Early initiation of antiretroviral therapy (ART) in acute HIV infection (AHI) is effective in limiting seeding of the HIV viral reservoir, but little is known about how the resultant decreased antigen load affects long-term antibody development after ART. We report here that Env-specific plasma antibody levels and antibody-dependent cellular cytotoxicity (ADCC) increased during the first 24 weeks of ART and correlated with antibody levels persisting after 48 weeks of ART. Participants treated in AHI stage 1 had lower Env-specific antibodies levels and ADCC activity on ART than those treated later. Importantly, participants who initiated ART after peak viremia in AHI developed elevated cross-clade ADCC responses detectable one year after ART initiation even though clinically undetectable viremia was reached by 24 weeks. These data suggest that there is more germinal center activity in the later stages of AHI and that antibody development continues in the absence of detectable viremia during the first year of suppressive ART. Development of therapeutic interventions that can enhance earlier development of germinal centers in AHI and antibodies after ART initiation could provide important protection against the viral reservoir that is seeded in early treated individuals.
Anti-HIV antibody development up to one year after antiretroviral therapy initiation in acute HIV infection

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Competing Interests

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Abstract

Early initiation of antiretroviral therapy (ART) in acute HIV infection (AHI) is effective in limiting seeding of the HIV viral reservoir, but little is known about how the resultant decreased antigen load affects long-term antibody development after ART. We report here that Env-specific plasma antibody levels and antibody-dependent cellular cytotoxicity (ADCC) increased during the first 24 weeks of ART and correlated with antibody levels persisting after 48 weeks of ART. Participants treated in AHI stage 1 had lower Env-specific antibodies levels and ADCC activity on ART than those treated later. Importantly, participants who initiated ART after peak viremia in AHI developed elevated cross-clade ADCC responses detectable one year after ART initiation even though clinically undetectable viremia was reached by 24 weeks. These data suggest that there is more germinal center activity in the later stages of AHI and that antibody development continues in the absence of detectable viremia during the first year of suppressive ART. Development of therapeutic interventions that can enhance earlier development of germinal centers in AHI and antibodies after ART initiation could provide important protection against the viral reservoir that is seeded in early treated individuals.
Introduction

Initiation of antiretroviral therapy (ART) during acute HIV infection (AHI) is associated with multiple benefits, including decreased HIV reservoir size (1-6), and preservation of a homogenous viral reservoir with few escape mutations (7). While it was postulated that the benefits of very early ART initiation would allow for post-treatment control, a treatment interruption study in participants from Thailand who initiated ART in AHI stage 1, reported that all participants experienced viral rebound (8). However, the HIV-specific CD8 T cell response and Env-specific antibody (Ab) titers during viral rebound both correlated negatively with the maximum peak viral load reached during rebound, suggesting that these responses might partially control viral replication post-ART. We have found that early ART in AHI leads to the preservation of functional immune memory, but the decreased antigen burden also leads to lower numbers of memory T cells after long-term ART (unpublished observations). Similarly, about half of the people treated in AHI stage 1 either do not develop detectable HIV-specific Abs or serorevert within the first 24 weeks of ART (9). Beyond these limited observations, the impact of early treatment on the development of Abs against HIV has not yet been characterized.

During HIV infection, seroconversion does not occur until the time of peak viremia in stage 3 (S3) of acute HIV infection (AHI) (10, 11). The first free Abs detected in the blood, which recognize gp41 (12), can be cross-reactive to gut flora and show relatively high somatic mutation, indicating that they are likely derived from polyreactive memory B cells activated by Env protein during the early immune response (13, 14). Abs against gp120 arise later and are initially non-neutralizing (12). Using mathematical modeling it was determined that the early Ab response to HIV does not significantly affect viral load (12), and Ab-selected escape mutations
are not identified until development of autologous neutralizing Abs 3-12 months after infection (15-17). However, other antibody functions such as antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) mediated inactivation may potentially provide some protection prior to neutralization (18-21).

In this study, we sought to determine how ART affects the initial development of Env-specific Abs in participants of the Thai RV254 cohort who initiated treatment in the earliest stages of AHI. We analyzed changes in plasma Env-specific Ab levels, ADCC titers, ADCP scores, and plasma neutralization between the time of ART initiation and initial control of virus during the first 24 weeks of ART. Further, to look at the potential for Ab development after viral suppression, we measured changes in Ab levels and function after a subsequent 24 weeks of clinically suppressed virus.
Results

To characterize the effects of ART administration in AHI on the development of Ab responses directed against HIV, we measured plasma Ab levels and ADCC titers in 52 participants of the RV254 Thai cohort who were diagnosed in AHI and immediately started on ART (22). All participants were males of similar age, infected mostly with the CRF01_AE HIV clade, and the stage of infection was classified using the 4th generation HIV test (10, 11)(Table 1). With this staging the median time to S1 is 2 days shorter than for Fiebig I, resulting in lower median viral loads in S1, and S2 is reached approximately 5 days later (11). Classification for S3 and later is equivalent to Fiebig staging, with S3 being coincident with peak viral load (median 13 days after initial viral detection) and S4/5 encompassing the time of viral decline and establishment of set point viral load (10, 23). Participants treated in S1 of AHI had significantly lower viral loads (VLs) at diagnosis than other participants (p<0.001 vs S2, p<0.0001 vs S3, p<0.05 vs S4/5)(Table 1, Figure 1A). Ab levels and ADCC responses were measured in plasma samples from participants in the RV304 Thai cohort who had untreated (n=4) or treated (n=8) chronic HIV infection (CHI) for reference.

