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RESEARCH ARTICLE

HIV-specific T-cell Responses and Generalized Activation in HIV-1 Infected Long-term Non-progressors and Progressors from South India

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Abstract: ***Background*:** Anti-viral cytokine expressions by cytotoxic T-cells and lower activation rates have been reported to correlate with suppressed HIV replication in long-term non-progressors (LTNP). Immune mechanisms underlying disease non-progression in LTNP might vary with HIV-1 subtype and geographical locations.

***Objective*:** This study evaluates cytokine expression and T-cells activation in relation to disease non-progression in LTNP.

***Methods*:** HIV-1 Subtype C infected LTNP (n=20) and progressors (n=15) were enrolled and flowcytometry assays were performed to study HIV-specific CD8 T-cells expressing IL-2, IFN-γ, TNF-α and MIP-1β against gag and env peptides. CD4+ T-cell activation was evaluated by surface expression of HLADR and CD38.

***Results*:** Proportions of cytokines studied did not differ significantly between LTNP and progressors, while contrasting correlations with disease progression markers were observed in LTNP. CD4+ T-cell activation rates were significantly lower in LTNP compared to progressors which indicate the potential role of T-cell activation rates in disease non-progression in LTNP.

***Conclusion*:** LTNP and progressors showed similar CD8+ T-cell responses, but final conclusions can be drawn only by comparing multiple immune factors in larger LTNP cohort with HIV-1 infected individuals at various levels of disease progression. A possible role of HIV-1 subtype variation and ethnic differences in addition to host-genetic and viral factors cannot be ruled out.

Keywords: HIV LTNP, HIV non-progression, antiviral cytokine response in HIV LTNP, T-cell activation in HIV LTNP, HLADR, CD38.

1. INTRODUCTION

Differences in the rate of human immunodeficiency virus (HIV) disease progression in the absence of anti-retroviral therapy (ART) have been widely contributed to effective cytotoxic T- lymphocyte (CTL) responses [1, 2]. CD8+ T-cell responses were observed to coincide with a decline in viral replication during acute HIV and simian immunodeficiency virus (SIV) infections [3, 4] and depletion of these CD8+ T-cells results in dramatic increase of SIV plasma viral load (PVL) [5]. Polyfunctionality of CD8+T-cells by means of expression of multiple effector functions resulting in cytolysis and their capacity to perform degranulation has been attributed to the spontaneous control of viral expansion [6-8]. In the absence of ART, progressive loss of CD8+ T-cell responses has been reported, which results in ultimate loss of viral control in most of the chronically infected HIV patients [9], for reasons that remain unclear.

However, there exists a small group (~5%) of HIV infected population who remains healthy without significant changes in CD4+ T-cell count or rise in HIV-RNA levels for over 10 years even in the absence of ART, known as long-term non-progressors (LTNP). LTNP were found to be heterogeneous, when it comes to the degree of virological control, with most LTNP having low to medium levels of viremia [10-12]. Availability of routine viral load testing further divided controller population and leads to the identification of elite controllers (EC), who are typically defined

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as HIV infected people maintaining PVL below the limit of detection by a series of viral load testing in the absence of ART [13]. The presence and prolonged maintenance of strong broadly directed HIV-specific CD8+ T lymphocyte responses during chronic phase were reported to be more in LTNP compared to progressors [14-16]. A number of evidences have shown that the functionality of CD8+T-cells might be responsible for the extended replication control of HIV in LTNP [1] because of that considerable attention has been directed towards HIV-specific CD8+ T-cells [17-19]. LTNP exhibit increased frequencies of polyfunctional T-cells which are able to perform multiple effector functions simultaneously, and are thought to be responsible for superior viral control [20]. However, conflicting results have also been reported questioning the possible role of CD8+ T cell responses in viral control and its relation with HIV non-progression [9, 21]. Defects in phenotypic [22-24] and functional properties [25, 26] of HIV-specific CD8+ T-cells and their inability to expand *in vitro* [21] have also been described.

Viral replication driven generalized immune activation is now established as the main mechanism behind $CD4^+$T-cell depletion [27]. It has been widely postulated, that loss of regenerative capacity of immune system due to high T-cell turnover is caused by accelerated proliferation, expansion, and death of T-cells during the course of HIV infection [28]. Immune activation is one of the more well-examined features in non-progressors and has been found to be lower in elite controllers (EC) and viral controllers (VC) compared to progressors [29-31]. Susceptibility of T-cells to HIV-1 infection is reduced with less CD4+ T-cell activation rates and it can lead to better disease prognosis [15, 32]. Activation profile of LTNP was similar to SIV infected sooty mangabeys and African green monkeys which also showed no signs of increased immune activation or high T-cell turnover despite high viral loads [33]. Contrarily, there are also reports stating that there are no differences in immune activation between EC and LTNP [34] and EC, LTNP and progressors [35]. Data on HIV infected LTNP and their immune tolerance capabilities are limited from a country like India which has diverse ethnicities and remains scarce from southern India. Hence, in this study, we characterized and compared HIV-specific CD8+ and CD8- T-cell responses by means of their cytokine expression profile in LTNP and progressors. Moreover, we also correlated the cytokine expression with their respective disease progression markers such a CD4+ T-cell count, CD4% and plasma viral load (PVL). Further, we extended our study to explore and compare the frequencies of T-cell activation and also compared them with disease progression markers.

2. MATERIALS AND METHODS

2.1. Subjects

In this cross-sectional study, HIV-1 Subtype C infected individuals attending YRG CARE medical center were screened based on their $CD4^+$ T-cell counts and length of HIV infection. Of these, a cohort of LTNP (n=20), defined as patients who had a durable maintenance of peripheral CD4+ T-cell counts of >500 cells/mm^3 for more than 7 years in the absence of ART and progressors (n=15) defined as patients who had $CD4^+$ T-cell counts of 300-500 cells/mm^3, 3-5 years post infection without receiving ART were enrolled. This study was approved by the institutional review board and duly signed written informed consent forms were obtained from all the willing participants.

2.2. Specimens and Cell Stimulation

According to the standard procedure, peripheral blood mononuclear cells (PBMCs) were harvested from EDTA-treated peripheral blood using ficoll-paque density gradient centrifugation method and cryopreserved at $\leq$-140 ^{0}C until testing. Before stimulation, PBMCs were thawed and rested overnight at 37^{o}C in 5% CO_2 environment, incomplete culture medium (RPMI 1640 supplemented with 10% FBS and 1% penicillin-streptomycin). At least 1 million PBMCs were added with 2µL of co-stimulatory antibodies, antiCD28/49d (BD Biosciences, USA) and then stimulated with peptides (15 mers overlapping 11) corresponding to full length HIV-1 consensus C *gag* and *env* (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH, USA) at a final concentration of 2µg/ml each. PBMC were then incubated at 37^{o}C in 5% CO_2 environment for 6 hours. Golgi plug (BD Biosciences) was added to cells after 2 hours of stimulation. PBMCs stimulated with 1µg/ml staphylococcal enterotoxin B (SEB) were included as a positive control and unstimulated PBMCs as a negative control.

2.3. Immunfluorescence Staining and Flowcytometric Analysis

Following incubation, cells were surface stained with anti-CD8 ECD (Cytostat / Coulter Clone) and incubated in dark at room temperature for 20 mins. Cells were then washed and permeabilized using 1X PERM 2 (BD Biosciences, San Jose, CA, USA), incubated for 10 mins. Following washing, cells were stained intracellularly with anti-IFN-γ FITC (Beckman Coulter Inc.), anti-IL-2 PE (Beckman Coulter Inc.) and anti-CD3 PerCP-Cy5.5 (BD Biosciences, San Jose, CA, USA) antibodies. Simultaneously, another set of same specimens were added with anti-MIP-1β FITC (BD Biosciences, San Jose, CA, USA), anti-TNF-α PE (Beckman Coulter Inc.) and anti-CD3 PerCP-Cy5.5 (BD Biosciences) antibodies, incubated for 20 mins in dark at room temperature. Cells were then washed and fixed using 1% paraformaldehyde.

