Evolution of Broadly Cross- Reactive HIV-1 Neutralizing Activity: Therapy Associated Decline, Positive Association with Detectable Viremia and Partial Restoration of B-cell Subpopulations


AIDS Research Unit, Institut d’Investigacions Biomediques August Pi i Sunyer, Barcelona, Spain. HIVACAT, Barcelona, Spain. IrsiCaixa AIDS Research Institute, Hospital Germans Trias i Pujol, Badalona, Barcelona, Spain. Infectious Diseases Unit, Hospital Clinic, Barcelona, Spain. Citometry and Cell Sorting Facility, CEK, IDIBAPS, Barcelona, Spain

Authorship note: Eloisa Yuste and Victor Sanchez-Merino contributed equally to this work.

Word count: Abstract (249 words), text (4107 words)

Running title: HIV Neutralization Evolution and B-cells

Key words: HIV-1; Neutralization breadth; Evolution; Viral loads; B-cells,

#Corresponding author contact information: Victor Sanchez-Merino,

vmsanchez@clinic.ub.es

JVI Accepts, published online ahead of print on 4 September 2013
Copyright © 2013, American Society for Microbiology. All Rights Reserved.
Little is known about the stability of HIV-1 cross-neutralizing responses. Taking into account that neutralization breadth has been positively associated to plasma viral load, there is no explanation for the presence of broadly neutralizing responses in a group of patients on treatment with undetectable viremia. In addition, the B-cell profile responsible for broadly cross-neutralizing responses is unknown. Here we study the evolution of neutralizing responses and the B-cell subpopulation distribution in a group of patients with broadly cross-reactive HIV-1 neutralizing activity. We studied neutralization breadth evolution in a group of six previously identified broadly cross-neutralizers and six control patients during a 6-year period with a previously described minipanel of recombinant viruses from five different subtypes. B-cell subpopulation distribution during the study was also determined by multiparametric flow-cytometry. Broadly cross-neutralizing activity was transient in four broadly cross-neutralizers and stable, up to 4.6 years, in the other two. In four out of five broadly cross-neutralizers who initiated treatment, a neutralization breadth loss occurred after viremia had been suppressed for as much as 4 to 20 months. B-cell subpopulation analyses revealed a significant increase in the frequency of naïve B cells in broadly cross-reactive samples, compared with samples with less neutralization breadth (increased from 44% to 62%). We also observed a significant decrease in tissue-like and activated memory B cells (decreased from 19% to 12% and from 17% to 9% respectively). Our data suggest that HIV-1 broadly cross-neutralizing activity is variable over time and associated with detectable viremia and partial B-cell restoration.
INTRODUCTION

Most successful vaccines induce neutralizing antibodies and their role in protective immunity is well established (1). Due to viral capacity to evade antibody recognition, an antibody-based HIV-1 vaccine will likely require the induction of broadly neutralizing antibodies (bNabs). Development of an effective HIV-1 vaccine is even more challenging considering that the virus has evolved several mechanisms to evade antibody mediated neutralization (1-4). Despite these mechanisms, many HIV infected individuals are able to generate neutralizing antibodies (NAbs). In addition, some chronically-infected patients are able to mount a strong cross-reactive neutralizing response with the ability to neutralize several HIV-1 isolates from different clades (5-8). The percentage of patients able to develop bNabs is low but higher than initially estimated. In some studies, sera from 10 to 25% of the patients displayed broadly neutralizing activity (5-9).

Antibody responses against viral envelope glycoproteins emerge during the first 2 weeks of HIV-1 infection. However, these antibodies are non-neutralizing and fail to inactivate the infecting virus (10, 11). Autologous neutralizing antibodies expand during the first months of infection (12) and cross-neutralizing antibody responses have been shown to emerge on average at 2.5 years after infection (13). The subsequent evolution of these responses in HIV-1 infected patients is not well understood. Neutralization breadth has been positively correlated with plasma viral load (5, 9, 13, 14). However, this correlation contrasts with our report in which broad neutralizing responses were detected in patients on combination antiretroviral therapy (cART), despite having undetectable viremia (15). A better understanding of how broadly cross-reactive
neutralizing activity (bCrNA) develops and evolves in infected patients may provide important
clues for vaccine design. To date, most of the studies analyzing the breadth of neutralizing
responses in HIV-1 infected patients have been cross-sectional. Only a few studies have carried
out a follow up of these responses and none of these studies included patients on cART (5, 12,
13, 16, 17).

