

Blood and tissue HIV-1 reservoirs display plasticity and lack of compartmentalization in virally suppressed people

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Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This manuscript from Pardons and colleagues tackled critical questions with respect to (1) the difference in phenotypic signatures between the HIV-1 blood and tissue reservoir (with a focus placed on translation-competent reservoir cells) and (2) their compositions, characterized by the integrity of the proviral genomes (intact versus defective).

It is important to note that in this study, longitudinal clinical samples, including blood (B_T0 and B_T1), lymph node (L_T1), and gut (G_T1) biopsies, which correspond to respective patients were utilized, allowing the possible investigation of the temporal evolution of the HIV-1 reservoir (T0: 6 out of 9 patients; T1: 4 out of 9 patients).

This work was initiated with the performance of HIV Tat mRNA-containing lipid nanoparticle (Tat-LNP) for the detection of p24+ cells, referred to as translation-competent reservoir cells in this work, and observed interhost variations (variations between patients) as measured by the proportion of p24+ cells and intact proviruses.

Furthermore, Pardons and colleagues observed distinct phenotypic signatures across different CD4 T cell subsets between blood and tissue cells. Notably, the authors claimed that the majority of the translation-competent reservoir cells (GC Tfh cells marked as CXCR5highPD1high) are contributed by peripheral CD4 T cells.

Pardons and colleagues continued to depict the landscape of the provirus genomic integrity in reservoir cells, corresponding between blood and tissues using HIV-PULSE assay. Identical proviral genomes can be sequenced and identified, as it is claimed that this phenomenon results from the clonal expansion of reservoir cells. The authors further characterized the co-receptor usage of proviruses and observed that, except for the patient MRC21, all proviruses were CCR5-tropic.

As a final note, based on the calculation of the clonality and the phylogenetic analysis, the authors claimed that the propensity for the manifestation of clonal expansion is comparable between reservoir cells in blood and in tissues, and reservoir cells can disseminate across various anatomical compartments. Overall, this paper deals with a very important and timely topic: investigating long-standing questions about HIV-1 reservoirs.

Although this paper presents key findings, there are a few issues that the authors may try to clarify, as suggested below:

Comments:

1. One of the major concerns arising, in my opinion, is the presence of a wide range of varieties among patients enrolled

in this study. In lines 132-135, Pardons et al. concluded that the proportion of intact proviruses was similar between reservoir cells from blood and lymph nodes. However, the same conclusion cannot be made if we look at MRC01, 15, and 16, especially MRC15, in which no intact proviruses were detectable (Figure 2C). In addition, based on Figure 3B, it seems that more intact proviruses can be sequenced in blood cells than cells isolated from tissues.

Here my suggestion for the authors is to either provide statistical significance on Figure 2C or rephrase the conclusion with a minor tune.

2. It is not clear to me why in the first panel from the right-hand side in Figure 2E, the bar representing GC Tfh from blood was missing; however, this subset of the cells showed a prevalence in the stacked bar chart, representing p24+ cells in Figure 2F.

3. It is not clear to me that, in Figure 2C, the proportion of intact proviruses in MRC08 dominates more than others; however, an opposite observation was observed in Figure 3C.

4. Here I would have a theoretical concern regarding the average pairwise distance analysis shown in Figure 3E. According to the plot, I assume that the authors consider B_T0, B_T1, L_T1, and G_T1 as four independent events for the data analysis. However, physiologically speaking, B_T0 and B_T1 are both the data obtained from blood and theoretically, the initial distance between these 2 datasets should already be closer as compared to either L_T1 or G_T1. Can the authors comment on it?

5. Line 213: please double-check whether “CCR4-tropic” is what the authors attempt to express.

6. Using the HIV-PULSE assay, the authors detected 33 sequences unique in B_T1 (Figure 4A). Based on the conclusion made by the authors that the probability of ongoing viral replication is negligible, can the authors comment why these 33 sequences cannot be identified in B_T0 as longitudinal samples were applied here?

Reviewer #2

(Remarks to the Author)

Pardons et al, studied the composition of the viral reservoir in blood, lymph nodes, and gut samples from 9 people with HIV on long term ART. They used a novel approach using Tat mRNA-containing nanoparticle together with Panobinostat to reactivate the virus for phenotyping by HIV-flow. Near full length HIV sequencing was done to compare sequence distribution between time points in blood or between compartments. The manuscript is well written and will be of interest to the community working on HIV reservoirs. The main innovation is in the methods that allow for reactivation of HIV presumably without modifying the phenotype of the cells using Tat mRNA-containing nanoparticle together with panobinostat. However, the findings are not novel and mostly confirm what has been shown in other studies. The study is also limited in the number of participants included and by the low number of p24+ cell recovered.

Major

The major limitation of the study is the limited sampling. Few participants were included and very few p24+ cells were recovered. That makes statistical analysis impossible, and firm conclusions are difficult to make about difference in phenotypes between compartments. As stated by the authors, the difference in phenotype of p24+ between blood and lymph nodes mostly reflect the different composition of the CD4 T cell compartment in those locations. Thus, it is an expected result.

Provincial sequencing was performed by either HIV-PULSE or STIP-Seq assays for characterizing total and inducible reservoirs, respectively, and downstream analysis of clonal populations. However, the methodology used to classify proviruses as clonal is unclear, including when comparing sequences obtained from the two methods. What criteria were used in the ONT long-read HIV-PULSE data and the Illumina STIP-Seq data? In addition, the authors previously published that the accuracy of their HIV-PULSE pipeline is 99.99% (1 error per 9 kb genome; <https://doi.org/10.1093/nar/gkad790>), much greater than the 5-10% error rate that is typical of ONT. Given the error-prone nature of ONT sequencing, it would be helpful to provide additional validation of the UMI-based system with respect to proviral sequencing accuracy performed on the study samples included in this manuscript.

The results are mostly confirming what had been previously described. Below are some examples:
Gantner et al. (Immunity. 2023) The phenotype of productively infected cells differed between blood and lymph nodes.
Collora et al. (Immunity. 2022) HIV-infected cells are enriched in GZMB+ cytotoxic effector memory Th1 cells.
Chaillon et al. (J Clin Invest. 2020) identical, intact HIV RNA populations in blood after cessation of therapy, which repopulated tissues throughout the body.
Pardons et al (Nature Communications 2023). P24+ cells are enriched in peripheral GZMA+ CD4 T cells.

Minor

Line 31, please specify that this is on ART.

Reviewer #3

(Remarks to the Author)

In this study, Pardons et al. characterizes the HIV-1 CD4+ cell reservoir isolated from blood, gut and lymph nodes. The research group utilizes HIV PULSE and STIP seq to phenotypically characterize HIV total and inducible reservoirs within PLWH. While the authors have taken enormous effort to characterize matched tissue and blood samples and used innovative methods to understand reservoir dynamics in PLWH, several concerns remain; namely i) a lack of detailed methods and ii) making overt generalizations need to be prevented due to limited sample size and cell numbers.

