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Research Article

Low Expression of $CD4^+CD25^+$ T Regulatory Cells is Associated with High Level of Immune Activation in HAART Naïve HIV-1 Infected Persons

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ABSTRACT

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Regulatory T cells (Tregs) are a subset of T cells that play a key role in immunoregulation. The naturally occurring $CD4^+CD25^+$ Tregs constitute 2–10% of cluster of differentiation-4^+ ($CD4^+$) T cells in humans. Recent studies have shown that persistent immune activation drives human immunodeficiency virus (HIV) disease progression. The mechanisms leading to immune activation are not well understood, and strategies to dampen it are urgently needed. The current available information does not sufficiently describe whether $CD4^+CD25^+$ Treg cells are helpful in HIV infection by controlling immune activation. A better understanding of the mechanisms of T-cell activation in HIV infection, as well as the contribution of Tregs to its control might increase therapeutic options for HIV-infected persons and provide an alternative method for monitoring HIV disease progression. This study evaluated the expression of $CD4^+CD25^+$ Treg cell populations in HIV-infected patients and correlated it with level of $CD8^+$ T-cell activation, absolute $CD4^+$ count and viral load. The study design was cross sectional and descriptive with samples of 32 HIV-positive patients and 32 healthy controls. There was significantly lower $CD4^+CD25^+$ Treg populations in HIV-infected patients (median 1.04%) as compared with the control group (median 3.1%), $p < 0.05$. There was elevated $CD8^+$ T-cell activation in the HIV-infected patients (median 3.8%) as compared with the control group (median 1.3%), $p < 0.05$. A significant negative correlation between $CD4^+CD25^+$ Treg-cell population and level of $CD8^+$ T-cell activation was observed among the patients in this study (rho = -0.798, $p < 0.001$). This correlation was, however, not significant in the healthy control group (rho = - 0.411, $p = 0.061$). Correlations between $CD8^+$ T-cell activation and viral load with $CD4^+$ absolute counts were all significant ($p < 0.001$). However, the correlation was the strongest between $CD4^+$ T-cell absolute count and $CD8^+$ T-cell activation. HIV infection was characterised by low expression of $CD4^+CD25^+$ Treg cells and increased immune activation as well as a decline in absolute $CD4^+$ T-cell count and increased viral replication. The level of $CD8^+$ T-cell activation was a better predictor of $CD4^+$ T-cell count decline than viral load.

Keywords: Regulatory T cells, Cluster of differentiation 8^+ ($CD8^+$T) cells, Viral load, Immune activation, Immunoregulation, Human immunodeficiency virus, Viral replication

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INTRODUCTION

Human immunodeficiency virus (HIV) research regarding cluster of differentiation-4$^+$ (CD4$^+$) T-cells in the past 10 years has largely focused on the Th1 and Th2 lineages but now HIV research increasingly focuses on the immune balance and function of other cellular immune subsets, such as regulatory T cells (Tregs) (Zhu *et al.,* 2012) Treg cells, which are characterised by Forkhead Box Protein 3 (FoxP3$^+$) and CD25 (IL-2Rα) expression, represent an important subset that mediates immunoregulation by controlling the proliferation of different immune cell subsets (Rudensky *et al.,* 2011) Naturally occurring (thymus derived) CD4$^+$CD25$^+$ Treg cells constitute 2–10% of peripheral CD4$^+$ T-cells in humans and play a key role in establishing and maintaining self-tolerance and immune homeostasis (Shevach *et al.,* 2002) These specific functions of CD4$^+$CD25$^+$ Treg cells are derived from the regulation of the proliferation of different immune cells (Sakaguchi *et al.,* 2008) The suppressive capacity of Treg cells is widely dependent and influenced by several factors, such as IL-2, inhibitory cytokines (IL-10, TGF-β, or IL-35), CD152, CTL (Cytotoxic T Lymphocytes) -associated antigen 4 (CTLA-4), and GITR (glucocorticoid-induced tumour necrosis factor receptor) (Seddiki *et al.,* 2014). Based on this, Treg cells may participate in the immune regulation in human autoimmune diseases, cancer, allograft rejections and virus infection (Cooles 2013 *et al.,* Beyer 2006 *et al.,* Tang *et al.,* 2013 and Veiga-Parga *et al.,* 2013).

