

Persistent pneumococcal colonisation in antiretroviral-treated HIV infection is associated with nasal inflammation

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This manuscript, "HIV-associated epithelial-immune cell dysfunction in the nasal mucosa promotes pneumococcal carriage despite ART," investigates nasal mucosal immunity in people living with HIV (PLHIV) who are stable on long-term antiretroviral therapy (ART), with a particular focus on their susceptibility to pneumococcal colonization. Using a combination of flow cytometry, cytokine profiling, and single-cell RNA sequencing, the study characterizes epithelial and neutrophil phenotypes, epithelial-neutrophil interactions, and transcriptional signatures in the nasal mucosa. The authors report that PLHIV exhibit impaired neutrophil function, signs of epithelial barrier disruption, and increased markers of immune exhaustion, despite viral suppression. The study offers novel insights into tissue-specific immunity in HIV and its potential implications for infection risk and vaccine responsiveness.

The study is described as cross-sectional; however, some data appear to have been collected at different time points (e.g., over several years or ART durations). It would be helpful if the authors clarified whether these samples were collected from the same individuals longitudinally or from different individuals at each time point. This distinction is important for interpreting any temporal or ART-duration-related comparisons and understanding whether observed differences reflect within-individual changes or inter-individual variability.

Please can the authors provide why these participants were attending the clinics? Were they routine visits, did they have any other underlying illnesses, or were they attending for medical care?

The use of nasal curettes is appropriate for obtaining high-quality mucosal samples for transcriptomic and cellular immune profiling. However, this method can be more invasive and prone to inter-operator variability compared to flocked swabs. It would be helpful if the authors provided additional detail on standardization procedures for sample collection and whether any participants experienced discomfort or bleeding. Furthermore, given the possibility of blood contamination with curettage, the authors should clarify whether any quality control steps were taken to assess or mitigate this, especially in RNAseq or cytokine analyses.

Although the sample size (n=57 PLHIV, n=51 controls) appears modestly adequate, subgroup comparisons (e.g., between carriers and non-carriers within PLHIV) become underpowered. Some findings may be due to chance, especially in the context of functional assays with smaller n.

In lines 96-98, the authors say, "Neutrophil abundance was significantly higher in PLHIV-ART>1yr compared to HIV- adults, but only marginally higher compared to PLHIV-ART<3m (Fig. 1c)." However, this was not significant. The authors need to rephrase this sentence to reflect this.

Throughout the results section, the authors need to provide the p-values where comparisons are made.

The cohort of PLHIV, despite long-term ART and viral suppression, may still represent individuals with varying degrees of immune reconstitution. The manuscript does not adequately address potential confounding due to differences in CD4 count, ART duration, co-infections, or mucosal inflammation unrelated to HIV.

The methodology used for detecting pneumococcal carriage is not described in sufficient detail. The criteria for defining "carriage" are not well established in the text.

In the results section, in the text, the authors make mention of CD66b+ neutrophils, CD3+ T cells and CD4+ monocytes; however, the figure has it labelled as neutrophils, T cells and monocytes. The figure needs to be changed to match the description in the text.

In Table 1, there is an asterisk on socioeconomic status; however, there is no corresponding footnote below the table describing what the asterisk means.

Line 145 – What were the viruses tested? How were they tested? This needs to be included in the methods section.

References: Reference 17 is duplicated and again referenced as reference 42.

This is a well-conceived and important study that addresses persistent gaps in our understanding of mucosal immunity in PLHIV. However, revisions are required. With these changes, the manuscript will make a valuable contribution to the field.

Reviewer #2

(Remarks to the Author)

As people living with HIV (PLHIV) remain at an elevated risk of *Streptococcus pneumoniae*, even when on long-term antiretroviral therapy (ART), the authors investigated the upper respiratory mucosal immune landscape in HIV-uninfected adults and PLHIV at different stages of ART.

Using multiparameter flow cytometry, single-cell transcriptomics, and neutrophil functional assays they evaluate immune cell composition, epithelial-immune communication, and their relationship with pneumococcal carriage.

