

## Increased Frequency of Circulating CCR5<sup>+</sup> CD4<sup>+</sup> T Cells in Human Immunodeficiency Virus Type 2 Infection<sup>∇</sup>

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**CCR5 expression determines susceptibility to infection, cell tropism, and the rate of human immunodeficiency virus type 1 (HIV-1) disease progression. CCR5 is also considered the major HIV-2 coreceptor in vivo, in spite of broad coreceptor use in vitro. Here we report a significantly increased proportion of memory-effector CD4 T cells expressing CCR5 in HIV-2-infected patients correlating with CD4 depletion. Moreover, HIV-2 proviral DNA was essentially restricted to memory-effector CD4, suggesting that this is the main target for HIV-2. Similar levels of proviral DNA were found in the two infection categories. Thus, the reduced viremia and slow rate of CD4 decline that characterize HIV-2 infection seem to be unrelated to coreceptor availability.**

Human immunodeficiency virus type 2 (HIV-2) immunodeficiency is characterized by slow disease progression with limited impact on the survival of the majority of infected adults (20, 33, 45). The rate of CD4<sup>+</sup> T-cell decline is much slower in HIV-2 than in HIV-1 disease, and there is a low plasma viral load irrespective of disease stage (2, 4, 9, 17, 20, 32, 41, 43).

The factors contributing to the suggested decreased rate of virus production in HIV-2 infection remain largely unknown.

In spite of the promiscuity of coreceptor usage exhibited by HIV-2 in in vitro experimental settings (8, 13, 16, 22, 29, 39, 40), several lines of evidence show that CCR5 and CXCR4 are the major coreceptors for HIV-2 infection in vivo (6, 24, 25).

TABLE 1. Cohort characterization

Characteristic	Value for group		
	Healthy controls	HIV-2-positive patients <sup>c</sup>	HIV-1-positive patients <sup>c</sup>
No. of subjects (no. male/no. female)	21 (9/12)	22 (6/16)	23 (10/13)
Age (yr) <sup>a</sup>	42 ± 4 (21–84)	46 ± 3 (21–64)	37 ± 3 (20–68)
Ethnicity			
White	17	8	13
Other	4	14	10
HIV transmission category			
Heterosexual	n.a. <sup>b</sup>	17	18
Homosexual or bisexual	n.a.	1	3
Intravenous drug user	n.a.	1	2
Blood transfusion	n.a.	3	0
CD3 count in cells/μl <sup>a</sup>	1,844 ± 165 (767–3,488)	1,305 ± 119 (427–2,629)*	1,454 ± 158 (378–3,554)*
% CD3 <sup>a</sup>	73 ± 1.8 (58–84)	71 ± 2.3 (48–87)	74 ± 1.9 (55–90)
CD4 count (cells/μl) <sup>a</sup>	1,174 ± 108 (581–2,387)	551 ± 73 (38–1,207)***	498 ± 61 (86–1,300)***
% CD4 <sup>a</sup>	46 ± 1.8 (33–64)	29 ± 2.8 (4–49)***	28 ± 2.4 (9–52)***
CD8 count (cells/μl) <sup>a</sup>	632 ± 71 (234–1,236)	729 ± 76 (180–1,456)	861 ± 100 (269–2,007)
% CD8 <sup>a</sup>	24 ± 1.5 (13–38)	40 ± 2.9 (24–70)***	44 ± 2.4 (28–64)***
Viremia (RNA; no. of copies/ml) <sup>a</sup>	n.a.	409 ± 190 (200–4,006) <sup>d</sup>	75,508 ± 30,429 (118–548,631) <sup>e</sup>
Proviral DNA (copies/10 <sup>6</sup> PBMC) <sup>a</sup>	n.a.	1,760 ± 823 (5–10,725) <sup>f</sup>	2,481 ± 863 (61–12,565) <sup>f</sup>

<sup>a</sup> Data are means ± standard errors of the means with limits in brackets.

<sup>b</sup> n.a., not applicable.

<sup>c</sup> Significance in comparison with healthy control results is represented as follows: \*,  $P < 0.05$ ; \*\*\*,  $P < 0.0001$ . There were no significant differences between the HIV-2 and HIV-1 cohorts except for the viremia results ( $P < 0.0001$ ).

