

**Title:** Resistance to antibody neutralization in HIV-2 infection occurs in late stage disease and is associated with X4 tropism

**Running head:** Antibody neutralization and escape in HIV-2

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## ABSTRACT

**Objectives:** To characterize the nature and dynamics of the neutralizing antibody (NAb) response and escape in chronically HIV-2 infected patients.

**Methods:** Twenty-eight chronically infected adults were studied over a period of 1-4 years. The neutralizing activity of plasma IgG antibodies against autologous and heterologous primary isolates was analyzed using a standard assay in TZM-bl cells. Co-receptor usage was determined in GHOST cells. The sequence and predicted 3D-structure of the C2V3C3 Env region were determined for all isolates.

**Results:** Only 50% of the patients consistently produced IgG NAbs to autologous and contemporaneous virus isolates. In contrast, 96% of the patients produced IgG antibodies that neutralized at least two isolates of a panel of six heterologous R5 isolates. Breadth and potency of the neutralizing antibodies were positively associated with the number of CD4<sup>+</sup> T cells and with the titer and avidity of C2V3C3-specific binding IgG antibodies. X4 isolates were obtained only from late stage disease patients and were fully resistant to neutralization. The V3 loop of X4 viruses was longer, had a higher net charge and differed markedly in secondary structure compared to R5 viruses.

**Conclusions:** Most HIV-2 patients infected with R5 isolates produce C2V3C3-specific neutralizing antibodies whose potency and breadth decreases as the disease progresses. Resistance to antibody neutralization occurs in late stage disease and is usually associated with X4 viral tropism and major changes in V3 sequence and conformation. Our studies support a model of HIV-2 pathogenesis in which the neutralizing antibodies play a central role and have clear implications for the vaccine field.

**Key words:** HIV-2 infection; neutralizing antibodies and disease progression; X4 tropism and resistance to neutralizing antibodies; C2V3C3 neutralizing domains.

## Introduction

In contrast to HIV-1 infected patients, the majority of HIV-2-infected individuals have low or absent viremia, reduced general immune activation, and generally exhibit a much slower rate of CD4 decline and disease progression [1-7]. Hence, HIV-2 infection is viewed as an example of how the human immune system can control HIV replication and disease progression. The control of virus replication in HIV-2 patients might be related to a more effective and preserved antiviral innate, cellular and humoral immune responses [8-10]. Unlike HIV-1, most chronically infected HIV-2 patients elicit the production of potent and broadly neutralizing antibodies suggesting that these antibodies might directly contribute to suppress virus replication and control disease progression [11-16]. However, the neutralizing antibody response has still not been documented in disease progressing patients with low CD4<sup>+</sup> T cell counts and X4 isolates. Data is also lacking on the longitudinal dynamics of the neutralizing antibody response within a single individual, on the nature and sequence of the neutralizing epitopes and on HIV-2 resistance to antibody neutralization. This information is crucial for vaccine production and to understand the impact of neutralizing antibodies in viral evolution and pathogenesis.

The antibody specificities that mediate HIV-2 neutralization *in vivo* are still elusive. Using different methods, the V3 region in the envelope gp125 has been identified as a potent neutralizing domain by some investigators [12, 13, 17-21]. Other weakly neutralizing epitopes were identified in V1, V2, V4 and C5 regions as well as in the CD4-binding site in gp125 and in the COOH-terminal region of the gp41 ectodomain [12, 13, 18, 19, 22]. Recently we found that mice immunized with a C2V3C3 recombinant polypeptide from a HIV-2 CCR5-using isolate produce potent neutralizing antibodies against R5 viruses but not against X4 viruses[23]. This suggested that the

C2V3C3 region comprises a potent neutralizing domain that is differently presented in the envelope complex of X4 and R5 viruses and established a new association between HIV-2 tropism and susceptibility to antibody neutralization. Whether this neutralizing domain is formed in natural HIV-2 infection and whether susceptibility to antibody neutralization in HIV-2 patients is related with virus tropism is still not known.

The aims of this study were to characterize the evolution and dynamics of the NAb response (autologous and heterologous) and escape in chronically HIV-2 infected patient and identify neutralizing determinants in the envelope glycoproteins.

## **Material and Methods**

### **Study population.**

Twenty eight HIV-2 patients attending different hospitals in Lisbon were analyzed in this study. The characteristics of the patients are shown in Table 1. Samples were collected 1-4 times per year over a four year period. HIV-2 viremia in the plasma was quantified with a quantitative-competitive reverse transcriptase-PCR assay as described elsewhere [24]. Ethical approval was obtained from Hospital Curry Cabral Ethics Committee ethics committee and each participant gave written informed consent before entry into the study.

### **Purification and quantification of IgGs**

The serum samples were diluted (1:1 ratio) in 500 mM NaCl (binding buffer) and mixed with equal volume (200 µl) of protein G Sepharose<sup>Tm</sup> 4 Fast Flow (GE Healthcare Europe). The beads were washed three times with binding buffer and one time with phosphate buffered saline (PBS) and the antibodies were eluted with 200 µl of 100 mM glycine-HCl elution buffer (pH 2.7) for 30 s. The beads were then centrifuged for 30 s

and the acid-eluted solution containing IgG was quickly removed and placed into a separate tube containing 1 M Tris (pH 9.0) buffer to reach pH 7.0 to 7.4. IgG nephelometry Turbox kits (Orion Diagnostica's Turbox plus, Finland) were used to evaluate the concentrations of IgG fractions, following the manufacturer's instructions.

