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Successful HAART Is Associated with High B-Chemokine Levels in Chronic HIV Type 1-Infected Patients

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ABSTRACT

Chemokine receptors are used by HIV-1 for entry into $CD4^+$ T cells. The β-chemokines are capable of inhibiting HIV replication. This study measured β-chemokine macrophage inflammatory protein (MIP)-1α and MIP-1β levels and determined the CCR5 and CXCR4 expression on T cells in HIV-1-infected patients treated with HAART. The time of known HIV infection and time of HAART use were similar between failure and successful groups. The $CD4^+$ T cell nadir was 163 vs. 251 cells/mm^3, $p = 0.07$, for failure and successful groups, respectively. The successfully treated group, when compared with the failure group, had a higher median $CD4^+$ T cells count (667 vs. 257 cells/mm^3; $p = 0.003$) as well as higher spontaneous MIP-1α (median of 4390 vs. 802 pg/ml, $p = 0.03$) and MIP-1β (median of 2416 vs. 1117 pg/ml, $p = 0.001$) levels. The untreated patients had a higher number and intensity of CCR5- and CXCR4-expressing T cells. Higher levels of chemokines were not related to nadir $CD4^+$ T and current $CD8^+$ T cell counts. Successfully treated patients were able to produce higher amounts of β-chemokines and normalize the coreceptor overexpression on T cells. These findings may have clinical implications, such as a new strategy of using chemokines as adjuvants in anti-HIV therapy.

INTRODUCTION

CHEMOKINES ARE SUBSTANCES chemotactic to leukocytes that regulate the activation and recruiting of monocytes, neutrophils, and others cells to inflammatory sites, binding to chemokine receptors.[1,2] The members of this family belong to two major groups, based on the position of two cysteine residues: the chemokine CC and CXC.[2,3] The chemokine CC binds to nine chemokine receptors CC, designated CCR1–CCR9, and the chemokine CXC binds to five chemokine receptors CXC, designated CXCR1–CXCR5.[4] The CCR5 is linked to a G protein (GPCR), with seven transmembrane residues that regulate the circulation and effector function of leukocytes. Some chemokine receptors are used by HIV-1 as coreceptors with the CD4 molecule *in vitro*, but only CCR5 and CXCR4 seem to be used *in vivo*.[5]

Natural ligands to the CCR5 receptor are the β-chemokine macrophages inflammatory protein (MIP)-1α, MIP-1β, and RANTES (regulated on activation, normal T cell expressed and secreted).[2,3,6,7] The CCR5 and CXCR4 coreceptors explain cellular tropisms by different virus strains, resistance to infection by some highly exposed individuals, and the varying rates of HIV disease progression.[8,9] The chemokine receptor interaction and its ligands block and downregulate the host cell surfaces and, thereby, effectively inhibit HIV infection.[8,10] The β-chemokines are capable of inhibiting the replication of HIV[11] by competing with virus binding to the CCR5 coreceptor.[4,12]

The CCR5 coreceptor is important in the pathogenesis of HIV-1 disease.[2] Naturally occurring polymorphisms of the CCR5 gene generated by single point mutation, deletion of the function receptor, or reduced expression of the gene also play a role in the resistance to HIV-1 infection and progress of the disease.[13] The expression of CCR5 and CXCR4 appears to be extremely important in determining the susceptibility of T cells to HIV-1 infection. However, the infection rate and severity of the disease are controlled not only by the variability of CCR5 expression but also by the availability of an agonist.[13]

HIV pathogenesis involves both the response of cytotoxic T cells (CTL, as the cytolytic response) to viral proteins and the release of β-chemokines by $CD8^+$ T cells (the suppression response), which are important in the anti-HIV response.[11] In addition, $CD4^+$ T cells are also an important source of the β-chemokines,[14,15] especially among HIV-1-exposed but un-

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infected persons,[16,17] and among patients whose infection follows a slow progression to disease.[18]

The use of highly active antiretroviral therapy (HAART) results in a rapid decline of viral load, an initial immune reconstitution, and a decrease in the risk of further morbidity and death.[19] Despite the general success of this strategy, many individuals benefit only temporarily because of therapeutic failure caused by the emergence of drug-resistant viruses.[19] Few data have been published evaluating subjects who experience a failure to HAART and the expression of chemokine receptors on their T cells. Thus, we hypothesized that β-chemokine production may be upregulated in HIV-infected subjects, and that this fact may be modified after HAART use.[19] In the present study, we examined the effect of HAART on the expression of CCR5 and CXCR4 HIV coreceptors and we compared the production of MIP-1α and MIP-1β taken from different groups of HIV-1-infected patients who had either experienced a failure or a success after HAART use.

