Dendritic cells restore CD8+ T cell reactivity to autologous HIV-1

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Abstract

Recall T cell responses to HIV-1 antigens are used as a surrogate for endogenous cellular immune responses generated during infection. Current methods of identifying antigen-specific T cell reactivity in HIV-1 infection use bulk PBMC, yet ignore professional antigen presenting cells (APC) that could reveal otherwise hidden responses. In the present study, peptides representing autologous variants of MHC class I-restricted epitopes from HIV-1 Gag and Env were used as antigen in IFN\(\gamma\) ELISpot and polyfunctional cytokine assays. Here we show that DC enhance T cell reactivity at all stages of disease progression, but specifically restored T cell reactivity after combination antiretroviral therapy (cART) to early infection levels. Type-1 cytokine secretion was also enhanced by DC and was most apparent late post-cART. We additionally show that DC reveal polyfunctional T cell responses after many years of treatment, when potential immunotherapies would be implemented. These data underscore the potential efficacy of a DC immunotherapy that aims to awaken a dormant, autologous HIV-1-specific CD8\(^+\) T cell response.
Importance

Assessment of endogenous HIV-1-specific T cell responses is critical for generating immunotherapies for subjects on cART. Current assays ignore the ability of dendritic cells to reveal these responses and may therefore underestimate the breadth and magnitude of T cell reactivity. As DC do not prime new responses in these assays, it can be assumed that the observed responses are not detected without appropriate stimulation. This is important because dogma states that HIV-1 mutates to evade host recognition, and that CD8<sup>+</sup> cytotoxic T lymphocyte (CTL) failure is due to the inability of T cells to recognize the autologous virus. The results presented here indicate that responses to autologous virus are generated during infection but may need additional stimulation to be effective. Detecting the breadth and magnitude of HIV-1-specific T cell reactivity generated in vivo is of the utmost importance for generating effective DC immunotherapies.
Introduction

HIV-1-specific CD8+ T cell responses are effective at imposing immunological pressure in acute infection, as evidenced by the high turnover and mutation rates in virus populations (1-3). However, the failure of CTL to control virus in chronic infection results in progression to AIDS that can be attributed to several factors. Viral evolution, specifically in CTL epitopes, can interfere with recognition by naïve CD8+ T cells, resulting in a limited repertoire of T cell-mediated immune responses against the mutated regions (4). In the absence of an effective CTL response that is specific for these mutated epitopes, the virus persists and disease progression continues (5, 6). To more fully understand the mechanisms of viral pathogenesis and develop effective treatments for HIV-1-infected subjects, we must evaluate how mutations within regions of T cell recognition affect HIV-1-specific T cell responses.

Alterations in T cell homeostasis during chronic infection largely impact naïve T cell subsets and partially result from decreases in thymic output (7-9). Progressive infection is also accompanied by decreases and dysfunction in the naïve CD8+ T cell subset despite increases in total CD8+ T cells (10, 11). These perturbations in the naïve CD8+ T cell repertoire could reduce the number and likelihood of mutated epitopes being recognized. This may explain the decreases observed in HIV-1-specific CTL activity in chronic infection, presumably as a result of viral mutations (5, 12). It remains to be elucidated, however, if these responses are not generated, or if they are generated but are not detected due to insufficient antigen presentation and/or stimulation in readout assays.
While many aspects of the immune system become dysfunctional in chronic HIV-1 infection and remain dysfunctional even when subjects receive cART, myeloid dendritic cells (DC), the most potent antigen presenting cells (APC), retain the ability to process and present antigen (13, 14) and to stimulate HIV-1-specific IFN-γ production in CD8+ (15-17) and CD4+ T cells (18). These DC require activation with proinflammatory cytokines and an additional stimulation such as that provided by CD40 ligand (CD40L) on activated CD4+ T cells. The resulting mature DC express high levels of the T cell costimulatory molecules CD80 and CD86 and the maturation marker CD83, and secrete the proinflammatory molecule IL-12p70 (15, 16, 19, 20). These cells may be a valuable tool in analysis of the antigen recognition repertoire of CD8+ T cells or in immunotherapies that strive to enhance a dysfunctional HIV-1 recall CD8+ T cell response. Indeed, we have previously shown that CD40L-matured DC stimulation reveals CD8+ T cell responses to consensus MHC class I-restricted HIV-1 epitopes that are otherwise masked in subjects on cART (15, 21). It is currently unclear if DC can also reveal responses to the subject’s own, unique (autologous) virus, and if this DC enhancement changes with treated HIV-1 infection. As a successful immunotherapy will likely enhance the breadth and magnitude of the autologous HIV-1-specific T cell response (22-24), it is pertinent to ascertain the best method of detecting and enhancing these responses.

The aim of this study was to longitudinally evaluate CD8+ T cell reactivity to a broad array of autologous HIV-1 Gag and Env MHC class I founder and variant epitopes pre- and post-cART and to determine the effects of DC addition on the breadth and magnitude of these responses. We also determined the effects of epitope mutations on
the generation of HIV-1-specific immune responses and analyzed how DC can reveal otherwise masked responses for these mutated epitopes. The findings presented here demonstrate a unique ability of DC to reveal HIV-1-specific responses to a multitude of autologous epitope variants and to enhance CD8 T cell cytokine profiles after long-term treatment, thus supporting the use of these cells in an immunotherapy for HIV-1-infected subjects on cART.

Materials and Methods

Study participants

Three HIV-1 infected subjects (designated as S2, S3 and S8) were chosen from the MACS, a natural history study of men who have sex with men for which the methodologies have been described previously (25, 26). Human subject approval was obtained from the University of Pittsburgh Institutional Review Board. These subjects were chosen based on their prolonged enrollment in the study (>10 years), typical course of disease progression, favorable response to combination antiretroviral therapy (cART), and the presence of at least one common HLA allele. Positivity for HLA A*2402 was confirmed by high resolution PCR genotyping (Tissue Typing Laboratory, University of Pittsburgh Medical Center). All three subjects were enrolled in the MACS prior to seroconversion to HIV-1. Seropositivity was confirmed by positive enzyme-linked immunosorbent assay (ELISA) for the presence of HIV-1 p24 and a Western blot with bands corresponding to at least two of the Gag, Pol, and Env proteins (25). Blood specimens and epidemiological and clinical data were collected at each visit, as described previously (27).
Clinical and virologic characteristics

At each biannual visit, plasma samples and peripheral blood mononuclear cells (PBMC) were collected from the study subjects and were stored at -80°C and -140°C, respectively. T cell phenotypes were determined by flow cytometry as previously described (28, 29). HIV-1 plasma viremia was determined by extracting RNA using a COBAS® Ampliprep Instrument (Roche Diagnostics, Indianapolis, IN) and performing RT-PCR on a COBAS® Taqman® 48 Analyzer (Roche Diagnostics) using the COBAS® Ampliprep/COBAS® Taqman® HIV-1 test. This assay is capable of detecting 20 to 1 x 10^6 HIV-1 RNA copies/ml of plasma. Negative, low positive, and high positive controls were used in each extraction and amplification per the manufacturer’s instructions.