<sup>d</sup> HIV-2 viremia was quantified by a reverse transcriptase PCR-based test (41); the results were below 200 RNA copies/ml (cutoff) for 19 out of the 21 patients studied. In these cases the cutoff value was used.

<sup>e</sup> HIV-1 viremia was quantified by reverse transcriptase PCR (Ultrasensitive Test; Roche Molecular Systems, Branchburg, NJ). The cutoff was 50 RNA copies/ml.

<sup>f</sup> Proviral DNA was quantified by real-time PCR and was detected in all 17 HIV-2-infected patients and all 16 HIV-1-infected patients investigated.

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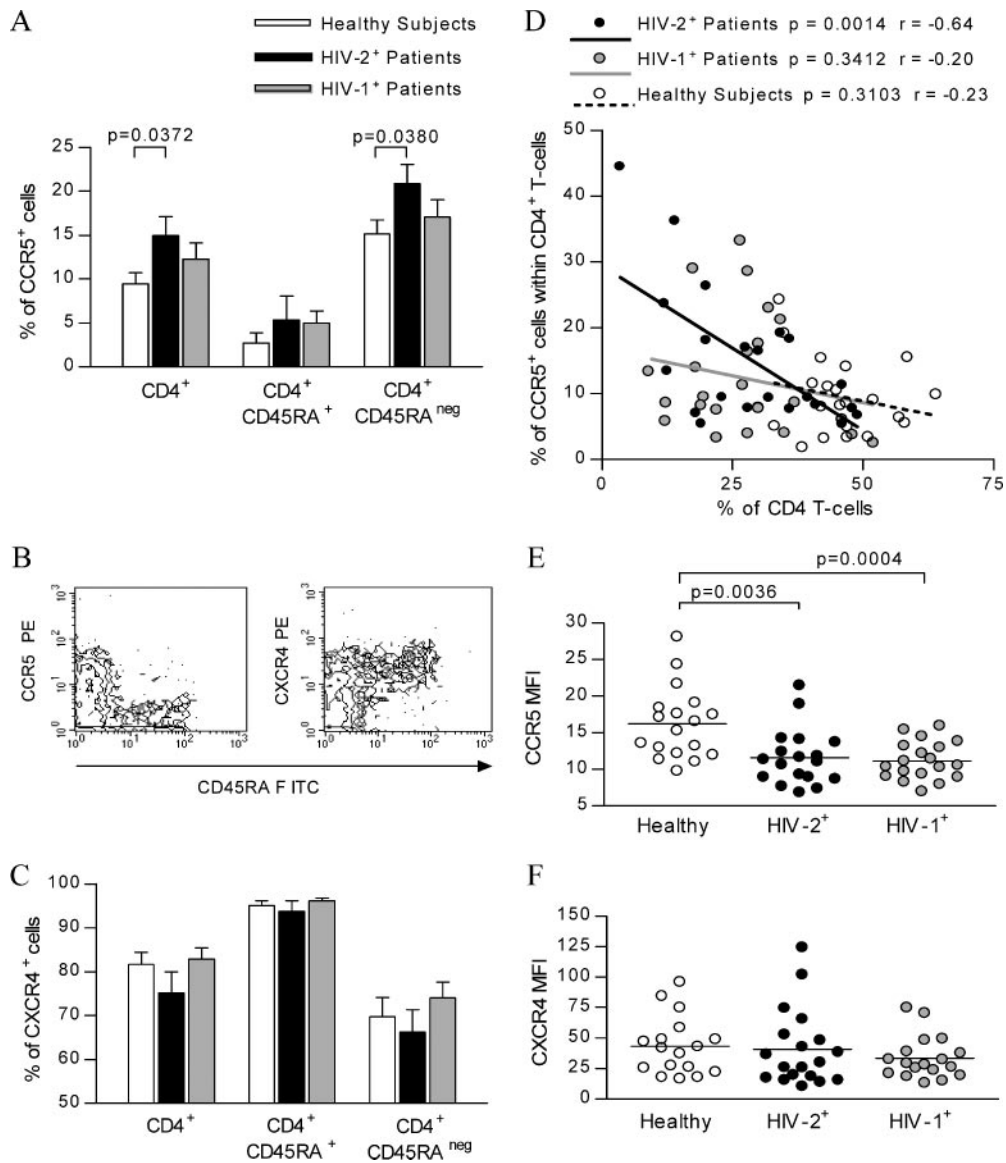