For activation profile, thawed and rested cells were surface stained with anti-HLADR FITC (BD Biosciences), anti-CD38 PE (BD Biosciences, San Jose, CA, USA), anti-CD4 ECD (Cytostat / Coulter Clone) and anti-CD3 PerCP-Cy5.5 (BD Biosciences) and incubated in dark at room temperature for 20 mins. Cells were then washed and fixed using 1% paraformaldehyde. Both cytokine expression profile and activation profile were identified by acquiring PBMC in Cytomics FC500 5-color flowcytometer (Beckman Coulter, Fullerton, Fullerton, CA). A minimum of 200000 events was acquired for cytokine expression and a minimum of 300000 events were acquired to study immune activation. Fig. (**1**) represents the gating strategy followed for studying the cytokine expression profile of CD8+ T-cells and CD8- T-cells. Fig. (**2**) represents the gating strategy followed for studying CD4+ T-cell and CD4- T-cell activation profile. Acquired

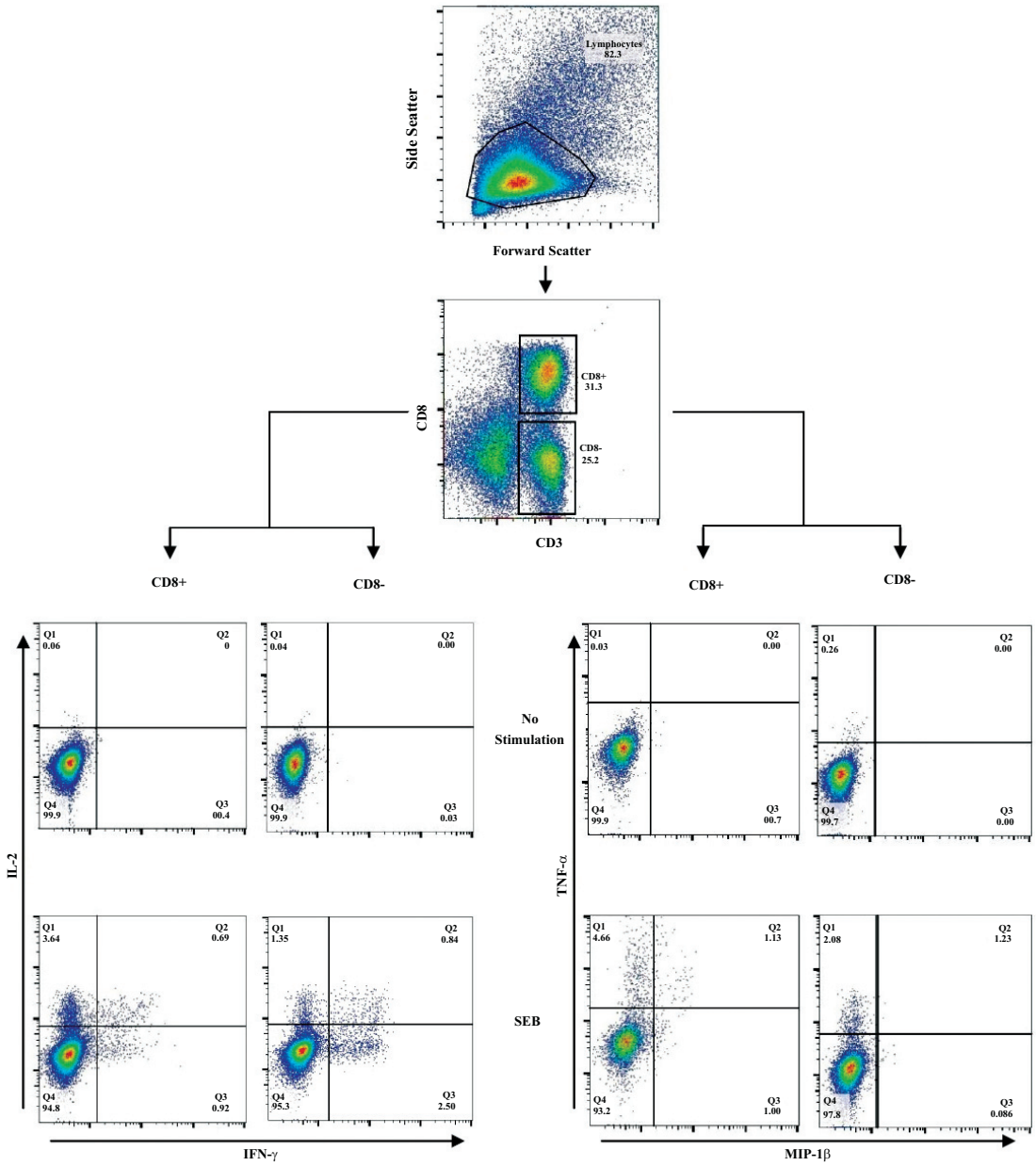


Fig. (1). Schematic representation of gating strategy used for ICS analysis. Representative plots showing HIV-specific CD8+ and CD8- T-cell responses against HIV peptides *gag* and *env*. Culture medium alone (no stimulation) was used as negative control and SEB stimulation (positive control) was used as positive control.

cells were analyzed using FlowJo v7.2 (Tree Star Inc., Ashland, Oregon, USA). Gates defining the positive and negative expression of surface antigens were combined by boolean gating strategy (as indicated in FlowJo software) to generate all possible combinations of antigen expression, as previously described [4].

2.4. Laboratory Monitoring Parameters

Absolute CD4+ T-cells counts were obtained using FACS count (BD Biosciences, San Jose, CA, USA) and PVL were quantified using m2000rt Abbott Real-Time PCR (Abbott Molecular Inc, IL, USA). Complete blood count testing was performed using Sysmex XT 1800i (Sysmex Corporation, Kobe, Japan).

2.5. Statistical Analyses

Rho (correlation coefficients) were calculated using Spearman's correlation for assessing the correlations of CD8+ and CD8- T-cell responses and activation with disease progression markers. Correlations could not be calculated if there were less than two observations in the subset of patients. Comparison of activation rates between two groups was calculated by two sided t-tests for difference in means, comparisons between the three groups were calculated by analysis of variance analysis (ANOVA). Box plots were used to visually present response profiles to the measured cytokines. All statistical analysis was performed using Stata 12.0 Statistical Software (College Station, TX) with an alpha value of 0.05.

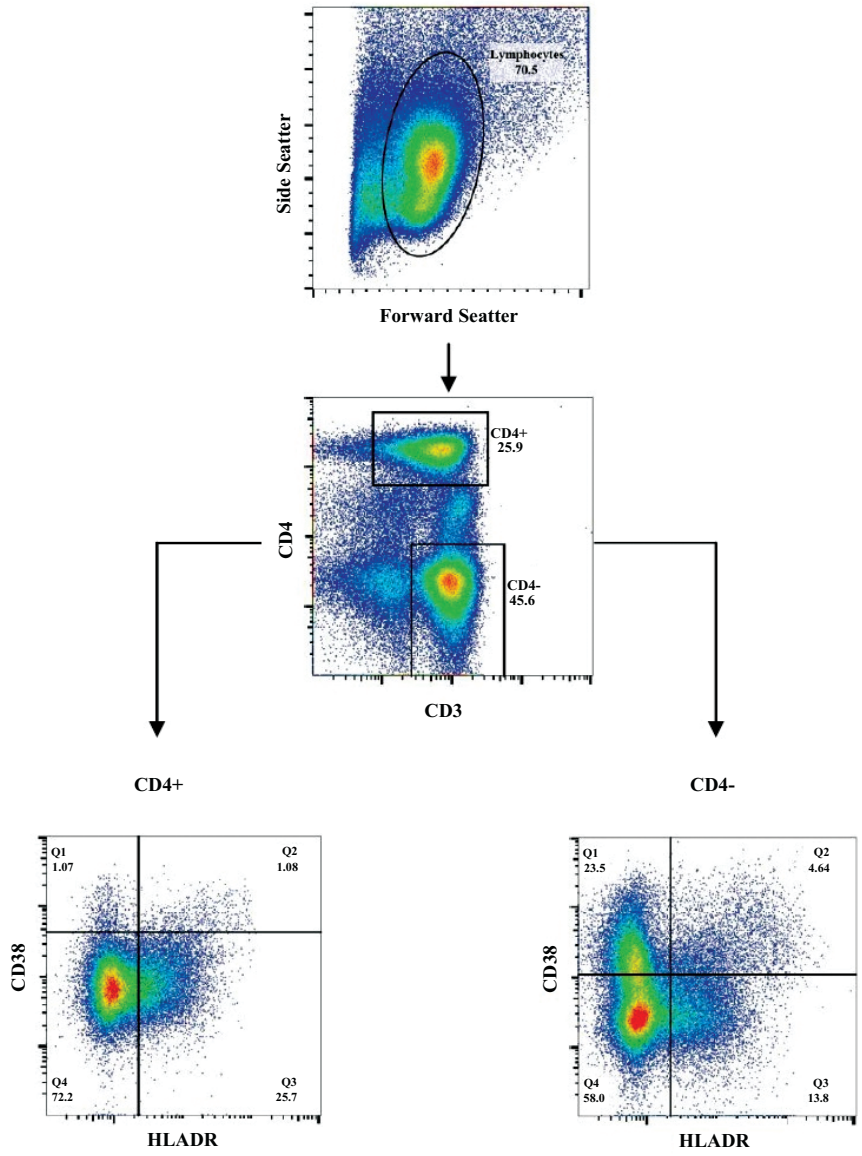


Fig. (2). Schematic representation of gating strategy used for activation rates are shown, quantifying the expression of CD38 and HLADR markers on the surface of CD4+ and CD4- T-cells.