Flow cytometry revealed that long-term ART does not restore nasal mucosal immunity with increased neutrophils in ART+ HIV+ donors and decreased CD4 T cells. As one might expect, activated neutrophil subsets were enriched in nasal mucosa compared to blood especially in ART+ HIV+ donors correlating with myeloperoxidase (MPO) levels but not proinflammatory cytokines. However neutrophil chemo-attractants were only positively associated with nasal neutrophil abundance in HIV- donors suggesting dysregulation of neutrophil recruitment pathways.

ScRNA-seq was then carried out on nasal immune cells neutrophils derived from HIV- and HIV+ART+ donors. Multinichenet was first used to infer to-cell communication and revealed that epithelial cell - neutrophil interactions were significantly expanded in HIV+ donors showed strong ligand-receptor signalling pathways associated with neutrophil recruitment from basal, secretory, goblet, squamous, and ciliated epithelial subsets. Airway epithelial cells were therefore concluded to contribute to neutrophil recruitment and activation. Compared to HIV- donors, neutrophils from HIV+ donors who had been on ART for more than year showed elevation of mitochondrial stress-associated genes, whereas donors who had been on ART for less than 3 months showed elevated levels of antiviral response genes. CEMiTool was then used to identify co-expressed gene modules enriched in neutrophils across the study groups indicating enrichment in homeostatic regulation pathways as well as those associated with cytokine signalling, interferon response, antigen presentation, and immunoregulatory.

Functional competence of blood neutrophils was then assessed by measuring phagocytic uptake and oxidative burst assays. No difference in phagocytic uptake was recorded but oxidative burst capacity was significantly lower in both HIV+ groups suggesting potential impairment in killing capacity.

Similar to neutrophils, CD4 T cells derived from both HIV+ donor groups also showed elevated levels of genes associated with immune activation, chemokines and age associated genes.

Finally, the authors showed that in HIV+ART+>1yr donors (but not PLHIV-ART<3m donors), neutrophil abundance was significantly higher in individuals with pneumococcal carriage compared to those without with a similar pattern observed for CD14+ monocytes.

Strengths

A very clear strength of the study is the recruitment of 132 adults from Malawi including HIV+ patients who have only recently started ART (within 3 months), those who have been taking ART for over a year and HIV- controls. Nasal mucosal cells, nasal lining fluid, and peripheral blood were collected from all participants which allowed this study to be possible. Thus, this study is as physiologically relevant as is possible in a human setting.

- cells for nasal immunity and notably were detected in the single cell analysis of nasal cells (Fig 3).
- If single-cell transcriptomic profiling was done, what specific helper CD4 T cells were reduced?
- The authors have been open about acknowledging the several limitations such as the limited sample for single-cell analyses, functional studies being restricted to blood-derived neutrophils and lack of assessment of microbial community composition, which may shape mucosal immunity and carriage risk.

Weaknesses

1. It odd that myeloid antigen presenting cells were not investigated as they showed up in flow cytometry analysis (Figure 1) as well as scRNA-seq analysis which would account for an enrichment of genes encoding protein associated with cytokine signalling, interferon response, antigen presentation, and immunoregulatory. Why were markers for myeloid antigen presenting cells not included such as CD1c, CD11c, XCR1 for dendritic cells and CD68, CD163, FXIIIa for macrophages. APCs are an important mucosal immune constituent.

2. Figure 1: The authors state that flow cytometry and single-cell transcriptomic profiling was carried out. What single-cell transcriptomic profiling platform was used and where is the data? Figure 1 extended Figure 1 only show flow cytometry data.

3. Neutrophil functional assay was conducted with blood neutrophils rather nasal. However, this assay would likely be impossible to conduct using the small numbers that can be derived from the nasal mucosa. As the authors state in the discussion, neutrophils are generally short-lived and nasal neutrophils almost certainly traffic from peripheral blood, so the use of blood derived neutrophils is justified. Direct assessment of mucosal neutrophil function does remain an important future direction. Please could the authors speculate how this might be achieved.

4. The study is over reliant on gene expression data. Some functional data is carried out on neutrophils but nothing on T cells. The T cell part feels a little bit 'stuck on the end'. I wonder if this should be removed and make the manuscript solely about neutrophils given these cells where some functional assays have been carried out? Or would it be possible to provide

more information about the specific Th subsets between study groups and/or memory status.

