ABSTRACT
Infection with HIV results in discordant immunological response characterized by dysregulation of cytokines and a decrease in CD4+ T cells. As HIV progresses to AIDS, there is a switch from Th1 to Th2 cytokines. In this study, plasma cytokine levels were compared between newly diagnosed HIV-positive HAART naïve patients and HAART undergoing patients. Additionally, the association between plasma cytokine levels and CD4+ T cell count was examined. 160 HIV-positive individuals were enrolled for the study, of which 80 were HAART naïve and 80 were undergoing HAART for over six months since diagnosis. The plasma cytokine levels of IFN-γ, IL 2, IL 4 and IL 10 were analysed using ELISA and CD4+ T cell counts were assessed using flow cytometry. Comparing the HAART naïve patients to HAART undergoing group, the plasma cytokine concentrations of IL 4 and IL 10 was considerably higher in HAART naïve patients while IFN-γ and IL 2 concentration was greater in HAART undergoing patients. Similarly, while examining the relationship between CD4+ T cells and plasma cytokine levels, it was discovered that IFN-γ significantly correlated with CD4+ T cells in HAART-naive individuals as opposed to IL 2, IL 4, and IL 10. Therefore, an increase in Th1 cytokines over Th2 cytokines and the restoration of immune response are indicators of successful anti-retroviral therapy.

Keywords: Pro inflammatory cytokines, anti inflammatory cytokines, HIV, HAART, T cells.

1. INTRODUCTION
Cytokines are crucial immune system mediators that assist in regulating immunological responses like immune cell growth, inflammation, and responsiveness. Variation in the amounts of cytokines, which are released by lymphocytes and macrophages, is a key factor in determining health [1]. Human immunodeficiency virus (HIV) infection results from a rapid decline in CD4+ T cells and dysfunctional cytokine production. The changes in cytokine levels during HIV infection can be inhibitory, stimulatory, or bifunctional [2]. Prior researches [3, 4] have shown that the transition from T-helper type 1 (Th1) to T-helper type 2 (Th2) cytokines occurs over the course of HIV infection, results in decreased production of interleukin 2 (IL 2) and interferon gamma (IFN-γ) and higher levels of interleukin 4 (IL 4) and interleukin 10 (IL 10) and this unbalanced production of cytokines is partially responsible for the progression of HIV infection to AIDS. When intracellular infection is detected, Th1 response is generated, whereas Th2 response is for extracellular pathogens. CD4+ T cells are essential for cellular immune response because they secrete cytokines, and the cytokine levels in plasma are indicative of the nature of immune response. Given that HIV is an intracellular infection, a lack of CD4+ cells leads to poor Th1 cytokine release [5]. IL 2 is a pro inflammatory Th1 cytokine and deficiency in its production leading to decreased CD4+ T cell count, is one of the first immunological defects of HIV infection in the individual [6]. IFN-γ is associated with T cell activation and acts by inhibiting HIV replication. With the onset of HIV infection, decreased IFN-γ secretion leads to chronic HIV infection during which HIV rapidly induces the production of Th2 cytokines. IL 4 secretion results in B cell activation and antibody production while IL 10 inhibits T cell proliferation and macrophage activation thus suppressing the synthesis of Th1 cytokines [7, 8]. Such dysregulation in cytokine production contributes to HIV disease progression to AIDS by impairing cellular immune responses.
Highly active antiretroviral therapy (HAART) is a treatment regimen consisting of at least three antiretroviral drugs such as nucleoside reverse transcriptase inhibitor (NRTI), non-nucleoside reverse transcriptase inhibitor (NNRTI) and integrase strand transfer inhibitor (INSTI). Successful HAART results in marked improvement in clinical and immunological status of the patient by suppressing the viral load and increasing CD4+ T cell counts [9]. The impact of HAART on plasma cytokine levels has not been fully determined despite our understanding of Th1 and Th2 cytokine roles in immunopathogenesis of HIV infection. According to pre-HAART research, cytokine expression variations have accelerated the progression of immunodeficiency, while a successful HAART treatment has significantly raised plasma IFN-γ and decreased IL-10 levels [10]. The effectiveness of HAART could be impacted by pro and anti-inflammatory cytokines involved in the regulation of immune response in HIV infection. The aim of this study was to evaluate the plasma cytokine levels and to assess its relationship between CD4+ T cells in HIV-positive HAART naïve patients and HIV-positive HAART undergoing patients.

