB infection (12-16). By contrast, a shift towards anti-inflammatory cytokines released by Th2 cells, such as IL-4, IL-10, and IL-6, can lead to the deterioration of the host immune system by HIV. This triggers the reactivation of LTB to ATB (17,18). Acquisition of the immune activation status caused by MTB notably accelerates the progression of

HIV infection by enhancing viral replication (5,19-21). This suggests that determination and analysis of the cytokine expression profile during HIV/MTB co-infection may be essential in understanding the mechansim underlying HIV progression.

Although several attempts have been made to evaluate the pattern of cytokine expression in individuals with HIV either with or without MTB co-infection (22-27), further investigations are required to elucidate its association with TB, especially in HIV- and TB-endemic countries. Therefore, the present study aimed to analyze the expression profile of cytokines, including IL-17A, IFN-γ, TNF, IL-2, IL-10, IL-6, and IL-4, in individuals with HIV with or without MTB co-infection. The aim of the present study was to faciliate the future development of potential biomarkers for predicting disease progression in patients with HIV and HIV-MTB-co-infection.

Materials and methods

Study design and participants. The present comparative study was approved by The Health Research Ethics Committee, Faculty of Medicine, Universitas Padjadjaran (approval no. 834/UN6.KEP/EC/2018; Bandung, Indonesia) and conducted in accordance with the Declaration of Helsinki. The recruitment of study participants was conducted at Hasan Sadikin Central Hospital, Bandung between August 2018 and August 2019. A total of 70 participants were recruited with mean age of 35±8 years; 56 males and 14 females. Written informed consent was obtained from all study participants.

Patients aged ≥18 years with a positive diagnosis of HIV according to the national algorithm testing sequence by The Ministry of Health of the Republic of Indonesia were included as participants (28). Patients with pregnancy, malignancy and/or autoimmune conditions were excluded. The serum of participants was collected and stored at -80˚C before analysis at The Immunology Research Unit, Faculty of Medicine, Universitas Padjadjaran.

TB status was determined based on the presence of classical clinical symptoms of TB (chronic cough, weight loss, and night sweats) as well as the result of the acid-fast staining test by Ziehl-Neelsen method, MTB culture by using Lowenstein-Jensen medium and IFN-γ release assay (IGRA). LTB was defined as a positive IGRA result without clinical or radiographic findings of ATB. Subsequently, 70 participants were divided into three categories: i) HIV-ATB (n=19), ii) HIV-LTB n=21) and iii) HIV-alone (n=30).

Acid-fast staining test by Ziehl-Neelsen method. Samples were stained with 0.3% Carbol-fuchsin for 5 min. The stain was washed off with running water. Next, the slide was covered with 3% acid alcohol for 1 min and rinsed with running water. For counterstaining step, the slide was covered with 0.1% methylene blue stain for 20 sec followed by washing with running water and air-drying. Finally, the slide was examined microscopically under a high power objective. A positive result from the acid-fast staining test was determined if a minimum of one acid-fast bacili in 100 fields was found.

MTB culture. The sputum sample was mixed with an equal volume of 4% NaOH solution in a centrifuge tube, vortexed, and incubated for 15 min at room temperature. After adding 14 ml of sterile phosphate-buffered saline (PBS) at pH 6.8, the mixture was centrifuged at 3,000 g/min for 15 min at room temperature. To re-suspend the sediment, the supernatant was discarded, followed by adding 1 ml of PBS (pH 6.8). The sediment was inoculated into vials containing Lowenstein-Jensen (L-J) medium and incubated at 37˚C for 8 weeks and inspected weekly. A positive result from MTB culture was determined if at least one colony was detected.

