B cell, CD8⁺ T cell and gamma delta T cell lymphocytic alveolitis alters alveolar immune cell homeostasis in HIV-infected Malawian adults [version 1; referees: 2 approved with reservations]

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Abstract

Background: HIV infection is associated with increased risk to lower respiratory tract infections (LRTI). However, the impact of HIV infection on immune cell populations in the lung is not well defined. We sought to comprehensively characterise the impact of HIV infection on immune cell populations in the lung.

Methods: Twenty HIV-uninfected controls and 17 HIV-1 infected ART-naïve adults were recruited from Queen Elizabeth Central Hospital, Malawi. Immunophenotyping of lymphocyte and myeloid cell populations was done on bronchoalveolar lavage fluid and peripheral blood cells.

Results: We found that the numbers of CD8⁺ T cells, B cells and gamma delta T cells were higher in BAL fluid of HIV-infected adults compared to HIV-uninfected controls (all p<0.05). In contrast, there was no difference in the numbers of alveolar CD4⁺ T cells in HIV-infected adults compared to HIV-uninfected controls (p=0.7065). Intermediate monocytes were the predominant monocyte subset in BAL fluid (HIV-, 63%; HIV+ 81%), while the numbers of classical monocytes was lower in HIV-infected individuals compared to HIV-uninfected adults (p=0.0006). The proportions of alveolar macrophages and myeloid dendritic cells was lower in HIV-infected adults compared to HIV-uninfected controls (all p<0.05).

Conclusions: Chronic HIV infection is associated with broad alteration of immune cell populations in the lung, but does not lead to massive depletion of alveolar CD4⁺ T cells. Disruption of alveolar immune cell homeostasis likely explains in part the susceptibility for LRTIs in HIV-infected adults.
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Competing interests: No competing interests were disclosed.

How to cite this article: Mwale A, Hummel A, Mvaya L et al. B cell, CD8 + T cell and gamma delta T cell lymphocytic alveolitis alters alveolar immune cell homeostasis in HIV-infected Malawian adults [version 1; referees: 2 approved with reservations] Wellcome Open Research 2017, 2:105 (doi: 10.12688/wellcomeopenres.12869.1)

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Grant information: This work was supported by the Wellcome Trust [105831]; and the Bill and Melinda Gates Foundation [OPP1125279]. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Introduction

HIV-infected individuals have increased susceptibility to lower respiratory tract infections (LRTIs)\(^1\),\(^2\), which account for 75–98% of lung complications in antiretroviral therapy (ART)-naïve HIV-infected adults worldwide\(^3\). Predisposition to LRTIs is largely attributed to HIV-induced impairment of lung immunity, including reduced frequency of respiratory antigen-specific alveolar CD4\(^+\) T cells\(^4\),\(^5\) as well as impaired alveolar macrophage function\(^6\),\(^7\). HIV infection is also associated with CD8\(^+\) T cell alveolitis, a condition characterized by the influx of HIV-specific CD8\(^+\) T cells into the lung\(^8\),\(^9\). While these immune cell perturbations partly underlie propensity for LRTIs in HIV-infected individuals, the impact of HIV infection on the composition and functions of other immune cell populations in the lung is not well defined.

Several studies have reported alterations in the proportions and functions of different immune cell populations in peripheral blood in HIV-infected individuals\(^10\),\(^11\),\(^12\). While peripheral blood CD4\(^+\) T cell depletion and an increase in CD8\(^+\) T cells are hallmarks of progressive untreated chronic HIV infection\(^13\), depletion of B cells\(^14\),\(^15\) and aberrant NK cell function and redistribution from CD56\(^+\) towards CD56\(^-\) subsets has been observed during early and chronic HIV infection\(^16\). Two major human \(\gamma\delta\) T cells subsets (designated V\(\delta 1\) or V\(\delta 2\)) are also altered in HIV-infected individuals, with an increase in the V\(\delta 1\) subset and a decrease in the V\(\delta 2\) subset\(^17\). Furthermore, increased proportions of non-classical and intermediate monocytes and depleted myeloid and plasmacytid dendritic cell subsets have been reported in individuals with high plasma HIV viral load\(^18\),\(^19\),\(^20\).