B cell activation, somatic hypermutation, and class-switching occur within germinal centers (GCs) of secondary lymphoid organs during the development of humoral responses (24). Plasma CXCL13 levels, which can be used as a marker of GC activity (25), were measured at enrollment and were higher with later stage of infection at diagnosis (p<0.05 S1 vs S3 and S4/5)(Figure 1B). We observed similar kinetics for the magnitude of plasma gp41-specific and gp120-specific Ab responses, both of which were significantly higher in participants in S4/5 than those in earlier stages of AHI (p<0.05), albeit much lower than in participants in CHI (Figure 1C). The quality
of the Ab response against HIV, rather than the Ab quantity, has been associated with better viral
control in elite controllers and with vaccine protection in the RV144 trial in Thailand (26-29).

Very few participants had positive ADCP phagocytic scores in AHI (Figure 1D), but participants
in S4/5 had significantly higher ADCC titers against CRF01_AE-infected targets than those in
earlier stages of AHI (p<0.05)(Figure 1E). Finally, as neutralizing antibodies do not arise until 3-
12 months after HIV infection, only low titer, non-specific inhibition of HIV strains was
measured in plasma from participants in AHI (Figure 1F). Together, these data suggest that
despite HIV DNA being measurable in the lymph nodes as early as AHI S1 (1), measurable GC
activity and Ab development is delayed until after peak viremia in AHI.

Viral suppression after ART initiation

It is clear that ART initiation has potent effects on the initial reduction of virus replication, but
complete viral suppression can take months. As expected from their lower VL at ART initiation
(Figure 1A), participants treated in S1 of infection had significantly shorter time to first
undetectable VL (≤20 copies/mL) than those treated in S3 and S4/5 of AHI (p<0.01)(Figure 2A).
By the 24-week visit, all but one participant had undetectable VL (≤20 copies/mL), and all
participants had VL ≤20 copies/mL at 36- and 48-weeks. To estimate the antigen load for each
participant, we calculated the VL area under the curve (AUC), which was defined as the sum of
the post-diagnosis AUC and an imputed AUC, which was modeled on data from the RV217
untreated acute infection cohort and accounts for the level of viremia before diagnosis (23, 30).
We found that there was no significant difference in the VL AUC between participants who
initiated treatment in S2, S3, and S4/5 of AHI (Figure 2B and Supplemental Figure 1A).
Analysis of single copy VLs revealed that the majority (84%) of participants who initiated
treatment in S2 of AHI or later had detectable virus at week 24 using the ultrasensitive assay, but these levels decreased between week 24 and week 48 (S2 and S4/5: p<0.05; 56% detectable) (Supplemental Figure 1B). Together, these data indicate that participants who initiated treatment in S3 and S4/5 of AHI had similarly higher antigen exposure during AHI and early ART, but after 24 weeks of ART there was almost no antigen present in the blood (S3: 1.6 copies/mL at week 24 and 0.63 copies/mL at week 48; S4/5: 0.76 copies/mL at week 24 and 0.20 copies/mL at week 48).

**Env-specific antibody levels increase during the first 6 months of ART**

To determine whether Ab development occurs after ART initiation, Ab levels and function were analyzed after 24 and 48 weeks of ART. Participants treated in S1, S2, and S3 of AHI had elevated gp41-specific Abs at week 24 compared to enrollment (p<0.05), but those treated in S1 had significantly lower Ab levels at weeks 24 and 48 than participants treated in later stages of AHI (p<0.05) (Figure 2C); gp41-specific Ab levels were lower in participants treated in AHI than those treated in CHI. For gp120-specific Abs, participants treated in both S1 and S2 had lower levels than those treated at later stages (S1: p<0.0001, S2: p<0.05 vs S4/5), even though participants treated in S2 had an increase in gp120-specific Ab levels at week 24 (p<0.01) (Figure 2D). Both gp41- and gp120-specific Ab levels strongly correlated between weeks 24 and 48 (gp41: r=0.90, p<0.0001; gp120: r=0.86, p<0.0001) (Figure 2E), even though participants tended to have lower Ab levels at week 48 compared to week 24 (Figure 2, C and D).

