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Original Research Article

Comparison of CD4 and CD8 counts and ratio in HIV negative pulmonary tuberculosis patients with normal healthy controls

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ABSTRACT

Background: There is an equivocal contention that Tuberculosis may be a cause of non-HIV-associated CD4+ T cell lymphopenia. In HIV negative patients, CD4+ and CD8+ T cell count suppression has been associated with TB infection. Prediction of HIV coinfection in newly diagnosed pulmonary TB patients with negative HIV status by estimation of CD4, CD8 count and CD4:CD8 ratio.
Methods: Newly diagnosed pulmonary TB patients comprising of 30 numbers with negative HIV status were subjected for estimation of CD4, CD8 counts and ratio for prediction of HIV coinfection. Equal number of healthy controls was also included in the study for comparison of the values.
Results: Significantly lower CD4 and CD8 counts among pulmonary TB infected HIV negative patients as compared with healthy controls was found. The CD4:CD8 ratio was normal when compared with healthy controls.
Conclusions: The present study highlights the importance of estimation of CD4+ and CD8+ T cell counts and ratio in newly diagnosed pulmonary TB patients with negative HIV status. Prediction ability in combination with early detection and appropriate management play major role in evading emergence of drug resistance among the HIV-TB coinfected patients.

Keywords: CD4 counts, CD8 counts, Coinfection, HIV, Pulmonary TB

INTRODUCTION

Human immunodeficiency virus infection has become a pandemic far more extensive than what was predicted even a decade ago. The global spread has been so swift that no country has been spared and the pace of the epidemic is increasing in India.[1] Tuberculosis remains the most common opportunistic infection and is the commonest cause of death in HIV infected patients. Clinical presentation of TB in early HIV infection resembles to that observed in immune-competent persons but in later stage, the clinical presentation of TB can be atypical.[2] The hallmark of HIV infection is a chronic illness characterized by a progressive decline in cell mediated immune function i.e., progressive deficiency of T lymphocytes referred to as helper or inducer T-cells. Infection with HIV leads to protracted disease and depletion of CD4+ T cells in most cases resulting in AIDS.[3] Adequate cell-mediated immunity is the crucial host defence against *Mycobacterium. tuberculosis*. The

primary target for *M. tuberculosis*, the alveolar macrophage, can also be infected with HIV and they exacerbate HIV replication in macrophages. Immune responses in TB induce cytokines that enhance the replication of the HIV and thus drive the patient into the full picture of AIDS. There is evidence that TNF-α and other immunological mediators released in TB lead to transactivation of the HIV provirus and its subsequent replication. In addition, TB causes a CD4+ T-cell lymphopenia, which may synergize with that induced by the HIV. Whatever the cause, the occurrence of active TB in the HIV-positive patient has very serious consequences, worsening the prognosis of HIV infection.[4]

The interaction between HIV and TB in persons coinfected with them is bidirectional and synergistic. The course of HIV infection is accelerated subsequent to development of TB and the inverse relationship between HIV viremia and CD4+T cell count gets shifted to the right. Accelerated HIV progression is partly attributable to the increased systemic immune activation in patients with HIV-TB. TB is associated with increased risk of progression to AIDS, overlapping toxicities, drug interactions and complications. HIV has 100-fold risks for development of all forms of TB with a yearly risk of 7-10% versus lifetime 5-15% in HIV negative patients. There is also increased risk of death regardless of CD4+ T cell count. They are difficult to diagnose because of smear negative, extra pulmonary TB and disseminated forms. CD4+ T-cell lymphopenia is a pivotal immune aberration in HIV patients and important changes in CD8+ T cell counts also occur in this disease.

The depletion of CD4+ T cells, which is a main feature of AIDS, is certainly an important contributor to the increased risk of reactivation of latent TB and susceptibility to new *M. tuberculosis* infection. There is also some evidence that CD8+ T cells play a role in the control of latent TB. HIV specific CD8+ T lymphocytes play a key role in the initial reduction of viremia during acute infection, but become increasingly dysfunctional and exhausted under conditions of chronic antigen persistence. CD8+ T cells have been implicated in the control of chronic HIV replication as suggested by studies on simian immunodeficiency virus (SIV) viremia in non-human primates.[5] CD8+ T cells also play a part in protective immunity against mycobacteria and are seen to accumulate at the site of mycobacterial infections, forming a cuff at the periphery of epithelioid cell granulomas. Despite the pivotal importance of CD4+ and CD8+ T lymphocytes in mycobacterial immunity, very few researchers have studied changes in peripheral blood counts of these cells in the setting of TB.