### **Virus isolation and co-receptor usage**

Virus were isolated by co-culture of patient PBMCs with PBMCs from uninfected individuals as described elsewhere [25]. Co-receptor usage of the HIV-2 isolates was determined in GHOST cells expressing CD4 and CCR1, CCR2b, CCR3, CCR4, CCR5, CXCR4, Bonzo/STRL33 or BOB/GPR15. Virus replication in these cells was determined with an in-house enzyme-linked immunosorbent assay (ELISA) for detection of HIV-2/SIV antigen [26]. CCR5 and CXCR4 usage were confirmed by entry inhibition assays using the CCR5 antagonists Maraviroc and TK779 and the CXCR4 antagonist AMD3100 as described elsewhere [27].

### **Neutralization assay**

The neutralizing activity of IgG antibodies against HIV-2 primary isolates was analyzed in quadruplicate using a luciferase reporter gene assay in TZM-bl cells as reported previously [23, 28, 29]. Briefly, the eluted IgG samples (concentrations tested ranged from 0.05 to 100 µg/ml) were mixed with 2 to 15 ng of HIV-2 isolates (autologous and heterologous). After 1 h incubation at 37°C the IgG-virus mixture was added to the cells. Forty-eight hours later, cells were lysed directly in the culture plate and 100 µl of ONE-Glo luciferase assay substrate reagent (Promega, Madison, USA) was added. Plates were immediately analyzed for luciferase activity on a luminometer. Neutralizing activity was displayed as the percent inhibition of viral infection (luciferase activity) at

each antibody concentration compared to an antibody-negative control: percent inhibition =  $[1 - (\text{luciferase with antibody} / \text{luciferase without antibody})] \times 100$ . Fifty percent inhibitory concentrations ( $IC_{50}$ ) were calculated through a dose-response curve fit with nonlinear function (four-parameter logistic equations) using GraphPad Prism version 5.1 software (San Diego, CA).

### **Homology modelling**

Protein structure coordinates of C2V3C3 patient sequences were produced with SWISS-MODEL homology modelling server [30-32] in automated mode using as template the structure of an unliganded and fully-glycosylated SIV gp120 envelope glycoprotein (PDB file 2BF1) [33]. Secondary structure was assigned with DSSP [34, 35] and imported to the correspondent loaded structure within Pymol (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC, <http://www.pymol.org/pymol>.) using in house built scripts, after which 3D- images of the superimposed closely related models were produced.

### **Statistical analyses**

Statistical analyses were performed using GraphPad Prism version 5.01 (San Diego, CA) with a level of significance of 5%. The Mann Whitney U was used to compare means between variables. Contingency tables were analyzed with Fisher's exact test. To study how two variables varied together, linear regression was performed and Spearman correlation coefficients were computed.

## Results

### Plasma viremia is associated with lower CD4<sup>+</sup> T cell counts and X4 tropism

Twenty eight chronically HIV-2 infected patients were studied between 2003 and 2006. Nineteen patients were on antiretroviral therapy with protease and/or reverse transcriptase inhibitors (Table 1). Viral load was undetectable (<200 HIV-2 RNA copies  $\mu\text{l}^{-1}$ ) throughout the study period in 24 patients; median viral load in the remaining 4 patients that had a positive viral load on at least one occasion was 5,525 copies (range, 484-160,559) (Table 1). Median number of CD4<sup>+</sup> T cells at study entry was 363 cells  $\mu\text{l}^{-1}$  (range, 15-1523). The median number of CD4<sup>+</sup> T cells was significantly higher in aviremic patients compared with viremic patients (440 cells  $\mu\text{l}^{-1}$ , range 66-1,523 vs 63 cells  $\mu\text{l}^{-1}$ , range 15-342;  $P = 0.0127$ ).

Twenty-five new primary HIV-2 isolates were obtained from twelve patients. Eight patients harbored viruses that preferentially or exclusively used CCR5 whereas isolates from the remaining 4 patients preferentially or exclusively used CXCR4 (Table 1). Out of ten X4 isolates, three isolates from three different patients were able to use  $\geq 5$  co-receptors whereas R5 isolates could use only one or two additional co-receptors (Table 1). Patients infected with X4 isolates tended to have lower median number of CD4<sup>+</sup> T cell counts than patients infected with R5 isolates but this did not reach statistical significance (median number of CD4<sup>+</sup> T cells in patients harboring X4 isolates, 78 cells  $\mu\text{l}^{-1}$ ; range, 15-342 vs 270 cells  $\mu\text{l}^{-1}$ ; range, 43-615;  $P = 0.0868$ ). Strikingly, however, all patients infected with X4 isolates had detectable viral load on at least one occasion whereas patients infected with R5 isolates were aviremic. These results revealed a significant association between plasma viremia and X4 tropism ( $P = 0.002$ ).