QuantiFERON-TB Gold Plus ELISA (QFT-Plus) testing. IGRA using QFT-Plus (Qiagen GmbH) was performed according to the manufacturer's protocols as previously described (29). In brief, 1-ml-heparinized blood samples were incubated at room temperature (17-25˚C) before transfer to four QFT-Plus Blood Collection Tubes as follows: Nil, containing heparin; TB Antigen Tube 1 (TB1), comprising early secreted antigenic target (ESAT)-6 and culture filtrate protein (CFP)-10 peptide; TB2, containing ESAT-6 and 10 kDa CFP-10 peptide and mitogen, contained phytohemagglutinin, a non-specific stimulant of T-cells. The aliquoted tubes were inverted to mix 10 times before 16-24 h incubation at 37˚C, followed by centrifugation at 3,000 x g for 15 min at room temperature. Furthermore, 50 µl working strength conjugate, 50 µl plasma samples and 50 µl standards were added to the appropriate ELISA well plate. The ELISA plate was incubated at room temperature for 120 min, followed by washing using wash buffer and incubation at room temperature for 30 min. A total of 50 µl enzyme-stopping solution was added to each well. The optical density was measured using the BioTek ELx800™ Absorbance Microplate Reader (BioTek® Instruments, Inc.) at 450 and 650 nm and analyzed using the QFT Plus analysis software (ver.2.71; Qiagen GmbH). A positive test for IGRA was defined as concentration of TB1 and/or TB2 minus Nil ≥0.35 IU/ml or ≥25% of the Nil value.

Cytokine determination by cytometric bead array (CBA). IL-17A, IFN-γ, TNF, IL-10, IL-6, IL-4 and IL-2 levels were quantified in plasma samples using the BD CBA Human Th1/Th2/Th17 Cytokine kit (BD Biosciences) based on the manufacturer's protocols. Briefly, 50 µl each mixed captured beads, standard solutions, unknown samples and detection reagent were added to each assay tube, followed by 3 h incubation at room temperature with protection from light. The samples were washed with 1 ml wash buffer and centrifuged at 200 x g for 5 min at room temperature. The bead pellet of each assay tube was resuspended with 300 µl wash buffer after aspirating the supernatant. The cytokine concentration in each sample was measured using the BD FACSLyric™ Flow Cytometry System (BD Biosciences) and analyzed using the FCAP Array™ Software (ver.3.0; BD Biosciences). The theoretical detection limits were 18.9, 3.7, 3.8, 4.5, 2.4, 4.9 and 2.6 pg/ml for IL-17A, IFN-γ, TNF, IL-10, IL-6, IL-4 and IL-2, respectively.

CD4 analysis. CD4 cell count was performed using the PIMA™ CD4 assay (Abbott Pharmaceutical Co. Ltd.). Briefly, ~25 µl venous blood sample was added to the PIMA™ CD4 cartridge. In the case of finger prick, the sample was directly collected onto the cartridge. PIMA™ CD4 cartridge was inserted immediately into the Alere PIMA CD4 analyzer (Alere, Inc.) and $CD4^+$ cell count was automatically calculated.

Table I. Baseline characteristics of the study population.

Variable	Total (n=70)	HIV-active tuberculosis (n=19)	HIV-latent tuberculosis (n=21)	HIV (n=30)
Mean age, years	35±8	39±8	33±8	36±7
Sex (%)				
Male	56 (80.0)	16 (84.2)	14 (66.7)	26 (86.7)
Female	14 (20.0)	3 (15.8)	7 (33.3)	4 (13.3)
Viral load, copy/ml (%)				
Not detected	58 (82.9)	15 (78.9)	17 (80.9)	26 (86.7)
<1,000	9 (12.8)	3 (15.8)	3 (14.3)	3 (10.0)
≥1,000	3 (4.3)	1 (5.3)	1 (4.8)	1 (3.3)
CD4 count, cells/μl (%)				
<350	31 (44.3)	15 (78.9)	5 (23.8)	11 (36.7)
≥350	39 (55.7)	4 (21.1)	16 (76.2)	19 (63.3)

HIV, human immunodeficiency virus.

Table II. Concentration of cytokine production.

Cytokine, pg/ml	HIV-ATB (n=19)	HIV-LTB (n=21)	HIV (n=30)	P-value
IL-17A	3.26 (0.00-120.46)	1.74 (0.00-15.5)	3.07 (0.00-52.52)	0.281
IFN-γ	0.94 (0.00-17.12)	0.00 (0.00-1.25)	0.09 (0.00-3.23)	<0.001
TNF	0.35 (0.00-11.99)	0.07 (0.00-5.08)	0.24 (0.00-55.14)	0.018
IL-10	3.24 (0.77-27.46)	0.73 (0.24-7.17)	1.06 (0.48-10.18)	<0.001
IL-6	3.93 (0.94-21.92)	1.13 (0.1-15.63)	1.12 (0.54-11.44)	<0.001
IL-4	0.59 (0.00-43.81)	0.35 (0.00-1.49)	0.39 (0.00-3.91)	0.210
IL-2	0.16 (0.00-4.69)	0.02 (0.00-1.87)	0.02 (0.00-2.07)	0.006

Data are presented as median (range). HIV, human immunodeficiency virus; ATB, active tuberculosis; LTB, latent tuberculosis.