Reviewer #3

(Remarks to the Author)

This interesting paper assesses the relationship between nasal mucosal neutrophil function and immune composition to pneumococcal colonisation in people with HIV (PWH) treated with either 3 months or >1 year of ART. The major strengths are the novelty of the hypothesis, the new insights generated into an important issue of streptococcal infection and the use of multiple new technologies to gain insights into mucosal immunity in the nose. The weaknesses are that this is a cross sectional study and therefore only describes associations, the group with PWH on ART for <3 months are a heterogeneous group and therefore may not be easy to interpret, there may be other confounders to the findings such as CD4 count, duration on ART and the presence of viremia. the cohort is overall very young and therefore relevance to other age groups is unclear.

Major concerns

1. Study design – the study focuses on three groups – HIV uninfected, PWH on ART<3 months (24% virus detected) and PWH on ART >1yr (10% virus detected). I am concerned that the ART<3 months includes a heterogeneous group, specifically those that are and aren't viremic and who may and may not be undergoing inflammatory immune reconstitution. Ideally a group preART would have been far better to include. The authors should highlight this weakness in the discussion
2. I struggled to understand why mucosal immunity would be more dysfunctional in people on ART>1 year compared to <3 months. This intuitively didn't make a lot of sense. One explanation is that the PWH on ART<3 months was a heterogeneous group. On line 336, the authors propose that they observed evidence of 'T-cell exhaustion, senescence and apoptosis.....on long term ART'. However, this type of dysfunction is usually much worse in PWH without ART. The unsuspected findings that PWH on ART>1 year had more dysfunction and more colonisation needs to be more clearly discussed
3. The authors should analyse the impact of viremia or no viremia on the parameters measured. This might explain why the ART<3 month group often are closer to HIV uninfected than the ART>1 year group. This relationship was difficult to understand eg figure 1c and 2b as two examples of immune markers where HIV on ART<3 months looked closer to HIV uninfected participants
4. Certain clinical parameters such as nadir and current CD4 and duration on ART may impact the findings,
 - a. CD4 count may play a very important role in nasal immunity – including both current and nadir CD4. The authors should assess the impact of current and nadir CD4 as a confounder for the findings of changes in nasal immunity
 - b. The duration on ART is also quite variable, ranging from 37 to 111 months. The authors should assess whether duration on ART influences any of the key parameters of immune dysfunction
5. The authors largely describe correlations and therefore are unable to infer causation. Have the authors treated any participants with antibiotics to clear colonisation? Have any participants been immunised against pneumococcus and if yes, can the authors compare vaccinated and unvaccinated groups? The addition of an intervention such as antibiotics or vaccination would greatly strengthen the findings

Minor concerns

6. The authors quote that there is a 30-100 fold increase in invasive pneumococcal disease in PWH on ART compared to HIV uninfected people, can they also include some background information on the relationship between colonisation and invasive disease
7. The authors examine a subset of participants with scRNAseq. This is reasonable given the cost of scRNAseq. Why were 3 participants chosen per group? What was the statistical rationale for this? Also, how were the 3 participants selected. These details should be included to ensure there wasn't bias in the selection of the subset which could have impacted the findings
8. The figures show comparison between all three groups, both statistically significant and non-significant comparisons. I think it would be better to either write ns for the non-significant comparisons, or not show the p value at all. By including all the p values, significant and non-significant, it's hard to digest the truly significantly different findings
9. Figure 1 g-l show no significant differences between the three groups. Perhaps these panels could move to extended data?
10. Figure 4a – are these analyses adjusted for total cell number? Could one explanation of the low level of connections for HIV negative donors is that there were fewer cells analysed?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have sufficiently addressed my comments and revised the manuscript.
Recommend for publication.

Reviewer #2

(Remarks to the Author)

The authors have addressed my concerns. I congratulate them on this nice study.

Reviewer #3

(Remarks to the Author)

The authors have addressed all my concerns in a satisfactory manner.