The role Treg cells on HIV infection is still inconclusive as these cells can be involved both in the promotion as well as in the prevention of disease progression. Recent studies have suggested that Tregs might play a role in the dynamics of immune activation during HIV-1 infection (Ascher *et al.,* 1991). Some findings point to a beneficial effect through suppression of chronic immune activation and inhibition of activated CD4$^+$ T cells and consequent control of viral replication. On the other hand, Treg cells may play a detrimental role since the inhibition of specific HIV immune response through suppressive action can promote viral persistence in the host (Groth *et al.,* 2008; Simonetta *et al.,* 2013).

Although there is consensus among most researchers that persistent immune activation is a leading factor in HIV-disease progression, current available information does not sufficiently answer the question whether CD4$^+$CD25$^+$ Treg cells are helpful or detrimental in HIV infection as well as their role in HIV-associated immune activation. Thus, this study set out to investigate the CD4$^+$CD25$^+$ Treg-cell populations in HIV-infected patients and correlate it with level of CD8$^+$ T-cell activation, absolute CD4$^+$ count and viral load. The study used CD8$^+$ cellular immune activation surface markers (CD8$^+$/CD38$^+$) because CD8$^+$ cellular activation, as opposed to CD4$^+$ activation, is more predictive of long-term immunologic responses as CD4$^+$ cells are infected by HIV and are more likely to be removed through apoptosis (Zhang *et al.,* 2011).

MATERIALS AND METHODS

This study was conducted at the Academic Model for Providing Access to Healthcare (AMPATH) HIV care clinics in Moi Teaching and Referral Hospital (MTRH). AMPATH was started in the year 2000 as part of collaboration between Moi University (MU), MTRH, and a consortium of American Universities led by Indiana University of the United States. AMPATH is the largest HIV/AIDS treatment and care programme in Kenya. MTRH is the second largest hospital in Kenya and serves referral patients from Rift Valley and Western Kenya. The hospital also serves as a teaching hospital for the College of Health Sciences and School of Medicine at MU among other higher learning institutions. It is located along Nandi Road in Eldoret town. AMPATH aims at providing comprehensive care for HIV-infected patients, enhancing education and research in HIV/AIDS. The Regional Blood Transfusion Centre (RBTC), located near MTRH, also located in Eldoret was used to recruit healthy controls for the study.

In a cross sectional study design, HIV-negative and HIV-positive study participants above 18 years with no prior exposure to antiretroviral medications were evaluated. The study population comprised HIV-infected patients attending the AMPATH HIV clinic and healthy controls, who were recruited from healthy blood donors at the RBTC in Eldoret. HIV-infected patients on highly active antiretroviral therapy (HAART) [including previous treatment with antiretroviral drugs (ARVs), prevention of mother to child transmission

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of HIV (PMTCT) prophylaxis] and those who were pregnant were excluded from the study.

