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A study of T/NK cell distribution in HIV positive adults by flow cytometry

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Abstract
Background: HIV attacks a specific type of immune system cell such as CD4 helper cell or T cell in the body. Also, natural killer (NK) cells play a key role in immune response against HIV infection. The present study was undertaken to evaluate the variation of T/NK cells in HIV positive adults by flow cytometry and its correlation to disease status.
Method: A total of 100 HIV positive cases >18 years of age diagnosed as per National AIDS Control Organization (NACO) guidelines were included. The data was collected from patients regarding demographic profile, clinical spectrum and T/NK cells parameters.
Results: WHO staging of patients showed that maximum patients belong to Stage III (33%) followed by Stage II (29%), Stage I (27%) and Stage IV (11%). Majority of patients had lower CD4 count <350 (39%). Mean CD4 and CD8 count was 423.81±320.17cells/µl and 1090.58±723.03cells/µl respectively. The mean CD4/CD8 ratio was 0.56±0.41. Mean NK cells were 234.23±121.67 cells/µl. The association of HIV staging and CD4 count showed that patients with CD4 count <200 were more in Stage III, which was statistically significant, (P<0.05). Patients in Stag IV had lower NK cells (136.76±98.21) compared to Stage I (523.12±231.76) showed statistically significant difference, (P<0.05). CD4 cells showed statistically significant positive relation with NK cells, hemoglobin, TLC, neutrophils, lymphocyte, platelets, MCV and MCHC, (P<0.05).
Conclusion: The findings of present study, reveal that activation levels of NK cells are elevated and coordinated in HIV infections. Additionally, elevated NK cells are associated with reduced CD4+ T cell percentages and higher viral loads, in a HIV infection.

Keywords: HIV, natural killer, CD4 or T cell, flow cytometry, hemoglobin, neutrophils, lymphocyte

Introduction
World Health Organization (WHO) indicates that almost 33.2 million people are living with HIV infection worldwide [1]. The natural history of infection with HIV is characterized by the progressive loss of immunological function. The mechanism responsible for this immune dysregulation is unknown, but immune activation is positively associated with viral load and negatively associated with CD4 count [2]. However, in the majority of cases, the development of AIDS parallels the decline in CD4+ T cells, which play a vital role in the regulation of the immune response. HIV specifically targets and binds to the CD4 antigen and chemokine receptor 5 (CCR5) or CXC chemokine receptor 4 (CXCR4) on CD4+ T cells to replicate, ultimately causing the destruction and deterioration of the immune system. The monitoring of CD4+ T cells during the course of HIV infection is therefore a crucial component in the monitoring of disease progression and the response to ARV therapy [3, 4].

CD8+ (cytotoxic) T cells and CD4+ Helper T cells, are generated in the thymus and express the T cell receptor. HIV infection is characterized by CD4 T cell depletion, CD8 T cell expansion, and chronic immune activation that leads to immune dysfunction. The dynamics of CD4 and CD8 T cells are altered in many ways during HIV infection. Although both show evidence of increased proliferation and preferential loss of the naïve subset, there is depletion of CD4 T cells and expansion of CD8 T cells. CD4+/CD8+ T cell ratio serves as a more reliable indicator of the immune status of the patient in comparison to the sole CD4+ T cell count. CD4+/CD8+ T cell ratio, which is affected by the variation of both T cell subsets. Despite the controlling of the viral load, the ratio might still remain at low levels which is representative of immune activations. In such a setting, a higher CD4+/CD8+ T cell ratio reflects a more normalized immune response of the individual [5, 6].

Natural killer (NK) cells are a subset of lymphoid cells that function as important mediators of the innate immune defence against viruses and tumour cells. They constitute 15% of peripheral-blood lymphocytes and are also found in the liver, peritoneum, and placenta. NK cells can secrete T-helper 1 (Th1)-associated pro-inflammatory cytokines like interferon-gamma (IFNg) and tumor necrosis factor-alpha (TNFa). NK cell mediated antibody-dependent cellular cytotoxicity (ADCC) has been associated with protection from infection and disease progression and is an important mechanism to control HIV infection [7]. However human NK cells are commonly defined to two subsets, CD56 dim CD16 + and CD56 bright CD16−subpopulations. Chronic HIV infection is associated with reduced proportion and absolute number of CD3−CD56+ NK cells compared to healthy controls, as well as increased proportion of CD56dimCD16+ NK cell subset [8].