Sequencing of autologous plasma-and cellular-derived HIV-1 gag and env

Five post-seroconversion time points for S2 and S8, six post-seroconversion time points for S3, and one post-cART time point for S2 and S3 were chosen for HIV-1 gag p17-p6 and env gp120 sequencing. HIV-1 RNA was obtained from freeze-thawed plasma for all pre-cART time points. Under cART, plasma viremia was reduced to <20 copies/ml in all subjects. We therefore sequenced HIV-1 cultured from latently-infected CD4+ T cells obtained during cART using a previously-described method (30-32). The presence of HIV-1 in culture supernatants was evaluated every 3 days by p24 ELISA (Zeptometrix, Buffalo, NY). Cultures were terminated and supernatants were collected when the concentration of p24 reached or exceeded 20,000 pg/ml. While this technique
was attempted using cells derived after cART in subject S8, we were unable to induce sufficient virus production for sequencing.

Viral RNA was extracted from plasma or cell culture supernatants using a viral RNA mini kit (Qiagen, Valencia, CA). cDNA synthesis was performed using Nef3 (33) and RT2 (34) primers with SuperScript III Reverse Transcriptase (200 U/ml; Invitrogen, Carlsbad, CA). Endpoint dilution methodology was used to dilute reactions to ~1 copy per 3-5 reactions followed by direct sequencing to avoid detection of PCR-induced mutations (35). Multiplex first-round PCR was performed with the Gag1 (34) and RT2 primers to amplify \textit{gag} and the Ed3 (36) and Nef3 (33) primers to amplify \textit{env}-gp120.

Singleplex second round PCR was performed with Gag2 (34) and RSP15R (37) primers for \textit{gag} and gp120 forward (38) and reverse (38) primers for \textit{env}. PCR products were run on a QIAxcel automated electrophoresis system (Qiagen, Valencia, CA) and Sanger sequencing was performed on samples with positive bands (High Throughput Genomics Center, Seattle, WA).

\textit{Identification of epitopes and peptide synthesis}

Six known HLA A*24-restricted HIV-1 Gag and Env epitopes were identified for each subject using the Los Alamos Database (http://www.hiv.lanl.gov). The combined epitope prediction model within The Immune Epitope Database (39, 40) was used to identify seven additional predicted A*2402-restricted epitopes within autologous sequences from the three study subjects. This prediction model ranks potential epitopes within an input sequence based on predicted proteasomal cleavage, TAP transport, and MHC class I affinity using the netMHCpan prediction method (39-41). A PEPscreen
custom library representing the dominant epitope variants (frequency >0.25) that evolved in each study subject was synthesized (Sigma-Aldrich). Each peptide was resuspended in 100μl DMSO and further resuspended in AIM V media at a final concentration of 1 mg/ml or 100 μg/ml. Peptides were stored at -80°C.

Generation of monocyte-derived dendritic cells (DC)

PBMC from each study subject under cART were obtained by leukapheresis. Monocytes were isolated from PBMC by Percoll (GE Healthcare Life Sciences, Uppsala, Sweden) density separation. Immature DC were generated by culturing monocytes in IMDM containing 10% FBS with GM-CSF and IL-4 (both 1000 U/ml; R&D Systems, Minneapolis, MN). On day 5, immature DC were treated with 0.5 μg/ml soluble CD40L (Enzo, Farmingdale, NY) for 48h. Maturation status of the DC was confirmed by expression of CD83, CD86, and CCR7 as determined by flow cytometry.

IFNγ ELISpot assay

IFNγ production was measured by a standard overnight ELISpot assay. Briefly, 96-well nitrocellulose plates (EMD Millipore, Billerica, MA) were coated with anti-IFNγ monoclonal antibody (10 μg/ml; Mabtech, Stockholm, Sweden) and incubated overnight at 4°C. Plates were washed and blocked with IMDM supplemented with 10% heat-inactivated FBS for 2h at 37°C. PBMC (1x10^5/well) obtained early post-seroconversion (early post-SC; <6 months post-SC), late post-seroconversion (late post-SC; >7 years post-SC and <6 months before cART), early post-cART (<6 months post-cART), and late post-cART (>13 years post-cART) were tested in singlet or duplicate for reactivity to
peptides representing the autologous HIV-1 epitope variants that evolved by the time point of PBMC sampling. Responders were stimulated overnight at 37°C with peptide alone (5 μg/ml) or 1x10^4 autologous DC pre-pulsed with peptide (5 μg/ml) in IMDM supplemented with 10% heat-inactivated FBS. Responders in media alone or with DC alone served as negative controls and a peptide pool consisting of CMV, EBV, and flu (CEF) peptides was used as a positive control for both responder conditions. ELISpot plates were washed and processed as described previously (15, 42). Spots were counted using an automated ELISpot plate reader (AID, Straßberg, Germany) and are shown as the number of background-subtracted antigen-specific spot-forming cells (SFC) per 10^6. Background was calculated as the mean number of SFC/10^6 in duplicate control wells without peptide plus 2 standard deviations.