There is an equivocal contention that TB may be a cause of non-HIV-associated CD4+ T cell lymphopenia, with some available studies.[6] CD4+ T cell lymphopenia is a well-defined risk factor for the development of active TB in patients infected with HIV. In HIV negative patients, CD4+ and CD8+ T cell count suppression has been associated with TB infection.[7] Enumeration of CD4+ has a great role in initiating and monitoring of HIV treatment. However, it has been documented that there is significant role of CD4: CD8 ratio in predicting HIV co-infection in newly diagnosed TB.[8]

It has been suggested that TB patients have a microenvironment that facilitates HIV infection by i) increasing the expression of co-receptors CXCR4 and CCR5 regulated by *M. tuberculosis* products; ii) increasing pro-inflammatory cytokines, especially TNF; and iii) down-regulation of CCL5. It has also been shown that *M. tuberculosis* and its cell wall component, lipoarabinomannan (LAM) may activate replication of HIV in provirus-carrying cells by inducing TNF and IL-6 production through the NF-kB pathway, which in turn triggers transcriptional activation of the long terminal repeat (LTR) promoter and supports replication of HIV.[5]

The aim of the present study was to predict HIV coinfection in newly diagnosed pulmonary TB patients with negative HIV status by flow cytometrical estimation of CD4, CD8 count and CD4:CD8 ratio. This is the first study from North East India to look at T-lymphocyte subsets in HIV-negative patients with pulmonary TB since the advent of flow cytometry.

METHODS

The present study was conducted in the Department of microbiology, in a Tertiary Care Centre in North East India over a period of one year from January 2013 to December 2013. Ethical clearance was duly obtained from the Institute Ethical Committee for conducting the study. Samples were collected from patients attending DOTs clinic of the hospital referred from various OPD and Indoors. Clinical samples including sputum and blood were taken from the patients following the guidelines of RNTCP India after obtaining due informed consent.

Attending DOTs clinic: Patients presenting with sign and symptoms of TB were subjected to sputum smear microscopy employing fluorescent microscope at DOTs clinic. Any patient having high risk behaviour, other sexually-transmitted infections, or opportunistic conditions suggestive of HIV infection were referred to ICTC for further evaluation.

The newly diagnosed TB patients who were referred to ICTC for determination of HIV status and if negative were subjected for estimation of CD4, CD8 counts and CD4:CD8 ratio for prediction of HIV coinfection. Equal number of healthy controls with known TB and HIV negative status was also included in the study for comparison of the values.

Patients attending DOTS clinic: Two numbers of sputum sample were collected in sterile leak-proof, disposable,

appropriately labeled containers without any fixatives as per RNTCP guidelines.[9] The collected specimens were subjected for demonstration of Acid Fast Bacilli using fluorescent microscopy employing Auramine O stain and grading was done accordingly.

Patients attending ICTC: 2-3ml blood collected aseptically in a clean sterile tube for HIV testing after counselling and obtaining due informed consent. The collected sample was tested for HIV antibody (HIV1/HIV2) using rapid diagnostic test kits following the NACO, India guidelines. The kits used were COMB AIDS, PAREEKSHAK TRILINE, SD BIOLINE HIV- ½.

Patients with newly diagnosed pulmonary TB with negative HIV status: Sample: 3ml blood sample collected in a clean sterile EDTA vial was subjected for estimation for CD4 and CD8 counts and ratio using Flow cytometer, Cytomics FC 500MPL, manufactured by Beckman Coulter and procured by the institute. Fresh sample were collected from thirty patients with newly diagnosed Pulmonary TB with negative HIV status. Equal number of age and sex matched healthy controls with known HIV and TB negative status were included in the study. The sample collected in EDTA vial was used within half an hour; else refrigerated the sample at 4°C and used it within 24 hours.

