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SARS CoV-2 mRNA vaccination exposes latent HIV to Nef-specific CD8$^+$ T-cells

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Abstract

Efforts to cure HIV have focused on reactivating latent proviruses to enable elimination by CD8$^+$ cytotoxic T-cells. Clinical studies of latency reversing agents (LRA) in individuals treated with antiretroviral therapy (ART) have shown increases in HIV transcription, but without reductions in virologic measures, or evidence that HIV-specific CD8$^+$ T-cells were productively engaged. Here, we show that the SARS-CoV-2 mRNA vaccine BNT162b2 activates the RIG-I/TLR – TNF – NFκb axis, resulting in transcription of HIV proviruses with minimal perturbations of T-cell activation and host transcription. T-cells specific for the early gene-product HIV-Nef uniquely increased in frequency and acquired effector function (granzyme-B) in ART-treated individuals following SARS-CoV-2 mRNA vaccination. These parameters of CD8$^+$ T-cell induction correlated strongly with significant decreases in cell-associated HIV mRNA, suggesting killing or suppression of cells transcribing HIV. These results are, to our knowledge, the first observation of an intervention-induced reduction in a measure of HIV persistence, accompanied by precise immune correlates, in ART-suppressed individuals. However, we did not observe significant depletions of intact proviruses, underscoring challenges to achieving (or measuring) HIV reservoir reductions. Overall, our results support prioritizing the measurement of granzyme-B-producing Nef-specific responses in latency reversal studies and add impetus to developing HIV-targeted mRNA therapeutic vaccines that leverage built-in LRA activity.

Lifelong antiretroviral therapy (ART) has transformed HIV infection into a manageable chronic condition, but there is no safe cure[1-3]. A two-pronged strategy to reactivate latent HIV reservoirs and enhance HIV-specific cytotoxic T-lymphocytes (CTL) has been proposed to 'shock and kill' residual infected cells[4,5], but trials to date have neither decreased residual HIV RNA nor reduced inducible reservoirs[6-8]. Insufficient latency reversal and/or deficiencies in CD8$^+$ CTL responses are

thought to be factors in these outcomes. Determining whether meaningful latency reversal has been achieved is confounded by the fact that HIV RNA (often used to measure latency reversal) does not necessarily equate to antigen expression[9]. Querying $CD8^+$ T-cells for evidence of recent antigen recognition, following a therapeutic intervention, may provide a more direct way of assessing whether these cells have been engaged.

In order to optimally leverage HIV-specific $CD8^+$ T-cell response measures for this purpose, we must identify which HIV antigens become visible to $CD8^+$ T-cells following proviral reactivation, and which $CD8^+$ T-cell functions best reveal recognition of these antigens. Our previous *in vitro* and observational clinical studies support the idea that $CD8^+$ T-cells specific for the early HIV gene product Nef interact with HIV reservoirs to a degree not seen in those specific for late gene products (e.g. Gag)[10,11]. We reasoned that this most likely reflects Nef-mediated immunoevasion via reduction of surface MHC-I levels[12], where – immediately following proviral activation – a window of opportunity exists for robust MHC-I-mediated presentation of epitopes derived from early gene products, while late gene products are expressed only after this immunoevasion mechanism is in place. This has been modeled *in vitro*, where Nef-specific $CD8^+$ T-cells can respond to reactivated cells prior to the onset of Gag expression[13]. The impact of Nef-mediated immunoevasion on reservoir surveillance *in vivo* has also been highlighted by a recent study associating superior Nef-mediated MHC-I downregulation in effector memory $CD4^+$ T-cells with their status as a sanctuary for intact HIV proviruses on ART[14]. An additional line of evidence supporting that Nef-specific $CD8^+$ T-cells can preferentially respond to residual antigen expression on ART is that these Nef-specific responses are disproportionately skewed towards granzyme B release (gzm-B)[10], a hallmark of recent *in vivo* antigen recognition as well as a key mediator of the desired cytopathic activity[15-17]. Based on the above, we hypothesized that Nef-specific $CD8^+$ T-cells would both increase in frequency and be further skewed towards gzm-B production following receipt of SARS CoV-2 mRNA vaccination.

The impact of vaccination on HIV latency has been the subject of previous studies but has not yet been extended to mRNA vaccines. Günthard and colleagues demonstrated transient increases in HIV plasma viral load following influenza vaccination[18]. It was subsequently shown that the vaccine combinations of Influenza/Hep B and Pneumococcus/Hep B drove transient increases in cell-associated HIV RNA, whereas a number of other vaccines and vaccine combinations did not (e.g. measles-mumps-rubella, Varicella zoster virus, and tetanus-diptheria). Increases in cell-associated HIV RNA were accompanied by modest and transient increases in HIV-p24-specific $CD8^+$ T-cell responses, as measured by IFN-γ ELISPOT, but whether this was associated with subsequent decreases in measures of HIV persistence was not assessed[19]. Here, as in other studies that reported HIV reactivation following vaccination with the recall antigens tetanus[20,21], and cholera[22], the relative roles of adaptive versus innate immune responses is unclear – in particular given that influenza- and tetanus-specific $CD4^+$ T-cells are known to harbor portions of HIV reservoirs[23]. SARS CoV-2 vaccination provided a unique opportunity to assess the impact of a novel class of vaccines (mRNA) on the HIV reservoir, in a scenario where most study participants were antigen naïve – and to incorporate cutting-edge virologic assays, including measuring intact proviruses.

In the current study, we show that the Pfizer/BioNTech BNT162b2 SARS CoV-2 mRNA vaccine induced HIV reactivation from the PBMCs of SARS CoV-2-naïve ART-treated people with HIV *ex vivo*. This was associated with innate immune sensing of mRNA and downstream activation of NFκb. Remarkably, this HIV reactivation occurred without detectable T-cell activation, and with minimal perturbation of host transcription. In contrast, the inactivated-virus influenza vaccine induced robust T-cell activation and host transcriptional changes, without detectable HIV reactivation in our assay. *In vivo* results confirmed our primary hypothesis, showing unique increases in T-cell responses targeting early gene products – predominately Nef. These increases

were further pronounced when assessed by gzm-B ELISPOT. Importantly, each of the T-cell response metrics that showed significant increases following the first vaccine dose in turn showed strong correlations with subsequent decreases in cell-associated HIV RNA. Although we consider this significant decrease in a virologic measure of HIV persistence along with precise immune correlates to be an important milestone, ultimately HIV reservoir sizes were not significantly reduced. This points to additional barriers to either achieving or measuring reservoir reductions, even when a degree of success is achieved with the proximal goals of shock and kill.