Intracellular cytokine staining

PBMC were also evaluated for polyfunctional cytokine secretion in response to peptide alone or peptide-loaded DC. PBMC obtained from each subject during late infection (<6 months pre-cART), early post-cART (<6 months post-cART), and late post-cART (>13 years post-cART) were thawed and resuspended in AIM V with CD28/CD49d FastImmune™ co-stimulatory reagent (BD Biosciences), Golgistop (BD Biosciences), Golgiplug (BD Biosciences), and CD107a-FITC H4A3 (BD Pharmingen). Peptides representing variants of known HLA A*24 HIV-1 Gag and Env epitopes that were in circulation at the time of PBMC sampling (“contemporaneous variants”) were added to PBMC or pre-loaded into DC before adding to PBMC at a final concentration of 5 μg/ml. Cells were incubated for 6h at 37°C, washed with PBS, and stained with
LIVE/DEAD Aqua Viability Dye (Invitrogen) for 30 minutes at room temperature in the dark. Cells were washed and stained for surface expression of CD3-PerCP clone SK7 (BD Biosciences), CD4-APC-Cy7 clone SK3 (BD Biosciences), and CD8-PerCP-Cy5.5 clone SK1 (BD Biosciences). Cells were again washed and fixed with 100 μl Cytofix/Cytoperm (BD Biosciences) and then incubated with 1X Perm/Wash buffer (BD Biosciences) for 30 minutes at room temperature in the dark. Cells were washed and resuspended in 50 μl 1X Perm/Wash buffer containing IFN-γ-Alexa Fluor 700 clone B27 (BD Pharmingen), TNFα-eFluor 450 clone MAb11 (eBioscience), and MIP-1β-APC clone D21-1351 (BD Pharmingen) and incubated for 20 minutes at room temperature in the dark. Cells were washed, resuspended in PBS, and analyzed on a BD LSR Fortessa flow cytometer using BD FACSDiva software. Data were analyzed using FlowJo version 9.6.4. The gating strategy is shown in Supplementary Figure 3 and multifunctional CD8+ T cells were identified using Boolean gating. Polyfunctional data were analyzed using SPICE version 5.3 (43).

Statistical analyses

Evaluation of the differences in IFNγ production with and without DC in response to individual peptides and the differences in mean IFNγ production of all peptides combined was performed using a two-way ANOVA with Šidák’s multiple comparisons post-test. All graphs and statistical analyses were generated using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA).

Results

Clinical characteristics of the study participants
In the present study, 3 HIV-1 infected subjects from the Multicenter AIDS Cohort Study (MACS) were chosen for longitudinal analysis. These subjects were chosen based on their prolonged enrollment in the study (>15 years), typical course of disease progression, and the presence of at least 1 common HLA allele, which we determined to be HLA A*2402. For each subject (S2, S3, and S8), HIV-1 plasma viral loads and CD4⁺ and CD8⁺ T cell counts were determined biannually for >15 years post-seroconversion (Figure 1). All three subjects progressed to AIDS (<200 CD4⁺ T cells/µl) between 7.0 and 8.25 years after infection and subsequently received and responded favorably to cART. Subject S2 had several rebounds in viral load shortly after implementation of cART, which possibly indicated treatment adherence issues. At most other post-cART time points, however, viral load was below the limit of detection (<20 copies/ml) in this subject as well as in subjects S3 and S8.

Changes in HIV-1 Gag and Env epitopes in chronic infection

A proposed mechanism by which infected subjects fail to control HIV-1 replication is the lack of cytotoxic T lymphocytes (CTL) that recognize the patient’s own virus (“autologous virus”) due to mutations within CTL epitopes that ablate MHC class I affinity and/or T cell recognition (4, 44). We therefore longitudinally assessed the changes in predicted MHC class I affinity of Gag and Env epitope variants derived from the autologous HIV-1 sequences of subjects S2, S3, and S8.

HIV-1 was sequenced from plasma obtained from 5 to 6 post-seroconversion, pre-cART time points in all three subjects. Additionally, HIV-1 was isolated from CD4⁺ T cells at one post-cART time point in subjects S2 and S3. We were unable to isolate HIV-
13 from CD4+ T cells in subject S8, potentially due to the low HIV-1 viral load in this subject at all stages of disease progression. The source of HIV-1 likely did not affect the resulting sequences, as virus recovered from plasma has been shown to be identical to virus obtained from CD4+ T cells after short-term culture (45). We performed single genome HIV-1 gag p17-p24 and env gp120 sequencing on the virus obtained from each subject at each time point. Nucleotide sequences were then translated to their corresponding amino acid sequences and we identified 6 known Gag and Env HLA A*24-restricted epitopes that were documented in the Los Alamos HIV Immunology Database (http://www.hiv.lanl.gov/content/immunology) and 7 HLA A*2402-restricted predicted epitopes using the Immune Epitope Database combined predictor with the netMHCpan MHC class I prediction method (39-41). Using the autologous Gag and Env sequences from each subject, we were able to generate a library of epitope variants that evolved throughout infection. To narrow the number of epitope variants and to eliminate focus on the dominant variants that evolved throughout infection, only variants that had a frequency of greater than 0.25 within the variant pool at any given time point were included in our study. The Gag epitopes studied and the variants that evolved in vivo are shown in Table 1. In all Gag epitopes that evolved throughout infection, there was a switch in the dominant form of the epitope, whereby the founder epitope became less frequent than the variant that evolved later in infection (Figure 2A). In subjects S2 and S3, for whom we obtained post-cART sequences, the dominant variant at the last pre-cART time point was the only variant in circulation post-cART; accordingly, this last time point (>20 years post-SC) is not shown in Figure 2. Interestingly, not all known and predicted epitopes evolved throughout infection, despite each subject being infected.
for >7 years without receiving cART. Of note, the predicted AFSPEVIPMF (AF10) and
known DYVDRFYKT (DT9) Gag p24 epitopes did not evolve in any of the subjects and
the predicted Gag p17 RFAVNPGLL (RL9) epitope presented major variants in all 3
subjects. Not surprisingly, most, but not all variants had a lower predicted affinity for the
HLA A*2402 molecule in comparison to the founder variant that was present at the first
post-seroconversion time point.

We then evaluated amino acid changes in the 3 known and 4 predicted Env
epitopes (Table 2). In the Env epitopes that mutated to produce variants in subjects S2
and S8, all resulted in the founder epitope being less frequent than the variant by the last
sequencing time point (Figure 2B). This was true in the mutated epitopes in subject S3
as well, except for the variant of the SI8 epitope, which only made up 31% of the SI8
epitope pool at its maximum. Two variants comprising of >25% of the epitope pool at a
given time point, but not more than 50%, were also noted in subjects S3 and S8.