FIG. 1. CCR5 and CXCR4 expression in circulating CD4<sup>+</sup> T cells from HIV-2- and HIV-1-infected patients as well as healthy controls. Freshly isolated PBMC were surface stained with anti-CCR5 (clone 2D7) or anti-CXCR4 (clone 12G5) monoclonal antibodies (phycoerythrin conjugated; BD Biosciences, San Jose, CA), acquired, and analyzed in a FACSCalibur flow cytometer using CellQuest software (BD Biosciences). Percentages of CCR5- and CXCR4-expressing cells within total CD4<sup>+</sup> T cells (CD4<sup>+</sup>), a naïve CD4 subset (CD4<sup>+</sup> CD45RA<sup>+</sup>), and a memory CD4 subset (CD4<sup>+</sup> CD45RA<sup>neg</sup>) are shown in panels A and C, respectively. Bars represent means  $\pm$  standard errors of the means. Representative contour plots of the flow cytometric analysis of CD4<sup>+</sup> gated T cells of an HIV-2-infected patient with 291 CD4<sup>+</sup> T cells/ $\mu$ l are illustrated in panel B. (D) Correlation between the percentage of CCR5<sup>+</sup> cells within the CD4 subset and the frequency of circulating CD4<sup>+</sup> T cells. Each dot represents one individual. The Pearson correlation coefficients for the different cohorts are shown. The median fluorescence intensities (MedianFI) of the whole population of CCR5- and CXCR4-expressing CD4<sup>+</sup> T cells are shown in panels E and F, respectively. Each dot represents one individual; bars represent means. Statistical analysis was performed using GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA). The data were compared using an unpaired *t* test. Spearman's correlation coefficients were used to determine the correlations between two variables. *P* values < 0.05 were considered significant and are depicted in the figure.

In HIV-1-infected patients, CCR5 expression determines susceptibility to infection, cell tropism, and the rate of disease progression and is currently an important target of new antiretroviral drugs (10, 11, 21, 27, 34, 35).

There are scanty data available on CCR5 and CXCR4 expression in HIV-2-infected patients. A previous study of a Senegalese cohort reported lower CCR5 expression in HIV-2 than in HIV-1 infection, but as these patients, unlike our co-

horts, were not stratified according to CD4 depletion or viremia, direct comparison is problematic (38).

We analyzed here CCR5 and CXCR4 expression in freshly isolated peripheral blood mononuclear cells (PBMC) from untreated HIV-2- and HIV-1-infected subjects who were currently living in Portugal and attending outpatient clinics in Lisbon and who exhibited no known ongoing opportunistic infections or tumors. The epidemiological and clinical features

TABLE 2. Primer and probe sequences<sup>a</sup>

Primer or probe	Sequence <sup>b</sup>
<b>HIV-2</b>	
Forward primer .....	5'-CGC GAG AAA CTC CGT CTT G-3'
Reverse primer .....	5'-CAC ACA ATA TGT TTT AGC CTG TAC TTT TT-3'
Probe .....	5'-FAM-CCG GGC CGT AAC CT-MGB-3'
<b>HIV-1</b>	
Forward primer .....	5'-GGG AGA ATT AGA TCG ATG GGA AA-3'
Reverse primer .....	5'-CTG CTT GCC CAT ACT ATA TGT TTT AAT TTA-3'
Probe .....	5'-FAM-CCC TGG CCT TAA CCG AAT T-MGB-3'
<b>Albumin</b>	
Forward primer .....	5'-TGC ATG AGA AAA CGC CAG TAA-3'
Reverse primer .....	5'-ATG GTC GCC TGT TCA CCA A-3'
Probe .....	5'-FAM-TCA CCA AAT GCT GCA CAG A-MGB-3'
<b>CCR5<sup>c</sup></b>	
Forward primer .....	5'-TTC ATT ACA CCT GCA GCT CT-3'
Reverse primer .....	5'-CAC AGC CCT GTG CCT CTT CTT CTC ATT TCG-3'

<sup>a</sup> Primers and probes were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA) and checked against the Los Alamos HIV database.