### Antibody neutralization is related with disease stage and viral tropism



Autologous neutralization, employing IgG samples, was investigated in twelve HIV-2 patients. Autologous NAb activity was found in six (50%) patients infected with R5 isolates (Figure 1A). The autologous NAb activity was sustained over time (up to 4 years in patient 20) in three patients that were followed longitudinally suggesting that their R5 isolates were unable to escape neutralization. Most patients produced NAbs with  $IC_{50}$  in the micromolar range (median  $IC_{50}$  = 3.91  $\mu$ g/ml; range = 0.05-38.0  $\mu$ g/ml). Patient 18 was the exception with  $IC_{50}$  in the nanomolar range (50 ng/ml) (Figure 1B). Of note, we found no autologous neutralizing activity in the four patients infected with X4 isolates (patients 10, 19, 27 and 28) as well as in two patients infected with R5 isolates (patients 1 and 7) (Figure 1A).

The neutralizing activity of plasma IgGs from 28 patient samples (one time point per patient, year 2003) was tested against 11 heterologous primary isolates, three X4 and eight R5. All X4 isolates (PT19-03, PT27-03 and PT28-03) and two R5 isolates (PT01-03 and PT07-03) resisted neutralization with up to 100 $\mu$ g/ml of purified IgG. These isolates were named X4-resistant (X4-R) and R5-resistant (R5-R). With the exception of patient 28 that did not neutralize any of the isolates, all patients produced IgG antibodies that neutralized at least two out of six R5-sensitive isolates (R5-S) (Table 2). In total, of the 164 IgG/R5-S virus combinations only 26 (16%) were negative for neutralization. Remarkably, the majority of the patients (18/27; 68%) could neutralize all R5-S isolates. NAb  $IC_{50}$  range was 0.06-49.08  $\mu$ g/ml indicating wide variability in the neutralizing response between patients and that some patients elicit very potent heterologous NAb responses (Table 2). A strong negative correlation was found between the breadth of response and median  $IC_{50}$  (Spearman rank,  $r = -0.7209$ ;  $P < 0.0001$ ) (Figure 2A) indicating that the most potent neutralizing antibodies are also those with higher breadth. Importantly, the number of  $CD4^+$  T cells was negatively

associated with median  $IC_{50}$  (Spearman rank,  $r = -0.155$ ;  $P = 0.0279$ ) and positively associated with breadth of neutralizing response (proportion of viruses neutralized) (Spearman rank,  $r = 0.5249$ ;  $P = 0.0041$ ) (Figures 2B and C). Thus, a sustained and potent autologous and heterologous NAb response targeting exclusively R5 isolates is found in most chronically infected HIV-2 patients. The breadth and potency of neutralizing antibodies decrease as HIV-2 disease progresses and X4 viruses emerging in late stage disease are fully resistant to antibody neutralization.

### **The NAb response in HIV-2 patients is directed to the C2V3C3 envelope region**

Previously, we have shown that most of our patients produce high titers of IgG antibodies that bind to polypeptides comprising the C2V3C3 region in envelope gp125 (rpC2-C3 polypeptide) and the gp36 ectodomain (rgp36 polypeptide) [36]. To search for the envelope determinants of neutralization we have analyzed the association between the neutralizing and binding antibodies. Remarkably, a significant inverse association was found between the median  $IC_{50}$  of heterologous IgG NAbs and the titer (Spearman  $r = -0.4729$ ,  $P = 0.0262$ ) and avidity (Spearman  $r = -0.6136$ ,  $P = 0.0024$ ) of IgG antibodies binding to the rpC2-C3 polypeptide (Figure 3A). This association was not found for rgp36 binding antibodies (NAb  $IC_{50}$  vs IgG binding titer, Spearman  $r = -0.3968$ ,  $P = 0.0675$ ; NAb  $IC_{50}$  vs IgG avidity, Spearman  $r = -0.3199$ ,  $P = 0.1466$ ) (Figure 3B). The results suggest that the IgG NAbs in HIV-2 patients target the C2V3C3 envelope region.

### **V3 correlates of X4 tropism and neutralization resistance.**

To investigate the interplay between the NAb response and the evolution of virus isolates in each individual and to try to gain some insight into the molecular basis of

neutralization sensitivity and escape in HIV-2, the consensus C3V3C3 amino acid sequences of the patient isolates were aligned with the reference HIV-2ALI sequence (an R5 isolate) (Supplemental Digital Content 1). Clonal nucleotide sequences of these isolates were previously published in Borrego et al. [37]. The most interesting amino acid changes possibly associated with neutralizing susceptibility were found in the V3 loop. NAb sensitive R5 isolates (R5-S) had a median V3 loop net charge of 7 (range, 6-7) which was lower than the V3 loop charge of NAb resistant isolates (R5-R and X4-R) (median 9, range 8-11) (Supplemental Digital Content 2). In addition, all X4-R isolates had a 1-3 amino acid insertion at the tip of the V3 loop.