Major Comments:

For Figure 1, with regards to cells obtained from the gut, please provide a better pictorial representation of the procedure used.

For the data presented, it is hard to conclusively determine whether phenotypic differences are to the effect of Tat-LNP/PNB as the data sets do not have cells obtained from HIV-ve participants (for figures 2A, supplementary figure 2). For cells isolated from gut samples, were the authors able to obtain T-cells from HIV-ve donors as these would be informative and important controls for the sample set.

For the line (126-127), Tat-LNP/PNB treatment exhibited a more profound impact on the lymph node CD4 T-cells, why is that?

Is there a reason why for certain participants (such as MRC08), translation competent cells (p24+/106 cells, Figure 2B) do not correlate with the % of intact/total HIV+ DNA that is higher in the lymph nodes (Figure 2C)? Is this due to sample sizes? Line 180: "Almost all p24+ cells from the blood and lymph nodes expressed Bcl2" – this is a very unspecific statement and also neglects the fact that a vast majority of the cells sampled are positive for Bcl2.

For figure 2, the authors note that there is an absence of TEM cells that are p24+. Is this due to the Tat-LNP/PNB treatment negatively affecting TEM viability? Did the authors compare T-cell subset composition before and after treatment?

It would be informative to provide the average and standard deviations for figure 2B and 2C. Furthermore, the fraction of intact proviruses (intact/total HIV), as measured by IPDA in the sorted live CD3+CD4+/- fraction, was also similar between both anatomical sites (means = 10.5% and 8.5% in blood vs lymph nodes)." is not fully supported by the data. This is because: Figures 2B and 2C clearly shows that the blood and lymph nodes are very different for each donor: by 5 folds in MRC08, MRC01, even up to hundreds of folds in MRC15. The authors averaged across all five donors, but did not conduct any significance test, and didn't even present the "10.5% and 8.5%" data as a figure. The data sample size, only 5 donors, is too small to make any statistically significant conclusion.

While it can be justified as to why the authors pooled cell numbers for figure 2E (due to understandable lack of cells from these sample sets), it would be informative to also show the cell counts per participant.

For supplementary Figure 3B, it's a little confusing as to what the dark and light gray bars represent? is it untreated vs. tat LNP/PNB treated or is it p24+ vs p24-ve.

For sequencing analysis of intact vs. defective HIV, please provide how many cells were used for the sequencing per participant. In Methods, line 468 says "8 µL", and line 487-488 does not say HIV genomic DNA from how many cells are used for each PCR reaction.

For Figure 4A, it is interesting that a majority of the intact sequences were seen in blood T0 and T1 samples compared to the gut and lymph nodes. Is there a reason as to why more intact sequences were seen in these groups?

The statements are contradictory on line 247-250: In the first half of the sentence, the authors say that "no significant differences were observed between blood and tissues". In the latter half of the sentence, the authors say that "the clonality estimate for each participant was systematically lower in the lymph nodes compared to the blood". Since there is no significant difference, the authors should avoid making definitive statements. This point extends to other conclusions in the paper, considering the statement in line 664: "No statistically significant differences were observed in any of the pairwise comparisons run in this study."

Minor concerns:

In line 52: "these molecules lack of specificity" should be "these molecules lack specificity".

In line 58: "Few studies have attempted to compare the HIV-1 proviral landscape between blood and tissues (19-27)." should be "Many studies..." as there are 9 such studies cited in this sentence.

Figure 2D has no percentage labels

Figure 3B: what does the percentage on the horizontal axis mean? Are they percentages of the number of distinct sequences or total sequences?

The meaning for the horizontal axis in Figure 4A is unclear and should be labeled. Do they represent each donor?

Reviewer #4

(Remarks to the Author)

In this manuscript, Marion Pardons, Laurens Lambrechts, and colleagues investigated the composition and phenotypes of HIV-infected cells in blood and tissues. They capitalize on novel technologies developed by their group, including HIV-flow, HIV-Pulse, STIP-seq, and the use of Tat-LNP + Panobinostat to reactivate HIV gene expression. The manuscript is eloquently written, and the conclusions are well supported by the experimental data generated. The main finding is that p24 positive cells present a distinct phenotype between blood and lymph nodes. While TEM with a cytotoxic (GZMA) phenotype make up most of the p24 positive cells in blood, TCM CXCR5+/- cells represent most of p24 positive cells in lymph nodes. This work adds to the importance of dissecting reservoir features between blood and tissues.

Strengths: the Authors use cutting edge technologies, especially the use of a more selective inducer of HIV expression (Tat-LNP). The observed differences in infected cell phenotype reflect important T cell biology. I especially appreciated the "anomaly" of participant MRC21, where the predominant CXCR4 phenotype was associated with a higher proportion of p24

positive cells in “naïve-like” T cells. The Authors did an exhaustive job in validating their experimental setup, especially regarding the impact of the Tat-LNT+PNB stimulation on some markers (e.g. GZMA).

Weaknesses: I wish the authors provided direct evidence that the same HIV-infected clones have different phenotype based on their microenvironment. I understand the challenges of doing STIP-seq from lymph node cells, but it would have added strength to the study, allowing also to compare TCR sequences between compartments. Lastly, the observations regarding proviral features, clonality, lack of ongoing replication, and compartmentalization, all confirmed previous findings, representing hallmark features of HIV-infected cell population on ART.

Major comment:

I encourage the Authors in providing better contextualization of their findings regarding the different phenotypes between blood and tissues, as they reflect key features of T cell biology. TEMs are the circulating counterpart of many TCM that mostly reside or are confined in secondary lymphoid organs. Along the same lines, how much of the lack of proviral variant overlap between blood and tissues is due to sampling versus the presence of HIV-infected tissue resident cells? Why did the Authors not use markers for tissue resident T cells (CD69, CD103) in their phenotyping panel? I understand that these markers do not capture all TRM, but they would have helped. Lastly, could the Authors speculate on the potential role of CD4 antigen specificity on the phenotype of these p24+ T cells? I am referring to recent works from Collora et al (PMID: 35320704) and Weymar et al (PMID: 36070690)

Minor comments:

Line 163 “Circular Tfh cells” probably needs to be corrected.

Line 256 “intermixed for all participants” I would clarify with “intermixed within each participant”.

Line 425 “stored on ice until freezing”, probably needs to be corrected with “processing”.

Figure 1 and following. Spell out NS (no stim) as it could be confused with “not significant”

Figure 3A. “Totals” at the bottom of the grey table --> Total

Reviewer #5

(Remarks to the Author)

Overall impressions:

This is a well conducted study and a well written manuscript which will add valuable knowledge to the field. The data support the conclusions and the methodology is sound. Some minor points of clarification are warranted.

Noteworthy results:

1. Persistence of clonal populations of HIV infected cells across longitudinal timepoints.
2. Persistence of clonal populations of HIV infected cells across anatomical compartments.
3. Phenotypically distinct populations of translation-competent reservoir cells in different anatomical compartments.
4. Ongoing replication tissues under ART was not observed.

Significance to the field:

This work contributes to the growing understanding of HIV persistence using cutting-edge methodology allowing collection of previously unmeasurable data.

