

RELATIONSHIP BETWEEN PLASMA CYTOKINE LEVELS AND CD4 T CELL COUNTS DURING ACUTE HIV INFECTION AMONG HIV/AIDS PATIENTS ATTENDING NAKURU PROVINCIAL GENERAL HOSPITAL, KENYA

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ABSTRACT

Cytokines play an important role in immunity, inflammation and hematopoiesis. They are rapidly produced by a variety of cell types and secreted in response to specific and non-specific stimuli. They play a central role in the pathogenesis of many diseases including Human Immunodeficiency Virus and Acquired Immunodeficiency Disease Syndrome (HIV and AIDS). HIV most often infects CD4 cells, which are important part of the immune system. The aim of this study was to determine the relationship between cytokines measurable in plasma and CD4 T cell counts during acute HIV infection. Eighty individuals were recruited for this study that comprised of forty newly diagnosed with HIV-1; twenty HIV negative individuals; and twenty HIV positive individuals currently on highly active antiretroviral therapy (HAART). Cytokines were measured using multiplex cytokine immunoassay while CD4 T cell counts were done by flowcytometry. Data analyses were performed using Graph Pad Prism 6. Spearman Rank tests were used to test for correlations. Statistical analysis were done using SPSS version 17. The study showed that CD4 T cell counts and all detected plasma cytokines were negatively correlated among the treatment naïve HIV patients.

Keywords: Plasma Cytokines, Human Immunodeficiency Virus, CD4 T cell counts.

INTRODUCTION

The human immunodeficiency virus has become one of the world's most serious health and development challenges (UNAIDS, 2013). Since the start of the epidemic, around 78 million people have become infected with HIV and 39 million people have died of AIDS-related illnesses. In 2013, there were 35 million people globally living with HIV of which 24.7 million were from sub-Saharan Africa (UNAIDS, 2014). The Human Immunodeficiency Virus (HIV) targets the immune system and weakens the surveillance and defense system of the body against infections, resulting in HIV infected individuals becoming more susceptible to a wide range of infections normally cleared by the immune system of a healthy individual (WHO, 2013).

The amount of human immunodeficiency virus (HIV) in the blood gets very high in the acute HIV infection stage yet some people who get infected don't notice anything and is easy to overlook the signs of acute HIV infection. Exposure to the blood of someone in the acute phase of infection is more likely to result in infection than exposure to someone with long-term infection. The risk of passing HIV infection through sexual activity is also much higher during the early stage of acute infection (AIDS infonet). Acute HIV infection is a critical phase of infection when irreparable damage to the immune system occurs (Gay, 2011).

Cytokines are small glycoproteins produced by a number of cell types predominantly leukocytes, the regulate immunity, inflammation and hematopoiesis. They regulate a number

of physiological and pathological functions including innate immunity, acquired immunity and a plethora of inflammatory responses (Khan, 2008). CD4+ T helper cells play a vital role in the immune system by secreting cytokines, which regulate the immune response. Cytokines are secreted by T cells when an intracellular infection is detected as in case of HIV infection. HIV is known to infect T cells that have CD4+ receptors present on their surface. These cells, among others in the immune system, secrete cytokines. Cytokines are crucial for fighting off infections and in other immune responses (Dinarello, 1999). However, they can become dysregulated and pathological in inflammation, trauma, and sepsis (Dinarello, 2000). The levels of cytokines present in the plasma become an indicator of the nature of the immune response.

METHODOLOGY

Study site and study population

The study was carried out in the Nakuru Provincial General Hospital (PGH), Kenya after the approval by the hospital's administration. Consent was sought from the study group by the researcher with the assistance and guidance of the staff of the Centre for Comprehensive Care (CCC) before commencement of the study. Only those who gave consent were enrolled in the study and they completed a written informed consent form in accordance with the Helsinki Declaration (59th WMA General Assembly, 2008).