Phagocytic scores were elevated at week 24 for participants who were treated in S3 (p<0.01) and S4/5 (p=0.13) (Supplemental Figure 1C), though there were no significant increase in the number
of participants with positive phagocytic scores (scores >11). Significantly more participants treated in S2 and S3 of AHI had measurable CRF01_AE-specific ADCC responses after 24 weeks of ART than at the time of ART initiation (Figure 2F). Consistent with low Env-specific Ab levels, less than 50% of the participants treated in S1 developed measurable ADCC responses (Figure 2F). In contrast, despite lower levels of gp120-specific Ab levels in S2 compared to S3 and S4/5, there was no difference in the average potency of the CRF01_AE-specific ADCC responses (Figure 2G). As observed with the gp41-specific and gp120-specific Abs, there was a strong correlation between the CRF01_AE-specific ADCP and ADCC responses at week 24 and week 48 of ART (r=0.83 and r=0.85, respectively, p<0.0001)(Figure 2H and Supplemental Figure 1D). To determine if the increased Ab functionality was due to changes in Ab binding, we measured Ab binding to CRF01_AE infected cells by flow cytometry. Similar to increases in gp120-specific Ab levels, Ab binding increased between week 0 and week 24 in participants treated in S2 or later (p<0.05), and tended to decline by week 48 (Supplemental Figure 1E). The level of Ab binding at week 48 correlated well with gp120-specific Ab levels, and weakly with phagocytic scores and ADCC responses (Supplemental Figure 1F).

In contrast to increases in ADCP and ADCC activity, no increased neutralization was measured after early ART initiation (Supplemental Figure 1G). Plasma neutralization was measured for participants who initiated treatment in S3 or later using pseudoviruses produced with an ART-resistant backbone to minimize viral inhibition mediated by plasma ART (Supplemental Figure 1H). Low titer, non-specific inhibition of two HIV strains (MN.3 and 40646v01) and MuLV (negative control) was detected and was likely due to plasma ART rather than HIV-specific Abs.
To determine how antigen levels affected development of the Ab response after ART, we analyzed correlations with VL measures. We identified weak correlations between single copy VL and Env-specific Ab levels after 24 and 48 weeks of ART (Supplemental Figure 2), suggesting that ongoing low-level viremia does not significantly contribute to Ab levels during early ART. Moreover, when considering participants treated in all stages of AHI, there were only modest correlations between VL AUC and all Ab measures at week 48 that were driven by the AHI stage at ART initiation (Figure 3A). When analyzed at the level of the individual stages, VL AUC had a significant correlation with Env-specific Ab levels and ADCP, but not ADCC, only in participants who initiated ART in S4/5 of AHI (gp41: r=0.78, p<0.05; gp120: r=0.81, p<0.05; ADCP: r=0.92, p<0.01)(Figure 3B and Supplemental Figure 3, A-C). Interestingly, after 48 weeks of ART the levels of Ab binding also correlated with gp120-specific Ab levels and ADCP, but not ADCC (Supplemental Figure 3D). Our data suggest that viral antigen load after ART (VL AUC) may be associated with development of HIV-specific binding Ab levels but not ADCC only in participants treated in S4/5 of AHI, and other factors must contribute to the development of ADCC responses in participants who initiate treatment in AHI.

**Development of increased cross-clade antibody responses**

To further understand how the Ab response develops after early ART initiation, we measured cross-clade ADCC responses. As participants in this study were infected with CRF01_AE and AE/B recombinant viruses, cross-clade activity was measured against targets infected with the Clade C strain TV1, which has recently been used in the HVTN 702 vaccine study and clusters separately from the CRF01_AE CM235 virus (31). We found that few participants developed cross-clade ADCC responses during the early stages of AHI, but over 50% of participants in
S4/5 had Clade C-specific ADCC responses (Figure 4A). After 24 weeks on ART, an increased proportion of participants who initiated treatment in S3 had detectable Clade C-specific ADCC responses (64%, p=0.13). Intriguingly, while there was little change in the magnitude of CRF01_AE-specific ADCC responses between weeks 24 and 48 (Figure 2F), participants treated in S4/5 of AHI had significant increases in the magnitude of Clade C-specific ADCC responses after 48 weeks of ART (Figure 4B). Despite having similar times to viral suppression and VL AUC (Figure 2, A and B), participants treated in S4/5 of AHI had higher cross-clade ADCC titers after 48 weeks of ART than those treated earlier in AHI (p<0.05), with all participants treated in S4/5 developing measurable cross-clade ADCC potency (Figure 4, A and B). There was a trend toward weak correlation between the VL AUC and cross-clade ADCC responses at week 48 of ART (r=0.21, p=0.16) and a trend toward modest correlation for participants treated in S4/5 of AHI when stratified by stage (r=0.57, p=0.12)(Supplemental Figure 4, A and B).

