The dominant source of CD4$^+$ and CD8$^+$ T-cell activation in HIV infection is antigenic stimulation

James W.T. Cohen Stuart$^{1,3}$
Mette D. Hazenberg$^2$
Dörte Hamann$^2$
Sigrid A. Otto$^2$
Jan C.C. Borleffs$^3$
Frank Miedema$^3$
Charles A.B. Boucher$^1$
Rob J. de Boer$^4$

1 Department of Virology, Eijkman-Winkler Institute, University Medical Center Utrecht
2 Department of Clinical Viro-Immunology, CLB, and the Laboratory for Experimental and Clinical Immunology and Department of Human Retrovirology, Academic Medical Center, University of Amsterdam
3 Department of Internal Medicine, University Medical Center Utrecht
4 Department of Theoretical Biology, University Utrecht, the Netherlands.

(Accepted for publication in the Journal of Acquired Immune Deficiency Syndromes)
Abstract

To distinguish between antigenic stimulation and CD4+ T-cell homeostasis as the cause of T-cell hyperactivation in HIV infection, we studied T-cell activation in 47 patients before and during HAART. We show that expression of HLA-DR, CD38 and Ki67 on T cells decreased during HAART but remained elevated over normal values until week 48 of therapy. We confirm previous reports that T-cell activation correlates positively with plasma HIV RNA levels (suggesting antigenic stimulation), and negatively with CD4 count (suggesting CD4+ T-cell homeostasis). However, these correlations may be spurious, i.e. misleading, due to the well-established negative correlation between CD4 count and plasma HIV RNA levels. To resolve this, we computed partial correlation coefficients. Correcting for CD4 counts, we show that plasma HIV RNA levels contributed to T-cell hyperactivation. Correcting for plasma HIV RNA levels, we show that CD4+ T-cell depletion contributed to T-cell activation. Correcting for both, the activation of CD4+ and CD8+ T cells remained positively correlated. Since this suggests that the the CD4+ and CD8+ T-cell activation is caused by a common additional factor, we conclude that antigenic stimulation by HIV or other (opportunistic) infections is the most parsimonious explanation for T-cell activation in HIV infection. Persistence of HIV antigens would explain why T-cell activation fails to revert to levels of healthy individuals after 48 weeks of therapy.

Keywords: Activation, Proliferation, T lymphocytes, CD4, CD8, Antiretroviral therapy.
Introduction

The T lymphocytes of HIV infected individuals have increased expression of activation markers HLA-DR and CD38 (1-11) and increased proliferation rates. The latter has been demonstrated using two different techniques. First, by determining the fraction of dividing cells via the expression of the nuclear antigen Ki67 (12), it was shown that T-cell proliferation rate is increased maximally two- to three fold in the CD4+ population, and 6-7 fold in the CD8 population (13-16). This limited increase in the division rate is consistent with studies measuring the replicative history of T cells by the average telomere lengths (17,18). The second technique, using deuterated glucose to label DNA in vivo, showed that the turnover of CD4+ and CD8- T cells in HIV infected patients is ~3 times higher than that of healthy individuals (19). Increased turnover of CD4+ and CD8+ T lymphocytes has also been observed in SIV infected macaques using BrdU to label DNA in vivo (20).

Two models have been proposed to explain the hyperactivation and increased proliferation of T cells in HIV-1 infection. One model contends that T-lymphocyte activation in HIV infection is driven by antigens from HIV and/or from other pathogens (15, 21,22). Alternatively, increased production of CD4+ T cells may be a homeostatic response to compensate for the loss of CD4+ T cells that are killed by HIV (23,24). The goal of this study was to determine which of these to mechanisms best explains the T-lymphocyte activation in HIV infection. We therefore performed a cross-sectional and a longitudinal analysis of the activation status of T lymphocytes, T-lymphocyte population density, and plasma HIV RNA levels in a large cohort of HIV infected patients before and during HAART.
Materials and methods