Statistical analysis. Kolmogorov-Smirnov normality test was performed using GraphPad Prism (ver.9.5.1; Dotmatics). Kruskall-Wallis and Dunn's post hoc analyses were used to compare the cytokine levels between >2 groups. Data are presented as the mean ± standard deviation. The result of cytokine analyses were presented as median (range). P<0.05 was considered to indicate a statistically significant difference.

Results

Baseline characteristics. The characteristics of the study participants are presented in Table I. Among 70 participants in the present study, 56 (80%) were male and 14 (20%) were female. The mean age of participants was 35 (25-43) years. The total participants with HIV-ATB, HIV-LTB, and HIV-alone that had a CD4 count <350 cells/μl were 15 (78.9%), 5 (23.8%) and 11 (36.7%), respectively.

Cytokine measurement. The comparison of IL-17A, IFN-γ, TNF, IL-10, IL-6, IL-4 and IL-2 levels in patients with HIV-ATB, HIV-LTB and HIV is presented in Table II. In the HIV-ATB group, the median level of all cytokines was higher compared with that in the HIV-LTB and HIV groups. Furthermore, a significantly higher median expression of IFN-γ, TNF, IL-10, IL-6 and IL-2 was observed in the HIV-ATB compared to HIV-LTB and HIV groups.

IFN-γ, TNF, IL-10, IL-6 and IL-2 were significantly higher in patients with HIV-ATB compared with those with HIV-LTB. (Fig. 1). Significant difference in IFN-γ, IL-10, IL-6 and IL-2 levels between patients in the HIV-ATB group and those in the HIV group was observed.

Cytokine levels in patients with CD4 count <350 cells/μl was next assessed (Table III). Median concentration of IFN-γ, IL-10, IL-6 and IL-2 was significantly different between HIV-ATB, HIV-LTB and HIV groups. IFN-γ, IL-10, IL-6, and IL-2 levels were also significantly higher in HIV-ATB compared to HIV-LTB and HIV groups. By contrast, only TNF and IL-10 levels differed significantly between the HIV-ATB, HIV-LTB, and HIV groups in patients with with CD4 count ≥350 cells/μl (Table IV). However, post hoc analysis revealed no statistical difference (data not shown). For CD4 count <350 cells/μl, IFN-γ, IL-10 and IL-6 levels were significantly higher in HIV-ATB compared with HIV-LTB and HIV groups (Fig. 2).

Table III. Cytokine production stratified by CD4 count <350 cells/μl.

Cytokine, pg/ml	HIV-ATB (n=15)	HIV-LTB (n=5)	HIV (n=11)	P-value
IL-17A	6.23 (0.00-120.5)	1.27 (1.21-10.16)	3.37 (0.00-17.12)	0.270
IFN-γ	0.97 (0.00-17.12)	0.00 (0.00-0.44)	0.06 (0.00-1.17)	<0.001
TNF	0.48 (0.00-11.99)	0.43 (0.00-0.96)	0.16 (0.00-4.75)	0.240
IL-10	3.24 (0.77-27.46)	0.66 (0.36-1.65)	0.99 (0.48-4.30)	0.001
IL-6	4.35 (0.96-21.92)	1.13 (0.15-1.54)	1.06 (0.54-2.26)	<0.001
IL-4	0.70 (0.00-8.27)	0.31 (0.00-0.95)	0.39 (0.00-2.40)	0.260
IL-2	0.16 (0.00-4.69)	0.00 (0.00-0.48)	0.00 (0.00-0.48)	0.030

Data are presented as median (range). HIV, human immunodeficiency virus; ATB, active tuberculosis; LTB, latent tuberculosis.