Principle: Flow cytometry is a laser-based, biophysical technology employed in cell counting, cell sorting, biomarker detection and protein engineering, by suspending cells in a stream of fluid and passing them by an electronic detection apparatus. It allows simultaneous multiparametric analysis of the physical and chemical characteristics of up to thousands of particles per second. A beam of laser light of a single wavelength is directed onto a hydrodynamically focused stream of liquid.

A number of detectors are aimed at the point where the stream passes through the light beam: one in line with the light beam (Forward Scatter or FSC) and several perpendicular to it (Side Scatter or SSC) and one or more fluorescence detectors. Each suspended particle from 0.2 to 150 micrometers passing through the beam scatters the ray, and fluorescent chemicals found in the particle or attached to the particle may be excited into emitting light at a longer wavelength than the light source.

This combination of scattered and fluorescent light is picked up by the detectors, and, by Analysing fluctuations in brightness at each detector (one for each fluorescent emission peak), it is then possible to derive various types of information about the physical and chemical structure of each individual particle. FSC correlates with the cell volume and SSC depends on the inner complexity of the particle. This is because the light is scattered off of the internal components of the cell.[10]

Procedure: Anticoagulated 100µl of blood as sample and 100µl of ImmunoTrol as control was mixed with 10 µl TetraCHROME antibodies (CD45-FITC/CD4-PE/CD8-ECD/CD3-PC5) and vortexed for 5 sec. Then incubated at room temperature for 20 min in dark. Thereafter 1ml of Versalyse (Lysate Reagent) was added and vortexed for 5sec and incubate at room temperature for 10 min in dark. Then 1ml of Phosphate Buffered Saline (ISOFLO) and 100µl of flowcheck Fluorospheres were added and vortexed for 5 sec. Acquisition performed on Cytomics FC 500.MXP Software on list mode data utilized to determine the lymphocyte gate.FL1/FL2 contour plots finally employed for four colour analysis

The CD4+ and CD8+ T cell count of the respective samples were recorded covering both subjects and control included in the study. The ratio of CD4 and CD8 also estimated and recorded accordingly.

Normal range

The CD4+ T cell count of 400-1600 cells/µl, CD8+ T cell count of 200-900 cells/µl and ratio of CD4+:CD8+: (1.2 to 2):1 were regarded to be normal range as per manufacturer's instructions.

Statistical interpretation

Mean and Standard Deviations (SD) were calculated for all the results obtained in the study. Degree of Freedom (DF) and significance of statistical association were calculated from standard probability (p-value) chart. Among all the software available, the results generated in present study were statistically analyzed using MedCalc for Windows, Version 12.5 (MedCalc software, Ostend, Belgium) and the significance of p value was determined if it's less than 0.05.

RESULTS

A total of 2651 patients attended DMC under RNTCP where 253 (9.54%) were positive for examination of Acid Fast Bacilli. Patients with risk factors for HIV comprising 115 (45.4%) numbers were referred to ICTC where 2 (0.79%) were detected HIV positive.

Estimation of CD4 and CD8 count and CD4:CD8 ratio

Thirty numbers of newly diagnosed pulmonary TB patients were subjected for estimation of CD4 and CD8 counts in the study. Equal number of subjects used for the same comprising of age and sex matched healthy individuals as controls.

Result of CD4, CD8 count and CD4:CD8 ratio in Test group

The values of CD4, CD8 count and CD4:CD8 ratio among the 30 newly diagnosed pulmonary TB positive and HIV status negative patients which was determined by employing flowcytometry is depicted in Table 1.

Table 1: Pattern of CD4, CD8 count and ratio in test group.