Results

mRNA vaccines induce HIV reactivation *ex vivo* with minimal T-cell activation

SARS-CoV-2 mRNA vaccination induces transient systemic innate immune responses *in vivo*, which include the activation of TLR, RIG-I, and other inflammatory signaling pathways, providing potential latency reversal stimuli[24-26]. We therefore first assessed whether the exposure of *ex vivo* PBMCs or purified $CD4^+$ T-cells from ART-suppressed donors (**Extended Data Table 1**) would release HIV RNA if exposed to SARS-CoV-2 mRNA vaccines *ex vivo*. PBMC samples were from SARS-CoV-2 naïve individuals, with most cryopreserved prior to 2020. Cells from an initial participant were used to establish a dose-response curve, which showed peak HIV RNA release after stimulation with either the Pfizer BioNTech BNT162b2 or the Moderna mRNA-1273 vaccines at 1-5% of the culture volume – with the former showing greater induction (**Fig. 1A**). This approach was extended to 5 additional donors, testing the 1% and 5% doses of both mRNA vaccines, alongside 2% v/v of Fluzone™ quadrivalent inactivated-virus influenza vaccine, as well as previously established optimal concentrations of the latency-reversing agents Bryostatin-1 and romidepsin and the mitogen PHA. We observed significant reactivation across this cohort following 1% BNT162b2 treatment (**Fig. 1B)**, with a lesser degree of overall reactivation with 5% BNT162b2 treatment ($p < 0.05$ by paired t-test) and less consistent reactivation with 1% mRNA-

1273 treatment ($p = 0.12$ by paired t-test). Treatment with influenza vaccine did not induce detectable release of HIV RNA in this *ex vivo* system.

Established latency reversing agents (LRAs) performed relatively poorly in this assay, which we attribute to cell-free RNA being a particularly rigorous measure of latency reversal (ex. romidepsin has previously been shown to induce detectable cell-associated but not cell-free RNA[27]). Though induction of HIV release was marginal in response to bryostatin-1 and PHA, flow cytometric analysis of CD69 expression confirmed the very high levels of T-cell activation expected with these agents[27] (**Fig. 1C**). Influenza vaccine treatment was also associated with appreciable $CD4^+$ T-cell activation, despite a lack of viral RNA release. Interestingly, neither of the SARS CoV-2 mRNA vaccines induced T-cell activation by this measure (**Fig. 1C**). Thus, in this *ex vivo* experimental system the SARS CoV-2 mRNA vaccine BNT162b2 induced HIV latency reversal without the T-cell activation typically observed with most potent latency-reversing agents.

To probe the mechanisms underlying mRNA vaccine-induced latency reversal, we purified $CD4^+$ T-cells from three of the samples studied in **Fig. 1B & C** and subjected these to bulk mRNA sequencing (RNA-Seq) along with corresponding influenza vaccine (Fluzone^{TM}) treated samples. The majority of variance between samples could be attributed to treatment effects, though with the 1% BNT162b2 samples showing relatively little divergence from untreated (No Treatment) samples (**Fig. 1D**). Correspondingly, treatment with the CoV-2 mRNA vaccines yielded relatively few numbers of differentially expressed genes (DEGs) compared to untreated samples: 71 DEGs for 1% BNT162b2 and 193 DEGs for Moderna's mRNA-1273 (adj. p-value < 0.05). The Fluzone^{TM} influenza vaccine had a substantially greater impact on the host transcriptional profile, with 3,424 DEGs.

Despite limited overall transcriptional perturbations, gene set enrichment analyses (GSEA) implicated biologically relevant signaling pathways that largely overlapped between the BNT162b2 and mRNA-1273 treatments (**Fig. 1E**). Retinoic acid-inducible gene I (RIG-I)-like receptors are the primary innate immune receptors of viral RNA which, when stimulated, induce type I interferons (IFN) and pro-inflammatory cytokines[28]. The results clearly implicated both the pathway itself and the downstream IFN response in these mRNA vaccine treatments. BNT162b2 further implicated toll-like receptor (TLR) signaling. TLR-7 and TLR-8 comprise additional innate immune sensors of foreign ssRNA. They are predominately expressed by myeloid lineage cells, such as monocytes and pDCs, where signaling results in the release of type I IFN as well as TNF. TLR-7 agonists, including GS-9620, have been established as HIV LRAs. We have previously shown that these act predominately by driving the release of TNF, which induces NFκb activation and resulting proviral transcriptional initiation in HIV-infected $CD4^+$ T-cells[29]. The observed activation of TNF signaling via the NFκb pathway implies an analogous mode of action for BNT162b2 (**Fig. 1E & F**). Of note, these *ex vivo* transcriptional perturbations are in agreement with those observed in the days following *in vivo* vaccination with BNT162b2 - where RIG-I like receptor signaling, TLR and inflammatory signaling, and the type I IFN response feature prominently[30].

mRNA vaccination drives selective increases in HIV-Nef-specific $CD8^+$ T-cell responses

To assess if HIV reactivation occurred *in vivo* following first and second vaccine doses, we queried HIV-specific T-cell responses for evidence of antigenic stimulation using activation induced marker (AIM) assays (assessing co-induction of CD69 and CD137) at baseline (Visit 1, V1) and ~2 weeks after SARS-CoV-2 mRNA vaccine dose 1 (Visit 2, V2; median, range: 17, 14-24 days) and dose 2 (Visit 3, V3; median, range: 16, 14-24 days) in a cohort of 13 antiretroviral therapy (ART) treated adults with plasma HIV RNA below the limits of detection by a standard clinical

assay (**Table 1**). As an aside, we first note that direct virologic assessments of HIV expression following initial vaccination would have been valuable – but would require sample timepoints from the days following vaccination, which were not collected. We did collect these early timepoints following mRNA vaccine boosters (third dose), where we observed significant increases in cell-associated HIV RNA were observed following receipt of mRNA vaccine boosters (third dose) (**Extended Data Table 2, Extended Data Fig. 1**). Returning to T-cell responses following initial vaccine doses, we observed the expected inductions of SARS-CoV-2-Spike-specific $CD4^+$ and $CD8^+$ T-cell responses following vaccine dose 1 (V2), which were further enhanced following dose 2 (V3), from means of CD8 – 0.03% AIM+ (V1) to 0.08% AIM+ (V2) and 0.11% AIM+ (V3); and CD4 - 0.04% AIM+ (V1) to 0.07% AIM+ (V2) and 0.13% AIM+ (**Fig. 2A-C**). Corresponding with this, SARS-CoV-2 anti-S serology tests showed reactivity in 2/13 individuals at V1, 10/12 at V2, and 13/13 at V3. In contrast, no significant changes were observed in HIV-Gag-specific $CD8^+$ or $CD4^+$ T-cells, HIV-Nef-specific $CD4^+$ T-cell responses, nor cytomegalovirus (CMV)-pp65-specific responses (included as an irrelevant control) (**Fig. 2B & C**), nor in anti-HIV gp120 antibody levels (**Extended Data Fig. 2**). However, we did observe trends towards increases in HIV-Nef-specific $CD8^+$ T-cell responses following first vaccine dose, from a mean of 0.06% AIM+ (V1) to 0.09% AIM+ (V2) – p = 0.06 (**Fig. 2B**).

