# Chemokine Receptors Expression on T Cells and Response to HAART Among Chronic HIV-1-Infected Subjects

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**Abstract:** Chemokines receptors are used by HIV-1 for entry into $CD4^+$ T cells. The β-chemokines are capable of inhibiting HIV replication. This study determined the CCR5 and CXCR4 expression on T cells in HIV-1-infected patients treated with HAART. The successfully treated group (plasma viral load <400 copies/mL), when compared with the failure group (plasma viral load >400 copies/mL), had higher median $CD4^+$ T cells count (583 and 245 cells/mm$^3$; respectively, $p< 0.0001$). The failure patients had higher numbers and intensity of CCR5 and CXCR4-expressing T cells. Successfully treated patients were able to normalize the co-receptors expression-over on T cells. The viremic group showed higher CCR5 expression on $CD4^+$ T cells and lower number of cells; CCR5 expression was normalized in the aviremic group; the naïve group showed lower CCR5 expression and higher numbers of CD4 T cells; all groups showed normal CXCR4 expression compared to healthy controls. These findings may have clinical implications, since down-regulation of these co-receptors could be an adjuvant strategy for anti-HIV treatment.

**Keywords:** HIV-1, chemokines, co-receptors, CCR5, CXCR4, antiretroviral therapy.

## INTRODUCTION

Chemokines are chemotactic substances that regulate the activation of leukocytes, promote the recruiting of monocytes, neutrophils and other cells to inflammatory sites, binding to chemokine receptors [1]. The members of this family belong to two major groups, based on the position of two cystein residues: the chemokine CC and CXC [2]. The chemokine CC binds to nine chemokine receptors CC, named CCR1 to CCR9 and the chemokine CXC binds to five chemokine receptors CXC, designed CXCR1 to CXCR5 [2]. The CCR5 is linked to a G protein (GPCR), with seven trans-membrane residues that regulate the circulation and effectors function of leukocytes. Some chemokine receptors are used by HIV-1 as co-receptors with CD4 molecule *in vitro*, but only CCR5 and CXCR4 seem to be used *in vivo* [3].

The CCR5 co-receptor is important in the pathogenesis of HIV-1 disease [4]. It was identified as the HIV-1 co-receptor whose activity is blocked by MIP-1α/CCL3, MIP-1β/CCL4, and RANTES/CCL5 chemokines [5]. The CXCR4, with its ligand, SDF-1/CXCL12, endowed with anticoreceptor activity [5]. 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 progression to disease [6]. Individuals homozygous for a 32 base pair deletion in their CCR5 gene (referred to as CCR5-D32), are almost completely resistant to HIV infection. The mutation is a 32 base pair deletion corresponding

to the second extracellular loop of the 7-transmembrane G-coupled protein receptor in the CCR5 gene, which causes a frame shift, leading to a premature termination of translation and the resulted protein encoded by this mutant lacks three transmembrane segment of the receptor. Such a truncated protein is nonfunctional [18=7]. Homozygous status is associated with slower progression [8].

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 [19]. The CCR5 molecule is expressed on the surface of cells which present CD26$^{high}$, CD45RO and CD95 molecules (characteristic of memory cells). It is present on activated T cells (particularly on $T_H1$ type), monocytes, macrophages, dendritic cells, eosinophils, basophils and platelets [10].

The CXCR4 receptor is also important, but few polymorphisms have been ascribed to this *locus*. In contrast, SDF-1, which is located in the SDF-1β fragment, is characterized by G substitution for A in the 801 position (in accordance to *GenBank* L36033) in the gene sequence (SDF-1-3´UTR-801 G-A or SDF-1-3´A) [11-12].

These genetic polymorphisms could be used as genetic markers to detect individuals at higher risk of developing either a faster disease progression or therapeutic failure. Pharmacogenetic is very likely to underlie future therapies for HIV-1 infection, and current patients with multi-resistance to the existing antiretroviral agents could also benefit from this approach. These developments also underscore the importance of continuing the investigation of new therapies targeted to the host in order to inhibit the HIV-1 entry into the host cells [13]. Based on the fact that the HIV disease progression has been related to specific genetic po-

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lymorphisms, we decided to study the expression of chemokine receptors among HIV-1-infected subjects and the HAART response.