Despite tremendous advances in the diagnosis and management of HIV infection in the present era of basic and clinical infectious diseases research, the constantly high mortality rate of the patients remains a challenge for scientists. With the revolutionary introduction of flow cytometry as a clinical diagnostic technique, a new light was shed on the way of clinical practice not only for the diagnosis and monitoring of the infection, but also to set up more effective therapeutic protocols based on the immunophenotyping of the immune cells [9]. Multi-parameter flow cytometry is a powerful tool for rapid, accurate analysis of large number of blood cells in a short time. Moreover, the abilities to study multiple parameters simultaneously in a quantitative manner, in a thousand of cells give a distinct advantage makes this technique suitable to analyse T/NK in HIV positive individuals [10]. Therefore, the present study was conducted to evaluate the variation of T/ NK cells in HIV positive adults by flow cytometry and its correlation to disease status in tertiary care hospital.

Materials and Methods

The present cross-sectional study was conducted in total 100 HIV positive cases above 18 years of age diagnosed as per National AIDS Control Organization (NACO) guidelines in the Department of Pathology at tertiary care hospital from December 2018 to October 2020. Patients less than 18 years of age, patients currently suffering from any opportunistic infection, malignancy like Kaposi sarcoma and lymphoma, pregnant and lactating women and individuals unwilling to give consent were excluded from the study. The study was approved by the Institutional Ethical Committee and written informed consent was taken from the patients.

The selected subjects were visited, and questionnaire was administered which consisted of two parts. The first part included socio-demographic details such as age, sex, occupation, marital status, religion, education, socio-economic status (according to the standard of living index and BG Prasad's classification), and per capita income. The second part consisted of clinical examination, hematological and serological findings with outcome. The questionnaire was validated by translation into the local language and reviewed by a group of experts.

Complete history and other details of the patients were collected from ART Centre. Hematological parameters (complete hemogram-hemoglobin estimation, total RBC count, RBC indices, packed cell volume, total leucocyte count, total lymphocyte count, Differential leucocyte count, platelet count, CD4, CD8 and NK cells) were done by collecting 5 ml of venous blood sample from antecubital vein in EDTA prefilled tubes/bulbs. The analysis was done by the automated Hematology analyzer Beckman coulter: 5-part Analyzer. Erythrocyte sedimentation rate was measured by Westergren's method. Peripheral smears were studied after staining with Leishman's stain. For serology a venous blood sample was collected from patients and transported to the laboratory immediately. The samples were coded according to standard operating procedure to eliminate the bias in the study. CD4 T cell, CD8 and NK cell were studied in laboratory by flow cytometry. The data of all cases was collected in a specially designed proforma and later transformed to master chart and subjected to analysis.

Statistical Analysis

All data analysis had been done by using SPSS (version 22) for windows. The initial measures of each group were compared with the final measures of the study period and compared between the groups by using student t test, chi square test and Pearson coefficient. P value <0.05 was considered statistically significant.

Observations and Results

A total of 100 HIV positive cases above 18 years of age diagnosed as per NACO guidelines were enrolled in the study, of them maximum were in the age group of 41-50 years (28%) followed by 51-60 years (23%) with male predominance (64%). The mean age of patients was 46.28±10.28 years. Most of the patients had primary schooling (38%) and majority of patients belongs to Class IV (37%) SES as shown in table 1.