We took two approaches to further assess this increase in Nef-specific $CD8^+$ T-cell responses following the first vaccine dose, given that it was on the margin of statistical significance : i) We performed these same AIM assays on an independent cohort (n = 15) and ii) We re-assessed T-cell responses in this original cohort by gzm-B ELISPOT, a more selective readout of cells that have recently encountered antigen *in vivo*[15,31,32]. Our independent validation cohort was based in Vancouver, Canada, and had samples from baseline (pre-vaccine, V1) and ~4 weeks after both SARS-CoV-2 mRNA vaccine dose 1 (V2; median 31, range 28-37 days) and dose 2 (V3; median 30, range 27-32) (**Table 1**)[33]. Due to Canada's decision to delay second SARS-CoV-2 vaccine

doses due to limited initial vaccine supply the time between first and second vaccine doses was significantly longer for the Vancouver (median 54, range 49-61 days) compared to New York cohort (median 27, range 21-57 days). As such, the V3 results are not directly comparable. For the Vancouver cohort, we observed a significant increase in HIV-Nef-specific $CD8^+$ T-cell responses following the first vaccine dose from a mean of 0.05% AIM+ (V1) to 0.09% AIM+ (V2) – $p = 0.03$ (**Fig. 2D**). No such increases were observed for HIV-Gag or CMV-pp65, while SARS CoV-2-Spike-specific $CD8^+$ T-cell responses were induced as expected (**Fig. 2D**). Thus, AIM results from the validation cohort further supports our hypothesis by showing unique boosting of HIV-Nef-specific $CD8^+$ T-cell responses following the first dose of SARS CoV-2 vaccine.

Both effector and memory $CD4^+$ and $CD8^+$ T-cells readily produce IFN-γ *in vitro* in response to their cognate antigens, evidencing either past or ongoing antigen exposure. Granzyme B (gzm-B) production following short-term *in vitro* stimulation, however, is a distinguishing feature of virus-specific effector $CD8^+$ T cells that have recently encountered antigen *in vivo*, through either infection or vaccination (with induction from memory $CD8^+$ T cells requiring >24 hours of *in vitro* stimulation)[15-17]. To further test the hypothesis that SARS-CoV-2 mRNA vaccination can reactivate HIV expression, we assessed gzm-B and IFN-γ responses in parallel by ELISPOT, using peptide pools spanning each of: HIV-Gag, HIV-Env, HIV-Pol, HIV-Nef, HIV-Tat, HIV-Rev, HIV-Vif/Vpr/Vpu (combined pool), CMV-pp65, and SARS-CoV-2-Spike[10,34]. Amongst the HIV-specific responses, increases were uniquely observed in gzm-B-producing responses to the early gene products Nef and Rev – spiking between V1 (baseline) and V2 (vaccine dose 1) from means of 71 and 50 spot forming units (SFU)/10^6 PBMC to 220 and 129 SFU/10^6 peripheral blood mononuclear cells (PBMCs), respectively (Nef – 3.1-fold increase, $p = 0.002$, Rev – 2.6-fold increase, $p < 0.05$) (**Fig. 3A & B**). No inductions of Nef- or Gag-specific T-cell responses were observed following SARS-CoV-2 mRNA vaccination in a cohort of HIV-negative individuals, ruling

out HIV- SARS-CoV-2-Spike cross-reactivity as a driver of these increases (**Extended Data Fig. 3A & B**). As expected, we observed inductions of SARS-CoV-2-Spike-specific T-cell responses as measured by either gzm-B (means: V1 - 42 SFU/10^6 PBMCs, V2 - 51 SFU/10^6 PBMCs, V3 - 71 SFU/10^6 PBMCs) or IFN-γ (means: V1 – 13 SFU/10^6 PBMCs, V2 - 47 SFU/10^6 PBMCs, V3 - 114 SFU/10^6 PBMCs), and a lack of significant changes in CMV-pp65-specific responses (**Fig. 3**). These findings support the hypothesis that the first dose of SARS-CoV-2 mRNA vaccine induces HIV reactivation that is preferentially sensed by early-gene-product specific T-cells, driving an effector functional profile.

HIV RNA decreases in association with post-vaccine Nef-specific T-cell responses

The *in vivo* sensing of reactivated HIV by gzm-B-releasing Nef and/or Rev-specific T-cells may result in elimination of some HIV-infected cells, specifically those that were poised for vaccine-induced reactivation. This could also explain the lack of a clear boosting effect on Nef/Rev-specific T-cell responses following second vaccine doses (**Figs. 2 & 3**). Alternatively, activated HIV-specific $CD8^+$ T-cell responses may suppress HIV transcription, through incompletely understood mechanisms[35]. To approach the potential impact of T-cell engagement on HIV-infected cells, we first measured changes in residual cell-associated HIV RNA from baseline, following each vaccination. Levels of polyadenylated HIV RNA decreased significantly over the course of the study (V1 to V3) as measured by two different sets of primers and probes, targeting either the 5' or 3' region of the HIV genome. The target of the 5' primers/probes is only present in unspliced HIV RNA, whereas that of the 3' primers/probes is present in all splicing isoforms (**Fig. 4A**). 5' HIV RNA decreased from a mean of 2,027 copies/10^6 CD4 cells at V1 to 1,257 copies/10^6 CD4 cells at V3 (1.6-fold decrease $p = 0.03$); 3' HIV RNA decreased from a mean of 541 copies/10^6 CD4 cells at V1 to 351 copies/10^6 CD4 cells at V3 (1.5-fold decrease $p < 0.05$) (**Fig. 4B**).

Interestingly, for 3' HIV RNA, these changes showed a strong inverse correlation with Nef-specific gzm-B-producing T-cell responses at V2 ($r = -0.73$, $p = 0.006$), and significant but weaker inverse correlations with Nef- or Rev-specific gzm-B-producing T-cell responses at V3 (**Fig. 4C & E, Extended Data Table 3**). Changes in 5' HIV RNA showed evidence of a similar pattern, with the relationship with Nef-specific gzm-B-producing responses at V2 near the threshold of significance. This fits with the expected observations of killing of cells transcribing HIV RNA by Nef-specific gzm-B-producing T-cells given that only the 3' primer/probes directly detect Nef-encoding spliced transcripts, whereas the 5' primer/probes would also detect transcripts from cells where unspliced HIV RNA predominates. No correlations were observed between gzm-B-specific responses to late gene products (Gag, Pol, or Env) nor IFN-γ-producing responses to any gene product, and either HIV RNA measure (**Fig. 4C, Extended Data Table 3**). AIM assay results showed agreement with the gzm-B ELISPOT results, with Nef-specific $CD8^+$ ($r = -0.76$, $p = 0.006$) and – to a lesser extent – $CD4^+$ T-cell responses ($r = 0.66$, $p = 0.02$) correlating inversely with changes in 3' HIV mRNA (**Fig. 4D & E, Extended Data Table 4**). Thus, each of the HIV-specific T-cell responses shown to be significantly increased following SARS-CoV-2 vaccine dose 1 (**Figs. 2 & 3**), were in turn correlated with reductions in HIV RNA. This supports a model whereby the induced T-cell responses either suppressed viral transcription[35], and/or eliminated some of the transcriptionally-competent HIV-infected cells, with the demonstrated cytotoxic functionality (gzm-B) perhaps suggesting the latter[36].

