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The Association Between Pro-Inflammatory Cytokines and Antiretroviral Therapy Resistance-Related Mutations in HIV-Positive Patients

Dwitya Elvira[1], Rizka Nadia[2], Raveinal Raveinal[1]

[1]Andalas University Medical Faculty, Dr. M. Djamil General Hospital, Clinic of Internal Medicine, Allergy and Immunology Subdivision, Padang, Indonesia
[2]Andalas University Medical Faculty, Dr. M. Djamil General Hospital, Clinic of Internal Medicine, Padang, Indonesia

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Corresponding Author: Dwitya Elvira, Andalas University Medical Faculty, Dr. M. Djamil General Hospital, Clinic of Internal Medicine, Allergy and Immunology Subdivision, Padang, Indonesia
Phone: +62 37771 **E-mail:** dwityaelvira@med.unand.ac.id **ORCID:** orcid.org/0000-0003-0227-7735

Abstract

Objective: Human-immunodeficiency virus (HIV) infection initiates the dysregulated production of pro-inflammatory cytokines. Moreover, unsuccessful treatment for HIV might be due to the drug resistance mutations (DRM) against anti-retroviral therapy (ART). This study aimed to compare pro-inflammatory cytokines levels of HIV patients, who received first line ART and patients, with mutations of M184V and K103N.

Materials and Methods: This was a case-control study which involved eighty patients with AIDS who received first-line anti-retroviral therapy for at least 6 months. The respondents were divided into two groups of patients with HIV with DRM and the ones with HIV without DRM. M184V and K103N drug resistance mutations to ART were analyzed using reverse transcription-polymerase chain reaction (RT-PCR) meanwhile tumour necrosis factor alpha (TNF-α) and interleukin (IL)-6 as pro-inflammatory cytokines were measured using enzyme linked immunosorbent assay.

Results: Most of the patients were men with an average age of 35.5 ± 9.2 years. The median levels for TNF-α and IL-6 were found 43.3 (4.3-96.1) pg/mL and 46.2 (15.5-158.1) pg/mL, respectively. The results showed that the DRM group got higher values compared to the non-DRM group, but there was no statistically significant difference found between both groups.

Conclusion: There were no differences in pro-inflammatory cytokines between the DRM AIDS patients group compared to the non-DRM who received first-line ART with mutations of M184V and K103N.

Keywords: Cytokine, drug resistance, HIV/AIDS patient, IL-6, mutation, TNF-α

Introduction

The human immunodeficiency virus type-1 (HIV-1) infection, the acquired immunodeficiency syndrome (AIDS) are the major health threats worldwide. Based upon the official data from the World Health Organization (WHO), in 2019, an estimated 38 million people suffer from human immunodeficiency (1-3).

Combining first line antiretrovirals was the most vital advancement in treating HIV infection (2). In successfully treated patients, chronic inflammation often persists (2). Antiretroviral therapy has been found to be effective in HIV infection and transmission (2). WHO recommends the antiretroviral treatment (ART) to the patients after the diagnosis (3,4). ART reduces the viral load to an undetectable extent while partially restoring the immunological function (4). However, the rate of unsuccessful treatments is considered high (4). Unsuccessful treatment might be responsible for the selection of drug resistance mutations (DRM) in the viral genome, which might be archived in the cellular reservoir (4). DRM might be detected in proviral DNA, even when viral RNA is suppressed, by sequencing extracted DRM archived in proviral DNA. It might be expressed upon virologic failure and have impact on the management of long-term ART (4).

ORCID: D. Elvira: 0000-0003-0227-7735, R. Nadia: 0000-0001-9216-4448, R. Raveinal: 0009-0003-9100-3329

Based upon to the WHO about HIV Drug Resistance Report (2019) (5), the ubiquity of ART drug resistance is 3-29%. Of these populations, the ubiquity of nucleoside reverse-transcriptase inhibitor (NRTI) resistance is 21-91% and that of non-NRTI (NNRTI) resistance is 50-97%. Zuo et al. (6) reported that the M184V mutation caused resistance to lamivudine and K103N was the most common mutation in almost all NNRTI class drugs. Zou et al. (7) reported that NRTI resistance was caused by the M184V mutation (62.04%), whereas that of the NNRTI resistance was caused by K103N mutation (41.90%).

The combination of selective immune and anti-HIV drug pressures to create the evolution of HIV resistance is one of the main health concerns of the highly active antiretroviral therapy (HAART) (8). The concern is that it could hamper the effect of successful HAART (8). Therefore, it is vital to explore the other target molecules to fight HIV-1. Studies reported that the cell profile was altered during HIV-1 infection, leading to the abruption of $CD4^+/CD8^+$ T, and the cytokine network, including tumor necrosis factor alpha (TNF-α) (8).