<sup>b</sup> FAM, 6-carboxyfluorescein; MGB, minor groove binding.

<sup>c</sup> Fifty nanograms of genomic DNA was amplified by PCR using 25 µl of Platinum PCR SuperMix (Invitrogen, Carlsbad, CA), 300 nM primer (each), and 5 mM MgCl<sub>2</sub> and was run on a 2% agarose gel with DNA bands stained with ethidium bromide.

of these cohorts, as well as of healthy controls, are summarized in Table 1. Of note, HIV-2 and HIV-1 cohorts exhibited similar levels of CD4 depletion but striking differences in viremia.

CCR5 and CXCR4 expression were assessed by flow cytometry in freshly isolated PBMC as previously described (42).

HIV-2-positive patients exhibited a higher frequency of CCR5<sup>+</sup> cells within the CD4<sup>+</sup> subset that reaches statistical significance than that seen with healthy subjects (Fig. 1A). In HIV-2 patients CCR5 expression was also largely confined to the memory CD4<sup>+</sup> CD45RA<sup>-</sup> population, as illustrated in the representative contour plot of Fig. 1B.

The frequencies of CXCR4<sup>+</sup> cells within CD4 T cells showed no significant differences among the three cohorts, although there was a trend to lower frequencies in the HIV-2 cohort in both the naïve and memory subsets (Fig. 1C).

A significant correlation between the frequency of CCR5<sup>+</sup> cells within the CD4 subset and the degree of CD4 depletion was observed with the HIV-2 cohort that was not observed with HIV-1-positive patients (Fig. 1D).

We have previously shown that CD4 depletion is directly linked to immune activation in both HIV-2 and HIV-1 infections in spite of the striking differences in viremia (15, 42). Since CCR5 is up-regulated upon T-cell activation, we looked for a possible correlation between the frequency of CCR5<sup>+</sup> cells and the expression of HLA-DR, a marker widely used to quantify immune activation in HIV disease (15). A significant correlation was observed with the HIV-2 cohort ( $r = 0.68$ ;  $P = 0.0009$ ) that was not found with HIV-1-positive patients ( $r = 0.25$ ;  $P = 0.2814$ ).

TABLE 3. HIV-2 proviral DNA in CD4 naïve and memory subsets

Case	No. of copies of proviral DNA/10 <sup>6</sup> PBMC	No. of CD4 cells/µl	Result for indicated subset <sup>a</sup>			
			CD4 <sup>+</sup> memory		CD4 <sup>+</sup> naïve	
			No. of copies of proviral DNA/10 <sup>6</sup> PBMC	% CCR5	No. of copies of proviral DNA/10 <sup>6</sup> PBMC	% CCR5
1	57	181	95	7.45	5	0.86
2	206	629	1,541	11.21	5	2.08

<sup>a</sup> Freshly isolated PBMC were successively gated using CD4<sup>+</sup> and CD45RA<sup>+</sup> (naïve) or CD45RA<sup>-</sup> (memory); the subsets were purified using FACSAria (BD Biosciences), with purity higher than 98%.

These data illustrated the link between the expansion of CCR5<sup>+</sup> cells and immune activation in HIV-2 infection. The lower frequency of circulating CCR5<sup>+</sup> cells in HIV-1 infection compared to HIV-2 results despite the similarities in heightened immune activation may be related to a continuous depletion of the CCR5 pool in association with the high level of viremia (19, 23).

The frequency of CCR5<sup>+</sup> cells may be underestimated due to binding-induced receptor internalization (26, 30). In fact, the assessment of the median fluorescence intensity (MedianFI) of CCR5<sup>+</sup> CD4<sup>+</sup> T cells revealed similar and significant down-regulation results for both HIV-2- and HIV-1-infected cohorts in comparison with the results seen with healthy subjects (Fig. 1E). This contrasts with the absence of differences in MedianFI of CXCR4<sup>+</sup> CD4<sup>+</sup> T cells for the three cohorts (Fig. 1F). It is noteworthy that HIV-2 infection has been associated with high levels of production of RANTES, MIP-1α, and MIP-1β (1, 18, 28), possibly contributing to the CCR5 down-regulation.