To explore further the determinants of tropism and neutralization susceptibility in HIV-2, the 3-D structure of C2V3C3 amino acid sequences from R5 and X4 isolates were determined by homology modelling using the structure of an unliganded SIV gp120 envelope glycoprotein as template [33]. The V3 loops of X4-R isolates fit in two major structural motifs, a  $\beta$ - $\alpha$ - $\beta$  motif with higher  $\beta$ -sheet content present in patients 10, 27 and 28 and a helix-loop-helix motif with higher  $\alpha$ -helix content present in patient 19 (Supplemental Digital Contents 3 and 4). The V3 loops of R5-S isolates fit into two main motifs that were markedly different from those of X4-R isolates. One structural pattern present in patients 2, 18 and 20 was characterized by absence or low amounts of regular secondary structural elements; the other was characterized by a high percentage of extended  $\beta$ -strands (patients 6, 11 and 16) (Supplemental Digital Contents 4 and 5). However, V3 loop structures from neutralization resistant R5 isolates (patients 1 and 7) were similar to one of the structures found in R5-S isolates Supplemental Digital Contents 4 and 6).

## Discussion

In this study the autologous and heterologous neutralizing IgG antibody responses were investigated in 28 HIV-2 infected patients from Portugal followed for a period of 1-4 years. Patients covered the full spectrum of disease, a significant proportion being severely immune compromised [19 (68%) patients had lower than 500 CD4<sup>+</sup>T cells  $\mu\text{l}^{-1}$ ; eight (29%) had lower than 200 CD4<sup>+</sup> T cells  $\mu\text{l}^{-1}$ ]. While, as expected, most patients (8/12, 67%) harbored viruses that preferentially used CCR5, four (33%) patients harbored X4 isolates and plasma viral load was detectable only in these patients. Viremic patients with X4 viruses had a significantly lower number of CD4<sup>+</sup> T cells compared with aviremic patients providing further support for the association between viral X4 tropism and HIV-2 disease progression [16, 38].

Similar to other studies, most (68%) patients elicited potent neutralizing antibodies against the majority of R5 isolates (neutralization sensitive R5 isolates termed R5-S) [11-14]. This is a significantly higher proportion compared to chronic HIV-1 patients in which only 10-31% make such broad NAb [39-42]. As shown here, the potent and broad neutralizing activity in the plasma of HIV-2 patients is essentially due to the IgG antibody fraction. Patients with the most potent neutralizing IgGs were also those with higher neutralizing breadth as found previously in HIV-1 subtype B infected individuals [43] and HIV-2 patients from Guinea Bissau [11]. Importantly, breadth and potency of the neutralizing antibodies were positively associated with the number of CD4<sup>+</sup> T cells. Hence, CD4<sup>+</sup> T cell depletion and disease progression leads to a significant decrease in neutralizing activity in HIV-2 infected patients. This may be due to a selective decrease in NAb production or maturation associated with the expansion of regulatory CD4<sup>+</sup> T cells [44] which negatively regulate B cell function [45-47].

Autologous neutralizing IgG antibody responses were found in half of the patients that were tested and this response was sustained over the years in some patients. Of note, one patient produced very potent autologous NAb that inhibited viral replication at nanogram concentration (patient 18,  $IC_{50}$ , 50 ng/ml). The results confirm that some chronic HIV-2 infected patients can elicit autologous neutralizing antibodies [13, 15, 16] and demonstrate that this neutralizing activity is due to the IgG antibody fraction and can be very potent and sustained over the course of infection. This is in contrast to HIV-1 infection where autologous NAb are undetectable in most chronically infected patients because the virus easily escapes neutralization by these antibodies during acute infection [40, 48-50]. Remarkably, however, a significant proportion (50%) of our patients could not produce neutralizing antibodies targeting their autologous and contemporaneous virus isolates. This was neither due to an intrinsic immune defect nor to low levels of antibody production since with one exception (patient 28) all patients elicited neutralizing antibodies against heterologous virus isolates. Rather, the results are consistent with effective virus escape from autologous antibody neutralization which has not been detected before in HIV-2 infected patients. Importantly, most (4 of 6, 67%) of the patients with evidence of resistance to autologous antibody neutralization were infected with CXCR4-using isolates. These isolates were also fully resistant to neutralization by heterologous IgGs. Thus, neutralization- resistance in HIV-2 isolates is strongly associated with X4 virus tropism. Assuming that these 4 patients were originally infected with R5 isolates, our results suggest that the selective pressure exerted by the neutralizing antibodies in HIV-2 patients' favors the selection of neutralization resistant X4 isolates. This is in contrast to HIV-1 where X4 viruses are usually easier to neutralize than R5 viruses [51-53] and escape from antibody neutralization has only rarely been associated with changes in viral tropism [53-56]. The

association between X4 tropism and resistance to antibody neutralization hasn't been detected previously because late stage disease HIV-2 patients with low CD4<sup>+</sup> T cell counts and X4 viruses have not been enrolled in previous studies. It has however been observed in mice immunized with envelope antigens derived from an R5 HIV-2 isolate [23]. These mice were unable to elicit antibodies against X4 isolates. Our studies support a model of HIV-2 pathogenesis in which a significant decrease in the number of CD4<sup>+</sup> T cells and selective expansion of regulatory T cells occurring after many years of infection leads to the production of less potent neutralizing antibodies which favors the emergence of neutralization escape mutants able to use CXCR4 and other alternative co-receptors and infect other cell types. These X4 viruses replicate faster and are more pathogenic and promote faster disease progression [16, 57].