We next used computational prediction algorithms to evaluate the potential effects
of these amino acid substitutions on the affinity of these epitope variants for the common
HLA allele amongst the 3 subjects, HLA A*2402. Of the 4 epitopes that evolved in
subject S2, 2 evolved to variants with lower predicted affinity (KF9 and RI10) and 2
evolved to variants with higher predicted affinity (SI8 and MI10). In subject S3, 6
epitopes produced major variants throughout infection. Of these, 3 mutated to produce
variants with lower predicted MHC class I affinity (SI8, KF9, and MI10), 2 epitopes
produced variants with higher MHC class I affinity (RI10 and LI9), and 1 epitope
produced variants with lower and higher predicted MHC class I affinity (FF9). In
subject S8, 4 epitopes contained amino acid changes, with 2 producing variants with
lower predicted MHC class I affinity (LY10 and KF9) and 2 produced variants with higher predicted MHC class I affinity (FF9 and MI10). Therefore, these findings suggest that Gag and Env epitope variants with lower MHC class I affinity were not specifically selected for during disease progression or were at a high cost to viral fitness and were therefore removed from the viral pool. Escape mutations in Gag, but not Env, may have incurred significant fitness costs as has been described previously (46). Nonetheless, within the quasispecies that remained in circulation, variants with a higher MHC class I affinity than the founder epitope were as predominant as variants with a lower affinity than the founder epitope.

DC-mediated enhancement of autologous HIV-1-specific T cell responses

We showed above that HIV-1 Gag and Env epitope variants may evolve to higher and lower MHC class I affinities. This has obvious implications in the generation of T cell responses to these variants. However, we have shown previously that CD8⁺ T cell responses generated in vivo to HIV-1 peptide antigens may not be detected in standard in vitro assays without the addition of a mature, antigen-loaded DC (47). We therefore confirmed the recognition of the known and predicted epitopes and assessed the impact of autologous HIV-1 epitope evolution on longitudinal T cell responses and on the ability to detect them with the addition of DC at early post-seroconversion (early post-SC; <6 months post-SC), late post-seroconversion (late post-SC; >7 years post-SC and <6 months before cART), early post-cART (<6 months post-cART), and late post-cART (>13 years post-cART) time points. To do this, the founder peptides and variants shown in Tables 1 and 2 were synthesized and used to represent autologous MHC class I-
restricted HIV-1 peptide antigens, with “founder” epitopes designated as autologous epitope variants that were in circulation early post-SC, and “variants”, which evolved after the early post-SC time point. PBMC obtained from each time point were stimulated with peptide alone or peptide-loaded mature, autologous DC in an overnight IFN\(\gamma\) ELISpot assay. Only peptides representing the autologous HIV-1 epitope founders and variants that evolved by the time of PBMC sampling were used. At both post-cART time points, PBMC from subjects S2 and S3 were tested against autologous epitope variants as determined by sequencing reactivated cell-associated virus derived from the early post-cART time point. We were unable to reactivate virus in subject S8, so we therefore tested PBMC against the epitope variants that were in circulation immediately prior to cART, as HIV-1 derived from the cART reservoir has been shown to be phylogenetically similar to the virus that was in circulation prior to treatment (48).

PBMC responses of varying magnitude were detected to all founder epitopes and all epitope variants in at least one time point in subject S2 (Figure 3). Included in these were peptides predicted to be epitopes, thereby confirming recognition of these peptides by PBMC. The use of antigen-loaded DC significantly increased PBMC reactivity observed early post-SC to 13 out of 14 founder epitopes (p values shown in Supplementary Tables 1 and 2). By late post-SC, the effect of DC on IFN\(\gamma\) production had waned, with increased reactivity detected to only 6/14 founder epitopes and only 3/7 variants. This was not unexpected, as the subject had progressed to AIDS. The effect of DC on PBMC reactivity decreased even further by the early post-cART time point, with DC enhancing responses to only 3 founder epitopes and 2 variants. In total, 12 of 14 founder epitopes exhibited the lowest PBMC reactivity at the early post-cART time point.
Subsequently, in 11 of 14 founder epitopes, DC-enhanced IFNγ production rebounded after many years of treatment at the late post-cART time point, coincident with recovery during long-term cART.

We observed a similar pattern in reactivity in subject S3. PBMC responses were detected to all founder epitopes and all variants in at least one time point (Supplementary Figure 1), indicating priming to these variants occurred in vivo. At the early post-SC time point, the presence of antigen-loaded DC in the ELISpot assay significantly increased IFNγ production in response to 12 out of 13 founders (p values shown in Supplementary Tables 1 and 2). DC-enhancement to 11 founders decreased by the late post-SC time point, and significant enhancement was observed to only 5 founders and only 4 of 10 variants. Unlike in subject S2, the effect of DC on PBMC reactivity rebounded early after treatment, at the early post-cART time point, showing a significant enhancement in response to 12 founders and 9 variants. This effect and IFNγ production was largely maintained after many years of treatment, at the late post-cART time point, with 11 founders and all 10 variants inducing significantly greater reactivity with antigen-loaded DC compared to PBMC stimulated with peptide alone.

Analysis of subject S8 also produced similar findings. At the early post-SC time point, antigen-loaded DC significantly increased IFNγ production in response to 11 out of 13 founders (Supplementary Figure 2; p values shown in Supplementary Tables 1 and 2). We observed decreases of variable magnitude in DC enhancement for 9 of 13 founders at the late post-SC, time point, with DC significantly increasing IFNγ production in response to only 6 of 13 founders. This was the first time point at which late-evolving epitope variants were evaluated, and DC were unable to increase PBMC
reactivity to any of these 6 variants. However, DC enhancement was restored to reactivity that was equal to or greater than that observed early post-SC in response to all founders for which we observed a decrease after the early post-SC time point. Additionally, DC-enhanced responses to 5 of 6 variants were increased late post-cART. These increases were mostly maintained late post-cART.

There were no correlations identified between IFN\textsubscript{γ} production and predicted MHC class I affinity in any subject or time point, with or without DC (data not shown).

In summary, the data presented here suggest that HIV-1-specific responses are generated \textit{in vivo} to founder epitopes and late-evolving epitope variants. Recall PBMC reactivity was enhanced by the use of antigen-loaded DC in the ELISpot assay and was detected during chronic infection and after many years of suppressive cART. These responses and the enhancing effect of DC was detected at all time points but was of a lesser efficacy late post-SC when the subjects had progressed to AIDS and were in poor immunological health, and to an extent at the early post-cART time point in subject S2, when this patient experienced a rebound in viral load while under cART. Nevertheless, our findings show that autologous HIV-1 epitope variants induce primary immune responses \textit{in vivo}, regardless of minute changes in affinity for MHC class I, but may not be detected in immunological assays without the use of a potent APC.

