# Flexible use of CCR5 in the absence of CXCR4 use explains the immune deficiency in HIV-1 infected children

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**Design:** CCR5-using HIV-1 (R5 viruses) are usually isolated during acute infection from both adults and children. We have recently demonstrated that R5 viruses with a flexible use of CCR5 (called R5broad) can be detected in children close to birth and are predictive of a fast immunological failure. The aim of the present work was to investigate viral phenotype variation during disease progression in HIV-1 infected children, six slow and eight fast progressors.

**Methods:** A total of 74 viral isolates obtained sequentially from 14 HIV-1 infected children were tested for their ability to infect U87.CD4 cells expressing a set of six different CCR5/CXCR4 chimeric receptors or wild-type coreceptors. The sensitivity of 35 R5 viruses to inhibition with the CC-chemokine RANTES (regulated upon activation, normal T-cell expressed and secreted) was evaluated in a peripheral blood mononuclear cells based assay.

**Results:** Viral evolution to R5broad or to R5X4 phenotype occurred with one exception, in all children, although at a different time point according to rate of disease progression. Immune deficiency in the children was significantly associated with the appearance of R5broad phenotype or R5X4 viruses. Analysis of the sensitivity to inhibition by RANTES revealed a significant correlation between the R5broad phenotype and an augmented resistance to this CC-chemokine.

**Conclusion:** We demonstrate that the viral evolution to a more flexible CCR5-use is sufficient to explain the immunological failure in the absence of CXCR4 usage. These results warrant detailed analysis of the R5 phenotype in forthcoming clinical studies introducing CCR5 inhibitors for the treatment of pediatric HIV-1 infection.

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## Introduction

About 20% of HIV-1 infected and untreated infants develop severe immunodeficiency and AIDS-related symptoms within the first year of age, whereas others progress more slowly and have a well preserved immune system for many years [1–4]. Children generally display an accelerated progression to AIDS compared with adults, and survival time is considerably shorter.

As in adults, overt immune deficiency of infected children often occurs without the emergence of CXCR4-using virus variants (X4 or R5X4) [5–10]. It was shown that CXCR4-using viruses possibly emerge in some children as a consequence of the severe immune deficiency [11], which strongly supports the hypothesis that the intrinsic variation of R5 viruses itself may drive disease progression.

We have recently demonstrated that viruses with a more flexible use of CCR5 (R5broad), measured as the ability to use a set of different CCR5/CXCR4 chimeric receptors in addition to wild-type CCR5, are transmitted from infected mothers to their newborn [12]. The presence of these viruses in the infected newborns was significantly associated with a fast progression to severe immunological failure within 3 years of age. Furthermore, we showed that in adults R5broad viruses were associated with $CD4^+$ T-cell decline [13].

Several studies have suggested that in-vivo chemokine production may confer protection against both HIV-1 infection and progression to AIDS [14–16]. However, it was demonstrated that viruses might switch coreceptor usage in the presence of CCR5-specific or CXCR4-specific chemokines or small molecule inhibitors *in vivo* and *in vitro* [9,17–19]. Accordingly, we and other groups have suggested that, in addition to the level of CC-chemokine production in the host, the sensitivity of the virus to CC-chemokine inhibition may influence disease progression favoring the emergence of escape variants [9,13,20–22].

Here we demonstrate that viral evolution to a broader coreceptor usage (either R5broad or R5X4) is common during disease progression and is associated with immunological failure in HIV-1-infected children. Conceivably, difference in the sensitivity to RANTES inhibition of R5narrow and R5broad viruses could drive the phenotypic evolution. This information is relevant for the use of small CCR5 inhibitors in the treatment of pediatric HIV-1 infection and warrants detailed phenotype testing prior to clinical trials.