Study population
The activation and proliferation status of T cells was analyzed in 47 patients from the previously described CHEESE study cohort (26) with a sustained plasma HIV RNA response to levels < 50 copies/ml. Briefly, this is a randomized study comparing antiviral efficacy of zidovudine (Retrovir, Glaxo-Wellcome, Research Triangle Park, N.C.) plus lamivudine (Epivir, Glaxo-Wellcome, Research Triangle Park, N.C.) plus saquinavir-soft-gelatin-capsules (SQV-SGC, Fortovase, Hoffmann-La Roche, Inc., Nutley, New Jersey) versus zidovudine plus lamivudine plus indinavir (Crixivan, Merck, West Point, Pa), in HIV-1 infected patients. Antiretroviral naive patients were eligible for study treatment if at the moment of screening plasma HIV RNA levels were at least 10,000 copies/ml and/or if CD4 counts were less than 500 cells / µL and/or if they had a history of HIV related symptoms (CDC stage B or C). During 48 weeks of treatment, the virologic and the CD4 count response was not different between the two treatment arms (data not shown). Of the selected patients, 25 were from the indinavir arm and 22 from the SQV-SGC arm.

Healthy Controls
As controls for the expression of Ki67+ on T lymphocytes, cryopreserved PBMC’s from five HIV-seronegative blood bank donors were used. As controls for expression of CD38 and HLA-DR on T cells, freshly isolated PBMC’s from 12 healthy HIV-seronegative donors were used.

Blood sampling
Blood samples were obtained at baseline, and every 4 weeks through week 24, and every 8 weeks from week 24 through week 48 of treatment.

Plasma Viral Load
Plasma HIV RNA levels were measured using an investigational version of the ultra sensitive quantitative reverse transcriptase polymerase chain reaction (RT-PCR) assay (Amplicor HIV-1 Monitor, Roche Diagnostic Systems). The lower limit of detection was 50 copies / ml.
### Table 1. Correlation between viral load and T-cell activation/proliferation.

<table>
<thead>
<tr>
<th>Activation Marker</th>
<th>( r )</th>
<th>Controlled for CD4</th>
</tr>
</thead>
<tbody>
<tr>
<td>%Ki67 (CD4)</td>
<td>0.47 ( $^$ )</td>
<td>0.46 ( $^$ )</td>
</tr>
<tr>
<td>%HLA-DR (CD4)</td>
<td>0.22 ( $^$ )</td>
<td>0.20 ( $^\bullet )</td>
</tr>
<tr>
<td>%Ki67 (CD8)</td>
<td>0.47 ( $^$ )</td>
<td>0.54 ( $^$ )</td>
</tr>
<tr>
<td>%HLA-DR (CD8)</td>
<td>0.27 ( $^$ )</td>
<td>0.26 ( $^$ )</td>
</tr>
<tr>
<td>%CD38 (CD8)</td>
<td>0.64 ( $^$ )</td>
<td>0.60 ( $^$ )</td>
</tr>
</tbody>
</table>

\( $^\bullet \): p<0.05  
\( $^\$ \): p<0.001

### Table 2. Correlation between CD4 count and T-cell activation/proliferation.

<table>
<thead>
<tr>
<th>Activation Marker</th>
<th>( r )</th>
<th>Controlled for CD4</th>
</tr>
</thead>
<tbody>
<tr>
<td>%Ki67 (CD4)</td>
<td>-0.58 ( $^$ )</td>
<td>-0.55 ( $^$ )</td>
</tr>
<tr>
<td>%HLA-DR (CD4)</td>
<td>-0.46 ( $^$ )</td>
<td>-0.43 ( $^$ )</td>
</tr>
<tr>
<td>%Ki67 (CD8)</td>
<td>-0.30 ( $^\bullet )</td>
<td>-0.12, p=0.3</td>
</tr>
<tr>
<td>%HLA-DR (CD8)</td>
<td>-0.12 ( $^\bullet )</td>
<td>-0.03, p=0.3</td>
</tr>
<tr>
<td>%CD38 (CD8)</td>
<td>-0.42 ( $^$ )</td>
<td>-0.32 ( $^$ )</td>
</tr>
</tbody>
</table>

\( $^\bullet \): p<0.05  
\( $^\$ \): p<0.001
Table 3. Correlation between activation/proliferation in CD4+ and CD8+ T-cell subsets.