## MATERIAL AND METHODS

### Patients

Eighty-three HIV-1-infected individuals were recruited from the outpatient service of the Secondary Immunodeficiencies Clinic of the "Hospital das Clínicas da Faculdade de Medicina da Universidade de Sao 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, no active opportunistic infection or use of immunosupressive drugs. The HIV-1-infected patients were divided into three groups, according to their status regarding highly active anti-retroviral therapy (HAART) and level of viral load: Group I (failure therapy): 24 HIV-1-infected patients (viral load $\geq$ 400 copies/ml for 2 years after HAART); Group II (successful therapy): 22 HIV-1-infected patients (viral load < 400 copies/ml for 2 years after HAART); Group III (no HAART): 18 HIV-1-infected patients. Group IV (healthy controls): 23 HIV-1-seronegative individuals.

### Peripheral Blood Mononuclear Cell Cultures

PBMCs were collected in heparinized tubes and isolated using Ficoll-Hypaque density gradients (Amersham Pharmacia, Piscataway, NJ, USA). The cells were washed, adjusted to $2 \times 10^6$ cells/ml in RPMI 1640 medium supplemented with 10% fetal calf serum, and grown with or without 2.5 ug/ml of phytohemaglutinin (PHA) at 37 ℃, 5% $CO_2$ for 24 hours. The supernatant fluids were harvested and stored at -70 ℃ for the performance of chemokines assays.

### Genetic Determination of SDF-1-3'A, CCR5-D32 and CCR5-P-59029A/G

Genomic DNA was isolated from whole blood in EDTA anti-coagulant tubes. CCR5-D32 was determined by polymerase chain reaction using the primers: CCR5-D1 (5'-AAC/AGA/TCT/CAA/AAA/GAA/GGT/CT-3') and CCR5-D2 (5'-CAT/GAT/GGT/GAA/GAT/AAG/CCT/CAC/A-3') (Operon Technologies, Cologne, Germany). The SDF-1-3'A and CCR5-P-59029A/G polymorphism was determined by PCR using the primers: SDF-1: (5'-CAG/TCA/ACCC/TGG/GCA/AAB/CC-3' and SDF AGC/TTT/GGT/CCT/GAG/AGT/CC-3'); CCR2 (5'TTG/TGG/GCA/ACA/TGA/TGG-3' and 5'CTG/TGA/ATA/ATT/TGC/ACA/TTG/C-3') and CCR5-P (5'-CCC/GTG/AGC/CCA/TAG/TTA/AAA/CTC-3' and 5'-TCA/CAG/GGC/TTT/TCA/ACA/GTA/GG-3') followed by RFLP reaction using MspI, BsaB1 and BSP1268I, respectively, for each gene cited above.

### Antibodies for Flow Cytometry Analysis

Flow cytometry analyses were performed with freshly blood samples, using a Coulter® EPICS® XL-MCL™ Flow Cytometer (Beckman Coulter, Fullerton, CA). To determine co-receptor 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$^{FITC}$, and anti-CXR4$^{PE}$.

### Determination of Viral Load

Plasma HIV RNA levels were determined 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 patient characteristics or laboratory values among the four groups were evaluated with two-way Mann-Whitney's test and Kruskal-Wallis test. In both cases P values<0.05 were considered statistically significant.

## RESULTS

Table **1** depicts the demographical and immunological data of the infected groups. All 3 groups showed similar distribution of gender, and the average age was 40 years. The time of known HIV infection was lower for group III (no HAART), compared to group I and II ($p = 0.01$ and $p = 0.02$, respectively). The time of HAART was similar on treated groups, with a median of nine years. As expected, CD4 T cells count were decreased among failure patients compared to other groups. The median $CD8^+$ T cells number was similar, but the failure group had a higher median $CD8^+$ T cells count than the healthy controls ($p = 0.02$). The median HIV plasma viral load of the failure therapy group was 11600 copies/mL, while the median viral load was 12050 copies/mL for the HIV-1-infected untreated group ($p = 0.1$).

Table **2** discloses the genetic polymorphisms for the receptors. No differences were seen among groups.