The conclusions of this study are supported by the work presented.

Soundness of the methodology:

Lines 479 - 484

The authors of HIV-PULSE (PMID: 37819007) acknowledge that enrichment of proviral templates by PCR may introduce bias and that sequence polymorphisms may cause discrepancies in amplification efficiency. The authors have introduced novel primers for the current study. A discussion of whether amplification bias could impact the interpretation of their results is warranted.

Methodology overall is sound and up to the current standards of the field.

Detail in Methods:

Lines 466 - 478

Working primer concentrations should be included in addition to volumes for clarity and reproducibility in the IPDA section of the methods.

Additional comments:

Lines 355-358

This statement is vague - "...identified a few links...", "...certain clones of infected cells...".

I believe the data support the statement being made but I think this conclusion could be stated more clearly.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Response to Authors' Rebuttal

NCOMMS-24-54369A

First of all, I would like to thank Pardons et al. for their clear answers to my questions.

In this revised version of the manuscript, the authors have repainted the color code applied in Figure 2F (stacked bar charts) to avoid any confusion or misreading.

I concur with other reviewers' major concerns with respect to a small sample size that may cause statistical difficulties. On the other hand, I am aware of the limited availability of clinical samples, followed by the challenge of isolating primary cells from blood, matched lymph nodes, and gut microbiopsies, allowing addressing the critical question about HIV-1 reservoirs in different anatomical compartments. Certainly, a follow-up study, perhaps with an appropriate sample size to reach statistical significance, will be required, i.e., single-cell RNA-seq on large numbers of CD4 T cells as authors' response to the comment from Reviewer #4.

In addition, the statistical strategy offered by the authors (see the authors' response to my comment #4 and comments from Reviewer #3) is convincing to deal with this small number of patients in the current stage.

After taking into account the significance of the findings in this work, together with technical advances (HIV-Flow, HIV-PULSE, STIP-Seq, and Tat-LNP/PNB) generated from the lab, and the limitations, I am pleased to recommend this manuscript for publication.

Reviewer #2

(Remarks to the Author)

The authors have addressed my comments related to the sequencing and have argued their way around my other concerns about the low number of participants/p24+ cells and the novelty of the findings. At the very least, these limitations should be made explicitly clear in the manuscript with all appropriate references cited.

Reviewer #3

(Remarks to the Author)

In the revised manuscript, the authors addressed all of our concerns. Although no new data/samples were added, the revised text represents more appropriate conclusions. Moreover, the new statistical analyses now align well with the size of the sample and the new labelings on the figures make them clearer and easier to read. We have no further comments.

Reviewer #4

(Remarks to the Author)

The Authors have addressed all the concerns raised by the reviewers. I have no further comments or requests.

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Rebuttal

Reviewer #1 (Remarks to the Author):

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Furthermore, Pardons and colleagues observed distinct phenotypic signatures across different CD4 T cell subsets between blood and tissue cells. Notably, the authors claimed that the majority of the translation-competent reservoir cells (GC Tfh cells marked as CXCR5^{high}PD1^{high}) are contributed by peripheral CD4 T cells.

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As a final note, based on the calculation of the clonality and the phylogenetic analysis, the authors claimed that the propensity for the manifestation of clonal expansion is comparable between reservoir cells in blood and in tissues, and reservoir cells can disseminate across various anatomical compartments. Overall, this paper deals with a very important and timely topic: investigating long-standing questions about HIV-1 reservoirs.

We thank the reviewer for the positive evaluation of our work. Before addressing the specific comments, we would like to clarify that there seems to be a misunderstanding in the interpretation of Figure 2F. TEM CXCR5⁺ cells are the main contributors to the translation-competent reservoir in the blood. The germinal center Tfh cells (CXCR5^{high}PD1^{high}) are represented in dark pink and are solely found in lymph nodes, while the TEM CXCR5⁺ cells are depicted in red. It seems the reviewer might have accidentally switched the two colors.

Although this paper presents key findings, there are a few issues that the authors may try to clarify, as suggested below:

Comments:

1. One of the major concerns arising, in my opinion, is the presence of a wide range of varieties among patients enrolled in this study. In lines 132-135, Pardons et al. concluded that the proportion of intact proviruses was similar between reservoir cells from blood and lymph nodes. However, the same

conclusion cannot be made if we look at MRC01, 15, and 16, especially MRC15, in which no intact proviruses were detectable (Figure 2C). In addition, based on Figure 3B, it seems that more intact proviruses can be sequenced in blood cells than cells isolated from tissues. Here my suggestion for the authors is to either provide statistical significance on Figure 2C or rephrase the conclusion with a minor tune.

Due to the limited number of datapoints, we agree with the reviewer that a toning down is necessary. We have therefore adjusted the text: “While frequencies of p24+ cells were higher in the lymph nodes compared to the blood in 2/5 participants, the opposite trend was observed in the remaining individuals (means = 11.5 and 9.0 p24+ cells/10⁶ CD4 T cells in blood vs lymph nodes) (Figure 2B). Furthermore, the mean fraction of intact proviruses (intact/total HIV), as measured by IPDA in the sorted live CD3+CD4+/- fraction, was 10.5% in the blood and 8.5% in lymph nodes, with notable intra- and inter-individual variability (Figure 2C)”.

2. It is not clear to me why in the first panel from the right-hand side in Figure 2E, the bar representing GC Tfh from blood was missing; however, this subset of the cells showed a prevalence in the stacked bar chart, representing p24+ cells in Figure 2F.

Germinal center Tfh cells are localized within lymphoid tissues. Consistent with this, our findings confirm that GC Tfh cells are exclusively detected in the lymph nodes and are absent from the blood (Figure 2D and Supplementary Figure 2A). The red cluster in the unsupervised clustering approach represents TEM CXCR5- cells, and not GC Tfh cells. To avoid this confusion, the cluster corresponding to GC Tfh is now displayed in bright pink rather than in dark pink.

3. It is not clear to me that, in Figure 2C, the proportion of intact proviruses in MRC08 dominates more than others; however, an opposite observation was observed in Figure 3C.

This discrepancy is related to the quantification methods used. The IPDA method relies on the simultaneous detection of the Packaging Signal (PSI, Ψ) and the Rev Responsive Element (RRE) in Envelope (*env*) by digital PCR, which may lead to some defective proviruses being misclassified as intact (Reeves et al, Nat Commun, 2023; PMID: 37443365). In contrast, HIV-PULSE is a full-length approach, but the inclusion of a pre-amplification step that is necessary for efficient tagging and enrichment hinders accurate reservoir quantification and precise clonality assessment. For this reason, in Figure 3C, we focused on the proportion of intact HIV-1 among distinct proviral genomes, which means that each proviral sequence is counted only once, even if it was detected multiple times across different PCR reactions. Because of the intrinsic differences in the methodology used, we do not expect the proportions of intact HIV detected by IPDA and HIV-PULSE to correlate.