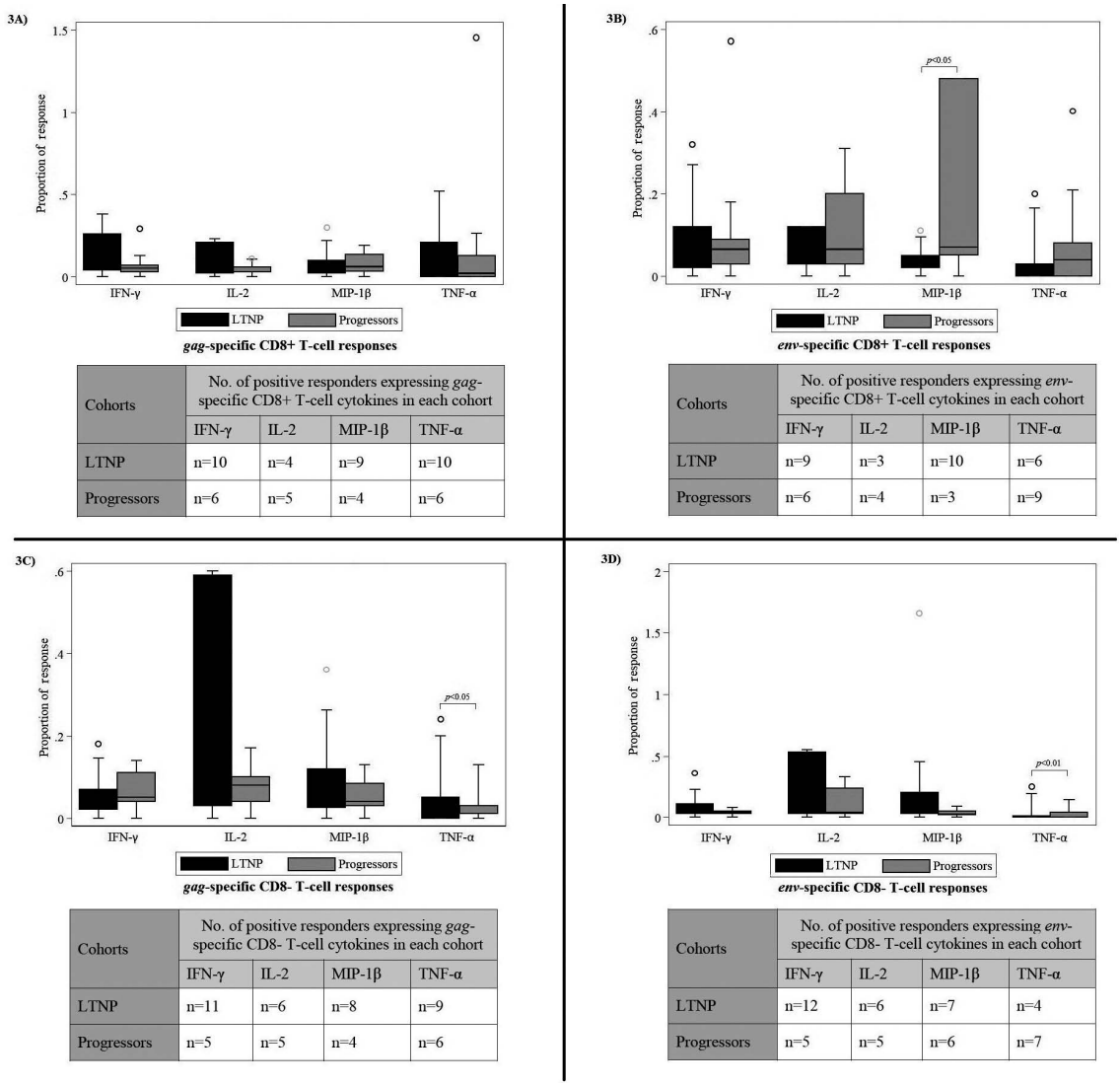
Table 1. Demographic and clinical characteristics of study participants.

Cohort	Participants	Percent Female	Median Age (IQR)	Median $CD4^+$ T-cell Count - cells/μL (IQR)	Median PVL - Copies/mL (IQR)	Median CD4% (IQR)
LTNP	n=20	80 %	37.5 (30-41.8)	773.5 (614-915.8)	1089 (133-2692.8)	29 (24.5-36.3)
Progressors	n=15	80 %	32 (30-42)	392 (344.5-425.5)	113989 (23548-282051)	19 (14-21.5)

3. RESULTS

3.1. Demographical Characteristics of the Study Population

In this study, female population comprised about 80% of the total population in both LTNP and progressors cohort. The mean age of LTNP and progressors were 37 and 35.6 years, respectively. There was no significant difference observed in the age groups of participants between the two cohorts. Demography and clinical characteristics of enrolled participants were collected from natural history data base as summarized in Table **1**.



Cohorts	No. of positive responders expressing *gag*-specific CD8+ T-cell cytokines in each cohort			
	IFN-γ	IL-2	MIP-1β	TNF-α
LTNP	n=10	n=4	n=9	n=10
Progressors	n=6	n=5	n=4	n=6

Cohorts	No. of positive responders expressing *env*-specific CD8+ T-cell cytokines in each cohort			
	IFN-γ	IL-2	MIP-1β	TNF-α
LTNP	n=9	n=3	n=10	n=6
Progressors	n=6	n=4	n=3	n=9

Cohorts	No. of positive responders expressing *gag*-specific CD8- T-cell cytokines in each cohort			
	IFN-γ	IL-2	MIP-1β	TNF-α
LTNP	n=11	n=6	n=8	n=9
Progressors	n=5	n=5	n=4	n=6

Cohorts	No. of positive responders expressing *env*-specific CD8- T-cell cytokines in each cohort			
	IFN-γ	IL-2	MIP-1β	TNF-α
LTNP	n=12	n=6	n=7	n=4
Progressors	n=5	n=5	n=6	n=7

Fig. (3). Two sided t-test showing the differences in functional profile of HIV-specific CD8+ and CD8- T-cells. (**3A**) & (**3B**): Frequency of various cytokine expressions of CD8+ T-cells against gag and env peptides showing significantly higher proportions of MIP-1β env-specific responses in progressors. (**3C**) & (**3D**): Similar cytokine expression profiles of CD8- T-cells. Significant responses were observed in gag-specific and env-specific TNF-α responses.

3.2. T-cell Responses

3.2.1. Total T-cell Responses

The quality and magnitude of specific response against HIV peptides *gag* and *env* were evaluated on CD8+ and CD8- T-cells. Expression of four cytokine markers (IL-2, IFN-γ, MIP-1β and TNF-α) were analyzed in both subsets of patients (LTNP and progressors). Here, the 'total' response is identified *i.e.* the sum of all cells positive for at least one marker, which provides the overall 'frequency' of responding cells among CD8+ and CD8-T-cells.

A sensitivity analysis performed comparing LTNP and progressors for HIV-specific CD8+ and CD8- T-cell frequencies of *gag* and *env* both individually and grouped together, revealed no significant differences between the two subsets. In addition, no significant difference was observed when total (adding both HIV-specific CD8+ and CD8- T-cell responses) HIV-specific T-cell response of *gag* was compared to *env* and vice versa.

3.2.2. Characteristics of HIV-specific T-cell Responses

Expression of cytokines such as IL-2, IFN-γ, MIP-1β and TNF-α by HIV-specific CD8+ and CD8- T-cells against *gag* and *env* peptides were compared between LTNP and progressors. Various combinations of HIV-specific T-cell subsets (*eg*. *gag*-specific CD8+IL2+, CD8+MIP-1β+, CD8-IL2+, CD8-TNFα+) were derived based on the respective cytokine expression. No significant differences were seen in the cytokine profile studied of all subsets between LTNP and progressors. Hence, only positive responders who showed a response of $\geq$0.02 in each subset in both the groups were considered and compared. Among the positive responders, significantly higher frequencies of *env*-specific CD8+MIP-1β+ T-cells (p=0.05) were found in progressors than in LTNP (Fig. **3A** & **3B**). Peculiarly, both *gag*-specific (p=0.05) and *env*-specific (p=0.01) CD8-TNF-α+ T-cell responses were significantly higher in LTNP compared to progressors (Fig. **3C** & **3D**).

In order to clarify whether PVL has any possible impact on the above results, LTNP were stratified into two sub-groups based on their control over HIV viremia: LTNP with PVL <2000 copies/mL (n=14) known as viremic controllers and LTNP with PVL >2000 copies/mL (n=6) known as viremic non-controllers [36] and similarly all possible subsets of HIV-specific CD8+ and CD8-T-cells were compared between LTNP with PVL <2000, LTNP with PVL >2000 and progressors.

When LTNP with PVL >2000 were compared with progressors, higher expression of *env*-specific CD8-IFN-γ+ T-cells was observed in LTNP (p=0.01). Also, collective responses of both *gag* and *env* showed that CD8- IFN-γ+ T-cells (p=0.01) and CD8-TNF-α+ T-cells (p=0.02) were more frequent in LTNP than in progressors. The analysis comparing all three groups (two LTNP sub-groups and progressors) also revealed a significantly higher frequencies of *env*-specific (p=0.02) and both *gag* and *env*-specific (p=0.04) CD8-IFN-γ+ T-cells in LTNP with PVL >2000. This helps to support the hypothesis that a rise in viral load influences T-cells to increase the expression of pro-inflammatory cytokines which might result in detrimental inflammation.