\textit{PBMC obtained after cART are restored in their ability to respond to HIV-1-specific DC stimulation}

We then longitudinally evaluated average PBMC reactivity with and without DC to founder and variant epitopes (Figure 4) as determined by our IFN\textsubscript{γ} ELISpot assay.
described above. Evaluating the data in this fashion enabled us to assess the overall change in the effect of DC on antigen-specific immune responses at each time point in infection. The mean PBMC reactivity to founder epitopes without DC was similar in subjects S2 and S3 (Figure 4, top left and top middle), with moderate mean IFNγ production early post-SC, followed by a decrease late post-SC. The low response was maintained early post-cART and rebounded to early post-SC levels at the late post-cART time point. In subject S8, however, the early post-SC PBMC response to founder epitopes was nearly undetectable. This was followed by an increase in mean reactivity late post-SC that was maintained early post-cART and slightly increased late post-cART (Figure 4, top right).

In all 3 subjects, mean reactivity with antigen-loaded DC was greater than the reactivity of PBMC with peptide alone at the early post-SC time point (Figure 4, top panels; p<0.001 for subjects S2 and S3, p=0.0012 for subject S8). This enhancement was eliminated late post-SC in all three subjects, with the average DC-enhanced reactivity showing no significant differences from PBMC stimulated with peptide alone. This low DC-stimulated response to founder epitopes was maintained in subject S2 early post-cART, but rebounded in subjects S3 and S8 to show a significant difference from PBMC alone (p=0.0008 for subject S3 and p=0.0006 for subject S8). By the late post-cART time point, all 3 subjects exhibited robust DC-enhanced IFNγ production in response to the founder epitopes that was of greater magnitude than peptide-stimulated PBMC (p<0.0001 for subject S2, p=0.0003 for subject S3, and p.0008 for subject S8).

A similar pattern was observed in mean IFN production to variant epitopes which were not evaluated for PBMC reactivity until the late post-SC time point (Figure 4,
bottom panels). In subject S2, DC had no significant effect on the mean PBMC reactivity late post-SC or early post-cART, but induced significant increases in IFN$\gamma$ production after many years of treatment ($p<0.0001$; Figure 4, bottom left). In subject S3, DC induced no significant increases in the mean PBMC response late post-SC, but induced gradual, significant increases in this response at the early post-cART ($p=0.0077$) and late post-cART time points ($p=0.0053$; Figure 4, bottom center). This same pattern was seen in subject S8 (Figure 4, bottom right), with DC inducing significant increases in mean PBMC reactivity to variant epitopes at the early post-cART ($p=0.0002$) and late post-cART ($p=0.0023$) time points.

Taken together, these data show a stark trend in the ability of DC to reveal HIV-1-specific T cell responses to Gag and Env founder and variant peptide epitopes. In all three subjects, DC enhancement of antigen-specific responses to founder epitopes was high early post-SC and then dropped in chronic HIV-1 infection. There was then a rebound in the DC-mediated increase in reactivity to founder and variant epitopes after cART when partial immune reconstitution and a reduction in viremia occurred.

**DC-mediated increases in CD8$^+$ T cell production of type-1 immune mediators**

We showed above that DC enhance IFN$\gamma$ secretion specific for autologous epitope variants at multiple stages of HIV-1 infection. Control of HIV-1 has been associated with a broad polyfunctional CD8$^+$ T cell response to HIV-1 antigens (49), particularly Gag (6, 50-52). A DC immunotherapy would aim to induce or reactivate such a response. These immunotherapies would be implemented in subjects who are likely to have been on cART for many years, so it is imperative to determine if an
endogenous polyfunctional CD8+ T cell response was generated, was retained during long-term cART, and is capable of being enhanced or reactivated by DC. We therefore evaluated intracellular polyfunctional CD8+ T cell reactivity in response to the autologous epitope variants that were in circulation at the late post-SC and post-cART time points and determined if this effect increased as subjects regained their health under suppressive treatment. We co-cultured PBMC obtained from each subject at the late post-SC, early post-cART, and late post-cART time points with autologous HIV-1 epitope variants alone or loaded onto DC (insufficient cell numbers were available for the early post-SC time point). For this analysis we only used variants of the previously known Gag and Env epitopes and excluded the predicted epitopes (Tables 1 and 2).

Following a 6 h co-culture, PBMC were evaluated for their expression of the type I-associated molecules IFNγ, CD107a, MIP-1β, TNFα, and IL-2 by flow cytometry. The gating strategy for identifying cytokine-secreting cells is shown in Supplementary Figure 3. All three subjects produced similar findings, with results from subject S2 shown here.

Late post-SC, DC often increased the percent of HIV-1-specific monofunctional CD8+ T cells (Figure 5) and specifically enhanced the breadth and magnitude of IFNγ production and CD107a translocation. We also observed an increase in the percent of CD8+ T cells producing MIP-1β and TNFα following DC stimulation, as well as in the number of peptide antigens that induced this bifunctional response. Tri-functional CD8+ T cells that stained positive for CD107a, MIP-1β, and TNFα were also more prevalent following DC stimulation and were observed in response to a higher number of the autologous HIV-1 antigens. The emergence of CD8+ T cells producing 4 immune
mediators in response to the DYVDRFYKT variant was observed following DC stimulation and was the only variant to induce CD8+ T cell production of 4 immune mediators. We observed an overall “shift to the left”, whereby more polyfunctional responses were seen with DC than without DC, and a higher breadth of peptides were inducing these polyfunctional responses.

We observed similar findings shortly after the initiation of cART (Figure 6). The breadth and magnitude of CD107a-positive CD8+ T cells was again increased following DC stimulation, as well as bifunctional CD8+ T cells that produced MIP-1β and TNFα. There was also a DC-mediated increase in the breadth and magnitude of IFNγ+/MIP-1β+/TNFα+/CD8+ T cells and CD107a+/MIP-1β+/TNFα+/CD8+ T cells. We again observed the emergence of CD8+ T cells staining positive for CD107a, IFNγ, MIP-1β, and TNFα when DC were used, again in response to DYVDRFYKT and now also in response to KYRLKHIIVW. At his time point, polyfunctional responses were more pronounced than at the late post-SC time point.

