HIV-1 Specific Antibody Titers and Neutralization among Chronically Infected Patients on Long-Term Suppressive Antiretroviral Therapy (ART): A Cross-Sectional Study

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Abstract

The majority of potent and broadly neutralizing antibodies against HIV-1 have been isolated from untreated patients with acute or chronic infection. To assess the extent of HIV-1 specific antibody response and neutralization after many years of virologic suppression from potent combination ART, we examined antibody binding titers and neutralization of 51 patients with chronic HIV-1 infection on suppressive ART for at least three years. In this cross-sectional analysis, we found high antibody titers against gp120, gp41, and the membrane proximal external region (MPER) in 59%, 43%, and 27% of patients, respectively. We observed significantly higher endpoint binding titers for gp120 and gp41 for patients with >10 compared to ≤10 years of detectable HIV RNA. Additionally, we observed higher median gp120 and gp41 antibody titers in patients with HIV RNA <50 copies/mL for ≤5 years. 22% of patients neutralized a HIV-1 primary isolate (HIV-1$_{JR-FL}$) and 8% neutralized a HIV-2/HIV-1 MPER chimera. Significantly greater HIV-1$_{JR-FL}$ neutralization was found among patients with >10 years of detectable HIV RNA (8/20 [40.0%] versus 3/31 [9.7%] for ≤10 years, p = 0.02) and a trend toward greater neutralization in patients with ≤5 years of HIV RNA <50 copies/mL (7/20 [35.0%] versus 4/31 [12.9%] for >5 years, p = 0.08). All patients with neutralizing activity mediated successful phagocytosis of VLPs by THP-1 cells after antibody opsonization. Our findings of highly specific antibodies to several structural epitopes of HIV-1 with antibody effector functions and neutralizing activity after long-term suppressive ART, suggest continuous antigenic stimulation and evolution of HIV-specific antibody response occurs before and after suppression with ART. These patients, particularly those with slower HIV progression and more time with detectable viremia prior to initiation of suppressive ART, are a promising population to identify and further study functional antibodies against HIV-1.

Introduction

A substantial amount of the antibody response in human immunodeficiency virus type 1 (HIV-1) infected individuals is directed against the envelope glycoprotein (Env) embedded on the viral surface [1]; however, only a minor fraction of these antibodies are able to recognize conserved epitopes on trimeric Env and thus elicit a consistent, broad, and potent neutralization of HIV-1 [2,3]. Distinguished epitopes prone to cross-neutralization include but are not limited to the membrane proximal external region (MPER) on gp41 [4,5], the CD4 binding site (CD4bs) [6,7], glycan based epitopes [8], variable loops 1 and 2 (V1/V2) [9], and the variable loop 3 (V3) region [10] on gp120. The majority of potent and broadly neutralizing HIV-1 monoclonal antibodies (mAbs) targeting these conserved regions were isolated from individuals with untreated acute or advanced chronic HIV infection when HIV RNA levels are highest [11].

Additionally, increased breadth and potency of isolated neutralizing antibodies were associated with low CD4+ T cell counts and high HIV RNA levels [3,12,13]. The direct correlation between high HIV RNA level and greater neutralization of HIV-1 specific antibodies was also observed among elite HIV controllers or suppressors (ES) not on antiretroviral therapy (ART) [14]. Doria-Rose and colleagues found that elite suppressors (with undetectable HIV RNA off ART) were less likely to generate broadly neutralizing antibodies than progressors or long term non-progressors with detectable HIV viremia [15]. Therefore, HIV-infected individuals with suppressed viremia (with or without ART) were considered poor candidates to evaluate for broadly neutralizing HIV-1 specific antibodies to novel epitopes [16].

HIV-1 envelope specific titers and neutralization clearly decrease after initiation of suppressive ART during acute infection [17–19]. However, a recent study reported high antibody titers with modest neutralization when ART was initiated several years

after established chronic infection [20]; thus, raising the possibility that HIV-1 specific immune responses evolve over time on ART. Additionally, it has been found that on suppressive ART, B cell counts increase, B cell subpopulations normalize, and B cell activation persists [21,22]. Recent evidence suggests that compartmentalized HIV replication and very low-level HIV viremia persist on suppressive ART [23–25]. We hypothesized that functional B cells responding to HIV antigen in lymphatic tissues, in the setting of immune recovery on ART, evolve a more effective humoral immune response. To improve our understanding of this type of autologous antibody response, we examined HIV-specific antibodies, neutralization, and effector functions among a population of patients on long-term suppressive ART with immune recovery. Although there is clear evidence from numerous non-human primate studies that neutralizing antibodies can prevent HIV-1 acquisition [26–31] little is known about their role in preventing or controlling established infection in humans [1,32–34]. Therefore, it is important to further the knowledge of humoral immunity in HIV-1 infected patients (with and without ART) and study the role of HIV-1 specific antibodies and their putative effector functions on virus transmission and pathogenesis.

Materials and Methods

Antibodies, viruses and peptides

Michael B. Zwick and Dennis R. Burton kindly provided mAbs Z13e1 [35] and b12 [36]. MAbs 1F7, 2G12, 4E10, and 2F5 were generously donated from Dietmar Katinger (Polymun Scientific) and Hermann Katinger [37]. MAb 17b was obtained through the IAVI NAC reagent repository after generous donation from James Robinson [38]. HIV-2/HIV-1 MPER chimeras were kindly provided by George M. Shaw [39]. MPER peptides were generously provided by Sampat Ingale and Philip Dawson [40].

Study population, serum samples, data collection, and ethics statement

We obtained sera from 51 adult patients consented and evaluated for participation in the Eramune 02 clinical trial (http://clinicaltrials.gov/ct2/show/NCT00976404) at the Northwestern University clinical site in Chicago, USA. Patients were assessed if they had current CD4+ cell counts ≥ 350 cells/µl, continuous suppression of HIV RNA <50 copies/mL for at least one year and <500 copies/mL for at least three years. We excluded patients from evaluation who had any of the following conditions: diagnosis of cancer within the last five years, history of autoimmune disease, immunologic therapeutic intervention within the past year, and any co-morbid condition with an expected survival of less than twelve months. As per Eramune 02 evaluation procedures, serum and cell pellets were obtained for evaluation of adenovirus antibodies and total proviral HIV DNA assessment. Clinical information including demographics, HIV diagnosis dates, current ART regimens, CD4+ cell counts, CD8+ cell counts, nadir CD4+ cell counts, HIV RNA levels, and dates of HIV suppression were collected by electronic chart review and patient interview.

