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Original Article

Immune activation markers in individuals with HIV-1 disease and their correlation with HIV-1 RNA levels in individuals on antiretroviral therapy

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ABSTRACT

Background: Currently CD4⁺ T lymphocyte counts and HIV-1 RNA levels are being utilized to predict outcome of human immunodeficiency virus (HIV) disease. Recently, the role of immune activation in HIV disease progression and response to treatment is being investigated. This study focused on the expression of CD38 and HLA-DR on lymphocyte subsets in various groups of HIV-infected individuals and to determine their association with HIV-1 disease progression.

Methods: Ninety-eight cases of patients with HIV/AIDS in different disease stages and twenty-four healthy HIV-negative individuals were included in the cross-sectional study. Their immune function and abnormal immune activation markers (CD38 & HLA-DR) were detected using a flowcytometer, and HIV-1 RNA levels in individuals receiving antiretroviral drugs were estimated.

Results: The immune activation marker levels were significantly different between patients with different disease stages ($P < 0.001$). A significant negative correlation was observed between peripheral blood CD4⁺ T cell counts and immune activation markers. Also, a significant positive correlation was observed between HIV-1 RNA levels and CD38⁺CD8⁺ T lymphocyte.

Conclusion: Immune activation markers (CD38 & HLA-DR) increase with disease progression. CD38⁺ on CD8⁺ T lymphocyte correlates well with HIV1 RNA levels in individuals failing on antiretroviral therapy.

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Introduction

Since the human immunodeficiency virus (HIV) disease process is highly complex, monitoring only the basis of clinical criteria is not sufficient. Hence, there is a requirement to identify laboratory markers that may help in monitoring progression of HIV disease and its response to antiretroviral treatment (ART). Absolute CD4⁺ T lymphocyte counts or its expression as a percentage of the total peripheral lymphocytes is a time-tested correlate that has been widely utilized in HIV disease staging.^{1–3} Currently, CD4⁺ T cell counts are being utilized as a marker not only for clinical staging but also for monitoring patients receiving ART. However, the CD4⁺ T cell count measurement alone is not adequate to predict either success and/or failure of ART regimens. CD4⁺ T lymphocyte counts are known to overlap among the various clinical stages of HIV disease, and the same is known to fluctuate considerably in an individual.⁴ The HIV-1 RNA level is another reliable parameter that can be utilized to monitor the ART outcome. But, HIV-1 RNA level estimation is a costly test for routine monitoring in India and, therefore, impractical to be used in developing countries like India.

Hence, we studied immune activation markers expressed by CD4⁺ and CD8⁺ T lymphocytes; because it has been seen that while most of the routine testing is based on CD4⁺ T cells, there is evidence pointing towards the need to evaluate the CD8⁺ T cell compartment, which may be important in elucidating the pathogenesis and disease progression.⁵ In this context, data suggest that there is an increased expression of immune activation markers, namely CD38 and HLA-DR, on CD4⁺ and CD8⁺ T lymphocytes in HIV infection.⁶ The present study was carried out to study the expression of these biomarkers on lymphocyte subsets in various groups of HIV-infected individuals and to determine their association with HIV-1 disease progression.

Material and methods

This cross-sectional study was conducted in a tertiary care institute in Pune, Maharashtra, India, from March 2010 to March 2011.

Study population

A total of 122 participants were enrolled in the five defined groups where group 1 included asymptomatic HIV-1-infected individuals with CD 4 counts >500 cells/ μ l and ART naive ($n = 25$), group 2 included HIV-1-infected individuals with symptomatic disease with CD 4 counts < 500 cells/ μ l and ART naive ($n = 25$), group 3 included HIV-1-infected individuals on ART for more than 1 year having HIV-1 RNA levels <1000 copies/ml ($n = 25$), and group 4 included HIV-1-infected individuals on ART for more than 1 year having HIV-1 RNA levels >1000 copies/ml ($n = 23$). Finally, group 5 included HIV-negative healthy controls ($n = 24$). Clinical details were noted. Written informed consent was obtained from each participant. Ethical clearance had been obtained from the institutional ethical committee.