Fig. (**1**) depicts the CCR5 and CXCR4 expression on $CD4^+$ T cells by flow cytometry. There was a difference: the naive patients showed decreased CCR5 expression compared to viremic subjects (p= 0,01) and to the control group (p= 0,007) (Fig. **1A**). Fig. (**1B**) shows that aviremic individuals had lower T $CD4^+/CCR5^+$ cells number compared to naïve subjects (p= 0,02) and to the control group (p= 0,001). Viremic patients had also decreased $CD4^+/CCR5^+$ T cells in relation to controls (p= 0,01).

Fig. (**2**) shows the results regarding T $CD8^+/CCR5^+$ acquisition by flow cytometry. There were significant differences in the CCR5 expression and in the number of CD4 + / CCR5 + T cells when the three groups of HIV-1 positive individuals and the control group were analyzed together by the Kruskal-Wallis method (p = 0.02 and p = 0002, respectively). In comparison, the Mann-Whitney test revealed (Fig. **2A**) that naïve patients presented lower CCR5 expression than viremic subjects (p = 0.01) and the control group (p = 0007). Aviremic individuals had fewer CD4 + T cells/CCR5+, compared to individuals without ARV (p = 0.02) and the control group (p = 0001). Viremic individuals compared to the control group also had a lower number of CD4+ T cells/CCR5+ (p=0.01) (Fig. **2B**).

Fig. (**3**) shows CD4+/CXCR4 + T lymphocytes acquisition by flow cytometry. There was no significant difference

**Table 1.    Demographical and Immunological Characteristics of HIV-1-Infected Subjects After Successful or Unsuccessful Use of HAART, and Healthy Control Subjects**

| Variable | Viremic VL > 400 | Aviremic VL ≤ 400 | No HAART | Control | P Value |
|---|---|---|---|---|---|
| **Gender**<br>Female/Male | n= 24<br>8/16 | n= 22<br>8/22 | n=18<br>13/16 | n=23<br>15/8 | - |
| **Age**<br>Mean, years | 43 ± 9,7 | 42 ± 8,8 | 38 ± 9 | 29 ± 9 | NS |
| **Time of HIV infection**<br>median, months (inter-quartile range) | 114,2<br>(37 – 140) | 104,6<br>(57 – 135) | 18,5<br>(15 – 152) | ---- | 0,011 |
| **Time of HAART**<br>median, months (inter-quartile range) | 8,7<br>(6,6 – 9,5) | 8,1<br>(4,1 – 9,8) | ----- | ---- | NS |
| **CD4$^+$ T cells**<br>median, months (inter-quartile range) | 245<br>(123 – 369) | 583<br>(396 – 765) | 414<br>(325 – 630) | 1110<br>(842 – 1382) | < 0,0001 |
| **CD8$^+$ T cells**<br>median, months (inter-quartile range) | 840<br>(707 – 1229) | 776<br>(560 – 1002) | 1074<br>(588 – 1518) | 614<br>(477 – 698) | 0,008 |
| **RNA plasma viral load**<br>median, copies/mL (inter-quartile range) | 11600<br>(4830 – 124500) | ---- | 12050<br>(3920 – 101100) | ---- | NS |

NS= not significant; *p* value= two-way Mann-Whitney's test. P values<0.05 were considered statistically significant; Successful: HIV-1-infected subjects who have plasma HIV RNA undetectable levels for more than two years after start of HAART. Failure: HIV-1-infected subjects who have plasma HIV RNA levels above detectable level for more than two years after HAART. Time of HIV infection: time period between the first known positive HIV testing and the start of follow up. HAART: Highly Active Anti-retroviral Therapy. VL: HIV plasma viral load.

in the CXCR4 expression on CD4 + T cells (Fig. **3A**). The absolute number of CD4+ T cells that expressed CXCR4 was not different among HIV-1-infected individuals, but the control group had higher absolute numbers of CD4 T cells that expressed CXCR4, compared to the other three groups studied, (p <0.05 for all comparisons) (Fig. **3B**).

Fig. (**4**) shows the CD8 + T lymphocytes and CXCR4 expression by flow cytometry. There was no difference in the CXCR4 expression and the absolute number of CD8+ T cells from HIV-1-positive individuals (Fig. **4A**, **B**). Meanwhile, individuals not on HAART showed a lower expression of CXCR4 on CD4 + T cells and a lower number of CD4+ T cells/CXCR4+, compared to the control group (p = 0.01 and p = 0.04, respectively) (Fig. **4A**).

