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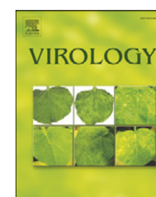

# Coreceptor usage by HIV-1 and HIV-2 primary isolates: The relevance of CCR8 chemokine receptor as an alternative coreceptor

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ABSTRACT

The human immunodeficiency virus replication cycle begins by sequential interactions between viral envelope glycoproteins with CD4 molecule and a member of the seven-transmembrane, G-protein-coupled, receptors' family (coreceptor).
In this report we focused on the contribution of CCR8 as alternative coreceptor for HIV-1 and HIV-2 isolates. We found that this coreceptor was efficiently used not only by HIV-2 but particularly by HIV-1 isolates. We demonstrate that CXCR4 usage, either alone or together with CCR5 and/or CCR8, was more frequently observed in HIV-1 than in HIV-2 isolates. Directly related to this is the finding that the non-usage of CXCR4 is significantly more common in HIV-2 isolates; both features could be associated with the slower disease progression generally observed in HIV-2 infected patients.
The ability of some viral isolates to use alternative coreceptors besides CCR5 and CXCR4 could further impact on the efficacy of entry inhibitor therapy and possibly also in HIV pathogenesis.
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## Introduction

The human immunodeficiency virus (HIV) replication cycle begins by sequential interactions between viral envelope glycoproteins and cellular receptors that ultimately lead to viral envelope and cell membrane fusion. The cellular receptors involved in these initial events are the CD4 molecule (Dalgleish et al., 1984; Klatzmann et al., 1984) and a member of seven-transmembrane, G-protein-coupled, receptors' (GPCRs) family, referred as coreceptor. At the present, twenty three of these GPCRs have been shown to act *in vitro* as coreceptors for human immunodeficiency viruses 1 and 2 (HIV-1 and HIV-2, respectively) and simian immunodeficiency virus (SIV): CCR1, CCR2b, CCR3, CCR4, CCR5, CCR8, CCR9, CCR10, CXCR2, CXCR4, CXCR5, CXCR6, CX3CR1, XCR1, FPRL1, GPR1, GPR15, APJ, ChemR23, CXCR7/RDC1, D6, BLTR and US28 (Broder and Jones-Trower, 1999; Neil et al., 2005; Shimizu et al., 2009; Simmons et al., 2000). Despite this array of potential coreceptors, only CCR5 and CXCR4 have been considered as major coreceptors and apparently the only that are relevant in HIV pathogenesis (Simmons et al., 2000; Y.J. Zhang et al., 1998). In fact, several reports corroborate the idea that CCR5 is important in HIV-1 transmission (Dean et al., 1996; Liu et al., 1996; Mummidi et al., 1998; Samson et al., 1996a). CCR5-using (R5) variants are also predominant during early stages of HIV-1 infection and only in approximately 40% of infected humans, viruses arise that can use CXCR4 in addition to (R5X4 strains), or sometimes instead of CCR5 (X4 strains) (Berger et al., 1998, 1999; Simmons et al., 1996). The emergence of such strains is associated with accelerated CD4+ T-cell loss and disease progression (Bjorndal et al., 1997; Connor and Ho, 1994; Connor et al., 1997; Richman and Bozzette, 1994).

The majority of the information regarding coreceptor usage by HIV strains derives almost exclusively from studies using HIV-1 isolates. However, we and others (Azevedo-Pereira et al., 2003; Bron et al., 1997; Guillon et al., 1998; McKnight et al., 1998; Reeves et al., 1999;

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Santos-Costa et al., 2009; Sol et al., 1997) have provided evidence that HIV-2 interaction with cellular receptors is remarkably different. HIV-2 isolates that are able to infect cells in the absence of CD4, the promiscuous use of chemokine receptors as coreceptors and the non-usage of either CCR5 or CXCR4 are notorious examples of the heterogeneous mechanisms by which HIV-2 interacts with and infects target cells.

CCR8 is a chemokine receptor that is expressed in different cell types including monocytes and T-lymphocytes (Goya et al., 1998; Roos et al., 1997; Samson et al., 1996b; Tiffany et al., 1997) and is preferentially detected on type 2 T-helper lymphocyte subpopulation (D'Ambrosio et al., 1998; Zingoni et al., 1998). There are also reports suggesting that CCR8 is also expressed by NK cells (Inngjerdingen et al., 2000), and nonhematopoietic cells such as endothelial cells (Haque et al., 2001), smooth muscle cells (Haque et al., 2004), and certain brain-derived cells (Jinno et al., 1998).

Furthermore, CCR8 has been described as a possible coreceptor for some HIV and SIV strains (Cilliers et al., 2005; Horuk et al., 1998; Isaacman-Beck et al., 2009; Lee et al., 2000; Liu et al., 2000; Ohagen et al., 2003; Rucker et al., 1997; Shimizu et al., 2009; Singh et al., 1999; Vodros et al., 2003; Willey et al., 2003). Due to its expression pattern, particularly in activated peripheral blood lymphocytes, monocytes and thymocytes, CCR8 could potentially have a role in HIV infection and pathogenesis and it may serve as a relevant HIV coreceptor *in vivo*.

In this report we analysed CCR5 and CXCR4 coreceptor usage by diverse HIV-2 and HIV-1. In addition to these two major coreceptors, we focused on the contribution of CCR8 as an alternative coreceptor in HIV-2 infection, using primary isolates obtained from patients at different disease stages, including sequential samples from specific patients. Additionally, we also address the capability of CCR8 to function as coreceptor for selected HIV-1 isolates. Our main objective was to understand to what extent CCR8 could be considered as a reliable alternative coreceptor for HIV-1 and HIV-2 primary isolates. We found that 26.2% (17/65) of the HIV-2 isolates tested are able to use this chemokine receptor to enter target cells regardless of the patient's clinical status, viral load or CD4+ T-cell counts. Furthermore, 56.7% (17/30) of the HIV-1 isolates are able to use CCR8 as coreceptor. We conclude that CCR8 could constitute a potential alternative coreceptor not only for HIV-2 but also for HIV-1 infection, a fact that may have implications for current therapeutic strategies that aim to block viral entry.