2. MATERIAL AND METHODS

2.1. Study population
This study was conducted at the Department of Microbiology, Dr. ALM Post Graduate Institute of Basic Medical Sciences, and University of Madras. A total of 210 study subjects from an antiretroviral therapy centre (ART centre) in Chennai, India were recruited for this study. The study population included 80 newly diagnosed HIV-positive HAART naïve patients that comprised the test group and 80 HIV-positive HAART experienced patients that served as the comparison group. The comparison group included patients who had been receiving HAART for over 6 months since diagnosis. 50 HIV seronegative healthy subjects served as the control group. This study was a comparative cross-sectional study and appropriate ethical clearance was obtained from the Institutional Human Ethical Committee (IHEC no: PGIBMS/Co/Tara/Human Ethical Com/Micro/12/150).

2.2. Sample collection
Informed consent forms were obtained from all the subjects before sample collection and data was collected using a questionnaire that included bio-data and the participant’s medical history. 5 ml of blood was drawn by venipuncture from all the subjects in the study. The blood samples were aliquoted in appropriate EDTA tubes for plasma cytokine analysis while whole blood was used for the enumeration of CD4+ T cells.

2.3. CD4+ T cells count
Whole blood was used for the enumeration of CD4+ T cells. 20µl of blood was incubated with CD4+ antibody and CD4+ T cell count was carried out by flow cytometry using Fluorescent Activated Cell Sorter (FACS count) system (BD™ Biosciences, USA) according to the manufacturer’s protocol [11].

2.4. Quantification of cytokines
Quantitative determination of cytokines in the plasma (Th1 subset: IFN-γ and IL-2, Th2 subset: IL-4 and IL-10) was carried out by ELISA with ELISA MAX™ Deluxe Sets (Biolegend, CA, USA) according to the manufacturer’s instructions. Briefly, 100µl of capture antibody specific to human IFN-γ, IL-2, IL-4, and IL-10 was coated onto the 96-well microtitre plate and incubated overnight at 4°C. Following incubation, the wells were blocked with 200µl of 1X Assay diluent for 1 h at RT. 100ul of plasma samples or standard was added to each well and incubated for 2 h at RT. Next, 100µl of detection antibody specific to human IFN-γ, IL2, IL4, and IL10 was added to the wells followed by 1 h incubation at RT. Thereafter, 100µl of Avidin Horseradish peroxidase (HRP) was added and incubated for 30 mins at RT. The microtiter plate was washed and 100µl of substrate solution for peroxidase was added and incubated in the dark for 30 min at RT. Lastly, stop solution was added to each well and the absorbance was read at 450 nm using a microplate reader (iMARK™ Microplate reader, Bio-Rad, USA). The plasma cytokine concentrations were calculated and expressed as pg/ml.

2.5. Statistical analysis
The resulting data were analyzed using Graph Pad Prism version 8.0 for Windows (Graph Pad, La Jolla, CA, USA). Cytokine levels are presented as mean ± standard deviation. The difference in the cytokine concentrations between newly diagnosed HIV-positive patients before commencement of HAART and HIV-positive patients undergoing HAART was analyzed using Student’s t-test. The relationship between plasma cytokine levels and CD4+ T cells was analyzed by employing the Pearson correlation and significant levels were considered at \( P < 0.05 \).
3. RESULTS

3.1. Sociodemographic characteristics
Males constituted 43% (n=91) of the study population, while 57% (n=119) were female participants. The mean age of the study subjects was 39.2 years and the sociodemographic profile of the study subjects are described in Table 1. Majority of the patients in the test groups (76%) had CD4+ T cell count ranging from 200 - 500 while the healthy controls (24%) had >700 CD4+ T cells.

3.2. Th1 cytokine levels and HAART
Significant differences were noted in the plasma levels of Th1 pro-inflammatory cytokines - IFN $\gamma$ (39.4 pg/ml) and IL 2 (53.32 pg/ml) before commencement of HAART and after undergoing HAART ($P < 0.05$, Student’s t test)(Fig.1a,Fig.1b). Group comparisons in Table 2 shows that plasma cytokine levels of IFN $\gamma$ (123.9 pg/ml) and IL 2 (113.3 pg/ml) were significantly higher after the commencement of HAART compared to the healthy control participants ($P < 0.05$, Student’s t test) (Fig 2).

3.3. Th2 cytokine levels and HAART
Significant differences were observed in the plasma levels of Th2 anti-inflammatory cytokines - IL 4 (58.9 pg/ml) and IL 10(45.2 pg/ml)(Fig.3a,Fig.3b) before the commencement of HAART and HAART experienced study subjects ($P < 0.05$, Student’s t test). Within the group comparisons as shown in Table 3, the plasma cytokine levels of IL 4 (15.7 pg/ml) and IL 10 (9.8 pg/ml) was found not to be significant in the HIV positive HAART undergoing patients compared to the control group ($P > 0.05$, Student’s t test)(Fig.4).