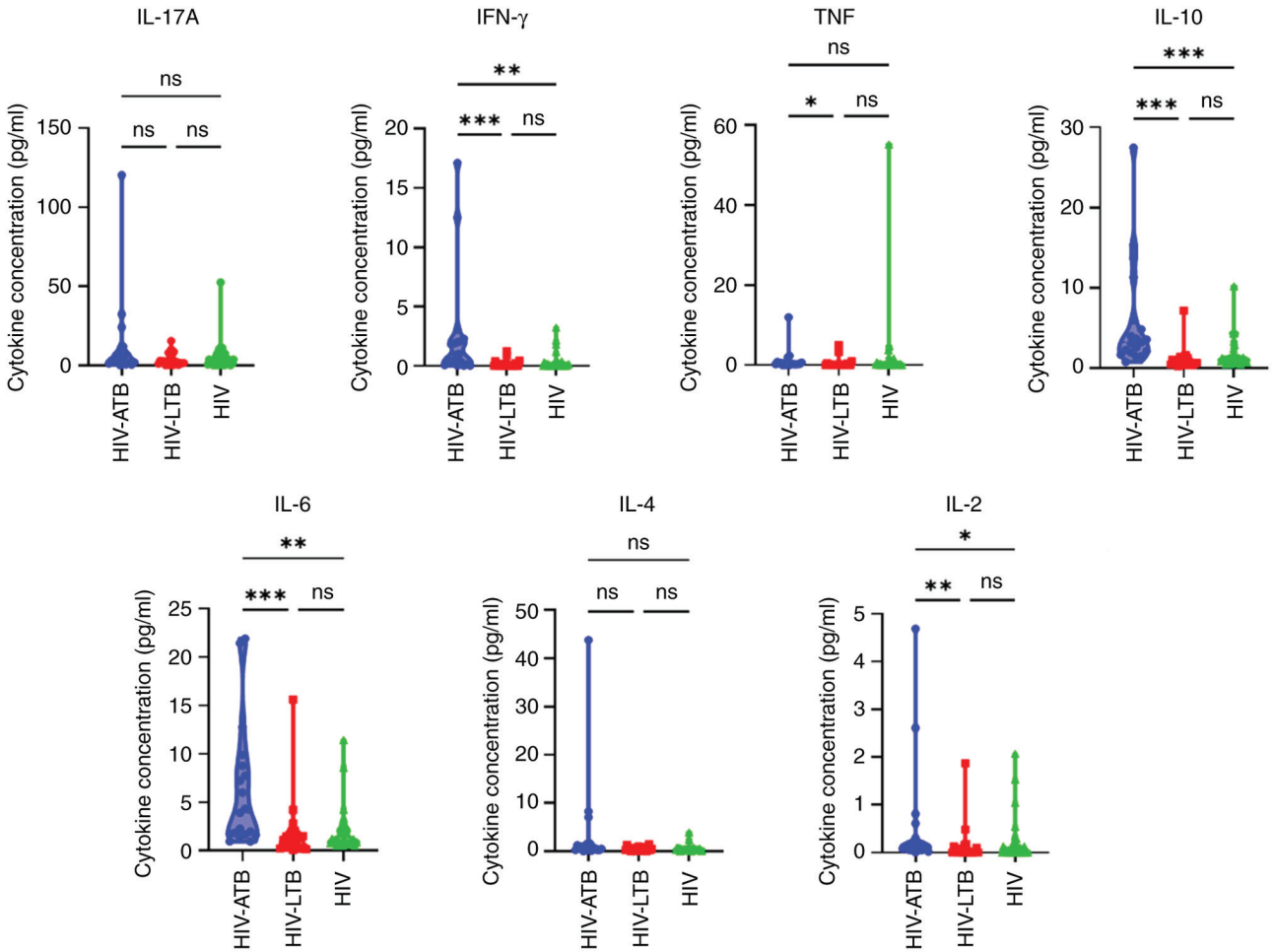

Figure 1. IL-17A, IFN-γ, TNF, IL-10, IL-6, IL-4 and IL-2 levels in HIV-ATB, HIV-LTB, and HIV groups. *P<0.05, **P<0.01 and ***P<0.001. HIV, human immunodeficiency virus; LTB, latent tuberculosis; ATB, active tuberculosis; ns, not significant.