3.2.3. Relationship Between T-cells Responses and Disease Progression Markers

When the cytokine expression profile of all the LTNP and progressors were correlated with disease progression markers (CD4+ T-cell count, CD4% and PVL), no significant differences were observed. However, when only positive responders were analyzed, CD4% positively correlated with *gag*-specific CD8-IL-2+T-cells (r=0.8; p<0.001) and *env*-specific CD8-IL-2+ T-cells (r=0.8; p<0.001) in LTNP. No correlations between IL-2 expressing CD8+ and CD8- T-cells and disease progression markers were observed in progressors which indicate that LTNP has T-cells with better proliferative capacity.

In LTNP, collective responses of CD8-IFN-γ+ T-cells against both *gag* and *env* (r=-0.7; p<0.001) showed a negative correlation with CD4+ T-cell count which might be a cause of IFN-γ induced inflammation. CD8+TNF-α+ T-cells that are *gag*-specific correlated positively with PVL (r=0.7; p<0.001) and *env*-specific CD8+TNF-α+ T-cells correlated negatively with CD4+ T-cell count in LTNP (r=-0.9; p<0.001). It signifies that TNF-α does have its activity and role even in minimalistic disease progression which might become increasingly significant factor when LTNP show signs of progression. Surprisingly, collective responses of CD8+MIP-1β+ T-cells against both *gag* and *env* (r=-0.6; p<0.001) exhibited a negative correlation against CD4% in LTNP (Table **2A**).

Among progressors, collective expression of CD8-IFN-γ+ T-cells against both *gag* and *env* showed positive correlation with CD4+ T-cell count (r=1; p<0.001) whereas *gag*-specific CD8+IFN-γ+ T-cells negatively correlated with PVL (r=-0.8; p<0.001), suggesting an increased CD8+ T-cell activity in progressors. Strong correlation was observed between *env*-specific CD8+MIP-1β+ T-cells and CD4+ T-cell counts (r=1; p<0.001) and CD4% (r=1; p<0.001). CD8+ MIP-1β+ T-cells that are *gag*-specific also showed strong direct correlation with CD4% (r=1; p<0.001) in progressors. A positive correlation was also observed between *env*-specific CD8-MIP-1β+ T-cells and PVL (r=0.8; p<0.001). Collective responses (against *gag* and *env*) of CD8+ T-cells expressing both IL-2 and IFN-γ (*i.e.* CD8+ IL2+IFN-γ+ T-cells) seen in progressors, showed a positive correlation with CD4% (r=0.9; p<0.001).

In progresors, *env*-specific CD8+TNF-α+T-cells correlated positively with CD4% (r=1; p<0.001). Unlike CD8- T-cells in LTNP, CD8+IL-2+ T-cells correlated positively with CD4+ T-cell count (r=1; p<0.001). It should be noted that IL-2 expression by CD8+ T-cells may act as a substitute for CD8- T-cells which are probably CD4+ T-cells, in progressors, to maintain the deprived proliferation capability of their CD4+ T-cells as a result of compromised immune systems functioning with depleted CD4+ T-cell population (Table **2B**).

Segregation of LTNP based on PVL reveals a direct correlation between *env*-specific CD8-IL-2+ T-cells and CD4% (r=0.9; p<0.001) in LTNP with PVL <2000. Like the above abnormal correlations of LTNP with MIP-1β expression, collective response of CD8+MIP-1β+ T-cells against both *gag* and *env* in LTNP with PVL <2000 showed a positive correlation with PVL (r=0.7; p<0.001) and negative correlation with that of CD4% (r=-0.7; p<0.001), while the LTNP with PVL >2000 showed a negative correlation between *env*-specific CD8+MIP-1β+ T-cells and CD4+ T-cell count. These results probably suggest that MIP-1β may not be an appropriate marker to study disease progression in HIV-1 subtype C infected LTNP.

3.3. Activation and Disease Non-progression

There were no striking differences in activation rates of CD4+ and CD4- T-cells between LTNP and progressors and hence, here too PVL stratification was applied, to study the difference between the populations on the basis of their respective viral loads. This showcased a nearly two fold decrease in CD4+ T-cell activation rates (CD4+ CD38+ HLADR+ T-cells) in LTNP with PVL <2000 copies/ mL (p=0.04) compared to progressors (Fig. **4A**). Similarly, CD4+ T-cell activation rates are also significantly lower in LTNP with PVL <2000 copies/mL (p=0.007) than LTNP with PVL >2000 copies/mL (Fig. **4B**). No such difference was found between LTNP with PVL >2000 copies/mL and progressors (p=0.2).

No significant difference was observed in CD4- T-cell activation rates between the groups but, it should be mentioned that CD4- T-cell activation in progressors is higher compared to their LTNP counterparts. These results clarify that PVL might be a significant factor in amplified activation thereby contributing more susceptible cells for HIV infection and replication resulting CD4+ T-cell depletion.

When all the LTNP were considered, CD4- T-cell activation rates had an inverse correlation with CD4+ T-cell count (r=-0.5; p<0.001) while, a significant negative correlation was observed between CD4+ T-cell activation rates and CD4% (r=-0.7; p<0.001) (Table **3**). PVL stratification revealed a significant negative correlation between CD4- T-cell activation rates and CD4+ T-cell counts (r=-0.6; p<0.001) in LTNP with PVL<2000 whereas, CD4 + CD38 +

Table 2A. Correlations between HIV-specific T-cell cytokine expressions from LTNP positive responders with their respective with their respective disease progression markers such as CD4+ T-cell count, PVL and CD4%.

LTNP	CD4+ T-cell Count		PVL		CD4%	
	Spearman (r)	*p*-value	Spearman (r)	*p*-value	Spearman (r)	*p*-value
***gag*-specific CD3+CD8-IFN-γ+**	-0.4	0.2	0.1	0.7	0.2	0.6
***gag*-specific CD3+CD8-IL2+**	0.5	0.3	0.2	0.7	0.8	***<0.001***
***env*-specific CD3+CD8-IFN-γ +**	-0.6	0.1	0.4	0.2	-0.4	0.2
***env*-specific CD3+CD8-IL2+**	0.5	0.3	-0.1	0.9	0.8	***<0.001***
CD3+CD8-IFN-γ+ (collective *gag* + *env*)	-0.7	***<0.001***	0.3	0.3	-0.2	0.4
CD3+CD8-IL2+ (collective *gag* + *env*)	0.2	0.6	-0.2	0.6	0.4	0.3
***gag*-specific CD3+CD8+IFN-γ+**	-0.1	0.8	0.1	0.8	-0.1	0.8
***gag*-specific CD3+CD8+IL2+**	-0.3	0.7	0.6	0.4	0.2	0.8
***env*-specific CD3+CD8+IFN-γ+**	0.1	0.9	0.4	0.2	0.3	0.4
***env*-specific CD3+CD8+IL2+**	-0.5	0.7	0.5	0.7	0.5	0.7
CD3+CD8+IFN-γ+ (collective *gag* + *env*)	-0.2	0.6	0	0.9	0.1	0.7
CD3+CD8+IL2+ (collective *gag* + *env*)	-0.4	0.6	0.9	0.1	0.2	0.7
***gag*-specific CD3+CD8-MIP-1β+**	0	0.9	0.1	0.8	-0.3	0.5
***gag*-specific CD3+CD8-TNF-α+**	0	0.9	-0.2	0.6	0.1	0.8
***env*-specific CD3+CD8-MIP-1β+**	-0.7	0.1	0.2	0.6	-0.4	0.4
***env*-specific CD3+CD8-TNF-α+**	-1	ns	0.6	0.4	-1	ns
CD3+CD8-TNF-α+ (collective *gag* + *env*)	-0.3	0.4	0.1	0.7	0	0.9
***gag*-specific CD3+CD8+MIP-1β+**	0.2	0.5	-0.2	0.7	0.2	0.6
***gag*-specific CD3+CD8+TNF-α+**	-0.2	0.5	0.7	***<0.001***	-0.4	0.3
***env*-specific CD3+CD8+MIP-1β+**	-0.5	0.1	-0.2	0.7	0.1	0.9
***env*-specific CD3+CD8+TNF-α+**	-0.9	***<0.001***	0.3	0.5	-0.7	0.2
CD3+CD8+MIP-1β+ (collective *gag* + *env*)	-0.3	0.3	0.5	0.1	-0.6	***<0.001***
CD3+CD8-TNF-α+ (collective *gag* + *env*)	-0.3	0.4	0.5	0.1	-0.3	0.5

ns = no significance

Table 2B. **Correlations between HIV-specific T-cell cytokine expressions from positive responders of progressors with their respective with their respective disease progression markers such as CD4+ T-cell count, PVL and CD4%.**