Study Design and Sampling

This was a prospective cross sectional study that involved selecting consenting individuals who attended the Voluntary counseling and Testing centre (VCT) and the Centre for Comprehensive Care at the hospital. Eighty consenting male and female of different ages were sampled according to Yamane (1967) to participate in the study and were later subdivided into three groups as recommended by Sudman (1976). Forty patients that included sixteen males and twenty four females were sampled from those recently diagnosed with HIV before they started antiretroviral treatment (treatment naïve HIV patients); twenty patients that included four males and sixteen females were sampled from HIV positive patients that were receiving treatment with highly antiretroviral therapy (HAART) while twenty participants that included nine males and eleven females were sampled from HIV negative individuals.

Plasma Collection

Whole blood was collected into EDTA treated tubes, stored at room temperature and then centrifuged for 10 minutes at 3000 revolution per minute (r.p.m). Plasma was pipetted and aliquoted into cryovials that were labeled with appropriate information of the individuals in the study group. The plasma was refrigerated at -20°C until when the entire cohort had been collected in order to analyze all the samples simultaneously.

Plasma Cytokines Determination

All the eighty labeled plasma samples were thawed before flow cytometry for cytokine detection. The types and quantities of cytokines were detected by flow cytometry using a multiplex assay system that included Becton and Dickinson Cytometric Bead Array (BD CBA), Human Inflammatory Cytokine kit and Becton and Dickinson Fluorescence Activated

Cell Sorter (FACSCalibur) flow cytometer (BD Biosciences, U.S.A). The workflow consisted of the following steps according to the recommended procedure (BD Biosciences, U.S.A):

Preparing Human Inflammatory Cytokines Standards

A vial of lyophilized Human Inflammatory Cytokine Standard was opened and the standard spheres transferred to a 1 ml polypropylene tube. The tube was labeled "Top Standard" (Tube 10). The standards were reconstituted with 2 ml of assay diluent and allowed to equilibrate for 15 minutes at room temperature. The reconstituted protein was gently mixed by pipette. Eight 12x75 mm tubes were labeled and arranged in the following order: 1:2 (Tube 9; 2,500 pg/mL), 1:4 (Tube 8; 1,250 pg/mL), 1:8 (Tube 7; 625 pg/mL), 1:16 (Tube 6; 312.5 pg/mL), 1:32 (Tube 5; 156 pg/mL), 1:64 (Tube 4; 80 pg/mL), 1:128 (Tube 3; 40 pg/mL) and 1:256 (Tube 2; 20 pg/mL). 300 µl of assay diluent was pipetted in each of the tubes. Serial dilution was performed by transferring 300µl from the Top Standard to the 1:2 dilution tube and mixed thoroughly by pipette, and continued by transferring 300µl from 1:2 tube to the 1:4 tube until to the 1:256 tube. One tube was prepared containing only assay diluent to serve as the 0 pg/mL negative control. This was labeled as Tube 1 and had no standard dilution. The standard curve for each protein covers a defined set of concentrations from 20 to 5,000 pg/mL (BD Biosciences, U.S.A).

Mixing Human Inflammatory Cytokine Capture Beads

Six Capture Beads that had been bottled individually were pooled before using them in the assay. Each capture bead suspension was vortexed before mixing. 10 µl aliquot of each capture bead, for each assay tube to be analyzed, was added into a single tube labeled "Mixed Capture Beads". Eighty assay tubes were to be analyzed and so the mixture comprised of the following capture beads: 800 µl (10 x 80) of interleukin 8 (IL-8), 800 µl of interleukin 1β (IL-1β), 800 µl of interleukin 6 (IL-6), 800 µl of interleukin 10 (IL-10), 800 µl of tumor necrosis factor (TNF) and 800 µl of interleukin 12p-70 (IL-12p70). The mixture of capture beads was vortexed thoroughly and was ready for transfer to the assay tubes.

Performing the Human Inflammatory Cytokine Assay

After preparing the standards and mixing the capture beads, the next step was to perform the assay. The mixed capture beads were vortexed and 50 µl added to all assay tubes. 50 µl of the sample was added to eighty labeled tubes each containing about 12.5 µl of plasma that included forty from HIV positive patients, twenty from HIV negative and twenty from HIV positive patients on HAART. The assay tubes were incubated for 1.5 hours at room temperature. After the incubation period, 1 ml of wash buffer was added to each assay tube and centrifuge at 200g for 5 minutes; the supernatant was aspirated and discarded. 50 µl of the human inflammatory cytokine phycoerythrin (PE) detection reagent was added to all assay tubes and incubated for 1.5 hours at room temperature. After this second incubation period, again 1 ml of wash buffer was added to each assay tube and centrifuged at 200g for 5 minutes; the supernatant was aspirated and discarded from the assay tubes and finally 300 µl of wash buffer was added to each assay tube to resuspend the bead pellet.