MATERIALS AND METHODS

Patients

Sixty-one HIV-1-infected individuals were recruited in the outpatient service of the Secondary Immunodeficiencies Clinic of the "Hospital das Clinicas da Faculdade de Medicina da Universidade de São Paulo" (HCFMUSP). Informed consent was obtained from individuals, and the research protocol was approved by the Ethical Committee Board. Inclusion criteria in this study were age over 18 years for both genders and no active opportunistic infection or use of immunosuppressive drugs. The HIV-1-infected patients were divided into three groups according to their status regarding HAART and level of viral load. Group I (failure therapy) included 23 HIV-1-infected patients (viral load ≥ 400 copies/ml for 2 years after HAART); group II (successful therapy) included 19 HIV-1-infected patients (viral load < 400 copies/ml for 2 years after ART); group III (no HAART) included 19 HIV-1-infected patients; and group IV (healthy controls) included 19 HIV-1-seronegative individuals.

Peripheral blood mononuclear cell (PBMC) cultures

PBMCs were collected in heparinized tubes and isolated using Ficoll-Hypaque density gradients[17] (Amersham Pharmacia, Piscataway, NJ). The cells were washed, adjusted to 2×10^6 cells/ml in RPMI 1640 medium supplemented with 10% fetal calf serum, and grown with or without 2.5 μg/ml of phytohemagglutinin (PHA) at 37°C, 5% CO_2 for 24 h. The supernatant fluids were harvested and stored at −70°C for the performance of chemokine assays.

β-Chemokines production

Measurement of MIP-1α and MIP-1β production was performed on PBMC supernatant fluids by enzyme immunoassays (EIA). Antibody matched pairs and respective standards were

TABLE 1. DEMOGRAPHIC AND IMMUNOLOGICAL CHARACTERISTICS OF HIV-1-INFECTED SUBJECTS AFTER SUCCESSFUL OR UNSUCCESSFUL USE OF HAART[a] AND HEALTHY CONTROL SUBJECTS

Variable	*Failure*[b] *VL > 400*	*Successful*[c] *VL ≤ 400*	*No HAART*	*HIV-uninfected controls*	p *value*[d]
Gender	*n* = 23	*n* = 19	*n* = 19	*n* = 19	
Women/men	3/20	5/14	5/14	11/8	
Age (mean, years)	43 ± 8.3	42 ± 9.2	38 ± 10	28 ± 7	<0.0001
Time of HIV infection[e]					
(median, months)	114.2	104.6	18.5	—	0.01
(percentile 25–75)	(37–140)	(57–135)	(15–152)		
Time of ART					
(median, years)	8.7	8.1	—	—	NS[f]
(percentile 25–75)	(6.6–9.5)	(4.1–9.8)			
T CD4					
(median, cells/mm^3)	257	667	414	1110	<0.0001
(percentile 25–75%)	(127–519)	(411–799)	(325–573)	(842–1382)	
T CD8					
(median, cells/mm^3)	899	698	1074	614	0.02
(percentile 25–75)	(785–1238)	(471–1002)	(542–1335)	(477–698)	
RNA plasma viral load					
(median, copies/ml)	4830	—	17,150	—	NS
(percentile 25–75)	(200–44,800)		(2690–172,600)		

[a]HAART, highly active antiretroviral therapy.
[b]Failure: HIV-1-infected subjects who have plasma HIV RNA levels above the detectable level for more than 2 years after HAART.
[c]Successful: HIV-1-infected subjects who have plasma HIV RNA undetectable levels for more than 2 years after start of HAART.
[d]*p* value = one-way Mann-Whitney test. *p* values <0.05 were considered statistically significant.
[e]Time of HIV infection: time period between the first known positive HIV testing and the start of follow-up.
[f]NS, not significant.

purchased from R&D Systems (Duoset ELISA development kit, Minneapolis, MN) and used according to the manufacturer's recommendation. The detection limit was 10 pg/ml for both chemokines. Optical density was measured with a 450-nm filter (Bio-Rad, Hercules, CA) and the concentration was determined using a standard curve developed with the GraphPrism software.