## Results

### *Patient's clinical and immunologic data*

The overall characteristics of the clinical, immunological and plasma viral load data of the patients from which the viruses were isolated are summarized in Table 1 and in more detail in Tables 2 and 3. The majority (72.3%; 47/65) of HIV-2 infected patients included in this cohort were symptomatic when blood sample was collected (Table 1). According to CDC classification system (CDC, 1992), symptomatic stage was defined as belonging to clinical categories B or C. The CD4 cell counts ranged from 50 to 964, with an average value of 338 T-CD4+ lymphocytes/µl of peripheral blood; 32.3% (21/65) of the patients had CD4+ cell counts below 200 and 58.5% (38/65) have levels of plasma viral load below 500 viral RNA copies/ml.

In the HIV-1 cohort (Table 1), 73.3% (22/30) of the patients were symptomatic; the CD4-cell counts range was 106–1455 (average: 455). The CD4 cell count was below 200 in 20% (6/30) and plasma viral load below 500 copies of viral RNA/ml was observed also in 20% (6/30) of the individuals included in the study.

### *CCR5, CXCR4 and CCR8 coreceptor usage by HIV-2 primary isolates*

Although HIV-2 primary isolates with broad coreceptor usage have been described, the frequencies with which these different coreceptors are used have not been conclusively determined, mainly because the majority of data available was obtained from studies based on small cohorts. In this study we analysed a total of 65 primary HIV-2 isolates, obtained from patients at different clinical stages and with different CD4+ T-cell counts and plasma viral load levels. We wanted to verify their capability to infect GHOST–CD4 cells individually expressing CCR5, CXCR4 and CCR8 coreceptors. The ability to use these coreceptors was analysed by the capacity to productively infect GHOST coreceptor-expressing cells, measuring viral progeny production by RT activity and scored as semi-quantitative results. The biotype of each isolate was assigned according to coreceptor usage profile (Table 2).

The results show that 62 out of 65 HIV-2 isolates are able to use CCR5 (95.4%), and CXCR4 mediated infection of 31 out of 65 strains (47.7%). Moreover, seventeen HIV-2 isolates are able to use CCR8 as coreceptor (26.2%). No replication was observed in GHOST–CD4 parental cell line (data not shown).

The effective usage of CCR8 was further confirmed in both GHOST–CD4–CCR8 and in peripheral blood mononuclear cells (PBMC) using blocking concentrations of I-309 (in GHOST–CD4–CCR8 cell line) or I-309 together with TAK-779 and AMD3100 (in PBMC). We selected a group of isolates (both HIV-1 and HIV-2) that showed a biotype characterized by high replication levels in CCR8 expressing cells (UCFL2018, UCFL2051, UCFL1007 and UCFL1016). As controls, we also included an R5 and an X4 isolate (ALI and UCFL2049, respectively). The results, summarized in Fig. 1, demonstrate that the replication of all the isolates in GHOST–CD4–CCR8 cell line was completely abrogated in the presence of 100 ng/ml of I-309 (Fig. 1A), confirming that CCR8 was the only coreceptor being used in that cell system. In PBMC, we used a combination of CCR5 and CXCR4-targeted inhibitors (TAK-779 and AMD3100, respectively) in the presence or absence of CCR8 ligand, I-309. In these experiments we used CD8-depleted PBMC in order to avoid any uncontrolled inhibition exerted by soluble factors eventually secreted by CD8+ T-cells. The results (Fig. 1B) reveal that I-309 inhibited the replication of these isolates in PBMC ($P<0.001$), indicating that CCR8 was efficiently used in this cell system.

Nevertheless, none of the HIV isolates tested was fully inhibited, even when combining I-309 with TAK-779 and AMD3100. This was particularly evident with UCFL2018 and UCFL1007 isolates, suggesting that these isolates could be using an additional coreceptor, besides CCR8, CCR5 and CXCR4, expressed in PBMCs.

**Table 1**
Patient's clinical, immunological and virological characteristics of the HIV-1 and HIV-2 cohorts included in the study.

| | Clinical stage[a] | | CD4 cell counts[b] | | Plasma viral load[c] | | |
|---|---|---|---|---|---|---|---|
| | % (n) | | % (n) | | % (n) | | |
| Cohort (n) | Asymptomatic | Symptomatic | ≤200 | >200 | ≥500 | <500 | ND |
| HIV-1 (30) | 26.7 (8) | 73.3 (22) | 20.0 (6) | 80.0 (24) | 76.7 (23) | 20.0 (6) | 3.3 (1) |
| HIV-2 (65) | 27.7 (18) | 72.3 (47) | 32.3 (21) | 67.7 (44) | 26.1 (17) | 58.5 (38) | 15.4 (10) |

[a] Clinical stage according to CDC classification (CDC, 1992); Asymptomatic = clinical stage A; Symptomatic = clinical stage B or C.
[b] Number of T-CD4 lymphocytes/µl.
[c] Copies of viral RNA/ml.

**Table 2**
CCR5, CXCR4 and CCR8 coreceptor usage by HIV-2 isolates and patient's clinical, immunological and virological data.