No measurable changes in HIV reservoir size following vaccinations

Measurable reductions in the frequencies of cells harboring HIV DNA would comprise more direct evidence that some infected cells had been selectively eliminated, but existing assays have important limitations. Total levels of HIV DNA provides a poor representation of the 'HIV reservoir' (defined as infected cells with the potential to reseed viremia), due to the fact that the large

304 majority of integrated viral genomes are defective (e.g. large deletions)[37]. A recently developed
305 duplex digital-droplet PCR (ddPCR) assay termed the intact proviral DNA assay (IPDA)
306 substantially improves upon this and provides a reasonable upper estimate of genomically intact
307 proviruses[38]. However, a further complexity is that the vast majority of these proviruses do not
308 reactivate and produce infectious virus even after maximal *in vitro* stimulation[38], and some may
309 be limited by chromosomal context from ever reactivating[39-41]. In applying the IPDA to quantify
310 intact proviruses, as well as the defective proviruses that yielded only packaging signal (Ψ) or rev
311 response element (RRE) amplification, we observed a lack of significant changes in any measure
312 across the three visits for the 11 participants that produced valid results (**Fig. 5A - C**) (2 individuals
313 showed characteristic detection failures likely attributable to HIV sequence diversity in the primer
314 or probe binding sites[42]).
315
316 We selected four individuals on the bases of clear inductions Nef-specific gzm-B releasing
317 responses (**Fig. 3**) and of sample availability, to measure the HIV reservoir using an alternative
318 method – the Tat/rev Induced Limiting Dilution Assay (TILDA)[43]. This assay quantifies the
319 frequencies of cells that can be induced by PMA/ionomycin to express *tat* or *rev* transcripts.
320 Samples from two study participants (PIDs 9 and 17) showed inducible cells trending higher
321 across visits, while this measure was unchanged in PID 15, and undetectable in PID 7. Although
322 overall interpretation is limited by the small 'n' available for this assay, it is notable that PID 9 had
323 the greatest magnitude increase in Nef-specific gzm-B responses in this study (**Fig. 3**), as well as
324 one of the more marked drops in cell-associated HIV RNA in **Fig. 4**. Thus, these results suggest
325 that – despite evidence for $CD8^+$ T-cell engagement – reductions in cells that could be induced
326 by PMA/ionomycin to produce transcripts were not achieved following SARS-CoV-2 mRNA
327 vaccination.
328
329

Discussion

Clinical trials of the shock and kill approach aimed at harnessing $CD8^+$ T-cells to reduce HIV reservoirs have provided evidence for increases in viral transcription, but without reductions in measures of viral persistence nor direct evidence of antigen expression and $CD8^+$ T-cell engagement[6-8]. The observations presented here advance the shock and kill concept, by providing evidence for the productive engagement of HIV-specific T-cells with their antigens in ARV-suppressed donors following receipt of an mRNA vaccine – resulting in significant reductions in cell-associated HIV RNA, a measure of HIV persistence. Of key importance, we demonstrate specific immune correlates of these *in vivo* reductions in cell-associated HIV RNA, namely increases in T-cell responses targeting the early HIV gene product Nef - especially $CD8^+$ T-cell responses and those releasing gzm-B following short-term *ex vivo* stimulation. These findings are novel in the context of an *in vivo* latency-reversing intervention and build upon our recent reports implicating Nef-specific T-cells as superior sensors of HIV reactivation, and gzm-B production as a hallmark of recent antigenic stimulation[15-17]. Our results indicate that incorporating these specific T-cell metrics in studies assessing latency reversal strategies will reveal evidence for *in vivo* engagement of T-cells that may have been missed by more conventional measures, such as Gag-specific IFN-γ responses.

A common interpretation for why past latency reversal studies have not reduced HIV reservoirs, despite increasing HIV transcription, is that $CD8^+$ T-cells were not effectively engaged, either because viral RNA did not lead to antigen expression[13], or because HIV-specific $CD8^+$ T-cells were insufficiently numerous or functional[44], or because these cells were unintentionally impaired by some latency reversing agents[45,46]. The current study allows us to move beyond these factors and ask, why HIV reservoirs were not reduced despite apparent engagement of HIV-specific $CD8^+$ T-cells with cytotoxic properties (gzm-B) and an associated reduction in cell-associated HIV RNA?

We propose the following non-mutually exclusive possibilities. The first is that HIV-specific $CD8^+$ T-cells were able to detect but not kill reservoir-harboring cells following vaccination. This is supported by from mounting evidence that reservoir-harboring cells may be intrinsically resistant to killing[47,48]. Two of the genes that have been implicated in this survival and in resistance to CTL, BCL2A1 (BCL-xL)[49,50], and BIRC3[51,52], were upregulated by *ex vivo* BNT162b2 treatment (**Fig. 1F**) raising the possibility that resistance to killing may be enhanced alongside HIV transcription, as previously observed with some other LRAs[53]. In this model, the reduction in cell-associated HIV RNA would be attributable to $CD8^+$ T-cell effector mechanisms that act to inhibit viral transcription – as has been implicated by other studies[35]. Another possibility is that the observed stimulation of T-cells and reduction in viral RNA was indicative of killing but involved too small of a fraction of intact or PMA/I-inducible proviruses to be detected by IPDA or TILDA. The third possibility is that appreciable $CD8^+$ T-cell-mediated killing of infected cells occurred but was counterbalanced by vaccine-induced clonal expansion of HIV-infected cells. Indeed, the infected-cell landscape is complex and dynamic on ART and recent studies have implicated gradual $CD8^+$ T-cell-mediated selection favoring cells with proviruses in poorly transcribed regions of the genome[41]. These possibilities can be addressed in either future mRNA vaccine studies that employ an expanded suite of reservoir quantification assays[54], or in other settings where $CD8^+$ T-cell responses show evidence of engagement.

mRNA vaccines targeting HIV antigens are under development for both prophylactic and therapeutic settings[55]. The objectives of the latter will be to induce immune-mediated control of viral rebound upon ART interruption and/or to drive reductions in HIV reservoirs. Our results suggest that innate immune sensing of the mRNA vaccine platform itself may contribute to the latter outcome by acting as a built-in LRA, thereby providing rationale for assessing the impacts of HIV mRNA vaccines on viral reservoirs, even in lieu of combination with additional LRAs. This LRA activity is analogous to the self-adjuvant effects of mRNA that have contributed to its status

as a compelling vaccine platform and - very likely - can similarly benefit from engineering to improve this activity[56]. If additional LRAs are to be combined with mRNA vaccines, their selection can also be guided to be synergistic with the LRA activity of mRNA vaccines by targeting non-overlapping mechanisms of latency. Such combinations can also be tested *ex vivo* as a key aspect of study design. The results of the current study also support the prioritization of Nef (and perhaps Rev) as the antigenic targets of therapeutic mRNA vaccines, as these may be more readily expressed by reactivated HIV. Although perhaps not critical, it is worth noting that the flexibility of mRNA vaccination platforms further provides a potential solution to managing Nef's genetic variability (relative to some other viral antigens such as Gag) by allowing for the potential to tailor immunogens to those prevalent in a given geographical region, or perhaps even within a given ARV-treated individual.