All patients included in this study provided written informed consent. The ERAMUNE 02 protocol and informed consent form were approved by the Northwestern University Institutional Review Board. Laboratory specimens were completely anonymized and unlinked from any patient identifying information.

Enzyme linked immunosorbent assay (ELISA)

Patient sera were screened for specific antibody titers against recombinant gp120$_{JR-FL}$, gp41$_{JR-FL}$ [35], and p24. To test whether any gp41 specific antibodies were directed against the MPER, antibody binding was assessed against three MPER mimetics: QIQQEKNMYELLALDKWASLWNWFDITKWLWYIKYG-VYIV – designated as MPER-p1 according to the MPER sequence of the HIV-2/HIV-1 Env chimera 7312-C1 – [41], LLELDKWASLWNWFDITNWLWYIKKKK – designated as MPER-p2, and NWFDITNWLWYIKKKK-NH2 – designated, as MPER-p3 – [35,42].

To further examine the antibody response against gp120, patient sera were evaluated for binding against recombinant gp120$_{JR-FL}$ mutants lacking either the V1/V2 loop (ΔV1/V2) or the V3 loop (ΔV3). Specific binding of human mAbs 2G12, 1F7, 2F5, Z13e1, 4E10, and a human serum control to the above-described antigens was determined in parallel. Direct binding ELISA was performed according to Gach and colleagues [43]. In brief, 96 well plates were coated with 1µg/mL of antigen (50 ng/well) over night a 4°C. Plates were washed, blocked and incubated with serially diluted serum samples and antibody controls for 1 hour at room temperature. Bound antibodies were detected with a HRP labeled goat anti human Fc antibody (Jackson ImmunoResearch) developed with TMB substrate and read with a BIOTEC plate reader.

Soluble CD4 (sCD4) inhibition assay

Sera from patients #12, #37, and #42 who had no significant decline in V1/V2 and V3 loop mutant binding were tested for the presence of CD4bs specific antibodies. For this approach, serially diluted sera and antibody controls were incubated with a constant concentration of sCD4 (10 µg/mL) to compete for binding with recombinant gp120$_{JR-FL}$ as described previously [43].

Serum antibody purification

HIV-1 patient serum samples were purified using 0.2 mL NAb protein A/G spin columns (Thermo Scientific) according to the manufacturer's instructions. Fractions containing the purified antibodies were further concentrated and buffer exchanged using Amicon Ultra 0.5 mL centrifugal filter (50 KDa) units (Millipore). IgG concentration was measured at OD$_{280}$ with a NanoDrop Spectrophotometer (Thermo Scientific).

Virus production and neutralization assay

Pseudotyped viruses (HIV-1$_{JR-FL}$, VSV-g pseudotyped HIV-1) were produced as described recently [44]. Replication competent viruses (HIV-2/HIV-1 chimeras) were generated identically but in the absence of the HIV-1 backbone plasmid pSG3ΔEnv. To evaluate patient samples for their neutralization potency, serum was heat inactivated and 0.2 µm filtered before virus incubation. In a preliminary neutralization experiment, crude serum samples were tested against pseudotyped HIV-1$_{JR-FL}$. Over 65% of the sera were able to neutralize HIV-1$_{JR-FL}$ with a reciprocal serum dilution factor ranging from 100 up to >2700 (data not shown). To minimize false positive results due to the presence of antiretroviral drugs (e.g. efavirenz) in serum, IgG was purified prior to testing. None of the purified patient samples neutralized a VSV-g pseudotyped HIV-1 virus control confirming successful removal of antiretroviral drugs during IgG purification [20]. In the next step, crude sera were screened for MPER neutralizing antibodies, as the antiretroviral drugs did not affect neutralization against HIV-2 chimeras. Single round infectivity assays were performed and evaluated as described by Walker and colleagues [45].

Antibody effector function assay

To test for antibody effector functions of neutralization positive patients, purified IgG fractions were serially diluted in the presence of green fluorescence protein labeled Env expressing virus like particles (VLPs). VLPs were opsonized for 1 hour at 37°C followed by a 1 hour incubation step with Thp-1 cells. Phagocytosis of VLPs was determined by flow cytometry as described previously [46].

Total proviral HIV DNA assessment

As part of the evaluation procedures for Eramune 02, we isolated PBMC pellets from each patient and performed a real time PCR assay for the semi-quantitative detection of HIV-1 cellular DNA. The methodology of this research assay has been described elsewhere [47].

Statistical analysis

We performed bivariate comparisons of $\log_{10}$-transformed serum antibody endpoint titers for demographic, clinical, and treatment characteristics using Fisher's exact or Mann Whitney Wilcoxon rank sum tests for dichotomous or continuous variables, respectively. As estimates of extent of HIV disease, burden of latent HIV reservoir, and replication, we compared mean HIV specific antibody titers for the following variables: a) nadir CD4+ cell count categorized as ≤ 200 versus >200 cells/µl, b) total proviral HIV DNA categorized as ≤ 150 and >150 copies/10^6 PBMCs, c) time with detectable HIV RNA categorized as ≤ 10 and >10 years, and d) time with HIV RNA <50 copies/mL categorized as ≤ 5 and >5 years. We also evaluated correlations between $\log_{10}$-transformed serum antibody titers and age, nadir CD4+ cell count, current CD4+ cell count, current CD8+ cell count, current CD4+/CD8+ ratio, current total proviral HIV-1 DNA level, time with detectable HIV RNA, time with HIV RNA <50 copies/mL, and adenovirus antibody titer using Pearson's correlation. We performed multivariable analyses of antibody neutralization controlling for age, race, sex, and nadir CD4+ cell count using logistic regression. All descriptive, comparative and correlative statistics were performed using SAS 9.3 or Graph Pad Prism 6.0. Test results were considered statistically significant for two-sided p-value <0.05.