| Isolate | Clinical stage[a] | CD4 cell count[b] | Plasma viral load[c] | Coreceptors used[d] | | | Biotype |
|---|---|---|---|---|---|---|---|
| | | | | CCR5 | CXCR4 | CCR8 | |
| ALI | Symptomatic | 491 | ND | +++ | − | − | R5 |
| UCFL2001 | Symptomatic | 230 | <500 | +++ | − | − | R5 |
| UCFL2003 | Symptomatic | 145 | 2373 | + | +++ | +++ | R5X4R8 |
| UCFL2004 | Symptomatic | 442 | 1418 | + | + | − | R5X4 |
| UCFL2005 | Symptomatic | 365 | <500 | + | + | + | R5X4R8 |
| UCFL2006 | Asymptomatic | 562 | <500 | + | + | + | R5X4R8 |
| UCFL2007 | Asymptomatic | 331 | <500 | + | − | + | R5R8 |
| UCFL2008 | Symptomatic | 52 | 2285 | +++ | +++ | +++ | R5X4R8 |
| UCFL2009 | Symptomatic | 527 | <500 | + | + | − | R5X4 |
| UCFL2010 | Asymptomatic | 456 | <500 | ++ | − | − | R5 |
| UCFL2012 | Symptomatic | 375 | 1268 | +++ | − | − | R5 |
| UCFL2013 | Symptomatic | 500 | 816 | + | − | − | R5 |
| UCFL2014 | Symptomatic | 213 | <500 | ++ | − | − | R5 |
| UCFL2015 | Symptomatic | 220 | <500 | + | + | − | R5X4 |
| UCFL2016 | Symptomatic | 213 | <500 | + | − | − | R5 |
| UCFL2017 | Symptomatic | 50 | 23,454 | ++ | ++ | ++ | R5X4R8 |
| UCFL2018 | Symptomatic | 392 | <500 | +++ | ++ | +++ | R5X4R8 |
| UCFL2019 | Symptomatic | 258 | 1870 | ++ | − | − | R5 |
| UCFL2020 | Symptomatic | 358 | <500 | + | + | − | R5X4 |
| UCFL2022 | Symptomatic | 578 | <500 | + | + | − | R5X4 |
| UCFL2023 | Symptomatic | 70 | <500 | ++ | − | − | R5 |
| UCFL2024 | Asymptomatic | 683 | <500 | + | − | − | R5 |
| UCFL2026 | Asymptomatic | 921 | <500 | +++ | − | − | R5 |
| UCFL2027 | Asymptomatic | 730 | <500 | ++ | − | − | R5 |
| UCFL2028 | Asymptomatic | 760 | <500 | +++ | − | − | R5 |
| UCFL2029 | Symptomatic | 230 | 3454 | + | + | + | R5X4R8 |
| UCFL2030 | Symptomatic | 346 | <500 | ++ | − | − | R5 |
| UCFL2031 | Symptomatic | 92 | <500 | +++ | − | − | R5 |
| UCFL2032 | Symptomatic | 106 | 1072 | − | + | − | X4 |
| UCFL2033 | Symptomatic | 139 | >100,000 | ++ | − | − | R5 |
| UCFL2034 | Asymptomatic | 432 | 9470 | ++ | − | − | R5 |
| UCFL2035 | Symptomatic | 292 | 6431 | +++ | ++ | − | R5X4 |
| UCFL2036 | Symptomatic | 195 | <500 | +++ | − | − | R5 |
| UCFL2037 | Symptomatic | 419 | <500 | +++ | − | + | R5R8 |
| UCFL2038 | Symptomatic | 318 | <500 | +++ | − | − | R5 |
| UCFL2039 | Symptomatic | 384 | <500 | + | + | − | R5X4 |
| UCFL2040 | Symptomatic | 91 | <500 | + | − | − | R5 |
| UCFL2041 | Symptomatic | 184 | <500 | ++ | − | − | R5 |
| UCFL2042 | Asymptomatic | 413 | <500 | + | + | − | R5X4 |
| UCFL2043 | Symptomatic | 324 | <500 | + | − | + | R5R8 |
| UCFL2045 | Asymptomatic | 267 | 1947 | +++ | − | − | R5 |
| UCFL2046 | Asymptomatic | 130 | <500 | +++ | − | − | R5 |
| UCFL2048 | Symptomatic | 59 | 1698 | + | − | ++ | R5R8 |
| UCFL2049 | Symptomatic | 84 | <500 | − | + | − | X4 |
| UCFL2050 | Asymptomatic | 697 | <500 | +++ | − | − | R5 |
| UCFL2051 | Asymptomatic | 605 | <500 | +++ | ++ | +++ | R5X4R8 |
| UCFL2053 | Symptomatic | 259 | <500 | +++ | − | − | R5 |
| UCFL2054 | Asymptomatic | 801 | <500 | + | + | − | R5X4 |
| UCFL2055 | Symptomatic | 127 | 13,883 | + | + | + | R5X4R8 |
| UCFL2056 | Symptomatic | 230 | 831 | + | + | + | R5X4R8 |
| UCFL2057 | Symptomatic | 219 | <500 | +++ | − | − | R5 |
| UCFL2058 | Symptomatic | 224 | <500 | ++ | − | − | R5 |
| UCFL2059 | Asymptomatic | 197 | <500 | +++ | +++ | − | R5X4 |
| UCFL2060 | Asymptomatic | 896 | ND | +++ | +++ | − | R5X4 |
| UCFL2061 | Asymptomatic | 964 | ND | +++ | + | − | R5X4 |
| UCFL2062 | Asymptomatic | 554 | <500 | + | − | − | R5 |
| UCFL2063 | Symptomatic | 304 | <500 | + | − | − | R5 |
| UCFL2064 | Symptomatic | 144 | >100,000 | + | ++ | − | R5X4 |
| UCFL2065 | Symptomatic | 263 | ND | + | + | + | R5X4R8 |
| UCFL2066 | Symptomatic | 126 | ND | + | + | − | R5X4 |
| UCFL2067 | Symptomatic | 186 | ND | +++ | + | +++ | R5X4R8 |
| UCFL2068 | Symptomatic | 96 | ND | − | + | − | X4 |
| UCFL2069 | Symptomatic | 485 | ND | + | − | − | R5 |
| UCFL2070 | Symptomatic | 108 | ND | +++ | ++ | − | R5X4 |
| UCFL2071 | Symptomatic | 74 | ND | + | + | + | R5X4R8 |

[a] Clinical stage according to CDC classification (CDC, 1992); Asymptomatic = clinical stage A; Symptomatic = clinical stage B or C.
[b] Number of T-CD4 lymphocytes/μl.
[c] Copies of viral RNA/ml; ND, not determined.
[d] Peak RT activity measured in culture supernatants during a 21 day-period after virus inoculation. − peak RT activity <10 pg/ml; + peak RT activity between 10 and 100 pg/ml; ++ peak RT activity between 101 and 1000 pg/ml; +++ peak RT activity >1000 pg/ml.

