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HIV-specific T-cell responses reflect substantive in vivo interactions with antigen despite long-term therapy

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Abstract

Antiretroviral therapies (ART) abrogate HIV replication; however, infection persists as long-lived reservoirs of infected cells with integrated proviruses, which re-seed replication if ART is interrupted. A central tenet of our current understanding of this persistence is that infected cells are shielded from immune recognition and elimination through a lack of antigen expression from proviruses. Efforts to cure HIV infection have therefore focused on reactivating latent proviruses to enable immune-mediated clearance, but these have yet to succeed in reducing viral reservoirs. Here, we revisited the question of whether HIV reservoirs are predominately immunologically silent from a new angle: by querying the dynamics of HIV-specific T-cell responses over long-term ART for evidence of ongoing recognition of HIV-infected cells. In longitudinal assessments, we show that the rates of change in persisting HIV Nef-specific responses, but not responses to other HIV gene products, were associated with residual frequencies of infected cells. These Nef-specific responses were highly stable over time, and disproportionately exhibited a cytotoxic, effector functional profile, indicative of recent in vivo recognition of HIV antigens. These results indicate substantial visibility of the HIV-infected cells to T-cells on stable ART, presenting both opportunities and challenges for the development of therapeutic approaches to curing infection.
Introduction

The needs for both a vaccine and a cure for HIV are underscored by the ongoing impact of this pandemic, which continues to cause close to 800,000 deaths annually (1). Antiretroviral therapy (ART) is capable of durably suppressing HIV replication and halting disease progression – for those able to access and adhere to these regimens. Infection persists, however, in reservoirs of CD4+ T-cells, and potentially other cell types (2–4), with integrated proviruses that re-seed systemic replication if ART is interrupted (2, 5–10). These proviruses often exist in a latent state, characterized by limited transcription and – presumably – a lack of antigen production. This gives rise to one of the central tenets in the study of HIV persistence, which postulates that the persistent reservoir (often called the ‘latent reservoir’) is not detected by the immune system in individuals on long-term ART. It follows that engaging the immune system to reduce HIV reservoirs depends upon latency reversal to re-expose the immune system to HIV antigen – the so-called “kick and kill” (or "shock and kill") strategy (11).

While latency undoubtedly diminishes immune recognition of viral reservoirs, several lines of evidence cast doubt on whether this is absolute in vivo, which would implicate additional contributors to viral persistence (12). Most notably, unspliced, and sometimes multiply spliced, HIV transcripts are readily detectable in peripheral blood mononuclear cells (PBMCs) of individuals on durable ART (13, 14). These observations have recently led some to propose amendments to the “latent reservoir” model, by introducing the idea of a continuum ranging from “deep latency” (no RNA produced) through to an “active reservoir” (15, 16). A key unresolved question, however, is whether these transcripts result in HIV-protein production, and thus enable immune recognition. Multiple factors limit the degree to which this can be inferred from direct measures of in vivo viral expression, including sampling difficulties – given that expression may be anatomically or temporally restricted – and the lack of equivalency between readily measurable features (ex. viral RNA) with bona fide antigen presentation (17, 18). We therefore hypothesized
that some level of antigen recognition by HIV-specific T-cells may occur in vivo in ART-suppressed individuals with undetectable viremia. We predicted that this would be reflected in relationships between the long-term dynamics of HIV-specific T-cell responses and measures of virologic persistence, including frequencies of infected cells as measured by HIV DNA.

Although the T-cell response to HIV infection has been generally well characterized, and is known to decay rapidly in the months following ARV initiation (19–21), there are a lack of well-powered studies that have addressed the long-term dynamics of these responses in association with virologic parameters. In a previous cross-sectional study, we observed a modest correlation between the magnitudes of T-cell responses to the HIV Nef protein with residual frequencies of infected cells (22), providing some initial suggestion that these responses may be maintained by antigen recognition. However, a recent longitudinal study reported that, while HIV-specific T-cell responses were highly stable on durable ART, no correlations were observed between response magnitudes and intact-inducible reservoir size as measured by quantitative viral outgrowth assays (QVOAs) across 18 individuals (23). The current study builds upon these earlier reports by uniquely assessing T-cell response dynamics over almost 3 years in association with multiple measures of viral persistence, in a cohort of 49 individuals on well-documented sustained ART. Using the scalable IFNγ enzyme-linked immune absorbent spot (ELISPOT) assay, we confirm that HIV-specific T-cell responses persist over years of well-documented suppressive ART. Strikingly, the persistence of T-cell responses to the HIV Nef protein (as measured by slopes of change) over 144 weeks were strongly and uniquely associated with the frequencies of infected cells that persisted on ART (as measured by HIV DNA), and these responses disproportionately exhibited a cytotoxic effector functional profile, indicative of recent in vivo antigen recognition. These results provide strong support for ongoing interactions between the immune system and the HIV reservoir over years of ART, with implications both for understanding HIV persistence, and for designing interventions aimed at curing infection.
Results