There was also a correlation between the Clade C-specific ADCC responses at weeks 24 and 48 (r=0.46, p<0.01)(Figure 4C), but it was weaker than the correlations seen for other Ab measures (Figure 2, E and H, and Supplemental Figure 5). Further, while the levels of gp120-specific Ab at week 24 correlated moderately with CRF01_AE-specific ADCC responses (week 48: r=0.72, p<0.0001), correlations with Clade C-specific ADCC responses were weaker (week 48: r=0.42, p<0.01)(Figure 4, D and E). Likewise, there was only a modest correlation between the CRF01_AE-specific and Clade C-specific ADCC responses at week 48 (r=0.42, p<0.01)(Figure 4F). Thus, the development of cross-clade antibodies was only weakly correlated with the autologous gp120 and ADCC responses. Lastly, increased Ab binding to Clade C infected cells was not detected after ART nor did Ab binding correlate with ADCC Ab titer (Supplemental Figure 4, C and D). Together these data suggest that participants who initiated treatment in S4/5
of AHI had a greater capacity to develop cross-clade ADCC antibodies from week 24 to week 48 of suppressive therapy.
Here we describe for the first time the development of cross-clade ADCC responses after viral suppression with early ART initiation specifically in S4/5 treated participants, suggesting a more developed GC response capable of supporting antibody evolution after peak viremia in AHI. Indeed, it was in S4/5 that we measured increased levels of Env-specific Abs, ADCC potency, and plasma CXCL13 levels at AHI. In addition to more developed germinal centers, the advanced Ab development in S4/5 treated participants after viral suppression in the blood may be a result of increased virus trapping by follicular dendritic cells (FDC) in the GCs. HIV immune complexes (ICs) are trapped and retained by FDCs in an infectious state for months for continued selection of high affinity B cells after viral control (32-38). Indeed, ongoing antibody maturation has been reported to occur over months after flu vaccination and SARS-CoV2 infection (39, 40). Deposition of virus on the FDC network is not evident until two weeks after SIV infection (41, 42), and minimal viral deposition has been shown as early as 1 month after symptoms in AHI (43), but the kinetics of HIV trapping and retention by FDCs in the earliest stages of AHI in humans, especially in the context of early ART initiation, is not known. However, the elevated levels of HIV-specific Abs found in participants in S4/5 of AHI could allow for greater IC formation and thus HIV trapping by FDCs, providing for more prolonged antigen exposure in the GC and enhanced Ab development in these participants after ART. As very low levels of virus have been detected on the FDC network even after 26 weeks of ART in SIV-infected nonhuman primates by RNAscope (44), it is possible that virus trapping on the FDC network could be measured in future studies that include collection of lymph node biopsies or in nonhuman primate studies to investigate if virus retention in the germinal center promotes Ab development after early ART initiation.
This study highlights the distinctions in Ab development in participants treated in different stages of AHI. Participants who were treated in S1 of AHI had lower gp41-specific Ab levels, and those who were treated in S1 and S2 of AHI had lower levels of gp120-specific Ab levels than individuals treated later in AHI. It has been shown that gp41-specific Ab responses arise earlier in AHI and have cross-reactivity with commensal bacteria in the gut, whereas the gp120-specific responses take longer to develop (12-14). These data suggest that participants who initiated ART in S1 and S2 of AHI did not have sufficient antigen load, duration of antigen exposure, or GC activity to induce significant de novo gp120-specific Ab production during the period of viral suppression after ART initiation. Delaying ART initiation for the sake of antibody development is not advised due to concomitant seeding of the viral reservoir, but identification of interventions that can be administered at ART initiation to stimulate GC development and promote antibody maturation in these early treated individuals could help provide better protection after treatment interruption.

As this study was done in a relatively homogenous group of participants, further work will be needed to determine if similar Ab kinetics are seen in women, people from other ethnicities or different ages, and individuals infected with other HIV clades. We focused our analysis on Ab function as isolation of HIV-specific memory B cells is likely to be challenging due to their extremely low frequency in early treated individuals. However, analysis of Env-specific B cell clones would clarify whether there is continued somatic hypermutation after ART and may be possible in nonhuman primate studies or individuals treated later in infection. We measured ADCC activity due to the correlations with protection found in the RV144 trial that was
completed in Thailand. Although a similar protection was not seen in the corresponding vaccine trial in South Africa, a recent study also found that antibody-mediated NK cell activation was associated with delayed rebound after treatment interruption in participants in Thailand (45), suggesting an important role of ADCC function in HIV control, at least in the context of HIV infection in Thailand. We also measured plasma neutralization and ADCP, which has been associated with reduced risk of HIV acquisition in clinical and pre-clinical studies (20, 21), but found little to no activity in these early treated participants. While we did not measure either Ab-dependent complement deposition, which is mediated in early acute HIV infection by IgM antibodies that wain after ART initiation, or Ab-dependent trogocytosis, which correlates with ADCP activity, these may be of interest in future studies.