<table>
<thead>
<tr>
<th>CD8+ T cells</th>
<th>CD4+ T cells</th>
<th>r</th>
<th>Controlled for CD4</th>
<th>Controlled for Viral Load</th>
<th>Controlled for CD4 and Viral Load</th>
</tr>
</thead>
<tbody>
<tr>
<td>%HLA-DR</td>
<td>%HLA-DR</td>
<td>0.58 §</td>
<td>0.62 §</td>
<td>0.56 §</td>
<td>0.62 §</td>
</tr>
<tr>
<td>%CD38</td>
<td>%HLA-DR</td>
<td>0.39 §</td>
<td>0.24 §</td>
<td>0.33 §</td>
<td>0.23 §</td>
</tr>
<tr>
<td>%Ki67</td>
<td>%Ki67</td>
<td>0.62 §</td>
<td>0.49 §</td>
<td>0.28 §</td>
<td>0.32 §</td>
</tr>
</tbody>
</table>

§ p<0.001

Monoclonal antibodies
Peridinin chlorophyll protein (PerCP)-labeled CD4, PerCP-labeled CD8 and phycoerythrin (PE)-labeled HLA-DR monoclonal antibodies were obtained from Becton Dickinson (San Jose, CA). Fluorescein isothiocyanate (FITC) labeled CD38 and FITC labeled Ki67 monoclonal antibodies were obtained from Immunotech (Marseille, France).

Flow Cytometry
The fraction of activated CD4+ and CD8+ T cells was determined by three color FACS analysis using monoclonal antibodies against CD4 (or CD8), CD38 and HLA-DR on heparin-anticoagulated venous blood (FACScan; Becton Dickinson Immunocytometry Systems, San Jose, CA). In 16 patients, the fraction of proliferating T cells was determined before therapy and at week 4,12, 24 and week 48 of HAART by measuring the expression of the nuclear antigen Ki67 on cryopreserved PBMC’s.

Statistical Analysis
The non-parametric Mann-Whitney-U-Test (Wilcoxon-Rank-Sum-W Tests) was used to compare patients with controls. Longitudinal changes of patient characteristics were tested using the non-parametric Wilcoxon
Matched Pairs Signed-Rank Test. Pearson’s correlation coefficients were computed to measure bivariate correlations. Partial correlations were calculated to analyze the correlation that remains between two variables after removing the correlation that is due to their mutual association with a third variable (51). Correlations were computed for pooled data of all time points (week 0-48) and for data of baseline only. Similar correlations were found for baseline and for the pooled data, although the p-values were generally higher in the baseline correlations probably due to a smaller sample size (Table 1-3). Reported p-values are two-sided. All statistical analyses were performed using SPSS for Windows (8.0.0). Non linear regression analysis was performed using Mathematica, version 2.1.

Results

Expression of activation markers before HAART
T cells expressing Ki67 were considered to be proliferating. Ki67 is a protein expressed by cells in the late G1 and the S, G2 and M phase of the cell cycle (12). T cells expressing HLA-DR were considered to be activated cells. The CD8+ T cells expressing CD38 were also to be considered activated (2,4,6-11). We confirm previous reports (1-11) that, prior to the start of HAART, the expression of HLA-DR and Ki67 on CD4+ T lymphocytes, and the expression of HLA-DR, CD38 and Ki67 on CD8+ T lymphocytes, is higher in HIV-1 infected patients as compared with healthy controls (see Figure 1).