We, therefore, undertook a comprehensive characterisation of the impact of HIV infection on immune cell populations in the lung. We obtained paired bronchoalveolar lavage (BAL) fluid and peripheral blood from HIV-uninfected and asymptomatic HIV-infected, antiretroviral therapy (ART)-naïve Malawian adults. We analysed and compared the proportions and numbers of CD4\(^+\) and CD8\(^+\) T cells, B cells, NK cell subsets, \(\gamma\delta\) T cells, monocytes, dendritic cell subsets, neutrophils and alveolar macrophages in samples from HIV-infected and uninfected individuals.

Methods

Study participants

The study was conducted at the Queen Elizabeth Central Hospital, a large teaching hospital in Blantyre, Malawi. Participants were recruited from the hospital’s voluntary counselling and testing (VCT) and ART clinics. They were adults aged ≥18yrs comprising healthy HIV-1-uninfected and asymptomatic HIV-1-infected volunteers with no clinical evidence of active disease and willing to undergo bronchoscopy and BAL for research purposes\(^21\). HIV testing was performed on whole blood using two commercial point-of-care rapid HIV test kits, Determine HIV 1/2 kit (Abbott Diagnostic Division) and Unigold HIV 1/2 kit (Trinity Biotech Inc.). A participant was considered HIV-uninfected if the test was negative by both kits or HIV-infected if the test was positive by both kits. If Determine and Unigold results were discordant, a third rapid test using Bioline HIV 1/2 kit (Standard Diagnostics Inc.) was performed to resolve the discordance. None of the participants were on ART at the time of recruitment to the study, but all initiated ART after sample collection according to the ‘test and treat’ Malawi national treatment guidelines. Exclusion criteria for the study were: current or history of smoking, use of immunosuppressive drugs, severe anaemia (Hb<8g/dl) and known or suspected pregnancy. The research ethics committee of Malawi College of Medicine approved the study under approval number P.03/16/1907 and all participants provided written informed consent.

Sample collection and experimental procedures

Bronchoscopy and BAL were performed on all participants as previously described\(^22\),\(^23\). The fluid was filtered using sterile gauze and centrifuged at 500g for 10min. The supernatant was removed, the cell pellet was resuspended and washed with PBS by spinning in a centrifuge at 500g for 10min. The supernatant was removed and discarded while the cell pellet was resuspended in complete media. Peripheral blood was also obtained from study participants for full blood count (FBC) and peripheral blood mononuclear cell (PBMC) isolation using density gradient centrifugation. Cell counts in BAL cells and PBMCs isolated from each sample were performed using a haemocytometer.

Immunophenotyping

Whole BAL cells (1 × 10\(^6\) cells) and PBMCs (1 × 10\(^6\) cells) were stained with predetermined optimal concentration of fluorochrome-conjugated monoclonal antibodies against human cell surface proteins. Two separate antibody panels targeting lymphocytic and myeloid cells were used. The lymphocyte panel consisted of anti-CD3 PE/Cy5, anti-CD4 BV421, anti-CD8 APC-Cy7, anti-CD19 PE, anti-CD56 APC, anti-TCR V\(\alpha\) FITC, and anti-CD45 PE-CF594. The myeloid panel consisted of anti-CD45 PE-CF594, anti CD14 BV421, anti-CD16 PE/Cy7 PC7, anti-HLADR PE/Cy5, anti-CD66 FITC, anti-CD206 APC, anti-CD11c APC/Cy7 and anti-CD123 BV510. Further details of the antibodies are in Supplementary Table 1. All samples were analysed using a BD LSRFortessa flow cytometer (Becton Dickinson, USA).

Statistical analysis

Statistical analyses and graphical presentation were performed using GraphPad Prism 5 (GraphPad Software, USA). We used FlowJo v10 software (Treestar, USA) to analyse flow cytometry data. The numbers of cell subsets in BAL fluid were estimated by calculating the proportion of a particular subset relative to the total number (1 × 10\(^6\) cells) of stained cells. In PBMCs, the absolute numbers were obtained by calculating the proportion of a particular subset relative to the full blood count (FBC) data. Data were analysed using Mann Whitney U test. Results are given as median and interquartile range (IQR). Differences were considered statistically significant when p<0.05.