HIV-Specific T-cell Responses are Readily Detectable Despite Long-Term ART

We previously assessed HIV-specific T-cell responses in participants from the AIDS Clinical Trials Group (ACTG) A5321 HIV Reservoirs Cohort Study (22), which consists of participants who initiated ART during chronic HIV infection and had subsequent well-documented, sustained virologic suppression (undetectable by clinical assay prior to and throughout the study period) (24). In our prior publication, PBMC samples from A5321 participants were assayed by IFN-γ ELISPOT at study entry, a median of 7 (range 4-15) years after ART initiation (22); here, we extended these results with batched analysis of samples from 24 and 168 weeks after study entry in a subset of 49 participants (Fig. 1 and Tables 1 & 2). These ELISPOT assays were performed using whole gene product pools composed of overlapping 15-mer peptides spanning: i) Gag, ii) Env, iii) Pol, iv) Nef, v) Tat, vi) Rev, vii) Nef/Tat/Rev (combined pool), and viii) cytomegalovirus (CMV) pp65 (control). In our prior study, as well as in the current study, responses against whole gene product peptide pools were background subtracted (thus, non-zero responses were >1x background), but no other ad hoc empirical cutoff was applied – consistent with other studies examining correlations with objectively reported T-cell responses as assessed by ELISPOT assay (25). In the current study, IFN-γ-producing HIV-specific T-cell responses were readily detected against Gag, Pol, and Nef, with mean, background-subtracted values at 24 weeks: 297.4, 173.1, and 107.7 SFU/10^6 PBMCs, respectively; and at 168 weeks: 169.2, 99.5, and 75.4 SFU/10^6 PBMCs, respectively (Fig. 2A - B and Table S1). Between this 24 to 168-week period, time-averaged responses against Gag were the highest, and significantly greater than responses to Env, Nef, Tat, and Rev (all p<0.05) (Table S2). Notably, T-cell responses directed against Tat and Rev were the lowest in magnitude, and negligible at both timepoints (Fig. 2B and Tables S1 & S2). The long-term persistence of HIV-
specific T-cell responses – primarily directed against Gag, Pol, and Nef – over years of ART thus provided initial support for these HIV-specific T-cells continuing to interact with antigen.

Dynamics of Nef-Specific T-cells Correlate with Virologic Parameters on Long-Term ART

To further characterize the long-term dynamics of HIV-specific T-cell responses in A5321 cohort participants on durable ART, we categorized participants’ IFN-γ ELISPOT responses from the batched 24 to 168 weeks post-study entry data as either increasing, decreasing, or not changing (pre-defined as ≤15% change), and observed considerable heterogeneity (Fig. S1). Notably, population-average responses to Nef, summed HIV, and CMV pp65 did not decline significantly over this 144-week time period, whereas responses to Gag, Env, and Pol all showed significant declines over time (Fig. 3A and Table S3). However, all HIV-specific T-cell responses demonstrated remarkable persistence, with the responses which showed a significant decline only averaging between 0.35% to 0.62% loss per week in IFN-γ ELISPOT assays (Table S3).

To determine whether ongoing antigen recognition by HIV-specific T-cells could be maintaining IFN-γ-producing HIV-specific T-cell responses, we next examined associations between the slopes of change of T-cell response magnitudes between 24 and 168 weeks post-study entry (based on absolute changes on a linear scale) with on-ART virologic parameters, including total cell-associated HIV DNA (CA-DNA), cell-associated HIV RNA (CA-RNA), and plasma HIV RNA by integrase single copy assay (iSCA). Due to a large number of statistical tests, we corrected for the false discovery rate at a stringent level of 0.05; additionally, we controlled for potential confounders in associations. The dynamics of responses to Nef were uniquely significantly associated with pre-ART viral loads before and after adjusting for CA-DNA at study entry (adjusted $r = 0.48$, $p = 0.012$ – Fig. 3B and Table S4), despite participants having been on ART for over a median of 7 years when responses were first measured. Strikingly, the slopes of change in Nef-specific responses were also unique in exhibiting highly significant direct associations with any on-ART virologic parameter after adjusting for potential confounding by pre-
ART plasma viral load, pre-ART CD4+ T-cell count, and years on ART at study entry – specifically, on-ART CA-DNA at study entry (adjusted $r = 0.50$, $p = 0.012$); slopes of change in Nef-specific responses were also uniquely associated with CA-RNA at study entry ($r = 0.46$, $p = 0.018$), though significance did not survive adjustment for confounders ($r = 0.40$, $p = 0.063$) (Fig. 3B and Table S4). These results indicate that higher frequencies of persistent infected cells (CA-DNA), and, potentially, higher levels of viral transcription (CA-RNA), were associated with greater maintenance of Nef-specific responses, consistent with some ongoing stimulation by infected cells. Slopes of change in HIV-specific T-cell responses were not associated with PD-1 levels on total CD4+ or CD8+ T-cells, nor were they associated with either age at study entry or years on ART at study entry (Fig. 3B and Table S4), though they generally correlated with each other (Table S5). Analyzing slopes of change in log$_{10}$-transformed T-cell response magnitudes, reflecting proportional changes in responses rather than absolute changes, revealed a significant association only between the dynamics of Nef-specific T-cell responses with on-ART CA-DNA at study entry (adjusted $r = 0.49$, $p = 0.029$ – Table S6). Proportional changes in HIV-specific responses generally correlated with each other (Table S7). Thus, whether dynamics were measured on an absolute or proportional change scale, Nef-specific response persistence was uniquely associated with HIV-infected cell frequencies.