Discussion

In the present study, IFN-γ, TNF, IL-10, IL-6 and IL-2 expression was significantly different in individuals infected with HIV-ATB compared with HIV-LTB.

Previous study showed that T cell cytokine profiles can be used to indicate certain disease states, including ATB and LTB. The secretory balance of pro- and anti-inflammatory cytokines generated by Th1 (IFN-γ, TNF and IL-2) and Th2 cells (IL-10, IL-6 and IL-4) during LTB is regulated to prevent reactivation into ATB (30). Its imbalance in patients testing positive for HIV with MTB co-infection affects the function of the immune system (19,31).

IFN-γ, TNF and IL-2 serve important roles in controlling the growth of MTB by activating innate and adaptive immune cells and modulating formation of granuloma (32,33). Compared with previous studies, a higher concentration of IFN-γ, TNF and IL-2 was here observed in HIV-ATB compared with

Table IV. Cytokine production stratified by CD4 count >350 cells/μl.

Cytokine, pg/ml	HIV-ATB (n=15)	HIV-LTB (n=5)	HIV (n=11)	P-value
IL-17A	1.48 (0.57-3.06)	2.11 (0.00-15.5)	3.26 (0.00-52.52)	0.660
IFN-γ	0.36 (0.01-1.89)	0.00 (0.00-1.25)	0.09 (0.00-3.23)	0.060
TNF	0.34 (0.23-0.35)	0.05 (0.00-5.08)	0.26 (0.00-55.14)	0.030
IL-10	2.97 (1.44-5.96)	0.74 (0.24-7.17)	1.10 (0.48-10.18)	0.008
IL-6	1.71 (0.96-21.45)	1.04 (0.10-15.63)	1.15 (0.54-11.44)	0.240
IL-4	0.37 (0.19-43.81)	0.37 (0.00-1.49)	0.39 (0.00-3.91)	0.880
IL-2	0.13 (0.20-0.21)	0.03 (0.00-1.87)	0.06 (0.00-2.07)	0.430

Data are presented as median (range). HIV, human immunodeficiency virus; ATB, active tuberculosis; LTB, latent tuberculosis.