Results

Study participants and samples

We recruited 20 HIV-uninfected healthy controls (median age [range] (32[18-52]); male:female, 12:8) and 17 asymptomatic HIV-infected adults (median age [range] (33 [24-58]); male:female, 8:9). The CD4 count (median [range]) was lower in HIV-infected adults compared to the HIV-uninfected controls (365[218-541]) vs. 731[541-888] cells/ul, p=0.0024). The main characteristics of the participants are summarised in Table 1. Not all experimental assays were performed on all study participants.
CD8+ T cells, B cells and γδ T cells contribute to HIV-associated lymphocytic alveolitis

We investigated the impact of HIV infection on the proportion and numbers of lymphocyte populations using flow cytometry. The gating strategy is illustrated in Figure 1. We found that the proportions and numbers of lymphocytes in BAL fluid were higher in HIV-infected adults compared to HIV-uninfected (median 20.8% vs. 8.5%, p=0.0004 and median 1 × 10^6 vs. 2.7 × 10^6 cells/100ml of BAL fluid, p=0.0005, respectively) (Figure 2A and 2B). We next determined the cell subsets that were responsible for the increased frequency of lymphocytes in the alveoli. We found that the proportions and numbers of CD8+ T cells (median, 68% vs. 32%, p<0.0001 and median 7 × 10^6 vs. 7 × 10^3/100ml of BAL fluid, p<0.0001, respectively) and B cells (median 1.8% vs. 0.8%, p=0.0014 and median 7 × 10^6 vs. 1 × 10^4/100ml of BAL fluid, p<0.0001, respectively) in BAL fluid were higher in HIV-infected adults compared to HIV-uninfected controls (Figure 2C, 2D, 2E and 2F). We found that the proportions and numbers of γδ T cells were also higher in BAL fluid from HIV-infected adults compared HIV-uninfected controls (median 1.4% vs. 0.8%, p=0.036 and median 1 × 10^5 vs. 2 × 10^4/100ml of BAL fluid, p=0.0002, respectively) (Figure 3A and 3B).

Table 1. Demographics of the study participants.

<table>
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<tr>
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<th>HIV-uninfected controls (n=20)</th>
<th>HIV-infected ART-naive (n=17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years), median (range)</td>
<td>32(18-52)</td>
<td>33(24-58)</td>
</tr>
<tr>
<td>Sex (M:F)</td>
<td>12:8</td>
<td>8:9</td>
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<tr>
<td>CD4 count (cells/μl), median (IQR)</td>
<td>731(541-888)</td>
<td>365(218-541)</td>
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In contrast, the proportions of CD4+ T cells and NK cells in BAL fluid were lower in HIV-infected adults compared to HIV-uninfected controls (CD4+ T cell, median 2% vs. 4%, p<0.0001; NK cells, median 1% vs. 2%, p<0.0001) (Figure 2C and Figure 3C). However, the numbers showed no difference in CD4+ T cells (median 1.1 × 10^6 vs. 1.0 × 10^6/100ml of BAL fluid, p=0.7065) and NK cells (median 5.4 × 10^5 vs. 4.9 × 10^5/100ml of BAL fluid, p=0.8911) between HIV-infected adults and HIV-uninfected controls (Figure 2D and Figure 3D). These findings demonstrate that HIV infection has a differential impact on alveolar lymphocyte populations.