After many years of suppressive cART, at the late post-cART time point, we observed a pronounced “shift to the left” in the cytokine profile of HIV-1-specific CD8+ T cell reactivity (Figure 7). We noted increases in the percent of monofunctional CD8+ T cells that stained positive for CD107a and IFNγ and in the number of peptides that induced production of these mediators following DC stimulation. As seen during the late post-SC and early post-cART time points, the breadth and magnitude of MIP-1β+/TNFα+/CD8+ T cells was increased with the use of DC, as well as a myriad of other bi- and tri-functional cytokine profiles. The CD107a+/IFNγ+/MIP-1β+/TNFα+ cytokine profile was again revealed and enhanced by DC.
In summation, the addition of DC to the HIV-1 variant-specific intracellular cytokine assay revealed CD8+ T cells that were producing multiple immune mediators at all three time points, but with a more pronounced polyfunctional profile after initiation of cART. These observations therefore show the capacity of DC to reveal otherwise masked recall T cell responses specific for autologous virus even after many years of treatment at a time point when DC immunotherapy would likely be implemented.

Discussion

CTL play a vital role in controlling HIV-1 infection (53, 54). The failure to control viremia in chronic infection has been attributed to a decline in CTL responses that are specific for the autologous virus (5, 12, 55-60). Indeed, mutations within CTL epitopes that ablate T cell recognition or MHC class I affinity and would therefore prevent the generation of a primary CD8+ T cell response against that epitope. In this study, we employed a method of detecting and enhancing T cell responses specific for autologous HIV-1 epitope variants at multiple stages of infection, before and after cART. Here we show that DC reveal broad and robust IFNγ ELISpot responses regardless of disease progression, but most specifically after long-term suppressive cART. These results were expanded using a DC-enhanced, polychromatic flow cytometry assay to detect multiple immune mediators in response to autologous HIV-1 epitope variants that were in circulation immediately prior to or after cART (contemporaneous variants). By using this approach, we observed DC enhancement of multiple cytokine production by
HIV-1-specific CD8+ T cells that was associated with suppression of viremia subsequent to cART.

The known and predicted HLA A*24-restricted epitopes used in our study exhibited various patterns of evolution. While many of the epitopes incurred amino acid changes throughout infection, there was no universal effect of these changes on the predicted MHC class I affinity, indicating HIV-1 evolution did not specifically evade MHC class I loading by antigen presenting cells (APC) or expression on the surface of infected cells, or that the mutations affected the proteolytic processing required to result in peptide loading. This observation would suggest that APC priming of naïve CD8+ T cells could have occurred in vivo if the T cell receptor (TCR) repertoire was sufficient to recognize these antigens. Indeed, we detected PBMC responses to >95% of variants irrespective of the predicted MHC class I affinity, showing amino acid mutations within epitopes did not ablate priming to these variants in vivo. Moreover, IFNγ production to variants of novel predicted epitopes was of a similar magnitude to the response against variants of known epitopes, therefore highlighting the potential of these predicted epitope regions to be classified as HLA A*2402-restricted epitopes.

We observed no correlation between the predicted MHC class I affinity of known or predicted epitope variants and IFNγ production. These findings are not surprising, as we have previously reported detection of recall T cell responses to autologous HIV-1 antigens with high, medium, and low experimental MHC class I affinities and multiple time points in disease progression (61). While decreased binding of mutated epitopes to MHC class I is thought to be a primary mechanism by which HIV-1 and SIV evade CD8+ T lymphocyte responses (4, 12, 62-70), our detection of IFNγ production to the epitope
variants in this report would challenge this conclusion. We do recognize, however, that
detecting IFNγ production is not necessarily indicative of a cytolytic CD8+ T cell
response that can control infection (71-74). The 3 subjects used in our study exhibited
conventional characteristics of HIV-1 disease progression, and therefore resident CTL
were unable to control viral replication in chronic infection. A CTL response with high
breadth and magnitude, specifically to Gag proteins, has been associated with SIV and
HIV-1 control (53, 54, 75-77). However, high-avidity CD8+ T cells have been detected
in progressive infection and therefore point to poor immune selection pressure exerted by
these cells (72). Indeed, the PBMC responses to autologous epitope variants described in
this study were broad and within a wide range of magnitudes despite the continuation of
disease progression, thus indicating ineffective selective pressure on the virus. We
postulate that a primary response to these epitope variants was generated in vivo,
although this response was likely of variable efficacy, as disease progression continued
and many of the epitope variants persisted throughout infection. Future studies should
focus on changes in cytolytic effector function against a select number of epitope variants
throughout HIV-1 infection. Nonetheless, these findings provide an in-depth
understanding of the breadth of T cell responses that are generated to autologous HIV-1
antigens regardless of MHC class I affinity.

We previously reported on the ability of mature, autologous DC to reveal and
enhance HIV-1-specific T cell responses in subjects on cART (21) and on their
usefulness in detecting and generating responses specific for HIV-1 variants with variable
MHC class I affinities (61). Not much is known, however, about the ability of DC to
enhance responses during progressive infection. After detecting moderate responses to
autologous epitope variants in PBMC, we determined the effects of DC addition on longitudinal CD8+ T cell IFNγ production throughout untreated and treated HIV-1 infection. Addition of DC to PBMC did not enhance the number of epitope variants that were recognized, as the breadth was already >95% when evaluating PBMC, but the magnitude was significantly enhanced at most time points in each subject.

Regardless of disease progression, DC were functional at enhancing IFNγ production against autologous epitope variants as judged in our overnight ELISpot assays. More importantly, we observed similar responses to variants that evolved early and late post-seroconversion, thus supporting the ability of DC to present mutated epitopes to their cognate CD8+ T cells. When we evaluated the “effect” of DC enhancement on the PBMC response over time, we saw striking similarities between the three subjects. There was an enhancing effect of DC observed early post-SC, followed by a drastic reduction late post-SC, and a restoration at the early post-cART or late post-cART time point. There are multiple mechanisms by which DC enhance recall T cell responses to HIV-1 (15, 16, 19), including high IL-12p70 production, expression of T cell costimulatory molecules CD80 and CD86, and expression of the DC maturation marker CD83. Our current findings show that DC-mediated enhancement of T cell responses is restored after many years of cART. These results suggest a DC immunotherapy that enhances endogenous HIV-1 reactivity could be effective in persons on cART. Such a treatment would involve the ex vivo generation of monocyte-derived DC, followed by antigen-loading, and injection back into the patient to reactivate quiescent recall T cell populations or to prime new CTL from naïve precursors (78).
We hypothesize that these findings are indicative of the immunological health of the subjects during infection. Within the first 6 months after seroconversion, which is where our early post-SC PBMC were derived, T cell counts in our three subjects were within normal ranges. It can be assumed, then, that immunological function was relatively intact, and therefore T cells at this time point could respond to DC stimulation. Late post-SC, however, an environment of immune dysfunction persisted and T cells were less able to respond to an antigen-specific DC stimulation. In two of the three subjects, this dysfunction was reversed early after cART and T cell responses to DC stimulation were restored. Interestingly, we did not see this in subject S2 until late post-cART. This may be due to the delay in the reduction of viral load and consequently in the reconstitution of immunological function following the administration of cART (Figure 1). In all three subjects, however, cells regained or retained the ability to respond to DC stimulation late post-cART, thus suggesting a DC-mediated therapy for subjects on long-term treatment may be successful in reinvigorating quiescent memory responses.