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Thank you for allowing us to resubmit the manuscript. We thank the reviewers for their constructive comments and queries. [We have addressed all the queries, point-by-point, below.](#)

Reviewer 1

Query 1: *Please can the authors provide why these participants were attending the clinics? Were they routine visits, did they have any other underlying illnesses, or were they attending for medical care?*

[All participants in this study were recruited either from voluntary testing and counselling services at the Gateway Voluntary Counselling and Testing Centre or from routine ART clinics at the Lighthouse ART Clinic in Blantyre, Malawi. HIV-uninfected participants were enrolled from the surrounding community in Blantyre. Importantly, all individuals were otherwise “healthy” and asymptomatic, with no evidence of infection or chronic illness, as confirmed by standard clinical assessments. We have clarified this in the Cohort characteristics and sampling section \(Page 4, lines 76–81\).](#)

Query 2: *The use of nasal currettes is appropriate for obtaining high-quality mucosal samples for transcriptomic and cellular immune profiling. However, this method can be more invasive and prone to inter-operator variability compared to flocked swabs. It would be helpful if the authors provided additional detail on standardization procedures for sample collection and whether any participants experienced discomfort or bleeding.*

Response: [While we acknowledge the potential for blood contamination and inter-operator variability during sample collection, all procedures were performed by well-trained staff with extensive experience in collecting nasal cells using a nasal curette for research purposes. Participants did not report significant discomfort during sampling. In the rare cases where blood contamination was observed \(<1% of all samples\), the affected samples were excluded from analysis. We have clarified this in the Nasal sample collection section \(Page 14, lines 418–419\).](#)

Query 3: *Furthermore, given the possibility of blood contamination with curettage, the authors should clarify whether any quality control steps were taken to assess or mitigate this, especially in RNAseq or cytokine analyses.*

Response: As noted above (Query 2 response), we applied several quality control measures to ensure that only high-quality nasal samples were included in the single-cell and nasal serological analyses. All samples with visible blood or mucus were excluded from processing or analysis. For single-cell RNA sequencing, we used the Seurat analysis pipeline to exclude cells with low complexity ($nFeature_RNA < 100$) and those containing reads mapped to haemoglobin genes. Importantly, no reads mapped to haemoglobin were detected in our dataset, indicating an absence of blood contamination. These quality control metrics have been provided in the analysis R scripts (seurat_pipeline.R) available on our GitHub account. For nasal lining fluid assays, samples with clear blood contamination, though rare, were also excluded.

Query 4: *Although the sample size ($n=57$ PLHIV, $n=51$ controls) appears modestly adequate, subgroup comparisons (e.g., between carriers and non-carriers within PLHIV) become underpowered. Some findings may be due to chance, especially in the context of functional assays with smaller n .*

Response: Even though the sample size for the subgroup analysis was relatively small, the results of the functional assays are consistent with the overall findings of the manuscript, making it unlikely that they occurred by chance. Nonetheless, this limitation has been acknowledged in the discussion section (Page 13, lines 374–376).

Query 5: *In lines 96-98, the authors say, “Neutrophil abundance was significantly higher in PLHIV-ART>1yr compared to HIV– adults, but only marginally higher compared to PLHIV-ART<3m (Fig. 1c).” However, this was not significant. The authors need to rephrase this sentence to reflect this.*

Response: We have rephrased the sentence on Page 4, line 101, for clarity. The revised sentence now reads:

“Neutrophil abundance was significantly higher in PLHIV-ART>1yr compared to HIV– adults, but only marginally higher compared to PLHIV-ART<3m, however, this was not statistically significant.”

Query 6: *Throughout the results section, the authors need to provide the p-values where comparisons are made.*

Response: We have adhered to the Nature Communications reporting guidelines, which do not require inclusion of in-text p-values within the manuscript.

Query 7: *The cohort of PLHIV, despite long-term ART and viral suppression, may still represent individuals with varying degrees of immune reconstitution. The manuscript does not adequately address potential confounding due to differences in CD4 count, ART duration, co-infections, or mucosal inflammation unrelated to HIV.*

Response: While CD4 count is primarily associated with impaired systemic immunity, our analysis showed no relationship between CD4 count or ART duration and markers of nasal inflammation (including neutrophil and monocyte abundance). This indicates that, in this study, CD4 count and ART duration were not associated with nasal inflammation. To minimise potential confounding, participants were carefully selected: individuals with acute infections, chronic diseases (including cancer, diabetes, hypertension, tuberculosis, and COPD), or recent antibiotic use were excluded, except for co-trimoxazole prophylaxis in PLHIV.