Effect of HAART on plasma viral load, CD4 count and CD8 count
The median plasma viral load decreased from 40.000 copies/ml to <50 copies/ml in 16 weeks (p<0.001). The CD4 count increased from 301±28 at baseline to 507±40 cells/µl at week 48 (p<0.001). The increase of CD4 count during the first 4 weeks of therapy was higher (2.0 cells/mm³/day) as compared with the mean CD4 count rise during later 4 week intervals (0.38 cells/mm³/day), in agreement with a biphasic response pattern of the CD4+ T cells to HAART (27). The CD8 count decreased from 1050±70 cells/mm³ at baseline to 870±60 cells/mm³ at week 48 (p=0.023).
Figure 1. The effect of HAART on the expression of activation antigens on T lymphocytes. Mean values are shown. Bars represent standard error of the mean. The dotted lines represent mean expression levels in healthy HIV-seronegative controls. Figure 2a and 2b show the expression of HLA-DR and Ki67 on CD4+ T cells, respectively. Figure 2c, 2d and 2e show the expression of HLA-DR, CD38 and Ki67 on CD8+ T cells, respectively. At week 48, the mean expression levels of the activation markers on T cells were still significantly higher than in healthy controls (p<0.05 each comparison of patients vs controls (Mann-U-Whitney test)).
Effect of HAART on expression of HLA-DR, CD38 and Ki67 on T lymphocytes

The expression of all activation markers on CD4+ and CD8+ T cells gradually decreased during HAART (all p values < 0.005). At week 48 however, the mean expression levels were still significantly higher than in healthy controls, even though all patients had plasma HIV RNA levels below 50 copies / ml for a median period of 32 (range 0-44) weeks (Figure 1), consistent with the findings of others (28-32).

Correlation between CD4 count, plasma viral load and percentage of activated and proliferating T cells

It has been established before that T-cell activation markers are positively correlated with the plasma viral load, and negatively with CD4 counts (11,14). For all 3 activation markers, we report similar correlations (Table 1 and 2). However, these correlations may be spurious, due to the indirect effect of the negative correlation between CD4 count and plasma HIV RNA levels (at baseline r = -0.4, p=0.04). We therefore corrected for the negative correlation between CD4 count and plasma HIV RNA by computing partial correlations. The positive correlations that were observed between the plasma viral load and the expression of activation markers on T lymphocytes (Table 1) are hardly affected by controlling for the indirect effect of the CD4 count. Apparently, independent of homeostatic effects via the CD4 count, the plasma HIV RNA level has a true contribution to the T-cell hyperactivation. This supports the model that antigenic stimulation plays a role in T-cell hyperactivation during HIV infection. Similarly, the negative correlation between the CD4 count and activation of CD4+ T cells, persists after controlling for the plasma HIV RNA load. This suggests that there is a true additional role for CD4 homeostatic effects on CD4+ T-cell activation. Thus, in the CD4+ T-cell compartment, both mechanism seem to play a role. The negative correlation between the CD8 activation markers and the CD4 count however largely disappears (HLA-DR, Ki67, Table 2) when controlling for plasma HIV RNA. Apparently, activation of CD8+ T cells is largely due to plasma HIV RNA levels, and is not directly related to the CD4 count. In contrast to the inverse relation between CD4 count and the percentage of activated CD4+ T cells, which suggests homeostasis (Table 2), no
Association was observed between the CD8 count and percentage of CD8+ T cells expressing HLA-DR or CD38 (r = 0.12 and r = -0.01, respectively, p > 0.05). This seems natural as homeostasis is not expected to play a role in the expanded CD8 population. A weak positive correlation between CD8 count and the percentage of Ki67 expressing CD8 cells was observed (r = 0.22, p = 0.047).