Convenience sampling technique was used to recruit 32 HIV-infected patients as the study subjects, and 32 HIV-negative participants as the control group. Specific levels of $CD4^+CD25^+$ Treg cells were determined and correlated with activated $CD8^+$ $CD38^+$ cells in the two groups. Laboratory analysis to determine the levels $CD4^+$ absolute counts, $CD4^+CD25^+$ Treg cells and activated $CD8^+$ T lymphocytes was performed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Blood samples were analysed within 24 hours of collection. The $CD4^+CD25^+$ Treg cells were stained using anti-CD25 PE, anti-CD3 APC and anti-$CD4^+$ FITC. Activated $CD8^+$ T-cell were stained with anti-CD38 PerCP, anti-CD3 APC and anti-$CD8^+$ FITC. All monoclonal antibodies were obtained from BD Biosciences (BD Biosciences, San Jose, CA, USA). Samples were processed using fluorescence-activated cell sorter (FACS) lyse/no wash method before data acquisition by BD FACSCalibur cytometer. During processing, 50 mL of the blood sample in a tube was vortexed, and then 20 mL of the antibodies added in each tubethen vortexed again and incubated for 15 min. After staining and following incubation, cells were lysed with 450 mL of BD FacLyse reagent and incubated for at least another 15 min. Live gating was used to collect 10,000 events by forward scatter (FSC) and side scatter (SSC) cytogram with a gate set on the $CD3^+$ lymphocyte fraction.

The percentage of $CD4^+$ T-cells expressing $CD25^+$ and percentage of $CD8^+$ T-cells expressing CD38 and surface markers was then read using the Cellquest pro® software (BD Biosciences, San Jose, CA, USA). All the data was recorded on standard case report forms, a tool developed for this study. Statistical analysis on the data was performed using statistical package for social sciences (SPSS) version 20.0 (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0, Armonk, NY: IBM Corp). Nonparametric methods were used to statistically evaluate the data and compare data sets as the data was not normally distributed. Significance of the results was set at $p < 0.05$.

Quantification of Plasma HIV-1 RNA Viral Load was performed by reverse transcription polymerase chain reaction (PCR) using Roche Amplicor 1.5 assay (Roche Diagnostics, Branchburg, NJ, USA). The minimum detection level by the assay was 400 copies/mL. Samples with undetectable virus load were excluded from statistical analysis.

The study was given ethical approval by Institutional Research and Ethics Committee (IREC) from MU/MTRH (approval number *000388*). The study procedures did not interfere in any manner with the routine clinical care provided to patients enrolled in the study. Informed and voluntary consent by each study participant was obtained before any data was collected. The purpose, procedures, and risks and benefits of the study were also discussed with the study participants. The participants were given adequate opportunity to discuss and contemplate their participation and retained the right to decline to answer individual questions or to discontinue study participation without jeopardy. Privacy was maintained and all information was treated confidentially.

RESULTS

The median age of the 32 HIV-positive study participants was 34 (IQR 27–41) years, whereas that of the 32 HIV negative controls was 31.5 (IQR 23–39.5) years. No statistically significant difference was found in the distribution of age ($p = 0.092$) between the two study groups. The statistically significant difference was however observed in the $CD4^+$ absolute count between the healthy controls and the HIV-infected group ($p < 0.05$). The median HIV viral RNA load was 54,875 cpm (IQR 6,631.5–116,905.0). The demographic and clinical characteristics of the study participants are shown in Table 1.

Table 1: Demographic and clinical characteristics of study participants

Characteristics	HIV Patients ($n = 32$)	Healthy Controls ($n = 32$)	p Value
Median age in years	34	31.5	0.092
Median CD4 count	683	872.5	<0.05
Median HIV RNA	54,875	N/A	N/A

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The study showed significantly lower $CD4^+CD25^+$ Treg frequencies in HIV-infected patients as compared with the control group ($p < 0.001$) as shown in Figure 1. Medians found in the $CD4^+$ cells expressing $CD25^+$ in the HIV-infected individuals and HIV negative controls were 1.04% and 3.1%, respectively.