Sample Acquisition and analysis of data

The samples were acquired on the flow cytometer. The assay setup procedure was followed according to the protocol by the manufacturers (BD Biosciences, U.S.A) and the appropriate

acquisition template was available. Each sample was vortexed for 3-5 seconds immediately before acquiring on the flow cytometer. Tube 1 (0 standard) was vortexed and ran in setup mode according to the protocol. Sample acquisition continued by running Tube 2 (20 pg/mL), followed by Tube 3 (40 pg/mL), and so on throughout Tube 10 (Top Standard). The test samples were run after the standards. Data for the detection of individual proteins was analyzed using BD Cell Quest software, according to the recommended protocol (BD Biosciences, U.S.A).

CD4 T cell Enumeration

All the 80 labeled blood samples were thawed before the flow cytometry for the enumeration of CD4 cells and refrigerated after. Enumeration of CD4 cells was performed as follows: TruCount tubes (BD TruCOUNT tubes) were labeled with identification number. 20uL of CD3/CD8/CD45/CD4 monoclonal antibody reagent (BD MultiTEST), for determining percentages and absolute counts of human helper/inducer and suppressor cytotoxic T lymphocytes in erythrocyte-lysed whole blood was put into the bottom of each appropriately labeled tube. 50uL of anticoagulated whole blood was put into the bottom of the tubes. The tubes were vortex gently to mix, and then incubated for 15 minutes in the dark at room temperature (20-25C). 450uL of FACS lysing solution was added to the tubes, vortexed gently to mix and incubated for 15 minutes in the dark at room temperature (20-25C). The samples were then processed and analyzed in the FACSCalibur flow cytometer using BD Cell Quest software. The results were reported as the percentage of positive cells per lymphocyte population and as the number of positive cells per microliter of blood (absolute count). The results were acquired through a computer printout.

RESULTS

In this section please present the results including tables, figures, numbers and graphs (if any). Font Size 12, Times New Roman, single spaced. All the subheadings in this section should be in font size 12 Bold, Times New Roman, single spaced. The first letter of each word in subheading should be capital. For tables please use font size 10. Tables/graphs or figures should be named as Table 1/ Figure 1/ Graph 1 and be given in center of the page.

Plasma Cytokine Profiles of the study population

The types and quantity of cytokines were determined in the study population. Five types of plasma cytokines: Interleukin 12p70 (IL-12p70), Tumor Necrosis Factor (TNF), Interleukin 10 (IL-10), Interleukin 6 (IL-6) and Interleukin 1 β (IL1 β) were detectable in the study population. IL-12p70, TNF, IL-10 and IL-6 were detected in treatment naïve HIV patients; in HIV positive patients on HAART and in HIV negative patients. IL-1 β was detected in treatment naïve HIV patients and in HIV positive patients on HAART but none was detectable in HIV negative patients (Table 1).

The mean concentrations of IL-12p70 (3.317 ± 4.441 pg/mL), TNF (7.707 ± 13.40 pg/mL), IL-10 (2.794 ± 4.437 pg/mL) and IL-6 (6.629 pg/mL) were higher in the treatment naïve HIV patients compared to the HIV positive patients on HAART (0.5593 ± 1.397 pg/mL, 1.975 ± 4.980 pg/mL, 0.6918 ± 1.628 pg/mL and 2.602 ± 4.620 pg/mL) and the HIV negative patients (0.2815 ± 0.5145 pg/mL, 0.7895 ± 2.247 pg/mL, 0.1630 ± 0.5032 pg/mL, 0.5135 ± 1.206 pg/mL) respectively. The mean concentration of IL-1 β (5.401 ± 10.14 pg/mL) was higher in

the treatment naïve HIV patients compared to HIV positive patients on HAART (0.7028 ± 4.445 pg/mL). There was no detectable IL-1 β in HIV negative patients (Table 1).