Immune system activation can be seen from the cytokines produced during the inflammatory process (9). HIV infection causes dysregulation of cytokines *in vivo* and *in vitro* and induces TNF-α, TNF-beta (TNF-β), interleukin (IL)-1 and IL-6, causing HIV virus replication in T-cells and monocyte-derived macrophages CXCL-10 cells. TNF-α and IL-6 (pro-inflammatory cytokines) and IL-10, IL-4, and transforming growth factor beta (TGF-β) (anti-inflammatory cytokines) were notably present in HIV patients (9). The elevation was more marked before the commencement of HAART, which gradually declining by one year after HAART, even the level of TNF-α and TGF-β are failed to return to normal compared to control (9).

The association between the M184V and K103N mutations of the HIV virus and pro-inflammatory cytokines such as IL-6 and TNF-α has never been studied. Furthermore, this mutation is associated with drug resistance, leading to therapy failure. Therefore, the aim of this study was to investigate the comparison between pro-inflammatory cytokines (IL-6 and TNF-α) and HIV drug resistance mutations (especially M184V and K103N mutation) on HIV patients receiving first-line antiretroviral therapy.

Materials and Methods

Research Design

The research was conducted based upon a retrospective review of secondary data including 80 HIV-diagnosed patients as a sample from the department of infectious and tropical disease at Dr. M. Djamil General Hospital, Padang. The patients were chosen via consecutive sampling. The research samples had inclusion criteria: Being aged ≥18 years, taking first-line ART for >6 months, and giving consent to be research participant. The antiretroviral regimen consisted of two NRTI and one NNRTI. This research was conducted in a period of 6 months, from January to June 2022. This research has followed the CONSORT guidelines and the associated checklists. The research protocol was approved by the Health Research Ethics Committee RSUP Dr. M. Djamil Padang (decision no: LB.02.02/5.7/157/2022, date: 26.04.2022).

Cytokine and HIV-1 Pol Gene Amplification and Sequencing

Human enzyme linked immunosorbent assay kit, Elabscience was applied to calculate cytokine (IL-6 and TNF-α) using the quantitative sandwich enzyme immunoassay technique. QIAamp viral RNA mini kit (QIAGEN, Hilden, Germany) was applied to extract HIV-1 RNA from plasma. The amplification of the pol gene region used self-designed primers.

The first round forward and reserve primer were (RT): 5'-TTTYAGRGARCTYAATAARAGAACTCA-3' and (RT): 5'-CCTCITTYTTGCATAYTTYCCTGTT-3', respectively.

The second round forward and reverse primers were (RT): 5'-TTYTGGGARGTYCARYTAGGRATACC-3'andreverse (RT): 5'-GGYTCTTTGRTAAATTTGRATATGTCCA-3', respectively.

The region sequenced included a complete RT region of the pol gene. The derived nucleotide sequences were submitted to "HIV db Program: Sequence Analysis" in the Stanford University HIV drug-resistance database (http://hivdb.stanford.edu/hiv) for quality assessment and DRM interpretation.

$CD4^+$ T-cell Count

Enumeration of absolute $CD4^+$ T-cell count was performed using automated flow cytometry to check the immune status of the patients. Absolute CD4 count is obtained by multiplying the CD4 percentage based upon flow cytometry results, with the total measured white blood cell count.

Statistical Analysis

All patient characteristics, including sex, age, duration of HAART treatment, and CD4 counts, were summarized using either mean or median values. Bivariate analysis was performed using a computerized statistical program, to analyze the correlation between mutations in pro-inflammatory cytokines in patients receiving reverse transcriptase inhibitor therapy. The independent t-test was applied for normally distributed data. The Mann-

Whitney U test was used for non-normally distributed data. In order to investigate the association between the duration of treatment, CD4 extents, and viral load with pro-inflammatory cytokines (IL-6 and TNF-α) in patients receiving reverse transcriptase inhibitor therapy, the Pearson correlation test was used for normally distributed data and the Spearman correlation test for non-normally distributed data.

Results

Demographic Characteristics

There were 80 patients, of which 74 were males (92.5%) and 6 were females (7.5%) (Table 1). The patients were divided by age groups. The average age of the patients was 35.5 years, the majority of which were 31-40 years (ranging from 6 months to 168 months). The average length of treatment obtained was 43.3 months (ranging from 6 months to 168 months). We found that 32 (40%) and 48 patients (60%) had number of $CD4^+$ T-cell ≤250 cells/mm^3 and >250 cells/mm^3, respectively. The average $CD4^+$ T-cell count was 298.6 cells/mm^3 (ranging from 18 to 956 cells/mm^3). In this study, we found 8 patients (10%) had HIV with mutations of M184V and K103N, while 72 (90%) of the subjects did not have any mutations.