Lab no. (DMC)	Lab no.	CD4 count	CD8 count	CD4:CD8
1095	ILMN/01	505	438	1.15:1
1099	ILMN/04	378	260	1.45:1
2091	ILMN/05	366	307	1.19:1
2089	ILMN/09	274	508	0.73:1
2653	ILMN/10	359	181	1.98:1
2696	ILMN/11	500	207	2.41:1
2697	ILMN/12	209	58	3.60:1
2907	ILMN/13	388	377	1.02:1
2909	ILMN/14	508	455	1.11:1
2757	ILMN/15	84	43	1.9:1
1841	ILMN/16	173	186	0.93:1
1374	ILMN/17	497	419	1.18:1
3269	ILMN/18	215	219	0.98:1
2193	ILMN/19	262	180	1.45:1
1456	ILMN/20	630	878	0.71:1
2851	ILMN/21	201	162	1.24:1
3014	ILMN/22	516	445	1.15:1
03	ILMN/23	402	361	1.11:1
04	ILMN/24	530	466	1.13:1
138	ILMN/25	784	279	2.81:1
208	ILMN/26	561	269	1.06:1
214	ILMN/27	466	459	1.01:1
109	ILMN/28	235	172	1.36:1
157	ILMN/29	195	489	0.39:1
72	ILMN/30	504	225	2.24:1
2519	ILMN/31	277	116	2.38:1
280	ILMN/32	193	246	0.78:1
235	ILMN/33	137	42	3.26:1
284	ILMN/34	44	117	0.37:1
290	ILMN/35	169	87	1.94:1

Result of CD4, CD8 count and CD4:CD8 ratio in Control group

The values of CD4, CD8 count and CD4:CD8 ratio among 30 healthy controls who were age and sex matched with test group was determined by employing flowcytometry is depicted in Table 2.

Table 2: Pattern of CD4, CD 8 count and ratio in control group.

Lab no.	CD4 count	CD8 count	CD4:CD8
ILMN/02	1373	655	2.09:1
ILMN/03	1335	889	1.5:1
ILMN/06	552	1149	0.48:1
ILMN/07	1236	1092	1.13:1
ILMN/08	920	925	0.99:1
ILMN/36	664	586	1.13:1
ILMN/37	348	285	0.99:1
ILMN/38	443	787	1.13:1
ILMN/39	372	328	1.13:1
ILMN//40	472	343	1.37:1
ILMN/41	426	392	1.08:1
ILMN/42	515	361	1.42:1
ILMN/43	369	403	0.91:1
ILMN/44	539	354	1.52:1
ILMN/45	835	698	1.19:1
ILMN/46	550	478	1.15:1
ILMN/47	351	346	1.01:1
ILMN/48	595	989	0.60:1
ILMN/49	681	598	1.13:1
ILMN/50	507	556	0.91:1
ILMN/51	364	410	0.88:1
ILMN/52	675	401	1.68:1
ILMN/53	922	753	1.22:1
ILMN/54	959	423	2.26:1
ILMN/55	498	510	0.97:1
ILMN/56	757	409	1.85:1
ILMN/57	1282	1029	1.24:1
ILMN/58	970	780	1.24:1
ILMN/59	1019	810	1.25:1
ILMN/60	416	371	1.12:1

Interpretation of CD4, CD8 counts and ratio

CD4 Count: The study group which comprised of 30 patients, the mean CD4 count was 355.40±177.69 cells/µl, 95% confidence interval for the mean was 289.05 to 421.75. Among the control group which comprised of 30 subjects, the mean CD4 count was 698.17±315.59 cells/µl, 95% Confidence Interval for the mean was 580.32 to 816.01. The distribution statistics of the CD4 count for the two groups are depicted in the form of Box and whisker plot in Figure 1. For comparison between the 2 groups Independent samples t-test was employed. The P value was $P < 0.0001$, which was statistically significant.

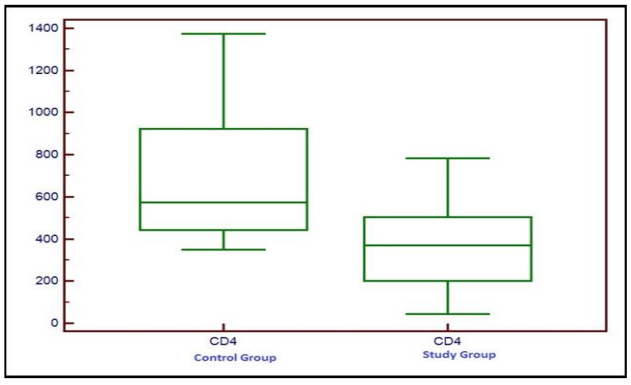

Figure 1: Box and whisker plot comparing the CD4 count between study group and control group.