Query 8: *The methodology used for detecting pneumococcal carriage is not described in sufficient detail. The criteria for defining “carriage” are not well established in the text.*

Response: We used standard WHO-recommended protocols for pneumococcal carriage detection. To provide further clarity, we have added additional methodological details, including a citation of our previously published work on pneumococcal detection and carriage density calculations (**Page 16, lines 464–471**).

Query 9: *In the results section, in the text, the authors make mention of CD66b+ neutrophils, CD3+ T cells and CD4+ monocytes; however, the figure has it labelled as neutrophils, T cells and monocytes. The figure needs to be changed to match the description in the text.*

Response: We have corrected the labelling in Figure 1b (**Page 22**) to accurately reflect that neutrophils were identified as CD66b⁺, T cells as CD3⁺, and monocytes as CD14⁺.

Query 10: *In Table 1, there is an asterisk on socioeconomic status; however, there is no corresponding footnote below the table describing what the asterisk means.*

Line 145 – What were the viruses tested? How were they tested? This needs to be included in the methods section.

Response: We have corrected Table 1 by adding an asterisk in the legend to highlight socioeconomic status and by removing the asterisk that was previously included for HIV viral load. In addition, we have added a section in the Methods (RT-qPCR for identification of respiratory viruses) describing how respiratory viruses were tested (**Page 16-17, lines 481–493**).

Query 11: *References: Reference 17 is duplicated and again referenced as reference 42.*

Response: This has been corrected.

Reviewer 2

Query 1: *If single-cell transcriptomic profiling was done, what specific helper CD4 T cells were reduced?*

Response: In Figure 3d (**Page 25**), we show that CD3⁺ T cells in the nasal curette are predominantly CD8⁺ T cells and natural killer T cells. This finding is consistent with our flow cytometry data (**Figure 1e, Page 22**), which demonstrate that CD4⁺ T cells are present at low frequencies in nasal currettes, likely due to the sampling method, and is in agreement with previous publications using nasal curette samples. Single-cell RNA sequencing did not further resolve this minor CD4⁺ T-cell subset, as the majority of CD3⁺ T cells were CD8⁺ and natural killer T cells. However, our unpublished flow cytometry data from a larger sample size of nasal currettes (to be reported in a separate manuscript) indicate that CCR6⁺ and CXCR3⁺ CD4⁺ T cells comprise the majority of this minor CD4⁺ T-cell population.

Query 2: *Its odd that myeloid antigen presenting cells were not investigated as they showed up in flow cytometry analysis (Figure 1) as well as scRNA-seq analysis which would account for an enrichment of genes encoding protein associated with cytokine signalling, interferon response, antigen presentation, and immunoregulatory. Why were markers for myeloid antigen presenting cells not included such as CD1c, CD11c, XCR1 for dendritic cells and CD68, CD163, FXIIIa for macrophages. APCs are an important mucosal immune constituent.*

Response: The identification of myeloid antigen-presenting cells during cell annotation was based on the expression of canonical markers shown in Figure 3c (Page 25) and validated by previous single-cell studies of nasal cells (doi.org/10.1016/j.cell.2021.07.023; Nature Medicine, 2024). In our nasal single-cell dataset, CD1c was not constitutively expressed in dendritic cells, although CD11c and XCR1 expression coincided with dendritic cell subsets identified in the data. Macrophages were also clearly defined by the expression of CD68, CD163, and F13A1 (FXIIA). However, we did not focus our analysis on antigen-presenting myeloid cells because the larger flow cytometry dataset (**Figure 1d, Page 22**) showed no significant group differences. This does not diminish their importance as a key mucosal immune constituent.

To clarify, our CEMiTool and differential expression single-cell analyses were performed in a cell cluster-specific manner, focusing either on neutrophils or T cells. Accordingly, the enrichment of genes encoding proteins linked to cytokine signalling, interferon responses, antigen presentation, and immunoregulatory functions reflects neutrophil- or T cell-specific biology as resolved by single-cell RNA sequencing.