To try to identify the determinants of antibody neutralization in the HIV-2 envelope, we have first analyzed the nature of the association between the patient's neutralizing antibodies and antibodies binding to recombinant polypeptides comprising the C2, V3 and C3 regions (rpC2-C3) or the gp36 ectodomain (rgp36). Remarkably, a significant direct association was found between the potency of heterologous IgG NAb and the titer and avidity of C2V3C3-specific binding IgG antibodies. These results suggest that the neutralizing IgG antibodies in HIV-2 patients are mostly directed to the C2V3C3 region in gp125. It remains unclear whether C2V3C3-specific antibodies actually neutralize HIV-2 or simply represent a marker for other Env-specific neutralizing antibodies such as those that target V1, V2, V4, C5 and CD4-binding site in gp125 [12, 13, 18, 19, 22]. However, the observation that mice immunized with the C2V3C3 polypeptide elicit a potent and broadly neutralizing antibody response supports the former hypothesis that the C2V3C3 region is a potent neutralizing domain in HIV-2 [23].

To further investigate the molecular and structural determinants of neutralization sensitivity and resistance in the C2, V3 and C3 regions of HIV-2 we analyzed an extensive number of clonal sequences of the C2V3C3 region obtained from our patients. Neutralization resistant isolates (X4 isolates and two R5 isolates) had a higher charged V3 loop compared to neutralization sensitive R5 isolates. In addition, X4 isolates had a larger V3 loop due to a 1-3 amino acid insertion. The results confirm that the charge and size of the V3 loop are crucial determinants of CCR5 and CXCR4 use by HIV-2 [58]. More importantly, they suggest that resistance to antibody neutralization in X4 isolates is also determined by the charge and size of the V3 loop. Early studies have shown that amino acids FHSQ (V3 loop positions 315-318) and WCR (positions 329-331) in the HIV-2 V3 stem interact with one another to form a conformational neutralizing epitope [18, 19]. The 1-3 amino acid insertions found in the X4-R isolates were placed immediately after the FHSQ motif and may prevent the proper assembly of this epitope thereby conferring resistance to the V3-directed neutralizing antibodies.

Alternative V3 conformations that may be responsible for the selective interactions with CCR5 or CXCR4 have been identified in HIV-1 [59]. HIV-1 escape from antibody neutralization is usually not related with cell tropism because the neutralizing antibodies that target the V3 region are able to bind to both V3  $\beta$  hairpin conformations [59, 60]. We showed that the V3 loop structures of X4-R and R5-S HIV-2 primary isolates are markedly different which supports a direct role of V3 loop conformation in the different susceptibility of these viruses to antibody neutralization. Hence, in contrast to HIV-1, R5 to X4 transition in HIV-2 primary isolates seems to involve a significant change in V3 loop charge, size and conformation which might prevent efficient binding of the neutralizing antibodies that target this region.

In conclusion, most HIV-2 patients infected with R5 isolates produce C2V3C3-specific neutralizing antibodies whose potency and breadth decreases as the disease progresses. Resistance to antibody neutralization occurs in late stage disease and is associated with X4 viral tropism and major changes in V3 loop sequence and conformation. Our studies support a new model of HIV-2 pathogenesis in which the neutralizing antibodies play a central role and have clear implications for the vaccine field.

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## Figure legends

**Figure 1-** Autologous IgG neutralizing activity in HIV-2 patients. A) Neutralizing activity, expressed in inhibition of viral infectivity, is shown against autologous and contemporaneous R5 [gray bars] or X4 isolates [pink bars] from each patient. An excess of IgG was used in this experiment (100 µg/ml). B) The median IC<sub>50</sub> (in µg/ml) was determined for all IgG/virus pairs that gave a positive neutralization result as shown in panel A.

**Figure 2-** Relationship between CD4<sup>+</sup> T cell counts and potency and breadth of neutralizing antibodies (IgG fraction) in HIV-2 patients. A) Association between potency and breadth of heterologous neutralizing antibodies; B) Association between the number of CD4<sup>+</sup> T cells and potency of neutralizing antibodies; C) Association between the number of CD4<sup>+</sup> T cells and breadth of neutralizing antibodies. P and r values refer to the Spearman rank correlation test. A linear regression line is also shown.

**Figure 3-** Relationship between neutralizing and binding antibody reactivity and avidity in HIV-2 infection. The potency (median IC<sub>50</sub>) of heterologous neutralizing antibodies (IgG fraction) was plotted as a function of binding IgG titer (black circles) and avidity (red circles) against polypeptides rpC2-C3 (panel A) and rgp36 (panel B). A linear regression line is shown.

## **List of Supplemental Digital Content**

**Supplemental Digital Content 1.pdf** - Co-receptor usage and amino acid sequence of C2, V3 and C3 domains of sequential isolates from HIV-2 infected patients.

**Supplemental Digital Content 2.doc**- Susceptibility to antibody neutralization, co-receptor usage and V3 loop charge and size of viruses analyzed in this study.