4. Here I would have a theoretical concern regarding the average pairwise distance analysis shown in Figure 3E. According to the plot, I assume that the authors consider B_T0, B_T1, L_T1, and G_T1 as four independent events for the data analysis. However, physiologically speaking, B_T0 and B_T1 are both the data obtained from blood and theoretically, the initial distance between these 2 datasets should already be closer as compared to either L_T1 or G_T1. Can the authors comment on it?

We thank the reviewer for this insightful suggestion. Following discussions with our statistics department, we have determined that it would indeed be more appropriate to treat the four datasets as paired rather than as independent, as was initially done. Non-parametric Wilcoxon tests were performed for the analysis of the percentage of intact proviruses (Figure 3C), whereas parametric paired t-tests were performed for the analysis of the average pairwise distance (Figure 3E) and the clonality estimate (Figure 4B). Because non-parametric Wilcoxon tests and parametric paired t-tests

can not handle missing values, those were removed from the analyses. Furthermore, when possible, a mixed-effects analysis was also performed. This model can handle missing values, by estimating effects based on the available data from each individual rather than discarding missing data. For the average pairwise distance, no statistical difference was observed between any of the groups when performing paired analyses, both with the paired t-tests and the mixed-effects analysis.

We now added a supplementary data table (Supplementary Data 1) showing all pairwise comparisons for each statistical test performed for Figures 3C, 3E, and 4B.

5. Line 213: please double-check whether “CCR4-tropic” is what the authors attempt to express. We thank the reviewer for noticing this mistake. This is now corrected by CXCR4-tropic.

6. Using the HIV-PULSE assay, the authors detected 33 sequences unique in B_T1 (Figure 4A). Based on the conclusion made by the authors that the probability of ongoing viral replication is negligible, can the authors comment why these 33 sequences cannot be identified in B_T0 as longitudinal samples were applied here?

We appreciate the reviewer’s insightful question. Of the 33 different clonal sequences detected only in B_T1, 15 sequences originate from individuals for whom no T0 sample was available (Supplementary Figure 5; specifically, MRC16, MRC29, and MRC31). We are now mentioning in the legend of Figure 4A that not all participants contributed to all time points and anatomical compartments (line 822). For the remaining sequences, it is possible that they were not identified in B_T0 due to sampling bias. Additionally, some low-abundance clones at T0 may have expanded between T0 and T1, facilitating their detection at T1 while being missed at T0.

Reviewer #2 (Remarks to the Author):

Pardons et al, studied the composition of the viral reservoir in blood, lymph nodes, and gut samples from 9 people with HIV on long term ART. They used a novel approach using Tat mRNA-containing nanoparticle together with Panobinostat to reactivate the virus for phenotyping by HIV-flow. Near full length HIV sequencing was done to compare sequence distribution between time points in blood or between compartments. The manuscript is well written and will be of interest to the community working on HIV reservoirs. The main innovation is in the methods that allow for reactivation of HIV presumably without modifying the phenotype of the cells using Tat mRNA-containing nanoparticle together with panobinostat. However, the findings are not novel and mostly confirm what has been shown in other studies. The study is also limited in the number of participants included and by the low number of p24+ cell recovered.

We are pleased to hear that the reviewer finds the manuscript well written and relevant to the HIV reservoir research community. We recognize that the small number of participants may pose a limitation in our study. However, it is important to highlight the challenges involved in recruiting participants agreeing on giving a lymph node and the technical-related challenges, therefore limiting access to this kind of samples. Additionally, while the recovery of p24+ cells in our study may seem low, this is the first study to successfully detect and characterize lymph node-derived p24+ cells by flow cytometry after latency reversal in ART-suppressed individuals. We believe that our phenotyping data on p24+ cells derived from lymph nodes have not been described previously. Below, we outline why our data differ from the studies referenced by the reviewer.

Major

The major limitation of the study is the limited sampling. Few participants were included and very few p24+ cells were recovered. That makes statistical analysis impossible, and firm conclusions are difficult to make about difference in phenotypes between compartments. As stated by the authors, the difference in phenotype of p24+ between blood and lymph nodes mostly reflect the different composition of the CD4 T cell compartment in those locations. Thus, it is an expected result.

One could argue that HIV reservoir cells may share intrinsic phenotypic signatures that promote their long-term persistence, such as the expression of survival or cytotoxic molecules, regardless of their anatomical location. For instance, p24+ cells could preferentially express markers like GZMA in both blood and lymph nodes. However, our findings suggest that the phenotype of p24+ cells is shaped more by their local environment than by intrinsic characteristics. This is now mentioned in the discussion on lines 331-334.

Proviral sequencing was performed by either HIV-PULSE or STIP-Seq assays for characterizing total and inducible reservoirs, respectively, and downstream analysis of clonal populations. However, the methodology used to classify proviruses as clonal is unclear, including when comparing sequences obtained from the two methods. What criteria were used in the ONT long-read HIV-PULSE data and the Illumina STIP-Seq data?

Below, we outline the criteria used for clonal classification within each independent assay and explain the approach taken when integrating HIV-PULSE and STIP-Seq datasets to identify overlaps. More details about clonal classification are now included in the Methods section on lines 698-715.

HIV-PULSE

For clonal classification with the HIV-PULSE assay, we followed the methodology detailed in our original publication (PMID: 37819007). To link sequences across different runs as done for this manuscript (e.g., for overlaps between timepoints or compartments), **>99.99% sequence identity** following MAFFT alignment was required, ensuring robust identification of clones.

STIP-Seq

The STIP-Seq assay relies on single-cell sorting of p24-expressing cells, followed by recovery of proviral genomes and their associated integration site. Identification of clones in this dataset is simplified: p24+ cells are defined as clonal if proviruses share **100% sequence identity**, with further validation provided by matched integration sites.

Integrating HIV-PULSE and STIP-Seq data to detect clonal overlaps

For each participant, proviral genomes from STIP-Seq and HIV-PULSE were aligned to HXB2 using MAFFT (v7.471). These alignments were manually inspected, and clones were identified based on the following criteria:

- 1) We considered a proviral overlap between HIV-PULSE and STIP-Seq proviruses if sequences were 100% identical.
- 2) If no exact match was observed, we still considered a proviral overlap between HIV-PULSE and STIP-Seq proviruses if identity was greater than 99.5% and they spanned the same deletion junction sites.

In addition, the authors previously published that the accuracy of their HIV-PULSE pipeline is 99.99% (1 error per 9 kb genome; <https://doi.org/10.1093/nar/gkad790>), much greater than the 5-10% error rate that is typical of ONT. Given the error-prone nature of ONT sequencing, it would be helpful to provide additional validation of the UMI-based system with respect to proviral sequencing accuracy performed on the study samples included in this manuscript.

We appreciate the comments regarding sequencing accuracy and are pleased to provide some extra data.