In summary, our findings advance HIV cure research in two important ways. First, we have identified and provided mechanistic insights into LRA activity mediated by innate immune recognition of mRNA vaccines. Although this activity - incidental in the context of SARS-CoV-2 mRNA vaccines - was insufficient to drive measurable reservoir reductions, there are multiple ways that future studies may intentionally optimize and leverage this built-in LRA activity to enable reservoir reductions by mRNA vaccines encoding HIV antigens. Second, we have uncovered specific measures as sensitive indicators of T-cells engagement with HIV antigens *in vivo,* following treatment with an LRA. Broad inclusion of these in future latency reversal studies will help identify cases where such engagement has occurred, enabling the field to evaluate and then push beyond this milestone, towards achieving and measuring reductions in HIV reservoirs.

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Contributions

E.M.S, S.T, D.C.C, L.L, P.Z., D.B., F.D., T.W.C., A.B., M.C.D., M.P.P, R.M.L, G.Q.L, A.R.W, Z.L.B and R.B.J. conceived, designed and analyzed the experiments. M.C, C.G., G.B.E., C.D.J., T.J.W., M.C.D., H.R.L., S.S., N.M-G., C.J.S., E.M.S, and S.T. designed and executed clinical protocols. E.M.S, S.T, D.C.C., M.P.P., D.C.C, S.N., L.L, P.K, J. W., A.D., T-W.C., E.M., K.B., R.B.J, M.P.P and G.Q.L carried out the experiments. R.B.J wrote the manuscript with input from all co-authors. The order of co-first authors are presented based on earliest involvement with the current project.

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Figure Legends

Fig. 1. The BNT162b2 mRNA vaccine stimulates the RIG-I/TLR – TNF-α – NFκb axis and activates HIV transcription *ex vivo*. A. qPCR measurements of HIV RNA in supernatants, 48 hours following *ex vivo* treatment of PBMCs from an ART-treated participant with the indicated concentrations of BNT126b2 (Pfizer BioNTech) or mRNA-1273 (Moderna) mRNA vaccines. **B**. Extension of results from **A** to n = 6 ART-treated participants, adding treatments with 2% volume/volume Fluzone™ influenza vaccine, 25 nM bryostatin-1, 40 nM romidepsin, or 2 μg/ml phytohemagglutinin-L (PHA). **C**. Flow cytometry data from the same samples harvested for **B**.

Shown are %CD69^{+} (activated) following gating on viable CD4^{+} T-cells. **C - E**. Bulk mRNA-seq data was generated using a subset of the samples plotted in **B**. **C**. Principal component analysis (PCA). The results show that transcriptional profiles of BNT126b2- and mRNA-1273-treated cells are more similar to 'No treatment' and to each other than Fluzone™-treated cells. **E**. Gene set enrichment analyses showing pathways activated following mRNA vaccine treatments. **F**. Heatmap of 67 genes in the leading edge for the TNFA_SIGNALING_VIA-NFKB pathway, comparing BNT126b2 to No treatment.

Fig. 2. Sustained increases in SARS-CoV-2-specific CD4^{+} and CD8^{+} T-cell responses and transient increases in HIV Nef-specific CD8 T-cells following COVID mRNA vaccination. A. Representative gating schematic for TCR-dependent activation induced marker (AIM)+ populations (CD69+CD137+) after stimulation with HIV, SARS-CoV-2 or CMV gene products. **B & C.** Combined AIM+ CD8^{+} and CD4^{+} T-cells results for n=13 ARV-treated donors at baseline (V1), and ~2 weeks after vaccine dose 1 (V2) or vaccine dose 2 (V3). CD8+ - **B**, CD4+ - **C**. Data points represent means of duplicates. P values were calculated by one-tailed Wilcoxon matched pairs signed rank test, adjusted for multiple comparisons using the Holm method. **D.** Results are analogous to panel B, but performed on a confirmatory cohort from Vancouver, Canada.

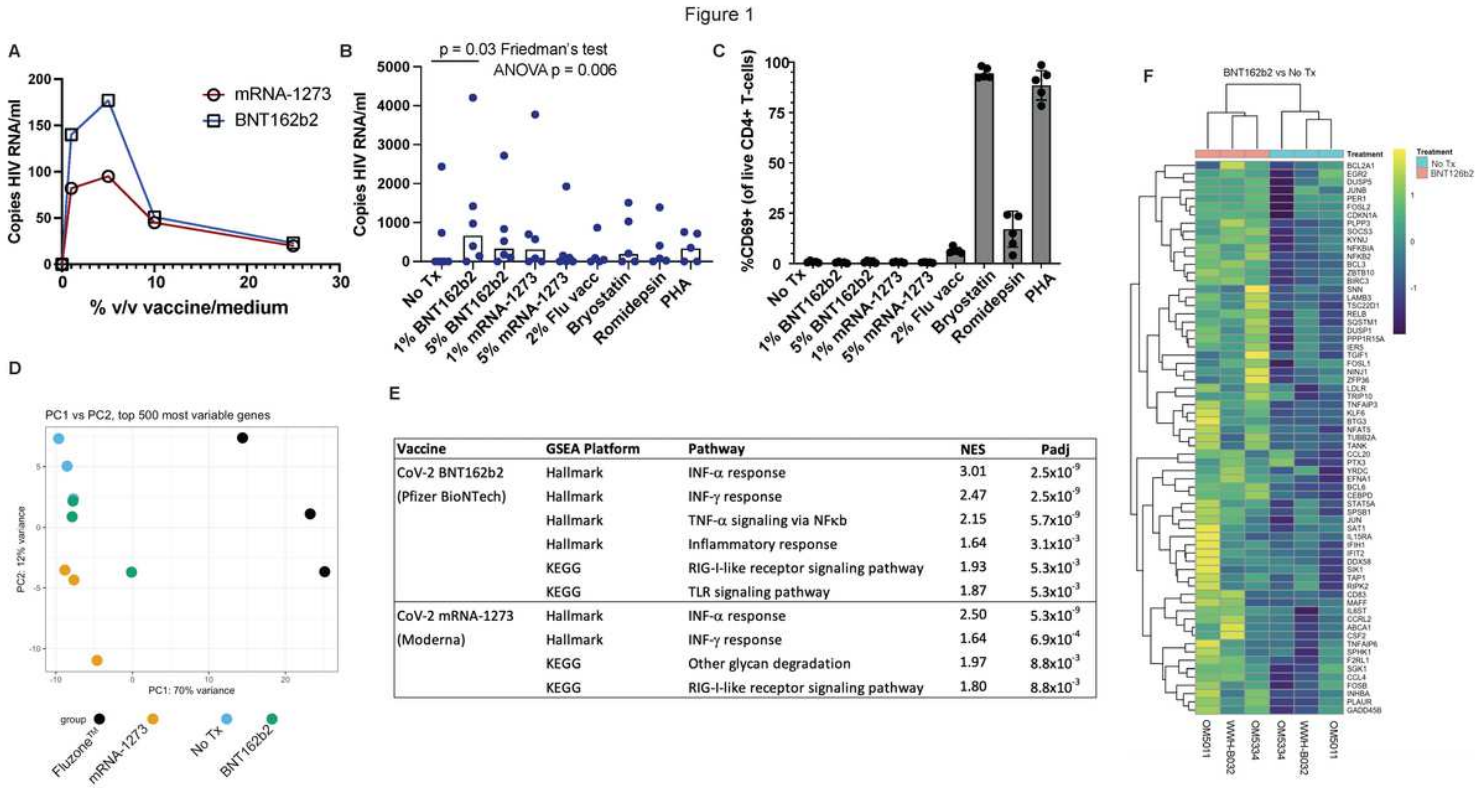
Fig. 3. Transient increases in granzyme B T-cell responses to early HIV-gene products following the first dose of SARS-CoV-2 mRNA vaccination. A & C. Representative ELISPOT results measuring granzyme B (Gzm-B) (**A**), or IFN-γ (**C**). Peptide stimulations are plated in duplicates. **B & D.** Combined ELISPOT results ARV-treated donors at baseline (V1), and ~2 weeks after vaccine dose 1 (V2) or vaccine dose 2 (V3). Gzm-B – **B**, IFN-γ – **D**. n = 13 donors for V1 and V3, and n = 12 for V2 (donor 14 did not provide a V2 sample). Data points represents