Query 3: *Figure 1: The authors state that flow cytometry and single-cell transcriptomic profiling was carried out. What single-cell transcriptomic profiling platform was used and where is the data? Figure 1 extended Figure 1 only show flow cytometry data.*

Response: We used the 10x Genomics Chromium Controller platform for single-cell gel bead-in-emulsion (GEM) preparation, together with the 10x 5' v2 library preparation protocol. This has been indicated in the Methods section, and we have amended the Results section sentence under "HIV alters nasal immune cell composition despite long-term ART" (**Page 4, line 96**) for clarity. In addition, we confirm that Figures 3, 4, 5, and 6 present single-cell transcriptomic data.

Query 4: *Neutrophil functional assay was conducted with blood neutrophils rather nasal. However, this assay would likely be impossible to conduct using the small numbers that can be derived from the nasal mucosa. As the authors state in the discussion, neutrophils are generally short-lived and nasal neutrophils almost certainly traffic from peripheral blood, so the use of blood derived neutrophils is justified. Direct assessment of mucosal neutrophil function does remain an important future direction. Please could the authors speculate how this might be achieved.*

Response: Our laboratory is currently developing approaches to improve cell recovery and yield by employing a combined curette-swab collection method. This strategy is intended to facilitate downstream applications, including neutrophil functional assays.

Query 5: *The study is over reliant on gene expression data. Some functional data is carried out on neutrophils but nothing on T cells. The T cell part feels a little bit 'stuck on the end'. I wonder if this should be removed and make the manuscript solely about neutrophils given these cells where some functional assays have been carried out? Or would it be possible to provide more information about the specific Th subsets between study groups and/or memory status.*

Response: While the study is primarily focused on neutrophils due to their indispensable role in pneumococcal carriage, the T-cell analysis is integral to the overall narrative. We used the T-cell transcriptomic data to demonstrate that the senescence-associated

secretory phenotype (SASP) observed in neutrophils is linked to broader effects within the nasal environment of PLHIV on long-term ART. Consistent with this, our T-cell data reveal stress and apoptotic responses in T cells from PLHIV on long-term ART. With respect to the specific Th subsets, we kindly refer to our response to Query 1.

Reviewer 3

Query 1: *Study design – the study focuses on three groups – HIV uninfected, PWH on ART<3 months (24% virus detected) and PWH on ART >1yr (10% virus detected). I am concerned that the ART<3 months includes a heterogenous group, specifically those that are and aren't viremic and who may and may not be undergoing inflammatory immune reconstitution. Ideally a group preART would have been far better to include. The authors should highlight this weakness in the discussion.*

I struggled to understand why mucosal immunity would be more dysfunctional in people on ART>1year compared to <3 months. This intuitively didn't make a lot of sense. One explanation is that the PWH on ART<3 months was a heterogenous group. On line 336, the authors propose that they observed evidence of 'T-cell exhaustion, senescence and apoptosis.....on long term ART'. However, this type of dysfunction is usually much worse in PWH without ART. The unexpected findings that PWH on ART>1 year had more dysfunction, and more colonisation needs to be more clearly discussed

Response:

The primary aim of this study was to immunologically investigate why PLHIV on ART >1 year exhibit persistently high pneumococcal colonisation. Elevated carriage prevalence in PLHIV-ART>1yr has been consistently reported over the past 14 years (<https://doi.org/10.1097/qad.0000000000000755>, <https://doi.org/10.1371/journal.pone.0100640>, <https://doi.org/10.1093/cid/cis842>, <https://doi.org/10.1038/s41467-020-15786-9>), including studies directly comparing HIV-uninfected individuals, PLHIV pre-ART, and PLHIV on ART of varying durations. We also independently confirmed this in our published BMC Medicine case-control study, showing that PLHIV-ART>1yr have higher pneumococcal carriage compared to both HIV-uninfected and PLHIV-ART<3m (<https://doi.org/10.1186/s12916-024-03631-5>). This manuscript, therefore, addresses the unexpected and important finding of sustained high carriage in PLHIV-ART>1yr.