We report here critical insights into Ab development in early treated individuals and the development of cross-clade Ab responses during ART. Participants treated in S2 or later of AHI had sufficient antigen load in the lymph nodes to develop CRF01_AE-specific Abs in the first 24 weeks of ART while those treated in S1 did not. But only those treated in S4/5 showed increased cross-clade ADCC potency after 48 weeks of ART, suggesting that GC development was more advanced in these individuals. However, the GC activity and antigen load were not sufficient to foster significant development of neutralizing Ab responses in these participants, and as such all individuals who start treatment in AHI may benefit from concurrent administration of therapeutic vaccination or other interventions that target GC development and antibody maturation when ART is initiated. It is unknown whether continued development of Ab depth and breadth after ART would be seen in participants treated in chronic infection. Individuals in chronic infection have higher Ab levels and ADCC responses before ART, and also have altered B cell phenotypes
and increased collagen deposition in lymph nodes (47) which could affect further antibody development after ART. However, the prospect of continued antibody development after ART initiation even in chronic infection is intriguing as recent studies suggest that the HIV reservoir is dominated by viruses that are circulating at the time of ART initiation (48, 49). Thus, maturation of the Ab response against the HIV reservoir at time of ART initiation could have important implication for HIV remission studies and is in line with recent data showing that autologous IgG antibodies block outgrowth of a substantial but variable fraction of viruses in the latent reservoir for HIV-1 (50). Thus, any Ab development after ART initiation could produce better antibodies to control virus after treatment interruption and is worth further study.
Methods

Study participants

Plasma samples from 64 participants were analyzed to determine HIV-specific Ab development in acute HIV infection and after early ART initiation. Fifty-two of the participants were enrolled in the RV254/SEARCH 010 cohort at the Thai Red Cross AIDS Research Centre in Bangkok, Thailand (clinicaltrials.gov NCT00796146)(4). This study enrolls individuals who are diagnosed with HIV in the earliest stages of acute infection and who are offered ART as part of a separate protocol (NCT00796263). For the purpose of the current study, participants were categorized into stages 1-5 of AHI based on previously reported staging strategies (10, 11)(Table1): S1 - positive HIV RNA, non-reactive 4G generation (4G) immunoassay (IA), non-reactive 3G IA; S2 - positive HIV RNA, reactive 4G IA, non-reactive 3G IA; S3 - positive HIV RNA, reactive 4G IA, reactive 3G IA, negative western blot (WB); S4 - positive HIV RNA, reactive 4G IA, reactive 3G IA, intermediate WB; S5 - positive HIV RNA, reactive 4G IA, reactive 3G IA, positive WB except p31. Longitudinal samples from the time of enrollment (week 0), 24-week visit, and 48-week visit were analyzed for all participants, as available. Samples from participants with untreated chronic HIV infection (n=4) and treated chronic HIV infection (n=8; median 44 months ART, IQR: 25-116 months) from the RV304/SEARCH013 cohort (NCT01397669) were also analyzed.

Measurement of HIV-1 RNA

Plasma VL was measured quantitatively with the Roche Amplicor v 1.5 ultrasensitive assay with a lower limit of quantification of 50 copies/mL (Roche Diagnostics), before replacement with the COBAS TaqMan HIV-1 Test v2.0 (Roche Diagnostics) with a lower limit of quantification of 20
copies/mL. Plasma VL was measured at the time of enrollment, every 2 weeks from enrollment through week 4 of ART, every 4 weeks from week 4 through week 24 of ART, and every 12 weeks thereafter. Single copy HIV-1 RNA levels were measured retrospectively using ultrasensitive hybrid real time/digital PCR, as previously described (51, 52). AUC was calculated by adding the AUC measured in the study starting at ART initiation and the estimated AUC at each AHI stage derived from the trendline of viral load in untreated AHI (23, 30).

**Measurement of plasma CXCL13 levels**

Plasma CXCL13 levels were measured using Luminex technology with a ProcartaPlex multiplex immunoassay (Assay MXH49YW)(Life Technologies Corporation). Samples were run according to the manufacturer’s instructions and cytokine standards were provided by the manufacturer. A Bioplex-200 system was used to acquire samples, and the data was analyzed with the BioPlex Manager Software (Bio-Rad Laboratories).

