HIV

Autologous neutralizing antibodies increase with early antiretroviral therapy and shape HIV rebound after treatment interruption

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Early initiation of antiretroviral therapy (ART) alters viral rebound kinetics after analytic treatment interruption (ATI) and may play a role in promoting HIV-1 remission. Autologous neutralizing antibodies (aNAbs) represent a key adaptive immune response in people living with HIV-1. We aimed to investigate the role of aNAbs in shaping post-ATI HIV-1 rebound variants. We performed single-genome amplification of HIV-1 *env* from pre-ART and post-ATI plasma samples of 12 individuals who initiated ART early after infection. aNAb activity was quantified using pseudoviruses derived from the most common plasma variant, and the serum dilution that inhibited 50% of viral infections was determined. aNAb responses matured while participants were on suppressive ART, because on-ART plasma and purified immunoglobulin G (IgG) demonstrated improved neutralizing activity against pre-ART HIV-1 strains when compared with pre-ART plasma or purified IgG. Post-ATI aNAb responses exerted selective pressure on the rebounding viruses, because the post-ATI HIV-1 strains were more resistant to post-ATI plasma neutralization compared with the pre-ART virus. Several pre-ATI features distinguished post-treatment controllers from noncontrollers, including an infecting HIV-1 sequence that was more similar to consensus HIV-1 subtype B, more restricted proviral diversity, and a stronger aNAb response. Post-treatment control was also associated with the evolution of distinct N-glycosylation profiles in the HIV-1 envelope. In summary, aNAb responses appeared to mature after early initiation of ART and applied selective pressure on rebounding viruses. The combination of aNAb activity with select HIV-1 sequence and reservoir features identified individuals with a greater chance of post-treatment control.

INTRODUCTION

For most people living with HIV-1, viral load rebounds rapidly after treatment interruption (*1*, *2*); however, there are rare individuals, termed post-treatment controllers (PTCs), who are able to suppress HIV-1 for a prolonged period of time after treatment interruption. These individuals are considered an ideal example of durable HIV-1 control and have the potential to provide substantial insight into the "natural" mechanisms of functional cure and sustained HIV-1 remission (*3*). We have previously shown that the timing of antiretroviral therapy (ART) initiation has a profound impact on the chances of becoming a PTC after treatment interruption (*4*). Specifically, early initiation of ART has been found to restrict the size and diversity of the HIV-1 reservoir (*5*, *6*) while preserving B cell antiviral immunity (*7*, *8*). Early ART initiation also alters viral rebound kinetics after analytic treatment interruption (ATI) and appears to play a role in reducing the barrier to HIV-1 remission (*4*, *9*). However, little is known about the underlying mechanisms by which early-treated individuals delay or control viral rebound and how rebounding variants are selected from the pool of intact proviruses.

Neutralizing antibodies (NAbs) represent a key adaptive immune response against a broad range of viruses (*10*) and play a major role in the immune response to HIV-1 (*11*, *12*). In the setting of HIV-1 infection, the primary target for autologous NAbs (aNAbs) is the trimeric envelope glycoprotein (Env) expressed on the surface of the virion and on the membrane of productively infected cells. These HIV-1–specific aNAbs initially arise during acute infection, with more potent aNAbs developing over the course of several months (*12*–*15*). In response, HIV-1 has developed formidable defense mechanisms that hinder the ability of the host to elicit an effective neutralizing antibody response. These include in part the conformationally dynamic nature of Env, a high mutation rate in response to selective immune pressure, the steric shielding of conserved epitopes by glycans, and the low spike density on the virion surface (*16*). Continuous viral replication in the absence of ART allows for rapid viral escape (*13*, *14*) and renders the humoral response largely ineffective in controlling HIV-1 infection (*16*, *17*).

Despite this, there is evidence that aNAbs can play a role in maintaining immune pressure for individuals with chronic HIV-1 infection, including the emergence of aNAb-resistant variants in individuals who started ART at the chronic stage of infection who experience viral rebound (*18*). A recently published report also demonstrated that, in these individuals who started treatment at the chronic stage, in vitro viral outgrowth assays modified to incorporate autologous antibodies selected for outgrowth variants that are resistant to neutralization and were more closely related to rebounding variants detected in vivo during treatment interruption

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(*19*). In this study, we used single-genome plasma viral sequencing of longitudinal time points and pseudovirus generation to investigate the role of aNAbs in viral rebound variant selection in a cohort of early-treated PTCs and post-treatment noncontrollers (NCs) undergoing an ATI. The results offer insight into the maturation of the humoral immune responses after early ART and the impact of aNAbs on the selection of rebounding viral variants.

RESULTS

aNAb responses mature during early initiation of ART

We evaluated 12 participants from the ACTG A371 study (six PTCs and six NCs) who initiated ART during early infection and subsequently underwent an ATI. The PTCs and NCs had comparable baseline demographics and laboratory characteristics (tables S1 and S2). The inclusion criteria for participants in this study are summarized in table S3. For each participant, we obtained a median of 75 (range from 18 to 172) single-genome *env* sequences from the pre-ART and post-ATI time points (table S4). We first assessed the evolution of aNAb responses immediately before ART initiation and shortly after the ATI. aNAb activity was evaluated using pre-ART, on-ART, and post-ATI plasma tested against pseudoviruses created with either pre-ART or post-ATI viral Env (Fig. 1A). To create the early post-ATI pseudoviruses, we incorporated single-genome *env* sequences from the earliest time point with detectable low viremia to minimize the extent of viral evolution. In pre-ART, the median viral load was 4.4 $\log_{10}$ HIV-1 RNA copies per ml, and only a weak aNAb activity was detected against contemporaneous pre-ART virus (Fig. 1B, red arrow). All participants were virologically suppressed (for a median of 44 weeks) before ATI. The median early and late post-ATI viral loads were 1.7 to 2.2 $\log_{10}$ HIV-1 RNA copies per ml, respectively, for PTCs and 3 to 3.6 $\log_{10}$ HIV-1 RNA copies per ml, respectively, for NCs. aNAb titers were calculated for pre-ART and post-ATI pseudoviruses incubated with plasma from both the pre-ART and post-ATI time points (Fig. 1B and fig. S1). Although there was little evidence of aNAb responses against contemporaneous viruses at the immediate pre-ART time point, there was evidence that aNAb responses increased during the period of suppressive ART, because aNAb titers against pre-ART viruses were significantly (paired P = 0.002) higher immediately after the treatment interruption [pre-ART versus early post-ATI median half-maximal inhibitory dilution (ID_{50}) plasma neutralizing titers ($1/x$): 19 versus 353; Fig. 2]. To measure aNAb activity using plasma containing ART, we performed two additional studies. First, we tested on-ART plasma against pseudoviruses with a modified *pol*-containing drug resistance mutations to the participants' ART. The results for six participants show that, compared with pre-ART plasma, on-ART plasma demonstrated significantly higher neutralizing titers versus pre-ART virus [median ID_{50} plasma neutralizing titers ($1/x$) in these six participants: 21 versus 496; paired P = 0.03; Fig. 2 and fig. S1]. To further confirm this observation, immunoglobulin G (IgG) was purified from pre-ART, on-ART, and early post-ATI plasma for four participants (two PTCs and two NCs) with sufficient plasma and tested for neutralizing activity against pre-ART and early post-ATI viruses. The results from these experiments also demonstrate that aNAb activity matures during early ART (fig. S2).