means of duplicates. P values were calculated by one-tailed Wilcoxon matched pairs signed rank test, adjusted for multiple comparisons using the Holm method.

Fig. 4. Cell-associated HIV RNA decreased across SARS-CoV-2 mRNA vaccination, in inversely correlating with Gzm-B T-cell responses to early gene products. A. Position of RT-dPCR primer/probes. The 3' primer/probes target all unspliced and spliced isoforms of HIV poly(A) RNA, whereas the 5' primer/probes only target unspliced. **B**. Cell-associated HIV RNA for n = 13 ARV-treated donors at baseline (V1), and ~2 weeks after vaccine dose 1 (V2) or vaccine dose 2 (V3). **C & D.** Depiction of Spearman correlations between proportional changes (V3/V1) in HIV RNA and magnitudes of indicated T-cell responses measured at indicated visits by ELISPOT (**C**) or AIM assay (**D**). Circle sizes are proportional to P values, and color to Spearman's R, as indicated in the scales shown. Analyses are for the n = 12 individuals who completed all 3 visits (donor 14 missed V2). The rings around each circle indicate the threshold P value of 0.05. **E.** Plots of the most significant correlations from **C** (left panel) **& D** (right panel).

Fig. 5. No consistent changes in HIV reservoir measures following COVID vaccinations. A – C. Copies of the indicated HIV DNA species for ARV-treated donors at baseline (V1), and ~2 weeks after vaccine dose 1 (V2) or vaccine dose 2 (V3). P values were calculated by one-tailed Wilcoxon matched pairs signed rank test comparing V1 with V3. n = 13 at V1 and V3 and n = 12 at V2 (PID 14 missed V2). **D**. Tat/rev induced limiting dilution assay results for the n = 4 study participants tested.

Figures



Vaccine	GSEA Platform	Pathway	NES	Padj
CoV-2 BNT162b2	Hallmark	INF-α response	3.01	$2.5x10^{-9}$
(Pfizer BioNTech)	Hallmark	INF-γ response	2.47	$2.5x10^{-9}$
	Hallmark	TNF-α signaling via NFκb	2.15	$5.7x10^{-9}$
	Hallmark	Inflammatory response	1.64	$3.1x10^{-3}$
	KEGG	RIG-I-like receptor signaling pathway	1.93	$5.3x10^{-3}$
	KEGG	TLR signaling pathway	1.87	$5.3x10^{-3}$
CoV-2 mRNA-1273	Hallmark	INF-α response	2.50	$5.3x10^{-9}$
(Moderna)	Hallmark	INF-γ response	1.64	$6.9x10^{-4}$
	KEGG	Other glycan degradation	1.97	$8.8x10^{-3}$
	KEGG	RIG-I-like receptor signaling pathway	1.80	$8.8x10^{-3}$

Figure 1

The BNT162b2 mRNA vaccine stimulates the RIG-I/TLR – TNF-a – NFkb axis and activates HIV transcription ex vivo.