Table 1: Socio-demographic profile of the patients

Socio-demographic details		Frequency	Percentage
Age groups (In years)	<20	03	3.0
	21-30	11	11.0
	31-40	14	14.0
	41-50	28	28.0
	51-60	23	23.0
	>60	21	21.0
Gender	Male	64	64.0
	Female	36	36.0
Education	Illiterate	11	11.0
	Primary	38	38.0
	Secondary	29	29.0
	Higher secondary	13	13.0
	Graduate & above	09	09.0
Socioeconomic status (SES)	I	09	9.0
	II	11	11.0
	III	21	21.0
	IV	37	37.0
	V	22	22.0

The majority of patients had fever (58%) followed by loss of appetite (54%), cough (51%) and pallor (41%) while 36% patients were asymptomatic as depicted in figure 1.

Distribution of patients according to WHO staging showed that most of the patients belongs to stage III (33%) followed by stage II (29%), stage I (37%) and stage IV (11%).

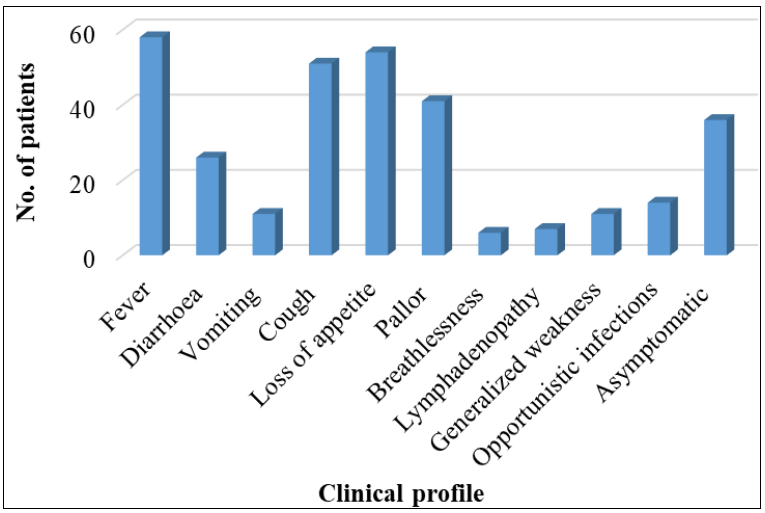

Fig 1: Clinical profile distribution among patients

Most of the patients had CD4 count 201-350 (39%) followed by CD4 count 51-200 (33%), 16% had CD4 count ≤50 and 12% had CD4 count >350. The mean CD4 and CD8 count was 423.81±320.17 cells/µl and 1090.58±723.03 cells/µl respectively. The mean CD4/CD8 ratio was 0.56±0.41 and the mean NK cells were 234.23±121.67cells/µl.

Patients with CD4 count <200 were more in Stag III and showed statistically significant difference, (P<0.05). Patients with age >40 years had greater CD4 count compared to age <40 years but showed no statistical difference, (P>0.05). Similarly, gender, socioeconomic status and education showed no statistically significant difference with respect to CD4 count, (P>0.05) as shown in table 2.

Table 2: Association of demographic profile and HIV staging with CD4 count

HIV staging and Demographic profile		CD4 count		P value
		<200 (N=49)	≥200 (N=51)	
HIV staging	Stage I	07	20	<0.05
	Stage II	14	15	
	Stage III	19	14	
	Stage IV	09	02	
Age in years	<40	18	11	>0.05
	≥40	31	40	
Gender	Male	34	30	>0.05
	Female	15	21	
SES	I, II & III	18	23	>0.05
	IV & V	31	28	
Education	Illiterate	07	04	>0.05
	Literate	42	47	

From the figure 2, it was observed that patients in Stag IV had lower NK cells (136.76 ±98.21) as compared to Stage I (523.12 ±231.76) which showed statistically significant difference, (P<0.05).

CD4 cells showed statistically significant positive relation with CD8 cells, CD4:CD8 ratio and NK cells with p value of <0.001.