Antibodies for flow cytometry analysis

The following monoclonal antibodies (mAb) were purchased from Caltag (USA): anti-$CD3^{PC-5}$, anti-$CD4^{FITC}$, and anti-$CD8^{FITC}$. The anti-$CCR5^{PE}$ and anti-$CXCR4^{PE}$ mAbs were purchased from R&D Systems (Minneapolis, MN).

Flow cytometry analysis

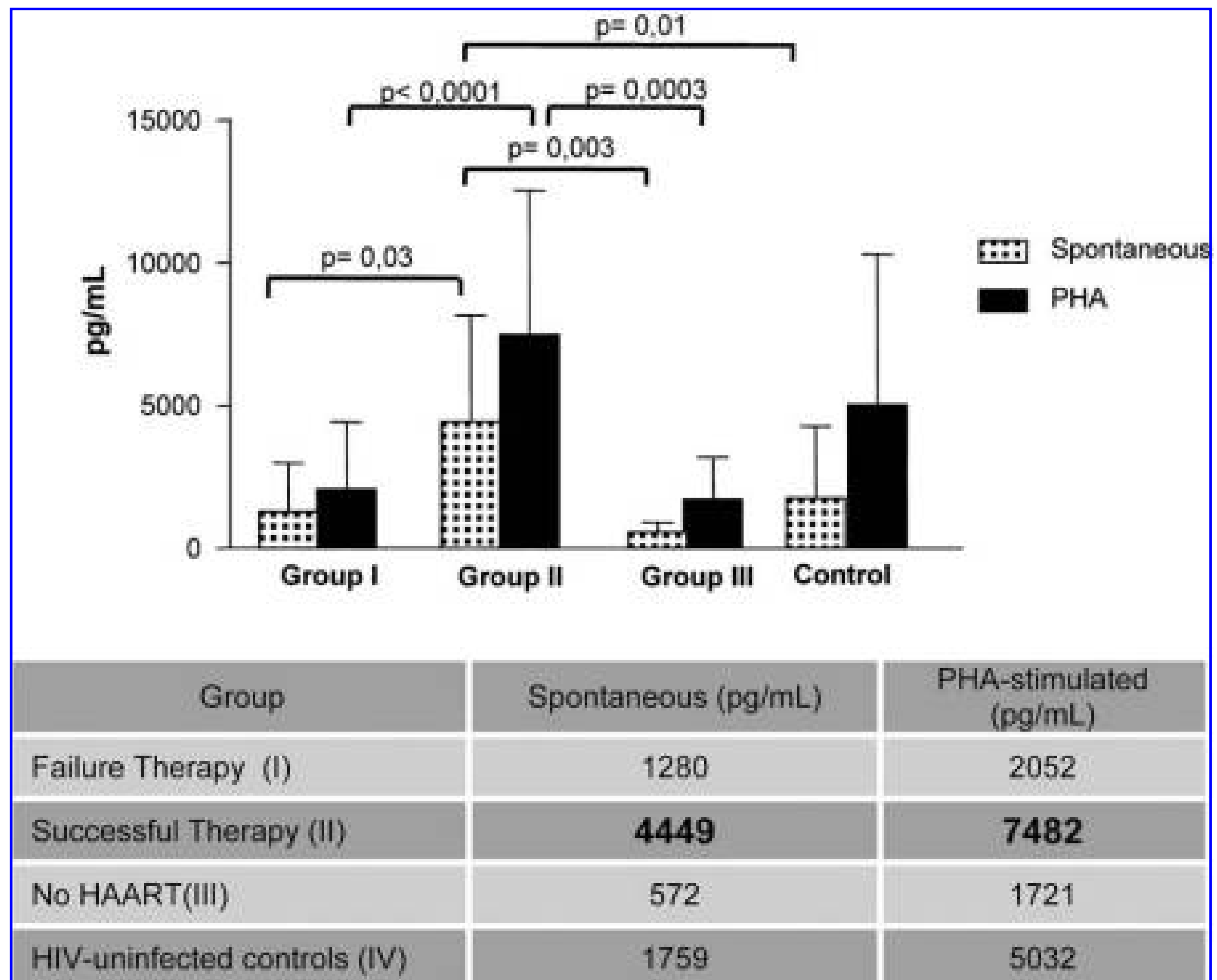
Flow cytometry analyses were performed with fresh blood samples, using a Coulter EPICS XL-MCL Flow Cytometer (Beckman Coulter, Fullerton, CA). To determine coreceptor expression on $CD4^+$ and $CD8^+$ T cells, total lymphocytes were first identified by forward and side scatter. The cells were then gated for $CD4^+$ or $CD8^+$ expression. The resulting bivariate plots of CCR5 (R5) and CXCR4 (X4) staining were then analyzed according to the isotype-matched controls. The following combinations were analyzed: (I) anti-$CD3^{PC-5}$, anti-$CD4^{FITC}$, anti-$CCR5^{PE}$; (II) anti-$CD3^{PC-5}$, anti-$CD4^{FITC}$, anti-$CXR4^{PE}$; (III) anti-$CD3^{PC-5}$, anti-$CD8^{FITC}$, anti-$CCR5^{PE}$; (IV) anti-$CD3^{PC-5}$, anti-$CD8^{FITC}$, anti-$CXR4^{PE}$.