Progressors	CD4+ T-cell Count		PVL		CD4%	
	Spearman (r)	*p*-value	Spearman (r)	*p*-value	Spearman (r)	*p*-value
***gag*-specific CD3+CD8-IFN-γ+**	0.90	0.10	0.70	0.20	-0.40	0.60
***gag*-specific CD3+CD8-IL2+**	-0.30	0.60	0.40	0.50	-0.20	0.70
***env*-specific CD3+CD8-IFN-γ +**	0.20	0.70	0.20	0.70	-0.70	0.20
***env*-specific CD3+CD8-IL2+**	-0.60	0.30	-0.70	0.20	-0.40	0.50
CD3+CD8-IFN-γ+ (collective *gag* + *env*)	1.00	***<0.001***	0.40	0.30	-0.30	0.40
CD3+CD8-IL2+ (collective *gag* + *env*)	-0.70	0.10	-0.20	0.70	-0.50	0.30
***gag*-specific CD3+CD8+IFN-γ+**	0.10	0.90	-0.80	***<0.001***	0.70	0.10
***gag*-specific CD3+CD8+IL2+**	-0.60	0.30	-0.50	0.40	-0.60	0.30
***env*-specific CD3+CD8+IFN-γ+**	-0.10	0.90	-0.60	0.20	0.70	0.10
***env*-specific CD3+CD8+IL2+**	1.00	***<0.001***	0.80	0.20	0.20	0.80
CD3+CD8+IFN-γ+ (collective *gag* + *env*)	0.00	0.90	-0.60	0.10	0.60	0.20
CD3+CD8+IL2+ (collective *gag* + *env*)	-0.30	0.50	-0.60	0.10	0.00	1.00
***gag*-specific CD3+CD8-MIP-1β+**	0.30	0.70	0.60	0.40	0.90	0.10
***gag*-specific CD3+CD8-TNF-α+**	-0.50	0.30	-0.60	0.20	0.20	0.70
***env*-specific CD3+CD8-MIP-1β+**	0.40	0.40	0.80	***<0.001***	0.30	0.50
***env*-specific CD3+CD8-TNF-α+**	0.20	0.70	0.30	0.50	-0.20	0.60
CD3+CD8-TNF-α+ (collective *gag* + *env*)	-0.30	0.40	-0.30	0.30	-0.20	0.50
***gag*-specific CD3+CD8+MIP-1β+**	0.40	0.60	0.40	0.60	1.00	***<0.001***
***gag*-specific CD3+CD8+TNF-α+**	0.40	0.40	-0.20	0.70	-0.30	0.60
***env*-specific CD3+CD8+MIP-1β+**	1.00	***<0.001***	0.50	0.70	1.00	***<0.001***
***env*-specific CD3+CD8+TNF-α+**	0.60	0.10	-0.20	0.60	0.80	***<0.001***
CD3+CD8+MIP-1β+ (collective *gag* + *env*)	0.00	1.00	0.30	0.60	0.10	0.90
CD3+CD8-TNF-α+ (collective *gag* + *env*)	0.40	0.30	-0.10	0.70	-0.10	0.80

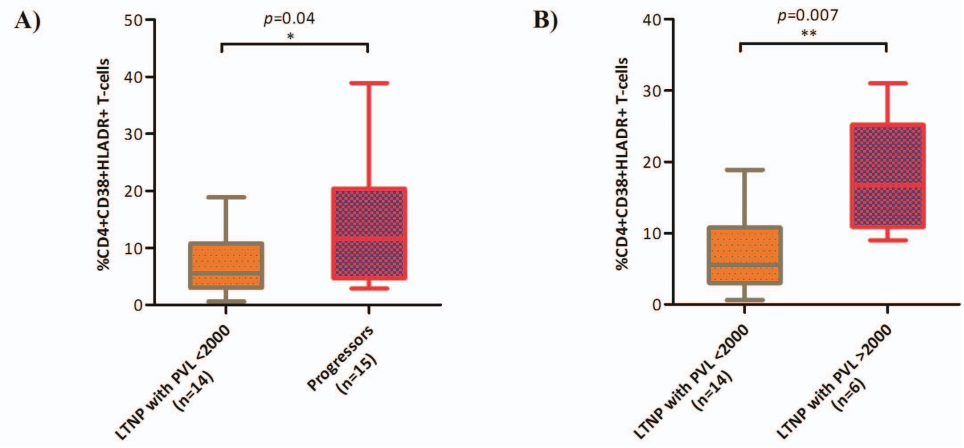


Fig. (4). 4A) Box plot showing a significant difference in activation rates between LTNP with PVL<2000 copies/mL and progressors. **4B**) Similar plot showing the difference in activation rates between LTNP with PVL <2000 copies/mL and LTNP with PVL >2000 copies/mL.

Table 3. Detailed comparison of activated T-cell subsets in LTNP and progressors with their respective disease progression markers such as CD4+ T-cell count, PVL and CD4%.

Cohort	CD4+ T-cell Count		PVL		CD4%	
	Spearman (r)	*p* value	Spearman (r)	*p* value	Spearman (r)	*p* value
LNTP						
CD4+CD38+HLADR+ T-cells	-0.3	0.1	0.7	***<0.001***	-0.7	***<0.001***
CD4-CD38+HLADR+ T-cells	-0.5	***<0.001***	0.0	0.8	0	0.9
Progressors						
CD4+CD38+HLADR+ T-cells	0.1	0.7	0.1	0.6	0.1	0.8
CD4-CD38+HLADR+ T-cells	0.1	0.7	0.1	0.7	0.4	0.2

HLADR + T-cells correlated inversely with CD4% (r=-0.8; $p<0.001$) in LTNP with PVL >2000. No significant correlations were found between activated T-cells of progressors and their respective disease progression markers.

4. DISCUSSION

HIV-specific immune responses have been attributed to the effective control of viremia [31, 37]. As part of these adaptive response mechanisms, CD4+ T-cell responses, CD8+ T-cell responses and neutralizing antibodies are the main factors playing a central role in viral control [38, 39]. A consistent association has always been found between control of infection and CD8+ T-cell responses. During primary HIV infection, expansion of HIV-specific CD8+ T-cell responses corresponds with the decrease in viremia [40, 41]. Virus infected host cells are terminated by the presence of classic CD8+ T-cell responses and *gag*-specific CD8+ T-cell responses have been shown to correlate well with reduced PVL [42, 43]. Studies on macaque models showed *in vivo* depletion of CD8+ T-cells are unable to control acute SIV infection [44] and elevated viral loads in SIV infected macaques with chronic infection [45, 46].

Numerous studies have highlighted the importance of immune tolerance in HIV disease non-progression in LTNP. CD8+ T-cells act as potential medications in lowering PVL levels and that it strongly correlates in facilitating immune control in LTNP [47, 48]. Unlike progressors, LTNP has been found to possess robust, polyclonal T-cell responses and that are maintained for longer duration during HIV infection, which directly correlates with their decreased viral load [14, 16]. Broadly reactive CD8+ T-cell responses against conserved sequences of *env*, *gag* and *pol* genes have also been reported in LTNP [47, 49].

In this study, HIV-specific responses of CD8+ T-cell and CD8- T-cell (probably CD4+ T-cells) responses against *gag* and *env* were analyzed in LTNP and progressors. Interestingly, there were no significant differences in the frequencies of IL-2 and IFN-γ in both CD8+ and CD8- T-cells against both *gag* and *env* peptides and many LTNP failed to mount a significant response to HIV peptides. Though contrary to many studies, this is in line with a finding revealing that the role of *gag*-specific CD8+ T-cells expressing IL-2 and IFN-γ in durable control of is limited [50]. When only positive responders were considered, frequency of TNF-α expressing CD8-T-cell against *gag* and *env* individually, was significantly higher in LTNP. This shows a striking contrast with other studies [9, 51], where TNF-α expression was shown to be significantly superior in HIV infected patients with progressive infection. Here, increasing PVL in LTNP might

have played a major role in higher TNF-α expression, since LTNP with PVL >2000 also expressed significantly higher frequencies of TNF-α in CD8- T-cells against collective of both peptides. Effect of gradual CD4 T-cell depletion by means of TNF related apoptosis [52, 53] might result in increased TNF-α expression in LTNP. Another possible explanation is that 20% of LTNP involved in this study were HIV infected for more than 10 years and it is certain that they might be on the verge of losing their LTNP status as a result of compromised tolerance. This can be concluded only by longitudinal studies and quantitative analysis of TRAIL and other apoptosis related markers in LTNP comparing with other groups of HIV-infected individuals.

LTNP with PVL >2000 witnessed a spiked frequency of *env*-specific and both *gag*-specific and *env*-specific CD8-IFN-γ+ T-cells. IFN-γ expression by LTNP despite PVL, being elevated which is in line with other studies [1, 14, 54], but here it is CD8-T-cells but not CD8+ T-cells. CD8- T-cells are not classical cytotoxic T-cells, but still express IFN-γ. Helper T-cells exhibiting cytotoxicity have already been characterized in persistent viral infections [55-57] and similar phenomenon has also been explained in PBMC from rare HIV infected individuals and LTNP [39, 40]. Hence, this can be hypothesized that CD8- T-cells might be stressed to perform cytolytic activities in order compensate the impaired CD8+ T-cell cytotoxic responses in LTNP (4) which is not even observed in progressors, possibly indicating the presence of completely impaired CD8+ as well as CD8- T-cells.