While the initial version of ONT sequencing was known to be error-prone, with an error rate ranging from 5-10%, the latest ONT methodology consistently achieves a Q20 score, corresponding to an error rate of approximately 1% (PMID: 35789207). Since the publication of HIV-PULSE, substantial technological improvements in ONT sequencing have been implemented, including new sequencing chemistries (LSK109 vs LSK114), alternative flow cell designs (R10.3 vs R10.4.1) and updated base calling models (Guppy v.5.0.17 vs v.6.5.7). In addition, we made a minor adjustment to the HIV-PULSE PCR protocol by replacing the LongAmp enzyme used during pre-amplification with PrimeSTAR GXL (PS-GXL), as documented in the method section. We are now including a benchmarking figure showing the improvements in sequencing accuracy achieved by switching from R10.3 to R10.4.1 flow cells and from the LongAmp enzyme to the PS-GXL enzyme (Supplementary Figure 7A). In brief, we conducted experiments on UMI bin centroid accuracy (not to be confused with the 99.99% megabin accuracy mentioned by the reviewer) using the J-Lat 10.6 cell line. PCR products obtained with old (LongAmp) and updated (PS-GXL) pre-amplification protocols were sequenced using different ONT technologies (R10.3, LSK109, Guppy v.5.0.17 vs R10.4.1, LSK114, Guppy v.6.5.7). While the original conditions (R10.3 flow cell, LongAmp enzyme) show a mean bin centroid accuracy of 99.91%, the same PCR protocol with updated ONT technology (R10.4.1 flow cell, LongAmp enzyme) improved this accuracy to 99.93%. The main improvement between the updated technologies lies in the required bin coverage to meet the Q30 accuracy goal, with the original HIV-PULSE R10.3 data needing at least 15 reads while more

than 5 reads suffice with the R10.4.1 data. Moreover, the replacement of the enzyme (R10.4.1 flow cell, PS-GXL enzyme) further enhanced accuracy to 99.97%, leaving only ~3 residual errors per 9 kb genome, predominantly substitutions.

To further validate the robustness of the HIV-PULSE data, we performed a cross-validation using the 37 sequences (25 distinct) that were overlapping between HIV-PULSE and STIP-Seq (see Supplementary Figure 7B). Here, we considered the short-read based STIP-Seq genomes as 'ground-truth' references. Among 37 HIV-PULSE genomes, 29 were found to have 100% agreement with their STIP-Seq reference counterpart. For the remaining 8 sequences, minor discrepancies (an average of 1.6 base pair substitutions) were observed. Importantly, these discrepancies did not affect the proviral classification or clonal linkage. The mean 'megabin' accuracy of 99.99% aligns with the earlier reported value referenced by the reviewer from the original publication.

Based on these results, we are confident that the HIV-PULSE pipeline performs with the required accuracy and does not perform different when applied to the samples in this study.

The results are mostly confirming what had been previously described. Below are some examples:

- Gantner et al. (Immunity. 2023) The phenotype of productively infected cells differed between blood and lymph nodes.
We would like to point out that this study was performed on viremic samples and not under ART suppression.
- Collora et al. (Immunity. 2022) HIV-infected cells are enriched in GZMB+ cytotoxic effector memory Th1 cells.
While lymph nodes are the primary focus of our study, this anatomical site was not included in the study by Collora et al. Additionally, it is important to note that a limited number of HIV RNA+ cells from ART-suppressed individuals were identified (9 in the non-stimulated condition and 43 in the antigen-stimulated conditions), further highlighting the challenges associated with detecting latently infected cells in individuals on ART.
- Chaillon et al. (J Clin Invest. 2020) identical, intact HIV RNA populations in blood after cessation of therapy, which repopulated tissues throughout the body.
The study conducted by Chaillon et al. concentrated on HIV envelope sequencing rather than full-length sequencing. The novelty of our study lies in the use of a full-length HIV sequencing approach with a long-read sequencing assay, which allowed us to obtain an unprecedented number of HIV genomes (>3000). It is also important to note that the study by Chaillon et al did not collect blood prior to death, impeding the comparison of HIV sequences between blood and tissues.
- Pardons et al (Nature Communications 2023). P24+ cells are enriched in peripheral GZMA+ CD4 T cells.
Our previous study indeed showed that blood-derived p24+ cells are enriched in GZMA+ cells. One of the primary objectives of this new study was to assess whether p24+ cells from lymph nodes also show preferential expression of cytotoxic molecules, which does not appear to be the case.

Minor

Line 31, please specify that this is on ART.

This is now added to the text on line 31.

Reviewer #3 (Remarks to the Author):

In this study, Pardons et. al characterizes the HIV-1 CD4+ cell reservoir isolated Blood, gut and lymph nodes. The research group utilizes HIV PULSE and STIP seq to phenotypically characterize HIV total and inducible reservoirs within PLWH. While the authors have taken enormous effort to characterize matched tissue and blood samples and used innovative methods to understand reservoir dynamics in PLWH, several concerns remain; namely i) a lack of detailed methods and ii) making overt generalizations need to be prevented due to limited sample size and cell numbers.

We appreciate the reviewer's recognition of our efforts to characterize matched tissue and blood samples employing innovative approaches. We value the feedback and provide detailed responses to the reviewer's comments and concerns below.

Major Comments:

For Figure 1, with regards to cells obtained from the gut, please provide a better pictorial representation of the procedure used.

We have now updated Figure 1 to provide a better representation of the procedure used for the gut samples, including the number of biopsies used, as well as buffers and enzymes used to obtain a single-cell suspension. We also mention that cells from gut samples were not stimulated and were immediately used for downstream assays (phenotype by flow cytometry and virological assays).

For the data presented, it is hard to conclusively determine whether phenotypic differences are to the effect of TatLNP/PNB as the data sets do not have cells obtained from HIV-ve participants (for figures 2A, supplementary figure 2). For cells isolated from gut samples, were the authors able to obtain T-cells from HIV-ve donors as these would be informative and important controls for the sample set.

HIV negative participants were not included in this study, as our primary focus was to characterize the phenotype of p24+ cells and compare it to the phenotype of p24- cells. Although the inclusion of HIV negative participants would have informed on the overall impact of HIV on cell subsets composition, we believe this is out of the scope of this study. However, for each participant, we included a non-stimulated condition in parallel of the Tat-LNP/PNB treatment, allowing to assess the impact of Tat-LNP/PNB stimulation on the expression of the markers included in our study. These results are described on lines 138-150 and are shown on Supplementary Figure 2.

For the line (126-127), Tat-LNP/PNB treatment exhibited a more profound impact on the lymph node CD4 T-cells, why is that?

The mechanical dissociation of lymph nodes is a rather long process that can generate more mechanical force than the gentle methods typically used to isolate PBMCs from blood, potentially leading to higher rates of cell damage. This is now specified in the text on lines 128-130.

Is there a reason why for certain participants (such as MRC08), translation competent cells (p24+/106 cells, Figure 2B) do not correlate with the % of intact/total HIV+ DNA that is higher in the lymph nodes (Figure 2C) ? Is this due to sample sizes ?