Figure 2. Figure 2a and 2b show the negative correlation between CD4 count and expression of HLA-DR and Ki67 on CD4+ T cells, respectively, suggesting homeostatic effects in the CD4+ T-cell population. Black dots represent baseline data, white dots represent data from week 4 through week 48 of therapy. Figure 2c shows the correlation between expression of HLA-DR on CD4+ T cells and on CD8+ T cells. Figure 2d shows the correlation between expression of Ki67 on CD4+ T cells and on CD8+ T cells. Figure 2a and 2c: n = 461; Figure 2b and 2d: n = 79.
The rate of decay of T-cell activation markers during HAART
To determine whether a relation exists between decreasing plasma HIV RNA levels and T-cell activation during HAART, we estimated the second phase slope (week 4-48) of HIV RNA from plasma for each patient by linear regression analysis. In addition, the decay rates of the activation markers on T cells were estimated assuming that the percentage of activated T cells at baseline is $a$, and that T-cell activation decreases with rate per day $c$ to a level of healthy individuals $b$. We estimated $a$, $b$ and $c$ by fitting equation $y = b + a[\exp(-ct)]$ to the measurements of T-cell activation, where $y$ is the percentage of activated T cells and $t$ is time. No significant correlations were found between the rate of decline of plasma HIV RNA and the decay rates $c$ of HLA-DR and Ki67 expression on CD4$^+$ T cells, or the decay rates of HLA-DR, CD38 and Ki67 expression on CD8$^+$ T cells (each absolute Pearson’s coefficient $< 0.37$, each $p$ value $>0.24$). These findings indicate that the daily decrease of plasma HIV RNA plays a limited role in the decay rate of T-cell activation.

To determine the influence of increasing CD4 counts on CD4$^+$ T-cell deactivation during HAART, we also estimated the daily increase of the CD4 count during HAART for each patient. Because of the biphasic pattern of CD4 count increase, the speed of increase was estimated for the first phase (week 0-4), and the second phase (week 4-48), using linear regression analysis. During both phases, no significant correlations were observed between the daily increase in CD4 count and the decay rates of expression of HLA-DR and Ki67 on CD4$^+$ T cells (each absolute Pearson’s coefficient $< 0.24$, each $p$ value $>0.45$). These findings indicate that the daily increase of the CD4 count plays a limited role in the decay rate of T-cell activation.

Correlation between activation and proliferation status of CD4$^+$ and CD8$^+$ cells
We confirm observations by Sachsenberg et al. (14) that Ki67$^+$ expression on CD4$^+$ and CD8$^+$ T cells is positively correlated (Table 3, Figure 2d). Similarly, the percentage of HLA-DR$^+$ CD4$^+$ T cells was positively correlated with the percentage of HLA-DR$^+$ or CD38$^+$ CD8$^+$ T cells (Figure 2c). This suggests that CD4$^+$ and CD8$^+$ T-cell activation is driven by a common mechanism.
The fact that the positive correlation between CD4\(^+\) and CD8\(^+\) T-cell activation persists after controlling for the indirect effect of CD4 count (Table 3), suggests that other factors than CD4 homeostasis drive CD4\(^+\) and CD8\(^+\) T-cell activation. This positive correlation however also persists when we control for plasma HIV RNA level, and when we control for both CD4 count and plasma HIV RNA levels. These findings indicate that additional factors may play a role in T-cell activation, such as immune activation by other infections or HIV antigens that are not correlated with the plasma HIV RNA load.

Discussion

The aim of this study was to determine the mechanisms involved in increased activation and division of T lymphocytes in HIV infected patients. We found a negative correlation between the CD4 count and the percentage of activated CD4\(^+\) T cells, which remains after controlling for plasma HIV RNA load. Observations like this suggest a homeostatic response of the CD4\(^+\) T-cell population to compensate for the CD4\(^+\) T-cell depletion in HIV infection (23,24). Several other observations however argue against a general role for homeostasis in the increased activation of CD4\(^+\) and CD8\(^+\) T cells in HIV infection. First, the activation and proliferation is also in the expanded CD8\(^+\) T-cell population. Second, because the expression of activation markers on CD4\(^+\) and CD8\(^+\) T cells remains positively correlated after controlling for the CD4 count (Table 3), factors other than CD4\(^+\) T-cell depletion appear to play a role in driving the activation of both CD4\(^+\) and CD8\(^+\) T cells. Third, the percentage of CD4\(^+\) T cells expressing HLA-DR and Ki67 decreased rapidly after the start of HAART eventhough CD4\(^+\) T cells were still depleted (15). Fourth, at no time point during therapy, the decrease in the expression of HLA-DR and Ki67\(^+\) on CD4\(^+\) T cells was correlated to the increase in CD4 count (data not shown).