Determination of viral load

Plasma HIV RNA levels were determinated using the Roche Amplicor assay following the manufacturer's instructions (Roche Amplicor HIV-1 Monitor Test, Roche Molecular Systems, Inc., Branchburg, NJ).

Statistical analysis

Possible differences in patients characteristics or laboratory values from the four groups were evaluated with a



Group	Spontaneous (pg/mL)	PHA-stimulated (pg/mL)
Failure Therapy (I)	1280	2052
Successful Therapy (II)	**4449**	**7482**
No HAART(III)	572	1721
HIV-uninfected controls (IV)	1759	5032

FIG. 1. MIP-1α levels after 24 h among the HIV-1-infected subjects and controls. Successful: treated HIV-1-infected subjects who have had undetectable plasma HIV RNA levels for more than 2 years after start of HAART. Failure: treated HIV-1-infected subjects who have had detectable plasma viral loads for more than 2 years after HAART. Successful group vs. failure group and no use of HAART group. HAART, highly active antiretroviral therapy.

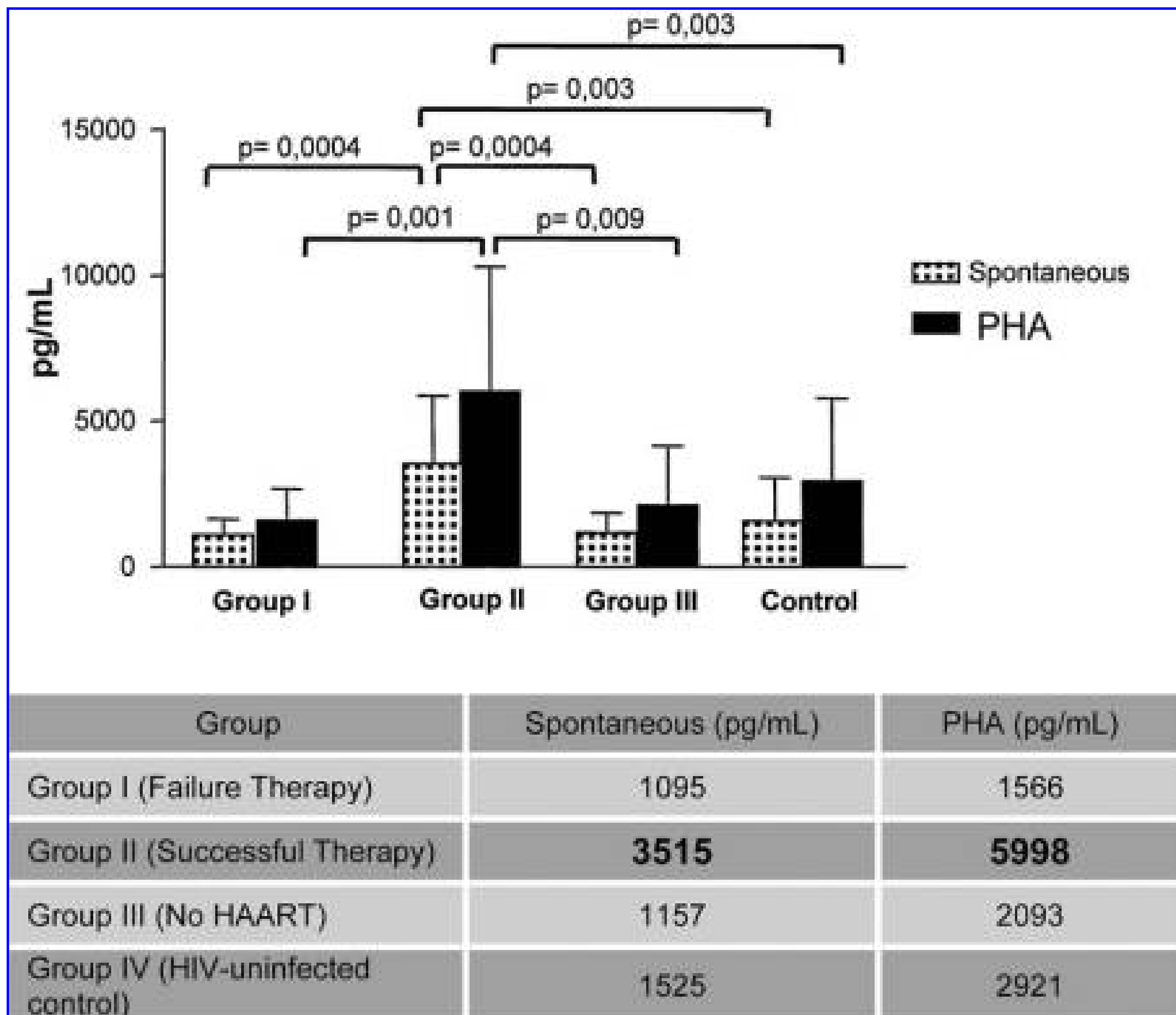
one-way Mann–Whitney test and Kruskal–Wallis test. In both cases p values < 0.05 were considered statistically significant.

RESULTS

Table 1 depicts the demographic and immunological data of the infected groups. All three HIV-1 infected groups showed similar distribution in gender, and the average age was 40 years. The time of known HIV infection was lower for group III (no HAART) compared to groups I and II ($p = 0.01$ and $p = 0.02$, respectively). The median $CD8^+$ T cell number was similar, but the failure group had a higher median $CD8^+$ T cell count than the healthy controls ($p = 0.02$). The median HIV plasma viral load of the failure therapy group was 4830 copies/ml and that of HIV-1 infected untreated group was 17,150 copies/ml ($p = 0.1$).