We believe there is not direct comparison possible between these two measurements for different reasons: (i) Figure 2B displays the frequency of p24+ cells per million CD4 T cells, while Figure 2C presents the intact-to-total ratio, which depends on the overall size of the reservoir. For instance, even if a sample would contain a large number of intact-inducible proviruses, a high total reservoir size would still result in a relatively low intact-to-total ratio. (ii) Our previous studies have shown that, following reactivation with PMA/ionomycin or Tat-LNP/PNB, 90% of the inducible proviruses have a

PSI/MSD defect and only 10% are putatively intact (Figure 3A from PMID: 38110433). Therefore, p24+ cells do not only reflect the population of cells carrying an intact provirus.

Line 180: “Almost all p24+ cells from the blood and lymph nodes expressed Bcl2” – this is a very unspecific statement and also neglects the fact that a vast majority of the cells sampled are positive for Bcl2.

We agree with the reviewer that our statement could be made clearer. We now specify: “Almost all p24+ cells from the blood and lymph nodes expressed Bcl2, in line with the majority of CD4 T cells being Bcl2+ in these anatomical sites (Supplementary Figure 2B)”.

For figure 2, the authors note that there is an absence of TEM cells that are p24+. Is this due to the Tat-LNP/PNB treatment negatively affecting TEM viability? Did the authors compare T-cell subset composition before and after treatment?

T cell subset composition was compared between the non-stimulated condition and the Tat-LNP/PNB treatment. These data are shown on Supplementary Figure 2 and are described in the text on lines 138-150. Frequencies of CD4 T cell subsets (TN, TCM, TEM, TTd) were comparable between non-stimulated and treated cells in both anatomical compartments. Therefore, the absence of p24+ cells with a TEM phenotype in the lymph nodes do not appear to be attributed to a negative impact of Tat-LNP/PNB treatment on TEM viability.

It would be informative to provide the average and standard deviations for figure 2B and 2C. Furthermore, the fraction of intact proviruses (intact/total HIV), as measured by IPDA in the sorted live CD3+CD4+/- fraction, was also similar between both anatomical sites (means = 10.5% and 8.5% in blood vs lymph nodes)." is not fully supported by the data. This is because: Figures 2B and 2C clearly shows that the blood and lymph nodes are very different for each donor: by 5 folds in MRC08, MRC01, even up to hundreds of folds in MRC15. The authors averaged across all five donors, but did not conduct any significance test, and didn't even present the “10.5% and 8.5%” data as a figure. The data sample size, only 5 donors, is too small to make any statistically significant conclusion.

The mean and standard deviation for Figures 2B and 2C are now added to the graphs. We agree that the relatively low number of participants limits the ability to draw statistically significant conclusions, necessitating a more cautious interpretation of the results. We have therefore rephrased our statements: “While frequencies of p24+ cells were higher in the lymph nodes compared to the blood in 2/5 participants, the opposite trend was observed in the remaining individuals (means = 11.5 and 9.0 p24+ cells/10⁶ CD4 T cells in blood vs lymph nodes) (Figure 2B). Furthermore, the mean fraction of intact proviruses (intact/total HIV), as measured by IPDA in the sorted live CD3+CD4+/- fraction, was 10.5% in the blood and 8.5% in lymph nodes, with notable intra- and inter-individual variability (Figure 2C)”.

While it can be justified as to why the authors pooled cell numbers for figure 2E (due to understandable lack of cells from these sample sets), it would be informative to also show the cell counts per participant.

We appreciate the suggestion from the reviewer. The numbers of p24+ cells per participant are now included in a table on the side of the graphs (Figure 2E). We would also like to refer to Supplementary Figure 3D, where p24+ cells on the UMAPs are color-coded per participant, and to Supplementary Table 3, where the number of CD4 T cells analyzed per participant using the HIV-Flow assay is indicated.

For supplementary Figure 3B, it's a little confusing as to what the dark and light gray bars represent? is it untreated vs. tat LNP/PNB treated or is it p24+ vs p24-ve.

The light and dark grey colors were indeed comparing p24- vs p24+ cells on Supplementary Figure 3B. To avoid confusion with other bar plots from the same panel of figures that use the same color scheme to represent non-stimulated vs Tat-LNP/PNB treatment, we now only use dark grey to represent both p24- and p24+ cells, as the phenotype of those cells was analyzed following Tat-LNP/PNB treatment. The legend below the axis indicates that the comparison is done between p24- and p24+ cells.

For sequencing analysis of intact vs. defective HIV, please provide how many cells were used for the sequencing per participant. In Methods, line 468 says "8 μ L", and line 487-488 does not say HIV genomic DNA from how many cells are used for each PCR reaction.

We have now included a table (Supplementary table 3) indicating the total number of CD4 T cells analyzed per assay and per participant for blood and lymph nodes. Because of the presence of debris and residual epithelial cells in the gut CD45⁺-enriched fraction, cell counting was challenging and was not performed before making a dry pellet. Therefore, we can not determine how many gut cells were used in the IPDA and HIV-PULSE assays.

For Figure 4A, it is interesting that a majority of the intact sequences were seen in blood T0 and T1 samples compared to the gut and lymph nodes. Is there a reason as to why more intact sequences were seen in these groups?

Supplementary Figure 5 highlights that these shared blood T0/T1 intact sequences were only found in two individuals (MRC15, MRC25). Furthermore, because of the high availability of blood cells compared to lymph node and gut-derived cells, more sequences were retrieved from the blood compartment, increasing the likelihood to detect intact sequences that are shared between the two time points in the blood. We would like to point out that clones with intact sequences were also shared between blood T0 and lymph node T1, as well as blood T1 and gut T1.

The statements are contradictory on line 247-250: In the first half of the sentence, the authors say that "no significant differences were observed between blood and tissues". In the latter half of the sentence, the authors say that "the clonality estimate for each participant was systematically lower in the lymph nodes compared to the blood". Since there is no significant difference, the authors should avoid making definitive statements. This point extends to other conclusions in the paper, considering the statement in line 664: "No statistically significant differences were observed in any of the pairwise comparisons run in this study."

We acknowledge that the statements in this sentence may appear contradictory. Following discussions with our statistics department, we have determined that it would be more appropriate to treat the four datasets as paired rather than as independent, as was initially done. All statistics and p-values obtained for Figures 3C, 3E, and 4B are now presented in Supplementary Data 1. While paired analyses did not change conclusions for the analysis of the percentage of intact proviruses (Figure 3C) and average pairwise distance (Figure 3E), a significant difference is now observed between B_T1 and L_T1 for the clonality estimate's analysis ($p = 0.006$, parametric paired t-test; Figure 4B). We now adapted our statement based on this observation: "Clonality estimates were not significantly different between the two time points in the blood (medians = 46% for both time points, $p = 0.347$). Interestingly, the clonality estimate was consistently lower in the lymph nodes compared to the blood for each participant (medians = 24% for lymph nodes T1 and 46% for blood T1; $p = 0.006$).