**Table 3**
Data from HIV-2 sequential isolates obtained from follow-up patients.

| Patients | Virus | Elapsed time (weeks)[a] | Clinical stage[b] | CD4 cell count[c] | Plasma viral load[d] | Coreceptors used[e] | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | CCR5 | CXCR4 | CCR8 |
| Patient | UCFL2013 | | S | 500 | 816 | + | − | − |
| #1 | UCFL2014 | 52 | S | 213 | <500 | ++ | − | − |
| | UCFL2015 | 41 | S | 220 | <500 | + | + | − |
| Patient | UCFL2018 | | S | 392 | <500 | +++ | ++ | +++ |
| #2 | UCFL2019 | 90 | S | 258 | 1870 | ++ | − | − |
| Patient | UCFL2027 | | A | 730 | <500 | ++ | − | − |
| #3 | UCFL2028 | 61 | A | 760 | <500 | +++ | − | − |
| Patient | UCFL2030 | | S | 346 | <500 | ++ | − | − |
| #4 | UCFL2031 | 246 | S | 92 | <500 | ++ | − | − |
| Patient | UCFL2034 | | A | 432 | 9470 | ++ | − | − |
| #5 | UCFL2035 | 57 | S | 292 | 6431 | +++ | ++ | − |
| | UCFL2036 | 30 | S | 195 | <500 | +++ | − | − |
| Patient | UCFL2037 | | S | 419 | <500 | +++ | − | + |
| #6 | UCFL2038 | 73 | S | 318 | <500 | +++ | − | − |
| Patient | UCFL2045 | | A | 267 | 1947 | +++ | − | − |
| #7 | UCFL2046 | 79 | A | 130 | <500 | +++ | − | − |
| Patient | UCFL2048 | | S | 59 | 1698 | + | − | ++ |
| #8 | UCFL2049 | 32 | S | 84 | <500 | − | + | − |
| Patient | UCFL2050 | | A | 697 | <500 | +++ | − | − |
| #9 | UCFL2051 | 78 | A | 605 | <500 | +++ | ++ | +++ |

[a] Elapsed time after previous blood withdrawal in sequential samples from the same patient.
[b] Clinical stage according to CDC classification (CDC, 1992); A, Asymptomatic = clinical stage A; S, Symptomatic = clinical stage B or C.
[c] Number of T-CD4 lymphocytes/µl.
[d] Copies of viral RNA/ml; ND, not determined.
[e] Peak RT activity measured in culture supernatants during a 21 day-period after virus inoculation. − peak RT activity <10 pg/ml; + peak RT activity between 10 and 100 pg/ml; ++ peak RT activity between 101 and 1000 pg/ml; +++ peak RT activity >1000 pg/ml.

Due to the large number of isolates tested, and the heterogeneity of this cohort, we analysed the correlation between HIV-2 coreceptor usage, patient's clinical stage and CD4+ T-cell counts. We aim to address two central questions: whether or not CCR5 usage is observed mainly in individuals with higher CD4+ T-cell counts and lower viral load; and whether the acquisition of CXCR4 usage could be related with more severe immunodeficiency (defined by CD4+ T-cell counts below 200 cells/µl) and with increased levels of plasma virémia (above 500 RNA copies/ml). The statistical analysis of the data presented in Table 2 indicates that differential usage of CCR5, CXCR4 or CCR8 by HIV-2 is not correlated ($P>0.05$) with the clinical stage or with the immunological/virological data of the infected patients. The values of $P$ obtained were: $P=0.522$ (CD4 T-cell counts vs. CCR5 usage), $P=0.149$ (CD4 T-cell counts vs. CXCR4 usage), $P=0.289$ (CD4 T-cell counts vs. CCR8 usage), $P=0.912$ (viral load vs. CCR5 usage), $P=0.056$ (viral load vs. CXCR4 usage) and $P=0.057$ (viral load vs. CCR8 usage).

From our results is remarkable that only three out of 65 HIV-2 tested (4.6%) show an X4 phenotype (exclusive CXCR4 usage), in contrast to 46.2% (30/65) of strains that only use the CCR5 coreceptor (R5 phenotype). This is particularly noteworthy considering that 47 out of 65 (72.3%) of the HIV-2 viruses tested were obtained from symptomatic patients (Tables 1 and 2). Furthermore, the R5 phenotype was observed regardless of patient's CD4 cell count and/or plasma viral load.

*Coreceptor usage by HIV-2 primary isolates obtained from sequential samples*

To further address the critical question about the evolutionary dynamics of coreceptor usage during disease progression, and whether X4 variants arise as a consequence of immune depletion, we analysed the evolution of coreceptor usage within several infected individuals from which follow-up blood samples could be obtained. A total of twenty sequential viruses isolated from nine different patients were studied, including four that were asymptomatic at the beginning of the follow-up. The elapsed time between consecutive samples ranged from thirty to 246 weeks (average: 76 weeks). The results (Table 3) reveal that only one patient had evolved from asymptomatic to symptomatic clinical stage (patient #5). A decrease in CD4+ T-cell counts was observed in six out of nine patients. All the initial isolates obtained from the earliest samples were R5 except those from patients #2 (R5X4R8) and #6 (R5R8). Interestingly, both isolates lost the ability to use CXCR4 and/or CCR8 coreceptors in sequential viruses obtained ninety and 73 weeks later, respectively.

Although the number of samples was too small to get a consistent analysis we may conclude that in this follow-up cohort there is no correlation between a decrease in CD4+ cell count and the acquisition of CXCR4 or CCR8 usage ($P>0.05$). Despite this disagreement between viral biotype and immunological status, it is noteworthy the evolutionary dynamics of viral biotype observed within isolates obtained from patient #5 (UCFL2034, UCFL2035 and UCFL2036). This patient was the only that showed a clinical evolution during the follow-up period from asymptomatic to symptomatic and viral biotype changed from R5 (UCFL2034) to R5X4 (UCFL2035), reverting again to R5 (UCFL2036). This coreceptor evolution was concomitant with a decrease in both plasma viral load and CD4+ T-cell counts. Interestingly, after isolation of UCFL2035, this patient initiated anti-retroviral therapy with a protease inhibitor and two nucleoside analogue reverse transcriptase inhibitors.

Also remarkable was the evolution of viral biotype observed in patients #2, #6 and #8. In all of them, CCR8 using variants appear to be cleared from the predominant viral population in peripheral blood. Although any assumption regarding the reasons underlying this evolution needs to be further tested, this observation may suggest that these variants could have intrinsic disadvantages, perhaps a lower viral fitness or an increased susceptibility to host immunological response.

