

East Africa Medical Journal Vol. 91 No. 8 August 2014

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ABSTRACT

Background: Interleukin-10, IL-2 and IFN- γ are some of the crucial cytokines associated with HIV infection and pathogenesis. While IL-2 and IFN- γ play critical roles in host resistance to infection, IL-10 inhibits the synthesis IFN- γ , IL-2 at mRNA and protein level; exacerbating damage to immune system.

Objective: To determine the levels of, changes in and correlation between CD4 count, viral load, IL-10, IL-2 and IFN- γ before HAART and at six months of HAART among HIV positive patients in Kigali; with a view to understand cytokine networks particularly in relation to HAART; and to see whether they can be used as alternative markers of the disease progression.

Design: Longitudinal study.

Setting: Kagugu, Kimironko, Biryogo, Gitega Health Centres and Centre Medico-Social Cornum; all located in Kigali.

Subjects: Thirty three (33) HAART initiation eligible HIV positive patients including 13 women and 20 men.

Results: A drop in viral load (though only a small number of patients achieved an undetectable viraemia); a recovery of CD4+ cells, a decrease in IL-10 (though it remained high for many patients especially those with unchanged viraemia); and an increase in IL-2 and IFN- γ indicated a successful HAART. A negative correlation between CD4 count and viral load and between CD4 count and IL-10 (but $r < -0.5$) was observed. IL-10 correlated positively and strongly with viremia ($r > 0.5$ at both time points: p -values < 0.05). There was no significant correlation between CD4 count, IL-2 and IFN- γ .

Conclusion: Results demonstrated the down-regulatory effect of IL-10 on Th1 cytokines and that a shift from Th1 to Th2 cytokine is associated with HIV disease progression. A successful HAART results in CD4+ cells recovery, drop in viraemia and IL-10 with up-regulation of Th1 cytokines. Also, findings show potential usefulness of IL-10 as a marker of HIV disease progression.

INTRODUCTION

Global statistics from UNAIDS indicate that nearly 35 million people are currently infected with human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS). About two million people are infected each year, while 25 million people have died from HIV/AIDS since the disease was first identified in the early 1980s (1).

Human Immunodeficiency Virus is a lentivirus belonging to the retroviridae family and is classified into two types, HIV-1 and HIV-2. HIV-1 comprises of

groups M (major), N (non-M non-O), and O (outlier). Due to a high level of genetic diversity, group M is further sub-classified into subtypes (A to D; F to H; J, and circulating recombinant forms). While HIV-2 remains essentially confined to West Africa, HIV-1 spreads around the world; with group M accounting for the vast majority of HIV infections (2,3).

Currently, viral load and CD4 count are the only markers employed for HIV disease progression assessment (4). Though less expensive compared to viral load, CD4 cells count does not always correlate with the viral load and disease progression and

this brings need for affordable alternative markers to assess the effects of treatment as well as disease progression (5). Recent studies suggest a powerful prognostic value for blood cytokines levels in different diseases (6). Cytokines are polypeptides produced by many different cell types mostly after their activation to mediate inflammatory and immune reactions (7).

Structural studies have shown that cytokines belong to one of the four groups: the hematopoietin family (participate to Haemopoiesis, for example, IL-2), the interferon family (one of their functions is to interfere with viral replication within a host cell, for example, IFN- γ), the chemokine family (Chemotactic molecules, for example, CCL14), or the tumour necrosis factor family (can induce cell death among other many functions, or example, TNF- α) (8).

Tumour necrosis factor alpha (TNF- α), IFN- γ , IL-1, IL-2, IL-6 and IL-10 are some of the cytokines affected during HIV infection and analysis of these cytokines may give an indication of the degree of immune activation, the extent of the immune response and disease progression (9). HIV infection has been associated with reduced production of the Th1 cytokines, especially IL-2. Increases in Th2 cytokine production particularly IL-4 and IL-10, restrict Th1 activity and are associated with HIV infection, as is a skewing of the CD4+ T cell population toward a Th2 phenotype (1).