Results

Study population

A total of 51 patients were evaluated for participation in the Eramune 02 clinical trial and included in this study (Table 1). The median age was 50 years (interquartile range [IQR] 46 – 55), 45/51 (88%) were male and 36/51 (71%) white race. The majority of patients were on non-nucleoside reverse transcriptase inhibitor (NNRTI) based ART ([29/51 [57%]) and 27/51 (53%) had ART regimens containing efavirenz. The median time since HIV diagnosis was 13.4 years (IQR 9.3 – 19.6), median time with detectable HIV RNA was 7.1 years (IQR 3.5 – 13.2), and median time with HIV RNA <50 copies/mL was 5.4 years (IQR 4.4 – 6.2). The median total proviral HIV-1 DNA was 159 copies/10^6 PBMCs (IQR 45 – 425), median nadir CD4+ cell count was 217 cells/µl (IQR 116 – 435), median current CD4+ cell count was 613 cells/µl (IQR 508 – 797), median current CD8+ cell count was 728 (IQR 509 – 1045) and median current CD4+/CD8+ ratio was 0.83 (IQR 0.58 – 1.24).

Overall antibody titers

Over half of the patients (59%) revealed antibody titers equal or higher than 1×10^6 (high) against recombinant gp120$_{JR-FL}$. Binding titers between 1×10^5 and 1×10^6 (medium) against

gp120$_{JR-FL}$ were observed in 33% of the patients whereas only 8% exhibited antibody titers below 1×10^5 (low) (Figure 1A). A similar distribution was seen for recombinant gp41$_{JR-FL}$ binding, where 43% of the patients revealed high binding titers greater than 1×10^6 followed by 51% with medium binding titers, and 6% with binding titers below 1×10^5 (Figure 1A). One fifth of the patients showed high titers against p24, followed by 33% with medium binding titers against the HIV-1 capsid protein (Figure 1A).

Antibodies to MPER and gp120 variable loops

Patient sera exhibited notable MPER specific antibody responses ranging from 1×10^4 to 5.5×10^4. MPER-p1 was recognized by 27% of the patients at an antibody titer $>1 \times 10^4$, followed by MPER-p3 with 20%, and MPER-p2 with 8%. Patient #26, revealed an exceptional antibody titer ($>2 \times 10^5$) against MPER-p1 (Figure 1B). 55% of the patients showed a $\geq 50\%$ decrease (ranging from 2-fold to 7-fold) in antibody titers against the ΔV1/V2 loop mutant compared to gp120 wild type (WT). In case of the second gp120 mutant, ΔV3, the effects were even more pronounced since 94% of the patients revealed a reduction ($\geq 50\%$) in antibody binding compared to the WT with a median drop in antibody levels of 7-fold (Figure 1C). These results confirm the presence of V1/V2 loop specific antibodies and, to a higher extent, V3 loop specific antibodies in the sera. In contrast, three of the patient serum samples (#12, #37, and #42) exhibited distinct antibody populations, which were not affected by the gp120 loop deletions (Figure 1C). Binding of antibody and serum controls against each above described antigen is summarized in Figure S1.

Antibodies to CD4bs

The presence of sCD4 did only marginally alter gp120 binding for patients #12, #37, and #42 (Figure 2A and 2B). We observed 1.5-fold (#12), 1.1-fold (#37), and 1.3-fold (#42) higher EC$_{50}$ values in the presence of sCD4 suggesting occurrence of CD4bs antibodies in these patients although at a low quantity (Table S1). Antibody control 2G12 revealed an EC$_{50}$ of 0.156 µg/mL in the presence of sCD4 compared to an EC$_{50}$ of 0.112 µg/mL in the absence of sCD4 (Figure 2C and D). On the contrary, mAb 1F7, a CD4bs specific antibody [43,48], exhibited a 12-fold higher EC$_{50}$ value in the presence of sCD4 (EC$_{50}$ = 0.052 µg/mL) than without (EC$_{50}$ = 0.624 µg/mL), indicating sCD4 competition. Binding enhancement was observed for mAb 17b (EC$_{50}$ = 3.726 µg/mL) where the presence of sCD4 decreased the EC$_{50}$ value more than 186-fold (EC$_{50}$ = 0.02 µg/mL).

Antibody neutralization and effector functions

22% of the patients were able to neutralize HIV-1$_{JR-FL}$ pseudotyped virions after IgG purification with a median IC$_{50}$ of 42.9 µg/mL (Table 2). Patient #48 exhibited a 14-fold lower IC$_{50}$ value than the median IC$_{50}$ value of all neutralizing patient samples. As shown in Table 2, four patients (7.8%) were able to neutralize MPER chimera 7312-C1 (subtype B HIV-1 MPER) with a serum dilution ranging from 1:108 to 1:125. No sign of neutralization was observed with the MPER clade C consensus variant 7312-C1C or the HIV-2 WT 7312A. Neutralization values of human mAb controls at a starting concentration of 5 µg/mL are shown in Table S2. Since patient #48 showed notable neutralization potency against HIV-1$_{JR-FL}$ the sample was further tested against a small panel of different HIV-1 clades (i.e. AE, B, C, and E) for potential cross-neutralization breadth (Table 3). Human mAbs b12 and 2G12 were included as a control at a starting concentration of 5 µg/mL. Patient #48 was able to neutralize eight out of twelve viruses including five viruses from clade B (HIV-1$_{MN}$, HIV-1$_{TRO.11}$, HIV-1$_{JR-CSF}$, HIV-1$_{SF-162}$, and

Table 1. Patient characteristics and results of antibody assays.

| Characteristic or Result (total n = 51)	
Age, median years (IQR)	50 (46,55)
Male sex (%)	45 (88)
White race (%)	36 (71)
Type of ART	
Boosted PI (%)	18 (35)
NNRTI (%)	29 (57)
Boosted PI+NNRTI (%)	2 (4)
Efavirenz in ART regimen	27 (53)
Nadir CD4+ count, median cells/μL (IQR)	217 (116,435)
Current CD4+ count, median cells/μL (IQR)	613 (508,797)
Current CD8+ count, median cells/μL (IQR)	728 (509,1045)
Current CD4+/CD8+ Ratio, median (IQR)	0.83 (IQR 0.58,1.24)
Total Proviral HIV DNA, median copies/10^6 PBMCs (IQR)	159 (45,425)
Total time from HIV diagnosis, median years (IQR)	13.4 (9.3,19.6)
Time with detectable HIV RNA, median years (IQR)	7.1 (IQR 3.5,13.2)
Time with HIV RNA <50 copies/mL, median years (IQR)	5.4 (4.4,6.2)
Adenovirus antibody, median titer (IQR)	43 (12,3704)
Antibody Epitope, median endpoint titer $\log_{10}$ (IQR)	
p24	5.2 (4.6,5.7)
gp120	6.1 (5.5,6.3)
gp41	6.0 (5.4,6.2)
MPER-p1	3.8 (3.3,4.1)
MPER-p2	3.4 (3.3,3.8)
MPER-p3	3.8 (3.4,4.0)
HIV-1$_{JR-FL}$ Neutralization	11 (22)