*CCR5, CXCR4 and CCR8 coreceptor usage by HIV-1 primary isolates*

In HIV-1 infection, the emergence of variants able to use other coreceptors besides CCR5 and CXCR4 has been referred occasionally and much less frequently than in HIV-2. In order to compare the pattern of coreceptor usage profile observed in the HIV-2 cohort we also investigate the contribution of CCR5, CXCR4 and CCR8 as coreceptors for diverse HIV-1 isolates. We are particularly interested in ascertaining the frequency with which CCR8 was used as viral coreceptor. With this aim we analysed thirty primary HIV-1 isolates obtained from patients at different clinical stages and with distinct CD4+ T-cell counts (Tables 1 and 4). The results, summarized in Table 4 and analysed according to the same criteria mentioned for Table 2, reveal that, as in HIV-2 group, CCR5 and CXCR4 are the main coreceptors used by HIV-1: 93.3% and 83.3% of the isolates were able to infect productively CCR5 and CXCR4-expressing cells, respectively. However, quite unexpectedly, but with statistical significance, we found that CCR8 could be used as coreceptor by seventeen out of thirty isolates tested (56.7%), more frequently than in HIV-2 group (26.2%; $P<0.001$). As already noticed in HIV-2 isolates, the use of this alternative coreceptor was observed regardless of the clinical stage, the plasma viral load or the CD4+ T-cell counts. Regarding CXCR4 usage by HIV-1 and HIV-2, it is noteworthy that the ability to infect CXCR4-expressing cells was more frequently observed in HIV-1 (83.3%) than in HIV-2 isolates (47.7%; $P<0.001$).

## Discussion

HIV infection *in vitro* could be mediated by a wide range of different seven-transmembrane G-protein coupled chemokine receptors. Despite the relative promiscuous usage of these coreceptors *in*

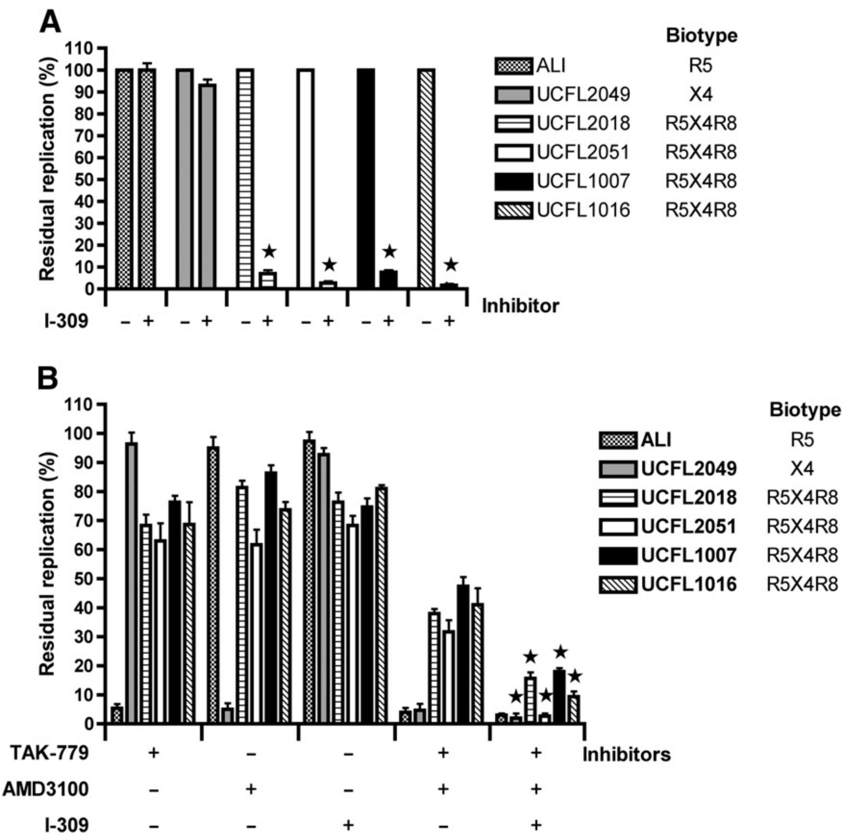


**Fig. 1.** Inhibition assay of CCR8-using HIV isolates. In these assays, HIV primary isolates were normalized by RT activity levels, and equivalent amounts of each isolate were added to GHOST–CD4/CCR8 [panel (A)] or CD8-depleted PBMC [panel (B)] in the presence or absence of the different inhibitors used: I-309 [panel (A)] or I-309, TAK-779 and AMD3100 [panel (B)], according to the tables. Data were analysed using GraphPad Prism software, version 4.0, and are representative of at least three independent assays. Data represent the mean of residual replication (%) ± standard error of the mean (bars). The biotype of each isolate is indicated according to coreceptor usage. (A) Inhibition assay of CCR8-using HIV isolates by I-309 using GHOST–CD4/CCR8 cell line; the table below the X-axis indicates the presence (+) or absence (−) of I-309. A star indicates statistical significant difference ($P<0.001$) between the medians of the percentage of residual replication in the absence and the presence of I-309. (B) Inhibition assay of CCR8-using HIV isolates by I-309 using PBMC; the table below the X-axis indicates the presence (+) or absence (−) of TAK-779, AMD3100 and I-309. A star indicates statistical significant difference ($P<0.001$) between the medians of the percentage of residual replication in the presence of AMD3100 + TAK-779 alone and in the combination of AMD3100 + TAK-779 + I-309.

*vitro* by certain HIV-1 and particularly HIV-2 strains (Broder and Jones-Trower, 1999; Bron et al., 1997; Guillon et al., 1998; McKnight et al., 1998; Morner et al., 1999; Owen et al., 1998), the majority of recent studies concerning coreceptor usage by HIV isolates have been focused in the two major chemokine receptors: CCR5 and CXCR4. Accordingly, only limited information is available regarding CCR8 usage (or other minor coreceptors) by primary HIV-1 and HIV-2 isolates. The role of alternative coreceptors, besides CCR5 and CXCR4, in HIV-1 infection is considered as being of limited importance *in vitro* and probably *in vivo* (Moore et al., 2004; Y.J. Zhang et al., 1998); accordingly, only CCR5 and CXCR4 are potential targets for therapeutic intervention. However, some exceptions have been reported (Cilliers et al., 2003, 2005; Deng et al., 1997; Edinger et al., 1998; Lee et al., 2000; Pohlmann et al., 1999; Shimizu et al., 2009; Willey et al., 2003; Xiao et al., 1998), revealing that some isolates can exploit alternate coreceptors *in vitro* although whether such coreceptors are used by HIV-1 *in vivo* remains unclear (Zhang and Moore, 1999). Nevertheless, the possibility that alternative molecules could have physiological relevance *in vivo* as a cofactor for HIV-1 infection remains open. The same is true concerning HIV-2 infection, where the more promiscuous usage of chemokine receptors as coreceptors has been widely referred *in vitro* (Bron et al., 1997; Guillon et al., 1998; McKnight et al., 1998). Additionally, the existence of primary isolates with atypical coreceptor usage profile (Azevedo-Pereira et al., 2003; Sol et al., 1997; Zhang et al., 2000), may further indicate that in HIV-2 the capacity to use other coreceptors besides CCR5 and CXCR4 should be potentially more common.