Viral variants that rebound immediately post-ATI are resistant to aNAb neutralization

We obtained a median of 23 (range from 6 to 38) single-genome sequences per participant at the early post-ATI time point and confirmed that viral diversity at this time point was extremely limited, with a median average pairwise distance (APD) of 0.17%. Compared with pre-ART viruses, early rebounding variants were found to be significantly more resistant to the neutralizing activity of early post-ATI plasma [between-group median ID_{50} plasma neutralizing titers ($1/x$): 353 versus 27; paired P = 0.04; Fig. 2]. Late post-ATI plasma neutralizing titers did not differ between early post-ATI and late post-ATI viruses (Fig. 2). Analysis of purified IgG isolated from the on-ART time points also demonstrated no neutralizing activity against early post-ATI viruses in contrast to the potent neutralization observed against pre-ART viruses (fig. S2).

PTCs are characterized by distinct HIV-1 sequences, reservoirs, and aNAb features

We first evaluated longitudinal changes in aNAb activity between PTCs and NCs. Both groups demonstrated similar overall trends in aNAb responses: (i) very low titers of pre-ART aNAbs against contemporaneous virus, (ii) maturation of aNAb responses on ART, (iii) early post-ATI rebounding virus resistant to contemporaneous aNAb activity, and (iv) increasing aNAb activity against pre-ART and early post-ATI virus over time (fig. S3). Single-genome near-full-length proviral sequencing at the pre-ATI (on-ART) time point was performed for a subset of four PTCs and five NCs with available samples. A median of four proviral sequences with intact *env* were obtained per participant. The HIV-1 reservoir size was modestly smaller in PTCs [PTCs versus NCs: median 2.3 versus 3.8 HIV-1 copies per 10^6 peripheral blood mononuclear cells (PBMCs) for total proviral genomes and median 0.35 versus 1.23 for intact proviral genomes; fig. S4]. Overall plasma and proviral diversity were fairly limited in this early-treated group of participants (fig. S5, A and B). The diversity of plasma viruses was not different in PTCs versus NCs (fig. S5C).

We next assessed the extent of sequence changes between the pre-ART and early post-ATI plasma HIV-1 viruses. The extent of this plasma virus divergence was strongly associated with the strength of aNAb activity during the early-ATI period against pre-ART viruses (Spearman r = 0.7, P = 0.02; Fig. 3), suggesting that more viral *env* mutations were needed to escape from stronger aNAb responses. In addition, greater proviral diversity appeared to increase the chances of viral escape, because there was a robust association between proviral diversity and eventual plasma viral divergence (r = 0.89, P = 0.02). Furthermore, higher plasma viral divergence was linked with increased post-ATI viral load (r = 0.8, P = 0.007), suggesting that greater viral divergence and escape can lead to higher degrees of viral rebound (Fig. 3).

Infecting (pre-ART) HIV-1 sequences from the PTCs were more genetically similar to the consensus subtype B sequence (PTC versus NC pre-ART plasma sequence root-to-tip distance: 0.09 versus 0.1; P = 0.02; fig. S6), and a shorter root-to-tip distance to consensus B virus was also associated with the development of stronger early post-ATI aNAb responses against pre-ART virus (r = −0.67, P = 0.01; Fig. 3). The combination of lower proviral diversity, infecting virus that is more genetically similar to consensus subtype B, and stronger aNAb response largely distinguished PTCs from NCs (Figs. 3 and 4). This suggests that, even among