Minor concerns:

- In line 52: "these molecules lack of specificity" should be "these molecules lack specificity".
This is now revised in the text.
- In line 58: "Few studies have attempted to compare the HIV-1 proviral landscape between blood and tissues (19-27)." should be "Many studies..." as there are 9 such studies cited in this sentence.
This is now revised in the text.
- Figure 2D has no percentage labels.
To avoid overcrowding the graphs, we did not add percentage labels. We would like to point out that all percentages for each participant included in these analyses can be found in Supplementary Figure 3A. This is now mentioned in the legend of the figure (line 796).
- Figure 3B: what does the percentage on the horizontal axis mean? Are they percentages of the number of distinct sequences or total sequences?
This is specified in the figure legend: "Proportions of different proviral classes observed among the distinct proviruses for each anatomical site".
- The meaning for the horizontal axis in Figure 4A is unclear and should be labeled. Do they represent each donor?
The axis of the small bar graph to the right shows the total number of distinct sequences per dataset. This is now specified in the legend of the figure (line 821).

Reviewer #4 (Remarks to the Author):

In this manuscript, Marion Pardons, Laurens Lambrechts, and colleagues investigated the composition and phenotypes of HIV-infected cells in blood and tissues. They capitalize on novel technologies developed by their group, including HIV-flow, HIV-Pulse, STIP-seq, and the use of Tat-LNP + Panobinostat to reactivate HIV gene expression. The manuscript is eloquently written, and the conclusions are well supported by the experimental data generated. The main finding is that p24 positive cells present a distinct phenotype between blood and lymph nodes. While TEM with a cytotoxic (GZMA) phenotype make up most of the p24 positive cells in blood, TCM CXCR5+/- cells represent most of p24 positive cells in lymph nodes. This work adds to the importance of dissecting reservoir features between blood and tissues.

Strengths: the Authors use cutting edge technologies, especially the use of a more selective inducer of HIV expression (Tat-LNP). The observed differences in infected cell phenotype reflect important T cell biology. I especially appreciated the “anomaly” of participant MRC21, where the predominant CXCR4 phenotype was associated with a higher proportion of p24 positive cells in “naïve-like” T cells. The Authors did an exhaustive job in validating their experimental setup, especially regarding the impact of the Tat-LNT+PNB stimulation on some markers (e.g. GZMA).

Weaknesses: I wish the authors provided direct evidence that the same HIV-infected clones have different phenotype based on their microenvironment. I understand the challenges of doing STIP-seq from lymph node cells, but it would have added strength to the study, allowing also to compare TCR sequences between compartments. Lastly, the observations regarding proviral features, clonality, lack of ongoing replication, and compartmentalization, all confirmed previous findings, representing hallmark features of HIV-infected cell population on ART.

We would like to thank the reviewer for his positive and thorough evaluation of our work. We understand the comment of the reviewer regarding the lack of direct evidence that same HIV-infected clones have different phenotype based on their microenvironment. While the STIP-seq assay would enable to track clones between compartments while assessing their phenotype, our ability to address the reviewer's question using STIP-Seq was constrained for several reasons: (i) the limited number of p24+ cells we obtained from each participant, especially in the lymph nodes; (ii) STIP-Seq requires methanol fixation, which interferes with the detection of certain epitopes. For example, GZMA expression is undetectable under methanol fixation, restricting our ability to measure it at the protein level. Although we were not able to address this question in this manuscript, we are currently performing single-cell RNA sequencing experiments on large numbers of CD4 T cells derived from blood and matched lymph nodes, with the intention to track clones of infected cells through their TCR. Finally, the exceptional number of HIV near full-length sequences obtained in this study (> 3000 sequences) through the high-throughput HIV-PULSE approach enabled us to validate previous findings with greater depth and precision. This is now mentioned on lines 389-391.

Major comment:

I encourage the Authors in providing better contextualization of their findings regarding the different phenotypes between blood and tissues, as they reflect key features of T cell biology. TEMs are the circulating counterpart of many TCM that mostly reside or are confined in secondary lymphoid organs. We thank the reviewer for this excellent suggestion. We are now offering improved contextualization of our findings within the framework of the various cellular subsets analyzed in our study. These modifications were implemented into the discussion and can be found on lines 290-330.

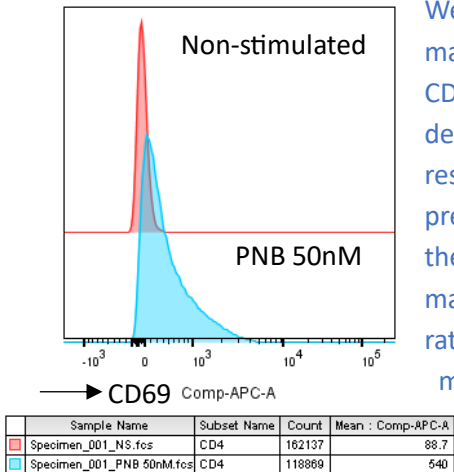
Along the same lines, how much of the lack of proviral variant overlap between blood and tissues is due to sampling versus the presence of HIV-infected tissue resident cells?

[REDACTED]

In our study, we identified 55 clones present in the gut, 37 of them being retrieved as well in the blood and 4 being shared with the lymph nodes. Whether the presence of TRM cells within tissues may be a factor responsible for the lack of proviral overlap between blood and tissue is an interesting point, although difficult to assess without sorting this specific subset. In an other cohort of individuals living with HIV, we assessed CD69 and CD103 expression ex vivo within gut-derived CD4 T cells and found that TRM cells (CD69+CD103+) accounted for 2 to 10% of the total CD4 T cell population. These findings are in agreement with published data from Yokoi et al (PMID: 36574662; Figure on the left), showing that proportions of CD4 TRM cells in control individuals are comparable to those obtained by our team. Therefore, we can not exclude that the existence of this subset of cells in tissues may account for a lack of proviral overlap between blood and tissues, although probably at a relatively low degree.

We have now discussed this on lines 371-377: “Interestingly, out of 55 infected clones detected in the gut, 37 were shared with blood. Several factors may explain why some sequences found in the gut were not detected in the blood: (i) the detection of four infected clones that were exclusively shared between gut and lymph nodes without being detected in the blood indicates that sampling limitations may have played a role; (ii) the direct infection of gut CD4 T cells with a resident memory phenotype, which comprise 2-10% of the total CD4 T cells in this anatomical site (PMID: 36574662), may further participate to the lack of proviral overlap between blood and tissues”.

Why did the Authors not use markers for tissue resident T cells (CD69, CD103) in their phenotyping panel? I understand that these markers do not capture all TRM, but they would have helped.



We understand the reviewer’s concern regarding the absence of markers of tissue resident T cells in our panel such as CD69 and CD103. While CD69 was planned to be included in the initial design, it appeared that its expression was upregulated in response to PNB 50nM stimulation (see Figure on the left). This precluded the possibility to include this marker in our panel, and therefore to have a look at tissue resident T cells. Although this may sound like a reason to stimulate CD4 T cells with Tat-LNP alone rather than with the combination Tat-LNP/PNB, we aimed for maximal latency reversal in this study. This is now clarified in the limitations on lines 408-409.