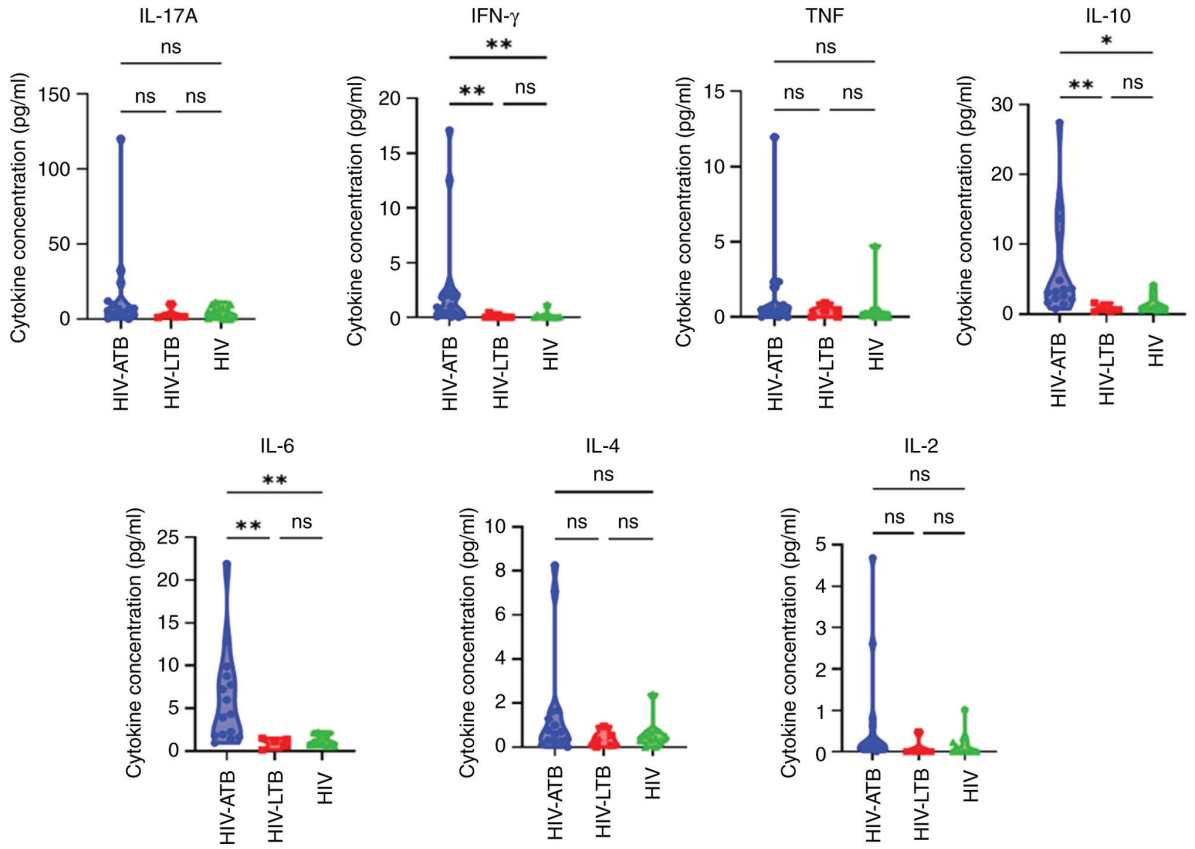

Figure 2. IL-17A, IFN-γ, TNF, IL-10, IL-6, IL-4 and IL-2 levels in HIV-ATB, HIV-LTB and HIV in patients with CD4$^+$ count <350 cells/μl. *P<0.05 and **P<0.01. HIV, human immunodeficiency virus; LTB, latent tuberculosis; ATB, active tuberculosis; ns, not significant.

HIV-LTB and HIV groups (15,31). This finding suggests that host protective responses against MTB infection by increasing the expression of pro-inflammatory cytokines is insufficient to prevent reactivation into ATB (34). In accordance with other reports, the present results also indicated that the expression of anti-inflammatory cytokines, particularly IL-10 and IL-6, in individuals with HIV-ATB was higher compared with that in HIV-LTB and HIV-alone (15,19,31). This could indicate that a shift towards the promotion of anti-inflammatory Th2 cytokines is associated with failure to curtail the growth of MTB in HIV-LTB, thereby facilitating progression into ATB.

Here, only IFN-γ, IL-10 and IL-6 were significiantly different in patients with HIV-ATB and HIV-LTB when the CD4 count was <350 cells/μl. Furthermore, expression of IFN-γ, IL-10 and IL-6 was not influenced by low CD4 count. The median concentration of IL-10 and IL-6 was higher than other cytokines in patients with HIV-ATB, suggesting a dominance of anti-inflammatory over pro-inflammatory cytokines.

To the best of our knowledge, the present study is the first to investigate expression of IL-17A, IFN-γ, TNF, IL-10, IL-6, IL-4 and IL-2 in individuals with HIV-ATB, HIV-LTB and HIV-alone.

There are certain limitations in the present study. The cross-sectional study design limited investigation of cytokine expression dynamics in the progression from LTB into ATB. In addition, there was a small sample size due to difficulty acquiring subjects that met the inclusion criteria within the

study period. Therefore, long-term cohort studies with larger subject population are required to observe changes in over time, especially when other associated opportunistic infections, such as aspergillosis and cryptococcosis are present (35).

In conclusion, the present study revealed differences in the cytokine profiles, particularly IFN-γ, IL-10 and IL-6, in patients with HIV-ATB and HIV-LTB, especially when the CD4 count was <350 cells/μl. However, further investigations are required to elucidate the mechanism of pro- and anti-inflammatory T cell cytokines in pathogenesis of immune activation during HIV-MTB co-infection.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

ARI, DKT and RW designed the study. ARI, AS, DKT confirm the auntheticity of all the raw data. AS, JH, NMDR and SM recruited study participants. AS, JH, NMDR and SM conducted experiments and collect study data. ARI, AS, JH, NMDR and SM analyzed the study data. ARI, AS, JH, NMDR, SM and DKT wrote the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by The Health Research Ethics Committee, Faculty of Medicine, Universitas Padjadjaran (approval no. 834/UN6.KEP/EC/2018; Bandung, Indonesia) and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants included in this study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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