Some studies on the secretion of cytokines in the course of HIV infection, particularly following HAART initiation have shown that CD4 count and IL-2 correlate positively; but correlate negatively with HIV load, IL-10 (10). While some studies suggested a positive correlation between CD4 count, IL-2 and IFN- γ ; others have claimed a negative correlation between this interferon and CD4+ cells count-IL-2 duo (11).

IL-2 permits rapid and selective expansion of effector T cells (both CD4+ and CD8+) activated by antigen, among many other functions. IL-2 is a growth factor for NK cells and promotes production of NK derived cytokines like TNF- α , IFN- γ and GM-CSF (12).

IFN- γ activates transcription of a large number of genes that play roles in anti-viral activity, apoptosis, antigen processing, MHC protein expression, and Th1 cells development (13).

IL-10 is an important cytokine with antiinflammatory properties besides TGF- β and IL-35 (14,15). IL-10 suppresses all functions of monocytes/macrophages, and this impairs the role of these cells in both innate and adaptive immunity (16). IL-10 inhibits Th1 cell cytokines synthesis including IFN- γ , IL-2, IL-3, and GM-CSF at mRNA and protein level; inhibits the expression of costimulators and class II MHC molecules on macrophages and dendritic cells and because of these actions, IL-10 serves to inhibit T

cell activation and terminate cell-mediated immune reactions (7).

Although several studies have been done to detail cytokine interactions in relation to HIV disease, these interactions are yet to be fully understood (17). In this study, the levels of CD4+ cells, IL-2, IL-10, and IFN- γ were assessed and correlated with viral load at the start of HAART and six months after HAART initiation among HIV positive patients in Kigali/Rwanda, where no such studies have been carried out. This study will contribute to a better understanding of the changes and interactions between various cytokines in the course of HIV infection; in relation to treatment. This study may also lead to identification of additional and hopefully more affordable markers for assessing HIV disease prognosis in relation to treatment.

MATERIALS AND METHODS

Scientific and Ethical Consideration: A scientific review, ethical clearance and a research permit were obtained from the National Health Research Committee of the Rwanda Biomedical Centre; the Rwanda National Ethics committee and the Rwanda Ministry of Education respectively.

Study Design: This was a six month (from August, 2013 to February, 2014) longitudinal study involving thirty three (33) HAART initiation eligible HIV+ patients (13 women and 20 men) recruited from Kagugu, Kimironko, Biryogo, Gitega Health Centres and Centre Medico-Social Cornum, all located in Kigali City. All patients were taking Tenofovir Disoproxil Fumarate Lamivudine and Efavirenz (tdf-3tc-efv) tritherapy.

Sample size and sampling strategy: A correlation of -0.5 ($r = -0.5$) between IL-10 and CD4+ T cells count was anticipated. The minimum sample size to estimate the same correlation with a Probability of Type I Error (α) of 0.05, a Power ($1 - \beta$) of 0.8 was 20 patients (sample size estimated using statstodo software available at www.statstodo.com).

Due to loss to follow up and death cases that are normally recorded at the selected health facilities; the sample size was raised to 40 HIV+ patients who came for HAART initiation. Any patient who came for HAART initiation was included in the sample size until the desired number of 40 patients was reached. At the end of the study, thirty three (33) patients were still under follow up (four patients died and three were lost to follow up during the study).

Data collection: Variables considered during this study included CD4 count, HIV plasma RNA, IL-10, IL-2 and IFN- γ . Measurements were done on whole blood (CD4 count) and on its plasma (cytokines and the viral load).

Blood was collected in 4 ml tubes containing EDTA at two time points namely before HAART (August, 2013) and six months following HAART initiation (February, 2014). Analyses were done at the Rwanda National Referal Laboratory according to the kits manufacturer's instructions. CD4 count was done using flow cytometry while the viral load and cytokines were measured using the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test and ELISA techniques, respectively.