CD8 Count: The study group which comprised of 30 patients, the mean CD8 count was 288.37±181.70 cells/µl, 95% Confidence Interval for the mean was 220.52 to 356.21. Among the control group which

comprised of 30 subjects, the mean CD8 count was 603.87±258.40 cells/ µl, 95% Confidence Interval for the mean was 507.38 to 700.36. The distribution statistics of the CD8 count for the two groups are depicted in the form of Box and whisker plot in Figure 2. For comparison between the 2 groups Independent samples t-test was employed. The P value was P < 0.0001, which was statistically significant.

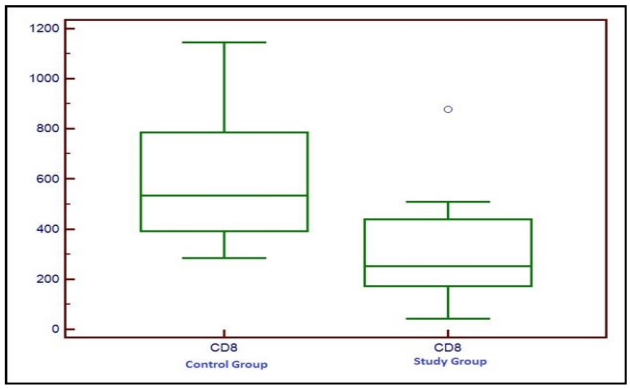

Figure 2: Box and whisker plot comparing the CD8 count between study group and control group.

CD4:CD8 Ratio: The study group which comprised of 30 patients, the mean CD4:CD8 ratio was 1.51±0.80, 95% Confidence Interval for the mean was 1.21 to 1.81. Among the control group which comprised of 30 subjects, the mean CD4:CD8 ratio was 1.21±0.40, 95% Confidence Interval for the mean was 1.06 to 1.36. The distribution statistics of the CD4:CD8 ratio for the two groups is depicted in the form of Box and whisker plot in Figure 3. For comparison between the 2 groups Independent samples t-test was employed. The P value was P = 0.0747, which was statistically not significant.

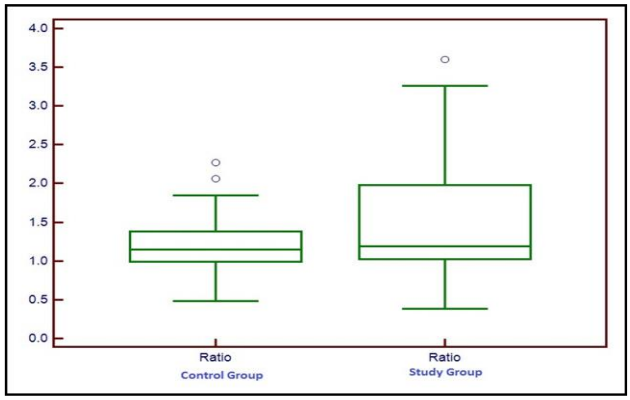

Figure 3: Box and whisker plot comparing the CD4:CD8 ratio between study group and control group.

DISCUSSION

The present study was a hospital based observational study conducted in the Department of Microbiology, in a Tertiary Care Centre in North East India for a period of one year from January 2013 to December 2013.