**Supplemental Digital Content 3.pdf** - 3D-structures of C2V3C3 envelope region from X4 isolates.

**Supplemental Digital Content 4.doc**- Percentage of the different elements of secondary structure present in the V3 loop of HIV-2 isolates.

**Supplemental Digital Content 5.pdf** - 3D-structures of C2V3C3 envelope region from neutralization-sensitive R5 isolates.

**Supplemental Digital Content 6.pdf** - 3D-structures of C2V3C3 envelope region from neutralization-resistant R5 isolates.

Figure 1

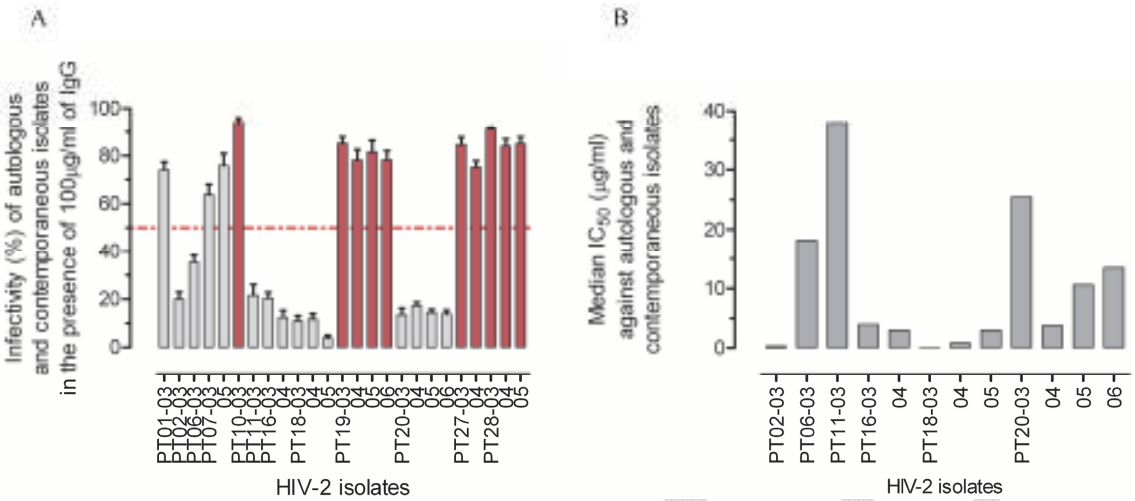
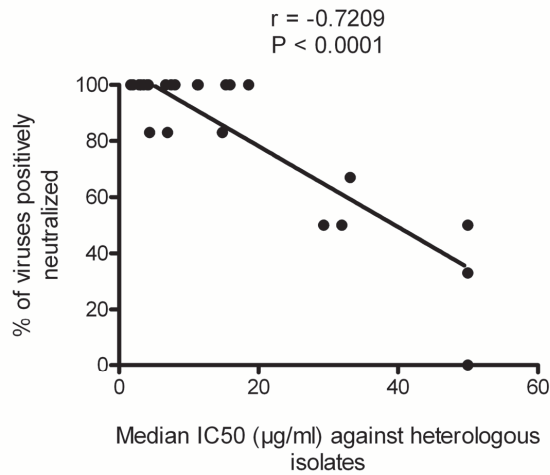
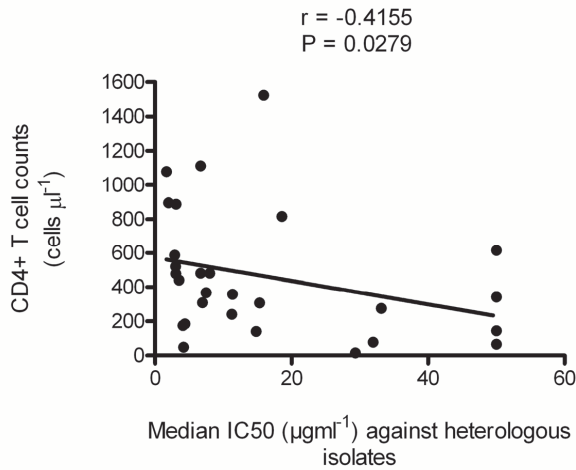