CD4 count: CD4 counts were performed using flow cytometry with Becton Dickinson FACSC alibur (Software: MultiSET V3.0.2). To 20µl of tritest (CD3/CD45/CD4 monoclonal antibodies) reagent in TruCOUNT tube, 50µl EDTA whole blood was added. Vortexing was done followed by a 15-minute incubation in the dark. This was followed by fixation and lysing for a further 15 minutes in dark. The sample analysis was then done.

Viral load determination: The viral load was measured using The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 test which is a nucleic acid amplification test for the quantitation of Human Immunodeficiency Virus Type 1 (HIV-1) RNA in human plasma done in three major steps: Specimen preparation to release viral nucleic acid, reverse transcription of the target RNA to generate complementary DNA, simultaneous PCR amplification of target cDNA and detection of cleaved dual-labeled oligonucleotide probe specific to the target. In this procedure, specimen preparation is automated using the COBAS® AmpliPrep Instrument; amplification and detection are automated using the COBAS® TaqMan® Analyzer.

Cytokines quantification: IL-10, IL-2 and IFN-γ were measured using ELISA techniques and this measurement involved addition of samples, standards and controls, conjugate, substrate-chromogen and stop solutions to microtiter wells (test plate for IL-10, test plate for IL-2 and test plate for

IFN-γ) coated with monoclonal antibodies specific to these cytokines; according to kits manufacturer's instructions. Optical densities were measured at 450 nm using a microtiter plate reader. Concentrations of the measured cytokines were determined using standard curves.

RESULTS

Specific objectives of this study were to determine the levels of, changes in and correlations between CD 4 count, viral load, IL-10, IL-2 and IFN-γ before HAART and at six months of HAART among HIV positive patients in Kigali/Rwanda. Thirty three (33) HIV positive patients participated to this study. These comprised of 20 (60.6%) men and 13 (39.4%) women with a mean age of 33 years. Results for cytokines are in pg/ml, CD 4 count in Cells/µl and the viral load in RNA copies/ml of blood. Significance of changes in the levels of measured variables was assessed using Wilcoxon signed rank test.

At the time of HAART initiation, 32 (97%) of the 33 study participants had CD4 count <350 cells/µl of blood. One patient (3%) had a CD4 count >350 cells/µl of blood. When categorized based on whether the patient had a CD4 count below 200 cells/µl of blood (indicating a very low CD4 count) or more than 200 cells/µl; 12 (36.3%) patients had less than 200 cells/µl while 21 (63.7%) patients were having CD4 count of 200 cells/µl and above. Seven men (21.2%) and 5 (15.2%) women had a CD4 count of less than 200 cells/µl whereas 13 (39.3%) men and 8 (24.2%) women had a CD4 count of 200 cells/µl of blood and above. The mean CD4 count was 213 cells/µl of blood.

After six months of HAART, 5 (15.1%) of the 33 patients had a CD4 count below 200 cells/µl namely 1 (3%) woman and four (12.2%) men. Twelve (36.3%) women and 16 (48.4%) men had a CD4 count of 200 cells/µl of blood and above. The mean CD4 count increased from 213 cells/µl of blood at HAART initiation to 369 cells/µl of blood after six months of treatment.