The frequency and phenotype of different B-cell subpopulations in patients with bCrNA is
another aspect that remains poorly understood. Previous reports have shown that HIV-1 infection
leads directly or indirectly to several perturbations on most immune system cells, including B
lymphocytes. It has been hypothesized that ongoing HIV-1 replication produces B cell
abnormalities, such as increase in the production of IgG (hypergammaglobulinemia) (18, 19),
increase in polyclonal activation (20), increase in cell turnover (20, 21), increase in expression of
activation markers (22, 23), increase in the differentiation of B cells in plasmablasts (4, 25),
augmented B-cell autoreactivity (26) and increase in the frequency of B-cell malignancies and
imbalance of different B-cell subpopulations (27, 28). Many of these defects (i.e. imbalance of
B-cell subpopulations) appear to be partly reversed after 12 months of antiretroviral therapy (29).

In a cross-sectional study (15), we have previously screened 508 serum samples from 364
patients (173 treated and 191 untreated) for broadly cross-reactive neutralizing activity using a
strategy based on the use of recombinant viruses. In this study (15), we identified 12 patients that
were capable of neutralizing viruses across 5 subtypes (termed from now on broadly cross-
neutralizers or bCrN). We were also able to confirm the presence of broadly IgG-associated
cross-reactive neutralizing responses in a group of patients on antiretroviral treatment, despite
having undetectable viremia (15). In the present study, we evaluate the evolution of neutralization breadth in a group of 6 previously identified bCrN and 6 control patients for a 6-year period. Our data suggest that broadly cross-reactive HIV-1 neutralizing activity is variable over time, and associated with detectable viremia and partial restoration of B-cell subpopulations.

**MATERIALS AND METHODS**

**Study participants**

Serum samples were obtained from HIV-1 infected patients treated at Hospital Clinic (Barcelona, Spain). Medical visits were scheduled at approximately 6-month intervals or more often as necessary for appropriate clinical care. bCrN individuals were identified previously (15) and control patients were selected so that they showed no significant neutralization breadth in the previous cross-sectional study. Control patients were also matched for similar age (25 to 57), CD4+ (291 to 759) and CD8+ (576 to 1698) counts and plasma viral loads (1.7 to 4.94 logs) (Table1). All the individuals gave informed written consent and the study was reviewed and approved by the Institutional Ethical Committee Board of the Hospital Clinic (Barcelona, Spain).

**Immunoglobulin G (IgG) purification**

IgGs were isolated from sera using Protein G HP spin trap (GE healthcare, UK) and extensively dialyzed with Spectra/Por® Float-a-Lyzer® G2 50-kDa cut off membranes (Spectrum Laboratories Inc., USA) following manufacturer’s instructions. IgG quantification was determined by a mini-Bradford assay on a microplate spectrophotometer (Tecan Trading AG, Switzerland).
Neutralization assays

Purified IgGs were tested at a 0.2mg/ml concentration (corresponding to a dilution range of 1/40 to 1/80) against a minipanel of 6 recombinant viruses from different tropisms and 5 different subtypes using TZM-bl cells, as previously described (15). Serial IgG concentrations (0.3 to 0.001mg/ml) from patients 181, 308 and 528 were also tested against the same recombinant virus panel. The minipanel of recombinant viruses was previously generated by replacing the env sequence of HIV NL4-3 with env sequences from isolates of 5 different subtypes (clades and Tier categorization are given in parenthesis): VI191 (A, Tier 2), 92BR025 (C, Tier 1B), 92UG024 (D, Tier 2), CM244 (AE, Tier 2), and AC10 (B, Tier 2)([15]). NL4-3 strain (clade B, Tier 1A) was included in the minipanel as a neutralization-sensitive control and an amphotropic vesicular stomatitis virus (VSV) Env pseudotyped on an HIV-1 core as a specificity control. Virus stocks were produced by transfection of 293T cells using the calcium phosphate method according to the manufacturer’s recommendations (ProFection mammalian transfection system; Promega, Madison, WI, USA). VSV pseudotyped virus stocks were produced by cotransfecting 293T cells with pNL4-3ΔenvFL (30) and pVSV-G plasmids as explained above. Neutralization activity for all purified IgGs was measured in triplicate as a reduction in infectivity using a luciferase reporter gene assay after what is considered a single-round infection of TZM-bl cells. A serum sample was considered to be capable of neutralizing a virus when purified IgGs from the corresponding serum reduced viral infectivity by a minimum of 50% at a 0.2mg/ml concentration. We considered that a serum sample displayed bCrNA when
the corresponding purified IgGs were capable of neutralizing viruses across 4 or more subtypes, out of the minipanel described above, with no significant neutralization of the VSV control.