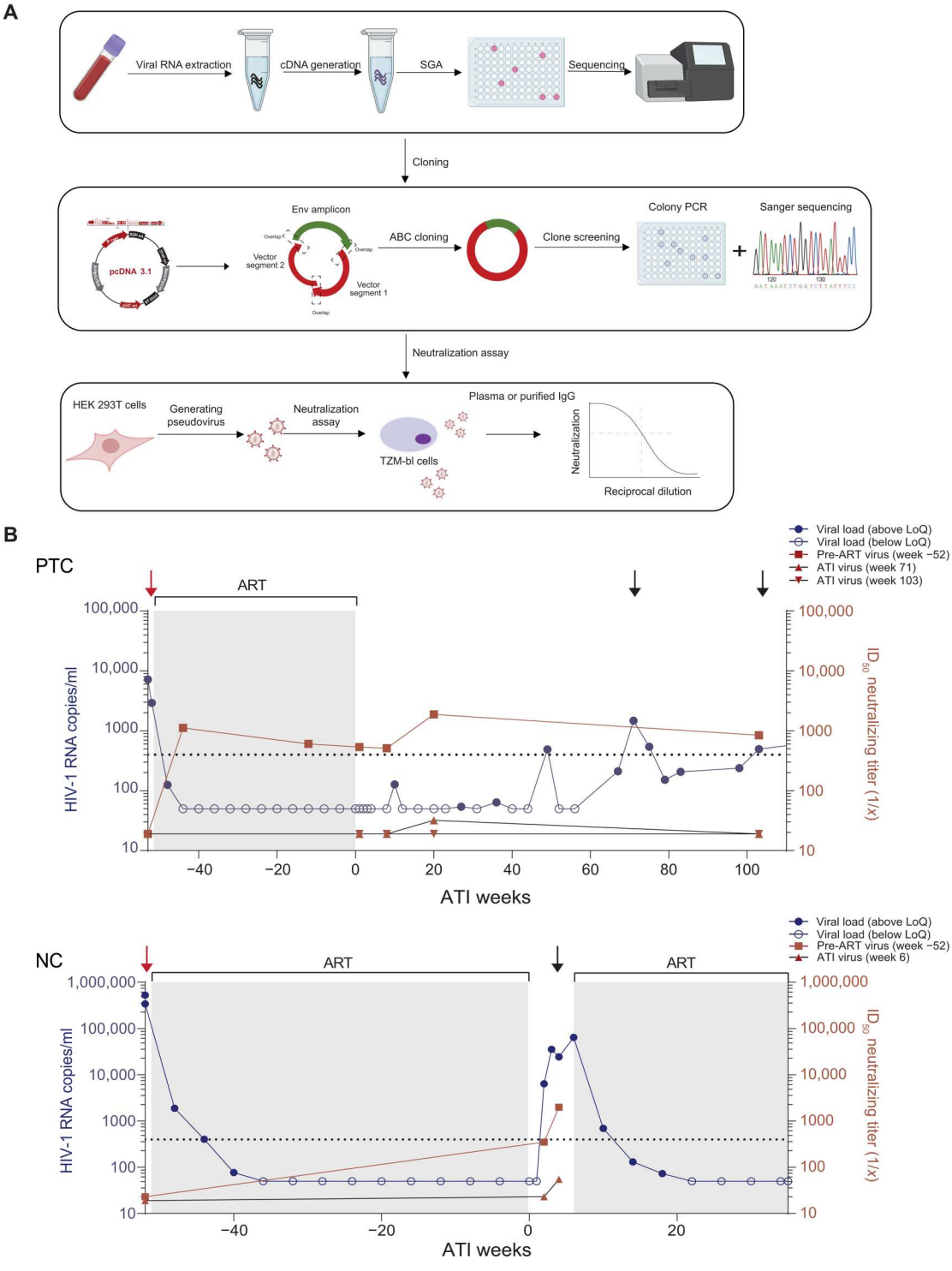

Fig. 1. Experimental design and examples of viral load and neutralizing titers for samples from a PTC and an NC. (**A**) The experimental workflow for evaluating aNAb responses is shown. SGA, single-genome amplification; HEK 293T, human embryonic kidney 293T. (**B**) aNAb titers are shown for representative participants, one PTC (top) and one NC (bottom), over time. Each data point represents aNAb titers for plasma collected from that time point against either pre-ART HIV-1 envelope (*env*) (red line) or post-ATI *env* (black line). Gray shaded areas represent time on ART; red and black arrows show pre-ART and post-ATI time points used for *env* isolation and construction of pseudoviruses, respectively; horizontal dashed lines represent the 400 HIV-1 RNA copies per ml threshold for the definition of post-treatment control; open circles represent viral load values that are below the assay limit of quantification.

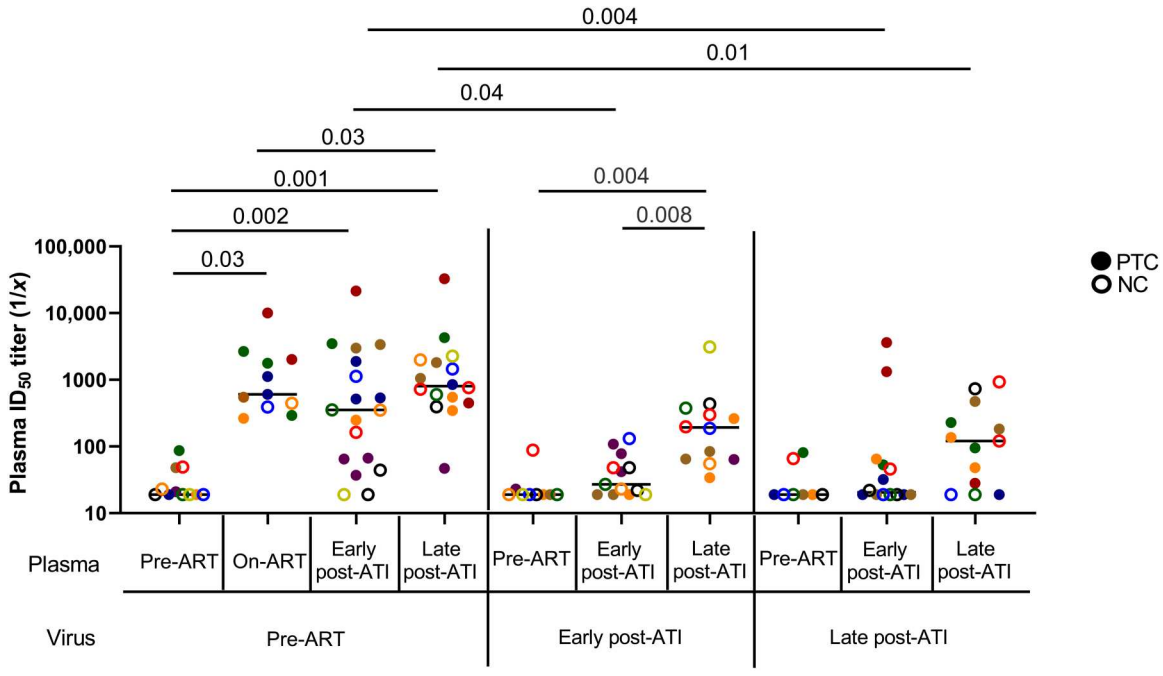

Fig. 2. Post-ATI plasma demonstrates improved neutralizing activity against pre-ART virus as compared with pre-ART plasma. One to three pseudoviruses were generated per participant time point, with the median value for each participant used for statistical comparison. Open circles represent PTC values, and closed circles represent NC values. No adjustment was made for multiple comparisons. Horizontal lines indicate median values, including all participants from the same time frame (including those with multiple measures within the same time frame).

individuals treated during early infection, there are certain viral and host characteristics that can be assessed before treatment interruption that may be used to identify those with a greater chance of post-treatment control.

Viral neutralization sensitivity to broadly neutralizing antibodies and heterologous immune serum were limited in PTCs and NCs

We evaluated the neutralization sensitivity profiles of pre-ART and early post-ATI HIV-1 *env* from PTCs and NCs using panels of broadly neutralizing antibodies (bNAbs) and heterologous clade B HIV-1$^+$ immune serum to evaluate tier phenotype (*20*). The bNAb panel consisted of antibodies targeting the CD4 binding site (b12, 3BNC117, and soluble CD4), the V3-glycan (10-1074 and PGT121), the V2-glycan (PG9 and PGDM1400), and membrane-proximal external region (MPER) (4E10 and 2F5) epitopes. Monoclonal antibody (mAb) 17b, which targets a co-receptor binding site epitope and can be used to define highly sensitive tier 1 viruses, was also used. Overall, PTC and NC pre-ART or post-ATI Env pseudoviruses demonstrated similar profiles of neutralization sensitivity to the mAb panel. Except for higher sensitivity to PG9 by PTC pre-ART virus and to 2F5 by NC pre-ART virus, no differences were observed, and no viruses were found to be sensitive to 17b (fig. S7, A and B). We also profiled the global neutralization sensitivity of PTC and NC viruses by testing against a panel of eight heterologous chronic HIV-1$^+$ clade B polyclonal immune serum samples. Env pseudoviruses from PTCs and NCs did not exhibit high degrees of sensitivity to heterologous HIV-1$^+$ immune serum, and we detected no differences in the mean neutralization titers between the two cohorts (fig. S7C). From these results, we conclude that Env pseudoviruses from both PTCs and NCs exhibit neutralization profiles that are consistent with a tier 2 phenotype, and no defined differences in the overall neutralization sensitivity could be detected.

We further profiled the potency and breadth of neutralizing antibodies in PTC plasma samples from week 24 post-ATI by testing plasma from 28 PTCs (including the 6 from this study; table S5) (*4*) against a panel of heterologous clade B tier 1 and tier 2 reference viruses (*21*). Although almost all plasma samples demonstrated potent neutralizing activity against the sensitive tier 1A virus MN-3, only limited neutralization activity was observed against tier 2 isolates (fig. S7D). These data suggest that post-treatment control is not specifically related to the development of exceptional broadly neutralizing antibodies.