Samples

Ten milliliters of peripheral venous blood was collected in a K3-EDTA vacutainer, in duplicate, for immunological and virological studies. Whole-blood samples were processed immediately using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, California USA.) for analysis of CD4⁺ and CD8⁺ T lymphocytes and activated subsets of CD4⁺ & CD8⁺ cells expressing CD38 and/or HLA-DR.

For HIV-1 RNA level estimation, whole blood was collected and plasma separation was performed by centrifuging at 72.67 $\times$ g for 10 min, followed by storage at -70°C .

Monoclonal antibodies

The following monoclonal antibodies were obtained from BDIS: anti-CD3 peridinin chlorophyll protein, anti-CD4 fluorescein isothiocyanate (FITC), anti-CD8 FITC, anti-CD38 phycoerythrin, and anti-HLA-DR APC (allophycocyanin). The combinations of fluorescent monoclonal antibodies used for identification of various cells included CD3/CD4/CD38 and HLA-DR for CD4⁺ cells, and CD3/CD8/CD38 and HLA-DR (combination) for CD8⁺ cells.

Flow cytometry procedure

All the reagents were brought to room temperature before use. Two Trucount tubes (BD) were taken and labeled—TUBE 1(CD4⁺ cells): CD3, CD4, CD38, HLA-DR—positive cells and TUBE 2 (CD8⁺ cells): CD3, CD8, CD38, HLA-DR—positive cells. Twenty microliters of monoclonal antibodies was added in combination to each marked tube. Peripheral blood (PB) was mixed thoroughly, and 50 μ l of it was added to each tube. Each tube was vortexed for 5–10 s to ensure thorough mixing of contents. These were incubated in dark for 15–30 min after which 450 μ l of 10 $\times$ lysis buffer was added to each tube. Tubes were again incubated in dark for duration of 10 min–24 h. Each tube was vortexed just before subjecting them to flow cytometry. A total of 10,000 cells were counted. Each lymphocyte subpopulation counted was expressed as an absolute number. Sample data were acquired and analyzed using Cell Quest software (BDIS). Before each run, calibration was carried out using BD CaliBRITE™ 3 Beads and BD CaliBRITE™ APC beads.

HIV-1 RNA level estimation

Plasma sample stored at -70°C was used to estimate HIV-1 RNA levels in study group 3 and 4 patients who were receiving ART. This was done by reverse transcriptase polymerase chain reaction using Roche Amplicor HIV-1 Monitor Test version 1.5 (Roche Diagnostics, Branchburg, NJ 08876, USA).

Statistical analysis

Comparison of study variables across the groups as well as between two groups was done using analysis of variance. A p value of <0.05 was considered statistically significant. Correlation analysis was performed using Pearson's coefficient.

Table 1 – Age and gender distribution in each study group.

Parameter	Group 1 N = 25	Group 2 N = 25	Group 3 N = 25	Group 4 N = 23	Group 5 N = 24
Mean age in yrs (SD)	33.64 (5.715)	39.12 (9.649)	37.72 (7.425)	35.52 (3.654)	36.36 (6.672)
Male: Female ratio	9: 16	23:2	16:9	21: 2	22 : 2
SD, standard deviation.					

Statistical analyses were carried out with computer program Microsoft Excel, version 7 (Microsoft Corporation, NY, USA) and SPSS version 16.0 (Statistical Package for the Social Science SPSS Inc. Chicago, USA).