## DISCUSSION

In addition to chemokines production data, genetic factors and immune phenotyping of T cells may add information regarding target cell interaction on HIV pathogenesis. With this aim, we analyzed the number and expression of

**Table 2.    Genetic Polymorphism for CCR5, CCR5-Promoter and SDF-1 Among HIV-1-Infected Subjects and Response to HAART**

| Receptor | Viremic (VL > 400 ) | Aviremic (VL ≤ 400) | No HAART | Control |
|---|---|---|---|---|
| **CCR5** | n= 14 | n= 22 | n= 18 | n= 23 |
| Wild type | 13 | 22 | 15 | 22 |
| Heterozygote | 1 | 0 | 2 | 1 |
| Homozygous | 0 | 0 | 0 | 0 |
| **CCR5-promoter** | n= 19 | n= 21 | n=14 | |
| Wild type | 6 | 3 | 6 | |
| Heterozygote | 13 | 15 | 5 | ND |
| Homozygous | 0 | 3 | 3 | |
| **SDF-1** | n= 8 | n= 22 | n= 6 | n= 10 |
| Wild type | 6 | 16 | 2 | 7 |
| Heterozygote | 2 | 5 | 3 | 3 |
| Homozygous | 0 | 1 | 1 | 0 |

ND: not done.
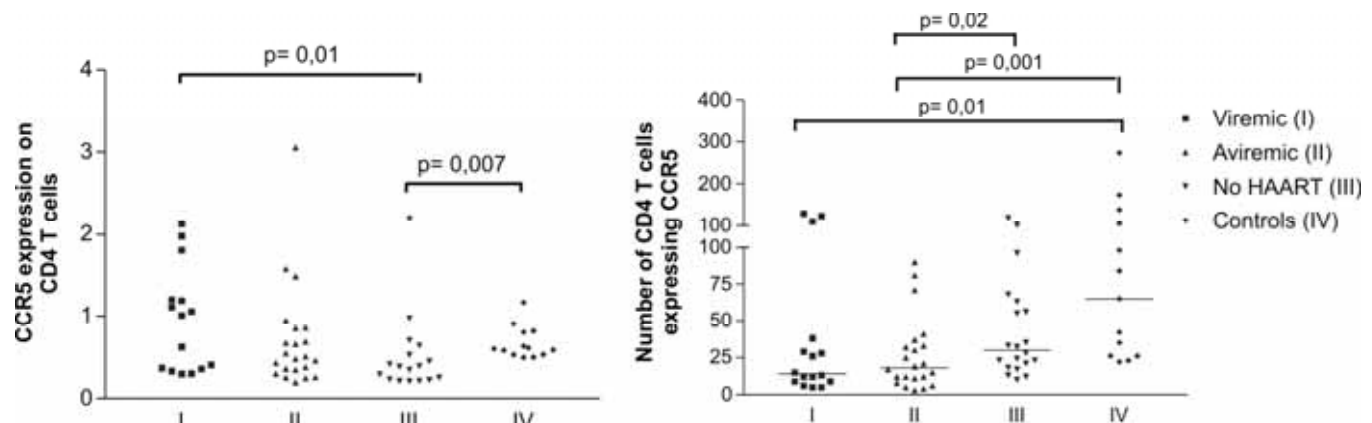


**Fig. (1). Results of the acquisition of CD4$^+$ T lymphocytes by flow cytometry.** (**A**) Expression of the CCR5 receptor was calculated based on the results obtained through the mean fluorescence intensity (MFI). (**B**) Absolute number of CD4$^+$ T cells expressing the receptor CCR5.
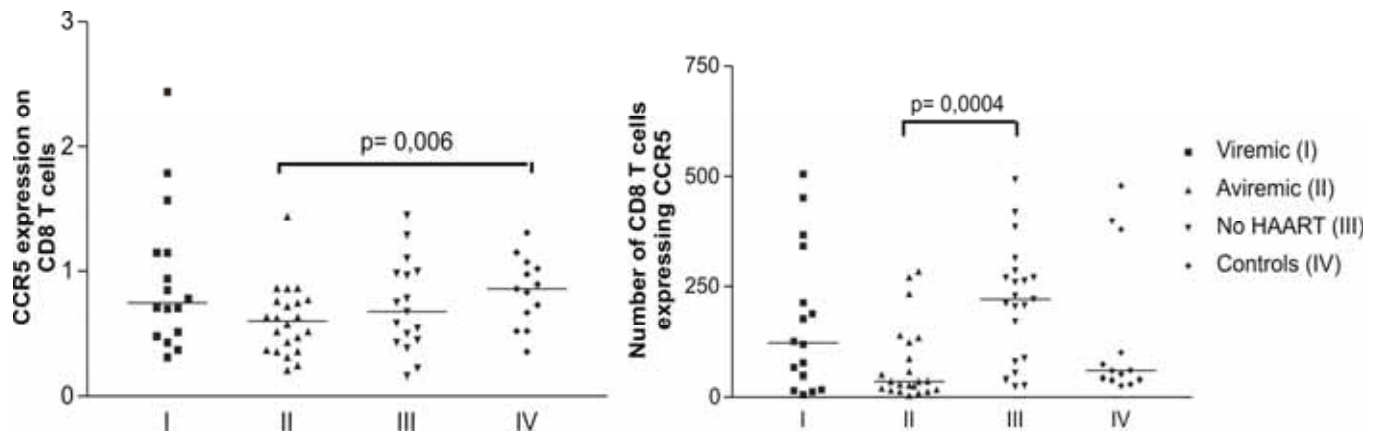
**Fig. (2). Results of the acquisition of CD8+ T lymphocytes by flow cytometry.** (**A**) Expression of the CCR5 receptor was calculated based on the results obtained through the mean fluorescence intensity (MFI). (**B**) Absolute number of CD8+ T cells expressing the receptor CCR5.
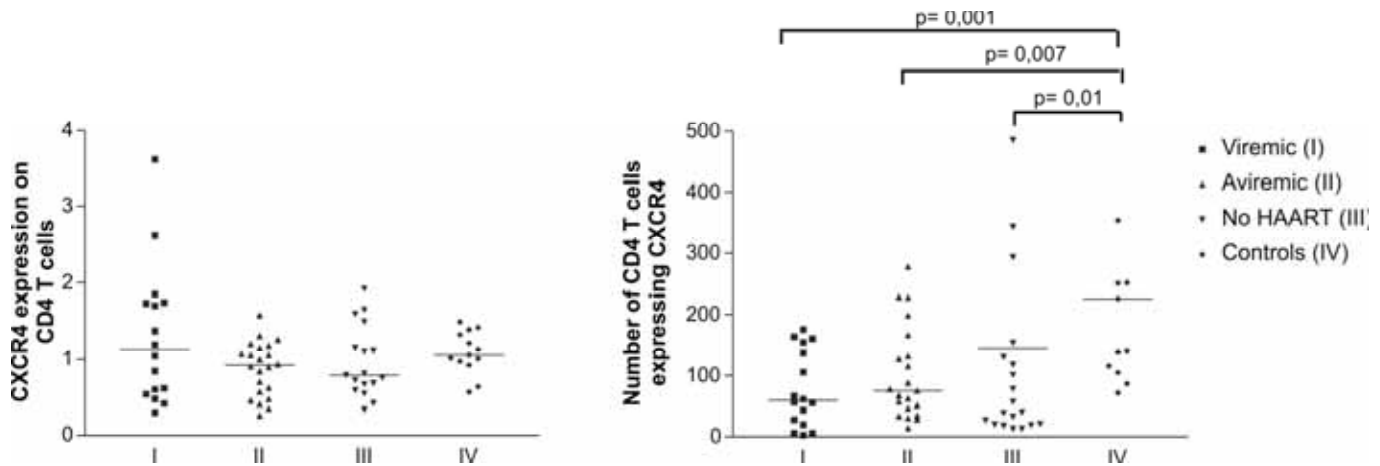