Figure 2

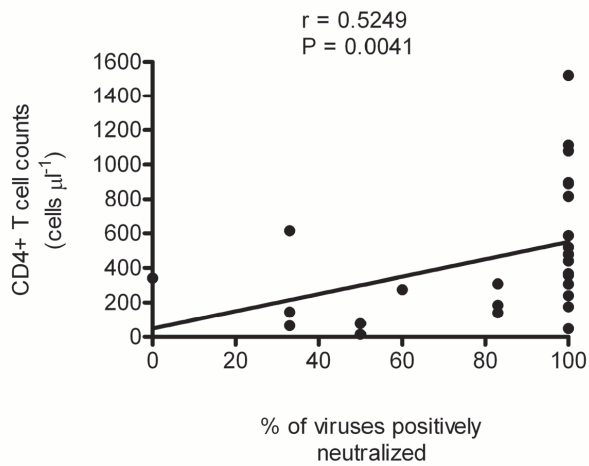
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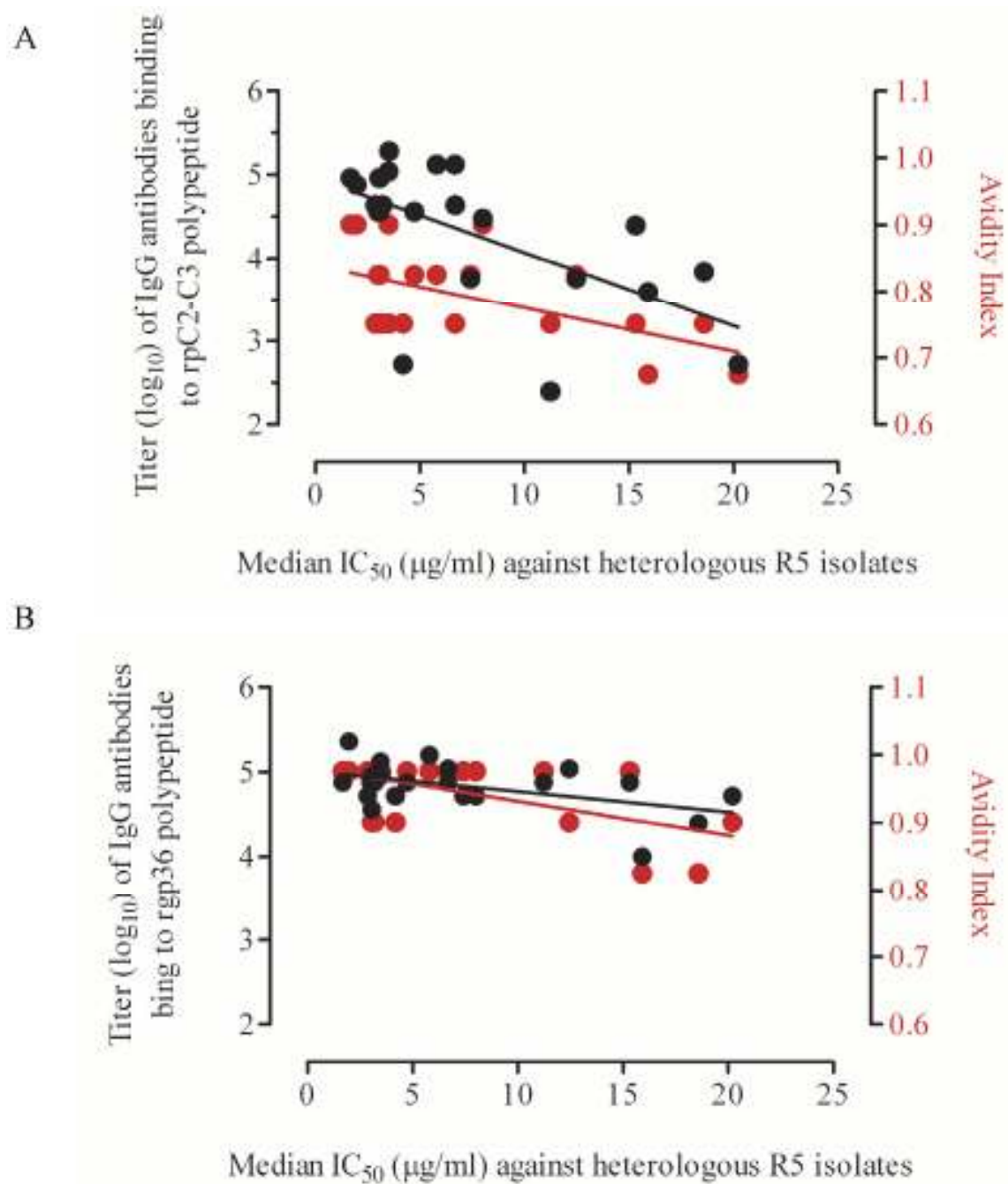
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**Figure 3**



**Table 1-** Characteristics of HIV-2-infected patients and virus isolates.

<b>Patient</b>	<b>Sampling year</b>	<b>CD4<sup>+</sup> T cells per <math>\mu</math>l</b>	<b>HIV-2 RNA copies per ml of plasma</b>	<b>Antiretroviral therapy</b>	<b>Major chemokine coreceptor usage (minor)<sup>†</sup></b>
1	2003	308	<200	ddI, d4T, IDV	R5
2	2003	358	<200	AZT, 3TC,NFV	R5
3	2003	307	<200	AZT, 3TC, IDV	na
4	2003	240	<200	3TC, d4T, IDV	R5
5	2003	480	<200	AZT, ddC	R5
6	2003	615	<200	AZT, 3TC, IDV	R5 (Bob, Bonzo)
7	2003	144	<200	3TC, d4T, NFV	R5 (R1)
	2005	43	<200	3TC, d4T, NFV	R5 (Bonzo)
8	2003	141	<200	AZT, 3TC, NFV	na
9	2003	226	<200	Combivir (3TC/AZT)	na
10	2003	48	160,559	ddI, AZT,SQV	X4 (R5, R1, R2b, R3, R4, Bob, Bonzo)
11	2003	66	<200	Drug naïve	R5 (R1, Bob)