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HIV-1$_{JR-FL}$), two clade C viruses (HIV-1$_{ZM53M}$, HIV-1$_{CAP045}$), and one clade E virus (HIV-1$_{93TH966.8}$). A similar pattern was seen for mAb b12, which was able to neutralize six viruses at a concentration below 5 μg/mL. However, b12 was not able to neutralize HIV-1$_{TRO.11}$ and HIV-1$_{93TH966.8}$ (clade B and clade E, respectively). Antibody 2G12 neutralized a total of five clade B viruses and none of the other clades tested at the highest concentration. None of the antibodies were able to neutralize the VSV-g pseudotyped HIV-1 virus control (data not shown).
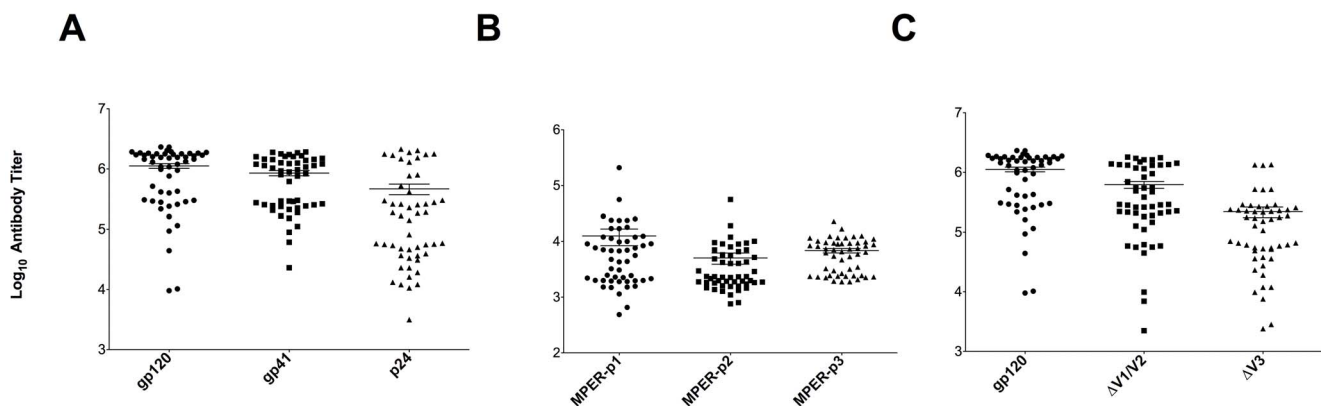
Figure 1. Serum mapping against different HIV-1 epitopes. Sera of HIV-1 patients were tested for binding against different HIV-1 antigens including recombinant gp120$_{JR-FL}$, gp41$_{JR-FL}$, and p24 (A). To check for MPER specific antibodies, serum binding against three different MPER peptides was evaluated (B). To gain better knowledge about the binding epitopes on gp120, sera were tested against the gp120 variable loop truncation variants ΔV1/V2 and ΔV3 (C). Endpoint titers of the patient samples were determined by calculating the highest serum dilution that gives a reading above the blank including three standard deviations.
doi:10.1371/journal.pone.0085371.g001
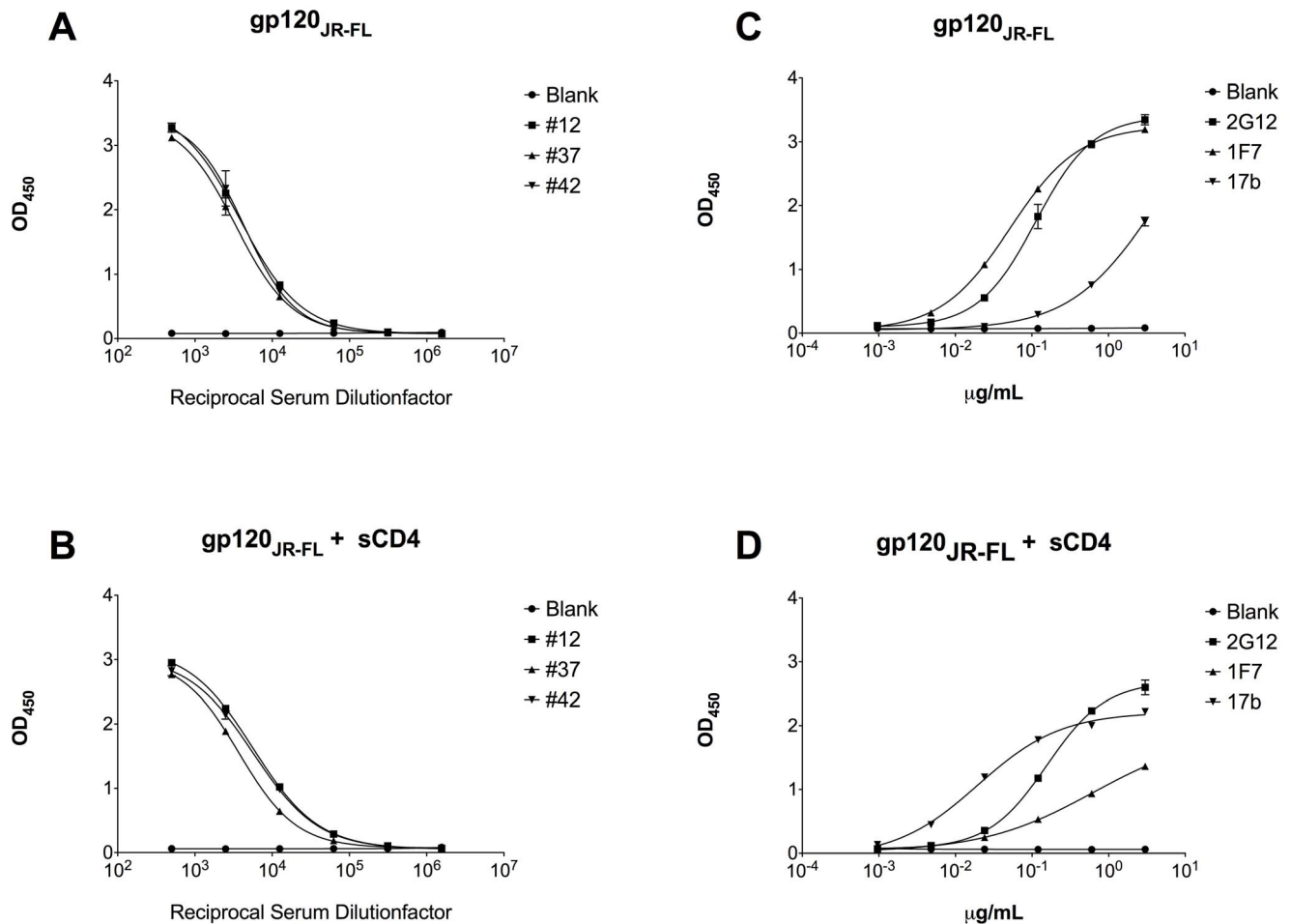
Figure 2. Soluble CD4 competition assay. Assay was performed with three HIV-1 positive serum samples and respective mAb controls (1F7, 2G12, and 17b) in the absence (A and C) or presence (B and D) of soluble CD4. The EC_{50} values (half maximal binding at reciprocal serum dilution or antibody concentration) were calculated and compared for significant differences.
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Next, we looked at antibody effector functions of purified neutralizing IgG fractions against green fluorescence protein labeled Env expressing VLPs. VLPs were phagocytosed very efficiently by all patient samples compared to the negative control IVIG. Patients #42, #18, and #10 revealed the highest phagocytosis levels starting at a concentration of 1.0 μg/mL. HIVIG was used as a positive control for successful phagocytosis of VLPs (Figure 3).