Figure 1. Representative flow cytometry plots for characterising lymphocytes in BAL fluid from an ART-naïve HIV-infected adult. BAL cells were stained with fluorochrome-conjugated antibodies.
Figure 2. Proportions and numbers of CD4+ T cells, CD8+ T cells and CD19+ B cells in BAL fluid from ART-naive HIV-infected compared to HIV-uninfected individuals. BAL cells were stained with fluorochrome-conjugated antibodies. A) Proportion of lymphocytes in BAL fluid. B) Numbers of lymphocytes in BAL fluid. C) Proportion of CD4+ and CD8+ T cells in BAL fluid. D) Numbers of CD4+ and CD8+ T cells in BAL fluid. E) Proportion of B cells in BAL fluid. F) Numbers of CD19+ B cells in BAL fluid. The horizontal bars represent median and 95% confidence intervals. Data were analyzed using Mann Whitney U test. (HIV-, n=20; HIV+ ART-, n=17).
Proportions and numbers of γδ T cells and NK cells in BAL fluid from ART-naïve HIV-infected compared to HIV-uninfected individuals. BAL cells were stained with fluorochrome-conjugated antibodies. A) Proportion of γδ T cell subsets in BAL fluid. B) Numbers of γδ T cell subsets in BAL fluid. C) Proportion of NK cell subsets in BAL fluid. D) Numbers of NK cell subsets in BAL fluid. The horizontal bars represent median and 95% confidence intervals. Data were analyzed using Mann Whitney U test. (HIV-, n=20; HIV+ ART-, n=17).

Differential impact of HIV infection on monocyte subsets in the alveolar and blood compartments

Next, we investigated the impact of HIV infection on the monocyte population in BAL fluid compared to peripheral blood. We found that irrespective of HIV status CD14+CD16+ intermediate monocytes were the predominant subset in BAL fluid, followed by CD14−CD16− classical monocytes and then CD14+CD16− non-classical monocytes (HIV-, Median 63% vs. 33% vs. 5%; HIV+, Median 81% vs. 13% vs. 9%) (Figure 5A and 5C). In blood, irrespective of HIV status, CD14++CD16low classical monocytes were the predominant monocyte subset, followed by CD14loCD16+ non-classical monocytes and then CD14+CD16+ intermediate monocytes (HIV-, median 74% vs. 18% vs. 9%; HIV+, median 73% vs. 23% vs. 8%) (Figure 5B and 5D).

Second, we compared the proportions and numbers of monocyte population in BAL fluid and peripheral blood between HIV-infected adults and HIV-uninfected controls. In BAL fluid, we found that the proportion and numbers of CD14+CD16+ classical monocytes were lower in HIV-infected adults compared to HIV-uninfected controls (median 13% vs. 33%, p=0.0002 and median 1 x 10⁵ vs. 2.8 x 10⁵ cells/100ml of BAL fluid, p=0.0001, respectively) (Figure 5C and 5E). In contrast, the proportion of CD14+CD16+ intermediate monocytes was higher in HIV-infected adults compared the
HIV-uninfected controls (median, 80% vs. 64%, p=0.0011) but the numbers were similar between the two groups (median 6.0 x 10^5 vs. 7.7 x 10^5 cells/100ml of BAL fluid, p=0.8628) (Figure 5C and 5E). In blood, we found that the numbers of CD14+ CD16^- classical monocytes (median 110 vs. 60 cells/1000 mm^3, p=0.0237), CD14^-CD16^- intermediate monocytes (median 20 vs. 6 cells/1000 mm^3, p=0.0362) and CD14^-CD16^- non classical monocytes (median 10 vs. 30 cells/1000 mm^3, p=0.0316) were higher in HIV-infected adults compared to HIV-uninfected controls (Figure 5F). These findings underscore differences in the composition and the impact of HIV infection on immune cells in the lung and systemic compartments.

Altered proportions of alveolar macrophages and dendritic cell populations in HIV-infected adults

Lastly, we investigated the impact of HIV on alveolar macrophages (AM), neutrophils and dendritic cell populations in BAL fluid. We found that the proportions of alveolar macrophages and myeloid dendritic cells were lower in HIV infected adults compared to HIV-uninfected controls (AM, median 73% vs. 80%, p=0.0109; mDC, median 0.6% vs. 0.9%, p=0.0036) (Figure 6A and 6C). The proportion of neutrophils and plasmacytoid dendritic cells was similar between HIV-infected adults and HIV-uninfected controls (neutrophils, median 0.34% vs. 0.14%, p=0.0789; pDC, median 0.04% vs. 0.05%, p=0.1947) (Figure 6A and 6C). The numbers of alveolar macrophages (median 2.0 x 10^6 vs. 2.2 x 10^6 cells/100ml of BAL fluid, p=0.8628), neutrophils (median 6.2 x 10^4 vs. 8.4 x 10^4 cells/100ml of BAL) and dendritic cells (mDC, median 2.1 x 10^5 vs. 2.7 x 10^5 cells/100ml of BAL fluid, p=0.2676; pDC, median 1.3 x 10^5 vs. 1.7 x 10^5 cells/100ml of BAL fluid, p=0.5328) were similar between the HIV-infected adults and HIV-uninfected controls (Figure 6B and 6D). Taken together, the findings show that chronic HIV-infection is associated with a disruption in the homeostatic proportions of alveolar macrophage and dendritic cell populations.