Amino acid changes occur in viral sequences of HIV-1 *env* over time

Given the observed differences in aNAb sensitivity between pre-ART and post-ATI rebound viruses, we characterized longitudinal amino acid sequence changes in the HIV-1 *env*. We aligned sequences from all three time points (pre-ART, early post-ATI, and late post-ATI), with the consensus pre-ART HIV-1 *env* as the reference. The alignments with amino acid changes compared with the pre-ART consensus sequence are shown for each participant (Fig. 5, A and B, and fig. S8), and a heatmap was created of amino acid changes across participants/time points (Fig. 5C). There was increasing accumulation of HIV-1 *env* mutations over time, with no differences between PTCs and NCs (fig. S9A). These changes were mainly in gp120, specifically the V1, C3, and V4 domains of HIV-1 *env* (fig. S9, B and C).

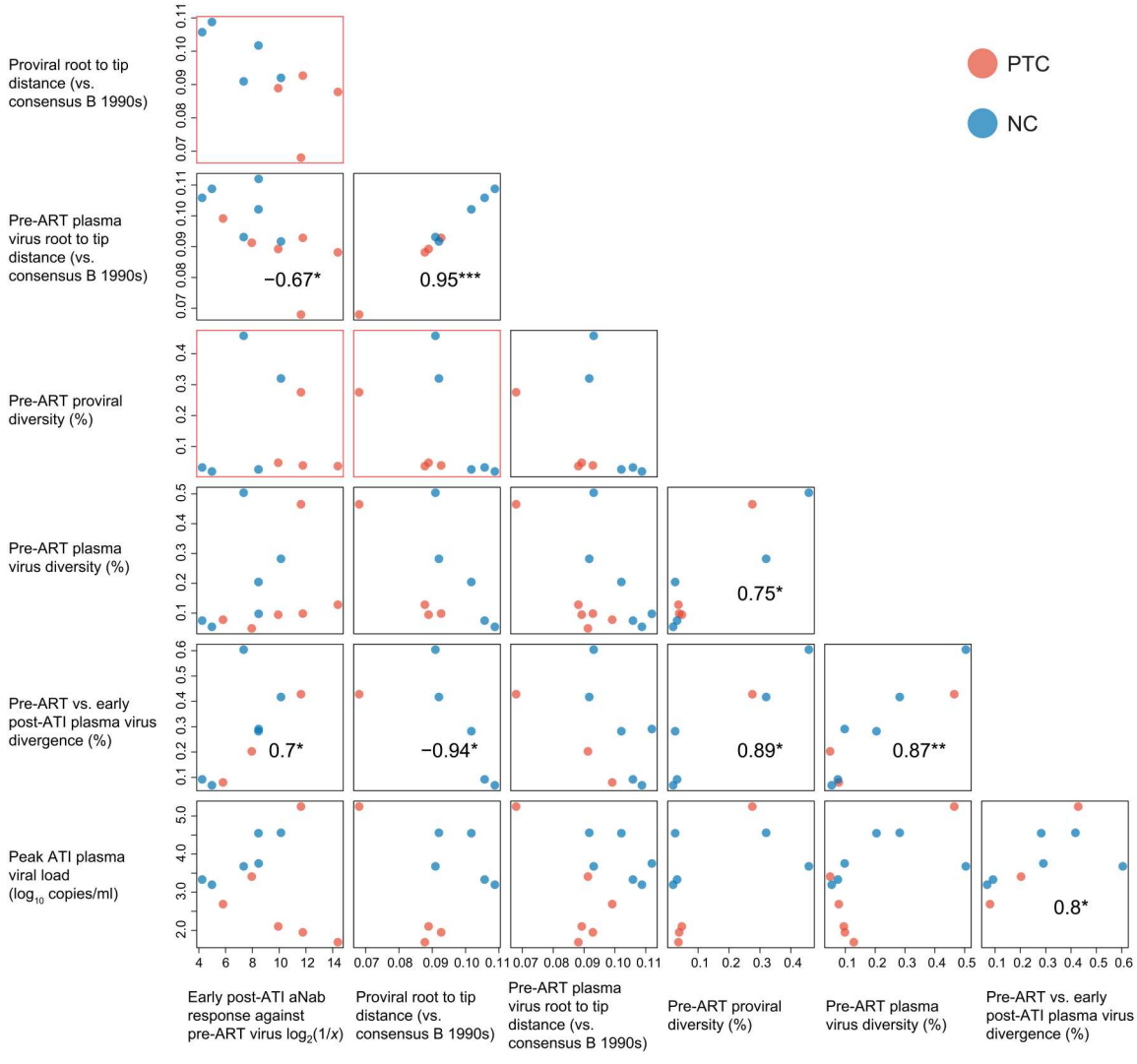

Fig. 3. Correlations of aNAb responses, proviral and plasma viral sequence divergence, diversity, and post-ATI viral load peak. Spearman rho values are shown with *P < 0.05, **P < 0.01, and ***P < 0.001. Red boxes denote factors that were used to categorize PTCs and NCs in Fig. 4.

HIV-1 Env N-glycosylated sites differ over time and between PTCs and NCs

N-glycosylated sites in HIV-1 Env play a critical role in viral evolution and immune escape against humoral immune responses (*22*). We assessed the number and longitudinal changes in N-glycosylated sites in HIV-1 Env and detected dynamic changes over time (fig. S10A). These changes were densely located in the gp120 domain of HIV-1 Env, specifically in V1 and V4 (fig. S10, B and C). Despite the similar degrees of aNAb activity between groups, PTCs and NCs demonstrated differences in their N-glycosylated sites in HIV-1 Env. This includes distinct locations for potential N-glycosylated sites between PTCs and NCs (Fig. 6A). In addition, we noted differing trends in N-glycosylated site numbers, with N-glycosylated sites decreasing in PTCs (P < 0.001) but remaining stable in NCs (Fig. 6B). In late post-ATI, NCs had a significantly higher number of potential N-glycosylated sites in Env than PTCs (P = 0.02).

DISCUSSION

Among the top priorities of the HIV-1 field is the search for therapeutic interventions that can lead to sustained ART-free HIV-1 remission. However, one of the key knowledge gaps in the field is our incomplete understanding of the mechanisms behind post-treatment control, including how early ART initiation increases the chances of HIV-1 remission. Early initiation of ART restricts the seeding of the HIV-1 reservoir (*5*), reduces inflammation (*23*), and preserves host immune responses (*24*). Although early ART has been shown to preserve numbers of HIV-1–specific B cells (*7*), the effect of early ART on the efficacy of humoral immune responses has been incompletely studied. We evaluated the development and impact of aNAbs in a cohort of early-treated PTCs and NCs. We found that aNAb responses matured while on suppressive ART and exerted selective pressure on the rebounding viruses. In addition, several pre-ATI features distinguished PTCs from NCs, including stronger aNAb responses, an infecting sequence more similar to consensus B, and more restricted proviral diversity.