Lastly, could the Authors speculate on the potential role of CD4 antigen specificity on the phenotype of these p24+ T cells? I am referring to recent works from Collora et al (PMID: 35320704) and Weymar et al (PMID: 36070690).

We thank this reviewer for this great proposition. To answer that question, we performed an extensive review of the literature concerning the phenotype and migration properties of CMV- and HIV- specific T cells.

CMV-specific T cells typically exhibit an effector memory phenotype (CD45RO+CCR7-CD27-) with a cytolytic profile [Appay 2000; Buggert 2020; Collora 2022]. These cells circulate at high frequencies in the blood [Gordon 2017] but are largely absent from the gut [Shacklett 2003], consistent with the observation that cytolytic T cells are selectively retained in the intravascular circulation [Buggert 2020]. Aligning with this, p24+ cells with a cytotoxic TEM profile were primarily detected in the blood in our study and may be more likely CMV-specific. In contrast, HIV-specific T cells predominantly exhibit a transitional memory phenotype (CD45RO+CCR7-CD27+), characterized by functional exhaustion and reduced cytotoxic responses compared to CMV-specific T cells [Buggert 2020; Collora 2022]. They are found at higher levels in lymph nodes than in blood [Altfeld 2002; Buggert 2020] and are detected more frequently in the gut compared to CMV-specific cells [Shacklett 2003]. Based on these observations, we speculate that p24+ cells with lower cytotoxic potential and a less differentiated phenotype, found across multiple anatomical compartments in our study, are more likely to be HIV-specific."

We are now discussing the link between antigen specificity and phenotype/anatomical location of the p24+ cells on lines 334-340.

Minor comments:

- Line 163 "Circular Tfh cells" probably needs to be corrected.
- Line 256 "intermixed for all participants" I would clarify with "intermixed within each participant".
- Line 425 "stored on ice until freezing", probably needs to be corrected with "processing".
- Figure 1 and following. Spell out NS (no stim) as it could be confused with "not significant"
- Figure 3A. "Totals" at the bottom of the grey table --> Total

All minor comments mentioned above have been corrected. Of note, the gut micro-biopsies were indeed stored on ice until freezing in cryostor10, as described in the methods section. Figure 1 now shows that we are starting from frozen material and not fresh biopsies.

Reviewer #5 (Remarks to the Author):

Overall impressions:

This is a well conducted study and a well written manuscript which will add valuable knowledge to the field. The data support the conclusions and the methodology is sound. Some minor points of clarification are warranted.

We are pleased to read that the reviewer considers our work as well conducted and adding valuable knowledge to the field. We would like to thank the reviewer for his comments and are providing a point-by-point response below.

Noteworthy results:

1. Persistence of clonal populations of HIV infected cells across longitudinal timepoints.
2. Persistence of clonal populations of HIV infected cells across anatomical compartments.
3. Phenotypically distinct populations of translation-competent reservoir cells in different anatomical compartments.
4. Ongoing replication tissues under ART was not observed.

Significance to the field:

- This work contributes to the growing understanding of HIV persistence using cutting-edge methodology allowing collection of previously unmeasurable data.
- The conclusions of this study are supported by the work presented.

Soundness of the methodology:

Lines 479 – 484: The authors of HIV-PULSE (PMID: 37819007) acknowledge that enrichment of proviral templates by PCR may introduce bias and that sequence polymorphisms may cause discrepancies in amplification efficiency. The authors have introduced novel primers for the current study. A discussion of whether amplification bias could impact the interpretation of their results is warranted.

We would like to clarify that the primers used in this study are not novel and were originally reported by Pinzone et al. (PMID: 30760706). They have also been used in the original HIV-PULSE manuscript (PMID: 37819007). These primers were selected based on their established performance in proviral template amplification by Pinzone et al. The issue of amplification bias, along with the potential effects of sequence polymorphisms on amplification efficiency, have been thoroughly discussed in our previous publication on the HIV-PULSE assay (PMID: 37819007) and by others (PMID: 34578455, 36074794). We have now included an additional sentence in the limitations paragraph on lines 401-403: “Primer design constraints and amplification biases towards shorter fragments in long-range PCR amplification, as previously discussed by our team and others, may have hindered the detection of additional overlaps”.

Methodology overall is sound and up to the current standards of the field.

Detail in Methods:

Lines 466 – 478: Working primer concentrations should be included in addition to volumes for clarity and reproducibility in the IPDA section of the methods.

Primers and probes concentrations are now added to the methods section.

Additional comments:

Lines 355-358: This statement is vague - "...identified a few links...", "...certain clones of infected cells....". I believe the data support the statement being made but I think this conclusion could be stated more clearly.

We acknowledge that this statement is vague, and we have now modified it: "Out of the 319 infected clones detected, we observed 115 overlapping clones between the blood and tissues, including three clones with an intact provirus, suggesting that clones disseminate in the body through the blood".

Reviewer #1 (Remarks to the Author):

First of all, I would like to thank Pardons et al. for their clear answers to my questions.

In this revised version of the manuscript, the authors have repainted the color code applied in Figure 2F (stacked bar charts) to avoid any confusion or misreading.

I concur with other reviewers' major concerns with respect to a small sample size that may cause statistical difficulties. On the other hand, I am aware of the limited availability of clinical samples, followed by the challenge of isolating primary cells from blood, matched lymph nodes, and gut microbiopsies, allowing addressing the critical question about HIV-1 reservoirs in different anatomical compartments. Certainly, a follow-up study, perhaps with an appropriate sample size to reach statistical significance, will be required, i.e., single-cell RNA-seq on large numbers of CD4 T cells as authors' response to the comment from Reviewer #4.

In addition, the statistical strategy offered by the authors (see the authors' response to my comment #4 and comments from Reviewer #3) is convincing to deal with this small number of patients in the current stage.

After taking into account the significance of the findings in this work, together with technical advances (HIV-Flow, HIV-PULSE, STIP-Seq, and Tat-LNP/PNB) generated from the lab, and the limitations, **I am pleased to recommend this manuscript for publication.**

Reviewer #2 (Remarks to the Author):

The authors have addressed my comments related to the sequencing and have argued their way around my other concerns about the low number of participants/p24+ cells and the novelty of the findings. At the very least, these limitations should be made explicitly clear in the manuscript with all appropriate references cited.

All limitations addressed by reviewer #2 are made explicitly clear in the manuscript. One citation was still lacking and is now referenced in the discussion section of the manuscript: "While the phenotype of p24+ cells in matched blood and lymph nodes has been studied in the context of viremic infection (Gantner et al, Immunity 2023), assessing it in ART-suppressed individuals has been challenging due to the lack of potent and specific LRAs to boost viral production in latently infected cells".

Reviewer #3 (Remarks to the Author):

In the revised manuscript, the authors addressed all of our concerns. Although no new data/samples were added, the revised text represents more appropriate conclusions. Moreover, the new statistical analyses now align well with the size of the sample and the new labelings on the figures make them clearer and easier to read. **We have no further comments.**

Reviewer #4 (Remarks to the Author):

The Authors have addressed all the concerns raised by the reviewers. **I have no further comments or requests.**