**Measurement of HIV-specific Ab levels**

HIV-specific Ab levels were measured as previously described (53, 54). High-binding half-area microplates (Grenier Bio-One) were coated with recombinant gp41 (HIV-1 Envelope, Prospec) or gp120 consensus CRF01_AE (Immune Technology Corp.) protein at a concentration of 1μg/ml in phosphate buffered saline (PBS) overnight at 4°C. The next day, plates were washed 5x (unless otherwise stated) with wash buffer (PBS + 0.05% Tween 20) and then blocked for 1h with PBS plus 10% (vol/vol) FBS at RT. The plates were washed again before plasma and standards were added in duplicate at different dilutions and incubated for 2h at RT. For standard curves, 2-fold dilutions of anti-gp120/gp160 (Clade 01_AE)(Immune Technology Corp., clone
26A4) or human HIV immunoglobulin (NIH AIDS Research and Reference Program, Division of AIDS, NIAID, NIH; received from Dr. Elias Haddad, Drexel University) was used. After subsequent washing, the plates were incubated for 1h at RT with 1μg/mL of biotin-conjugated anti–human IgG (Mabtech, clone MT78/145) or anti-mouse IgG (Mabtech) for the gp120 standard curve. The plates were then washed and incubated with streptavidin–horseradish peroxidase (Mabtech) for 1hr at RT. After a final wash, 50 μl of TMB substrate (Sigma-Aldrich, St. Louis, MO, USA) was added until appearance of color and the enzymatic reaction was stopped by adding 50 μl of 1M H₃PO₄. The absorbance was read at 450nM using a Versamax Tunable Microplate Reader (Molecular Devices). Absorbance values were converted to concentrations using the SoftMax Pro Software (Molecular Devices) to calculate a 4-parameter logistic fit of the standard curve. The mean concentration of duplicate wells was reported as units/mL (U/mL), with the gp120 standard curve being measured as ng/mL of anti-gp120/gp160 antibody and the gp41 standard curve being measured as μg/mL of total IgG in the HIV immunoglobulin standard.

**Infectious molecular clones (IMC)**

The HIV-1 reporter viruses used were replication-competent IMC designed to encode the env genes of CM235 (subtype A/E; GenBank No. AF259954.1) and TV-1 (subtype C; GeneBank No. HM215437) as previously described (55).

**Infection of CEM.NKR<sub>CCR5</sub> cell line with HIV-1 IMCs**

CEM.NKR<sub>CCR5</sub> cells were infected with HIV-1 IMCs as previously described (55). Briefly, IMCs were titrated in order to achieve maximum infection within 48-72 hours post-infection as
determined by detection of Luciferase activity and intra-cellular p24 expression. For each ADCC assay, we monitored the frequency of infected target cells by intracellular p24 staining. Assays performed using infected target cells were considered reliable if cell viability was ≥60% and the percentage of viable p24+ target cells on assay day was ≥20%.

**Luciferase ADCC assay**

We utilized a modified version of the ADCC luciferase assay (56). Briefly, CEM.NKRCCR5 cells were infected with HIV-1 IMCs as described above and used as target cells. For effector cells, cryopreserved PBMC obtained by leukapheresis from a HIV-seronegative individual (Fc-gamma-Receptor IIIa 158 V/F heterozygous) were thawed the day before the assay and rested overnight in RPMI 1640 medium supplemented with antibiotics and 10% fetal bovine plasma (R10), and with recombinant human IL-15 at a concentration of 10 ng/ml. Effector and target cells (30:1 ratio) were plated in opaque 96-well half-area plates and co-cultured with serial dilutions of plasma. Each plasma sample was assayed at six dilutions, starting at a dilution of 1:50, with duplicate wells set up for each dilution. For the 4-fold serial dilution scheme, plasma dilutions of 1:50, 1:200, 1:800, 1:3200, 1:12800, and 1:51200 were used. Co-cultures were incubated for 6 hours at 37°C in 5% CO2 in IL-15 supplemented R10. The assay readout is luminescence intensity (measured in relative light units, RLUs) generated by surviving target cells that have not been lysed by the effector population in the presence of ADCC-mediating plasma Abs. The monoclonal Ab palivizumab (Synagis), which mediates ADCC (57) but is specific for respiratory syncytial virus, and a cocktail of HIV-1 monoclonal Abs demonstrated to mediate ADCC [A32 (58), 2g12 (59), CH44 (60), and 7B2 (61)] were used as negative and positive controls, respectively. All mAbs were generated using IgG1 constant region containing
alanine substitutions (S298A, E333A, K334A) designed to enhance binding to Fc-gamma-receptor IIIa (FcyR3A)(62). A response was reported as positive if it was greater 15 specific killing at the first two dilutions after subtracting the average % specific killing observed by testing a panel of 11 sera collected from geographically matched seronegative subjects. The ADCC antibody titer, defined as the last dilution of plasma capable of mediating ADCC in our in vitro assay, was calculated by interpolation of the dilution curve intersected the positive cutoff of 15% specific killing.

**Antibody binding assay**

Ab binding to HIV infected cells was measured as described previously (63, 64). Briefly, 7.5 × 10^5 CM235 or TV1 IMC infected CEM.NKR<sub>CCR5</sub> cells were incubated with 100-fold diluted human plasma for 2 hours at 37°C followed by surface staining with anti-IgG secondary-PECy7 (Biolegend, clone M1310G05) and anti-CD4–APC (Biolegend, clone OKT4) for 20 minutes at room temperature. Cells were then resuspended in 100 μL Cytofix/Cytoperm and incubated for 20 minutes at 4°C, followed by staining with anti-p24–FITC antibody (Beckman Coulter, clone KC57) for 25 minutes at room temperature. Cells were washed and resuspended in 125 μL PBS–1% paraformaldehyde. The samples were acquired within 24 hours using a BD Fortessa cytometer (BD Biosciences), and plasma binding was quantified as the percentage of target cells positive for anti-IgG secondary antibody after background subtraction.