Figure 4. Representative flow cytometry plots for characterising myeloid cells in BAL fluid from an ART-naïve HIV-infected adult. BAL cells were stained with fluorochrome-conjugated antibodies.
Figure 5. Proportions and numbers of monocyte subsets in BAL fluid and peripheral blood from ART-naïve HIV-infected compared to HIV-uninfected individuals. BAL cells and PBMCs were stained with fluorochrome-conjugated antibodies. A) Flow cytometry representative plot of stained BAL sample from an HIV-uninfected control. B) Flow cytometry representative plot of stained peripheral blood sample from an HIV-uninfected control. C) Proportion of monocytes subsets in BAL fluid. D) Proportion of monocyte subsets in peripheral blood. E) Numbers of monocytes subsets in BAL fluid. F) Numbers of monocyte subsets in peripheral blood. The horizontal bars represent median and 95% confidence intervals. Data were analyzed using Mann Whitney U test. (BAL fluid, HIV-, n = 20; HIV+ ART-, n = 17; PBMC, HIV-, n=16; HIV+ ART-, n=14).
Figure 6. Proportions and numbers of alveolar macrophages, neutrophils and dendritic cells in BAL fluid from ART-naïve HIV-infected compared to HIV-uninfected individuals. BAL cells were stained with fluorochrome-conjugated antibodies. A) Proportion of alveolar macrophages and neutrophils in BAL fluid. B) Numbers of alveolar macrophages and neutrophils in BAL fluid. C) Proportion of dendritic cell subsets in BAL fluid. D) Numbers of dendritic cell subsets in BAL fluid. The horizontal bars represent median and 95% confidence intervals. Data were analyzed using Mann Whitney U test. (HIV-, n = 20; HIV+ ART-, n = 17).

Discussion
We report the broad impact of HIV infection on immune cell populations in the alveolar space beyond the well-characterised CD8+ T cell alveolitis observed in previous studies. We show that in addition to CD8+ T cells, B cells and γδ T cells are increased, while classical monocytes are decreased in BAL fluid from ART-naïve HIV-infected adults compared to HIV-uninfected individuals. We further show generalised disruption in the proportions of immune cell subsets including alveolar macrophages, CD4+ T cells, myeloid dendritic cells, intermediate monocytes and NK cells in BAL fluid of asymptomatic chronic HIV-infected adults.

Although HIV-infection was associated with accumulation of B cells and γδ T cells in BAL fluid, their contribution to pulmonary immunity during chronic HIV infection is incompletely understood. However, previous studies have reported HIV-associated impairment of function of these two cell subsets in peripheral blood. Consistent with what has been observed in the systemic circulation,
hyperglobulinemia has been reported in BAL fluid of HIV-infected adults\cite{22,23}, but the antibodies have impaired opsonic function\cite{24}. It is plausible that the HIV-associated increase in B cells in the lung results in increased antibody production and BAL fluid hyperglobulinemia. Furthermore, the increase in γδ T cells that we found in the present study supports the findings of Agostini et. al.\cite{25}, who showed that HIV-infected individuals with CD8+ T cell alveolitis had increased γδ T cells in BAL fluid, which were predominantly of the Vδ2 subset. However, HIV infection was also associated with anergic γδ T cells that were characterised by their substantially deficient response to phosphoantigens\cite{26,27,28}. Taken together, the findings of previous studies lead us to postulate that despite the increase in numbers, lung B cells and γδ T cells from HIV-infected individuals have impaired function as their blood counterparts.