Table 2 shows the serum IL-6 and TNF-α levels.

Analysis of Pro-inflammatory Cytokines with DRM

Table 3 demonstrates that there was no association between DRM and serum pro-inflammatory cytokines levels (IL-6 and TNF-α).

Table 4 shows that there was an association between CD4 extents and IL-6 extents (p=0.001) in patients receiving reverse transcriptase inhibitor at (r=0.364). Furthermore, there was no association between $CD4^+$ T-cell counts and TNF-α levels.

Discussion

This study is the first study to report the role of cytokines on HIV patients with drug resistance mutation of M184V and K103N. This study revealed that the most common characteristic among our AIDS patients was male gender, aged between 31 and 40 years. Despite the administration of rapid initial ART to all subjects, low $CD4^+$ T-cell counts (less than 200 cells/mm^3), with the lowest value of 18 cells/mm^3, were still observed. Table 1 also shows that there were 8 patients (10%) who had mutations of M184V and K103N and based on this study, it was also found that the average levels pro-inflammatory cytokines (TNF-α and IL-6) were higher in patients with AIDS who found to have mutations after receiving first-line ART compared to patients without mutations, but there was no significant difference between each group of cytokines. Cytokines have a salient role in governing the homeostasis of the immune system. HIV infection causes cytokine dysregulation *in vivo* and *in vitro* (10). The response of the immune system to HIV infection causes cytokine dysregulation and inflammation plays a salient role (10). This initiates the depletion of $CD4^+$ T-cells and an elevation in viral load. The cytokine storm that occurs in the acute phase of HIV infection predicts the viral set point and illness progression, with 66% of cases occurring at a set point of 12 months after infection (10). After that, there will be a chronic phase where several markers such as IL-6, acute phase protein, and D-dimer can be used as predictors for comorbidities, illness progression, and mortality in patients who are already on antiretroviral therapy (10).

During HIV infection, cytokines are produced by T helper-1, T helper-2 cells are, pro-inflammatory cytokines, and TNF. T helper-2 cells secrete IL-4 and IL-10 (11). Pro-inflammatory cytokines cause increased secretion of IL-1, IL-6, IL-8, and TNF-α (11). HIV infection is associated with a change in response from T helper-1 to T helper-2

Table 1. Basic characteristics

Variable	n (%)	Average (SD)
Sex		
Men	74 (92.5%)	
Women	6 (7.5%)	
Age (years)		35.5 (9.18)
≤30	27 (33.75%)	
31-40	34 (42.5%)	
41-50	13 (16.25%)	
>50	6 (7.5%)	
Duration of therapy		43.3 (30.4)
6-24	27 (33.8%)	
>24	53 (66.2%)	
CD4 counts (cell/mm^3)		298.6 (172.6)
≤250	32 (40)	
>250	48 (60)	
Mutations of M184V and K103N		
Mutations found	8 (10%)	
No mutations found	72 (90%)	

SD: Standard deviation

Table 2. Serum of TNF-α and IL-6 levels

Variable	Median	Average (SD)	Minimum	Maximum
IL-6 level (pg/mL)	30.945	51.1 (± 5.6)	2.8	231.72
TNF-α (pg/mL)	35.845	67.6 (± 8.4)	12.2	384.19

TNF-α: Tumour necrosis factor alpha, IL: Interleukin, SD: Standard deviation

Table 3. Correlation of cytokines and drug resistance mutations

Variable	Median	Minimum	Maximum	p-value
IL-6 extents (pg/mL)				0.994
No mutation	30.9	2.8	231.7	
Mutation	43.3	4.3	96.1	
TNF-α extents (pg/mL)				0.537
No mutation	34.8	12.2	384.2	
Mutation	46.2	15.5	158.1	

*p-value ≤0.05
TNF-α: Tumour necrosis factor alpha, IL: Interleukin

Table 4. Correlation of cytokines with length of therapy, CD4 counts, and viral load

Variable	Length of therapy	CD4 counts	Viral load
IL-6 extents (pg/mL)	0.074	0.001*(0.364)	0.542
TNF-α extents (pg/mL)	0.071	0.752	0.439

*p-value ≤0.05
TNF-α: Tumour necrosis factor alpha, IL: Interleukin

which leads to a declined production of IL-2 and IFN-γ and increased secretion of IL-4 and IL-10, as well as pro-inflammatory cytokines (IL-1, Il-6, IL-8, and TNF-α) (11). IL-6 secretion is also carried out by B-cells so that HIV infection trigers the production of IL-6 (11).