**ADCP assay**

ADCP was measured as previously described (65). Briefly, gp120 CM235 (NIH HIV Reagent Program, Division of AIDS, NIAID, NIH: Human Immunodeficiency Virus 1 (HIV-1) CM235
gp120 Recombinant Protein, ARP-12816, contributed by NIAID, DAIDS) was biotinylated at a biotin to protein ratio of 50 following manufacturer’s instructions (Thermo Scientific) and incubated with yellow-green streptavidin-fluorescent beads (Molecular Probes) for 2 hours at 37°C. 10μl of a 100-fold dilution of beads–protein was incubated 2h at 37°C with 100μl of 200-fold diluted plasma samples before addition of THP-1 cells (20,000 cells per well; Millipore Sigma). After 18h incubation at 37°C, the cells were fixed with 4% formaldehyde solution (Tousimis) and fluorescence was evaluated on a LSRII (BD Biosciences). The phagocytic score was calculated by multiplying the percentage of bead-positive cells by the geo mean fluorescence intensity of the bead-positive cells and dividing by $10^4$.

**Plasma neutralization assay**

Plasma neutralization was evaluated in a high-throughput TZM.bl pseudovirus assay, as previously described (66). Pseudoviruses (PSVs) were produced using an ART-resistant backbone vector (SG3ΔEnv/K101P.Q148H.Y181C; courtesy of Michael S. Seaman) shown to minimize the background inhibitory activity of antiretroviral drugs present in patient plasma (67). PSV Envs included HIV MN.3 (subtype B) and 40646v01 (CRF01_AE), and murine leukemia virus (MuLV) as a non-specific control. Plasma was diluted 1:10 in growth medium and serially diluted using the Biomek NXP liquid handler (Beckman Coulter). Titered plasma was transferred to 384-well culture plates and incubated with an equal volume of PSV for 45 min at 37°C. TZM-bl cells (3x10³ cell/well) mixed with DEAE-dextran were added to each well and incubated for an additional 48 h. Relative light units were detected with the SpectraMax Paradigm Microplate Reader (Molecular Devices) using the Bright-Glo Luciferase Assay System (Promega Corporation). Percentage neutralization (percentage reduction of relative light units in
the presence of plasma) was calculated for each plasma dilution. Neutralization dose–response curves were fitted by nonlinear regression using the LabKey Server, and the final titer is reported as the reciprocal of the dilution of plasma necessary to achieve 50% neutralization (50% inhibitory dose).

Statistics

Statistical analyses were performed using the Kruskal-Wallis test with Dunn’s multiple comparisons test to measure differences between groups. Comparisons between values at different times on ART were performed with the Wilcoxon matched-pairs signed rank test. Correlations were performed with the nonparametric Spearman test. When multiple comparisons were made for Ab measures within each AHI stage, the Benjamini-Hochberg Procedure was used to correct for a false discovery rate of 25%. Differences in proportion of responders were calculated using a chi-square test followed by the Marascuilo procedure. Statistical analyses were performed using GraphPad Prism (GraphPad Software Inc.) and R Statistical Software (Foundation for Statistical Computing). Significance was defined as p < 0.05 for two-sided testing.

Data availability

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Study approval
Informed consent was obtained from all participants prior to inclusion in the studies. All studies were approved by the Chulalongkorn University, Bangkok, Thailand, and Walter Reed Army Institute of Research, Silver Spring, MD, USA, institutional review boards.
Author Contributions

J.L.M. designed the experiments, analyzed the data, and wrote and edited the manuscript. J.P., J.N., S.B., and H.T. performed the experiments, analyzed data, and edited the manuscript. K.D., R.W.E., K.F.N., and M.Z. performed the experiments and analyzed data. R.M. provided guidance on methodology. E.K. managed participant recruitment and follow-up and edited the manuscript. S.P. and S.J. provided help in statistical analyses. S.M, S.C., P.T., P.Pr., N.R., B.N., and C.P.S. managed participant recruitment and follow-up. L.F. provided supervision. S.T., V.R.P., and E.K. provided resources. D.P.P., L.W. designed experiments and edited the manuscript. F.M. provided single copy viral load data. Y.L. and M.R. calculated the AUC. P.Ph. provided support for the clinical studies. N.P. provided support for the clinical studies and edited the manuscript. J.A. designed the clinical studies and edited the manuscript. S.V. provided supervision and edited the manuscript. G.F. and L.T. designed the experiments, wrote and edited the manuscript, and provided supervision.
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62. Shields RL, et al. High resolution mapping of the binding site on human IgG1 for Fc gamma RI, Fc gamma RII, Fc gamma RIII, and FcRn and design of IgG1 variants with improved binding to the Fc gamma R. *J Biol Chem.* 2001;276(9):6591-604.