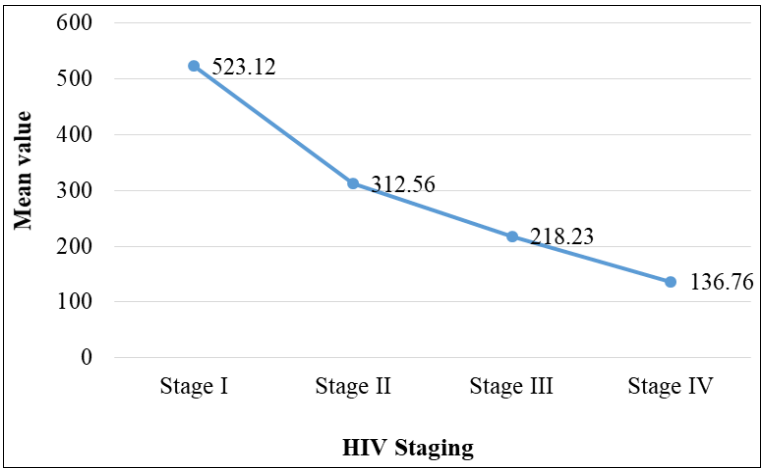

Fig 2: Association of HIV staging and NK Cells

Table 3 shows the mean hematological parameters distribution among patients.

Table 3: Hematological parameters distribution among patients

Hematological parameters	Mean ± SD
Hemoglobin (g/dl)	12.69 ± 2.43
TRBC	4.52 ± 4.17
HCT (%)	37.19 ± 6.30
MCV (fl)	91.13 ± 12.27
MCH (pg)	31.03 ± 5.39
MCHC (g/dl)	33.98 ± 1.78
RDW (%)	15.41 ± 2.92
TLC (per µl)	6871.75 ± 2221.22
Neutrophil (%)	54.88 ± 12.97
Lymphocyte (%)	32.82 ± 10.68
Monocyte (%)	8.35 ± 3.13
Eosinophils (%)	3.67 ± 3.49
Platelets ($X10^5$ µl)	2.49 ± 0.91
MPV (%)	9.05 ± 1.72
Erythropoietin level (IU/L)	22.26 ± 12.92

From the table 4, it was observed that the CD4 cells showed positive correlation with hemoglobin, TLC, neutrophils, lymphocyte, platelets, MCV and MCHC with statistically significant difference, (P<0.001).

Table 4: Association of hematological parameters and CD4 count

Hematological parameters		CD4 count		P value
		<200 (N=49)	≥200 (N=51)	
Hemoglobin (g/dl)	Normal	14	24	<0.001
	Decreased	35	27	
TLC (X 10^3 /µl))	Normal	19	40	<0.001
	Decreased	30	11	
Neutrophil count (%)	Normal	16	28	<0.001
	Decreased	33	23	
Lymphocyte (%)	Normal	11	20	<0.001
	Decreased	38	31	
Platelets (X 10^5 /µl))	Normal	17	42	<0.001
	Decreased	32	09	

Discussion

In the present study, maximum patients were in the age group 41-50 years (28%), followed by 51-60 years (23%). The mean age of the patients was 46.28 ±10.28 years. This is correlated with the study conducted by Kumawat S *et al.* [11] and Andgi AS *et al.* [12]. As per the recently released India HIV Estimation 2019 NACO report, overall, the estimated adult (15–49 years) HIV prevalence trend has been declining in India since the epidemic's peak in the year 2000 and has been stabilizing in recent years [13]. The estimate for this indicator was 0.22% (0.17–0.29%) in 2019. In the same year, HIV prevalence among adult males (15–49 years) was estimated at 0.24% (0.18–0.32%) and among adult females at 0.20% (0.15–0.26%). The national statistics of the HIV population showed that 59.5% of infected HIV population were composed of male population [4]. In current study also, 64% of patients were male, and 36% were females which is comparable with the previous studies [11, 12, 14, 15], since study was conducted in army hospital which comprises of predominately male population. 89% of patients were literate and 11% of patients were illiterate. This finding is similar to study conducted by Kumawat S *et al.* [11] while different from other studies [14, 16] as most patients in their studies were illiterate. Thus, it may be inferred that higher educational levels offered some protection against HIV through preventive measures for HIV. Anyone who is illiterate and educated below the secondary education level may not have adequate knowledge to protect themselves from STDs, including HIV/AIDS. Most of the patients belongs to Class IV (37%) SES, followed by Class V (22%), Class III (21%), Class II (11%), Class I (09%). Almost similar findings are noted by Kumawat S, *et al.*, [11] and Joge US *et al.* [14].