**Fig. (3). Results of the acquisition of CD4+ T lymphocytes by flow cytometry.** (**A**) Expression of the CXCR4 receptor was calculated based on the results obtained through the mean fluorescence intensity (MFI). (**B**) Absolute number of CD4+ T cells expressing the receptor CXCR4.
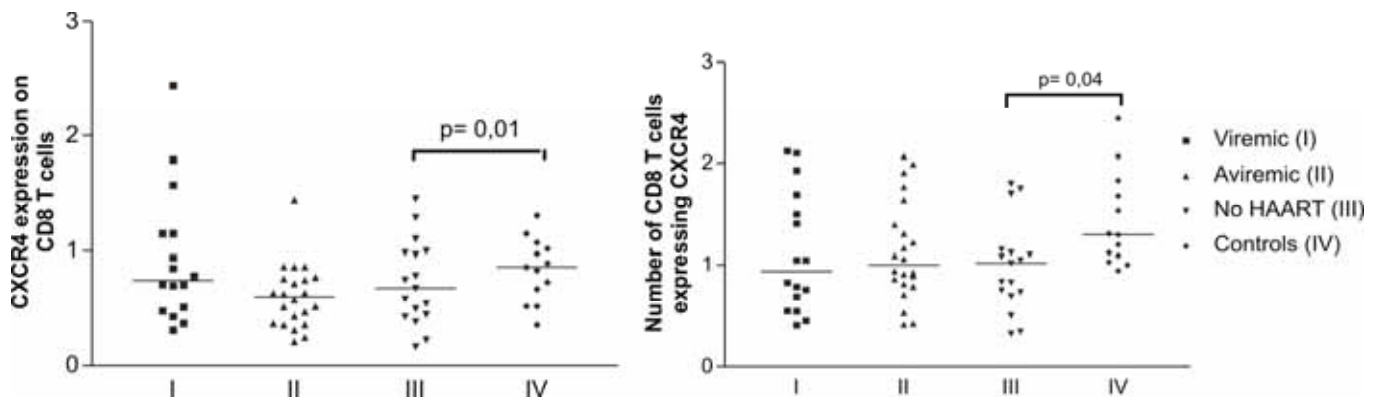


**Fig. (4). Results of the acquisition of CD8+ T lymphocytes by flow cytometry.** (**A**) Expression of the CXCR4 receptor was calculated based on the results obtained through the mean fluorescence intensity (MFI). (**B**) Absolute number of CD8+ T cells expressing the receptor CXCR4.

CCR5 and CXCR4 molecules on T cells, among HIV-1-infected infected subjects and their response to HAART. The results indicated that non-HAART users had lower expression of these receptors compared to viremic or non-HIV-infected controls. Thus, HAART leads to expression normalization, independently of plasma viral load. These findings indicate that the replication induced increasing expression of these receptors. In fact, after successful HAART, the CCR5 and CXCR4 expression, as well the production of beta-chemokines, was modified [14], what may reflect the peripheral memory or naïve T cells redistribution and/or decreasing immune activation [15, 16]. These modifications on molecules involved on viral tropism and anti-HIV activity may contribute to the emergence of virus variants when HAART fails.

Furthermore, our findings indicate that a well succeeded viral suppression leads to the progressive maturation and

functionality of the T cells, which are responsible for the apoptosis levels in those cells [17]. In this way, it may contribute to the reduction on T cell loss and the immune reconstitution after HAART [17]. This is possibly due to the increasing of T cell activation in HAART failures [18]. In contrast, the chemokine receptor polymorphism was not altered regarding the outcome of HAART.

These results also indicate that patients on therapeutic success had increased β-chemokines, what may have clinical implications [14]. Indeed, the antagonists of the CCR5 coreceptor are being developed as the first anti-HIV agents that act in the target cells of the host, and are currently under study in clinical stage [19]. Meanwhile, preliminary studies using these components showed that despite the decrease in the viral load of HIV-1 in plasma, there is the emergence of strains with the CXCR4 tropism [19]. More recently, the importance of co-receptor blockers was evaluated in a clinical trial, an antagonist of CCR5. A small molecule, named Maraviroc, blocks the region b-chemokine ligand, leading to the reduction of 1.6 log viral load as compared to the pretherapy level [20]. Indeed, the favorable response to the clinical use of the CCR5 antagonist, coupled with our results, showed that co-receptors expression may reflect the influence negatively viremia in the HIV-1 infected patients.

In conclusion, the naïve HAART individuals had higher CD4 + T cells and CD8 + that expressed CCR5 receptor. However, they had lower intensity of CCR5 expression, which may suggest decreased capacity of cellular infection; the viremic individuals showed no statistically significant difference, but there was higher CXCR4 and CCR5 expression on CD4 + T cells, which may indicate greater susceptibility to virions and hence HIV-1 replication. There was normalization of the CXCR4 and CCR5 expression on the surface of lymphocytes in aviremic individuals, allowing greater viral control. In this study, the therapeutic success proved to be unrelated to genetic polymorphisms of chemokine receptors.