## Methods

### Patients

This study was performed on 14 HIV-1 infected Italian children with documented disease progression, who acquired infection through mother-to-child transmission before the introduction of antiretroviral drugs used for prevention and were not breast-fed [23]. The children were selected on the basis of the following criteria such as documented progression of the disease, extensive clinical, immunological and virological follow-up from early age throughout the course of the infection, and availability of sequential viral isolates obtained at different stages of disease. None of the children received HAART during the study period. One child (*n.* 196) carried the heterozygous 32 bp deletion of the CCR5 gene [24].

The clinical and immunological stage of the children was defined according to the guidelines of the Centers for Disease Control (CDC) [25]. Children who experienced a severe decline of the $CD4^+$ T-cell counts entering CDC immunological category 3 within 3 years of age or died within 1 year were defined rapid progressor [12]. Children classified as CDC category 3 after 3 years or who did not enter this category within up to 10 years of follow up were defined slow progressor. Clinicians provided the children's follow-up data of complete blood cell counts and lymphocyte subsets. HIV-1 p24 antigen (Ag) determination in plasma or viral RNA load were not routinely collected and thus, excluded from the analysis.

Seventy-four virus isolates were obtained from peripheral blood mononuclear cells (PBMC) [9,23]. The time of first HIV-1 isolation varied among the children between 0 and 9 months of age (mean of 3 months). In one child (*n.* 204) HIV-1 was already detectable by viral isolation and PCR at 4 days of age. Between 2 and 9 viral isolates were available for each child mainly depending on the length of the follow-up before decease or start of HAART.

The Ethical Committee approved the use of samples according to national laws (in 1986, 1991 and 2008). An informed oral consent was obtained from the children's parents.

### Determination of viral phenotype and RANTES sensitivity

Virus stocks were used to infect human glioma U87.CD4 cells stably expressing the chemokine receptors CCR5 or CXCR4, or the 6 CCR5/CXCR4 chimeric receptors, as previously described [9,12]. Parental U87.CD4 cells, engineered to express CD4 cell but no chemokine receptor, were used as negative control. Chimeric receptors were obtained by replacing successively larger parts of CCR5 with corresponding parts of CXCR4 [26]. Viruses able to use only CCR5 as coreceptor were defined as R5narrow, whereas R5 viruses able to use chimeric receptors were defined as R5broad [13]. R5X4 viruses used the six chimeric receptors in different combinations and were not further classified.

PHA-activated PBMCs derived from HIV-seronegative blood donors were infected with viral supernatant in the

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presence or absence of RANTES (R&D Systems, Minneapolis, Minnesota, USA) as previously described [9]. The sensitivity to RANTES, expressed as inhibitory concentration 50 (IC50), was defined as the percentage of virus growth in the presence of the chemokine compared with that of the virus only. SDF-1 (R&D Systems), the ligand for CXCR4, was used as control. The R5 isolates were not inhibited with concentrations of 2 μg/ml of the chemokine.

### Statistical analysis

Mann–Whitney test was used to compare IC50 values of R5narrow vs. R5broad isolates, as well as of R5 isolates in the children, who experienced or not a viral switch to CXCR4 usage (defined as switch or nonswitch children, respectively). Correlation between viral phenotype and clinical or immunological CDC stage of infected children was analyzed by chi-squared test. Values below 0.05 were regarded as statistically significant.

## Results

### HIV-1 phenotypic evolution is common in infected children

With the aim of studying a link between evolution of viral phenotype and pediatric disease progression, we analyzed 74 sequential isolates obtained from 14 HIV-1-infected children with different rates of disease progression. During the first months of life in the early phase of infection 11 children carried a virus with R5narrow, two with an R5broad and one with an R5X4 phenotype (Table 1).