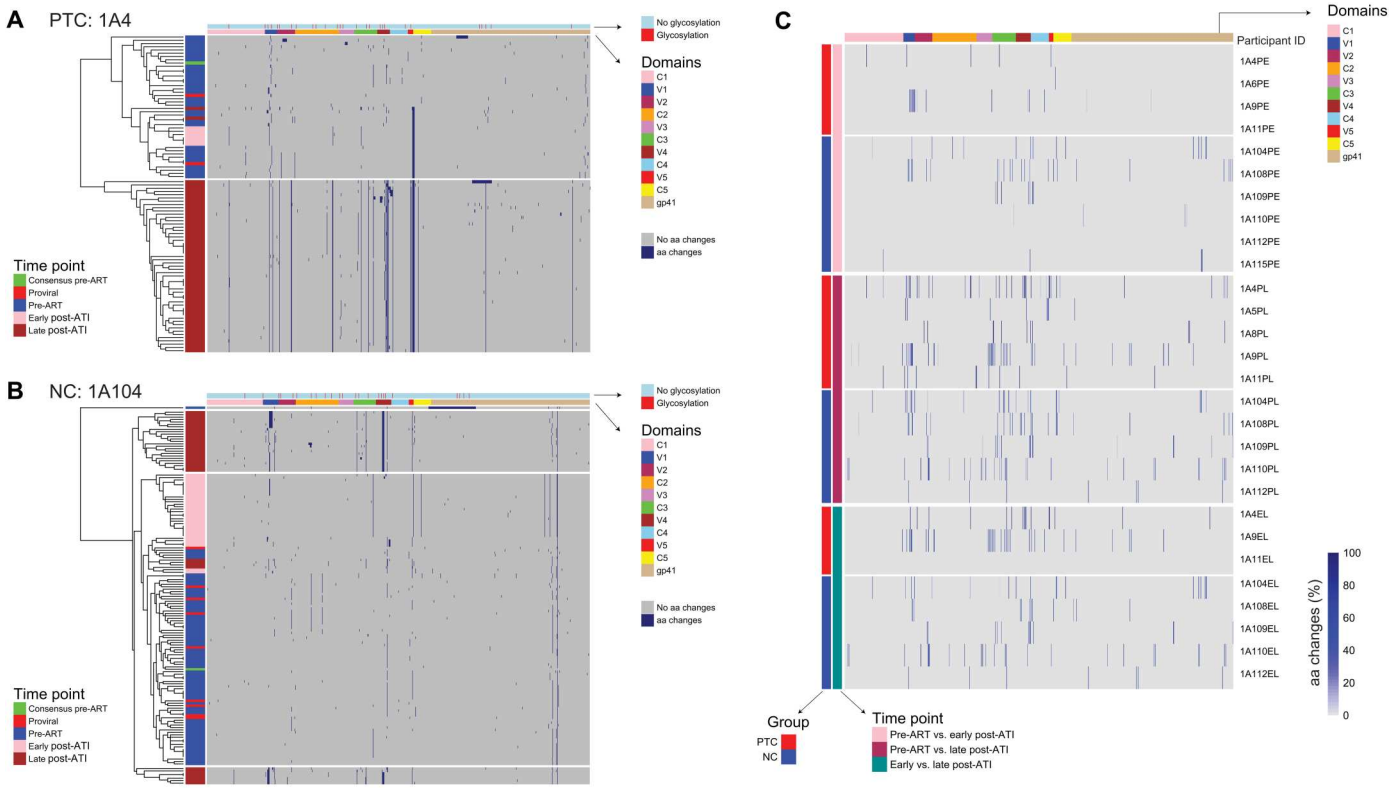

Fig. 5. Amino acid (aa) sequences in the HIV-1 envelope change over time for both PTCs and NCs. (**A** and **B**) Example of amino acid changes over time are shown for a PTC (A) and an NC (B). (**C**) Changes in amino acid sequences of the HIV-1 envelope are shown for different time points and different participants.

Post-treatment control was also associated with the evolution of distinct N-glycosylation profiles in the HIV-1 Env. The combination of aNAb activity with certain HIV-1 sequence and reservoir features may be able to identify individuals with a greater chance of post-treatment control.

Humoral immunity matures in response to persistent exposure to antigen. After HIV-1 infection, the initial antibody response is non-neutralizing and develops over to time to gain the capacity to neutralize (*25*). Thus, there have been questions regarding the impact that early ART may have on the maturation of the humoral immune response, especially given previous reports that ART treatment in chronically infected participants is associated with relatively low titers of HIV-1–specific IgG (*26*) and neutralizing antibody titers (*27*). Although ART suppresses viral replication to the extent that it is no longer detectable by commercial plasma HIV-1 viral load assays, there are several ways by which persistent antigenic exposure could be driving continued B cell maturation. ART by itself does not eliminate HIV-1–infected cells, and persistent RNA transcription and residual viremia are detectable in a large subset of individuals on ART when using an ultrasensitive assay (*28*, *29*). In addition, HIV-1 virions can be trapped and retained in follicular dendritic cells for many months after ART initiation (*30*), leading to prolonged antigen availability within germinal centers. A recent study on the effect of early ART in individuals with acute infection reported that antibody development persisted during suppressive ART (*31*), but this study did not evaluate aNAb responses and did not include a treatment interruption. In this study, we assessed the aNAb responses of 12 early-treated individuals, including the viral and plasma samples from pre-ART and early and late post-ATI time points. In contrast to previous studies in individuals who initiated treatment during the chronic stage of infection, we found that early initiation of suppressive ART allows for the maturation of the anti–HIV-1 aNAb response with strengthening of the on-ART and early post-ATI aNAb responses against pre-ART virus.

Historically, which viral variants rebound after ART interruption was thought to largely be due to a stochastic process. Compared to pre-ART virus, we found that rebounding HIV-1 variants were more resistant to contemporaneous post-ATI autologous plasma neutralization, suggesting that viral variants contributing to viral rebound do not arise purely from a stochastic process but are shaped by host immune pressures, including aNAb responses. These results provide an in vivo corollary to in vitro studies showing that the addition of autologous IgGs to viral outgrowth assays blocks the outgrowth of a large fraction of reservoir viruses and helped improve the prediction of which variants will rebound after ATI (*19*). Furthermore, there has also been recent evidence that rebounding HIV-1 variants may be more interferon resistant (*32*), and our results add to the data demonstrating that rebounding variants are selected by their ability to escape from host immune responses.