Contrary to other studies [1, 9], significant elevated frequency of MIP-1β by CD8+ T-cells against *env* were seen in progressors, while positive correlations between *gag*-specific and *env*-specific CD8+T-cells expressing MIP-1β with CD4+ T-cell count and CD4% were observed. Protection against HIV disease progression by β-chemokines such as MIP-1α, MIP-1β and RANTES differs with the relative affinity of chemokines with β-chemokine receptors of T-cells. Of the β-chemokines, MIP-1β has a very narrower range of immunological activity in terms of binding only with CKR5 [58-60] and not with other C-C chemokine receptors. It should be noticed that MIP-1β levels were elevated even in plasma of progressors which might have also reflected at the cellular level. Studies have reported an inverse correlation between β-chemokines and PVL and direct correlation with CD4+ T-cell counts regardless of the stage of HIV infection [61-63]. It should be noted that these studies do not exclude the effect of ART whereas in this study the progressor cohort were ART naïve. In line with this study, a positive association between β-chemokines and PVL along with activation was noted. It is suggested that increased frequency of β-chemokine positive T cells in HIV-1-infected female sex workers is likely to be a result of chronic T cell activation in these patients, which, in turn, may be driven by HIV-1 viremia [64]. However, evaluation of other β-chemokines along with MIP-1β is highly required to establish the underlying mechanism behind HIV disease non-progression in LTNP. Though there were no significant differences in IL-2 expression between two groups, direct correlations of HIV-specific CD8- T-cells expressing IL-2 with CD4% were observed in LTNP. This indicates an increased T-cell proliferation in LTNP compared to progressors.

This study did not draw any significant correlations with cytokine profile and disease non progression in LTNP. During acute infection, size or frequency of HIV-specific CD8+ T-cell responses and their negative correlation with PVL is interpreted as the effectiveness of these responses. But in persistent infection, efficacy and frequency of CD8+ T-cell responses are not clearly evaluated. It is because of the factor that proliferation of CD8+ T-cell responses occurs only in response to antigens and frequency of these responses is both a cause and an effect of the PVL. CD8+ T-cell responses that are efficient, proliferates rapidly and terminates HIV infected host cells which result in the reduction of antigenic stimulus [2]. Decay in the frequency of HIV-specific CD8+ T-cell responses after initiation of HAART, which suppresses PVL independent of host immune responses were strongly evidenced [65-67]. These studies clearly indicate the frequency of CD8+ T-cell responses were dependent on antigen load. Similarly, in this study lower HIV-specific CD8+ T-cell responses even in LTNP with PVL <2000 due to lack of antigenic stimulus cannot be ruled out. But this can be debated on the reason why progressors who are HAART naive cannot mount a significant response? This can possibly be explained by indicating that their basic immunological characteristics such as CD4+ T-cell counts and CD8+ T-cell counts present in lower levels which also might be of compromised quality. Hence, lower CD4+ T-cell counts might not provide adequate help for CD8+ T-cells to proliferate and mount a significant response.

This study agrees with the fact that contradictory phenomenon of CD8+ T-cell responses can only be concluded by studying critical attributes of these cells that would determine its efficiency such as CD8+ T-cell proliferation rate at a given PVL and the rate of CD8+ T-cell mediated lysis of infected cells at a given antigen load [2]. Controllers usually possess a lower CD4+ T-cell activation rates than progressors [68-70], while here the LTNP with PVL <2000 had significantly decreased CD4+ T-cell activation rates. Overall activation of CD4+ T-cells was lower in LTNP compared to progressors in this study, but when PVL categorization is applied, LTNP with PVL >2000 had high activation rates. CD4- T-cell activation rates (probably CD8+ T-cells) were quite similar in both LTNP and progressors. Categorization of LTNP with PVL could not draw any significant relevance.

Though a low CD4+ T-cell activation levels were seen in LTNP, it pulls a negative correlation with CD4%. Similarly CD4- T-cells also possess a negative correlation with CD4+ T-cell count in progressors. These factors seem contrasting when compared to other studies. Supporting these findings, elite controllers and HIV infected ART suppressed patients were reported to have similar activation rates of T-cells [71]. Even though CD4+ T-cell counts were maintained at normal levels, reports suggest that low PVL levels were strongly associated with a decline in CD4+ T-cell counts has been reported in few virologic controllers [71-73]. This observation can be applied to the observations in this study to possibly explain the negative correlations observed with activation rates and CD4+ T-cell frequencies in LTNP. Moreover, this study involved participants with much higher PVL levels, establishment of association between chronic inflammation and progressive immunodeficiency in LTNP, has to be considered similar to other findings [71, 74].

CD8+ T-cell activation rates might have an underlying association with gradual CD4+ T-cell depletion and clinical progression of HIV in untreated patients. Similarly, this study also reports CD4- T-cell activation (probably CD8+ T-cells) rates between LTNP and progressors which serve as a marker for immune deterioration in LTNP due to their choric nature of the infection and thus matching the CD4- T-cell activation rates with progressors. It is also observed that T-cell activation is directly associated with CD4+ T-cell decline than the extent of PVL levels in HIV-2 infected cases [75].

Microbial translocation as a result of high plasma LPS levels was attributed for high T-cell activation and factors alike influencing the negative correlation with CD4+ T-cell counts and CD4% cannot be excluded in this study. Here, no LPS levels were measured and in addition, the presence of other co-infections might have also influenced T-cell activation. Hence, data on evidences of microbial translocation and co-infections receives paramount importance even though LTNP maintained high CD4+ T-cell counts and low viral loads to decide the effects of activation in disease non-progression in LTNP. The presence of such data in this study could be advantageous to conclude the effect of microbial translocation in T-cell activation in LTNP.

With respect to activation, it is evident from this study that CD4+ T-cell counts and PVL levels may not serve as an absolute measure of viral pathogenesis and disease non-progression. Lack of major differences in T-cell activation profile between LTNP and progression and in comparison with their respective disease progression markers might be due to the longevity of HIV infection and chronic stages of the disease in which the specimens have been collected for this cross-sectional study. Besides CD4+ T-cell counts and PVL, it is suggested that in LTNP periodic screening of T-cell activation rates must be entertained, especially during the chronic stages. This helps in ascertaining the LTNP status and provides evidences of disease progression well before CD4+ T-cell depletion. This in turn might serve as an accurate predictor of CD4+ T-cell depletion which could improvise treatment strategies of HIV infected individuals.

Exploring the nature and degree of HIV-specific CD8+ and CD8- T-cell cytokine responses between LTNP and progressors reveals no major differences which are contrasting to other major studies yet interesting. It is unclear whether the results derived were because of selection of complete epitopes rather than particular region. But LTNP were shown to mount higher responses even to complete epitopes. Evidences also suggest that HIV-specific responses succeed only in a small number of people and fails in most. Taking these factors into consideration, studying and analyzing viral and genetic factors might draw a convincing conclusion for disease progression in LTNP especially in the region where this study is conducted. It can also be hypothesized that ethnic and HIV-1 subtype variations, as evidenced from earlier studies could be the likely factors influencing disease non-progression of LTNP. Activation rates can be used as an appropriate marker to monitor disease progression in LTNP, since it increases when the PVL levels are >2000 copies/mL.

CONCLUSION

Exploring the nature and degree of HIV-specific CD8+ and CD8- T-cell cytokine responses between LTNP and progressors reveals no major differences which are contrasting to other major studies yet interesting. It is unclear whether the results derived were because of selection of complete epitopes rather than particular region. But LTNP were shown to mount higher responses even to complete epitopes. Evidences also suggest that HIV-specific responses succeed only in a small number of people and fails in most. Taking these factors into consideration, studying and analyzing viral and genetic factors might draw a convincing conclusion for disease progression in LTNP especially in the region where this study is conducted. It can also be hypothesized that ethnic and HIV-1 subtype variations, as evidenced from earlier studies could be the likely factors influencing disease non-progression of LTNP. Activation rates can be used as an appropriate marker to monitor disease progression in LTNP, since it increases when the PVL levels are >2000 copies/mL.

LIMITATIONS

Our study has a small sample size considering the practical difficulties in enrolling LTNP cohort. This is particularly a problem in our study, once we further stratified the LTNP group based on PVL. A longitudinal study with LTNP enrolled when they are in potential LTNP stage, might be effective in studying the immune correlates since this is a cross-sectional and the length of infection of LTNP enrolled here is almost 10 years. Polyfunctionality cannot be studied since a 5-color flow cytometer was used and the resources are limited. HIV-specific activation was not studied which is also due to limited resources.

LIST OF ABBREVIATIONS

ART	=	Anti-retroviral therapy
CTL	=	Cytotoxic T- lymphocyte
HIV	=	Human immunodeficiency virus
LTNP	=	Long-term non-progressors
PBMCs	=	Peripheral blood mononuclear cells
PVL	=	Plasma viral load
SIV	=	Simian immunodeficiency virus

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the institutional review board of Y.R. Gaitonde Centre for AIDS Research and Education, VHS Hospital Campus, Taramani, Chennai, India.