## ACKNOWLEDGEMENTS

## REFERENCES

[1] Mueller A, Strange PG. The chemokine receptor, CCR5. Int J Biochem Cell Biol 2004; 36: 35-8.

[2] Rot, Andrian UHV. Chemokines in innate and adaptive host defense: basic chemokines grammar for immune cells. Annu Rev Immunol 2004; 22: 891-928.

[3] Oppermann M. Chemokine receptor CCR5: insights into structure, function, and regulation. Cell Signal 2004; 16:1201-10.

[4] Cocchi F, DeVico AL, Garzino-Demo A, *et al.* Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells. Science 1995; 270: 1811-5.

[5] Kuhmann SE, Hartley O. Targeting chemokine receptors in HIV: a status report. Annu Rev Pharmacol Toxicol 2008; 48: 425-61.

[6] 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 USA 2000; 97: 13812-7.

[7] Liu R, Paxton WA, Choe S, *et al.* Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. Cell. 1996; 86:367-77.

[8] Paxton WA, Kang S. Chemokine receptor allelic polymorphisms: relationships to HIV resistance and disease progression. Semin Immunol 1998; 10: 187-94.

[9] Tsimanis A, Kalinkovich A, Bentwich Z. Soluble chemokine CCR5 receptor is present in human plasma. Immunol Lett 2005; 96: 55-61.

[10] Lee B, Sharron M, Montaner LJ, *et al.* Quantification of CD4, CCR5 and CXCR4 levels on lymphocyte subsets, dendritic cells and differentially conditioned monocyte-derived macrophages. Proc Natl Acad Sci USA 1999; 96: 5215-20.

[11] Balter, M. Chemokine mutation slows progression. Science 1998; 279: 327.

[12] Winkler C, Modi W, Smith MW, *et al.* Genetic restriction of AIDS pathogenesis by an SDF-1 chemokine gene variant. ALIVE Study, Hemophilia Growth and Development Study (HGDS), Multicenter AIDS Cohort Study (MACS), Multicenter Hemophilia Cohort Study (MHCS), San Francisco City Cohort (SFCC). Science 1998; 279:389-93.

[13] Reiche EM, Bonametti AM, Voltarelli JC, Morimoto HK, Watanabe MA. Genetic polymorphisms in the chemokine and chemokine receptors: impact on clinical course and therapy of the human immunodeficiency virus type 1 infection (HIV-1). Curr Med Chem. 2007; 14: 1325-34.

[14] Brito A, Almeida A, Gonsalez CR, *et al.* Successful HAART is associated with high B-chemokines levels in chronic HIV-1-infected patients. AIDS Res Hum Retroviruses 2007; 23: 906-12.

[15] Giovannetti A, Ensoli F, Mazzetta F, *et al.* CCR5 and CXCR4 chemokine receptor expression and β-chemokine prodution during early T cell repopulation induced by higly active anti-retroviral therapy. Clin Exp Immunol. 1999; 118: 87-97.

[16] Nicholson JK, Browning SW, Hengel RL, *et al.* CCR5 and CXCR4 expression on memory and naive T cells in HIV-1 infection and response to highly active antiretroviral therapy. J Acquir Immune Defic Syndr 2001; 27: 105-15.

[17] Ensoli F, Fiorelli V, Alario C, *et al.* Decreased T cell apoptosis and T cell recovery during highly active antiretroviral therapy (HAART). Clin Immunol. 2000; 97:9-20.

[18] Steel A, Cox AE, Shamji MH, *et al.* HIV-1 viral replication below 50 copies/ml in patients on antiretroviral therapy is not associated with CD8+ T-cell activation. Antivir Ther 2007; 12:971-5.

[19] Westby M, Lewis M, Whitcomb J. Emergence of CXCR4-using human immunodeficiency virus type 1 (HIV-1) variants in a minority of HIV-1-infected patients following treatment with the CCR5 antagonist maraviroc is from a pretreatment CXCR4-using virus reservoir. J Virol 2006; 80: 4909-20.

[20] Fätkenheuer G, Pozniak AL, Johnson MA, *et al.* Efficacy of short-term monotherapy with maraviroc, a new CCR5 antagonist, in patients infected with HIV-1. Nat Med. 2005; 11: 1170-2.