HIV infection is associated with massive depletion of mucosal CD4+ T cells in the gut\cite{29,30} and gradual decline in peripheral blood CD4+ T cells\cite{31}. We have shown preserved mucosal CD4+ T cells in BAL fluid from chronic HIV-infected adults, even in those with depleted peripheral blood CD4+ T cells. Our findings are consistent with previous work that showed lung CCR5+CD4+ T cells are not massively depleted during HIV infection\cite{32}. The mechanisms behind this preservation of alveolar CD4+ T cells is unclear and warrants further investigation. However, Mahlknecht et. al. has shown that macrophages can prevent CD4+ T cell apoptosis in vitro via cell to cell contact using a mechanism that involves stimulation of nef-expressing CD4+ T cells with macrophage membrane-bound TNF\cite{33}. Nef in presence of TNF stimulation promotes activation of anti-apoptotic transcription factor NF-xB, resulting in blockade of caspase-8 activation and subsequent apoptosis\cite{34}. It is therefore plausible that alveolar macrophages could promote survival of CD4+ T cells in the lung through similar mechanisms, but this warrants further investigation. However, although alveolar CD4+ T cells are not massively depleted during chronic HIV infection, their functional capacity is perturbed\cite{35,36}.

Consistent with others\cite{37,38}, we have showed that CD16+CD14+ intermediate monocytes were the predominant subset in BAL fluid. CD16+ monocytes and AM have been shown to be permissive to HIV infection\cite{39,40}. The abundance of intermediate monocytes and AM in BAL fluid increases potential cellular targets for HIV. Our findings that AM are preserved during chronic HIV infection, may partly be attributed to the long life span of these cells\cite{37,38}, as well as their resistance to the cytopathic effects of HIV\cite{41,42}. In contrast, we observed a depletion in classical monocytes in BAL fluid from HIV-infected individuals. The mechanism for the selective depletion of classical monocytes is unclear, but might involve HIV-induced apoptosis\cite{43} or loss/downregulation of surface CD14\cite{44}. Alveolar macrophages originate from erythro-myeloid progenitors (EMPs), while monocytes originate from haematopoietic stem cells (HSCs)\cite{45}, hence the differential impact of HIV on these subsets might be due to the distinct nature of their source of origin. Presence of a wide array of HIV-permissive cells in the lung, including recruited and resident cells, could contribute to maintenance of local viral production and subsequent disruption of immune cell populations and homeostasis in this compartment.

A potential limitation of the study is that the numbers of BAL cell subsets are extremely difficult to measure with a very high degree of accuracy due to the variations in the dilution factor of epithelial lining fluid and differences in BAL fluid volume return. However, using a method utilised in previous studies\cite{46,47}, we calculated numbers of cell subsets using the BAL cell count obtained from a haemocytometer combined with proportions obtained by immunophenotyping. We have confidence in the reliability of this method to measure the numbers for the other cell subsets, as we have replicated the observation that the absolute number of CD8+ T cells is higher in HIV-infected adults compared with HIV-uninfected individuals\cite{48,49,50}.

In conclusion, our findings show that HIV infection is associated with broad alteration of immune cell populations in the lung. Disruption in immune homeostasis has been shown to lead to increased susceptibility to both infectious and non-infectious diseases. The broad alteration of immune cell populations in the lung in part explain the propensity to LRTI in HIV-infected individuals. However, the degree to which successful anti-retroviral therapy restores the composition of immune cells in the lung warrants further investigation.

Data availability
The data underlying the results presented in this manuscript are available from OSF: osf.io/ykve4.

Competing interests
No competing interests were disclosed.

Grant information
This work was supported by the Wellcome Trust [105831]; and the Bill and Melinda Gates Foundation [OPP1125279].