Maximum patients had fever (58%) followed by loss of appetite (54%), cough (51%), pallor (41%) and diarrhea (26%) which is correlated with the previous studies [14, 17]. The distribution of patients according to WHO staging of HIV Infection showed that most of the patients presented with Stage III (33%), followed by Stage II (29%), Stage I (37%) and Stage IV (11%) which is similar to the study conducted by Kumawat *et al.* [11] and Nayak *et al.* [15]. Majority of patients had lower CD4 count <350 (39%). The mean CD4, CD8 count, mean CD4/CD8 ratio and mean NK cell count was 423.81±320.17 cells/µl, 1090.58±723.03 cells/µl, 0.56 ±0.41 and 234.23±121.67cells/µl respectively. This finding is correlated with the study done by Antwal *et al.* [17]. The HIV staging, and CD4 cell count association showed that patients with CD4 cell count <200 were more in Stag III and showed statistically significant difference, (P<0.05). Kolber MA *et al.* [18] and Kaufmann G *et al.* [19] study observed a negative correlation between changes in CD4 and CD8 cell counts during the intensification of antiretroviral therapy. Margolick JB *et al.* study reported that CD8 counts increase whereas CD4 counts decline, however only viral load and CD4 cell count were considered the most relevant predictors for disease progression [20].

In the present study it was observed that patients in Stage IV had lower NK cells (136.76±98.21) compared to Stage I (523.12±231.76) showed statistically significant difference, (P<0.05). Jiang Y *et al.* [21] and Bächle SM *et al.* [22] observed that the percentage of NK cells in the HIV infection group was much lower than that of normal controls, (P<0.05). These findings may indicate that HIV infection leads to the impairment of CD4 T cells and causes NK cell counts to decline subsequently. The function of NK cells is determined by the integration of signals from several activating and inhibitory receptors. These receptors allow NK cells to recognize virally infected cells that have down regulated the expression of HLA class I molecules and up regulated stress molecules on their surface that serve as ligands for activating NK cell receptors. Furthermore, NK cells play an essential role in modulating the adaptive immune response to infection through their interaction with dendritic cells and the secretion of immunoregulatory cytokines. Recent studies have suggested that both the antiviral activity of NK cells and their immunoregulatory function can be impaired in HIV-1 infection [16, 23].

In the current study, CD4 cells showed a statistically significant positive relation with CD8 cells, (P<0.001), CD4:CD8 ratio (P<0.001) and positive relation with NK cells, (P<0.001) which is comparable with the study conducted by Jiang Y *et al.* [21]. It was observed that patients with higher haemoglobin had greater CD4 count and showed a statistically significant difference, (P<0.001) Similarly, TLC, Neutrophil count, lymphocyte count and platelets showed a statistically significant difference concerning CD4 count, (P<0.05). These findings are correlated with the previous studies [24, 25]. Anaemia in HIV patients is associated with increased morbidity and mortality. Haemoglobin concentration in HIV patients depends on several factors such as comorbidities, use of ARVs etc. Present study shows that CD4 count is an

essential determinant of haemoglobin concentration and low haemoglobin counts portend a bad prognosis in HIV patients as they are positively correlated with patients' immune status [25].

Conclusion

The present study found that the majority of patients had lower CD4 count <350 and patients with lower CD4 count had greater stage of HIV with statistically significant difference. CD4 cells showed statistically significant positive relation with NK cells. The CD4/CD8 ratio can be interpreted as a measure for the imbalance of the patient's immune system which captures essential information, additional to the cell count levels of both lymphocyte subtypes. The CD8 cell count is a marker for immune activation, an essential factor for disease progression. The findings reveal that activation levels of NK cells are elevated and coordinated in HIV infections. Additionally, elevated NK cells are associated with reduced CD4+ T-cell percentages and higher viral loads, in a HIV infection.

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