In this report we analysed the role of CCR8 chemokine receptor as coreceptor for HIV-1 and HIV-2 primary isolates. CCR8, formerly known as TER1, ChemR1 and CKR-L1, is the receptor for the chemokine CCL1/I-309 (Roos et al., 1997; Tiffany et al., 1997). Additionally, three virally encoded ligands are also known to interact with CCR8: vMIP-I and vMIP-II (encoded by HHV-8) and vMCC-I encoded by *Molluscum contagiosum* poxvirus (Dairaghi et al., 1999). The rationale underlying the inclusion of CCR8 as an alternative coreceptor mediating HIV-2 and HIV-1 infection, stems from the cellular and tissue distribution of this chemokine receptor *in vivo* that includes some major cellular targets of HIV infection. Namely, CCR8 expression has been detected on monocytes, thymocytes and peripheral blood T-lymphocytes with Th2 cytokine profile (D'Ambrosio et al., 1998; Napolitano et al., 1996; Tiffany et al., 1997; Zaballos et al., 1996; Zingoni et al., 1998). CCR8 usage by certain HIV strains was referred in earlier reports (Isaacman-Beck et al., 2009; Liu et al., 2000; Ohagen et al., 2003; Rucker et al., 1997; Shimizu et al., 2009; Singh et al., 1999; Willey et al., 2003). The majority of these reports used T-cell culture adapted (TCLA) strains and the total number of strains tested ranged from seven (Singh et al., 1999) to 22 (Rucker et al., 1997). In these studies, the percentage of viruses able to use CCR8 was quite heterogeneous, ranging from 12.5% (Isaacman-Beck et al., 2009) to 81.8% (Willey et al., 2003). Interestingly in this latter report, all the HIV-1 strains tested (including primary isolates) proved to be able to infect NP2/CD4 cell line expressing CCR8; some of them as efficiently as they infect CCR5 or CXCR4 expressing cells (Willey et al., 2003). Furthermore, CCR8 usage in primary cells was demonstrated for two HIV-1 isolates (Cilliers et al.,

**Table 4**
CCR5, CXCR4 and CCR8 coreceptor usage by HIV-1 isolates and patient's clinical, immunological and virological data.

| Isolate | Clinical stage[a] | CD4+ cell count[b] | Plasma viral load[c] | Coreceptors used[d] | | | Biotype |
|---|---|---|---|---|---|---|---|
| | | | | CCR5 | CXCR4 | CCR8 | |
| UCFL1001 | Symptomatic | 561 | 10,513 | − | + | − | X4 |
| UCFL1002 | Symptomatic | 348 | 19,568 | +++ | +++ | ++ | R5X4R8 |
| UCFL1003 | Symptomatic | 249 | 112,010 | +++ | ++ | − | R5X4 |
| UCFL1005 | Asymptomatic | 1003 | <500 | + | + | − | R5X4 |
| UCFL1004 | Asymptomatic | 1125 | <500 | +++ | ++ | − | R5X4 |
| UCFL1006 | Symptomatic | 227 | 280,738 | ++ | +++ | + | R5X4R8 |
| UCFL1007 | Symptomatic | 106 | 75,639 | +++ | ++ | ++ | R5X4R8 |
| UCFL1008 | Symptomatic | 238 | 172,720 | +++ | − | ++ | R5R8 |
| UCFL1009 | Symptomatic | 409 | 2,742,788 | + | ++ | + | R5X4R8 |
| UCFL1010 | Symptomatic | 274 | ND | +++ | ++ | − | R5X4 |
| UCFL1011 | Symptomatic | 1428 | 1595 | ++ | +++ | + | R5X4R8 |
| UCFL1013 | Asymptomatic | 446 | 1,876,150 | ++ | − | ++ | R5R8 |
| UCFL1014 | Symptomatic | 305 | 67,508 | ++ | + | − | R5X4 |
| UCFL1015 | Symptomatic | 259 | 120,345 | +++ | +++ | +++ | R5X4R8 |
| UCFL1016 | Asymptomatic | 453 | 6521 | +++ | +++ | +++ | R5X4R8 |
| UCFL1017 | Asymptomatic | 563 | 281,934 | ++ | − | − | R5 |
| UCFL1018 | Symptomatic | 451 | 192,969 | + | + | + | R5X4R8 |
| UCFL1019 | Symptomatic | 125 | 5765 | − | + | + | X4R8 |
| UCFL1020 | Symptomatic | 118 | 164,230 | ++ | + | ++ | R5X4R8 |
| UCFL1021 | Asymptomatic | 1076 | <500 | ++ | ++ | ++ | R5X4R8 |
| UCFL1022 | Symptomatic | 394 | 246,641 | +++ | ++ | ++ | R5X4R8 |
| UCFL1023 | Asymptomatic | 349 | <500 | + | + | − | R5X4 |
| UCFL1024 | Symptomatic | 187 | 387,659 | ++ | + | ++ | R5X4R8 |
| UCFL1025 | Asymptomatic | 578 | <500 | ++ | − | − | R5 |
| UCFL1026 | Symptomatic | 278 | 178,364 | +++ | + | − | R5X4 |
| UCFL1027 | Symptomatic | 451 | 70,557 | +++ | +++ | +++ | R5X4R8 |
| UCFL1028 | Symptomatic | 218 | 237,482 | +++ | + | − | R5X4 |
| UCFL1029 | Synptomatic | 1455 | <500 | +++ | + | − | R5X4 |
| UCFL1030 | Symptomatic | 183 | 56,457 | +++ | − | + | R5R8 |
| UCFL1031 | Symptomatic | 123 | 387,237 | ++ | ++ | − | R5X4 |

[a] Clinical stage according to CDC classification (CDC, 1992); Asymptomatic = clinical stage A; Symptomatic = clinical stage B or C.
[b] Number of T-CD4 lymphocytes/μl.
[c] Copies of viral RNA/ml; ND, not determined.
[d] Peak RT activity measured in culture supernatants during a 21 day-period after virus inoculation. − peak RT activity <10 pg/ml; + peak RT activity between 10 and 100 pg/ml; ++ peak RT activity between 101 and 1000 pg/ml; +++ peak RT activity >1000 pg/ml.