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements
The authors thank all study participants, Mrs Kunkeyani (MLW, Blantyre, Malawi), Mrs Kanyandula (MLW, Blantyre, Malawi) and staff of MLW and QECH for their support and co-operation during the study. We thank Prof David Russell (Cornell University, Ithaca, USA) for proof reading the manuscript.
Supplementary material

Supplementary Figure 1. Proportions of CD4+ T cells, CD8+ T cells, CD19+ B cells, γδ T cells and NK in peripheral blood from ART-naive HIV-infected compared to HIV-uninfected individuals. PBMC were stained with fluorochrome-conjugated antibodies.
A) Proportion of CD4+ and CD8+ T cells in peripheral blood. B) Proportion of B cells in peripheral blood. C) Proportion of γδ T cell subsets in peripheral blood. D) Proportion of NK cell subsets in peripheral blood. The horizontal bars represent median and 95% confidence intervals. Data were analyzed using Mann Whitney U test. (HIV-, n=16; HIV+ ART-, n=14).

Click here to access the data.

Supplementary Table 1. Details of fluorochrome-conjugated antibodies used in the study.

Click here to access the data.

References


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Current Referee Status: ? ?

Review by Graeme Meintjes

Mwale and colleagues report a cross-sectional study conducted in Blantyre, Malawi, in which 17 HIV-1 infected, ART naïve, asymptomatic adults and 20 HIV-1 uninfected adults were compared with respect to proportions and total number of leukocyte subsets in BAL obtained at bronchoscopy and in peripheral blood. The study is descriptive, providing a detailed profile of the leukocyte alterations associated with HIV-1 infection in these compartments.

My comments are:

1. More details regarding whether there was appropriate matching of cases and controls should be provided. It is unclear whether co-morbidities and environmental exposures that may impact lung immune responses were well matched across arms. For example, were respiratory diseases or symptoms present in any participants? Was a history of environmental exposures taken (eg. indoor smoke, occupational)? Were participants asked about previous TB, current TB symptoms and was any TB diagnostic work-up undertaken in those with symptoms? Was there a chest X-ray done in participants? Was any testing for TB infection (“latent TB”) undertaken?

2. The term alveolitis is used in the title. “Alveolitis” suggests a clinical syndrome characterized by lung inflammation focused on the alveoli (as is the case with CD8+ alveolitis in HIV-infected patients – this manifests with a clinical syndrome). The authors have described increased numbers of certain immune cell types in the alveoli. I do not think this represents alveolitis and suggest changing this term in the title.

3. In the abstract it is stated that HIV “does not lead to massive depletion of alveolar CD4+ T cells”. This is true for the participants studied but most had CD4 count in blood > 200 thus this statement should be qualified by recognising this may not apply to patients with severe CD4 depletion in blood.

4. In the Methods, the delay between taking the BAL sample and processing should be described. This may affect cell viability, especially neutrophils.
5. On page 3 for this statement: “In PBMCs, the absolute numbers were obtained by calculating the proportion of a particular subset relative to the full blood count (FBC) data.” Suggest providing details about which cell populations from the FBC data were used for denominators in calculating cell numbers from the FACS data.

6. Page 7: “In blood, we found that the numbers of CD14+ CD16lo classical monocytes (median 110 vs. 60 cells/1000 mm3, p=0.0237), CD14+CD16+ intermediate monocytes (median 20 vs. 6 cells/1000 mm3, p=0.0362) and CD14loCD16+ non classical monocytes (median 10 vs. 30 cells/1000 mm3, p=0.0316) were higher in HIV-infected adults compared to HIV-uninfected controls.” ... It appears the non classical monocytes were lower (10 vs 30) in HIV-infected adults not higher. Please check this.

7. Page 10: “We have shown preserved mucosal CD4+ T cells in BAL fluid from chronic HIV-infected adults, even in those with depleted peripheral blood CD4+ T cells.” The statement I underlined should be justified by data presented. Was a stratified analysis of those with CD4<200 or <350 conducted?

8. The authors acknowledge that the accuracy of measurement of cell numbers in BAL may be inaccurate because of variations in the dilution factor of epithelial lining fluid and differences in BAL fluid volume return. They do not provide details on any methods they used to standardise this measurement across participants. For example in the BAL sampling technique or in standardising to a constituent of ELF. Suggest discuss this in more detail.

Additional comments from my colleague Dr Muki Shey:

General comments
I was not certain whether clinically the presence of immune cells in the lungs of (HIV-infected) individuals constitute alveolitis, otherwise definition and contextualization was missing.