**Cell separation and flow-cytometric analysis of peripheral B cells**

Due to limited sample availability, we could only analyze 15 samples from 6 bCrN patients and 18 samples from 6 control patients before treatment (patients 181, 541 and 405: one sample each; patients 308, 488, 528, 108, 296 and 326: 3 samples each; patients 279, 363 and 423: four samples each and 5 samples from patient 363). We also analyzed frequencies of various B-cell subpopulations in 15 additional samples after initiating antiretroviral treatment from four bCrN (308 and 108: two samples each and 181 and 488: 3 samples each) and five controls (541, 279, 296, 326 and 405: one sample). Peripheral blood mononuclear cells (PBMC) were obtained by ficoll centrifugation (ACCUSPIN™ System-Histopaque®-1077, Sigma Diagnostics). PBMC were then aliquoted in 10 millions cells per vial and cryopreserved in liquid nitrogen until needed for flow cytometry. For flow-cytometric analysis PBMC were thawed and washed twice with phosphate-buffered saline (PBS) and then resuspended in RPMI medium supplemented with 10% fetal bovine serum for one hour at 37°C. Multiparametric surface staining was performed with the following reactive dye and antibodies: blue fluorescent reactive dye (live/dead stain, Invitrogen), anti-CD19 Percp Cy5.5, anti-CD3 Pacific Blue, anti-CD27 PE, anti-CD10-APC, anti-CD21 Fitc and anti-CD20 APC-H7 (all antibodies were purchased from BD, Beckton Dickinson). PBMC were incubated with antibodies at 4°C for one hour. Cells were then washed twice with 2 ml of PBS with bovine serum albumin (BSA 0.5%) and sodium azide 0.1%. After washing, cells were resuspended in 400µl FACS buffer containing 1% paraformaldehyde. Fluorescence-minus-one (FMO) and doublet exclusion controls were also used to delineate the populations of interest.
One to two million events were acquired on an LSRFortessa cell analyzer (BD, Beckton Dickinson) and data analyzed using FlowJo software (version 7.2.4; TreeStar Inc., USA). B-cell gating strategy is shown in supplementary Figure 1.

Statistics

Analyses were performed using GraphPad Prism 5 (GraphPad Software). Mann-Whitney U test were used for comparisons of continuous variables between groups. Simple comparisons were made with use of a two-sided alpha level of 0.05.
RESULTS

Neutralization breadth stability in bCrN and control patients

A group of six out of twelve bCrN patients previously identified (15), together with a six patient control group, were studied for a 6-year period. In the present study, time 0 corresponds to the last time point analyzed in the previous study, and a flow-chart indicating the study period for each patient is shown in Figure 1. The remaining six bCrN from the previous study were not included for sample availability reasons. Clinical characteristics of the six bCrN patients and controls at the beginning of the study, at time 0 and at the end of the study are described in the Materials and Methods section and shown in Table 1.

Detectable broadly cross-neutralizing responses, defined as neutralization of at least one virus included in the minipanel from at least four different subtypes, were observed in our cohort during the time of the study. Several groups have tested the neutralizing activity in a large group of samples on different virus panels and reported that screening for a reduced panel (6 viruses or less) of selected viruses provided similar information on the presence of cross-reactive neutralizing activity as screening for a large virus panel (31-34). The broadly neutralizing activity of the patients selected with our 6-virus panel was also confirmed previously with an extended panel including 25 additional viruses from different subtypes (15).

The stability of the bCrNA varied among patients. Patients 363, 488 and 541 showed neutralizing activity across 5 subtypes in only one of the time points analyzed (Figure 2). Especially significant were the cases of patients 181 and 308, who showed neutralizing activity
across 5 subtypes for 3.1 and 4.6-year periods respectively (Figure 2 and Supplementary Table 1). Patient 528 showed a significant cross-reactive neutralizing response (viruses from four or more subtypes neutralized) for a long period of time (5.2 years). However, the breadth of his antibody-mediated neutralizing response only reached 5-subtype neutralization intermittently (Figure 2 and Supplementary Table 1). In order to check for fluctuations in neutralization titers during the long periods of apparent neutralization stability, we made complete neutralization curves with samples corresponding to periods of more than three years of broad cross-reactive neutralizing responses (patients 181, 308 and 528). The IC50 values corresponding to these curves are shown in Table 2 and supplementary Figure 2. This new set of experiments showed that the corresponding neutralizing activity had some fluctuations in neutralization titers during the periods with broad cross-reactive neutralizing activity. However, the ability to neutralize viruses across 5 subtypes at an IgG concentration of 0.2mg/ml was not lost during these periods.

Only one patient in the control group (296) showed a transient bCrNA during the time of the study (neutralization across 4 subtypes at only one of the time points analyzed; Figure 3 and Supplementary Table 2). We found no significant direct correlation between stability of bCrNA during the study and nadir CD4+ T, nadir CD8+ T cell counts, peak viremina or time since first HIV positive serology (Figure 3 and Supplementary Table 2).

**Impact of viral load suppression in neutralization breadth**

Five out of six patients from both bCrN and control groups initiated combination antiretroviral therapy (cART) during the study reaching undetectable viremia (<50 copies/ml).
Patients 363 and 423 (a bCrN and control patient respectively) remained untreated (Figures 1, 2 and 3).