2005) suggesting that CCR8 may indeed prove to be a significant coreceptor for certain isolates of HIV-1 and HIV-2.

Our results demonstrate that CCR8 was effectively used by seventeen out of 65 HIV-2 isolates tested (26.2%). The usage of minor coreceptors, namely CCR8, in HIV-2 infection could be somewhat predicted by the well-known promiscuous usage of chemokine receptors that, in general, characterize HIV-2 strains (Bron et al., 1997; Guillon et al., 1998; McKnight et al., 1998). More surprisingly, however, was the frequency with which CCR8 was used by HIV-1 isolates: 56.7% of the strains tested were able to infect CCR8-expressing cells. This data raises once again the question of whether alternate molecules, such as CCR8, can *in vivo* contribute to HIV infection of natural target cells, at least under certain circumstances. This is particularly important when anti-CCR5 antagonists are being used in clinical practice and the coreceptor phenotype assays only detect CCR5 vs. CXCR4 variants. If an even minor viral population using CCR8 (or other coreceptors) is present in an infected patient, it will not be detected, with potential implications in therapeutic success and possibly also in the pathogenesis. However, it is important to emphasize that the capacity of HIV primary isolates to exploit coreceptors in indicator cell lines, such as GHOST–CD4+ cell lines, does not provide an exact indication of coreceptor usage *in vivo*. The levels of coreceptor expression in naturally occurring cells are, in general, lower than those observed in genetically engineered cell lines expressing CD4 and a chemokine receptor. Accordingly, the inference of *in vivo* coreceptor usage based on *in vitro* assay should be assumed with caution. Nevertheless, the findings presented in this report, together with previous data indicating that CCR8 can function efficiently as coreceptor for diverse HIV-1 and HIV-2 isolates, either in indicator cell lines or in primary cells (Cilliers et al., 2005; Horuk et al., 1998; Jinno et al., 1998; Lee et al., 2000; Liu et al., 2000; Rucker et al., 1997; Shimizu et al., 2009), highlight the potential relevance of this molecule for viral pathogenesis.

It is widely recognized that during later stages of HIV-1 infection, a significant proportion of viral variants that constitute the *quasispecies* within an infected individual, evolve in order to efficiently use CXCR4 (and probably other chemokine receptors) in addition to, or instead of, CCR5-using variants, which predominate during early stages of infection (Connor et al., 1997; Su et al., 1997). The emergence of X4 variants occurs in approximately half of the infected patients and is associated with accelerated disease progression and increased CD4+ T-lymphocytes depletion (Connor et al., 1997). These higher rates of disease progression and CD4+ T-cell depletion have been related to several viral factors, namely to an increase in replicative capacity and a more cytopathic phenotype of X4 viruses to primary CD4+ lymphocytes (Fouchier et al., 1996; Tersmette et al., 1989; van't Wout et al., 1998).

Our results reveal that the CXCR4 usage, either alone or together with CCR5 and/or CCR8 (R5X4 or R5X4R8 biotype, respectively), was more frequently observed in HIV-1 than in HIV-2 isolates (83.3% and 47.7%, respectively). Directly related to this is the finding that the non-usage of CXCR4 and/or CCR8 (R5 biotype) is significantly more common in HIV-2 isolates than in HIV-1 (46.2% and 6.7%, respectively). Even considering that *in vitro* isolation and propagation of HIV isolates may either reflect the full diversity of viral *quasispecies* or, instead, result in the selection of only a subset of HIV variants present in the patient's PBMC (those with the most fit phenotype under the *in vitro* culture conditions), this data suggests that the acquisition of CXCR4 usage could be more common in HIV-1 *quasispecies* and preservation of R5 biotype more frequently observed in HIV-2 viral population. Both features may be related with the slower disease progression generally observed in HIV-2 infected patients compared with HIV-1. Although tempting, this inference must also bear in mind the existence of intrinsic differences between our HIV-2 and HIV-1 patient cohorts, even considering that they both show a similar percentage of symptomatic patients, as well as a proportional number of patients with low CD4+ T-cell counts.

Furthermore, we cannot exclude the possible contribution of other coreceptors besides those included in this study (i.e. CCR5, CXCR4 and CCR8). Nevertheless, the emergence and predominance of a CXCR4-using viral population mostly observed in HIV-1 cohort, could be seen as an additional contribution to an accelerated disease progression. Specifically, the ability to infect naïve T-cells by X4 variants, which could occur early in T-lymphocyte ontogeny, may contribute to the described enhancement of T-cell depletion by X4 strains. Several studies addressing thymocyte development, have demonstrated that immature T-cell progenitor expresses high levels of CXCR4 (Berkowitz et al., 1998; Kitchen and Zack, 1997; Taylor et al., 2001; L. Zhang et al., 1998). As a result, infection of immature thymocytes by X4 strains may disrupt thymopoiesis leading to an impairment of T-cell development and to an accelerated T-cell depletion (Berkowitz et al., 1998; Kitchen and Zack, 1997; Taylor et al., 2001). Furthermore, HIV-1 X4 variants are associated with massive induction of apoptosis in bystander CD4+ T-cells; while R5 stains appear to induce lower levels of programmed cell death (Berndt et al., 1998; Biard-Piechaczyk et al., 1999; Herbein et al., 1998; Jekle et al., 2003). Accordingly, the predominance of non-CXCR4-using variants in HIV-2 individuals may help to better preserve CD4+ lymphocyte repertoire and immune system functionality than in individuals infected with HIV-1, where a more frequent predominance of CXCR4-using variants may be responsible for increased apoptosis and limited T-cell regeneration, with direct impact in CD4+ T-cell homeostasis and immune function.