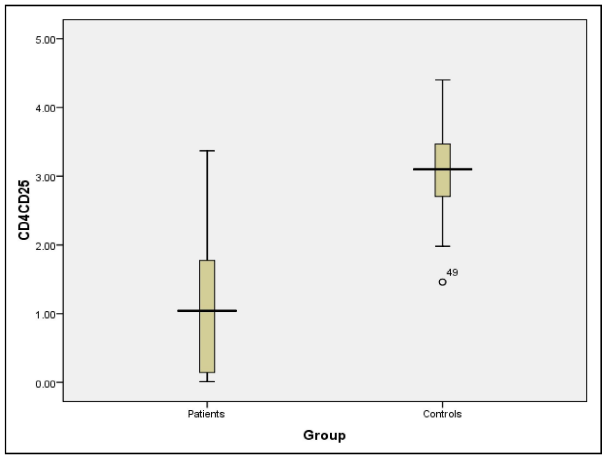


Figure 1: $CD4^+CD25^+$ Treg median populations, box plot by study group

There were also significantly elevated immune activation markers (CD38) in the HIV-infected patients as compared with the control group ($p < 0.001$) as depicted in Figure 2. Medians observed in $CD8^+$ T-cells expressing CD38 in the HIV-infected individuals and HIV negative controls were 3.8% and 1.3%, respectively.

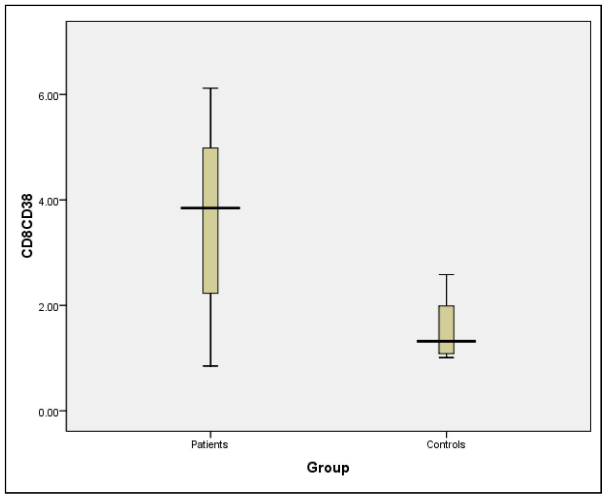


Figure 2: $CD8^+CD38^+$ T cell median populations, box plot by study group

When the $CD4^+CD25^+$ and $CD8^+CD38^+$ were correlated in both groups, it was observed that there was a significant negative correlation between $CD4^+CD25^+$ Treg cell population and $CD8^+CD38^+$ T-cell population among the HIV-infected patients (rho = -0.798, $p < 0.001$) as shown in Figure 3. However, the correlation between $CD4^+CD25^+$ Treg and $CD8^+CD38^+$ T-cell populations among the healthy controls was not statistically significant (rho = -0.411, $p = 0.061$) as indicated in Figure 4.

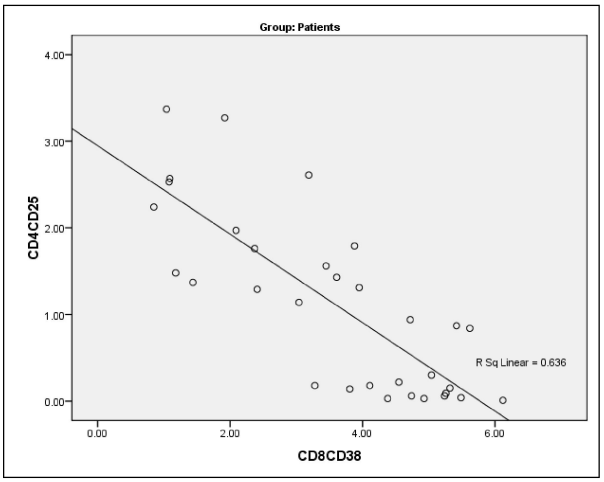


Figure 3: Correlation between $CD4^+CD25^+$ Treg and $CD8^+CD38^+$ T-cell populations in the HIV-infected patients group