The viral evolution was common in the infected children during disease progression. Indeed, the viral R5narrow phenotype evolved over time either to R5broad and/or R5X4 in all children, except one (*n.* 201) (Table 1). Progression from R5narrow to R5broad occurred in three rapid (*n.* 32, 224 and 380) and three slow progressors (*n.* 3, 190 and 306), but was followed by appearance of R5X4 only in one slow progressor (*n.* 3). Progression from R5narrow to R5X4 without an evident evolution through R5broad usage was observed in one rapid (*n.* 199) and in three slow progressors (*n.* 115, 136 and 145). Close to birth the R5broad phenotype was detected only in two rapid progressing children, who experienced a fast viral switch to CXCR4 use during follow-up. Of note is that the switch to CXCR4 usage appeared at an earlier age in rapid than in slow progressors (mean age: 19.6 vs. 64.5 months). The only exception, child *n.* 201 carried a virus with R5narrow phenotype until death, which occurred due to congenital CMV infection at 9 months of age. Progression of viral variation to R5 broad or R5X4 occurred despite mono or dual antiretroviral therapy.

### R5broad phenotype correlates with immune deficiency

The maintenance of R5 variants throughout infection and the association of CXCR4-using virus variants with disease progression suggests that the latter ones are either a cause of or evolve in response to progressive immune suppression. Our analysis showed a strong correlation between the viral phenotype of the 74 viruses tested, dissected into R5narrow, R5broad and R5X4, and the corresponding immunological CDC stage of the children ($P = 0.00047$, chi-squared test). A correlation of the clinical staging (CDC N, A, B, or C) was observed only when R5narrow and R5broad were considered together (R5 vs. R5X4, $P = 0.0147$, chi-squared test). We show that a severe $CD4^+$ T-cell decline can be established when R5 viruses have acquired a flexible use of the coreceptor without the need to switch to CXCR4 use.

### Chimeric receptor use and viral phenotype evolution are related to resistance to RANTES

We determined the sensitivity to RANTES inhibition of 35 R5 isolates obtained from 13 children as to understand if a selective pressure may drive the viral phenotype evolution. Our analysis showed that R5narrow viruses were inhibited with significantly lower concentrations of RANTES than R5broad viruses (mean IC50: 38 vs. 47 ng/ml; $P = 0.043$, Mann–Whitney test) (Fig. 1a). Furthermore, R5 viruses isolated from the seven children, whose virus progressed to CXCR4 usage during disease progression (defined as switch children), were inhibited with lower concentrations of RANTES than those of the six children who did not experience this phenotypic switch (mean IC50: 25 vs. 50 ng/ml; $P = 0.0029$ Mann–Whitney test) (Fig. 1b).

## Discussion

Here we show that the refined classification of R5 viruses into R5narrow and R5broad resolves the enigma of the R5 phenotype being associated with the state of immune deficiency. Indeed, in our cohort detection of R5broad in addition to R5X4 viruses but not R5narrow was significantly associated with immune deficiency either in slow or rapid progressors. These data are in line with those described for HIV-1-infected adults [13], with the important difference that the R5broad phenotype can be detected in the children already early in infection and be predictive of a fast immunologic failure, as we previously described in a large cohort [12].

It can be envisaged that a detailed analysis of the viral phenotype as the one used here would have possibly explained the establishment of the immune deficiency in the children without or before the viral change to CXCR4-usage described in the paper by Casper *et al.* [11]. In the former study, children were infected

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**Table 1. Clinical-immunological stage, disease progression and viral phenotype during follow-up of 14 children.**