In order to rule out the potential for IFN-$\gamma$-producing Nef-specific ELISPOT responses being enriched in low magnitude false positive responses, thereby influencing results, we performed a sensitivity analysis applying a rigorous positivity cutoff of >3x background. Data from a given participant were excluded if the results from both of the timepoints fell below this threshold (since going from detectable to below the threshold or vice versa are still biologically meaningful). Applying this cutoff, Nef-specific responses still exhibited remarkable stability, with no significant decline over time (Fig. S2 and Table S8). In correlation analyses, the magnitudes of correlations between the slopes of change in Nef-specific T-cell responses with on-ART virologic parameters
(CA-DNA and CA-RNA) were strengthened, relative to those observed without applying this cutoff (Table S9). Altogether, these results suggest that Nef-specific T-cell responses are preferentially maintained by ongoing interactions with HIV-infected cells, though all responses are likely maintained to some extent by ongoing HIV antigen recognition given their exceptional persistence.

Nef-Specific T-cells Exhibit Functional Profiles Consistent with Recent Antigen Recognition

We next investigated whether the functional properties of HIV-specific CD8+ T-cells would yield insights into their recent histories of in vivo antigen encounter. Data from human studies and animal models have highlighted ex vivo granzyme B production as a distinguishing feature of virus-specific effector CD8+ T-cells which have recently encountered antigen in vivo – either through infection or vaccination (26–30). While granzyme B production can be induced in memory CD8+ T-cells, this requires more than 24 hours of in vitro stimulation, whereas IFNγ is produced rapidly from both memory and effector CD8+ T-cells (26, 31, 32). Thus, ex vivo ELISPOT measurements of granzyme B have been established as an ‘immune diagnostic’ means of identifying effector responses to active infections (31, 33). To quantify the effector functionalities of HIV-specific T-cells on long-term ART, we performed batched granzyme B ELISPOT assays on week 24 and 168 samples (Fig. 4A). We focused on the Gag, Pol, and Nef peptide pools, having observed these to be the most immunogenic by IFNγ ELISPOT. Overall, granzyme B-producing HIV-specific responses were substantially lower in magnitude than IFNγ responses (Fig. 4B - 4C and Table S1). The mean, background-subtracted magnitudes of granzyme B responses relative to each other were: Nef>Pol>Gag (at both timepoints – Fig. 4B and Table S1), contrasting with IFNγ: Gag>Pol>Nef (Fig. 2B and Table S1). As with IFNγ, categorizing participants’ granzyme B responses as either increasing, decreasing, or not changing revealed heterogeneity (Fig. S3), though proportionally there were fewer decreasing responses, and the population-average levels of granzyme B responses were highly stable over time to all HIV gene
products (Fig. 4B and Table S3). In contrast to IFN\(\gamma\), we did not observe any significant correlations between the slopes of change of granzyme B responses with virologic measures of HIV persistence (Tables S10 & S12). These results may reflect the additional complexity that whereas both IFN\(\gamma\) and granzyme B-producing cells can be maintained by infected cells producing antigen, the latter are more likely to also perturb the virologic measures by eliminating infected cells (34).

To further assess the functional profiles of HIV-specific T-cell responses, we performed pairwise comparisons of granzyme B versus IFN\(\gamma\) responses for each of the gene products tested (Fig. 4C). At both timepoints, granzyme B response magnitudes to Gag, Pol, and CMV pp65 were substantially lower than IFN\(\gamma\) responses (all p<0.05) (Fig. 4C). Contrasting this, the magnitudes of granzyme B versus IFN\(\gamma\) responses to Nef were not significantly different from each other at either timepoint (p=0.100 at week 24, p=0.277 at week 168). These data indicate that in addition to being preferentially maintained over time, T-cell responses directed against the early HIV gene product Nef disproportionately exhibit effector functional profiles, as compared to the late gene products Gag and Pol (though granzyme B responses to these late gene products were still detected). Persistent HIV-specific granzyme B responses are indicative of recent antigen encounter, supporting the hypothesis that there is in vivo stimulation by HIV-infected cells despite suppressive ART.

The results showing that CMV pp65-specific T-cells exhibited lesser IFN\(\gamma\)-to-granzyme B ratios as compared to Nef-specific responses were somewhat unexpected, given that CMV infection is characterized by episodic low-level antigen exposure due to stochastic reactivation (35). Although our study hypothesized a similar scenario for HIV-infected cells, we had assumed that this would occur to a greater degree for CMV, driving more of an effector phenotype. Given that CMV-specific responses in HIV co-infected individuals have a robust CD4\(^+\) component (36), one explanation could be that this lesser ratio is the result of greater representation of CD4\(^+\)
versus CD8+ T-cells in the CMV versus Nef responses (with less granzyme B from the CD4+ T-cells) (37). To test this, we performed paired IFNγ and granzyme B ELISPOTs on PBMCs, CD8-depleted, and CD4-depleted samples in a subset of 6 participants, with depletions achieving substantial reductions in respective cell amounts (Fig. S4A). The results indicate that both CMV pp65 and HIV Nef responses were predominately CD8+ T-cell driven (Fig. S4B). Having ruled out a skewing towards a CD4+ T-cell response as a major driver in the higher IFNγ-to-granzyme B ratio for CMV pp65- versus Nef-specific responses, we suggest that this observation may reflect greater or more frequent antigen exposure of HIV Nef versus CMV pp65.