Patient characteristics and antibody comparisons

There were no statistically significant differences in any HIV-1 specific antibody endpoint binding titers for patients with nadir CD4+ cell count ≤ 200 cells/μl compared to those with >200 cells/μl for any of the epitopes analyzed. Patients with nadir CD4+ cell count >200 cells/μl experienced non-significantly greater HIV-1$_{JR-FL}$ neutralization with 8/29 [27.6%] compared to 3/22 [13.6%] for those with CD4+ cell counts ≤ 200 cells/μl exhibiting any activity, p = 0.31. In terms of total HIV-1 proviral DNA, there were also no statistically significant differences in any of the HIV-1 specific antibody endpoint binding titers between patients with ≤ 150 and >150 copies/10^6 PBMCs. Patients with total HIV-1 proviral DNA >150 copies/10^6 PBMCs tended to have greater neutralization with 8/26 [30.8%], compared to 3/25 [12.0%] for

those with ≤ 150 copies/10^6 PBMCs, exhibiting any activity against HIV-1$_{JR-FL}$ pseudotyped virus (p = 0.17).

We did find significantly higher endpoint binding titers for gp120$_{JR-FL}$ (p = 0.03), gp120$_{JR-FL}$ ΔV1/V2 (p = 0.003), gp120$_{JR-FL}$ ΔV3 (p = 0.01) and gp41$_{JR-FL}$ (p = 0.02) for patients with >10 compared to ≤ 10 years of detectable HIV RNA (Figure 4A to D). There were no statistically significant differences in HIV-1 specific antibody endpoint titers for patients with ≤ 5 compared to >5 years with HIV RNA <50 copies/mL, except gp41, for which we observed a higher median antibody titer in patients with HIV RNA <50 copies/mL for ≤ 5 years (6.1 versus 5.5 log$_{10}$, p = 0.02). A similar trend, although not statistically significant (p = 0.56), was observed for gp120 (Figure 5). We found significantly greater HIV-1$_{JR-FL}$ neutralization among patients with >10 years of detectable HIV RNA (8/20 [40.0%] versus 3/31 [9.7%] for ≤ 10 years, p = 0.02) and a trend toward greater neutralization in patients with ≤ 5 years of HIV RNA <50 copies/mL (7/20 [35.0%] versus 4/31 [12.9%] for >5 years, p = 0.08). In multivariable analyses controlling for age, race, sex, and nadir CD4+ cell count, the odds of HIV-1$_{JR-FL}$ neutralization were significantly greater for patients with >10 years of detectable HIV RNA (adjusted OR = 7.7; 95% CI: 1.4-43.3, p = 0.02) before ART initiation, and again a trend toward greater neutralization among those with ≤ 5 years of HIV

Table 2. Summary of patients with neutralizing activity against HIV-1$_{JR-FL}$ and/or HIV-2/HIV-1 7312-C1.

Patient ID #	IC$_{50}$* (µg/mL)		IC$_{50}$^		
	Virus		Virus		
	JR-FL	VSV-g	7312-C1	7312-C1C	7312A
9	34.1	> 200	< 100	< 100	< 100
10	56.0	> 200	< 100	< 100	< 100
11	125.0	> 200	< 100	< 100	< 100
12	42.9	> 200	108	< 100	< 100
18	127.3	> 200	116	< 100	< 100
26	> 200	> 200	109	< 100	< 100
32	65.5	> 200	< 100	< 100	< 100
35	41.5	> 200	< 100	< 100	< 100
41	38.7	> 200	< 100	< 100	< 100
42	17.5	> 200	< 100	< 100	< 100
48	3.0	> 200	125	< 100	< 100
50	77.8	> 200	< 100	< 100	< 100

*Polyclonal inhibitory concentration of purified IgG fractions
^ Reciprocal dilution factor of serum samples
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Table 3. Neutralization potency and breadth of HIV-1 patient #48 against a small panel of different HIV-1 viruses including clades AE, B, C, and E.