12	2003	851	<200	Drug naïve	na
13	2003	184	<200	AZT, 3TC, IDV	R5
14	2003	521	<200	Drug naïve	na
15	2003	897	<200	Drug naïve	na
16	2003	367	<200	Atripla (FTC/TDF/EFV)	R5 (Bob, Bonzo)
	2004	270	<200	Atripla (FTC/TDF/EFV)	R5 (Bob, Bonzo)
17	2003	480	<200	AZT, 3TC	na
18	2003	437	<200	d4T, 3TC, LPV	R5 (Bob, Bonzo)
	2004	400	<200	d4T, 3TC, LPV	R5 (Bob, Bonzo)
	2005	60	<200	d4T, 3TC, LPV	R5 (Bob, Bonzo)
19	2003	78	<200	TDF, ABC, LPV	X4
	2004	73	5,246	TDF, ABC, LPV	X4 (R2b)
	2005	85	<200	TDF, ABC, LPV	X4 (R1)
	2006	76	ND	TDF, ABC, LPV	X4 (Bonzo)
20	2003	275	<200	AZT, 3TC, ddI	R5 (Bob, Bonzo)
	2004	65	<200	AZT, 3TC, NFV	R5 (Bob, Bonzo)

	2005	122	<200	AZT, 3TC, NFV	R5 (Bonzo)
	2006	172	ND	AZT, 3TC, NFV	R5 (Bob, Bonzo)
21	2003	1523	<200	Drug naïve	na
22	2003	440	<200	Drug naïve	na
23	2003	1112	<200	Drug naïve	na
24	2003	478	<200	d4T, 3TC	na
25	2003	587	<200	Drug naïve	na
26	2003	1079	<200	Drug naïve	na
27	2003	15	<200	Drug naïve	X4
	2004	ND	484	AZT, 3TC, NFV	X4 (R5, R1, R2b, R3, R4, Bob)
28	2003	342	5,804	AZT, 3TC, NFV	X4 (R1)
	2004	265	4,792	AZT, 3TC, NFV	X4 (R5, R1, R2b, R3)
	2005	212	ND	AZT, 3TC, NFV	X4 (R1)

†, Tropism was determined based on p26 antigen production in GHOST/CD4+ cells expressing each of the indicated co-receptors. CCR5 and CXCR4 usage were confirmed by an entry inhibition assay in TZM-bl cells using the CCR5 antagonists Maraviroc and TK779 and the CXCR4 antagonist AMD3100; na, not applicable (virus was not isolated); ND, not done.; AZT, Zidovudine; ddI, didanosine; ddC, Zalcitabine; d4T,

Stavudine, 3TC, Lamivudine; FTC, Emtricitabine; ABC, Abacavir; EFV, Efavirenz;  
NFV, Nelfinavir; SQV, Saquinavir; LPV, Lopinavir; IDV, Indinavir.

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**Table 2-** Potency of IgG neutralizing activity of HIV-2 patients on a panel of six heterologous primary R5 isolates.

Patient	Neutralizing activity on heterologous primary HIV-2 isolates [Inhibitory concentration (IC <sub>50</sub> ), µg/ml]					
	PT02.03	PT06.03	PT11.03	PT16.03	PT18.03	PT20.03
1	1.18	3.19	29.11	>50	1.41	10.72
2	-	25.89	11.35	18.81	1.01	3.46
3	2.13	41.73	28.31	27.63	2.21	2.99
4	1.43	22.8	18.62	23.26	0.33	3.86
5	0.65	14.18	11.9	20.68	0.18	4.1
6	1.84	-	>50	>50	0.15	>50
7	0.07	>50	>50	>50	0.75	>50
8	12.46	24.62	17.18	>50	0.08	4.16
9	10.85	32.13	26.32	45.28	0.46	8.75
10	0.67	20.7	7.35	5.87	1.18	2.5
11	2.68	42.04	-	>50	>50	>50
12	0.3	16.74	2.99	3.14	0.82	3.84
13	0.75	40.52	4.71	>50	0.13	4.03

14	1.96	31.19	4.56	2.08	0.18	3.93
15	2.17	24.81	1.78	47.58	0.2	1.22
16	19.03	7.49	4.09	-	2.14	8.96
17	4.14	44.43	9.2	11.44	0.23	1.68
18	9.61	16.86	1.9	2.87	-	4.08
19	0.39	13.88	>50	>50	>50	3.78
20	2.79	33.14	>50	>50	14.93	-
21	8.88	49.08	22	0.73	15.93	15.88
22	1.05	38.28	7.89	1.76	0.37	5.24
23	1.37	43.12	8.63	18.64	1.42	4.74
24	0.81	12.07	8.98	2.37	0.06	3.74
25	2.36	16.04	7.82	1.42	0.07	3.38
26	1.33	15.96	4.86	1.98	0.07	1.37
27	4.26	5.91	>50	>50	>50	8.7
28	>50	>50	>50	>50	>50	>50

Red,  $\leq 0.1 \mu\text{g/ml}$ ; orange,  $0.1$  to  $1 \mu\text{g/ml}$ ; yellow,  $1$  to  $10 \mu\text{g/ml}$ ;  
green,  $\geq 10 \mu\text{g/ml}$ ; and white, not neutralized at any concentration  
tested.

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