Discussion

An important aspect of how HIV persists in individuals on long-term ART is through the evasion of immune recognition, predominately thought to be achieved through the maintenance of strict viral latency, with an additional aspect of anatomical sequestration. This perception that the reservoir is entirely latent has begun to shift lately, in response both to a new understanding of the dynamic nature of the HIV reservoir (driven by the clonal expansion of infected cells), and to new insights into ongoing viral transcriptional activity on ART (16, 38). To date, however, this has yet to prompt widespread re-consideration of the relationship between the HIV-specific T-cell response and the HIV reservoir. The current study provides evidence which challenges the prevailing model of a lack of reservoir immune surveillance, by indicating a level of ongoing antigenic stimulation of HIV-specific T-cells in ART-suppressed individuals. Nef-specific T-cells stood apart from those of other HIV gene products in this regard, supporting that early gene products (Nef, Tat, and Rev – of which only Nef was appreciably immunogenic [as also seen in other studies (23, 39)]) have lower thresholds to expression in a reactivation setting as compared to late gene products (Gag, Pol, and Env), which are expressed only after a cell has built up sufficient levels of Rev to drive nuclear export of unspliced and singly-spliced viral transcripts (40, 41). The preferential maintenance of Nef-specific T-cells was presented as a hypothesis of the
current study based both on this conceptual model, and on our previous observation that Nef-specific T-cells recognized cells reactivated from an in vitro latency model prior to recognition by Gag-specific T-cells, or detectable Gag expression (22). However, we also note the potential role of defective HIV proviruses in our observations - both in general, as a source of antigenic stimulation on ART, as well as through their potential to contribute to the unique relationship between total HIV DNA and Nef-specific T-cell responses. We have previously demonstrated that a subset of defective proviruses can give rise to antigen expression that can be recognized by HIV-specific T-cells (44), in-line with other studies which have reported protein expression. Of particular interest was a study which demonstrated that a defective provirus with a 2.4-kb internal deletion was nonetheless capable of producing Gag and Nef proteins (42). An alternative plausible explanation for our observations is that some antigens (e.g. Nef) may be more likely to be expressed from defective proviruses than others – driving the relationship with total HIV DNA. Our data do not provide direct insights into the relative roles of intact versus defective proviruses in maintaining HIV-specific T-cell responses on ART, but we suggest that this will be an interesting area for future investigation.

The dynamics of T-cell responses to other viruses elicited through vaccination or infection may be instructive towards understanding the persistence of HIV-specific T-cell responses. Exposure to transient antigen through vaccination with live yellow fever virus (YFV) induces an initial, robust virus-specific effector T-cell response, characterized by the expression of effector molecules such as granzyme B (45, 46). YFV-specific T-cells persist long after viral RNA is undetectable, capable of expressing IFNγ years after vaccination despite the lack of ongoing YFV antigen expression (45, 46). Unlike short-lived YFV-specific effector T-cells, however, this separate population of long-lived YFV-specific T-cells are incapable of expressing granzyme B without ongoing antigen stimulation (46). In contrast, CMV, or the murine equivalent, MCMV, is considered a “smoldering” infection characterized by persistent, low-level viral antigen expression
interrupted by intermittent productive viral reactivation events (35, 47). CMV infection induces a robust, long-lived CMV-specific T-cell response, capable of expressing both IFNγ and granzyme B, reflecting ongoing exposure to CMV-infected cells expressing antigen (39). Our data demonstrating that HIV-specific T-cells persist with an effector functional profile (capable of producing granzyme B) suggests that, like CMV-specific T-cells and unlike YFV-specific T-cells, HIV-specific T-cells periodically encounter their cognate antigens. Though additional studies are warranted, our data showing that the Nef-specific T-cell response exhibits a proportionally greater effector functional profile than the CMV-specific T-cell response additionally raises the possibility that Nef-specific T-cells encounter Nef antigen either to a greater extent or more frequently than CMV pp65-specific T-cells encounter their antigen.