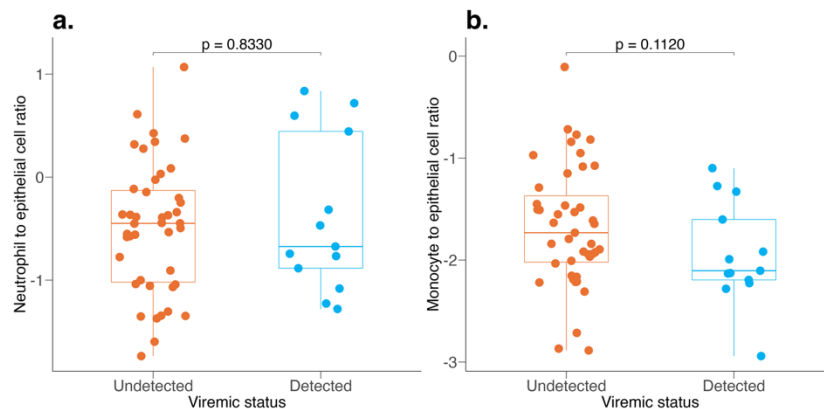
We are not suggesting that PLHIV-ART<3m lack nasal immune dysfunction. Indeed, we show that PLHIV-ART<3m exhibit lower nasal CD4⁺ T-cell frequencies, higher expression of T-cell exhaustion and senescence genes, and greater T-cell activation compared to PLHIV-ART>1yr. However, this T-cell dysfunction persists in PLHIV-ART>1yr when compared with HIV-uninfected individuals.

Finally, we have corrected the figure placement error in the submitted version. The appropriate figures (now **Figure 6g and 6h, Page 29**) have been inserted to match the corresponding results narrative.

Query 2: *The authors should analyse the impact of viremia or no viremia on the parameters measured. This might explain why the ART<3 month group often are closer to HIV uninfected than the ART>1 year group. This relationship was difficult to understand e.g. figure 1c and*

2b as two examples of immune markers where HIV on ART<3 months looked closer to HIV uninfected participants

Response: Our analysis of the impact of viraemia on markers of inflammation, including neutrophil and monocyte abundance, showed no significant differences. Refer to the figure below.

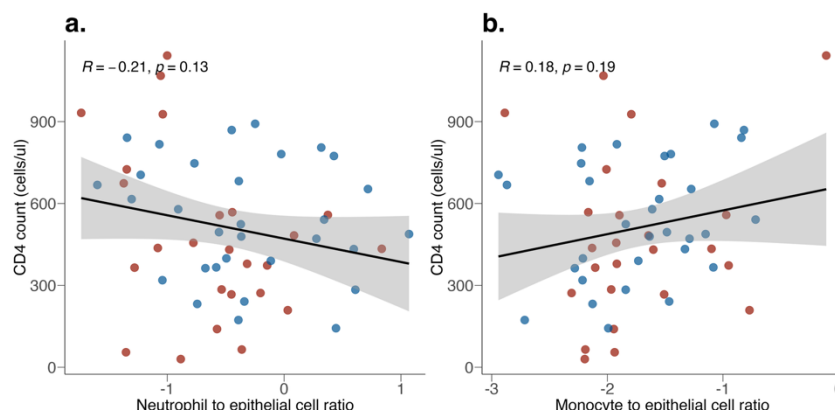


Consistent with published literature on the impact of HIV on T cells, our data show that T-cell-associated dysfunction is most pronounced in PLHIV-ART<3m, while profiles in PLHIV-ART>1yr are more comparable to those of HIV-uninfected individuals (refer to **Query 1 above**). In contrast, it is within the PLHIV-ART>1yr group that neutrophil dysregulation is most evident and is associated with pneumococcal carriage.

Query 3: Certain clinical parameters such as nadir and current CD4 and duration on ART may impact the findings,

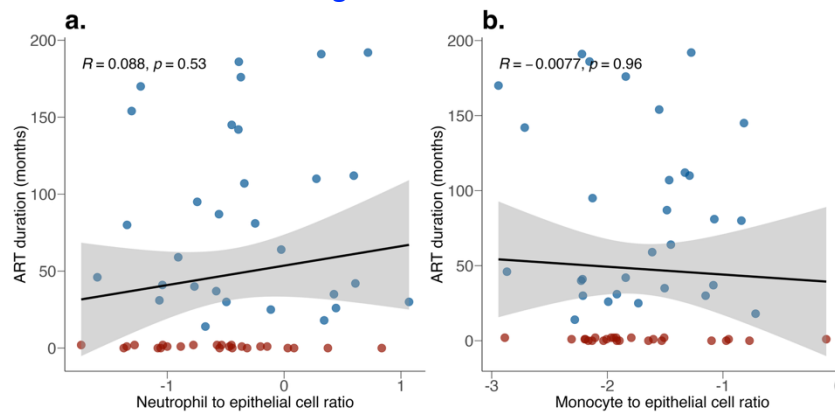
a. CD4 count may play a very important role in nasal immunity – including both current and nadir CD4. The authors should assess the impact of current and nadir CD4 as a confounder for the findings of changes in nasal immunity