Table 1: Plasma Cytokine Profiles of the study population

| | Treatment naïve HIV patients (N=40; 50%) | HIV Positive patients on HAART (N=20; 25%) | HIV Negative patients (N=20; 25%) |
|--------------------|--|--|-----------------------------------|
| Types of Cytokines | Quantity (Mean \pm SD) | Quantity (Mean \pm SD) | Quantity (Mean \pm SD) |
| IL-12p70 pg/mL | 3.317 \pm 4.441 | 0.5593 \pm 1.397 | 0.2815 \pm 0.5145 |
| TNF pg/mL | 7.707 \pm 13.40 | 1.9750 \pm 4.980 | 0.7895 \pm 2.247 |
| IL-10 pg/mL | 2.794 \pm 4.437 | 0.6918 \pm 1.628 | 0.1630 \pm 0.5032 |
| IL-6 pg/mL | 6.629 \pm 8.621 | 2.6020 \pm 4.620 | 0.5135 \pm 1.206 |
| IL-1 β pg/mL | 5.401 \pm 10.14 | 0.7028 \pm 4.445 | 0.0000 |

CD4 T cell Profile of the study population

Absolute CD4 T cell counts of the study population were assessed and their means analyzed. The HIV negative patients had the highest CD4 T cell count mean (1063.45 ± 971.705) and the lowest CD4 T cell count mean (364.03 ± 284.064) was found in the treatment naïve HIV positive patients. The HIV positive patients on HAART had a CD4 T cell count mean of 485.85 ± 232.278 (Figure 1). The distribution of CD4 T cell counts in both males and females were also analyzed in the study population. Among the treatment naïve HIV positive patients, the mean CD4 T cell count (458.54) was higher in the females than in the males (223.67); the mean CD4 T cell count (494.75) was also higher in the females than in males (450.25) among the HIV positive patients on HAART and finally, among the HIV negative patients, the mean CD4 T cell count (1292.27) was also higher in females than in males (783.78; Figure 1).