Figure 1 shows the MIP-1α results. The successful group had higher MIP-1α production at baseline (5-fold) and after PHA stimulation (6-fold) (4390 pg/ml vs. 8470 pg/ml, respectively) than the failure group (802 pg/ml vs. 1304 pg/ml), no ART patients (574 pg/ml vs. 1287 pg/ml), and healthy control subjects (517 pg/ml vs. 5032 pg/ml) ($p < 0.05$ for all comparisons).

Figure 2 shows the MIP-1β results. In general, the successful group had higher MIP-1β spontaneous production than the other groups, including healthy controls ($p < 0.05$ for all comparisons). Despite PHA stimulation, very little or no increase was observed for HIV-infected subjects, except for the successful group of patients, who presented a 3-fold increase.



Group	Spontaneous (pg/mL)	PHA (pg/mL)
Group I (Failure Therapy)	1095	1566
Group II (Successful Therapy)	**3515**	**5998**
Group III (No HAART)	1157	2093
Group IV (HIV-uninfected control)	1525	2921

FIG. 2. MIP-1β levels after 24 h among the HIV-1-infected subjects and controls. Successful: treated HIV-1-infected subjects who have had undetectable plasma viral loads for more than 2 years after HAART. Failure: treated HIV-1-infected subjects who have had detectable plasma viral loads for more than 2 years after HAART. Successful group vs. failure group and no use of HAART group. HAART, highly active antiretroviral therapy.