Results

Gender and mean age distribution in five study groups is tabulated in Table 1. There was a predominance of males among the study participants, except in group 1. CD4+ T lymphocyte counts were significantly different among the five groups ($p < 0.001$). CD 8 + T lymphocytes also showed significant differences across the five groups, being highest in asymptomatic HIV-infected individuals, i.e. group 1, and lowest in healthy volunteer, i.e. group 5 ($p < 0.001$). The CD4/CD8 T cell ratio differed significantly ($P < 0.001$) among the five groups with highest value recorded for normal controls (mean value 2.38) and lowest in individuals with symptomatic HIV disease (mean value 0.20) [Tables 2 and 3].

Expression of activation markers on CD4+ T lymphocytes

Mean proportion of CD4+ T lymphocytes expressing CD38 and HLA-DR individually showed significant difference across the 5 study groups ($p < 0.001$). Mean proportion of CD4+ T cells showing dual positivity for the activation markers, i.e. CD38 as well as HLA-DR, also showed significant differences across the groups ($p < 0.001$) [Table 4 & Fig. 1].

Expression of activation markers on CD8+ T lymphocytes

Mean proportion of CD8+ T lymphocytes expressing CD38 and HLA-DR individually showed significant difference among all the studied groups (p value < 0.001). Mean proportion of CD8+ T cells showing dual positivity, i.e. CD38 as well as HLA-DR, also showed significant differences across the groups ($p < 0.001$) [Table 5 & Fig. 2].

Table 2 – Mean CD4+ T lymphocyte count in each group.

Group	Mean	Standard deviation	ANOVA (p-value)
Group 1 (n = 25)	937.64	270.78	<0.001
Group 2 (n = 25)	149.00	108.72	<0.001
Group 3 (n = 25)	334.40	159.93	<0.001
Group 4 (n = 23)	134.35	98.70	<0.001
Group 5 (n = 24)	858.17	368.14	<0.001
Total (n = 122)	485.34	413.31	<0.001
ANOVA, analysis of variance.			

HIV-1 RNA viral levels & T-cell immune activation marker expressions

HIV-1 viral RNA level estimation HIV-1 viral RNA level estimation was done for study participants in group 3 and group 4. The HIV-1 RNA levels were below the detection limit of the assay kit (< 400 copies/ml) in group 3, whereas the values were > 1000 copies/ml in group 4 (median 51,066 copies/ml). CD4+ T cell proportions expressing CD38 or HLA-DR correlated poorly with the HIV-1 RNA levels in group 4. In contrast, there was a moderate positive correlation between the expression of CD38 on CD8+ T cells with the HIV-1 RNA levels in group 4 (Pearson's coefficient = 0.337), but it was poor for HLA-DR expression (Table 6).

Correlation of CD4+ T lymphocyte counts with immune activation marker expressions

On calculating the Pearson's correlation coefficient between CD4+ T lymphocyte counts and proportion values of CD8+CD38 + T cell, it was observed that there was a good negative correlation in group 2 (-0.526), a moderate negative correlation in group 4 (-0.251), and a moderate positive correlation in group 1 (0.346). Additionally, proportion of CD + T cell expressing HLA-DR showed a moderate negative correlation with CD4+ T cells in group 4 (Table 7). The correlation of proportion of CD + T cell expressing HLA-DR showed a moderate negative correlation with CD4+ T cells in group 4. Data analysis of the remaining groups revealed a poor correlation between the proportion of CD + T cell expressing HLA-DR and CD4+ T lymphocyte counts.

Discussion

The generalized immune cellular activation that happens almost immediately after HIV infection possibly has a crucial

Table 3 – Mean CD8+ T lymphocyte count across study groups.

Group	Mean	Standard deviation	ANOVA (p-value)
Group 1 (n = 25)	1278.92	879.29	<0.001
Group 2 (n = 25)	881.04	607.61	<0.001
Group 3 (n = 25)	949.48	505.21	<0.001
Group 4 (n = 23)	288.04	209.02	<0.001
Group 5 (n = 24)	461.92	282.69	<0.001
Total (n = 122)	782.35	652.60	<0.001
ANOVA, analysis of variance			

Table 4 – Mean proportion of activated CD4+ T lymphocytes amongst the different study groups.