There is no mention of TB infection in the participants or location where participants were recruited. Was there any screening for TB?

Some more discussion is necessary about the differences between cell numbers and proportions in BAL and Blood.

Specific comments
- Figure 1 needs revision: Different antibody fluorochromes are given in method section while different ones appear in the figure. For example, CD3 PE-Cy5 vs PerCP Cy5.5; and CD45 PE-Texas Red s CF594 (even though the two fluorochromes may be detected in the same channel, there needs to be consistency)

- Figure1: The CD4/CD8 axes labelling seems to be reversed. I am quite certain that the frequencies of CD4+ T cells cannot be more than frequencies of CD8+ T cells in HIV infected individuals

- The TCR Va chain is mentioned in the methods section while the TCR gd appears in the plots.
The gating of CD45+ cells looks more stringent in Figure 1 (for T cells) than in Figure 4 (for myeloid cells). Was there any reason for that?

The definition of different cell subsets by expression of markers may be necessary, for example the definition of NK T cells (CD3+CD56+?).

In the results, giving the various median values in the text which are in the figures seems a duplication.

HLA-DR antibody is mentioned in methods section but not shown either as a marker of activation or identification of myeloid cells. HLA-DR is an important marker of myeloid cell identification, especially in whole blood.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
No

Are the conclusions drawn adequately supported by the results?
Yes

**Competing Interests:** No competing interests were disclosed.

**Referee Expertise:** HIV-associated tuberculosis

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.
classical monocytes in HIV which resulted in an increased proportion but not absolute number of intermediate monocytes. The data is important and adds a significant contribution to the literature, in particular through providing clinically relevant samples in HIV in patients naïve to ART. While the data makes a valuable contribution to the literature some areas of further detail would be informative.

1. Background demographic data shows the control group are well matched in terms of age and gender. More details on the HIV-positive group are required. What steps were taken to exclude TB. Is there any data on baseline CXR or TB screening or is the assumption that patients were negative purely based on lack of symptoms? Is there any data on baseline HIV viral load?

2. Some further methodological details would aid interpretation. While the differences in proportions of cell subsets are clear interpretation of absolute numbers requires evidence that volumes of instillation and BAL recovery are similar. Can the authors report their standard methodology involved instillation of 200 mls in four aliquots, into the right middle lobe, or whatever? Have they specific data on the volume recovered ad its variability. Can they confirm there were low (<5%) numbers of bronchial epithelial cells or squamous cells?

Although the focus is analysis of cellular components have they any information on the permeability of the alveolar space in the two groups through measurement of albumin or a related marker? The authors appropriately remark in the discussion that these considerations limit interpretation of absolute numbers so this comment is meant only to provide detail not as a significant criticism.

3. The absolute number of monocytes in BAL should also be reported. In the abstract, the primary finding is presented as an increase in the proportion of intermediate monocytes in HIV BAL but in reality, the main finding appears to be a reduction in absolute number of classical monocytes which results in a relative rather than absolute increase in intermediate monocytes in HIV. Subsets of intermediate monocytes may also be defined by HLA-DR and the authors appear to have also used antibodies against HLA-DR. Did they find any differences in intermediate subsets by HLA-DR in their HIV positive population?

4. While reductions in non-classical monocytes are described by many groups the magnitude of the reduction in numbers of non-classical monocytes in the BAL is a little surprising since these cells are thought to be a source of alveolar macrophages. The authors suggest they can detect very few classical cells in BAL in contrast to the blood. Some more discussion of this point seems needed. Have the authors any data with alternative markers e.g. CCR2, CX3CR1 to confirm such low numbers? Or may they be missing some non-classical monocytes? This finding should be developed further and discussed a little further and related to other BAL-specific lung data.

5. In the discussion the authors highlight the different origins of monocytes and lung macrophages but some qualification of the differences in origin of lung macrophages in inflammatory settings and the potential for classical monocytes to contribute to lung macrophage numbers in inflammation may be pertinent.

6. In Figure 5D the HIV + dot has been labelled HIV – and needs to be altered.

Is the work clearly and accurately presented and does it cite the current literature?  
Yes
Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

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