| Child code | Age (months) | CDC stage[a] | CD4 cell count ($10^6$/l) | Therapy (months)[b] | Coreceptor usage | Chimeric receptor usage[c] |
|---|---|---|---|---|---|---|
| Rapid progressor | | | | | | |
| C32 | 3 | N1 | 2092 | | CCR5 | – |
| | 8 | N1 | 1535 | | CCR5 | – |
| | 10 | N1 | 656 | | CCR5 | – |
| | 42 | A3 | 264 | Mono (28) | CCR5 | FC2 |
| | 55 | A3 | 205 | Mono | CCR5 | FC2 |
| | 65 | B3 | 255 | Dual (59) | CCR5 | FC2, 4b |
| | 96 (D)* | | | | | |
| B193 | 4 | B2 | 1087** | | CCR5 | FC2, 4b |
| | 21 | C3 | 19 | Mono (12) | CCR5/CXCR4 | FC4b, 6, 7 |
| | 28 (D) | | | | | |
| B196 | 1 | A1 | 1865** | | CCR5/CXCR4 | FC4b, 7 |
| | 34 | C3 | 14 | Mono (9) | CCR5/CXCR4 | FC4b, 5, 6, 7 |
| | 44 (D) | | | | | |
| B199 | 2 | A1 | 2329 | | CCR5 | – |
| | 3 | A1 | 1953 | | CCR5 | – |
| | 7 | A1 | 1991 | | CCR5 | – |
| | 37 | B3 | 57 | Mono (28) | CXCR4 | FC4b, 7 |
| | 60 (D) | | | | | |
| B201*** | 2 | A1 | 934** | | CCR5 | – |
| | 3 | B2 | 934 | | CCR5 | – |
| | 6 | C3 | 613 | | CCR5 | – |
| | 9 (D) | | | | | |
| B204 | 0 | N1 | 1930 | | CCR5 | FC2, 4b |
| | 1 | A1 | 3216 | | CCR5/CXCR4 | FC4b, 7 |
| | 2 | B2 | 909 | | CCR5/CXCR4 | FC4b, 7 |
| | 33 | C3 | 4 | Mono (6) | CCR5/CXCR4 | FC4b, 6, 7 |
| | 37 (D) | | | | | |
| B224 | 1 | A1 | 2936** | | CCR5 | – |
| | 2.5 | A1 | 2936** | | CCR5 | – |
| | 5.5 | A3 | 300 | | CCR5 | – |
| | 18 | B3 | 680 | Mono (8) | CCR5 | – |
| | 43 | C3 | 535 | Mono | CCR5 | FC2 |
| | 74 | C3 | 423** | Dual | CCR5 | – |
| | 80 | C3 | 423** | Triple | CCR5 | – |
| B380 | 6 | B3 | 302 | Mono (4) | CCR5 | – |
| | 13 | B3 | 1248 | Dual | CCR5 | FC2 |
| | 19 | C3 | 770 | Dual | CCR5 | FC1, 2, 4b |
| | 26 | C3 | 533 | Dual | CCR5 | FC2, 4b |
| | 34 | C3 | 1987 | Dual | CCR5 | FC2, 4b |
| | 42 | C3 | 1667 | Dual | CCR5 | – |
| Slow progressor | | | | | | |
| C3 | 6 | N1 | 2920 | | CCR5 | – |
| | 18 | A1 | 3347 | | CCR5 | – |
| | 40 | B1 | 1512 | | CCR5 | FC2 |
| | 54 | B2 | 694 | Mono | CXCR4 | FC2, 4b, 5, 6, 7 |
| | 65 | B3 | 186 | Dual (56) | CXCR4 | FC2, 4b, 5, 6, 7 |
| | 76 | B3 | 131 | Dual | CCR5/CXCR4 | FC2, 4b, 5, 6, 7 |
| B115 | 3 | B1 | 1199 | | CCR5 | – |
| | 11 | | 1821 | | CCR5 | – |
| | 53 | | 591 | | CCR5 | – |
| | 119 | B2 | 608 | Mono (59) | CXCR4 | FC4b, 5, 6, 7 |
| B136 | 3 | A1 | 2193 | | CCR5 | – |
| | 34 | B1 | 1117 | | CCR5 | – |
| | 47 | | 873 | | CCR5 | – |
| | 60 | B2 | 358 | | CXCR4 | FC4b, 7 |
| | 64 | | 270 | | CCR5/CXCR4 | FC4b, 7 |
| | 67 | | 413 | Mono | CXCR4 | FC4b, 7 |
| | 76 | | 418 | Mono | CCR5/CXCR4 | FC4b, 7 |
| | 83 | B3 | 306 | Dual | CCR5 | – |
| | 92 | B3 | 357 | Dual | CCR5 | – |
| B145 | 1 | B1 | 2386 | | CCR5 | – |
| | 5 | B2 | 1769 | | CCR5 | – |