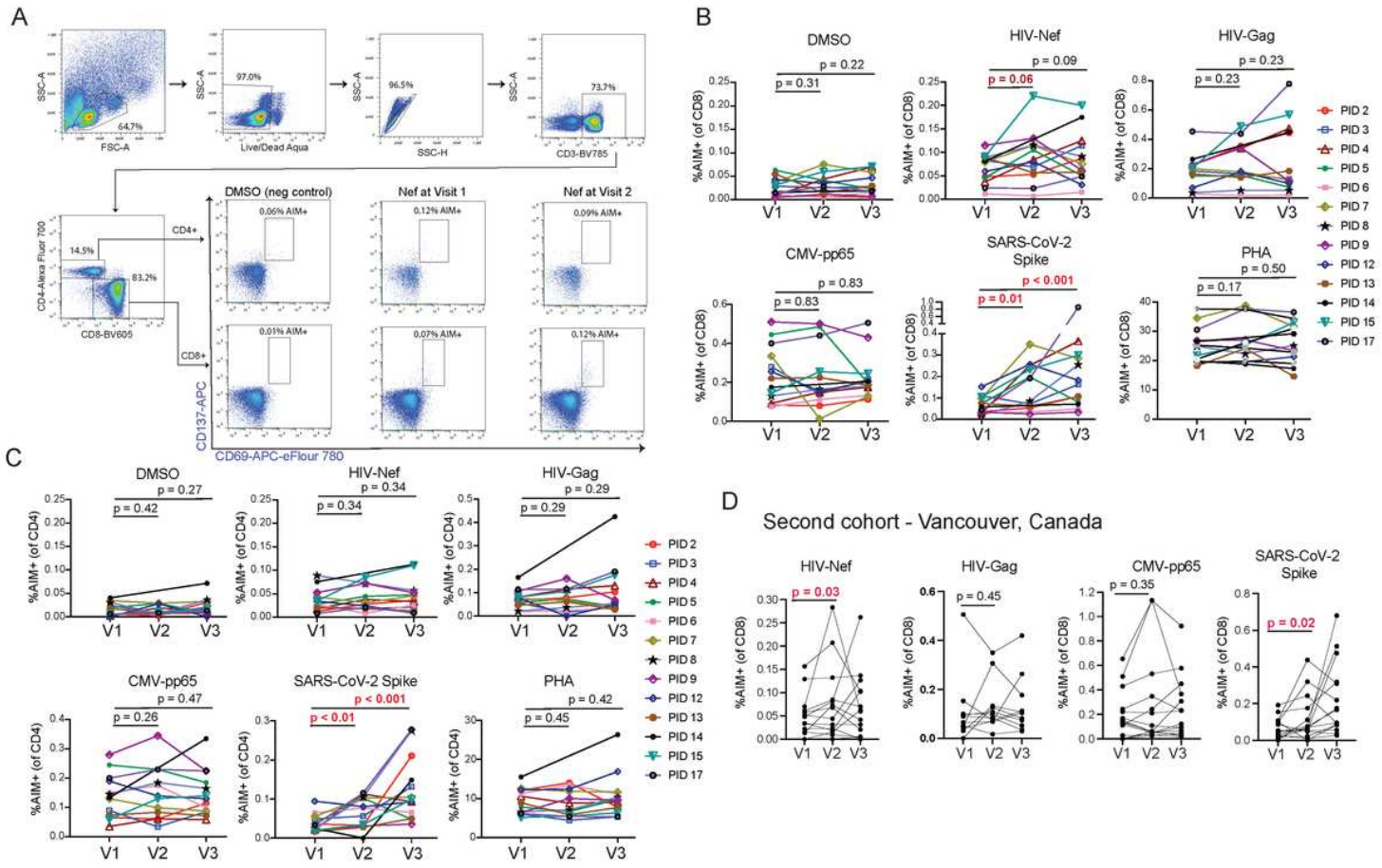


Figure 2

Sustained increases in SARS-CoV-2-specific CD4+ and CD8+ T-cell responses and transient increases in HIV Nef-specific CD8 T-cells following COVID mRNA vaccination.

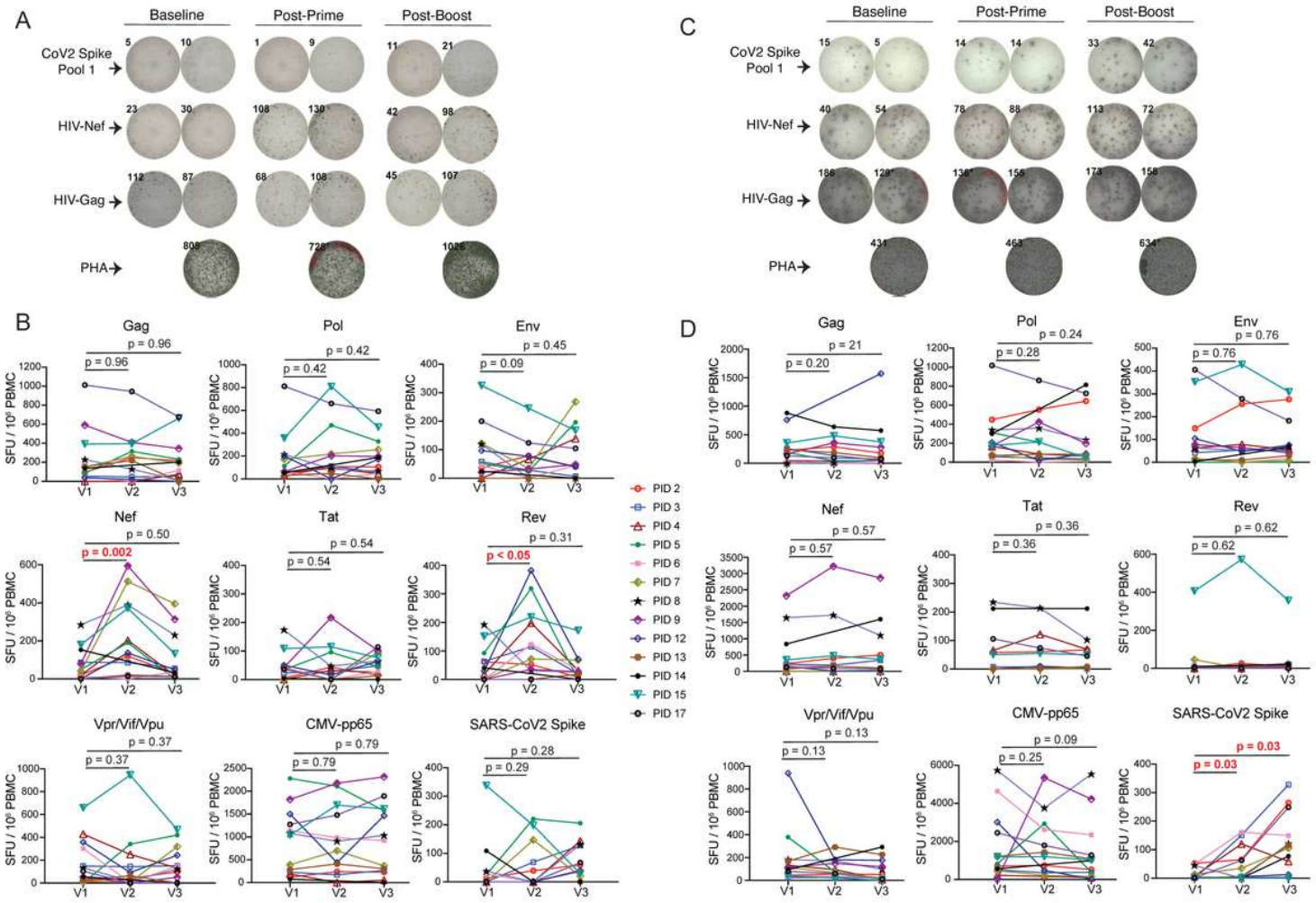


Figure 3

Transient increases in granzyme B T-cell responses to early HIV-gene products following the first dose of SARS-CoV-2 mRNA vaccination.