The presence of a persistent viral infection such as HIV infection stimulates the immune system, leading to its accelerated aging. Antiretroviral therapy can reduce systemic inflammation (12). De Pablo-Bernal et al. (12) reported that IL-6 returned to normal levels after ≥1 year of treatment and TNF-α remained high despite administration of antiretroviral therapy for 96 weeks (12). This is caused by TNF-α produced by different T-cells, particularly monocytes which are continuously activated during the infection (12). Inflammation persists even after the patient has received antiretroviral therapy, resulting in the damage of several tissues, which will eventually lead to other chronic illnesses, such as cardiovascular illness, an illness that is also caused by increased TNF-α production (12).

TNF-α has various roles in the regulation and development of the immune system including inflammation, differentiation, lipid metabolism, and apoptosis (8). TNF-α is produced by various immune cells activated by myeloid cells via macrophages and monocytes (8). Increased TNF-α has been associated with several pathological conditions such as inflammation, infection, autoimmune diseases, and cancer, as well as with the progression of HIV infection (8). Untreated patients with AIDS have increased TNF-α extents compared to healthy individuals of the same age (8).

Antiretroviral therapy was shown to increase the life expectancy of HIV patients (13). The use of antiretroviral therapy has been shown to decline viral load, elevate $CD4^+$ T-cell count, and to some extent alter cytokine profiles (13). Several studies indicated that decrease in TNF-α appeared to be correlated with the efficacy of HAART in 14 HIV patients who were observed for 12 months (13). Another study showed that TNF-α levels were low in patients who were already receiving ART therapy (13). However, Kumar et al. (8) found an elevation in TNF-α secretion in patients receiving antiretroviral therapy, which might be related to lipodystrophy-associated dyslipidemia that occurs in patients receiving antiretroviral therapy.

Several studies have showed that the expression and secretion of IL-6 were abnormal in patients with AIDS (14,15). HIV infection directly increases IL-6 secretion by monocytes and macrophages (14,15). IL-6 increases HIV replication in the latent phase of macrophages and interferes with macrophage function (14,15). Increased IL-6 also occurs in the reconstitution phase after antiretroviral administration, which might be related to the activation of the neuroendocrine system as it develops in the lipodystrophy syndrome (14,15). Connolly et al. (16) reported that TNF-α increased in the early phase of infection, then decreases in the final phase of the illness. TNF-α secretion had a direct association with the amount of HIV RNA and an inverse association with the $CD4^+$ T-cell count (16). Antiretroviral administration did not directly reduce TNF-α levels to normal values, even in patients with low viral loads and good $CD4^+$ T-cell response (16). In this study, we also found a significant correlation between $CD4^+$ T-cell counts with IL-6 cytokines level in the DRM group. IL-6, as a pro-inflammatory cytokine might be useful to accompanied CD4 as a biomarker measurement to evaluate and monitor drug resistance mutations on AIDS patients had been administered first-line ART. Okay et al. (17) examined IL-6, IL-1β, and TNF-α in 30 patients with HIV who had no comorbidities. Cytokine levels reported that

serum concentrations of IL-6, IL-1β, and TNF-α showed a remarkable decline after antiretroviral administration, but these were still above than those of the control group (17).

Study Limitations

It is important to ensure patient adherence to treatment and closely monitor their ARV consumption during medical visits. While $CD4^+$ T-cell count and viral load measurements are already standard practice in our management, this study suggests that measuring biomarkers such as pro-inflammatory cytokines is also crucial for maintaining treatment effectiveness.

Conclusion

This study concludes that there were no significant differences in pro-inflammatory cytokine levels between DRM HIV patients and non-DRM patients who received first-line ART with M184V and K103N mutations, despite observing higher cytokine levels in the DRM group. According to this study, $CD4^+$ T-cell count was found to be correlated with IL-6 level in the DRM group, thus monitoring $CD4^+$ T-cells with measurement of cytokines might be useful to evaluate drug resistance mutations in patients with AIDS who received first-line ART. Further research is required to investigate how mutations of ART might impact the cellular and humoral immune system.

Risk of DRM in our AIDS patients who received first line HIV therapy is still a challenging issue. It is important to ensure patient adherence to treatment and closely monitor their ARV consumption during medical visits. While $CD4^+$ T-cell count and viral load measurements are already standard practice in our management, this study suggests that measuring biomarkers such as pro-inflammatory cytokines is also crucial for maintaining treatment effectiveness.

Ethics

Ethics Committee Approval: The research protocol was approved by the Health Research Ethics Committee RSUP Dr. M. Djamil Padang (decision no: LB.02.02/5.7/157/2022, date: 26.04.2022).

Informed Consent: Each participant in the study provided informed consent.

Authorship Contributions

Surgical and Medical Practices: D.E., R.N., R.R., Concept: D.E., R.N., R.R., Design: D.E., R.N., R.R., Data Collection or Processing: D.E., R.N., R.R., Analysis or Interpretation: D.E., R.N., R.R., Literature Search: D.E., R.N., R.R., Writing: D.E., R.N., R.R.

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