In order to exclude the possibility that CCR5Δ32 mutations contribute to the low MedianFI of CCR5 (44), we screened the cohorts for the presence of this allele using the primers described in Table 2. None of the HIV-2- or HIV-1-infected patients exhibited the CCR5Δ32 allele. There were five healthy subjects heterozygous for CCR5Δ32. The exclusion of these individuals from the analysis resulted in an even more significant difference in the results of down-regulation of CCR5 MedianFI between HIV cohorts and healthy subjects ( $P = 0.0019$  for HIV-2 and  $P < 0.0001$  for HIV-1).

On the other hand, despite differing levels of viremia, we did not find significant differences between HIV-2 and HIV-1 proviral DNA levels, suggesting the presence of similar numbers of infected cells in the two infection categories (Table 1), in agreement with previous reports (3–5, 14, 31). Proviral DNA was assessed by absolute quantitative real-time PCR using an ABI PRISM 7000 sequence detection system (Applied Biosystems) with a detection range of 7 orders of magnitude and a sensitivity of five copies. Reactions containing 150 ng of genomic DNA extracted from 10<sup>6</sup> PBMC by use of an ABI PRISM 6100 nucleic acid extractor (Applied Biosystems), 25 µl of Platinum quantitative PCR SuperMix-UDG, 1 µl ROX reference dye (Invitrogen) (50×), 5 mM MgCl<sub>2</sub>, 300 nM primer (each), and 200 nM probe (Table 2) were run in duplicate. Albumin was used to standardize DNA input.

It is worth noting that no correlation was found between the

frequency of CCR5<sup>+</sup> cells within the CD4 subset and the levels of HIV-2 proviral DNA ( $r = 0.08$ ;  $P = 0.7483$ ).

In order to evaluate the possibility that the similar levels of proviral DNA in the presence of the dissimilar HIV-1 and HIV-2 viremia results might be due to differences in cell targets, we purified the naïve and the memory CD4 T cells from PBMC of two HIV-2 patients with different levels of CD4 depletion by high-speed cell sorting using FACSria (BD Biosciences).

As depicted in Table 3, the levels of HIV-2 proviral DNA documented in the naïve subset were minimal. Therefore, these data suggest that memory CD4 T cells are the main targets for HIV-2 infection in vivo, reinforcing the idea of a major role of CCR5 coreceptor in HIV-2 infection. This was in agreement with data on HIV-1 infection in which integrated proviruses are preferentially detected within the memory subset (7, 12, 36).

In summary, HIV-2-infected patients showed an increase in the proportion of CCR5<sup>+</sup> cells within the memory-effector CD4<sup>+</sup> T cells in correlation with the degree of CD4 depletion and immune activation. In contrast, in HIV-1 infection there was dissociation between CCR5 and other markers of immune activation which could be interpreted as an indirect evidence of depletion of the CCR5<sup>+</sup> cells by HIV-1. However, the HIV-2 proviral load was also mainly restricted to memory-effector CD4 T cells, suggesting these are the major HIV-2 targets, which is consistent with CCR5 being the main HIV-2 coreceptor in vivo. Moreover, the levels of HIV-2 proviral load were similar to those observed in untreated HIV-1-infected individuals, suggesting equivalent numbers of infected cells resulting from the two diseases in spite of viremia being undetectable in the majority of the HIV-2 patients.

The presence of reduced HIV-2 viremia seems to be unrelated to coreceptor availability. Since HIV-2 is no less cytopathic per se than HIV-1 (37), other host factors must be implicated in the control of viral replication in spite of significant proviral DNA levels in HIV-2-positive patients. The further investigation of the mechanisms contributing to this control of HIV-2 viremia in the absence of antiretroviral therapy may prove to be useful in defining complementary therapeutic strategies to control viral reservoirs in HIV-1.

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