Within the five bCrN that received cART and reached undetectable viremia during the follow-up, four showed significant declines in the levels of neutralization breadth (neutralization of viruses from five to three or fewer subtypes), with a delay in loss of breadth after viremia suppression of as much as 4 to 20 months (patients 541 and 308 respectively). The IC50 values corresponding to the periods of broad cross-neutralizing activity in patient 308 (Table 2 and supplementary Figure 2) indicated that the loss in neutralizing activity initiated coinciding with treatment associated viral loss but the ability to neutralize some of the virus from the minipanel was not lost until 20 months after viremia became undetectable. The dynamic link between neutralization breadth loss and decay of viremia in these patients could not be determined because there were no samples corresponding to intermediate time points available. For patient 528, neutralization breadth loss was not detected after suppression of viremia within the time of the study. This could be explained by the fact that this patient only reached undetectable viremia in the last time point analyzed (12.2 months since cART engagement; Figure 2). We did not observe significant differences between neutralization breadth before and after treatment in the control group. Due to their low neutralization breadth, we would probably need an extended panel of viruses to detect neutralization breadth losses in control patients.

Next, we investigated the potential relationship between neutralization breadth and plasma viral load levels in our patients. A univariate model analysis showed that the overall neutralization breadth was similar for plasmas from patients with detectable and undetectable
viremia (Figure 4A). Considering that it has been previously reported that virus from the secondary lymphoid tissue reservoir is not cleared until 6 months of potent therapy (35), we compared the levels of neutralization breadth in plasmas from viremic patients and patients with less than 6 months with undetectable viremia with the group of plasmas from patients with at least six months of undetectable viremia, and found that the neutralization breadth was significantly higher for the first group ($P=0.0495$; Figure 3B). This analysis confirmed that plasma viral load had a significant impact on neutralization breadth even when cART patients are included in the study.

**B-cell subpopulations before and after initiation of cART**

In order to confirm the previously reported effect of ART on B cell phenotypic profile, we evaluated frequencies of various B-cell subpopulations in bCrN and control patients before and after initiation of cART. The average times on treatment for the samples from bCrN and control patients were similar (17.6 ± 12.5 and 16.9 ± 12.7 months respectively). Based on previous studies (27), CD19+ B cells in peripheral blood of HIV infected individuals, can be divided into the following 6 subpopulations listed in order of increasing level of differentiation: immature/transitional (CD10+/CD27⁻), naïve (CD10⁻/CD27⁺/CD21hi), tissue-like memory (CD10⁻/CD27⁻/CD21lo), resting/memory (CD10⁻/CD27⁺/CD21hi), and activated/memory (CD10⁻/CD27⁺/CD21lo) B cells as well as plasmablasts (CD10⁻/CD27⁺⁺/CD20⁻/CD21lo).

B cell subpopulations analysis revealed the following changes in mean frequencies as a result of initiation of cART: immature transitional B cells decreased from 6% to 3%; naïve B
cells increased from 48% to 64%; tissue-like memory B cells decreased from 18% to 11%; activated memory B cells decreased from 15% to 9% and plasmablasts decreased from 0.07% to 0.04% (Figure 5). We did not observe any changes in the proportion of resting memory B cells (13%). Decreases in immature, tissue-like memory and activated memory B cells and the increase in naïve B cells were statistically significant. However, decrease in plasmablasts proportion did not reach statistical significance (Figure 5).

Despite having not found any increase in the percentage of resting memory B cells, these results are in good agreement with previous reports that describe a normalization of B-cell subpopulations after antiretroviral treatment, reflected by an increase in naïve and resting memory B cells and a decrease of the 2 apoptosis-prone subpopulations of B cells (immature transitional and mature activated B cells) (29, 36).

**B-cell subpopulations in samples with different neutralization breadths**

We analyzed next the frequencies of various B-cell subpopulations in our patients. In this analysis, we have only included samples before initiation of cART in order to avoid the effect of treatment associated suppression of viremia on B-cell phenotype and function described above and previously reported (29).

Data on all untreated patients were compiled and analyzed comparing samples from patients capable of neutralizing viruses across 4 or more subtypes and samples from patients that neutralize viruses from fewer than 4 subtypes. Analysis of the B-cell subpopulations revealed the
following mean frequencies in both groups: immature transitional B cells, 5% and 6%; naïve B
cells, 62% and 44%; tissue-like memory B cells 12% and 19%; resting memory B cells, 11% and
13%; activated memory B cells, 9% and 17% and plasmablasts 0.10% and 0.06% respectively
(Figure 6A and 6B). This analysis revealed a higher percentage of naïve B cells and plasmablasts
and a lower percentage of tissue-like memory, resting memory and activated memory B cells in
patients that neutralize viruses across 4 or more subtypes. However, as shown in Figure 6B, only
the differences in naïve, tissue-like memory and activated memory B cells were statistically
significant ($P= 0.0007$, $P= 0.0058$ and $P=0.0174$ respectively). No differences in the percentage
of immature B cells between both groups of samples were observed in this analysis. We have
also compared samples from patients capable of neutralizing across 5 subtypes and samples with
lower neutralization breadths. In this analysis, we obtained similar results, but the differences
were not statistically significant probably due to the small number of samples capable of
neutralizing across 5 subtypes (data not shown).