Do our results allow for any inferences into how frequently infected cells are recognized by HIV-specific T-cells in vivo? While numerous aspects of complexity introduce caveats to such an analysis (e.g. tissue distributions), our data do allow for side-by-side comparisons between the peripheral blood frequencies of infected cells with antigen-expression potential, and those of HIV-specific T-cells — which may be informative. The mean frequency of Nef-specific T-cells at week 24 of our study was 107.7/10⁶ PBMCs, whereas the median total frequency of HIV-infected cells (CA-DNA) was 515.7/10⁶ CD4⁺ T-cells (at week 0 — this was not measured at week 24, but CA-DNA is highly stable on long-term ART (48)), or roughly 103/10⁶ PBMCs. These infected cells, however, predominately contain defective proviruses (49), many of which are likely incapable of expressing antigens (44). It can therefore be reasonably estimated that, in most individuals, Nef-specific T-cells are at least as frequent as infected cells with the potential to express antigen. Our data indicating that the former are influenced by the latter therefore suggest that antigen expression is more likely to be a common versus a rare event in vivo, amongst infected cells with this potential. Further study is needed, however, and characterizing the clonal dynamics of HIV-specific T-cells may yield additional insights.
Although latency almost certainly contributes to viral persistence, our findings indicating that HIV reservoirs are not fully hidden from circulating cytotoxic T-cells raise the question of what additional mechanisms may be at play. We first consider the role of immune escape – the process by which HIV evades recognition by acquiring mutations in T-cell epitopes. Immune escape plays a critical role in limiting the overall efficacy of the HIV-specific T-cell response in untreated infection, and HIV reservoirs show clear evidence of past selection, in the form of extensive sequence variation in known T-cell epitopes (50). However, the question at hand pertains to HIV-specific T-cell responses that show evidence of being maintained by recent antigen recognition, indicating that they target epitopes which are intact in at least a portion of the reservoir. Further supporting this idea are the previous observations that: i) the fixation of escape mutations leads to the contraction of corresponding T-cell responses (51), and ii) the substantial majority of HIV-specific T-cells that remain detectable after years of ART target epitopes for which escape is not fixed in corresponding reservoir viruses (52, 53). As with latency, our data do not lead us to contest the idea that the fixation of escape mutations in the reservoir diminishes the overall potential for immune recognition, nor the value of therapeutic strategies to address either of these limitations. However, we are still left with the question of how to reconcile our findings indicating an appreciable level of ongoing in vivo recognition of infected cells by cytotoxic (granzyme B) T-cells, with the overall stability of HIV reservoir sizes.

We therefore draw from two recent findings in the field to propose how an HIV reservoir may persist without being fully hidden from circulating cytotoxic T-cells. The first derives from the recent demonstrations that the HIV reservoir is predominately composed of infected T-cells that have undergone clonal expansion (54–58), with different clones dynamically ‘waxing and waning’ over time (56). Thus, HIV-specific T-cells may frequently eliminate infected cells, only to have these replaced by clonal expansion of other reservoir-harboring cells. There have been somewhat
conflicting recent reports regarding this possibility – from groups that approached the question from different angles – highlighting the need for further study (44, 59, 60).

Second, we have recently reported that reservoir-harboring cells exhibit intrinsic resistance to T-cell mediated elimination (61), mediated in part by Bcl-2 over-expression, which antagonizes perforin/granzyme killing (62). In fact, while it has been generally assumed in our field that the encounter between an antigen-expressing HIV-infected cell and a functional (ex. perforin/granzyme releasing) CD8+ T-cell will result in elimination, this overlooks the role of the target cell as an active partner in the killing process. Multiple regulatory mechanisms exist, both in physiological and pathological states, by which target cells determine whether or not to undergo apoptosis, despite receiving a perforin/granzyme hit (63–65). Thus, one way to resolve our findings with others in the field is to propose that the recognition of HIV-infected cells by HIV-specific cytotoxic T-cells may occur with some frequency in vivo, but that this often does not result in target cell elimination. An intriguing possibility is that the combined effects of selection, based on intrinsic susceptibility to CD8+ T-cells, and clonal expansion of surviving cells may enable the evolution of a resistant reservoir, paralleling the phenomenon of ‘immunoediting’ in cancer (12). While latency reversal will likely be a critical component of curing HIV infection, our findings raise the hypothesis that – in lieu of an ideal latency reversing agent – reductions in HIV reservoirs may be achievable by boosting immune targeting of existing expression of early gene products (such as Nef, and in a manner that targets non-escaped epitopes) while enhancing cytotoxic function, limiting clonal expansion, and addressing resistance to cytotoxic T-cells in reservoir-harboring cells.

Methods

Study Design

Data for this manuscript were collected on a longitudinal cohort of participants who initiated ART during chronic HIV infection in AIDS Clinical Trials Group (ACTG) trials for treatment-naïve
individuals, and enrolled in the ACTG HIV Reservoirs Cohort Study (A5321) (24). A5321 cohort participants were recruited from 17 clinical research sites in the United States through the ACTG network. IFN\(_{\gamma}\) ELISPOTs were previously performed using samples from 99 participants at A5321 study entry (22), and a subset of 49 participants were selected from the original 99 for this longitudinal sub-study based on sample availability. All gene products and negative controls were tested in duplicate, with one replicate of PHA positive control. Assays performed under these same conditions have been previously validated in other participant cohorts. Outliers were not defined or excluded. Participants in the current sub-study had follow-up at least every 6 months following study entry, with documented sustained viral suppression (plasma HIV RNA levels <50 copies/mL by commercial assays starting at week 48 on ART and at all subsequent timepoints – Fig. 1). One participant had a large viral blip (>1,000 copies/mL) 43 weeks prior to their 168-week A5321 study timepoint, and data was right-censored for this participant after the 24-week A5321 study timepoint. Clinical data and paired plasma and PBMC samples were available from pre-ART and on ART study visits. We measured HIV levels (CA-DNA, CA-RNA, and plasma iSCA) and PD-1 levels (on CD4\(^+\) and CD8\(^+\) cells) on samples obtained at A5321 study entry (median 7 years on ART), and plasma HIV RNA levels and CD4\(^+\) T-cell counts were obtained from pre-ART clinical data. One participant later revoked consent for further testing and was excluded from analysis. We hypothesized a priori that the long-term dynamics of T-cell responses to the early HIV gene product Nef (measured by IFN\(_{\gamma}\) ELISPOT) would be associated with infected cell frequencies as measured by HIV DNA.