HUMAN AND ANIMAL RIGHTS

No animals were used in the study. All humans research procedures followed were in accordance with the standards set forth in the Declaration of Helsinki principles of 1975, as revised in 2008 (http://www.wma.net/en/20activities/10 ethics/10helsinki/).

CONSENT FOR PUBLICATION

Duly signed written informed consent forms were obtained from all the willing participants.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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REFERENCES

[1] Betts MR, Nason MC, West SM, *et al.* HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. Blood 2006; 107(12): 4781-89.

[2] Bangham CRM. CTL quality and the control of human retroviral infections. Eur J Immunol 2009; 39(7): 1700-12.

[3] Allen TM, O'Connor DH, Jing P, *et al.* Tat-specific cytotoxic T lymphocytes select for SIV escape variants during resolution of primary viraemia. Nature 2000; 407(6802): 386-90.

[4] Nemes E, Bertoncelli L, Lugli E, *et al.* Cytotoxic granule release dominates gag-specific CD4+ T-cell response in different phases of HIV infection: AIDS 2010; 24(7): 947-57.

[5] Davenport MP, Ribeiro RM, Perelson AS. Kinetics of virus-specific CD8+ T cells and the control of human immunodeficiency virus infection. J Virol 2004; 78(18): 10096-10103.

[6] Daucher M, Price DA, Brenchley JM, *et al.* Virological outcome after structured interruption of antiretroviral therapy for human immunodeficiency virus infection is associated with the functional profile of virus-specific CD8+ T cells. J Virol 2008; 82(8): 4102-4114.

[7] Almeida JR, Price DA, Papagno L, *et al.* Superior control of HIV-1 replication by CD8+ T cells is reflected by their avidity, polyfunctionality, and clonal turnover. J Exp Med 2007; 204(10): 2473-85.

[8] Seder RA, Darrah PA, Roederer M. T-cell quality in memory and protection: implications for vaccine design. Nat Rev Immunol 2008; 8(4): 247-58.

[9] López M, Soriano V, Lozano S, *et al.* no major differences in the functional profile of hiv gag and nef-specific cd8+ reponses between long-term nonprogressors and typical progressors. AIDS Res Hum Retroviruses 2008; 24(9): 1185-95.

[10] Madec Y, Boufassa F, Avettand-Fenoel V, *et al.* Early control of HIV-1 infection in long-term nonprogressors followed since diagnosis in the ANRS SEROCO/HEMOCO cohort. J Acquir Immune Defic Syndr 1999 2009; 50(1): 19-26.

[11] Rodés B, Toro C, Paxinos E, *et al.* Differences in disease progression in a cohort of long-term non-progressors after more than 16 years of HIV-1 infection. AIDS Lond Engl 2004; 18(8): 1109-16.

[12] Goudsmit J, Bogaards JA, Jurriaans S, *et al.* Naturally HIV-1 seroconverters with lowest viral load have best prognosis, but in time lose control of viraemia. AIDS Lond Engl 2002; 16(5): 791-93.

[13] Okulicz JF, Lambotte O. Epidemiology and clinical characteristics of elite controllers. Curr Opin HIV AIDS 2011 May; 6(3): 163-68.

[14] Boaz MJ, Waters A, Murad S, Easterbrook PJ, Vyakarnam A. Presence of HIV-1 Gag-specific IFN-γ+ IL-2+ and CD28+ IL-2+ CD4 T cell responses is associated with nonprogression in HIV-1 infection. J Immunol 2002; 169(11): 6376-85.

[15] Poropatich K, Sullivan DJJ. Human immunodeficiency virus type 1 long-term non-progressors: the viral, genetic and immunological basis for disease non-progression. J Gen Virol 2011; 92(Pt 2): 247-68.

[16] Migueles SA, Laborico AC, Shupert WL, *et al.* HIV-specific CD8+ T cell proliferation is coupled to perforin expression and is maintained in nonprogressors. Nat Immunol 2002; 3(11): 1061-68.

[17] Gea-Banacloche JC, Migueles SA, Martino L, *et al.* Maintenance of large numbers of virus-specific CD8+ T cells in HIV-infected progressors and long-term nonprogressors. J Immunol 2000; 165(2): 1082-92.

[18] Mothe B, Llano A, Ibarrondo J, *et al.* CTL responses of high functional avidity and broad variant cross-reactivity are associated with HIV control. PloS One 2012; 7(1): e29717.

[19] Ndhlovu ZM, Chibnik LB, Proudfoot J, *et al.* High-dimensional immunomonitoring models of HIV-1-specific CD8 T-cell responses accurately identify subjects achieving spontaneous viral control. Blood 2013; 121(5): 801-11.

[20] Rehr M, Cahenzli J, Haas A, Price DA, Gostick E, Huber M, *et al.* Emergence of polyfunctional CD8+ T cells after prolonged suppression of human immunodeficiency virus replication by antiretroviral therapy. J Virol 2008; 82(7): 3391-3404.

[21] Benito JM, López M, Lozano S, *et al.* Phenotype and functional characteristics of HIV-specific cytotoxic CD8+ T cells in chronically infected patients. J AIDS 2003; 34: 255-66.

[22] Champagne P, Ogg GS, King AS, *et al.* Skewed maturation of memory HIV-specific CD8 T lymphocytes. Nature 2001; 410(6824): 106-111.

[23] Appay V, Dunbar PR, Callan M, *et al.* Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. Nat Med 2002; 8(4): 379-85.

[24] Ghiglione Y, Falivene J, Ruiz MJ, *et al.* Early skewed distribution of total and hiv-specific cd8+ t-cell memory phenotypes during primary hiv infection is related to reduced antiviral activity and faster disease progression. PLoS ONE 2014; 9(8): e104235.

[25] Appay V, Nixon DF, Donahoe SM, *et al.* HIV-specific CD8+ T cells produce antiviral cytokines but are impaired in cytolytic function. J Exp Med 2000; 192(1): 63-76.

[26] Kostense S, Vandenberghe K, Joling J, *et al.* Persistent numbers of tetramer+ CD8+ T cells, but loss of interferon-γ+ HIV-specific T cells during progression to AIDS. Blood 2002; 99(7): 2505-11.

[27] Choudhary SK, Vrisekoop N, Jansen CA, *et al.* Low immune activation despite high levels of pathogenic human immunodeficiency virus type 1 results in long-term asymptomatic disease. J Virol 2007; 81(16): 8838-42.

[28] Deeks SG, Kitchen CMR, Liu L, *et al.* Immune activation set point during early HIV infection predicts subsequent CD4+ T-cell changes independent of viral load. Blood 2004; 104(4): 942-47.

[29] Kamya P, Tsoukas CM, Boulet S , *et al.* T cell Activation does not drive CD4 decline in longitudinally followed HIV-infected Elite Controllers. AIDS Res Ther 2011; 8(1): 20.

[30] Carbone J, Gil J, Benito JM, Fernandez-Cruz E. Decreased expression of activation markers on CD4 T lymphocytes of HIV-infected long-term non-progressors. Aids 2003; 17(1): 133-34.

[31] Gaardbo JC, Hartling HJ, Gerstoft J, Nielsen SD. Thirty years with HIV infection—nonprogression is still puzzling: lessons to be learned from controllers and long-term nonprogressors. AIDS Res Treat 2012; 2012.

[32] Bégaud E, Chartier L, Marechal V, *et al.* Reduced CD4 T cell activation and *in vitro* susceptibility to HIV-1 infection in exposed uninfected Central Africans. Retrovirology 2006; 3: 35.

[33] Broussard SR, Staprans SI, White R, Whitehead EM, Feinberg MB, Allan JS. Simian immunodeficiency virus replicates to high levels in naturally infected African green monkeys without inducing immunologic or neurologic disease. J Virol 2001; 75(5): 2262-75.

[34] Whittall T, Peters B, Rahman D, Kingsley CI, Vaughan R, Lehner T. Immunogenic and tolerogenic signatures in human immunodeficiency virus (HIV)-infected controllers compared with progressors and a conversion strategy of virus control. Clin Exp Immunol 2011 Nov; 166(2): 208-17.

[35] Vieillard V, Fausther-Bovendo H, Samri A, Debré P. French Asymptomatiques à Long Terme (ALT) ANRS-CO15 Study Group. Specific phenotypic and functional features of natural killer cells from HIV-infected long-term nonprogressors and HIV controllers. J Acquir Immune Defic Syndr 1999 2010; 53(5): 564-73.

[36] Okulicz JF, Marconi VC, Landrum ML, *et al.* Clinical outcomes of elite controllers, viremic controllers, and long-term nonprogressors in the us department of defense hiv natural history study. J Infect Dis 2009; 200(11): 1714-23.

[37] Chevalier MF, Jülg B, Pyo A, *et al.* HIV-1-Specific interleukin-21+ cd4+ t cell responses contribute to durable viral control through the modulation of hiv-specific cd8+ t cell function. J Virol 2011; 85(2): 733-41.