TABLE 2. ABSOLUTE NUMBERS OF CCR5 AND CXCR4 ON T CD4$^+$ AND CD8$^+$ LYMPHOCYTES OF THE HIV-1-INFECTED SUBJECTS AND HEALTHY CONTROLS[a]

	Failure[b] *VL > 400* (n = *16)* *Group I*	*Successful*[c] *VL ≤ 400* (n = *19)* *Group II*	p *value*[d] *I × II*	*No HAART*[e] (n = *16)* *Group III*	p *value* *I × III*	*II × III*	*Healthy uninfected controls* (n = *13)* *Group IV*	p *value* *I × IV*	*II × IV*	*III × IV*
CD3/CD4/R5 (cells/mm^3)	15 (11–74)	17 (12–35)	NS[f]	24 (18–45)	NS	NS	65 (26–122)	0.02	0.0007	0.01
CD3/CD4/X4 (cells/mm^3)	60 (24–147)	78 (50–183)	NS	36 (20–90)	NS	0.02	226 (111–429)	0.0001	0.0002	0.0005
CD3/CD8/R5 (cells/mm^3)	121 (33–278)	35 (23–135)	NS	208 (82–266)	NS	0.006	58 (38–240)	NS	NS	NS
CD3/CD8/X4 (cells/mm^3)	129 (61–183)	100 (65–185)	NS	47 (31–89)	0.01	0.007	132 (92–489)	NS	NS	0.0006

[a]Flow cytometric analysis of CCR5 and CXCR4 receptor expression on the CD4, CD8$^+$ T lymphocytes.
[b]Failure: untreated HIV-1-infected subjects who have had detectable plasma HIV RNA levels for more than 2 years after HAART.
[c]Successful: treated HIV-1-infected subjects who have had undetectable plasma HIV RNA levels for more than 2 years after start of HAART.
[d]*p* value = one-way Mann-Whitney's test. *p* values <0.05 were considered statistically significant.
[e]HAART, highly active antiretroviral therapy.
[f]NS, not significant.

Table 2 depicts the flow cytometry results. The number of CD4$^+$ and CD8$^+$ T cells expressing CCR5 or CXCR4 on the successful therapy group was similar to that on the failure therapy group ($p > 0.05$ for all comparisons). The untreated patients had lower CD4/X4 and CD8/X4 numbers compared to the successful group, their CD8$^+$ T cells presented higher levels of R5 than those of the successful therapy group, and also decreased levels of X4 when compared to treated groups ($p < 0.05$). The control group showed higher numbers of CD4/X4 and CD4/R5 T cells than the other groups.

Table 3 shows the mean fluorescence intensity (MFI) of the CCR5 and CXCR4 coreceptors on T lymphocytes. Both treated groups showed similar results. There was an overexpression of CD4/R5 T cells among the failure group compared to untreated patients. Those patients with no use of ART had lower MFI than the control group.

DISCUSSION

In this study, successfully treated HIV-1-infected subjects produced higher amounts of MIP-1α and MIP-1β levels than treated and failed HIV-1-infected patients, untreated HIV-1-infected patients, and healthy non-HIV-infected controls. A previous report showed that HIV-1-infected asymptomatic patients with CD4$^+$ T cells counts over 200 cel/mm^3 had higher levels of MIP-1α and MIP-1β production.[10] These chemokines are bi-

TABLE 3. MEAN FLUORESCENCE INTENSITY (MFI) CCR5 AND CXCR4 EXPRESSION ON T LYMPHOCYTES OF THE HIV-1-INFECTED SUBJECTS AND HEALTHY CONTROLS[a]

T CD3$^+$ cells	*Failure VL > 400* (n = *16)* *Group I*	*Successful VL ≤ 400* (n = *22)* *Group II*	p *value*[b] *I × II*	*No HAART* (n = *17)* *Group III*	p *value* *I × III*	*II × III*	*Healthy uninfected controls* (n = *13)* *Group IV*	p *value* *I × IV*	*II × IV*	*III × IV*
CD4/R5	1.03 (0.36–1.5)	0.5 (0.3–0.9)	NS[c]	0.38 (0.22–0.58)	0.01	NS	0.6 (0.53–0.8)	NS	NS	0.007
CD4/X4	1.12 (0.57–1.7)	0.92 (0.52–1.17)	NS	0.79 (0.6–1.3)	NS	NS	1.0 (0.94–1.35)	NS	NS	NS
CD8/R5	0.84 (0.64–1.79)	0.59 (0.35–0.76)	NS	0.67 (0.43–0.98)	NS	NS	0.85 (0.58–1.0)	NS	0.02	NS
CD8/X4	0.74 (0.49–1.5)	1.0 (0.79–1.5)	NS	1.02 (0.71–1.14)	NS	NS	1.3 (1.0–1.75)	NS	NS	0.01