We found that viral divergence correlates strongly with the development of stronger aNAb responses against pre-ART virus, which suggests either that more *env* mutations are needed to escape stronger aNAbs response or that stronger immune responses induce greater viral evolution. Previous studies have confirmed that

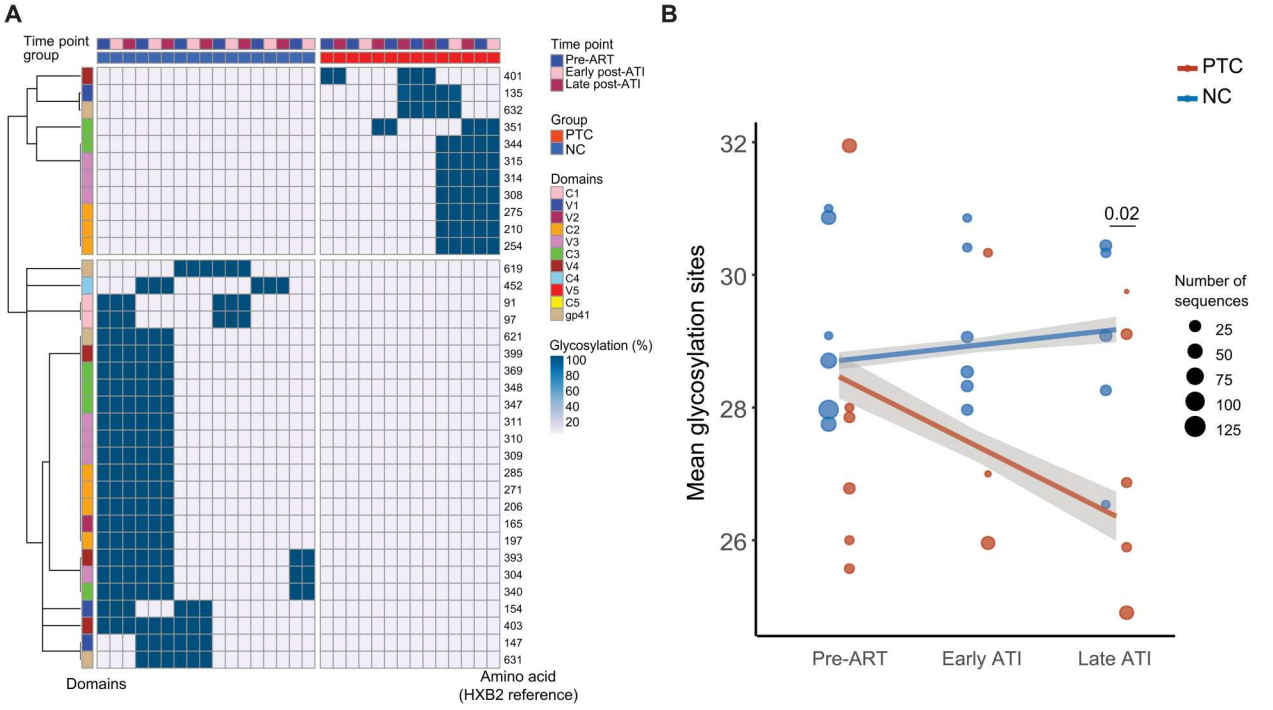

Fig. 6. PTCs and NCs have distinct N-glycosylation features. (**A**) A heatmap summarizing distinct N-glycosylation sites between PTCs and NCs is shown. (**B**) A comparison of the numbers of N-glycosylated sites between PTCs and NCs over time is shown. Data in (B) were analyzed using a generalized estimating equation to account for repeated measures, and Benjamini-Hochberg adjustment was made for multiple comparisons.

proviral sequences can be closely linked to post-ATI rebounding viruses (*33*). Our results further showed that the diversity of pre-ATI proviral sequences also correlates closely with the divergence of viruses from pre-ART to early post-ATI time points, suggesting that greater proviral diversity increases the chances of viral escape against host aNAb and other immune responses. We also found that increased escape can lead to higher initial viral loads during early post-ATI, providing evidence that aNAb responses may play a key role in post-treatment viral control.

Phylogenetic analysis showed that PTC viral sequences were more similar to the subtype B consensus. Given the early ART initiation and limited intra-host viral diversity, this appears to be an intrinsic property of the transmitted variant. The finding that a shorter genetic distance to consensus subtype B virus and development of stronger aNAb response against pre-ART virus points to both viral and immune factors in mediating post-treatment control. Specifically, these data suggest that robust aNAb responses may be more easily developed against infecting strains that are more similar to consensus subtype B. These findings are supported by previous studies showing that older HIV-1 sequences are more sensitive to heterologous neutralization, whereas continued evolution of HIV-1 may lead to increased neutralization resistance and suboptimal elicitation of neutralizing antibody responses (*34*–*37*). However, we note that the phenotypic analyses of pre-ART viruses from PTCs and NCs did not detect any differences in neutralization sensitivity profiles.

Individuals who initiated ART during early HIV-1 infection have consistently been identified as having an increased chance of HIV-1 remission after ART interruption (*3*, *4*, *38*). However, the mechanism behind post-treatment control remains largely undefined. Most studies of HIV-1 control have focused on HIV-1–specific T cell responses (*39*, *40*), and T cell responses are likely to play a critical role in reducing viral load to the set point during early infection (*41*). However, relatively little is known about the contribution of neutralizing antibodies in post-treatment control after early ART initiation. There are simian immunodeficiency virus studies in nonhuman primates that suggest that NAbs might contribute to post-infection viral suppression and infection control (*42*, *43*). A recent report identified robust post-ATI aNAb activity as an unusual characteristic of a PTC, although virus from only one initial post-ATI time point was tested in that study (*44*). In this study, we found that the combination of aNAb activity with certain HIV-1 sequence and reservoir features were able to identify individuals with a greater chance of post-treatment control. Although aNAb responses may represent only one component of an effective post-ATI immune response, these results provide important confirmation that key viral and immune parameters could be measured to identify those with a greater chance of post-treatment control.

The presence of a glycosylated shield appears to be an important mechanism for viral immune escape (*45*, *46*), and we identified distinct patterns of dynamic change in N-glycosylated sites between PTCs and NCs. Differential hotspots for changes in N-glycosylation were also identified within the V1 and V4 domains of gp120 that highlight key sites of humoral immune pressure that have been found in prior studies to lead to monoclonal neutralizing antibody resistance (*47*). Overall, these results suggest that distinct humoral immune responses may be present in the setting of HIV-1 remission that are reflected in differential pathways of intrahost viral evolution.

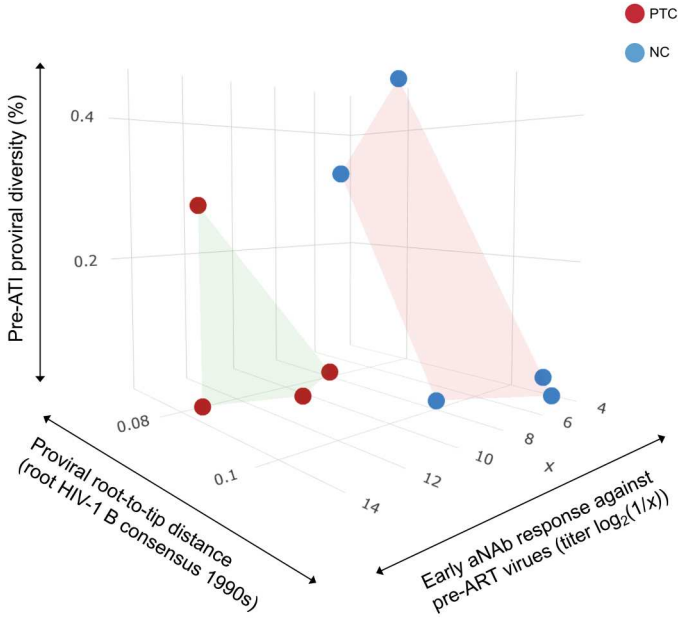

Fig. 4. PTCs can be differentiated from NCs by pre-ATI proviral diversity, pre-ATI proviral root-to-tip distance from HIV-1 subtype B consensus, and early post-ATI aNAb response against pre-ART viruses. Subtype B consensus was generated from sequences obtained between 1990 and 2000.