Figure 1. HIV-specific antibodies with ADCC function do not develop until Stages 4/5 of acute HIV infection. (A) Plasma viral load (VL) at time of diagnosis and ART initiation for participants at different stages of acute HIV infection (AHI) or those with untreated chronic HIV infection (CHI). (B) Levels of CXCL13 were measured in the plasma of participants prior to ART initiation at different stages of AHI or CHI by Luminex assay. (C) Levels of plasma gp41-specific and gp120-specific Ab levels were measured prior to ART initiation at AHI or CHI by ELISA. (D) Ab dependent cellular phagocytosis (ADCP) responses of plasma Abs prior to ART initiation at AHI or CHI were measured against CRF01_AE envelope. The cutoff for positive phagocytic scores is designated by a dotted line. (E) Ab dependent cellular cytotoxicity (ADCC) responses of plasma Abs prior to ART initiation at AHI or CHI were measured using target cells infected with CRF01_AE virus. (F) Plasma neutralization was measured on TZM.bl cells using pseudoviruses containing HIV 40646v01 (CRF01_AE), MN.3 (Clade B), or murine leukemia virus (MuLV; negative control). The plasma dilution necessary to achieve 50% neutralization (50% inhibitory dose – ID₅₀) is shown for plasma collected prior to ART initiation in AHI or CHI. Differences were measured by a Kruskal-Wallis test with Dunn’s multiple comparison test of AHI stages. n=12 for S1; n=17 for S2; n=14 for S3; and n=9 for S4/5. * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001
Figure 2. Continued antibody development after ART initiation in participants treated in S2 or later of AHI. (A) Time for VL to first reach undetectable levels (VL ≤ 20 copies/mL) after ART initiation for participants who initiated ART at different stages of AHI. (B) The log VL area under the curve (AUC) was calculated for each participant. Levels of gp41-specific (C) and gp120-specific (D) Ab levels were measured in the plasma by ELISA after 24 and 48 weeks of ART. (E) Correlations between Ab levels at W24 and W48 are shown for gp41-specific and gp120-specific Abs. (F) Proportion of participants who had measurable ADCC responses (responders) at each visit using target cells infected with CRF01_AE virus. (G) ADCC function of plasma Abs after 24 and 48 weeks of ART in participants who initiated treatment in AHI or CHI. (H) Correlation between ADCC Ab titers at W24 and W48. Differences in the proportion of participants responding at each visit were calculated using a chi-square test and the Marascuilo procedure, + significant difference in proportions. Differences in viral measures or Ab levels between AHI stages were measured by a Kruskal-Wallis test with Dunn’s multiple comparison test (black bars). Differences in antibody levels between visits were measured by a Wilcoxon matched-pairs signed ranks test (grey bars). For W0 and W24: n=12 for S1; n=17 for S2; n=14 for S3; and n=9 for S4/5. For W48: n=11 for S1; n=15 for S2; n=13 for S3; and n=9 for S4/5. * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001
Figure 3. Env-specific antibody levels correlate with viral load AUC only in participants treated in S4/5 of AHI. Correlations between VL AUC and levels of gp41-specific Abs, gp120-specific Abs, ADCP, and CRF01_AE-specific ADCC titers at W48 after ART initiation in all participants (A) and only those who initiated ART in S4/5 of AHI (B). Correlations were measured by Spearman correlation. n=11 for S1; n=15 for S2; n=13 for S3; and n=9 for S4/5.
Figure 4. Increased cross-clade ADCC antibody responses between 24 and 48 weeks of ART in participants treated in S4/5 of AHI. Cross-clade ADCC responses were measured in the plasma at W0, W24, and W48 after ART initiation in participants who initiated treatment in AHI or CHI using target cells infected with Clade C virus. (A) The frequency of participants who had measurable Clade C-specific ADCC responses (responders) within each group is shown at each visit. (B) Changes in Clade C-specific ADCC Ab titers at each visit are shown with stars representing median titers. (C) Correlation between Clade C-specific ADCC Ab titers at W24 and W48. (d-e) Correlations between gp120-specific Ab levels at week 24 and CRF01_AE- (D) or Clade C-specific (E) ADCC Ab titers at weeks 24 and 48. (F) Correlation between CRF01_AE- and Clade C-specific Ab titers at week 48. Differences in the proportion of participants responding at each visit were calculated using a chi-square test and the Marascuilo procedure, * significant difference in proportions. Differences between AHI stages were measured by a Kruskal-Wallis test with Dunn’s multiple comparison test (black bars). Differences in ADCC Ab titers between visits were measured by a Wilcoxon matched-pairs signed ranks test (grey bars). Correlations were measured by Spearman correlation. For W0 and W24: n=12 for S1; n=17 for S2; n=14 for S3; and n=9 for S4/5. For W48: n=11 for S1; n=15 for S2; n=13 for S3; and n=9 for S4/5. * p<0.05; ** p<0.01.
Table 1. Characteristics of participants included in this study.

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N/A: Not applicable
n.d.: Not determined