Response: While we did not collect nadir CD4 count data for the participants in this study, our analysis of current CD4 counts showed no significant associations with markers of nasal inflammation, including neutrophil and monocyte abundance. Refer to the figure below.



b. The duration on ART is also quite variable, ranging from 37 to 111 months. The authors should assess whether duration on ART influences any of the key parameters of immune dysfunction

Response: Our analysis of ART duration showed no significant associations with markers of nasal inflammation, including neutrophil and monocyte abundance. However, it is important to note that our study only assessed participants within two timeframes: 0–3 months on ART and >1 year on ART, leaving a gap between 3 and 12 months that was not evaluated. Refer to the figure below.



Query 4: The authors largely describe correlations and therefore are unable to infer causation. Have the authors treated any participants with antibiotics to clear colonisation? Have any participants been immunised against pneumococcus and if yes, can the authors compare vaccinated and unvaccinated groups? The addition of an intervention such as antibiotics or vaccination would greatly strengthen the findings

Response: All participants included in this study had not taken antibiotics for at least 2 weeks prior to enrolment, and we did not give any antibiotics as part of this study. However, participants in the PLHIV groups were receiving daily co-trimoxazole prophylaxis as part of the routine programmatic rollout. All study participants were adults and unvaccinated, as pneumococcal vaccination in Malawi is currently restricted to children under 5 years of age, following a 3+0 schedule (doses at 6, 10, and 14 weeks).

Query 5: The authors quote that there is a 30-100-fold increase in invasive pneumococcal disease in PWH on ART compared to HIV uninfected people, can they also include some background information on the relationship between colonisation and invasive disease.

Response: We have added the sentence “Pneumococcal carriage is a prerequisite for pneumococcal disease” on **Page 3, lines 46–47**.

Query 6: The authors examine a subset of participants with scRNAseq. This is reasonable given the cost of scRNAseq. Why were 3 participants chosen per group? What was the statistical rationale for this? Also, how were the 3 participants selected. These details should be included to ensure there wasn't bias in the selection of the subset which could have impacted the findings

Response: We have clarified the participant selection criteria for single-cell RNA sequencing in the Methods section. Participants were randomly selected using the same recruitment criteria and processes as the broader cohort. Only samples with an initial cell count >200,000, without visible mucus or blood contamination, were included and processed for single-cell library preparation (**Page 17, lines 505–508**). While we did not perform a formal sample size calculation for single-cell RNA sequencing, our approach was guided by

published studies indicating that a minimum of three biological replicates is sufficient to derive mechanistic insights, rather than for epidemiological generalisability.

Query 7: *The figures show comparison between all three groups, both statistically significant and non-significant comparisons. I think it would be better to either write ns for the non-significant comparisons or not show the p value at all. By including all the p values, significant and non-significant, its hard to digest the truly significantly different findings.*

Response: We have included all p-values in the figures, in line with Nature Communications reporting guidelines, to ensure transparency and avoid selective reporting of only significant findings.

Query 8: *Figure 1 g-i show no significant differences between the three groups. Perhaps these panels could move to extended data?*

Response: Although there were no significant differences among the groups in Figures 1g-i, we retained these panels in the main figure to provide context and to highlight that the observed differences were primarily in neutrophils, which are an important cell type for immunity against *Streptococcus pneumoniae*.

Query 9: *Figure 4a – are these analyses adjusted for total cell number? Could one explanation of the low level of connections for HIV negative donors is that there were fewer cells analysed?*

Response: We adjusted for both the total number of cells and the total number of samples per group. This information is detailed in the Multinichenet analysis R script, which is available on our GitHub account.