[38] Paranjape RS. Immunopathogenesis of HIV infection. Indian J Med Res 2005; 121(4): 240-55.

[39] Migueles SA, Tilton JC, Connors M. Advances in understanding immunologic control of HIV infection. Curr HIV/AIDS Rep 2004; 1(1): 12-7.

[40] Koup RA, Safrit JT, Cao Y, *et al.* Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J Virol 1994; 68(7): 4650-5.

[41] Saez-Cirion A, Jacquelin B, Barré-Sinoussi F, Müller-Trutwin M. Immune responses during spontaneous control of HIV and AIDS: what is the hope for a cure? Philos Trans R Soc Lond B Biol Sci 2014; 369(1645): 20130436.

[42] Kiepiela P, Ngumbela K, Thobakgale C, *et al.* CD8+ T-cell responses to different HIV proteins have discordant associations with viral load. Nat Med 2007; 13(1): 46-53.

[43] Levy JA. HIV pathogenesis: 25 years of progress and persistent challenges. Aids 2009; 23(2): 147-160.

[44] Okoye A, Park H, Rohankhedkar M, *et al.* Profound CD4+/CCR5+ T cell expansion is induced by CD8+ lymphocyte depletion but does not account for accelerated SIV pathogenesis. J Exp Med 2009; 206(7): 1575-88.

[45] Schmitz JE, Simon MA, Kuroda MJ, *et al.* A nonhuman primate model for the selective elimination of CD8+ lymphocytes using a mouse-human chimeric monoclonal antibody. Am J Pathol 1999; 154(6): 1923-32.

[46] Jin X, Bauer DE, Tuttleton SE, *et al.* Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques. J Exp Med 1999; 189(6): 991-98.

[47] Migueles SA, Connors M. Long-term nonprogressive disease among untreated HIV-infected individuals: clinical implications of understanding immune control of HIV. JAMA J Am Med Assoc 2010; 304(2): 194-201.

[48] Demers KR, Reuter MA, Betts MR. CD8+ T-cell effector function and transcriptional regulation during HIV pathogenesis. Immunol Rev 2013; 254(1): 190-206.

[49] Geldmacher C, Currier JR, Herrmann E, *et al.* CD8 T-cell recognition of multiple epitopes within specific Gag regions is associated with maintenance of a low steady-state viremia in human immunodeficiency virus type 1-seropositive patients. J Virol 2007; 81(5): 2440-48.

[50] Emu B, Sinclair E, Hatano H, *et al.* HLA class I-restricted T-cell responses may contribute to the control of human immunodeficiency virus infection, but such responses are not always necessary for long-term virus control. J Virol 2008; 82(11): 5398-407.

[51] Altfeld M, Addo MM, Kreuzer KA, *et al.* T (H) 1 to T (H) 2 shift of cytokines in peripheral blood of HIV-infected patients is detectable by reverse transcriptase polymerase chain reaction but not by enzyme-linked immunosorbent assay under nonstimulated conditions. J Acquir Immune Defic Syndr 1999 2000; 23(4): 287-94.

[52] Pasquereau S, Kumar A, Herbein G. Targeting TNF and TNF receptor pathway in hiv-1 infection: from immune activation to viral reservoirs. Viruses 2017; 9(4): pii: E64.

[53] Cummins NW, Badley AD. Mechanisms of HIV-associated lymphocyte apoptosis: 2010. Cell Death Dis 2010; 1(11): e99.

[54] Zimmerli SC, Harari A, Cellerai C, Vallelian F, Bart P-A, Pantaleo G. HIV-1-specific IFN-γ/IL-2-secreting CD8 T cells support CD4-independent proliferation of HIV-1-specific CD8 T cells. Proc Natl Acad Sci U S A 2005; 102(20): 7239-44.

[55] Khanna R, Burrows SR, Thomson SA, *et al.* Class I processing-defective Burkitt's lymphoma cells are recognized efficiently by CD4+ EBV-specific CTLs. J Immunol 1997; 158(8): 3619-25.

[56] Casazza JP, Betts MR, Price DA, *et al.* Acquisition of direct antiviral effector functions by CMV-specific CD4+ T lymphocytes with cellular maturation. J Exp Med 2006; 203(13): 2865-77.

[57] Zhou W, Sharma M, Martinez J, *et al.* Functional characterization of BK virus-specific CD4+ T cells with cytotoxic potential in seropositive adults. Viral Immunol 2007; 20(3): 379-88.

[58] Premack BA, Schall TJ. Chemokine receptors: gateways to inflammation and infection. Nat Med 1996; 2(11): 1174-78.

[59] Adams DH, Rlloyd A. Chemokines: leucocyte recruitment and activation cytokines. The Lancet 1997; 349(9050): 490-95.

[60] Ullum H, Cozzi Lepri A, Victor J, *et al.* Production of beta-chemokines in human immunodeficiency virus (HIV) infection: evidence that high levels of macrophage inflammatory protein-1beta are associated with a decreased risk of HIV disease progression. J Infect Dis 1998; 177(2): 331-36.

[61] Paxton WA, Neumann AU, Kang S, *et al.* RANTES production from CD4+ lymphocytes correlates with host genotype and rates of human immunodeficiency virus type 1 disease progression. J Infect Dis 2001; 183(11): 1678-81.

[62] Ferbas J, Giorgi JV, Amini S, *et al.* Antigen-specific production of RANTES, macrophage inflammatory protein (MIP)-1alpha, and MIP-1beta *in vitro* is a correlate of reduced human immunodeficiency virus burden *in vivo*. J Infect Dis 2000; 182(4): 1247-50.

[63] Cocchi F, DeVico AL, Yarchoan R, *et al.* Higher macrophage inflammatory protein (MIP)-1α and MIP-1β levels from CD8+ T cells are associated with asymptomatic HIV-1 infection. Proc Natl Acad Sci U S A 2000; 97(25): 13812-17.

[64] Jennes W, Sawadogo S, Koblavi-Dème S, *et al.* Positive association between beta-chemokine-producing T cells and HIV type 1 viral load in HIV-infected subjects in Abidjan, Côte d'Ivoire. AIDS Res Hum Retroviruses 2002; 18(3): 171-77.

[65] Ogg GS, Jin X, Bonhoeffer S, *et al.* Decay kinetics of human immunodeficiency virus-specific effector cytotoxic T lymphocytes after combination antiretroviral therapy. J Virol 1999; 73(1): 797-800.

[66] Kalams SA, Goulder PJ, Shea AK, *et al.* Levels of human immunodeficiency virus type 1-specific cytotoxic T-lymphocyte effector and memory responses decline after suppression of viremia with highly active antiretroviral therapy. J Virol 1999; 73(8): 6721-28.

[67] Casazza JP, Betts MR, Picker LJ, Koup RA. Decay kinetics of human immunodeficiency virus-specific CD8+ T cells in peripheral blood after initiation of highly active antiretroviral therapy. J Virol 2001; 75(14): 6508-16.

[68] Emu B, Sinclair E, Favre D, *et al.* Phenotypic, functional, and kinetic parameters associated with apparent T-cell control of human immunodeficiency virus replication in individuals with and without antiretroviral treatment. J Virol 2005; 79(22): 14169-78.

[69] Sáez-Cirión A, Lacabaratz C, Lambotte O, *et al.* HIV controllers exhibit potent CD8 T cell capacity to suppress HIV infection ex vivo and peculiar cytotoxic T lymphocyte activation phenotype. Proc Natl Acad Sci U S A 2007; 104(16): 6776-81.

[70] Owen RE, Heitman JW, Hirschkorn DF, *et al.* HIV+ elite controllers have low HIV-specific T-cell activation yet maintain strong, polyfunctional T-cell responses. AIDS Lond Engl 2010; 24(8): 1095-105.

[71] Hunt PW, Brenchley J, Sinclair E, *et al.* Relationship between T cell activation and CD4+ T cell count in HIV-seropositive individuals with undetectable plasma HIV RNA levels in the absence of therapy. J Infect Dis 2008; 197(1): 126-33.

[72] Mellors JW, Margolick JB, Phair JP, *et al.* Prognostic value of HIV-1 RNA, CD4 cell count, and CD4 Cell count slope for progression to AIDS and death in untreated HIV-1 infection. JAMA 2007; 297(21): 2349-50.

[73] Rodríguez B, Sethi AK, Cheruvu VK, *et al.* Predictive value of plasma HIV RNA level on rate of CD4 T-cell decline in untreated HIV infection. JAMA 2006; 296(12): 1498-506.

[74] Tesselaar K, Arens R, van Schijndel GMW, *et al.* Lethal T cell immunodeficiency induced by chronic costimulation via CD27-CD70 interactions. Nat Immunol 2003; 4(1): 49-54.

[75] Sousa AE, Carneiro J, Meier-Schellersheim M, Grossman Z, Victorino RMM. CD4 T cell depletion is linked directly to immune activation in the pathogenesis of HIV-1 and HIV-2 but only indirectly to the viral load. J Immunol Baltim Md 1950 2002; 169(6): 3400-6.