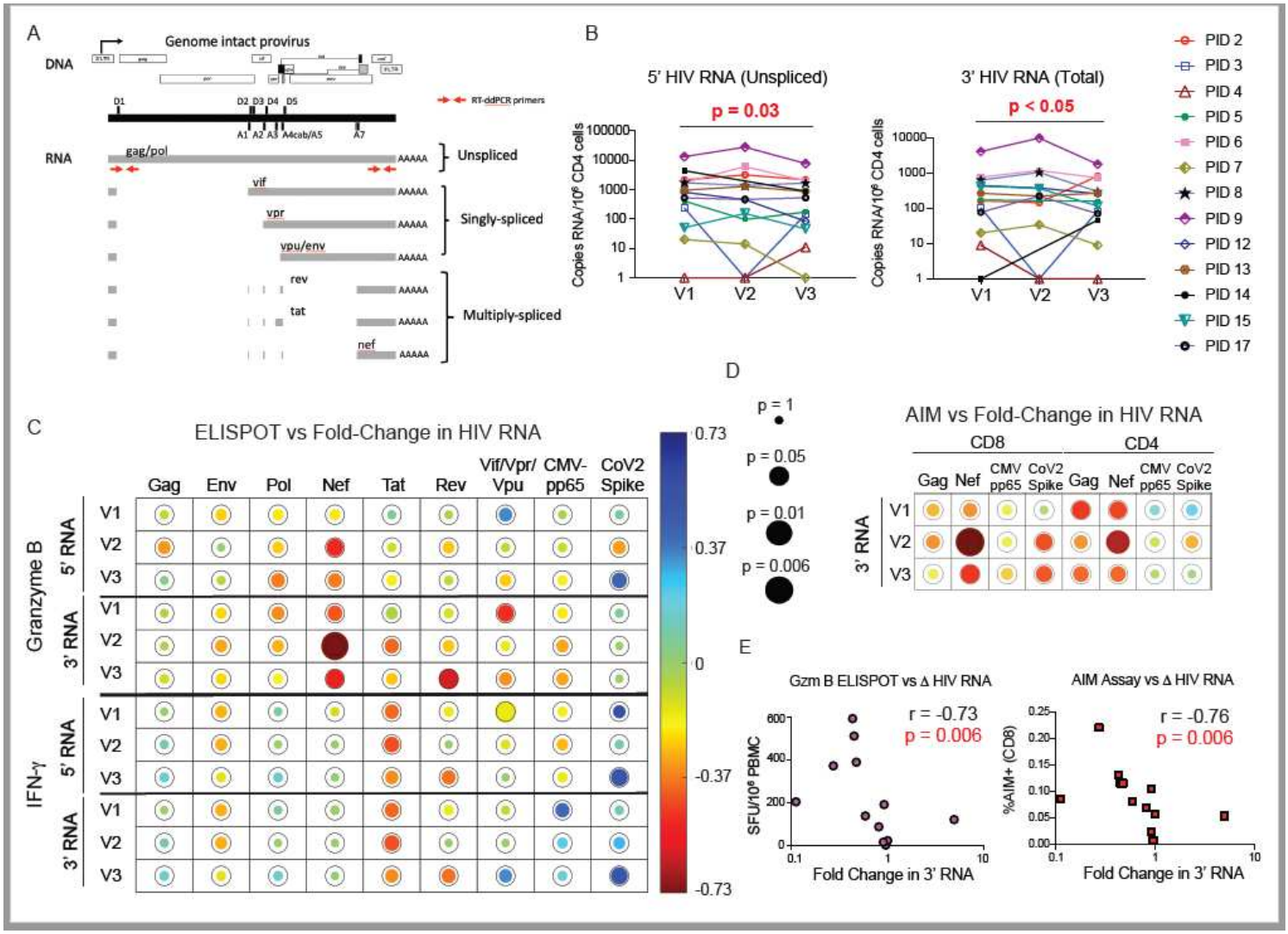


Figure 4

Cell-associated HIV RNA decreased across SARS-CoV-2 mRNA vaccination, in inversely correlating with Gzm-B T-cell responses to early gene products.

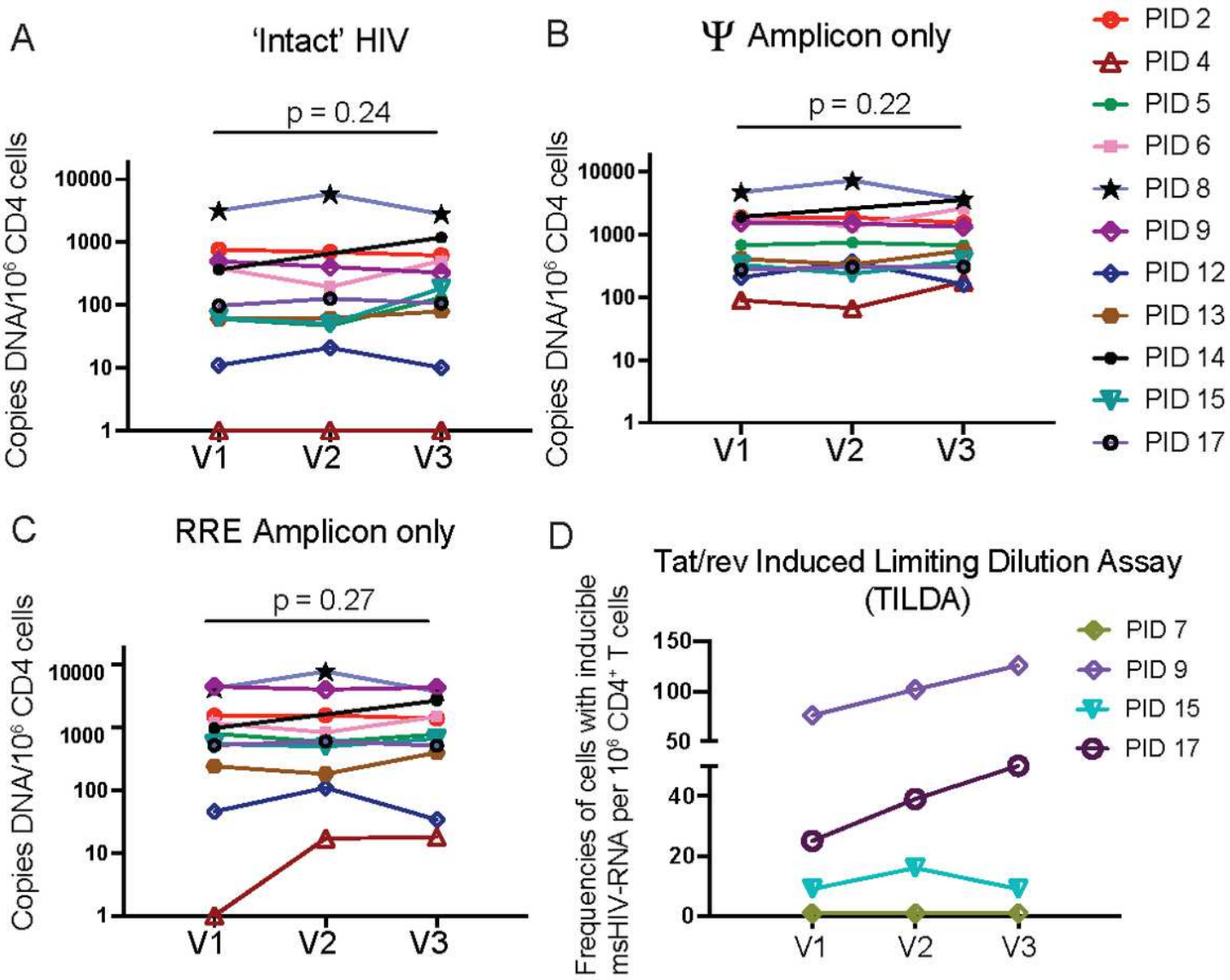


Figure 5

No consistent changes in HIV reservoir measures following COVID vaccinations.

Supplementary Files

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