This study has a few notable limitations. First, most participants of this study initiated ART around Fiebig stages 3 to 5 and received approximately 1 year of ART before treatment interruption (*48*). The strength and trajectory of aNAb maturation for individuals who initiated ART during the very earliest stages of infection (Fiebig stages 1 and 2) or after longer durations of ART are unknown. Additional studies are also needed to assess the role of aNAb responses in individuals undergoing treatment interruption after starting treatment during the chronic phase of infection. Last, because of the intensive nature of the assays, including longitudinal single-genome plasma/proviral sequencing and pseudoviral neutralization titers, our sample size was limited to six PTCs and six NCs.

In conclusion, we report a comprehensive assessment of the proviral plasma sequence and aNAb responses for a cohort of early-treated PTCs and NCs. Despite the early initiation of ART, aNAb activity was able to mature and exert selective pressure on rebounding variants. Our results also show that the combination of aNAb activity with certain HIV-1 sequence and reservoir features (sequence similarity to consensus B and lower proviral diversity) could differentiate PTCs from NCs, even among this more homogeneous population of early-treated individuals. These results provide a mechanistic framework to explain the ability of early ART initiation to lower the barrier to HIV-1 remission. Additional studies are needed to expand the analysis to other aspects of the antiviral immune response and to the study of aNAb responses in those who initiated ART during even earlier or at later stages of HIV-1 infection.

MATERIALS AND METHODS

Study design

We evaluated longitudinal samples collected from 12 participants (6 PTCs and 6 NCs) of the ACTG 371 study, a prospective ART treatment trial of acutely infected individuals (*49*). Participants were enrolled in early HIV-1 infection and initiated ART during the approximate Fiebig stages 3 to 5. Acute HIV infection was defined as detectable plasma HIV RNA ≥2000 copies per ml by reverse transcription polymerase chain reaction (RT-PCR) within 14 days before study entry and one of the following: (i) negative enzyme-linked immunosorbent assay (ELISA) within 14 days of study entry, (ii) positive ELISA but negative or indeterminate Western blot within 14 days of study entry, or (iii) positive ELISA and Western blot within 14 days of study entry but with documented negative ELISA or plasma RT-PCR (<2000 copies per ml) within the 30 days before study entry. Recent HIV seroconversion was defined as (i) positive ELISA and Western blot within 14 days of study entry but with documented negative ELISA or plasma RT-PCR (<2000 copies per ml) within days 31 to 90 before study entry or (ii) positive ELISA and Western blot plus a nonreactive, less-sensitive (detuned) ELISA in participants with a $CD4^+$ T cell count >200/mm^3 all documented within 21 days (≤14 days preferred) of study entry.

After a median of approximately 52 weeks of ART, participants who maintained a viral load below 50 HIV-1 RNA copies per ml underwent an ATI. We had previously identified PTCs in ACTG 371 as defined by those who were able to maintain post-ATI viral loads ≤400 copies per ml at two-thirds or more of time points for ≥24 weeks (*4*). NCs were participants who demonstrated viral rebound after ATI and did not meet the PTC criteria. Participants were selected randomly from ACTG 371 participants meeting these criteria and with sufficient stored plasma and PBMC samples. For each participant, pre-ART and post-ATI plasma samples were selected for RNA extraction and testing of aNAb titers. Less than 24 weeks after ATI was considered early post-ATI, and more than 24 weeks after ATI was defined as late post-ATI.

Single-genome sequencing and genetic analysis

The overall workflow for evaluating aNAb responses is outlined in Fig. 1A. Plasma viral RNA was extracted using either QIAamp Viral RNA Mini Kits (QIAGEN) or the single-copy assay viral extraction protocol for samples with very low viral loads (*50*). Single-genome sequencing (SGS) of HIV-1 pro-rt-*env* was performed with some modifications to previously described methods (*51*, *52*). Extracted plasma HIV-1 RNA was reverse-transcribed into cDNA in a reaction mixture of 5× SuperScript IV (SSIV) buffer, 2.5 mM deoxynucleoside triphosphate, 0.1 M dithiothreitol, RNaseOUT (40 U/μl), Invitrogen SuperScript IV Reverse Transcriptase (200 U/μl), and 10 μM antisense primer p9017 R (5′-TAAGTCATTGGTCTTAAAGGTA-3′) followed by addition of ribonuclease H (Thermo Fisher Scientific).

Before amplifying the synthesized cDNA, we performed end point dilution such that less than 25% of the plate wells had successful amplification. Nested PCR was performed with 10× PCR buffer, 50 mM $MgSO_4$, 2.5 mM deoxynucleoside triphosphate, 10 μM primers, and Platinum Taq DNA Polymerase High Fidelity (5 U/μl; Invitrogen). First-round PCR was performed with forward primer 1312-F (5′-TTATCAGAAGGAGCCACCCC-3′) and

reverse primer p9010R (5′-CATTGGTCTTAAAGGTACCTGAGG-3′). Two microliters of the first-round PCR product were added to the second-round PCR, which contained the forward primer 2065F (5′-TGTACTGAGAGACAGGCTAATTTTT-3′) and reverse primer *env*-in-R (5′-GTCTCGAGATACTGCTCCCACCC-3′). PCR amplicons were sheared, and Illumina TruSeq–compatible barcoded libraries were constructed and pooled.

We performed sequencing on the Illumina MiSeq platform with the 150–base paired–end module, and amplicons were assembled using the de novo assembler UltraCycler v1.0. Subsequently, the resulting single-genome sequences were aligned using ClustalW. To ensure that there was no cross-contamination of participants′ samples, we generated a neighbor-joining (NJ) tree including all sequences obtained and confirmed that all SGS clustered appropriately.

Near-full-length HIV-1 proviral sequencing

Single-genome, near-full-length proviral sequences were performed as previously described (*53*). Briefly, limiting-dilution proviral amplification was performed, and DNA was extracted from PBMCs using the QIAamp DNA Mini Kit (QIAGEN). Isolated DNA was amplified using limiting-dilution nested PCR amplification as described previously. PCR amplicons were sequenced using the Illumina MiSeq platform. Continuous HIV-1 proviral DNA sequences were assembled, and the sequences were aligned to HXB2 to identify sequence defects such as internal deletions, premature stop codons, out-of-frame mutations, internal inversions, and packaging signal defects. The sequences were also tested using the Los Alamos HIV-1 Sequence Database Hypermutated program to identify hypermutated sequences. Proviral sequences that lacked the defects mentioned previously were classified as intact (*54*).

env cloning and transformation

We cloned *env* sequences selected from the largest clonal or phylogenetic clusters using overlapping PCR based on ABC cloning as previously described (*55*). This method uses PCR reactions to combine an *env* gene with a modified pcDNA 3.1 vector. The pcDNA 3.1 vector backbone was amplified using primers to create a linear product with a 5′ and 3′ region homologous to the 3′ and 5′ regions of the previously amplified *env* gene, respectively. The PCR product was then gel-purified using the NucleoSpin Gel and PCR Cleanup Kit to remove traces of template DNA. The previously amplified *env* amplicons were then combined with the linearized vector in a PCR using primers positioned where the 3′ end of the vector overlapped with the 5′ end of the *env* gene product. The resulting product was a circularized vector containing the *env* gene. The combination reaction was done using Phusion DNA polymerase (New England Biolabs) (*55*).