Clade	Virus	Patient #48	b12	2G12
		IC$_{50}$* (µg/mL)	IC$_{50}$ (µg/mL)	
AE	C1080	> 200	> 5	> 5
B	MN	< 1.0	< 0.02	> 5
B	TRO.11	9.5	> 5	0.19
B	JR-CSF	12.9	0.26	0.4
B	SF162	< 1.0	0.02	0.14
B	JR-FL	3.4	0.03	1.1
B	PVO.4	> 200	> 5	0.41
C	DU156.12	> 200	> 5	> 5
C	93MW959	> 200	> 5	> 5
C	ZM53M	5.7	0.13	> 5
C	CAP045	6.0	1.1	> 5
E	93TH966.8	4.6	> 5	> 5

*Polyclonal inhibitory concentration of purified IgG fractions
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RNA <50 copies/mL (adjusted OR = 4.9; 95% CI: 0.95–25.6, p = 0.06).

Additionally, we found direct correlations between certain patient characteristics and HIV-1 specific antibody endpoint titers or neutralization levels as follows: age and MPER-p2 (r = 0.30, p = 0.03); current CD4+ cell count and MPER-p2 (r = 0.22, p = 0.12); time with detectable HIV RNA and gp120 (r = 0.28, p = 0.04), gp41 (r = 0.27, p = 0.05), and HIV-1$_{JR-FL}$ neutralization (r = 0.40, p = 0.004); total proviral HIV-1 DNA and MPER-p1 (r = 0.30, p = 0.04) and MPER-p2 (r = 0.31, p = 0.03).

Discussion

The recent discovery of potent and broadly neutralizing antibodies to HIV-1 from infected individuals has galvanized interest in protective or therapeutic interventions harnessing the humoral immune response [49]. Using the latest techniques, investigators have largely focused on natural production of these antibodies in acute or early untreated HIV infection where they arise in approximately 20% of infected individuals [50]. Several longitudinal studies of patients treated with ART during acute HIV-infection showed a decline in HIV-1 specific antibody levels over time. This was thought to be due to the rapid control of viremia and reduced time of antigen stimulation [17,51] as well as reduced B cell hyperresponsiveness [52]. In contrast, when ART was initiated several years after established chronic infection, there is persistence of antibody production against HIV-1 structural proteins [18,20] or in some cases even augmentation of antibody titers after ART treatment [53]. In our study, we observed exceptionally high antibody titers against recombinant gp120$_{JR-FL}$, and gp41$_{JR-FL}$ in the majority of patients on long-term suppressive ART. We found that antibody titers against gp120 and gp41 were significantly higher for patients who had longer time with detectable viremia (>10 years). Additionally, we found a trend toward lower median gp120 and gp41 antibody levels in patients who had greater time with undetectable viremia (>5 years). Our findings confirm the association between viremia and HIV specific

antibodies, but further illustrate the slow clearance of HIV-specific antibodies over longer times on suppressive ART. This could be due to continuous antigenic stimulation or persistent immune dysfunction and antibody production even when virus is controlled with ART [25,53].