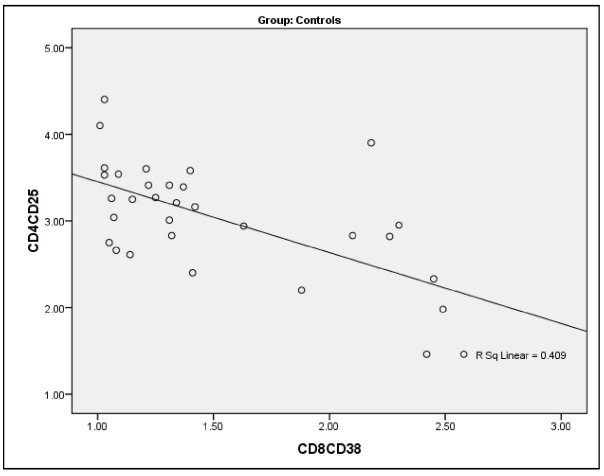


Figure 4: Correlation between $CD4^+CD25^+$ Treg and $CD8^+CD38^+$ T-cell populations in the healthy control group

Table 2: Correlation between $CD4^+$ absolute count, $CD4^+CD25^+$ Treg and $CD8^+CD38^+$ T-cell populations and viral load

	$CD4^+CD25^+$	$CD8^+CD38^+$	Viral Load
$CD4^+$ absolute count	Rho = 0.480, $p < 0.001$	Rho = -0.628, $p < 0.001$	Rho = -0.606, $p < 0.001$

Gender-specific differences in both $CD4^+CD25^+$ T regulatory cells and $CD8^+CD38^+$ T-cells were not statistically significant in both groups of HIV-infected patients and healthy controls ($p = 0.071$).

The correlations between $CD4^+CD25^+$ Treg population, $CD8^+$ T-cell activation and viral load with $CD4^+$ absolute count were all significant ($p < 0.001$). However, the correlation was strongest between $CD4^+$ absolute count and $CD8^+$ T-cell activation as indicated in Table 2.

DISCUSSION

This study investigated how HIV infection alters $CD8^+$ T-cell immune activation, and how this may correlate with $CD4^+CD25^+$ Treg populations, as well as viral load and absolute $CD4^+$ counts, an indicator of immune status. No statistically significant differences were found in the demographic characteristics between the two study groups. HIV infection appears to increase expression of activated $CD8^+$ T lymphocytes and reduce expression of $CD4^+CD25^+$ Treg cells.

Analysis of the data from this study revealed that the median $CD4^+CD25^+$ Treg population in this study was significantly lower in the HIV-infected patient group as compared with the healthy control group ($p < 0.05$), consistent with another similar study by (Lin *et al.,* 2011) The median $CD4^+CD25^+$ Treg population observed in the healthy controls was 3.1% of $CD4^+$ cells which is close to 4.47% reported from a previous study by Lin (2011)The median $CD4^+CD25^+$ Treg population in the HIV-infected patients was 1.3% of $CD4^+$ cells. This was lower compared with 3.58% reported by Lin (2011) the differences in median $CD4^+CD25^+$ Treg populations among healthy controls reported in different studies could be due to different protocols used in the studies. For instance, the study protocol in Lin's study used peripheral blood mononuclear cells (PBMC) samples, whereas the present study used whole blood samples

In this present study, the authors found that there was significantly increased immune activation in the HIV-infected patients as compared with the control group ($p < 0.05$) as supported by (Hazenberg *et al.,* 2003) who demonstrated that T cells in HIV-1 infected individuals displayed signs of excessive immune activation, compared with healthy controls. Immune activation *per se* might be important in the progressive depletion of T cells in HIV-infected individuals, and thus for the progression to AIDS (Paiardini *et al.,* 2013). The level of $CD8^+CD38^+$ T-cell activation in the controls, median 1.4% (1.1, 2.2) observed in the present study also closely compares with that described in a Dutch population, median (IQR) 2% (1–6) by (Kassu *et al*. 2001) In the HIV-infected participants, the median $CD8^+CD38^+$ T-cell activation was 3.8% (2.2, 5.0). This was lower compared with 8.8% (5.8–11.1) reported by (Weiss *et al*. 2010) The discrepancies in median $CD8^+CD38^+$ T-cell populations reported by different authors could be attributed to differences regarding study participant characteristics. For instance, Weiss included patients who had interrupted ART in his sample, an attribute associated with higher immune activation, whereas this present study only included ART naïve patients.