The resulting PCR product was visualized on an agarose gel and, upon confirmation of the correct-sized band, 1 μl of PCR product was transformed to *Escherichia coli* JM109 single-use competent cells (Promega). Recombinant clones were screened, and plasmids were verified by colony PCR using Platinum Taq Polymerase II and Illumina Sanger sequencing. Clones with intact HIV-1 *env* in the correct orientation were selected for pseudovirus generation use in neutralization assay.

Pseudovirus preparation and titration

The plasmids were purified using the Maxi Prep Invitrogen Kit (Thermo Fisher Scientific). Five micrograms of the Maxi Preps products and 10 μg of helper plasmid with an *env*-deficient HIV-1 backbone vector (pSG3ΔEnv) were transfected into 293T/17 human embryonic kidney fibroblast cells (the American Type Culture Collection) using the FuGENE protocol (Roche Molecular Biochemicals). In experiments testing plasma samples obtained during periods of ART treatment, pseudoviruses produced using an *env*-deficient backbone vector with three site-directed mutations to confer resistance to certain classes of ART drugs (SG3ΔEnv-K101P.Q148H.Y181C) were used. The 293T cells were maintained in Dulbecco′s modified Eagle′s medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U of penicillin per milliliter, and 100 μg of streptomycin per milliliter (DMEM complete). After 48 hours in culture, the supernatant was filtered through a 0.45-μm filter, aliquoted, and stored at −80°C until use. The infectious dose of each virus stock was determined by infecting TZM-bl cells with a range of viral dilutions and measuring luminescence as previously described (*56*). TZM-bl cells were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health.

IgG purification from plasma

One milliliter of plasma sample from each participant was subjected for IgG chromatography column purification using an rProtein G Sepharose Fast Flow antibody purification resin (Cytiva). Each resin chromatography column was washed five times with 1 ml of 1× phosphate-buffered saline. Purification columns were eluted with the Thermo Scientific Pierce IgG Elution Buffer (Thermo Fisher Scientific). The eluted fraction was concentrated using Millipore Amicon Ultra 4 Centrifugal Filters (Millipore). The purified IgG was quantified using the Bradford assay (Bio-Rad) with bovine serum albumin (Bio-Rad) as a standard in serial dilutions, measured at 595 nm. Purified IgG was applied for neutralization assays.

Neutralizing antibody assay

Neutralization assays were conducted using TZM-bl cells as previously described (*57*). Briefly, patient plasma was tested in duplicate in 96-well plates using a 1:20 dilution and serially diluted threefold seven times. Purified plasma IgG samples were tested using a primary concentration of 500 μg/ml and serially diluted threefold. HIV-1 Env pseudovirus was added to plasma or antibody serial dilutions, and plates were incubated for 1 hour at 37°C. TZM-bl cells were then added at 1×10^4 cells per well with DEAE-Dextran at a final concentration of 11 μg/ml. After 48 hours of incubation at 37°C, plates were harvested using Promega Bright-Glo luciferase, and luminescence was detected using a Promega GloMax Navigator luminometer. Plasma dilutions that inhibited 50 or 80% of viral infection were determined (ID_{50} and ID_{80} titers, respectively). Neutralization assays were conducted in a laboratory meeting Good Clinical Laboratory Practice quality assurance criteria.

Statistical analysis

Raw, individual-level data for experiments where $n < 20$ are presented in data file S1. We used the paired Wilcoxon signed-rank test for comparing the neutralizing antibody titers between different time points for pseudoviruses and plasma samples. No adjustments

were made for multiple comparisons because of the limited numbers of comparisons and the exploratory nature of this analysis. When a participant had multiple plasma time points tested for neutralizing activity during a study time period (e.g., early post-ATI), the mean neutralizing antibody titer was used for statistical analysis. Mann-Whitney *U* tests were also applied for comparing antibody titers between PTCs and NCs. These comparative statistics were performed in GraphPad Prism 9. R version 4.1.0 was used to compare sequences of each participant and for making heatmaps. To calculate and compare the amino acid and N-glycosylated site changes between different time points, we used the GenSig tool of the Los Alamos HIV-1 sequence database (*58*) and evaluated adjusted *P* values given the large numbers of comparisons.

Population genetic diversity was calculated as APD between the sequences in each group and at each time point. Divergence was defined as APD between the groups. NJ phylogenetic analyses rooted to HXB2 as well as diversity and divergence calculations were done using MEGA6. Root-to-tip distances were extracted from maximum likelihood trees, which were rooted to the consensus made from HIV-1 subtype B sequences from 1990 to 2000 (HIV-1-B consensus 1990s) using the generalized time reversible model. Spearman correlation was used to calculate the correlation between aNAb responses and diversity, divergence, divergence per day, and root-to-tip distance. Correlation plots and three-dimensional figures were generated using the R packages "psych" and "plotly."

We used the R package "DESeq2" to evaluate differential N-glycosylated sites between PTC and NC groups in a compressive way. DESeq2 is a package capable of detecting differentially expressed genes using unadjusted/raw expression matrices (*59*). Here, we generated the N-glycosylation expression probability matrix by calculating the probability of certain conserved N-glycosylated sites having asparagine (N) for each participant. We then subjected the matrix to the DESeq2 pipeline. Fold changes in N-glycosylation and adjusted *P* values in each possible N-glycosylated site were generated. Sites with an adjusted *P* value of <0.05 were selected for further analyses. We used the R package "Pheatmap" to generate a heatmap demonstrating differential N-glycosylated sites between PTC and NC groups. A principal components analysis plot was then generated to illustrate the distinctions in PTC and NC groups.

We used a generalized estimating equation to account for repeated measurements in each participant. The R package "geepack" was used to calculate between-group differences between PTCs and NCs at each time point. Benjamini-Hochberg *P* value adjustment was used to account for multiple comparisons in certain analyses. *P* values less than 0.05 were significant in all analyses.

Supplementary Materials

This PDF file includes:
Figs. S1 to S10
Tables S1 to S5
References (60 and *61*)

Other Supplementary Material for this manuscript includes the following:
Data file S1
MDAR Reproducibility Checklist

View/request a protocol for this paper from *Bio-protocol*.

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Autologous neutralizing antibodies increase with early antiretroviral therapy and shape HIV rebound after treatment interruption

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