[a]MFI of the CCR5 and CXCR4 on T lymphocytes among HIV-1-infected subjects and healthy controls.
[b]*p* value = one-way Mann–Whitney test. *p* values <0.05 were considered statistically significant.
[c]NS, not significant.

ologically important, particularly MIP-1β, which is a specific ligand for CCR5, blocking HIV entry. In addition, mutation in this gene has been associated with protection against HIV infection (in the homozygous state) and slower progression to AIDS (in the heterozygous state). In fact, MIP-1β does not bind to any other known chemokine receptor except CCR5, whereas MIP-1α also binds to another receptor.[8] Thus, it is reasonable to speculate that the beneficial effect of these proteins may be their binding to the CCR5 receptors, thus decreasing the HIV RNA plasma viral load. In addition, inhibitors of CXCR4 may also block HIV-1 infection.[20]

Although numerous immune and nonimmune cell types are secreting chemokines, CD4^{+} and CD8^{+} T cell-derived CCR5 chemokines have been demonstrated to be capable of inhibiting HIV infection.[7,8,21,22] Our findings may indicate higher CD4^{+} T cell numbers in the successful group, suggesting a better immune restoration.[23] Garzino-Demo *et al*.[8] found that in some cases chemokine levels correlated with CD4^{+} T cell numbers, but not with CD8^{+} T cells.

It is possible to argue that higher β-chemokine levels among successfully treated patients might reflect the higher CD4^{+} T cell numbers. However, if this observation were true, the control group would have had the highest production in our study. However, CD8^{+} T cells are potent sources of the β-chemokine, which was not different in this study. Thus, circulatory CD8^{+} T cell numbers were not important to induce the β-chemokine production. It was reasonable to infer that better specific CD8^{+} T cell clones are more important than their absolute number. Probably better immune restoration is very important for the antiviral response, and higher β-chemokine production by the T cells may be a critical issue. Despite some studies indicating CD4^{+} T cells as important sources of β-chemokines,[17,18] unfortunately we did not study which T cell subpopulation is the most important for their increased production. Thus, our results likely reflect the interaction between CD4^{+} and CD8^{+} T cells after immune restoration.

Another issue raised on this study was the CCR5 and CXR4 intensity of expression on T cells. The untreated group showed a higher number and increased expression of chemokine receptors compared with the treated cases and controls. Indeed, HAART leads to normalization of this expression, regardless of viral load. These findings indicate that viral replication induces upregulation of those receptors on T cells. In fact, after successful HAART, the expression of CCR5 and CXCR4 and β-chemokine production is altered in HIV-infected individuals, which suggests that their early modification during HAART reflects both peripheral redistribution of naive/memory T cell compartments and a decrease in T cell activation.[19] Such modifications in the expression of viral tropism and the production of antiviral molecules may play a role in the emergence of virus variants when a failure of HAART occurs.

The results of this study may indicate that an effective viral suppression can contribute to progressive normalization of maturational and functional T cell abnormalities responsible for the high levels of T cell apoptosis observed in HIV-1-infected individuals.[24] This, in turn, may contribute to a reduced rate of T cell loss and immune reconstitution during HAART.[24] This is probably due to the fact that these individuals presented with an elevated number of activated T cells as a result of HIV-1 pathogenesis.

Taken together, these results indicate that the successfully treated patients had an increase of β-chemokine levels, an observation that may have clinical implications. In fact, antagonists of the HIV-1 coreceptor CCR5 are being developed as the first anti-HIV agents acting on a host cell target; these small molecules are currently under clinical trials.[25] Preliminary reports have shown a decrease in RNA HIV plasma viral load, but also the emergence of CXCR4 strains.[25] The high production of β-chemokines has been associated with better viral control. The overexpression of coreceptors was associated with no HAART use and was normalized after the start of HAART, regardless of viral replication control. Thus, the clinical induction of β-chemokine production could be an additional approach for future studies in HIV replication control *in vivo*.

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