Our findings indicate that the frequency of naïve B cells was significantly higher in
patients capable of neutralizing viruses across 4 or more subtypes and close to the average 65%
reported for healthy individuals (37). In contrast, the frequency of tissue-like memory B cells was
significantly lower in patients with a broader neutralizing activity compared with patients with
less neutralization breadth, consistent with the concept that these are exhausted B cells induced
by chronic HIV-induced immune activation (38). The frequency of activated memory B cells was
also significantly lower in patients capable of neutralizing viruses across 4 subtypes or more
compared with patients with less neutralization breadth, indicating a reduction of the aberrant
increase in B-cell activation associated with chronic HIV-infection that was first reported several years ago (36).

HIV infection is associated with a number of perturbations in the B-cell compartment, including the overrepresentation of subpopulations of B cells in the blood that are thought to arise as a result of HIV-induced immune activation and CD4+ T cell lymphopenia. Taken together, these results showed that the balance within B-cell subsets in patients with bCrNA was partially restored compared with the proportions observed in patients with less neutralization breadth, including proportions closer to the ones reported for healthy individuals.

DISCUSSION

The inability to elicit broad and potent cross-reactive anti-HIV neutralizing antibodies by immunization has been a major obstacle for the development of an effective vaccine against HIV. However, we have evidence that the induction of this type of response is feasible since there are some chronically-infected patients with high titers of bNAbs (5). Currently, little is known about the stability of broadly neutralizing responses, the impact that the loss of viremia has on such response and about B-cell phenotype associated with this response. We carried out the present study in order to characterize the evolution of neutralization breadth and the impact of potent antiretroviral treatment in six bCrN. Our definition of bCrN is close to the definition used by Simek et al. for elite neutralizing activity, which considers elite activity to be “the ability to neutralize, on average, more than one pseudovirus at an IC50 titer of 300 within a clade group and across at least four clade groups” (33). While we were unable to completely satisfy these
criterias since our 6-virus panel included only single variants of subtypes A, C, D and AE, the bCrN included in the present study could neutralize viruses across 5 subtypes (A, B, C, D and AE). In addition, we have characterized the frequency of different B-cell subpopulations associated to bCrNA. This study describes the long-term stability of antibody-mediated neutralization breadth in a group of bCrN. Furthermore, this is the first report describing the impact of viremia decay associated to potent antiretroviral treatment on neutralization breadth.

Previous studies have hypothesized that the induction of broadly neutralizing antibodies requires prolonged exposure to the antigen and do not develop until two to four years post-infection (13). At this point, it is likely that is too late for these antibodies to have an effect due to the presence of a widespread infection and an irreversibly damaged immune system. The present study shows that, a normalized B-cell profile is also required to promote the development of broadly neutralizing responses. Based in our results, we suggest that the problematic induction of broadly neutralizing antibodies could be explained by the need to match two factors that seem to be incompatible: a prolonged antigen exposure characteristic of a chronic infection and a low damaged B cell profile. According to the present knowledge it has been suggested that, in order to be effective, broadly neutralizing antibodies should be in place before infection. The good news in the face of a preventive vaccine would be that the induction of broadly cross-reactive HIV-1 neutralizing antibodies might not be so problematic in non-infected individuals that have a healthy B-cell profile. However, the requirement of long periods of antigen exposure to induce broad neutralizing responses may still be an important obstacle for vaccine development and its relevance in non-infected individuals still needs to be determined.
According to numerous studies, the breadth of plasma cross-neutralizing antibody activities in HIV-1 infected subjects positively correlates with plasma viral load. However, in a previous report, we have confirmed the presence of a broad IgG-associated neutralizing response in patients on antiretroviral treatment, despite having undetectable viremia (15). In the present study, we show new evidence supporting a hypothesis that makes both observations compatible. We have studied the evolution of neutralization breadth in a group of bCrN showing that a delay in neutralization breadth loss occurs after viremia has been suppressed for as much as 4 to 20 months. As a result of this delay, in a cross-sectional study, aviremic patients with broad neutralizing responses can be found. Similar to the decline of neutralization breadth following cART engagement observed in the present study, a decline of CTL responses has also been described in chronically infected patients after initiation of treatment[39, 40]. This decline has also been associated with reduced viral replication and consequently with reduced CTL antigen stimulation. The identification of the factors associated with the maintenance of bCrNA, both in the presence and in the absence of viremia, may provide valuable information to improve the stability of an effective humoral immune response induced by vaccination. However, these studies are difficult to carry out due to the low percentage of bCrN patients within the group of HIV-1 infected patients (around 2%) (15).