Production of multiple cytokines by CD8+ T cells has been associated with CTL effector function and control of HIV-1 infection (6, 49, 50, 52, 79). We therefore expanded our analysis of DC-mediated enhancement of HIV-1-specific CD8+ T cell responses by evaluating intracellular cytokine secretion late post-seroconversion and during early and late cART time points with and without DC. Even after many years of cART, DC could reveal monofunctional and polyfunctional HIV-1-specific CD8+ T cell responses. These findings again support the notion that DC immunotherapy aimed at reinvigorating the dysfunctional CTL response can be effective. Our IFNγ ELISpot
analysis showed variable time-associated effects of DC addition as subjects received cART, but staining for multiple immune mediators gave a broader picture of how suppressive cART aids in the ability of T cells to respond to HIV-1-specific stimuli with a phenotype most associated with CTL effector function. For example, at all three time points, DC stimulation enhanced the breadth and magnitude of CD8\(^+\) T cells that stained positive for the degranulation marker CD107a, which is most associated with CTL function.

The data presented in this study highlight the ability of T cells to respond to autologous HIV-1 epitope variants at all stages of disease progression and support the use of DC immunotherapy in subjects on cART. This should be viewed with caution, however, as we have recently shown that recall, HIV-1-specific CTL may produce multiple immune mediators without effector function if stimulated with a related, but slightly different, antigen than the one to which it was originally primed (71). Not only do these cells fail to exert cytolytic function on target cells expressing the variant antigen, but they promote DC maturation and viral spread \textit{in vitro}. This suggests that DC immunotherapy that uses an antigen that is similar, but not exact, to the antigen to which the subject’s T cells were originally primed, may not induce an effective CTL response and may actually hinder viral clearance. It may be that the most effective way of inducing a potent CTL response specific for the autologous HIV-1 reservoir is to generate new CTL from naïve precursors (78). The effect of antigen-specific DC stimulation on naïve and memory T cells and on their ability to facilitate elimination of the HIV-1 reservoir needs to be evaluated.
Acknowledgements

We thank Dr. John Mellors for the Taqman® assay, Kelly Gordon, Dr. Ronald Fecek and Weimin Jiang for technical assistance, and the volunteers of the Pittsburgh site of the Multicenter AIDS Cohort Study. This work was supported in part by NIH grants R01-AI-40388, R37-AI-41870 and U01-AI-35041, and a T32-AI065380 fellowship to KNS.
References


42. Colleton BA, Huang XL, Melhem NM, Fan Z, Borowski L, Rappocciolo G, Rinaldo CR. 2009. Primary human immunodeficiency virus type 1-specific


Figure Legends

Figure 1. Clinical characteristics of the study subjects. HIV-1 plasma RNA and CD4+ and CD8+ T cell counts were determined biannually following seroconversion to HIV-1. CD4+ (green line) and CD8+ (blue line) T cell counts and viral load (red line) are shown longitudinally for subjects S2 (left panel), S3 (middle panel), and S8 (right panel). All three subjects progressed to AIDS (CD4+ T cell count below 200 cells/mm^3, solid line) and received cART (dotted line).

Figure 2. Evolution and changes in frequency of autologous HIV-1 Gag and Env epitope variants. HIV-1 gag and env single genome sequencing was performed at multiple post-seroconversion time points for subjects S2, S3, and S8, and one post-cART time point for subjects S2 and S3. Known and predicted epitopes were identified using the Los Alamos Database and netMHCpan, respectively (Tables 1 and 2), and the frequency of each variant within each epitope was determined for subjects S2, S3, and S8. (A) Changes in the frequency of variants of Gag p17 and p24 epitopes. (B) Changes in the frequency of variants of Env epitopes. The founder variant, or the variant that was present at the first post-seroconversion time point is shown in black. Major variants that evolved after the first post-seroconversion time point and have a frequency >0.5 at at least one time point are shown in red. Minor variants that evolved after the first post-seroconversion time point and have a frequency greater than 0.25 at at least one time point but less than 0.5 at all time points are shown in blue. The Gag p24 AFSPENVIPMF
and Gag p24 DYVDRFYKT epitopes did not evolve in any of the subjects and are therefore excluded from this graph.

**Figure 3.** Representative longitudinal HIV-1-specific IFNγ production IFNγ production in response to autologous antigen with and without DC. PBMC obtained from subject S2 early post-SC (<6 months after seroconversion), late post-SC (>7 years after seroconversion and <6 months prior to cART), early post-cART (<6 months after cART administration), and late post-cART (>13 years after cART administration) were stimulated with peptide antigen or antigen-loaded DC in an overnight IFNγ ELISpot assay. PBMC alone (No DC; open shape, dotted lines) and DC-stimulated PBMC (+DC; closed shape, solid lines) were evaluated for reactivity to autologous founder (black lines) and variant (red lines) peptide sequences representing known and predicted Gag and Env epitopes. Responses are shown as spot-forming cells (SFC) per 10^6 PBMC and are the mean +/- SD of duplicate ELISpot wells with the average background plus two standard deviations subtracted. P-values comparing ELISpot responses with and without DC are shown in Supplementary Tables 1 and 2. SC, seroconversion; cART, combined antiretroviral therapy.