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**Table 1** (*continued*)

| Child code | Age (months) | CDC stage[a] | CD4 cell count ($10^6$/l) | Therapy (months)[b] | Coreceptor usage | Chimeric receptor usage[c] |
|---|---|---|---|---|---|---|
| | 48 | B3 | 331 | | CXCR4 | FC1, 2, 4b, 5, 6, 7 |
| | 67 | | 28 | Mono (51) | CXCR4 | FC1, 2, 4b, 5, 6, 7 |
| | 74 | | 11 | Mono | CCR5/CXCR4 | FC1, 2, 4b, 5, 6, 7 |
| | 83 | | 6 | Dual | CXCR4 | FC1, 2, 4b, 5, 6, 7 |
| | 102 | C3 | 464 | Triple (96) | CXCR4 | FC1, 2, 4b, 5, 6, 7 |
| | 108 | | 594 | Triple | CXCR4 | FC1, 2, 4b, 5, 6, 7 |
| | 114 | C3 | 902 | Triple | CXCR4 | FC1, 2, 4b, 5, 6, 7 |
| B190 | 1 | N1 | 4339** | | CCR5 | – |
| | 2 | | 4339 | | CCR5 | – |
| | 11 | | 3421 | | CCR5 | FC2 |
| | 56 | | 516 | | CCR5 | FC2 |
| | 80 | | 924 | Dual (77) | CCR5 | FC2 |
| | 92 | N1 | 858 | Dual | CCR5 | – |
| B306 | 9 | A1 | 3374 | | CCR5 | – |
| | 16 | | 2194 | | CCR5 | – |
| | 40 | B1 | 1400 | | CCR5 | FC2, 4b |
| | 46 | | 435 | | CCR5 | FC2, 4b |
| | 52 | | 1290 | | CCR5 | FC1, 2, 4b |
| | 67 | B1 | 1563 | | CCR5 | FC2, 4b |

[a]Clinical CDC stage is defined according to the Center for Disease Control [25]: N = asymptomatic; A = mildly symptomatic; B = moderately symptomatic; C = severely symptomatic. 1, 2 and 3 define low to severe immunodeficiency according to age.
[b]One or a combination of two NRTIs (nucleoside reverse transcriptase inhibitors) were used as mono/dual therapy, and two NRTIs and one PI (protease inhibitor) for triple therapy. In parenthesis is indicated the age at which therapy was started if different from that of the sampling time.
[c]CCR5/CXCR4 chimeric receptors: FC-2, N-terminal and first transmembrane region of CCR5 exchanged for corresponding regions of CXCR4; FC-4, as FC-2 and first extracellular loop exchanged.
*(D) means deceased.
**When the $CD4^+$T-cell count was not available at the age of bleeding, the closest possible value was used instead.
***Child 201 died at 9 months of age due to congenital CMV infection.

predominantly with non-B subtype viruses as compared with only B-subtype infection in our cohort. Therefore, the evolution to broad R5 phenotype may be even more relevant in nonsubtype B infection, and would need further investigation.

In our pediatric cohort viral sensitivity to inhibition with the chemokine RANTES was higher for viruses with R5narrow phenotype and for those, which later would undergo a switch to CXCR4-usage. Obviously, in the presence of RANTES, the reduced sensitivity to RANTES inhibition could be an advantage for the virus. Resistance to RANTES may be achieved in two ways, either by using CCR5 in a different mode than the natural ligand or by switch to CXCR4 use. It is tempting to speculate that RANTES is driving viral evolution in