Parameter	Group 1 (n = 25)	Group 2 (n = 25)	Group 3 (n = 25)	Group 4 (n = 23)	Group 5 (n = 24)	ANOVA (p value)
Mean proportion of CD38 + CD4 + cells	0.74	0.76	0.69	0.87	0.64	<0.001
Mean proportion of HLA-DR + CD4 + cells	0.69	0.86	0.60	0.619	0.34	<0.001
Mean proportion of CD38 + & HLA-DR + CD4 + cells	0.50	0.65	0.41	0.54	0.29	<0.001

ANOVA, analysis of variance.

Fig. 1 – Mean proportion of activated CD4+ T lymphocytes amongst the different study groups.**Table 5 – Mean proportion of activated CD8+ T lymphocytes amongst different study groups.**

Parameter	Group 1 (n = 25)	Group 2 (n = 25)	Group 3 (n = 25)	Group 4 (n = 23)	Group 5 (n = 24)	ANOVA (p-value)
Mean proportion of CD38 + CD8 + cells	0.678	0.849	0.665	0.789	0.492	<0.001
Mean proportion of HLA-DR + CD8 + cells	0.715	0.876	0.660	0.589	0.281	<0.001
Mean proportion of CD38 & HLA-DR + CD8 + cells	0.501	0.763	0.445	0.524	0.259	<0.001

ANOVA, analysis of variance.

Fig. 2 – Mean proportion of activated CD8+ T lymphocytes amongst the different study groups.

Table 6 – CD38 & HLA-DR expression on CD4+ and CD8+ T lymphocyte with HIV-1 RNA levels in group 4.

	T lymphocyte population	Immune activation marker	Pearson's coefficient
Proportion values	CD4+	CD38+	–0.1454
		HLA-DR+	0.0268
		CD38 + HLA-DR+	–0.0391
	CD8+	CD38+	0.3373
		HLA-DR+	0.2066
		CD38 + HLA-DR+	0.2158

role in the maintenance of the asymptomatic state for long durations, or may lead to the initiation of clinical manifestations in HIV infected. Although CD4+T lymphocyte counts correlate well with HIV-1 disease stages,^{1–3} their estimation alone may not be sufficient to define clinical stages due to marked variability as the disease advances.⁴ Thus, there is a need for identification of additional tools for monitoring HIV disease progression.

The pattern of immune destruction in HIV disease has been shown to be progressive and not random. There is an initial rise in CD8+T cells, which is followed by decrease in the CD4/CD8 ratio. Subsequently, there is a progressive fall in CD4+ T cells, and lastly a drop in total lymphocytes counts.^{6–8} A similar pattern was observed by us as well. One of the early findings following HIV seroconversion is an elevation of the absolute CD8+ T lymphocyte count, without any obvious change in the CD4+ T cell population.⁶ In our study, a much higher mean CD8+T lymphocytes count was noticed in asymptomatic HIV-1–infected individuals as compared to the HIV-infected individuals who had symptomatic disease, although this difference among these groups was not statistically significant ($p > 0.05$). However, Ray et al.⁹ have reported a significant difference ($p < 0.05$) in CD8+ T lymphocyte count when asymptomatic HIV-infected individuals were compared with symptomatic disease with higher counts in the former. This could possibly be attributed to small sample size in our study groups.