Antibodies against gp41 are the first line of defense in acute HIV-1 infection [54]; however, the majority of gp41-binding antibodies produced in chronic HIV-1 infection are generated from memory B cells previously activated by non–HIV-1 antigens and thus unable to elicit a neutralizing immune response [55]. The MPER is one of the best-characterized regions on gp41 since it is the target of four broad and potent neutralizing antibodies including 2F5, 4E10, Z13e1, and 10E8 [56–59]. Moreover the MPER is highly conserved across clades and thus represents a promising vaccine target [4,60]. To investigate whether patients on long-term suppressive ART develop highly specific antibodies against the MPER, we tested their sera against three MPER mimetics. We found notable antibody titers (>1×10^4) against all peptides. Our titers were consistent with previously described MPER antibody titers from other individuals with chronic HIV-infection [41,61], but our patients were unique in that they all had achieved long-term virologic suppression on ART. Interestingly, patient #26 exhibited exceptionally high titers against MPER-p1 although we believe that most of the antibody response was directed against the N-terminus of the peptide as antibody titers against an overlapping MPER-p2 peptide were almost 30-fold lower. We also found that height of MPER binding titers did not necessarily correlate with neutralization. For example, patient #6 revealed an MPER specific antibody titer of 5.5×10^4 against both MPER-p1 and MPER-p2 but did not show any neutralization activity against HIV-2/HIV-1 7312-C1. One possible explanation for this is that these antibodies may recognize a linear, instead of a conformational, epitope. On the contrary, patient #48 exhibited the greatest neutralization (1:125) although the MPER specific titer was marginally lower than 1×10^4. We also observed overall higher titers against MPER-p3 compared to MPER-p2, which overlap in 13 MPER specific amino acids. We speculate the difference was due to enhanced exposure of accessible sites on the shorter peptide or avidity effects whereby the antibody interacts
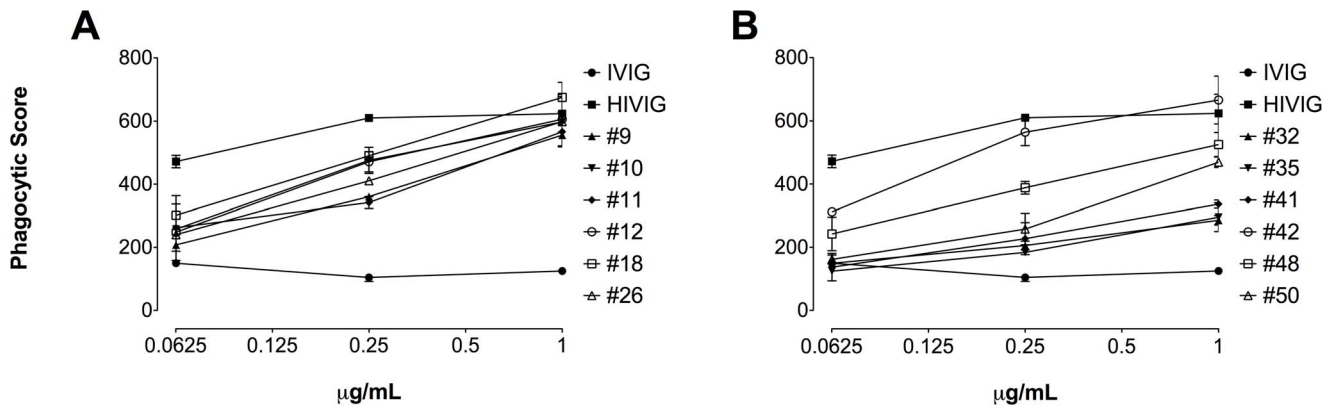
Figure 3. Phagocytosis assay. Phagocytic score of samples tested at different antibody concentrations is indicated. All purified IgG fractions and the positive control HIVIG revealed Fc-mediated effector functions compared to the negative control IVIG. The cut off value of confidence interval for IVIG has a phagocytic score of 194.77. All samples greater than the cut off are positive with 99% confidence.
doi:10.1371/journal.pone.0085371.g003
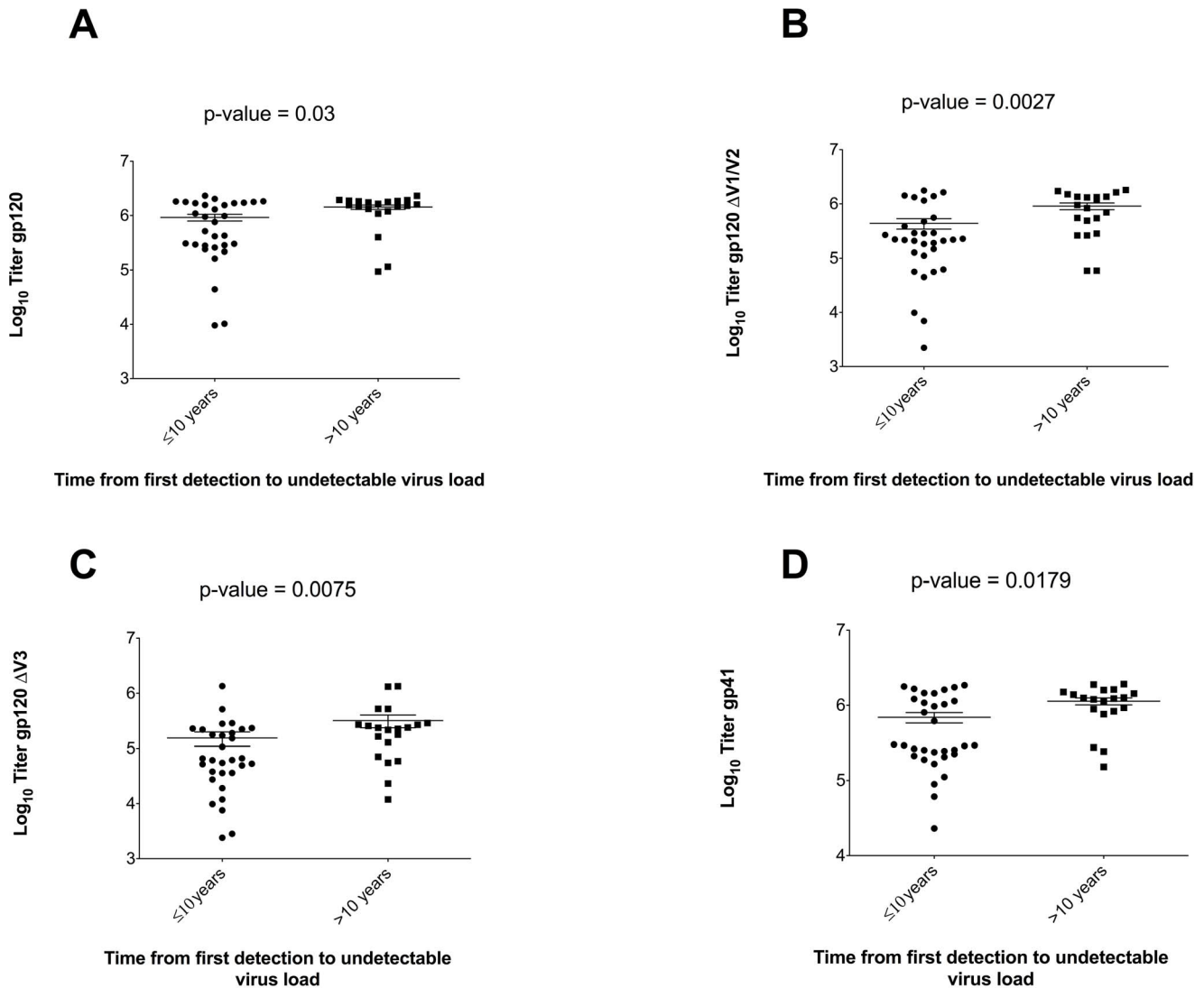
Figure 4. Comparison of HIV-1 specific antibody endpoint titers and time with detectable HIV RNA categorized as ≤10 and >10 years. Scatter plots of antibody endpoint titers to recombinant gp120$_{JR-FL}$, gp120$_{JR-FL}$ ΔV1/V2, gp120$_{JR-FL}$ ΔV3, and gp41 categorized by time with detectable HIV RNA show significantly higher titers for patients with >10 years of detectable viremia.
doi:10.1371/journal.pone.0085371.g004
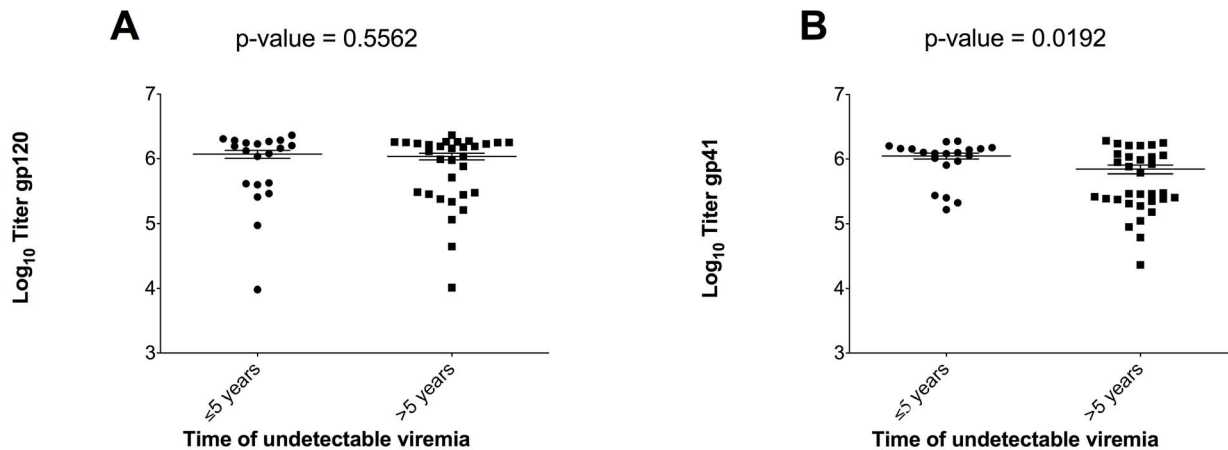
Figure 5. Comparison of HIV-1 specific antibody endpoint titers and time with HIV RNA<50 copies/mL categorized as ≤5 and >5 years. Median binding titers against gp120$_{JR-FL}$ (A) and gp41$_{JR-FL}$ (B) were both lower in patients with >5 years of undetectable viremia. doi:10.1371/journal.pone.0085371.g005