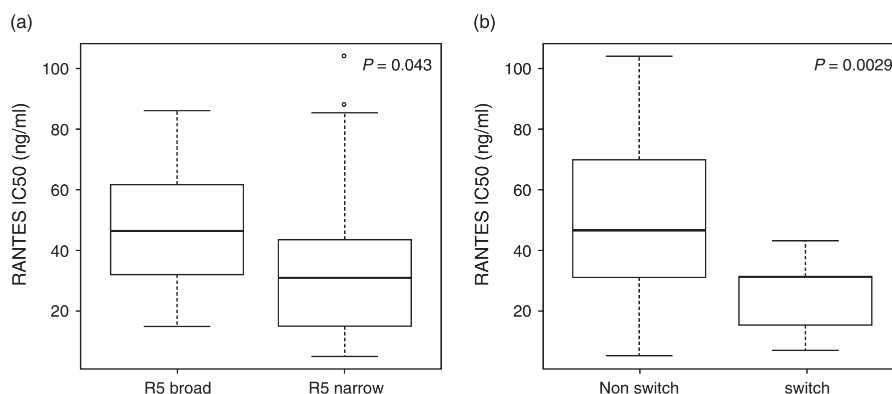


**Fig. 1. Sensitivity to RANTES of a) R5narrow and R5broad HIV-1 isolates from 13 children; b) R5 HIV-1 isolates from children whose virus evolved (switch) or not (nonswitch) to R5X4/X4 phenotype.** RANTES sensitivity is expressed as inhibitory concentration 50 (IC50). PHA-activated PBMCs derived from HIV-seronegative blood donors were infected with viral supernatant in the presence or absence of five steps of two-fold dilutions of RANTES starting from a concentration of 250 ng/ml, as previously described [9].

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these two directions. Whenever CXCR4-using viruses are present, they will be selected; if not present, R5 viruses with flexible use of CCR5 and RANTES resistant phenotype will have an advantage.

R5 isolates during advanced disease were repeatedly described to be resistant to chemokines or their analog [20,21,27], supporting the notion of a continuous evolution of the viral properties. Within the infected individual, virus variants may evolve towards a more efficient CCR5 usage and improved binding properties, possibly due to selection pressure exerted by the presence of CC-chemokines abundant in HIV-1-infected persons [14,28], competition for available target cells or the density of the coreceptor expression on the target cells. Accordingly, R5broad viruses may be considered escape variants as much as R5X4 viruses. Our results support the assumption that HIV-1 evolution is not restricted to a switch in coreceptor usage, but may include also alterations in the mode of use of the CCR5 receptor.

Indeed, it is recognized that binding of the viral envelope to its chemokine receptor, both CCR5 and CXCR4, activate multiple intracellular signaling cascades that modulate several cellular functions and affect viral infectivity and pathogenesis of infection (for a review see [29]). It remains to be understood if the higher infectivity of R5broad viruses may be a reflection of a different signaling induced in comparison to that of the R5narrow viruses. This differential intracellular signaling may in turn also contribute to the establishment of the immunodeficiency.

Others and we have previously shown that late R5 virus variants displayed a reduced sensitivity to entry inhibitors, such as TAK-779 and T-20, and a broadened ability to use CCR5/CXCR4 chimeric receptors compared with early R5 isolates [13,21,30]. It can be envisaged that a similar trend of increased flexibility of CCR5 use paralleled by augmented resistance to entry inhibitors is common in infants as in adults. If R5broad viruses show different susceptibility to small-molecule CCR5 inhibitors, such as maraviroc or others, compared with R5narrow viruses has still to be investigated. This notion is particularly important taking in consideration our results that R5broad viruses can be detected already in newborn babies. Attention should be paid to variation of R5 phenotype, as clinical trials introducing small CCR5 inhibitors for the treatment of pediatric HIV-1 infections are forthcoming.

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M.C. performed virus isolation and expansion of a consistent number of samples from the children, as well as the testing on the U87 cell lines of the vast majority of samples. She contributed largely to the writing of the manuscript; I.K. performed testing with the U87 cell lines of some of the children isolates and contributed with her expertise on the U87 cell lines expressing chimeric receptors; C.R. performed the RANTES inhibition assays; A.P. provided the samples and clinical data of children; E.M.F. contributed to the writing of the manuscript; G.S. designed the experimental outline, supervised the execution of the project and contributed largely to the writing of manuscript. All authors have revised the manuscript.

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