Several studies have demonstrated a significant recovery of B-cell numbers concomitant with a reduction in HIV-1 plasma viremia by cART. This increase has been associated with a normalization of B-cell subpopulations by reduction in the frequency of apoptosis-prone B-cell subpopulations associated with cART. The improved B-cell profile may also explain the benefits of cART in improving B-cell responses to specific immunogens. In contrast, in the present study
we have observed a decrease in the breadth of the neutralizing response against HIV concomitant with the improvement of the B-cell profile associated with a cART induced decay in viremia. We hypothesize that, even in the presence of an improved B-cell profile, a minimum level of antigen exposure is required to develop bCrNA.

Overall, our findings indicate that there are both immunological and virological determinants necessary to generate bCrNA. Our results indicate that, in addition to long periods of viremia, the presence of a normalized B-cell repertoire is required for the induction of broadly neutralizing responses.

**Acknowledgments:**

We thank Ana García and María T. García for technical assistance and Richard Diesso for assistance in preparing the manuscript. We also thank Julià Blanco and Jorge Carrillo for help on B cell results interpretation. TZM-bl cells were obtained from John C. Kappes, Xiaoyun Wu, and Tranzyme Inc through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. This work was supported by the Instituto de Salud Carlos III [FIS PS09/01459, FIS PS09/01297, FIS PS09/00283 and FIS PI10/02984]; Ayuda para el fomento de la traslación de la aplicación terapéutica de medicamentos de uso humano, huérfanos y terapias avanzadas [TRA-094]; Red de Investigación de SIDA [RD06/0006] to VSM; Ministerio de Sanidad [EC10-153]; SAF 2012-39075 to FG; Fundación para la Investigación y la Prevención del Sida en España [FIPSE 36780/08]; Ministerio de Ciencia y Tecnología to EY [RYC-2007-00788] and Portuguese Foundation for Science and Technology to CBF.
REFERENCES


FIGURE LEGENDS

Figure 1. Study flow-chart. bCrN: Broadly cross-neutralizer patients. Time 0 corresponds to the last time point analyzed in the previous study ([15]). The period included in the present study before and after the previous cross-sectional study (mean and range) is indicated. The periods in which some patients were on cART are also indicated.

Figure 2. Changes in neutralization breadth, CD4+ T cells and viral loads in the bCrN group. Neutralization breadth values (grey area, grey open circles and # subtypes crossed) indicate the number of viruses from different subtypes neutralized out of a previously described minipanel [15]. CD4+ abs indicate the number of CD4+ T cells/mm3 and are represented by a solid red line. Log (VL) indicate the levels of viral loads (copies/ml) and are represented by a dotted blue line. The periods of time in which some patients were on cART are indicated. Time is indicated as months (x axes). Time 0 corresponds to the last time point included in the previous study ([15]). Antibodies capable of neutralizing across 5 subtypes were detected in our cohort of bCrN patients for a period up to 4.6 years. 4 out of 5 bCrN patients that initiated cART showed a loss of neutralization breadth over time.

Figure 3. Changes in neutralization breadth, CD4+ T cells and viral loads in the patient control group. Neutralization breadth values (grey area, grey open circles and # subtypes crossed) indicate the number of viruses from different subtypes neutralized out of a previously described minipanel ([15]). CD4+ abs indicate the number of CD4+ T cells/mm3 and are represented by a solid red line. Log (VL) indicate the levels of viral loads (copies/ml) and are
represented by a dotted blue line. The periods of time in which some patients were on cART are indicated. Time is indicated as months within the present study (x axes). Time 0 corresponds to the last time point included in the previous study([15]).

**Figure 4. Breadth of the plasma NAb responses and plasma viral load levels.** A) Analysis of the overall neutralization breadth for plasmas from patients with detectable and undetectable viremia (bCrN and control patients). B) Comparison of neutralization breadth in plasmas from patients with at least 6 months of undetectable viremia with the group of plasmas from viremic patients and patients with less than 6 months of undetectable viremia. Horizontal bars within the point plots indicate the median subtype crossed ± SEM. Significance between groups is indicated above the groups. Mann-Whitney U tests were used for comparisons between groups. Simple comparisons were made with use of a two-sided alpha level of 0.05.

**Figure 5. Distribution of B-cell subpopulations before and after cART.** The percentages of cells in each of the six B-cell subpopulations for samples from patients before and after cART are shown. The mean frequency for each B-cell subpopulation is denoted by different colors. P values from comparison of B cell subpopulation are also shown. NS denotes not significant.