Table 1

Levels of CD4+ at HAART initiation and after six months of HAART. Results are presented based on sex and CD4 counts are categorised into CD4 equal or above 200 cells/µl of blood and CD4 below 200 cells/µl of blood. Wilcoxon signed rank test showed a statistically significant recovery of CD4+ following HAART

	Before HAAT			At six months of HAAT			Significant change following HAART
	Overall frequency (%)	Men (%)	Women (%)	Overall frequency (%)	Men (%)	Women (%)	
CD4 count of <200 cells/µl of blood	12(36.4)	7(21.2)	5(15.1)	5(15.2)	4(12.2)	1(3)	Yes
CD4 count of 200 cells/µl and above	21(63.6)	13(39.3)	8(24.2)	28(84.8)	16(48.5)	12(36.3)	

At HAART initiation, all study participants had high viremia. The median viral load was 23400 HIV RNA copies/ml of plasma with a mean log of 4.5. Men had a median viral load of 22950 RNA copies/ml and a mean log of 4.5 compared to 39500 RNA copies/ml and a mean log of 4.7 for women. After six months of HAART, the median viral load dropped to 661 RNA copies/ml with a mean log of 2.67. Viral load

median in men dropped to 612 RNA copies/ml and to a mean log of 2.71 whereas the median reached 767 RNA copies/ml with a mean log of 2.61 among women. When looked at individually, results show that six (18.1%) patients achieved undetectable viral load and five (15.1%) did not achieve any log decrease in their viral load despite HAART for six months.

Table 2

Viral load before HAART initiation and how it changed following HAART based on sex. Wilcoxon signed rank test showed a statistically significant change in viral load following HAART

	Before HAART			At six months of HAART			Significant change following HAART
	Overall	Men	Women	Overall	Men	Women	
Median Viral load	23400	22950	39500	661	612	767	Yes
Mean Log viral load	4.59	4.5	4.7	2.67	2.7	2.7	

Results are expressed in number of HIV RNA copies/ml of blood

As for cytokines levels before HAART and after six months of treatment; results show that the mean for IL-10 was 120.2 pg/ml and this dropped to 48.06pg/ml at six months of HAART. As for the two Th1 cytokines namely IL-2 and IFN- γ ; their means increased. Mean for IL-2 was 1.29 pg/ml at HAART initiation while the mean for IFN- γ was 0.84 pg/ml.

After six months of treatment, the two means raised 10.8 and 5.24 pg/ml respectively. When change in the levels of these three cytokines was assessed using Wilcoxon signed rank test, a statistically significant decrease in IL-10 as well as an increase in IL-2 and IFN- γ were seen.

Table 3

Levels of cytokines before HAART and at six months of treatment. The table shows overall means of the three cytokines measured in this study as well as means based on sex of participants. There was a statistically significant change in levels of measured cytokines

	Before HAART			At six months of HAART			Significant change following HAART
	Overall	Men	Women	Overall	Men	Women	
Mean IL-10	120.2	102.2	138.3	48.06	44.2	52.01	Yes
Mean IL-2	1.29	0.88	1.93	10.08	8.7	12.16	Yes
Mean IFN- γ	0.84	0.92	0.73	5.24	5.27	5.19	Yes

Results are expressed in pg/ml

Variables were finally correlated between themselves. A negative correlation ($r = -0.42$; and $r = -0.46$ at p values <0.05) was observed between CD4 count and HIV plasma RNA; before HAART and at six months of the treatment. While a negative correlation was found between CD4 count and IL-10 ($r < -0.5$ at p values <0.05), a positive correlation was seen between CD4 count and IL-2 as well IFN- γ (however, these correlations were all weak, hence non-significant

($r < -0.5$ with most of all p values > 0.05). As for the correlations between HIV plasma RNA or the viral load and these cytokines; there was a strong positive correlation with IL-10 ($r > 0.5$; all p values <0.05) compared to the correlation viral load-CD4 count ($r < -0.5$). We did not find a significant correlation between the viral load and the two The cytokines (IL-2 and IFN- γ).