Table 1: Sociodemographic profile of the study subjects

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>HAART naïve: (n = 80)</th>
<th>HAART undergoing: ( n = 80)</th>
<th>Control: (n = 50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in years (mean age)</td>
<td>37</td>
<td>43</td>
<td>36.9</td>
</tr>
<tr>
<td>Male</td>
<td>34</td>
<td>41</td>
<td>16</td>
</tr>
<tr>
<td>Female</td>
<td>46</td>
<td>39</td>
<td>34</td>
</tr>
<tr>
<td>CD4+ T cell count (mean)</td>
<td>227 cells/µl</td>
<td>501 cells/µl</td>
<td>990 cells/µl</td>
</tr>
</tbody>
</table>

Table 2: Th1 plasma cytokine levels of IFN $\gamma$ (pg/ml ) and IL 2(pg/ml) in HIV positive HAART naïve patients and HIV positive HAART undergoing patients

<table>
<thead>
<tr>
<th>Study group</th>
<th>IFN $\gamma$ (pg/ml)</th>
<th>IL 2 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAART naïve {a} (n = 80)</td>
<td>39.4 ± 29.401$^a$</td>
<td>52.32 ± 22.845$^d$</td>
</tr>
<tr>
<td>HAART undergoing {b} (n = 80)</td>
<td>123.90 ± 75.473$^b$</td>
<td>113.35 ± 100.272$^c$</td>
</tr>
<tr>
<td>Healthy control {c} (n = 50)</td>
<td>7.40 ± 1.419$^e$</td>
<td>4.51 ± 2.962$^f$</td>
</tr>
</tbody>
</table>

*{a} vs. {b} $P<0.05$, {a} vs. {c} $P<0.05$, {b} vs. {c} $P<0.05$, {d} vs. {e} $P<0.05$, {d} vs. {f} $P<0.05$ and , {e} vs. {f} $P<0.05$

Fig. 1: (a) Plasma cytokine levels of IFN $\gamma$ (b) Plasma cytokine levels of IL
3.4. Correlation between CD4+ T cells and plasma cytokine levels in HAART naïve patients

The relationship between CD4+ T cells and plasma cytokine levels in the newly diagnosed HIV-positive HAART naïve patients was analysed by Pearson correlation. Table 4 shows that there was a significant positive correlation observed between CD4+ T cells and IFN-γ ($r = 0.48$, $P < 0.05$) in the HAART naïve group. A negative correlation was noted between CD4+ T cells and IL 2 ($r = -0.25$, $P < 0.05$), IL 4 ($r = -0.39$, $P < 0.05$) and IL 10 ($r = -0.55$, $P < 0.05$) in patients with HIV before commencement of HAART.

Table 3: Th2 plasma cytokine levels of IL 4 (pg/ml) and IL 10 (pg/ml) in HIV positive HAART naïve patients and HIV positive HAART undergoing patients.

<table>
<thead>
<tr>
<th>Study group</th>
<th>IL 4 (pg/ml)</th>
<th>IL 10 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAART naïve {a} (n = 80)</td>
<td>58.95 ± 68.953$^a$</td>
<td>45.26 ± 26.180$^a$</td>
</tr>
<tr>
<td>HAART undergoing {b} (n = 80)</td>
<td>15.70 ± 24.052$^b$</td>
<td>9.89 ± 3.172$^b$</td>
</tr>
<tr>
<td>Healthy control {c} (n = 50)</td>
<td>5.06 ± 4.390$^c$</td>
<td>7.88 ± 14.083$^c$</td>
</tr>
</tbody>
</table>

*{a} vs. {b} $P<0.05$, {a} vs. {c} $P<0.05$, {b} vs. {c} $P>0.05$, {d} vs. {e} $P<0.05$, {d} vs. {f} $P<0.05$ and {e} vs. {f} $P>0.05$

Table 4: Correlation between CD4+ T cells and plasma cytokine levels in HAART naïve patients

<table>
<thead>
<tr>
<th>Study group</th>
<th>Parameters</th>
<th>$r$</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAART Naive (n=80)</td>
<td>CD4+T cells vs IFN-γ</td>
<td>0.48</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td></td>
<td>CD4+T cells vs IL 2</td>
<td>-0.25</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td></td>
<td>CD4+T cells vs IL 4</td>
<td>-0.39</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td></td>
<td>CD4+T cells vs IL 10</td>
<td>-0.55</td>
<td>$P &lt; 0.05$</td>
</tr>
</tbody>
</table>

Fig. 2: Th1 plasma cytokine levels difference in HAART naïve and HAART undergoing patients

Fig. 3: (a) Plasma cytokine levels of IL 4  (b) Plasma cytokine levels of IL 10
3.5. Correlation between CD4+ T cells and plasma cytokine levels in HAART undergoing patients

The association between CD4+ T cells and plasma cytokine levels was analyzed (Table 5) and no significant correlation between CD4+ T cells and IL 10 ($r = 0.18$, $P = 0.1$) was noted, whereas negative correlation was observed between CD4+ T cells and IFN $\gamma$ ($r = -0.37$, $P < 0.05$), IL 2 ($r = -0.43$, $P < 0.05$) and IL 4 ($r = -0.50$, $P < 0.05$) in HIV-positive patients undergoing HAART.