**Figure 6. Distribution of B-cell subpopulations in samples with different neutralization breadth.** A) The percentages of cells in each of the six B-cell subpopulations were measured for samples from patients capable of neutralizing viruses across 4 or more subtypes and patients that neutralize viruses from less than 4 subtypes. The mean frequency for each B-cell subpopulation is...
denoted by different colors B) Statistical analysis of each B cell subpopulation. Horizontal bars
within the point plots indicate the median percentage for each group ± SEM. Significance
between groups is indicated above the groups. Data corresponding to the same patient are
indicated with different symbols: control patients are indicated in black; patient 181, green
diamond; patient 308, red square, patient 363, blue circle; patient 488, pink asterisk; patient 528,
yellow triangle and patient 541, orange inverted triangle). Mann-Whitney U tests were used for
comparisons between groups. Simple comparisons were made with use of a two-sided alpha level
of 0.05.
Table 1. Clinical characteristics of the patients

<table>
<thead>
<tr>
<th>Patient code</th>
<th>Broadly cross-neutralizer patients</th>
<th>Control patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Risk group</td>
<td>MSM1</td>
<td>MSM1</td>
</tr>
<tr>
<td>Peak VL (log)</td>
<td>4.60</td>
<td>4.44</td>
</tr>
<tr>
<td>Nadir CD4+ cells/µl</td>
<td>266</td>
<td>410</td>
</tr>
<tr>
<td>Nadir CD8+ cells/µl</td>
<td>285</td>
<td>948</td>
</tr>
<tr>
<td>TPS (months)</td>
<td>185.3</td>
<td>127.4</td>
</tr>
<tr>
<td>Beginning of the study (±71 to -37 months)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>57</td>
<td>38</td>
</tr>
<tr>
<td>VL (log)</td>
<td>4.04</td>
<td>3.31</td>
</tr>
<tr>
<td>No. of CD4+ T cells/µl</td>
<td>567</td>
<td>506</td>
</tr>
<tr>
<td>No. of CD8+ T cells/µl</td>
<td>756</td>
<td>1270</td>
</tr>
<tr>
<td>cART ATP</td>
<td>ATP</td>
<td>ATP</td>
</tr>
<tr>
<td>VL (log)</td>
<td>&lt;1.7</td>
<td>&lt;1.7</td>
</tr>
<tr>
<td>No. of CD4+ T cells/µl</td>
<td>437</td>
<td>530</td>
</tr>
<tr>
<td>No. of CD8+ T cells/µl</td>
<td>857</td>
<td>1698</td>
</tr>
<tr>
<td>End of the study (±3 to 34 months)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cART ATP</td>
<td>ATP</td>
<td>ATP</td>
</tr>
<tr>
<td>VL (log)</td>
<td>&lt;1.56</td>
<td>&lt;1.56</td>
</tr>
<tr>
<td>No. of CD4+ T cells/µl</td>
<td>539</td>
<td>819</td>
</tr>
<tr>
<td>No. of CD8+ T cells/µl</td>
<td>1174</td>
<td>1380</td>
</tr>
</tbody>
</table>