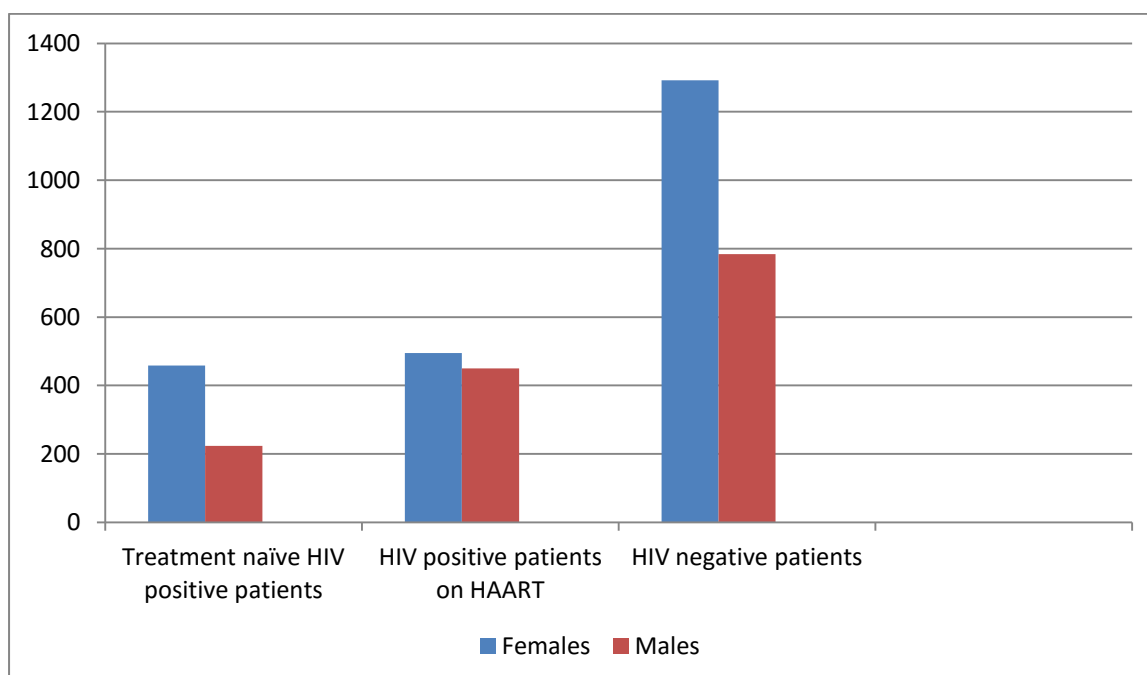


Figure 1: Means of the CD4 T cell counts of the study population

CD4 counts were grouped into three categories: <200 cells/mm³, 200-350 cells/mm³ and >350 cells/mm³ in order to assess the levels found in each group and gender. Among the treatment naïve HIV patients, 14 (9 males and 5 females) had CD4 T cell counts <200 cells/mm³; 5 (4 males and 1 female) had CD4 T cell counts 200-350 cells/mm³ and 21 (3 males and 18 females) had CD4 T cell counts >350 cells/mm³. Among the HIV positive patients on HAART, 3 (2 males and 1 female) had CD4 T cell counts <200 cells/mm³; 2 (females) had CD4 T cell counts 200-350 cells/mm³ and 15 (2 males and 13 females) had CD4 T cell counts >350 cells/mm³. All HIV negative patients (9 males and 11 females) had CD4 T cell counts >350 cells/mm³ (Table 2).

Table 2: CD4 T cell Profile of the study population

| CD4 counts (Normal range 410-1590 Absolute counts) | Treatment naïve HIV positive patients (N=40) | | HIV positive patients on HAART (N=20) | | HIV negative patients (N=20) | |
|---|--|-------------------------------|---------------------------------------|-------------------------------|------------------------------|--------------------------------|
| | Mean ± SD | 364.03±284.064 | Mean ± SD | 485.85±232.278 | Mean ± SD | 1063.45±971.705 |
| | Male (N=16) Mean =223.67 | Female (N=24) Mean =458.54 | Male (N=4) Mean =450.25 | Female (N=16) Mean =494.75 | Male (N=9) Mean =783.78 | Female (N=11) Mean =1292.27 |
| <200 | 9 (22.5%) | 5 (12.5%) | 2 (10.0%) | 1 (5.0%) | 0 (0.0%) | 0 (0.0%) |
| 200-350 | 4 (10.0%) | 1 (2.5%) | 0 (0.0%) | 2 (10.0%) | 0 (0.0%) | 0 (0.0%) |
| >350 | 3 (7.5%) | 18 (45.0%) | 2 (10.0%) | 13 (65.0%) | 9 (45.0%) | 11 (55.0%) |

(Normal absolute CD4 counts are according to the Nakuru Provincial General Hospital Laboratory)

Relationship between Plasma Cytokine Profiles and CD4 T cell Counts in the study population

The relationship between cytokine profiles and CD4 T cell counts was analyzed (Pearson correlation -2-tailed). CD4 T cell counts and all cytokines were negatively correlated among the treatment naïve HIV patients. CD4 T cell counts and IL-12p70 were negatively correlated ($r = -0.149$); CD4 T cell counts and TNF were negatively correlated ($r = -0.051$); CD4 T cell counts and IL-10 were negatively correlated ($r = -0.082$); CD4 T cell counts and IL-6 were negatively correlated ($r = -0.129$) and CD4 T cell counts and IL-1 β were also negatively correlated ($r = -0.058$; Table 3).

The relationship between cytokine profiles and CD4 T cell counts was also analyzed among the HIV negative patients and were found to be positively correlated. The correlation of CD4 T cell counts and IL-12p70 was $r = -0.273$; CD4 T cell counts and TNF was $r = 0.102$; CD4 T cell counts and IL-10 was $r = 0.123$ and the correlation of CD4 T cell counts and IL-6 was $r = -0.033$. There was no detectable IL-1 β in HIV negative patients (Table 3).

Finally, the relationship between cytokine profiles and CD4 T cell counts among the HIV positive patients on HAART was also analyzed. The correlation of CD4 T cell counts and IL-12p70 was $r = 0.072$; CD4 T cell counts and TNF was $r = 0.066$; CD4 T cell counts and IL-

10 was $r=-0.119$; CD4 T cell counts and IL-6 was $r=-0.143$ and the relationship between CD4 T cell counts and IL1 β was $r=0.022$ (Table 3).