Among the 253 pulmonary TB patients, 30 newly diagnosed pulmonary TB patients were subjected for estimation of CD4, CD8 count and CD4:CD8 ratio was calculated appropriately. Equal number of age and sex matched healthy controls were also included in the study for comparison of the values. In the present study, the mean CD4 count was 355.40±177.69 cells/µl, 95% Confidence Interval for the mean was 289.05 to 421.75 in the study group. The control group showed mean CD4 count 698.17±315.59 cells/ µl, 95% Confidence Interval for the mean 580.32 to 816.01. On comparison between the 2 groups by employing Independent samples t-test the P value was (P < 0.0001) which denotes significant CD4+ lymphopenia in the study group. This finding was similar to the observations made by Pilheu JA et al and Al-Aska AI et al, who reported mean of 341.25±142.73/mm^3 and 556.79±298.81 versus 1132.28±259.90 in the control group for CD4+ T cell count respectively along with CD8+ lymphopenia.[11,12] Similar to present study, Uppal et al and Davoudi et al, found CD4+ lymphopenia in patients with TB.[6,13] However, another study by Ainslie GM et al, found only CD4+ lymphocytes to be relatively depleted. In addition, Kony et al, found extra pulmonary and more especially military TB to have substantially lower CD4+ T cell counts.[14,15] Many hypotheses had been brought forward to explain the depletion of CD4+ cells which includes unusual response to infection and granuloma formation which plays a major role in containment of TB infection primarily in lungs may fail in individuals with a compromised immune system like HIV may exacerbate TB pathology through the manipulation of granulomas. This has been associated with the killing of CD4+ cells in the granuloma, probably resulting in a direct disruption of granuloma structure and abolition of the containment of infection which results in systemic forms.[5]

The mean CD8 count was 288.37±181.70 cells/µl, 95% Confidence Interval for the mean was 220.52 to 356.21 in the study group. The control group showed mean CD8 count 603.87±258.40 cells/µl, 95% Confidence Interval for the mean 507.38 to 700.36. On comparison between the 2 groups by employing Independent samples t-test P value was (P < 0.0001) which again denotes significant CD8+ lymphopenia in the study group. This finding was similarly observed by Pilheu JA et al and Al-Aska AI et al, who reported mean of 259.33±100.89/mm^3 and 1136.00±512.06 versus 1461.90±367.02 respectively.[11,12] In another study by Shijubo et al, they observed significantly decreased CD8+ T cell which is similar to our finding.[16] This finding could be related to the fact that protective immunity against TB requires CD8+ T cells which are seen to accumulate at the site of mycobacterial infection, forming a cuff at the periphery of epitheloid cell granulomas.[6] On the contrary, Uppal et al and Davoudi et al, reported higher CD8 values in patients with TB.[6,13]

The mean CD4:CD8 ratio in present study was 1.51±0.80, 95% Confidence Interval for the mean was 1.21 to 1.81. The control group showed the mean CD4:CD8 ratio was 1.21±0.40, 95% Confidence Interval for the mean was 1.06 to 1.36. On comparison between the 2 groups by employing Independent samples t-test the P value was (P = 0.0747) not significant which denotes that CD4:CD8 was not significantly reduced in the study group. This can be explained by the fact that due to significant reduction in both CD4 and CD8 counts; there is no significant reduction of the CD4:CD8 ratio. In a study by Han Pin Kuo et al, in the BAL of HIV negative TB patients, they observed significant increase in the CD4:CD8 ratio with respect to normal subjects (2.8±0.4 versus 1.3±0.2, p=0.03) which was similar to results reported by Singhal et al.[17,18] This finding is contrary to that observed by Ashtekar MD et al, Uppal et al and Davoudi et al, who reported significant reduction of the CD4:CD8 ratio which can be due to CD8 lymphocytosis with CD4 lymphopenia.[6,13,19]

CONCLUSION

In conclusion, present study found significantly lower CD4 and CD8 counts among Pulmonary TB infected HIV negative patients as compared with controls. This denotes that CD4, CD8 counts can be used as a tool for prediction of HIV coinfection in TB patients where preliminarily there may not be HIV infection. Unfortunately, we do not have follow-up studies of our patients with low CD4, CD8 counts, but those reported in the literature shows consistent improvement and often normalization in response to the administration of anti-tuberculous chemotherapy. As CD4 lymphopenia is reversible immunological phenomenon, appropriate treatment with strict compliance to anti-tubercular drug regimens as per protocol is important for effective management of such patients. Prediction ability in combination with early detection and appropriate management play major role in evading the emergence of drug resistance among the HIV-TB coinfected patients.

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Ethical approval: The study was approved by the Institutional Ethics Committee

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