Activated CD4+ T lymphocytes

A progressive depletion of CD4+ T lymphocytes in HIV disease has been associated with significant changes in *in vivo* lymphocyte phenotype. Here, with disease progression, there is a dramatic increase in HLA-DR and CD38 expressions on CD8+ T cells.^{10,11} There is an unusual increase in the expression of CD38 on circulating CD8+ T cells among HIV-1–infected individuals, which has been observed to persist throughout the course of disease among treatment

naïve.^{6,10–14} Subsequently, similar observations have been also documented in CD4+ T cells.^{15–18} We evaluated the expression of these two activation markers on CD4+ T lymphocytes in the study groups, which belonged to 4 separate clinical stages of HIV-1 disease, and showed that the relative proportion of CD4+ T cells expressing HLA-DR and CD38, either individually or together, is increased in both asymptomatic as well as symptomatic HIV-seropositive participants in comparison to the healthy controls, difference being statistically significant ($p < 0.05$). In addition, the relative co-expression of both markers, as well as for CD38 and HLA-DR alone, was higher in symptomatic patients than in asymptomatic subjects, this being statistically significant for HLA-DR alone as well as CD38 & HLA-DR co-expression ($p < 0.05$) only. This was expected as the higher HIV antigen load may have caused the naïve and the memory cells to get activated and try to eliminate the infectious agent. There has been one study from India conducted by Vajpayee et al.¹⁹ which is in agreement with our study.

Following ART, temporarily downregulation of expression levels of HLA-DR and CD38 on CD8 cells, and at times on CD4 cells, has been reported.^{13,20} We observed differences between the activation marker expression in the patients failing ART when compared to the patients on ART with virologic suppression, being higher in the former group, this being statistically significant for proportion of CD4+ T cell with CD38 expression ($p < 0.001$), but not for HLA-DR expression ($p > 0.05$).

Activated CD8+ T lymphocytes

Giorgi et al.¹² documented an increase in expression of CD38 on CD8+ T lymphocytes from HIV-1–positive patients for the first time. Similar studies followed which revealed that an increased CD38 expression on CD8+ T lymphocytes is a poor prognostic marker for HIV-1–infected,^{10–14} which predicts disease progression independent of the plasma HIV-1 RNA levels and PB CD4+ T cell counts.¹⁸ In addition, CD38 expression on CD8+ T cells has been shown to correlate with HIV-1 disease progression and predict PB CD4+ T cell decline.²¹

In agreement with aforementioned studies from West, we found difference in CD38 expression on CD8+ T lymphocytes between HIV seropositives and healthy controls to be highly significant ($p < 0.001$). Furthermore, similar highly significant difference of CD38 expression on CD8+ T lymphocytes between asymptomatic HIV-infected individuals and patients with symptomatic HIV-1 disease was observed ($p < 0.001$).

A marked reduction in the relative as well as absolute number of CD8+CD38+ T cells in HIV-1–infected patients has been observed after ART initiation.^{22,23} Persistent high CD8+CD38+ T lymphocyte levels have been associated with

Table 7 – CD4+ T lymphocyte counts with immune activation marker expressions.

Immune activation marker	Pearson's coefficient				
	Group 1	Group 2	Group 3	Group 4	Group 5
Proportion values -CD8+CD38+	0.346	–0.526	0.075	–0.251	–0.077
Proportion values -CD8+HLA-DR+	–0.192	–0.052	–0.181	–0.333	0.0006
Proportion values -CD8+CD38+HLA-DR+	–0.123	–0.523	0.043	–0.297	0.083

ART failure.^{24,25} A sustained upregulation of CD38 expression on CD8+ T cells from ART-treated HIV-1–infected patients, along with suppressed plasma viral RNA levels, has been suggested as a marker of residual viral replication.^{26,27} We observed significant difference in CD38 expression on CD8+ T cells between patients on ART showing virologic suppression and patients on ART showing virologic failure i.e. group 3 and group 4 ($p < 0.05$). This expression of CD38 on CD8+ T cell proportions in participants on ART with evidence of virologic failure (group 4) showed a moderate positive correlation with HIV-1 RNA levels. Thus, our observations highlight and once again underline the utility of expression of CD38 on CD8+ T lymphocytes as a potential biomarker to monitor HIV-1–infected individuals on ART.^{16,17}