## Conclusions

In summary, our data confirm that chemokine receptor CCR8 can function as coreceptor for a significant proportion of HIV-1 and HIV-2 primary isolates and may indeed be relevant as an alternative coreceptor, at least under certain circumstances and/or particular cell populations. We also conclude that the acquisition of CXCR4 usage could be more common in HIV-1 *quasispecies* and the preservation of R5 biotype more frequently observed in HIV-2 viral population. Both features could be related with the slower disease progression generally observed in HIV-2 infected patients compared with HIV-1.

## Material and methods

### Cells

Peripheral blood mononuclear cells (PBMCs), from HIV-uninfected donors, homozygous for wild-type *ccr5* gene [assessed as described (Azevedo-Pereira et al., 2003)] were obtained from buffy-coats by Ficoll–Hypaque density gradient centrifugation and stimulated with phytohemagglutinin (3 µg/ml) for 3 days and maintained in RPMI-1640 medium (Invitrogen, Paisley, UK) supplemented with 20 UI/ml of human recombinant Interleukin 2 (IL-2), 15% (v/v) inactivated Fetal Bovine Serum (Invitrogen, Paisley, UK), 50 µg/ml gentamicin and 2.5 µg/ml amphotericin B (Invitrogen, Paisley, UK). PBMCs used in all experiments reported here were obtained from one single pool of different buffy-coats to avoid inter-individual variations in HIV infection susceptibility. Human osteosarcoma cell line GHOST expressing different coreceptors and CD4 were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Paisley, UK) supplemented with 10% (v/v) inactivated Fetal Bovine Serum, 50 µg/ml gentamicin, 2.5 µg/ml amphotericin B, 1 µg/ml puromycin, 100 µg/ml hygromycin and 500 µg/ml G418 (Invitrogen, Paisley, UK).

### HIV isolates

All primary HIV-1 and HIV-2 isolates were obtained from infected patients' PBMC, by cocultivation with PHA-stimulated PBMC from uninfected individuals. Viral replication was assessed by reverse transcriptase activity in culture supernatants by an enzyme linked immunosorbent assay (Lenti-RT kit, Caviditech, Uppsala, Sweden). Viral stocks were established from low-passaged supernatants of infected PBMC and stored at −80 °C until further use.

### Infectivity assays

GHOST cell lines, expressing CD4 and chemokine receptors (CCR5, CXCR4 and CCR8), were seeded into 24-well plates on the day prior to infection, at a $1.5\times10^5$ cells/well. To assess chemokine usage, each cell line was inoculated with 1 ng of reverse transcriptase activity of each virus, in a final volume of 100 µl, and incubated for 3 h, at 37 °C, in the presence of 3 µg/ml of polybrene (Sigma-Aldrich, MO, USA). Cells were then washed and cultured in appropriate culture medium (500 µl/well). Viral replication was monitored by reverse transcriptase (RT) activity, by an enzyme linked immunosorbent assay (Lenti-RT kit, Caviditech, Uppsala, Sweden) or by the detection of Ag p24 (Vironostika HIV-1 Antigen; bioMérieux, Marcy l'Etoile, France) in culture supernatants every 3 days, during 21 days. The infectivity assays using GHOST cell lines also included the inoculation of GHOST–CD4 parental cell line as a control.

### Susceptibility to CCR5, CXCR4 and CCR8 inhibitors

The inhibitors TAK-779 (Baba et al., 1999) and AMD3100 (Donzella et al., 1998) specific for CCR5 and CXCR4, respectively, were obtained through the National Institute of Health (NIH) AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. The chemokine I-309, specific for CCR8 (Roos et al., 1997; Tiffany et al., 1997), was purchased from R&D Systems (Minneapolis, MN).

Virus sensitivity to these different inhibitors was performed using GHOST–CD4/CCR8 cell line or peripheral blood mononuclear cells (PBMC) and was based on the inhibition of viral production as described (Azevedo-Pereira et al., 2003). GHOST–CD4/CCR8 cells were seeded at $1.5\times10^5$ cells per well in 24-well plates and allowed to adhere overnight. Cells were incubated for 1 h at 37 °C with blocking concentrations of each inhibitor: 100 ng/ml of I-309 (Horuk et al., 1998), 100 nM of TAK-779 and 1.2 µM of AMD3100 (Bobardt et al., 2007). Viruses were then added as described in infectivity assays and incubated for 4 h in an inhibitor-containing medium. Cells were washed with PBS to remove unadsorbed viral particles and cultured in an appropriate medium containing the desired concentration of each inhibitor. Alternatively, these inhibition assays were also performed using CD8-depleted PBMCs as target cells, which were obtained from PBMCs after removal of CD8+ cells, using magnetic beads coated with anti-CD8 antibody (Dynabeads, Dynal, Invitrogen, Paisley, UK), according to the manufacturer's instructions.

Virus production was assessed by RT activity in culture supernatants, 7 days after infection. Residual viral replication (percentage) in the presence of an inhibitor was calculated as $100\times(RT_{Inhib}/RT_{control})$, $RT_{control}$ being the infection in the absence of inhibitors.

### Statistical analysis

Statistical analysis was performed using Epi info version 6.04 (CDC, Atlanta, USA) and SPSS software version 10 (SPSS Inc, Chicago, USA). The univariate analysis was tested using $\chi^2$ and 2-tailed Fisher's exact test in case of small sample size. Statistical significance was assumed when $P<0.05$.

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The GHOST indicator cell lines expressing CD4 and chemokine receptors CCR5, CXCR4 and CCCR8 (repository numbers: ARP078, ARP079 and ARP083, respectively) were obtained from the Programme EVA Centre for AIDS Reagents, NIBSC, UK, supported by the EC FP6/7 Europrise Network of Excellence, AVIP and NGIN consortia and the Bill and Melinda Gates GHRC-CAVD Project and was donated by Dr Littman and Dr. V KewalRamni (courtesy of NIH AIDS Research and reference Reagent Programme). The following reagent was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: AMD3100 and TAK-779.

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