The plasma viral load correlated positively with the expression of HLA-DR, CD38 and Ki67 on T cells. This positive correlation suggests that plasma HIV RNA load and HIV replication drive T-cell activation.
However, two of our observations suggest that additional factors play a role. First, even though at week 48 of HAART all patients had plasma HIV RNA loads below 50 copies/ml for a mean interval of 32 weeks, the level of T-cell activation and proliferation remained significantly higher than in healthy controls. Second, the decay rate of the percentage of activated and proliferating T cells was not correlated with the elimination rate of HIV RNA from plasma. Third, the expression of activation markers on CD4$^+$ and CD8$^+$ T cells remains positively correlated after controlling for plasma HIV RNA, suggesting that other factors than plasma HIV RNA contributes to T-cell activation.

What additional factors, apart from CD4 homeostasis and plasma HIV RNA levels, could contribute to the T-cell activation? The positive correlation between fractions of activated cells in the CD4$^+$ and CD8$^+$ T-cell population, which persist after controlling for the CD4 count and the plasma HIV viral load, suggests that CD4$^+$ and CD8$^+$ T-cell activation are governed by similar factors. Thus, we believe that the most parsimonious explanation for the hyperactivation of both CD4$^+$ and CD8$^+$ T-cell populations is antigenic stimulation. This may involve (long-lived) antigens from HIV and/or other (opportunistic) pathogens.

The negative correlation between the CD4 count and T-cell activation, which could be taken as evidence for a homeostatic response of the CD4$^+$ T-cell population, may also be explained by antigenic stimulation. A low CD4 count increases the risk of developing opportunistic infections with Pneumocystis Carinii, Cytomegalovirus (CMV) or Mycobacterium Avium Complex (33,34). Moreover, in the blood of patients with low CD4 counts signs of active CMV and Epstein-Barr virus replication have been observed (35-38). These (Opportunistic) infections are associated with increased T-cell activation (11,39-41). Antigenic stimulation also explains the positive correlation between the plasma viral load and T-cell activation. A high plasma viral load is associated with an increases the risk of developing opportunistic infections, and replication of HIV itself will also increase the antigenic load.

The slow decay of T-cell activation during HAART may be explained in two ways. First, the clearance of antigens from other anatomic compartments than the blood, e.g. lymphoid tissue, is expected to be slow
In line with this, we observed persistence of HIV p24 antigen in lymphoid tissue, after 18 months of HAART with plasma viral loads below 50 copies / ml (data not shown). Secondly, low level ongoing HIV replication during HAART may play a role. Based on theoretical considerations (45) and the detection of HIV mRNA in lymphoid tissue of patients on HAART with plasma viral load < 50 copies / ml (46-47), it has been hypothesized that a low level of HIV replication may occur during HAART. In addition, it has been demonstrated that the presence of episomal HIV-1 infection intermediates persist in patients with undetectable plasma HIV RNA levels during HAART (48).

There is a strong interaction between HIV replication and T-cell activation because productive HIV infection is largely restricted to CD4+ T cells that are activated (22,25). Several predator-prey type mathematical models of HIV infection describe this interaction, assuming that activated CD4+ T cells are the primary target cells of HIV (49). In contrast to our observations (Figure 1) however, the number of activated CD4+ T cells increases during HAART in these models. Thus, the number of target cells increases if HIV is suppressed. Our results therefore suggest that current mathematical models should be extended with mechanisms for CD4+ T-cell activation by HIV and/or other antigens. One such mechanism, obviously, would be to allow for the immune response to HIV (and/or other antigens).

In conclusion, our results suggest that antigenic stimulation is the dominant mechanism of T-cell activation in HIV infection, rather than CD4+ T-cell homeostasis. Persistence of HIV antigens, or low level ongoing HIV replication during HAART may explain why T-cell activation fails to revert to levels of healthy individuals after 48 weeks of therapy.

Acknowledgements

The authors greatly acknowledge the following individuals for their assistance: Andre Noest, Bert Bravenboer, Job Juttmann, Peter Koopmans, Frank Kroon, Pieter Meenhorst, Clarence Richter and Herman Sprenger.
References