with two peptides. All four patients with neutralizing MPER antibodies exhibited binding titers close to 1×10^4 or greater than 1×10^4 against MPER-p1. Identification of binding titers to this peptide could be a simple and valuable screening tool in future studies exploring neutralizing MPER antibodies.

Within the trimeric envelope spike, variable gp120 loops V1/V2 and V3 play dual roles in antibody recognition. They contain epitopes prone to neutralization and block key neutralizing epitopes on the CD4bs and sites of conformational change after CD4 binding [62,63]. The importance of these V1/V2 and V3 regions for the generation of broadly neutralizing antibodies has been investigated by several studies [64-66]. Our gp120 epitope analyses revealed a higher proportion of V3 loop (48/51; 94%) compared to V1/V2 loop (28/51, 55%) specific antibodies. Additionally, we observed a higher frequency of V1/V2/V3 specific antibodies among patients with less than 10 years of detectable viremia. This is in agreement with previous reports where early autologous neutralizing antibody responses predominantly target variable epitopes that are exposed on Env including V1/V2 and V3 [67,68]. Antibodies against V3 usually develop faster and reach higher titers compared to antibodies against other highly conserved epitopes such as CD4bs or MPER [69].

Interestingly, three of the patients showed an average one-fold reduction in binding against V1/V2 and 1.4-fold reduction in V3; however, when we checked for CD4bs antibodies, we observed only a marginal decrease in binding (1.5-fold to 1.1-fold) in the presence of sCD4. Nevertheless, two of these patients (#12 and #42) were able to neutralize HIV-1$_{JR-FL}$ in a single round infectivity assay suggesting either the occurrence of potent CD4bs-specific and V1/V2/V3-specific antibodies or the existence of neutralizing antibodies to other epitopes such as glycans [70,71].

We found a total of 12 patients (23.5%) with neutralization activity against HIV-1$_{JR-FL}$, HIV-2/HIV-1 7312 C1 or both. One patient had neutralization activity across both viruses (1.9%). Our neutralization results were similar to a recent study where 1.7% of patients on suppressive ART were able to neutralize a similar panel of viruses [20]. We observed significantly greater neutralization in patients with more time with detectable HIV viremia and a trend toward greater neutralization with higher nadir CD4+ cell counts and higher current total proviral HIV-1 DNA. Again, this illustrates an evolving partially effective antibody response as HIV disease advances with uncontrolled viremia. Alternatively, these patients were able to naturally control HIV progression and

avoid initiation of ART for longer periods of time due to an enhanced antibody response. Given the cross-sectional nature of our study, we are unable to fully understand the relationship between antibody neutralization and HIV replication over time.

Other mechanisms distinguished from antibody neutralization by Fab are Fc-mediated effector functions, such as phagocytosis and lysis, which also play an important role in the humoral immune response to HIV infection [72,73]. Essentially, the Fcγ portion of anti-HIV antibodies interact with the corresponding Fcγ receptor I or II on the cell surface of antigen presenting cells, thereby mediating HIV inhibition by a mechanism involving the phagocytosis and clearance of HIV-IgG immune complexes [46]. In our study, we found a positive correlation between antibody titers and phagocytosis levels. Patients #42, #18, and #10 showed the highest antibody titers against gp120 and gp41 along with the highest phagocytosis levels. Thus, our findings confirm that there are antibody-mediated effector functions present in addition to those leading to HIV neutralization.

In summary, our findings of high antibody binding titers to several structural epitopes of HIV-1 with antibody effector functions and neutralizing activity after long-term suppressive ART, suggest antigenic stimulation and antibody production continues for many years after systemic HIV RNA levels reach undetectable. Moving forward, the longitudinal antibody response before and after suppressive ART requires further research but we believe our study is an encouraging first step that warrants future investigation of key patient populations. Furthermore, screening for autologous broadly neutralizing antibodies in this population should be targeted at those with several years of detectable viremia prior to initiation of suppressive ART.

Supporting Information

Figure S1 Monoclonal antibody and serum control binding titers against various antigens. (A), gp120$_{JR-FL}$ wild type (B), gp120$_{JR-FL}$ V1/V2 loop deletion variant C), and gp120$_{JR-FL}$ V3 loop deletion variant (D). 2G12 and 1F7 only recognized gp120$_{JR-FL}$, whereas 2F5, Z13e1, and 4E10 interacted with gp41 and MPER. MPER-p2 was only recognized by mAb 4E10 since the epitopes of 2F5 and Z13e1 are missing No significant binding was detected against p24. The human serum control revealed only minor binding against all tested antigens. (TIF)

Table S1 EC$_{50}$ values of three potential CD4 binding site antibody- containing serum samples.
(DOCX)

Table S2 Neutralization assay with HIV-1$_{JR-FL}$ and HIV-2/HIV-1 MPER chimeras using monoclonal antibody controls.
(DOCX)

Acknowledgments

Author Contributions

Conceived and designed the experiments: JSG CJA. Performed the experiments: JSG VC NL GL. Analyzed the data: JSG CJA VC BB GL CK. Contributed reagents/materials/analysis tools: CJA BB DNF CK BHJ RLM. Wrote the paper: JSG CJA. Critically reviewed and edited the paper: JSG CJA VC GL BB NL DNF CK BHJ RLM.

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