Table 4
Correlation between variables measured in this study

	Correlation between measured variables			
	Before HAART		At six months of HAART	
	CD4 count	Viral load	CD4 count	Viral load
IL-10	-0.4	0.7	-0.2	0.7
IL-2	NS	NS	NS	NS
IFN- γ	0.4	NS	NS	NS
CD4 count		-0.42		-0.46

NS: Non significant correlation

DISCUSSION

While efforts have been made in providing ARV drugs to infected people particularly in Africa; monitoring of HIV positive patients to assess the response to ARV as well as infection progression remains a challenge especially due to scarcity of adequate facilities for the biological follow up. CD4 count facilities have been scaled up but the viral load measurement (which is the current most reliable test to assess the progression of the infection and response to treatment) is still limited to a small number of health facilities and this is particularly critical in Africa. Furthermore, cytokines networks in the course of HIV infection is yet to be fully understood. This study was done to examine the relationship between cytokines, viral load and CD4 count levels with a view to understand cytokines networks during HIV infection as well whether they can be used as alternative markers of the disease progression, easily implementable and affordable.

In the present study; 33 HIV positive patients were recruited and CD4 count, viral load, IL-10, IL-2, and IFN- γ were measured; changes were rated and correlations assessed both before HAART and at six months after initiation of treatment. Findings from this study have shown high levels of HIV plasma RNA and high concentration of IL-10 before HAART. This has also been described by Agarwale *et al* (18). Furthermore, results from his study demonstrated that low CD4 count was also accompanied by low levels of Th1 cytokines (IL-2, IFN-gamma) and high levels of IL-10 (a Th2 cytokine) as it is the case from the present study at HAART initiation.

In another study to assess IL-2 and IL-10 serum levels in HIV-1-infected patients with or without active anti-retroviral therapy, results similar to those of the present study were found. Higher levels of IL-10 have been detected in patients with low CD4 count, particularly those with CD4 count <200 cells/ μ l of serum and higher levels of IL-10 paralleled with

high viral load (19).

Another study by Ostrowski *et al* (20) suggested that HIV-1 may directly subvert specific immune responses by IL-10 induction. In this study, it was found that Circulating frequencies of CD4+ T cells constitutively producing IL-10 were significantly higher in individuals with infection progression or active HIV replication. HIV Gag antigen was observed to induce IL-10 production from CD4+ T cells but the IL-10 level was dramatically down regulated after HAART and these results are similar to those found in the present study where a statistically significant drop in IL-10 has been seen following HAART. A significant drop in the viral load as well as a statistically significant increase in secretion of IFN- γ following HAART found in this study, have also been documented by Sadeghi *et al* (21).

Paris *et al* (22) also found that a drop in the viral load was accompanied by a recovery of CD4 positive cells following HAART with a negative correlation between these two current HIV disease progression markers. A positive correlation that was found between IL-10 and viral load and CD4+ cells recovery that went on with a significant increase in IL-2 and IFN- γ (two Th1 cytokines) following HAART on the other hand, had been mentioned by Osakwe (23). Findings from this study have demonstrated the suppressive property of IL-10 on the synthesis of Th1 cytokines (IL-2 and IFN- γ) which was associated with high viraemia and a drop in CD4 count.

In conclusion, cytokines have a complex effect on the replication of HIV and conversely in infected individuals; HIV directly affects cytokine production. Different studies postulate that the progression of disease in HIV infected individuals may be controlled by the balance between the levels of type 1 (Th1) and type 2 cytokines (Th2). In this study, the down-regulatory effect of IL-10 on Th1 cytokines as well as a shift from Th1 to Th2 cytokines in the course of HIV disease progression have been demonstrated by a number of elements including: A strong positive correlation between viral load and IL-10; low CD4

count and low levels of the two Th1 cytokines which were associated with high levels of IL-10 and viremia; decrease in IL-10 and the viral load and a recovery of CD4 positive cells, increase in IL-2 and IFN- γ following HAART. This makes IL-10 a potential alternative marker of HIV disease progression.

ACKNOWLEDGMENT

This study was funded by the Centers for Disease Control and Prevention through the College of Medicine and Health Sciences of the University of Rwanda. The study also received technical support from the Rwanda National Reference Laboratory.

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