Table 3: Relationship between Plasma Cytokine profiles and CD4 T cell Counts of the study population

| Variable (Mean) | Treatment naïve HIV patients | Significance (r) | HIV Negative patients | Significance (r) | HIV positive patients on HAART | Significance (r) |
|-----------------|------------------------------|------------------|-----------------------|------------------|--------------------------------|------------------|
| CD4 counts | 364.600 | | 1063.45 | | 485.85 | |
| IL-12p70 | 3.31650 | -0.149 | 0.28150 | 0.273 | 0.55925 | 0.072 |
| TNF | 7.70700 | -0.051 | 0.78950 | 0.102 | 1.9750 | 0.066 |
| IL-10 | 2.79400 | -0.082 | 0.16300 | 0.123 | 0.69175 | -0.119 |
| IL-6 | 6.62850 | -0.129 | 0.38500 | 0.033 | 2.6020 | -0.143 |
| IL-1 β | 5.40050 | -0.058 | 0.00000 | - | 0.7028 | 0.022 |

DISCUSSION

The Acquired immunodeficiency syndrome (AIDS) is defined in terms of either a CD4⁺ T cell count below 200 cells per μL or the occurrence of specific diseases in association with an HIV infection (Chu and Selwyn, 2011). CD4 T cell count of 500-1000 cells/ mm^3 is considered a normal range for healthy individuals, while a CD4 T cell count of 200 cells/ mm^3 is diagnostic for AIDS (Ray *et al.*, 2006). In this study, 35% of the treatment naïve HIV positive patients had CD4 T cell counts <200 cells/ mm^3 , 12.5% had CD4 T cell counts 200-350 cells/ mm^3 and 52.5% had CD4 T cell counts >350 cells/ mm^3 , compared with HIV patients on HAART: <200 cells/ mm^3 =15%, 200-350 cells/ mm^3 =10% and >350 cells/ mm^3 =75% and also compared with HIV negative patients where all had CD4 T cell counts >350 cells/ mm^3 . The mean CD4 T cell count was significantly lower ($p=0.01$) in the treatment naïve HIV patients compared with HIV negative patients. Majority of the treatment naïve HIV positive patients had CD4 T cell counts below the normal range of 410-1590 cells/ mm^3 (Table 2), an observation similar to a study by Tudela *et al.*, (2014).

When comparing the relationship between the cytokine levels with CD4 T cell counts in the study population, all detectable cytokines in the treatment naïve HIV patients were found to be negatively correlated when compared with the HIV negative patients, with TNF, IL-10, IL and IL-1 β being highly negatively correlated ($r=-0.051$, -0.082 and -0.058 respectively (Table 3). This observation is in agreement with a study by Borge *et al.*, (2014). When comparing cytokines and CD4 T cell counts among the HIV patients on HAART, IL-12p70 and TNF and IL-1 β were found to have a positive correlation while IL10 and IL-6 were found to have a negative correlation (Table 3). This finding agrees with Bastard *et al.*, (2012), who found a negative correlation of IL-6 with CD4 T cell counts, although it disagrees with Stylianou *et al.*, (1999) who found IL-10 had a positive correlation in HAART patients. Given the nature of CD4 T cell counts as an important biomarker for HIV progression, this findings showed CD4 T cell counts were significantly lower in treatment naïve HIV patients versus HIV negative patients. This may contribute to the altered cytokine profile of the participants.

CONCLUSIONS

Cytokine profiling and CD4 T cell counts enumeration are useful biomarkers for HIV disease progress during the acute phase.

ACKNOWLEDGEMENTS

I am grateful to the administration of the Nakuru Provincial General Hospital (PGH), Kenya; to the staff of the Voluntary Counseling and Testing Centre and of the Centre for Comprehensive Care of the hospital. I am indebted to the staff of the PGH Virology Laboratory for their support and technical assistance. I acknowledge the technical assistance of BD Biosciences (Nairobi, Kenya) and am grateful for the scholarly and technical advices of Professor Michael Gicheru and Dr. Joseph Mwatha.

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