Along with CD38, expression of HLA-DR on CD8+ T cells has also been studied by some authors.^{28,29} These studies have elucidated the pattern of HLA-DR and CD38 co-expression on CD8+ T lymphocytes during various stages of HIV infection, and demonstrated that expression increases with advanced disease. Our study findings are in agreement with this in that the co-expression of CD38 and HLA-DR was significantly higher in HIV patients than controls ($p < 0.05$). Also, symptomatic HIV patients showed increased proportions of CD8+ T cells co-expressing CD38 and HLA-DR when compared to asymptomatic individuals, the difference being highly significant ($p < 0.001$). Kaushik et al.³⁰ from India have demonstrated similar significant differences in the expression of activated CD4+ and CD8+ T cell subsets HIV infected.

We observed highly significant difference in co-expression of CD38 and HLA-DR on CD8+ T cells in patients receiving ART for more than 1 year and showing virologic suppression, i.e. group 3 and symptomatic HIV individuals in group 2, being higher in the latter group ($p < 0.001$). When patients on ART showing virologic suppression were compared with patients on ART showing evidence of virologic failure, the difference in co-expression of CD38 and HLA-DR, although not statistically significantly ($p = 0.08$), was lower in the former study group. We attribute this to small sample size of study groups.

HIV-1 RNA levels & T lymphocyte activation markers

On analyzing the relationship of HIV-1 RNA levels with expression of immune activation markers in HIV-1–infected individuals on ART with evidence of virologic failure (group 4), we were not able to demonstrate any correlation with proportions of CD8+ T cells expressing CD38 and HLA-DR. Also the correlation of HIV-1 RNA levels was poor with the expression of these immune activation markers on CD4+ T cells.

Our findings are in agreement with similar reports from the West. In a recent study, similar to ours, a direct correlation of CD8+HLA-DR + CD38 + cells with plasma HIV-1 RNA levels in HIV-1–infected patients has been reported.³¹ Furthermore, it has been suggested that a rise of CD38 + mean fluorescent intensity on CD8+ T cells, if significantly higher than that observed in previous visit, even if not returning to initial baseline CD38 values, can provide an immediate indication for HIV-1 RNA level estimation.³²

Correlation of CD4+ T cell with immune activation

In our study, there was moderate negative correlation observed between CD4+ T cells and proportions of CD8+ T cells expressing CD38/HLA-DR alone or co-expressing both among individuals on ART with evidence of virologic failure. A similar good negative correlation was seen in symptomatic, ART-naïve individuals for proportions of CD8+ T lymphocytes expressing C38 alone as well those co-expressing CD38 and HLA-DR. Similar findings have been suggested by Wang et al.³³, from China.

Few studies from the West have demonstrated a negative correlation between CD38 + CD8+ T cells and CD4 central memory cells among virally suppressed HIV-1–infected individuals.^{34,35}

Conclusion

The role of immune activation has been studied well in the West, but studies on subtype C prevalent in the Indian subcontinent is the need of the hour. Hence, we carried out this study and have observed that expression of activation markers, such as CD38 and HLA-DR, increase on CD4+ and CD8+ cells in HIV infection as the disease progresses.

In patients receiving ART and responding to treatment, the expression of activation markers tend to be lower when compared with those not responding. Also, the levels of co-expressed CD38 + and HLA-DR + CD8+ T cells were higher in HIV-infected individuals, irrespective of clinical stage when compared to healthy individuals.

The limitations of our study include small sample size of the four subsets of HIV-1–infected participant groups and inability to follow-up.

Although there are two Indian studies which have studied the role of immune activation in HIV-1 infection, to our knowledge, this is the first study in India where immune activation markers were studied in adult patients receiving ART, along with those not receiving treatment, and were correlated with HIV-1 RNA levels. Further randomized control studies need to be carried out to assess the potential of activation markers in predicting response to treatment in HIV-1–infected individuals on ART.

Conflicts of interest

All authors have none to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.mjafi.2019.06.005>.

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