Virologic Assays

HIV CA-DNA and CA-RNA were measured by quantitative PCR (qPCR) assays in PBMCs using previously described methods (66). CA-DNA and CA-RNA values per million CD4\(^+\) T-cells were calculated by dividing the total CA-DNA or CA-RNA copies/million PBMCs [normalized for CCR5 copies measured by qPCR as published (66)] by the CD4\(^+\) T-cell percentage (× 0.01) reported
from the same specimen date or from a CD4⁺ T-cell percentage imputed using linear interpolation
to specimen dates before and after the CA-DNA or CA-RNA results. Cell-free HIV RNA was
quantified by iSCA in blood plasma (5 mL) (67).

**Immunologic Assays**

PBMCs obtained at A5321 study entry were stained with the following monoclonal antibodies to
evaluate surface PD-1 expression: CD3 APC-H7 (Cat. No. 560176), CD4 PC5 (Cat. No. 555348),
CD8 V450 (Cat. No. 560347), PD-1 (clone M1H4) A488 (Cat. No. 557860) (all from BD Biosciences, San Diego, California, USA), and Live/Dead Aqua (Invitrogen, Grand Island, New York, USA). Cells were fixed in 1% paraformaldehyde, and analyzed using a BD LSR Fortessa (FACSDiva) within 24 hours after staining. Lymphocytes were identified based upon size and granularity. The lymphocyte population was filtered through side scatter area versus side scatter height histogram to eliminate doublets from the analysis. Single cells were analyzed using Live/Dead Aqua dye exclusion and then CD4⁺ and CD8⁺ populations were defined based on dual expression with CD3. These two populations were plotted against PD-1. Fluorescence minus one (FMO) controls were used to define the PD-1⁺ T-cell populations.

**Peptide Pools**

The following sets of consensus HIV clade B 15 amino acid peptides (overlapping by 11 amino acids) were supplied by the NIH AIDS Research and Reference Reagent Program: Gag (cat # 8117), Env (cat # 9480), Pol (cat # 6208), Tat (cat # 5138), Rev (cat # 6445), and Nef (cat # 5189). All peptides were dissolved at 5mg/mL in 12.5% DMSO (Corning), and 87.5% PBS (Gibco). Peptides were pooled into whole gene product peptide pools and adjusted to a final concentration of 20μg/mL/peptide in PBS. A CMV pp65 PepMix peptide pool (JPT Peptide Technologies) was dissolved separately in DMSO and adjusted to a final concentration of 20μg/mL/peptide in PBS.

**IFNγ and Granzyme B ELISPOT Assays**

Multi-screen IP 96-well PVDF plates (Millipore) were either directly coated with 100μL/well of PBS + 0.5μg/mL primary anti-human IFNγ antibody (clone 1-D1K, Mabtech) overnight at 4°C, or first
primed with 20μL of 35% EtOH/well, and immediately washed 6x with 200μL ddH$_2$O and then coated with 100μL/well of PBS + 15μg/mL primary anti-human granzyme B antibody (clone GB10, Mabtech) overnight at 4°C. Granzyme B plates were washed 6x with 200μL PBS and blocked with RPMI 10% FBS (Gibco) (‘R-10’) at 37°C 5% CO$_2$. PBMCs were thawed and resuspended in R10 and added to plates at 100,000-200,000 cells/well. HIV peptide pools (20μg/mL/peptide) were added at 10μL/well for a final concentration of 1μg/mL/peptide in <0.5% DMSO. CMV pp65 peptide pools were added at 10μL/well for a final concentration of 1μg/mL/peptide in <0.5% DMSO. PHA was dissolved in DMSO and PBS to 200μg/mL, and then added to a final concentration of 1μg/mL as a positive control. 0.5% DMSO in PBS and R-10 media were used as negative controls. Plates were incubated for 18 hours at 37°C with 5% CO$_2$. Plates were washed 6x with 200μL PBS. Biotinylated secondary IFN$\gamma$ antibody (clone 7-B6-1, Mabtech) at 0.5μg/mL in PBS, or biotinylated secondary anti-granzyme B antibody (clone GB11, Mabtech) at 1.0μg/mL in PBS was added to the plates to a final volume of 100μL and incubated for 1 hour in the dark. Plates were then washed 6x with PBS and 0.5μg/mL of Streptavidin-ALP (Mabtech) was added to IFN$\gamma$ plates at 100μL/well, and 1μg/mL of Streptavidin-ALP (Mabtech) was added to granzyme B plates at 100μL/well and incubated for 1 hour. Plates were washed 6x with PBS and then color development substrate solution: 10.6mL of ddH$_2$O, 400μL 25x AP Color Development Buffer (Biorad), 100μL AP color reagent A (Biorad), and 100μL AP color reagent B (Biorad) was added to the plate at 100μL/well for 15 minutes. After removal of the color development substrate solution, 0.5% of Tween-20 in PBS was added at 100μL/well for 10 minutes. Plates were then washed with water, and left overnight to dry. Plates were counted using ImmunoSpot S6 Ultimate Analyzer and ImmunoSpot software (Cellular Technology Limited). As in our prior publication (22), ELISPOT responses against whole gene product peptide pools were background subtracted, but no other cutoff value was applied (the positivity criteria of 1] $>$50 SFU/million PBMCs after
background subtraction and 2] >2x above background in our prior publication were only applied to the responses for peptide pool matrix mapping).