### Table 5: Correlation between CD4+ T cells and plasma cytokine levels in HAART undergoing patients

<table>
<thead>
<tr>
<th>Study groups</th>
<th>Parameters</th>
<th>$r$</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAART undergoing</td>
<td>CD4+T cells vs IFN $\gamma$</td>
<td>-0.37</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td>(n=80)</td>
<td>CD4+T cells vs IL 2</td>
<td>-0.43</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td></td>
<td>CD4+T cells vs IL 4</td>
<td>-0.50</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td></td>
<td>CD4+T cells vs IL 10</td>
<td>0.18</td>
<td>0.1</td>
</tr>
</tbody>
</table>

4. DISCUSSION

This study was carried out to determine the preponderance of Th1 cytokines in HIV patients after the administration of HAART. Plasma cytokine levels in HAART naïve patients and HAART undergoing patients were quantified and their relationship with CD4+ T cells was analysed, in order to assess the effect of HAART on cytokine production. In comparison to Th1 plasma cytokines IFN $\gamma$ and IL 2, our study found higher levels of Th2 plasma cytokines IL 4 and IL 10 in HIV-infected patients who are yet to commence HAART. These results are in line with those of a related study conducted elsewhere [12]. Similarly higher plasma concentrations of IFN $\gamma$ and IL 2 in patients undergoing HAART for over six months since diagnosis in comparison with IL 4 and IL 10 was observed. This is consistent with research [13, 14] showing changes in pro-inflammatory and anti-inflammatory cytokines after HAART regimen that patients undergoing HAART have low plasma levels of IL 4 and IL 10. Likewise when analyzing the association between CD4+ T cells and plasma cytokine levels, a substantial positive correlation between CD4+ T cells and IFN $\gamma$ was noted in HAART naïve patients compared to IL 2, IL 4 and IL 10. No significant correlation was established between CD4+ T cells and the cytokine levels in HAART undergoing patients. These findings are consistent with a study of similar nature [15].

In the newly diagnosed HIV-positive HAART naïve patients, HIV immune activation is accompanied by the release of pro-inflammatory and anti-inflammatory cytokines by T cells, macrophages, and monocytes [16,17]. Increased production of IL 4 and IL 10 triggers a Th2 immune response, which in turn activates B cells and produces antibodies, creating a positive feedback loop that further drives the Th2 response and inhibits Th1 immunological activity [18, 19]. Our findings that there were substantial variations between Th1 (IFN $\gamma$ and IL 2) and Th2 (IL 4 and IL 10) plasma cytokine levels in HAART naïve patients confirm the aforementioned observation. HIV disease progression is significantly slowed down by HAART induced immunological reconstitution, notably the Th2 to Th1 shift [20]. Our study found higher levels of Th1 plasma cytokines (IFN $\gamma$ and IL 2) in patients on HAART, which was supported by earlier studies of a similar type [21, 22]. Our findings demonstrated that CD4+ T cell count were considerably lower in HIV-infected participants. While CD4+ T cell count is an essential biomarker for HIV progression the altered cytokine profiles of the HIV positive participants may have resulted from this. The findings of this study suggests that patients undergoing HAART experience significant recovery of CD4+ T cells, thus demonstrating immune reconstitution as described by earlier studies [23,24]. However, the methodology used in this study is...
insufficient to effectively address the Th1 to Th2 shift and vice versa after administration of HAART. It is unclear whether this observation is the result of a more sustained viral suppression as we did not measure the HIV viral load in our study individuals.

5. CONCLUSION
Our findings imply that HIV infection was characterized by dysregualtion of cytokine production and decline in CD4+ T cells. While significant levels of Th2 cytokines were found in HAART-naive patients, suggesting a switch from Th2 to Th1 cytokines indicative of a rapid progression to AIDS, our data show that HAART-induced immunological reconstitution resulted in increasing levels of Th1 cytokines. Overall, further research on pro and anti inflammatory cytokines is needed to maximise their value as indicators of HIV disease progression and treatment in HIV-infected individuals. For a more accurate assessment of how HAART affects cytokine levels, measurement of these cytokines might continue after prolonged treatment.

6. ACKNOWLEDGMENTS
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Conflict of interes
The authors have no conflict of interest in this study.

7. REFERENCES
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