**Figure 4.** ART restores the ability of DC to enhance HIV-1-specific reactivity. The average IFNγ ELISpot response of all founder (top panels) and variant (bottom panels) epitopes is shown at each time point for PBMC alone (open circle) and DC-stimulated PBMC (closed circle) in subjects S2 (left panels), S3 (middle panels), and S8 (right panels). Data are shown as the average background-subtracted IFNγ production of all
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Figure 5. DC-mediated increase in polyfunctional CD8+ T cell cytokine profiles late post-SC. Autologous monocyte derived DC were matured with CD40L and loaded with autologous HIV-1 epitope variants. PBMC derived late post-SC were incubated with peptide alone (no DC) or peptide-loaded DC (DC). For practicality, only the variants of known Gag and Env epitopes were used. PBMC with and without DC were also incubated with SEB or media alone for positive and negative controls, respectively. PBMC were then stained for CD107a, IFNγ, IL-2, MIP-1β, and TNFα. Background was determined as the percent of CD8+ T cells staining positive for the relevant cytokine in the negative control condition. Polyfunctional data were analyzed using SPICE version 5.3 (43). Data are shown as the percent of CD8+ T cells that are antigen-specific in response to each peptide evaluated for each cytokine/chemokine profile, as well as the overall number of immune mediators detected in response to each variant.

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**Figure 7. DC-mediated increase in polyfunctional CD8⁺ T cell cytokine profiles after long-term cART.** Autologous monocyte derived DC were matured with CD40L and loaded with autologous HIV-1 epitope variants. PBMC derived late post-cART were incubated with peptide alone (no DC) or peptide-loaded DC (DC). For practicality, only the variants of known Gag and Env epitopes were used. PBMC with and without DC were also incubated with SEB or media alone for positive and negative controls, respectively. PBMC were then stained for CD107a, IFNγ, IL-2, MIP-1β, and TNFα. Background was determined as the percent of CD8⁺ T cells staining positive for the relevant cytokine in the negative control condition. Polyfunctional data were analyzed using SPICE version 5.3 (43). Data are shown as the percent of CD8⁺ T cells that are antigen-specific in response to each peptide evaluated for each cytokine/chemokine profile, as well as the overall number of immune mediators detected in response to each variant.
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Figure 5. DC-mediated increase in polyfunctional CD8+ T cell cytokine profiles late post-SC. Antigen-specific monocyte-derived DC were stained with CD45R and loaded with autologous H1011 epitope variants. PBMC-derived late post-SC were incubated with peptide alone (no DC) or peptide-loaded DC (DC). For practicality, only the variants of known Gag and Env epitopes were used. PBMC with and without DC were also incubated with SEB or media alone for positive and negative controls, respectively. PBMC were then stained for CD107a, IFNγ, IL-2, MIP-1ß, and TNFα. Background was determined as the percent of CD8+ T cells staining positive for the relevant cytokine in the negative control condition. Polyfunctional data were analyzed using SPICE version 5.1 (1). Data are shown as the percent of CD8+ T cells that are antigen-specific in response to each peptide evaluated for each cytokine/chemokine profile, as well as the overall number of immune mediators detected in response to each variant.
Figure 6. DC-mediated increase in polyfunctional CD8 T cell cytokine profiles early post-AART. Autologous monocyte-derived DC were matured with CD40L and loaded with autologous HIV-1 epitope variants. PBMC-derived early post-AART were incubated with peptide alone (no DC) or peptide-loaded DC (DC). For practicality, only the variants of known CD8 and T cell epitopes were used. PBMC with or without DC were also incubated with media alone for positive and negative controls, respectively. PBMC were then stained for CD45RA, IFN-γ, IL-2, MIP-1β, and TNF-α. Background was determined as the percent of CD8 T cells staining positive for the relevant cytokine in the negative control condition. Polyfunctional data were analyzed using SPICE version 5.3 [G.L.]. Data are shown as the percent of CD8 T cells that are antigen-specific in response to each peptide evaluated for each cytokine/chemokine profile, as well as the overall number of immune mediators detected in response to each variant.
Figure 7. DC-mediated increase in polyfunctional CD8 T cell cytokine profiles after long-term cART. Antigenic monocyte-derived DC were matured with CD40L and loaded with analogous HBoV epitope variants. PBMC derived from post-cART were incubated with peptide alone (no DC) or peptide-loaded DC (DC). For practicality, only the variants of known Gag and Env epitopes were used. PBMC with and without DC were also incubated with MEB or media alone for positive and negative controls, respectively. PBMC were then stained for CD8, IFN-γ, IL-2, MIP-1β, and ENF. Background was determined as the percent of CD8 T cells staining positive for the relevant cytokine in the negative control condition. Polyfunctional data were analyzed using SPICE version 3.3 [1]. Data are shown in the percent of CD8 T cells that are antigen-specific in response to each peptide evaluated for each cytokine/chemokine profile, as well as the overall number of immune mediators detected in response to each variant.
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HXB2 location of each HIV-1 epitope within Gag p17 and p24

HXB2 consensus amino acid sequences for known and predicted HLA A*24-restricted HIV-1 Gag and Env epitopes. Known epitopes are in bold and non-bolded sequences show the HXB2 sequence of predicted CTL epitopes.

Subjects S2, S3, and S8 were chosen from the Multicenter AIDS Cohort Study for analysis.

Autologous epitope variants were identified by single-genome gag p17-p6 and env gp120 sequencing of autologous plasma-derived HIV-1. Underlined variants were the dominant form of the epitope (>55% of the variant pool) that was observed at the last time point sequenced.

The time at which the epitope variant was detected by single genome sequencing.

YPS, years post-seroconversion.

Predicted affinity for HLA A*2402 as determined using netMHCpan.

A lower value indicates a higher predicted affinity. nM, nanomolar
Table 2. Autologous variants of known and predicted HIV-1 Env epitopes that evolved throughout infection

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<th>Epitope location(^a)</th>
<th>Amino acid sequence(^b)</th>
<th>Subject(^c)</th>
<th>Autologous variant sequence(^d)</th>
<th>Time of evolution ((\text{YPS}))</th>
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aHXB2 location of each HIV-1 epitope within the Env protein

bHXB2 consensus amino acid sequences for known and predicted HLA A*24-restricted HIV-1 Gag and Env epitopes. Known epitopes are in bold and non-bolded sequences show the HXB2 sequence of predicted CTL epitopes.

cSubjects S2, S3, and S8 were chosen from the Multicenter AIDS Cohort Study for analysis.

dAutologous epitope variants were identified by single-genome gag p17-p6 and env gp120 sequencing of autologous plasma-derived HIV-1. Underlined variants were the dominant form of the epitope (>55% of the variant pool) that was observed at the last time point sequenced.

eThe time at which the epitope variant was detected by single genome sequencing.

YPS, years post-seroconversion.

fPredicted affinity for HLA A*2402 as determined using netMHCpan.

A lower value indicates a higher predicted affinity. nM, nanomolar