**CD8 and CD4-depleted PBMC Experiments**

Paired granzyme B and IFNγ ELISPOT assays were performed as described above, with the exception that PBMCs were counted and divided into three separate populations post-thaw: PBMCs, CD8-depleted PBMCs, and CD4-depleted PBMCs. These separate populations were plated at between 20,000 – 200,000 cells/well. CD8+ T-cells and CD4+ T-cells were depleted by positive selection (EasySep, STEMCELL Technologies). Samples were stained with anti-CD8 FITC (RPA-T8), anti-CD4 Pacific Blue (RPA-T4), anti-CD3 BV785 (SK7), anti-CD14 Alexa Fluor 647 (63D3) (all from BioLegend), and LIVE/DEAD™ Fixable Aqua dye (ThermoFisher L34966).

Flow cytometry data was collected using an Attune NxT flow cytometer, and data was analyzed using FlowJo (Treestar).

**Statistics**

Statistical analyses including univariate statistics and Spearman correlations and partial correlations (adjusting for potential confounders) were conducted in SAS University Edition. Correlation results were corrected post hoc for multiple comparisons by the false discovery rate method of Benjamini and Hochberg (68) using the SAS MULTTEST procedure (FDR option). For Figs. S1 & S3, responses were categorized as 'not changing' based on a ≤15% change in either direction; this threshold was arbitrarily pre-defined based on the frequency distribution of the percent change data. Slopes of change in Fig. 3B and Tables S4-S5 and S10-S11 were calculated based on absolute changes on a linear scale between weeks 24-168 post-A5321 study entry, excluding participants who had a change from 0 magnitude to 0 magnitude. Analyses for Tables S6-S7 and S12-S13 used slopes of change calculated based on proportional changes on a log_{10} scale between weeks 24-168 post-A5321 study entry, excluding participants who had a change from 0 magnitude to 0 magnitude; slopes reflecting a change from 0 magnitude to a non-
zero magnitude were analyzed as the highest rank, and slopes reflecting a change from a non-
zero magnitude to 0 magnitude were analyzed as the lowest rank. Statistical analyses including
one-way ANOVA, Friedman tests, and Wilcoxon signed-rank tests were conducted in GraphPad
Prism v.8.0. Plots for figures were made in GraphPad Prism v.8.0 and SAS University Edition. A
custom code was generated in MATLAB v.9.7 to produce the correlogram in Fig. 3B. All linear
mixed-effects models were conducted using the R 'lme4' package (69), with random intercepts
only on the participant level modeled for the random effects; multiple comparisons were made
where indicated using the R 'multcomp' package (70), adjusting for multiple comparisons using
Tukey's all-pair method. Linear mixed-effects models used log_{10}-transformed response data to
meet normality assumptions, treating zero-valued responses as missing data. Imputation was not
used to address missing data, as the degree of missingness was low. All statistical tests were
two-sided, $\alpha=0.05$.

Study Approval

Each ACTG A5321 clinical research site had the A5321 protocol and consent form, and its
relevant parental protocols and consent forms, approved by their local IRB, as well as registered
with and approved by the Division of AIDS (DAIDS) Regulatory Support Center (RSC) Protocol
Registration Office, prior to any participant recruitment and enrollment. Once a participant for
study entry was identified, details were carefully discussed with the prospective participant by
clinical staff at the site. The participant (or, when necessary, the parent or legal guardian if the
participant was under guardianship) was asked to read and sign the ACTG-approved protocol
consent form.

Author contributions: RBJ designed the study. EMS, ARW, RT, AST, SHH, TRD, ST, JKB,
TMM, AD, GQL, AG, PK, WDCA, JCC, and BM performed experiments. EMS, ARW, RT, ST,
SHH, JKB, CML, RJB, BM, JCC, and JWM analyzed data. RTG, DKM, JJE, and JWM provided
participant data. EMS, ARW, and RBJ wrote the manuscript. All authors contributed to the critical
revision of the manuscript. The order of the co-first authors was determined based upon the order in which they began working on the project.

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**References**


17. Mota TM et al. Integrated Assessment of Viral Transcription, Antigen Presentation, and CD8+ T Cell Function Reveals Multiple Limitations of Class I-Selective Histone Deacetylase Inhibitors during HIV-1 Latency Reversal. *Journal of Virology* 2020;94(9).


34. Yue FY et al. HIV-Specific Granzyme B-Secreting but Not Gamma Interferon-Secreting T Cells Are Associated with Reduced Viral Reservoirs in Early HIV Infection. *Journal of Virology* 2017;91(8).