1. MSM: Men that have sex with men; IDU: injecting drug user; HTX: heterosexual; 2. TPS: Time elapsed between first HIV-1 positive serology and time 0; 3. Time (months) before the last time analyzed in the previous study; 4. –: Untreated; 5. Last time (months) included in the previous study; 6. ATP: Atripla (efavirenz + emtricitabine + tenofovir); 7. The limit of detection was 1.7 log or 1.56 log (corresponding to 50 or 36 copies/ml respectively) depending on the used commercial kit; 8. Time (months) after the last time included in the previous study([15]).
Table 2. IgG neutralization data corresponding to periods with broad cross-reactive neutralizing responses in patients 181, 308 and 528 a.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Time b (Months)</th>
<th>V1 191 (A) Tier 2 c</th>
<th>NL4-3 (B) Tier 1A c</th>
<th>AC10 (B) Tier 2 c</th>
<th>92BR025 (C) Tier 1B c</th>
<th>92UG024 (D) Tier 2 c</th>
<th>CM244 (E) Tier 2 c</th>
</tr>
</thead>
<tbody>
<tr>
<td>181-025</td>
<td>-36.9</td>
<td>0.3</td>
<td>0.1</td>
<td>&gt;0.3</td>
<td>0.08</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>181-027</td>
<td>-28.2</td>
<td>0.3</td>
<td>0.3</td>
<td>&gt;0.3</td>
<td>0.08</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>181-029</td>
<td>-20.4</td>
<td>0.2</td>
<td>0.3</td>
<td>&gt;0.3</td>
<td>0.06</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>181-031</td>
<td>-14.9</td>
<td>0.3</td>
<td>0.3</td>
<td>&gt;0.3</td>
<td>0.1</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>181-032</td>
<td>-11.0</td>
<td>0.3</td>
<td>0.3</td>
<td>&gt;0.3</td>
<td>0.15</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>181-034</td>
<td>-4.8</td>
<td>0.2</td>
<td>0.08</td>
<td>&gt;0.3</td>
<td>0.2</td>
<td>0.08</td>
<td>0.08</td>
</tr>
<tr>
<td>308-028</td>
<td>-71.4</td>
<td>0.06</td>
<td>0.01</td>
<td>0.01</td>
<td>0.015</td>
<td>0.25</td>
<td>0.2</td>
</tr>
<tr>
<td>308-029</td>
<td>-61.3</td>
<td>0.04</td>
<td>0.004</td>
<td>0.006</td>
<td>0.02</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>308-030</td>
<td>-55.5</td>
<td>0.05</td>
<td>0.005</td>
<td>0.07</td>
<td>0.05</td>
<td>0.09</td>
<td>0.05</td>
</tr>
<tr>
<td>308-031</td>
<td>-49.1</td>
<td>0.05</td>
<td>0.007</td>
<td>0.05</td>
<td>0.05</td>
<td>0.08</td>
<td>0.04</td>
</tr>
<tr>
<td>308-033</td>
<td>-43.1</td>
<td>0.04</td>
<td>0.01</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>308-034</td>
<td>-39.5</td>
<td>0.04</td>
<td>0.007</td>
<td>0.06</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>308-036</td>
<td>-31.9</td>
<td>0.06</td>
<td>0.015</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>308-038</td>
<td>-22.3</td>
<td>0.008</td>
<td>0.006</td>
<td>0.06</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>308-040</td>
<td>-14.0</td>
<td>0.02</td>
<td>0.02</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>308-041</td>
<td>0.0</td>
<td>0.1</td>
<td>0.015</td>
<td>0.15</td>
<td>0.1</td>
<td>0.09</td>
<td>0.09</td>
</tr>
<tr>
<td>308-043</td>
<td>8.5</td>
<td>0.15</td>
<td>0.03</td>
<td>&gt;0.3</td>
<td>0.3</td>
<td>0.15</td>
<td>0.15</td>
</tr>
<tr>
<td>528-005</td>
<td>-31.2</td>
<td>&gt;0.3</td>
<td>0.01</td>
<td>0.1</td>
<td>0.05</td>
<td>0.015</td>
<td>0.04</td>
</tr>
<tr>
<td>528-006</td>
<td>-25.0</td>
<td>0.08</td>
<td>0.005</td>
<td>0.02</td>
<td>0.006</td>
<td>0.02</td>
<td>0.008</td>
</tr>
<tr>
<td>528-008</td>
<td>-16.3</td>
<td>0.2</td>
<td>0.004</td>
<td>0.08</td>
<td>0.003</td>
<td>0.02</td>
<td>0.006</td>
</tr>
<tr>
<td>528-009</td>
<td>-11.7</td>
<td>0.3</td>
<td>0.003</td>
<td>0.04</td>
<td>0.02</td>
<td>0.08</td>
<td>0.004</td>
</tr>
<tr>
<td>528-010</td>
<td>0.0</td>
<td>0.2</td>
<td>0.005</td>
<td>0.025</td>
<td>0.04</td>
<td>0.055</td>
<td>0.02</td>
</tr>
<tr>
<td>528-012</td>
<td>8.8</td>
<td>0.3</td>
<td>0.006</td>
<td>0.004</td>
<td>0.006</td>
<td>0.02</td>
<td>0.006</td>
</tr>
<tr>
<td>528-013</td>
<td>13.5</td>
<td>&gt;0.3</td>
<td>0.015</td>
<td>0.06</td>
<td>0.01</td>
<td>0.04</td>
<td>0.02</td>
</tr>
<tr>
<td>528-014</td>
<td>18.9</td>
<td>n.d. a</td>
<td>0.01</td>
<td>0.1</td>
<td>0.005</td>
<td>0.06</td>
<td>0.02</td>
</tr>
<tr>
<td>528-015</td>
<td>31.1</td>
<td>n.d. a</td>
<td>0.015</td>
<td>0.1</td>
<td>0.055</td>
<td>0.045</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Numbers indicate the concentration of purified IgG that reduces the infectivity by 50% (IC50). White indicates that 50% infectivity reduction was reached at the highest concentration tested (0.3mg/ml); yellow indicates that IC50 is ≤ 0.3 but ≥ 0.1mg/ml; orange indicates that IC50 is < 0.1 but > 0.01mg/ml; red indicates that IC50 is < 0.01. Time is indicated as months. Time 0 corresponds to the last time point included in the previous study. Clades and Tier classifications are indicated. Not done for sample availability reasons.

a Numbers indicate the concentration of purified IgG that reduces the infectivity by 50% (IC50).

b Time is indicated as months. Time 0 corresponds to the last time point included in the previous study.

c Clades and Tier classifications are indicated.

d Not done for sample availability reasons.