The most direct explanation for the cause of immune activation in the context of HIV infection is the result of innate and adaptive immune responses against the virus and its antigens. Not only are HIV antigens recognised by, and thus activate, T cells expressing virus-specific T-cell receptors and B cells bearing virus-specific surface immunoglobulin, but HIV components also bind to pattern recognition receptors, such as the Toll-like receptors 7 and 9 (Heil *et al.,* 2004) .

Supporting the important contribution of HIV replication in immune activation, this study showed a significant positive correlation between immune activation and viral load ($p < 0.001$). Several other studies have also shown that immune activation and inflammation correlated with the level of viraemia and

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that immune activation was significantly lower in HIV-infected patients, who controlled viral replication spontaneously or by HAART (Doisne *et al.,* 2004)

It was hypothesised in this study that $CD4^+CD25^+$ Treg cell depletion played a role in immune activation in HIV by failure of suppression of effectors $CD8^+$ and $CD4^+$ T-cells. The data from this study indicated that there was a significant inverse correlation between $CD4^+CD25^+$ Treg cell population and the level of $CD8^+$ T-cell activation among the patients in this study (rho = 0.798, $p < 0.001$) supporting this hypothesis. This correlation was however not significant in the healthy control group (rho = -0.411, $p = 0.061$). The fact that this correlation between $CD8^+$ T-cell activation and $CD4^+CD25^+$ Treg cell populations was not seen in HIV negative healthy control participants suggests that this effect may be HIV specific and requires the presence of persistent antigen to induce and activate the immune system.

This study also investigated the relationship between $CD4^+$ absolute count, the level of $CD8^+$ T-cell activation and viral load. There was a significant negative correlation between $CD4^+$ absolute count and the level of immune activation (rho = -0.628, $p < 0.001$) and a significant negative correlation between $CD4^+$ absolute count and viral load (rho = -0.606, $p < 0.001$). The correlation between $CD4^+$ absolute count and $CD8^+$ T-cell activation was stronger than the correlation between $CD4^+$ absolute count and viral load. Consistent with this finding, it has been observed in another study (Rodriguez *et al.,* 2006) that viral load is only an indirect contributor to the rate of progression to AIDS, that immune activation predicted changes in $CD4^+$ T-cells stronger and independent of viral load.

CONCLUSION

Emerging data from our study has therefore established that $CD4^+CD25^+$ Tregs are depleted in HIV infection, and this depletion, in conjunction with viraemia drives $CD8^+$ T-cell activation. The observed strong correlation between $CD8^+$ T-cell activation and absolute $CD4^+$ count implies that immune activation was better at predicting $CD4^+$ T-cell count decline than viral load and can therefore be used for monitoring treatment efficacy and disease progression in HIV. Our study design could not show the cause–effect relationship but gave a mere comparison of study groups and relationships of variables; this could be rigorously studied further using a study design with more control arms including cytokine assays to validate the observed results.

LIST OF ABBREVIATIONS

ARV – antiretroviral drug; CD – cluster of differentiation; FACS – fluorescence-activated cell sorter; HAART – highly active antiretroviral therapy; HIV – human immunodeficiency virus; PBMC – peripheral blood mononuclear cells; PCR – polymerase chain reaction; PMTCT – prevention of mother to child transmission of HIV; SPSS – statistical package for social sciences; @@@ Treg – regulatory T cell.

COMPETING INTERESTS

The authors declare that there are no competing interests regarding the publication of this paper.

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