Figures and figure legends

Fig. 1. ACTG A5321 Cohort participants achieved viral suppression prior to study entry and maintained viral suppression throughout the study period. Log_{10} plasma HIV RNA (copies/mL) by clinical commercial assays for ACTG A5321 Cohort study participants included in this longitudinal sub-study (n=49), followed from pre-ART initiation (ART initiated in other ACTG trials) through to the A5321 study 168 week timepoint. Limit of detection (LOD) for early clinical assays was 50 copies/mL, and for later clinical assays 40 copies/mL. Colored lines represent individual participants, with symbols indicating each clinical viral load measurement. X-axis break shows time post-ART initiation when all participants achieved initial viral suppression. Box plot shows the distribution of participants’ A5321 study entry timepoints relative to weeks post-ART initiation (minimum, Q1, median, Q3, maximum).
Fig. 2. HIV-specific T-cell responses are readily detectable ex vivo and persist on long-term ART, primarily directed against HIV Gag, HIV Pol, and HIV Nef. A. Representative IFN-γ ELISPOT results for two participants for both timepoints, with 2x10^5 PBMCs/well. B. Magnitudes of IFN-γ responses are shown for three on-ART timepoints for n=49 participants. Study entry timepoint data is shaded in gray because it was not performed in batch with 24 and 168 weeks timepoints. Each data point represents the mean SFU/10^6 PBMCs following background.
subtraction of negative control wells (duplicates). Vertical lines and error bars represent the mean and standard deviation for each gene product peptide pool.
Fig. 3. HIV-specific T-cell responses are highly stable on long-term ART, with HIV Nef-specific response dynamics uniquely associated with reservoir measures. A. Participant-specific slopes of change in T-cell responses from weeks 24 to 168 post-study entry. P-values
represent the significance level for the covariate time (in weeks) in linear mixed-effects models from Table S3. B. Correlogram depicting Spearman correlations between slopes of change in raw magnitudes of T-cell responses (from panel A) with virologic and immunologic parameters. Color scale bar represents magnitude of correlation coefficient. Circle size represents unadjusted p-values, corrected for the false discovery rate. Asterisks represent adjusted p-values: for HIV DNA controlling for pre-ART plasma HIV RNA, pre-ART CD4+ T-cell count, and years on ART at study entry, and for pre-ART plasma HIV RNA controlling for HIV DNA at study entry; all adjusted p-values are corrected for the false discovery rate (* <0.05, ** <0.01, *** <0.001).
Fig. 4. HIV-specific T-cells demonstrate cytotoxic ability, preferentially directed towards HIV Nef, evidencing recent in vivo antigen exposure. A. Corresponding granzyme B (GrB) and IFN$\gamma$ ELISPOT results for one participant at both timepoints, with $2 \times 10^5$ PBMCs/well. B. Magnitudes of granzyme B responses are shown for two batched on-Art timepoints for $n=49$ participants. Each data point represents the mean number of SFU/10$^6$ PBMCs following...
background subtraction of mean of negative control wells. Vertical lines and error bars represent
the mean and standard deviation for each gene product peptide pool. C. Pairwise comparisons
of granzyme B versus IFNγ responses for Gag, Pol, Nef, and CMV pp65 at both timepoints. P-
values calculated by Wilcoxon matched pairs signed-rank test.
Table 1. Characteristics of longitudinal sub-study participants - continuous variables

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<tr>
<td></td>
<td>Lower</td>
<td>Upper</td>
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<tr>
<td>Age at A5321 entry (years)</td>
<td>48</td>
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<td>74</td>
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<tr>
<td>Years on ART at A5321 entry</td>
<td>6.6</td>
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<td>HIV CA-DNA at A5321 entry (cps/10^6 CD4+ T-cells)</td>
<td>515.7</td>
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<td>HIV CA-RNA at A5321 entry (cps/10^6 CD4+ T-cells)</td>
<td>24.2</td>
<td>13.6</td>
<td>898.9</td>
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<tr>
<td>HIV plasma RNA via iSCA at A5321 entry (cps/mL)^A</td>
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<td>0.4</td>
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<td>%PD-1+ CD4+ cells at A5321 entry</td>
<td>36.75%</td>
<td>1.20%</td>
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<td>%PD-1+ CD8+ cells at A5321 entry</td>
<td>35.40%</td>
<td>0.70%</td>
<td>84.90%</td>
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<tr>
<td>Pre-ART plasma HIV-1 RNA (log_{10}cps/mL)</td>
<td>4.6</td>
<td>2.3</td>
<td>5.9</td>
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<td>Pre-ART CD4+ T-cell count (cells/mm^3)</td>
<td>287.5</td>
<td>15.5</td>
<td>708.5</td>
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^A: iSCA assay limit of detection 0.4 copies HIV per mL plasma
Table 2. Characteristics of longitudinal sub-study participants - categorical variables

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<td>Male</td>
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<td><strong>Race/Ethnicity</strong></td>
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<tr>
<td>Black (non-Hispanic)</td>
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<tr>
<td>Hispanic (regardless of Race)</td>
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<tr>
<td>White (non-Hispanic)</td>
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<td><strong>iSCA qualifier at A5321 entry(^A)</strong></td>
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<tr>
<td>Undetectable</td>
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<tr>
<td>Detectable</td>
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\(^A\)iSCA assay limit of detection 0.4 copies HIV per mL plasma