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HIV persistence in mucosal CD4$^+$ T cells within the lungs of adults receiving long-term suppressive antiretroviral therapy

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Background: The lungs were historically identified as one of the major anatomic sites for HIV replication in the pre-antiretroviral therapy (ART) era. However, their contribution to HIV persistence in individuals under suppressive ART remains understudied.

Design: We assessed HIV persistence and comprehensively characterized pulmonary mucosal CD4$^+$ T cells in HIV-infected (HIV$^+$) individuals receiving long-term suppressive ART versus uninfected participants.

Methods: Bronchoalveolar lavage (BAL), bronchial biopsies, and matched peripheral blood were obtained from $n = 24$ HIV-infected adults receiving long-term suppressive ART (median: 9 years) and $n = 8$ healthy volunteers without respiratory symptoms. HIV-DNA and cell-associated HIV-RNA were quantified by ultra-sensitive PCR, and lung mucosal CD4$^+$ T-cell subsets were characterized by multiparameter flow cytometry.

Results: The levels of HIV-DNA were 13-fold higher in total BAL cells compared to blood. Importantly, FACS-sorted CD4$^+$ T cells from BAL contained greater levels of HIV-DNA compared to peripheral CD4$^+$ T cells. BAL CD4$^+$ T cells in HIV$^+$ individuals were characterized mostly by an effector memory phenotype, whereas naive and terminally differentiated cells were underrepresented compared to blood. Furthermore, BAL CD4$^+$ T cells expressed higher levels of immune activation (HLA-DR/CD38) and senescence (CD57) markers. Importantly, BAL was enriched in T-cell subsets proposed to be preferential cellular HIV reservoirs, including memory CD4$^+$CCR6$^+$, Th1Th17 (CD4$^+$CCR6$^+$CCR4$^-$CXCR3$^+$), CD4$^+$CCR6$^+$CXCR3$^-$CCR4$^-$, and CD4$^+$CD32a$^+$ T cells.

Conclusion: The pulmonary mucosa represents an important immunological effector site highly enriched in activated and preferential CD4$^+$ T-cell subsets for HIV persistence during long-term ART in individuals without respiratory symptoms. Our findings raise new challenges for the design of novel HIV eradication strategies in mucosal tissues.

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Keywords: HIV persistence, lungs, mucosal CD4$^+$ T cells, pulmonary immunity

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Introduction

Despite suppressive antiretroviral therapy (ART), HIV-infected individuals suffer from a high burden of bacterial pneumonia, influenza, and tuberculosis [1], suggesting that lung immunity is not fully restored. The high prevalence of chronic lung diseases, notably chronic obstructive pulmonary disease, among HIV-infected individuals is associated with high mortality rates [1,2]. Importantly, HIV is an independent risk factor from tobacco for altered pulmonary immunity, despite suppressive ART [1].

HIV reservoirs are defined as cellular or anatomical sites in which potentially inducible, intact, replication-competent proviruses persist. Within lymphoid tissues, there are abundant target cells in close contact facilitating cell-to-cell spread of HIV, along with reduced antiretroviral penetration [3]. Meanwhile, cellular HIV reservoirs consist predominantly of $CD4^+$ T cells as they are exquisitely susceptible to infection [3]. HIV can persist as integrated latent proviral DNA in resting memory cells with long survival potential [4]. In addition to gut-associated lymphoid tissues (GALT), adipose tissues, and lymph nodes, anatomical HIV reservoirs include the central nervous system and testes due to their immune privilege and tissue barriers, which shield them from leukocyte trafficking within the systemic circulation [5].

The lungs differ from the central nervous system and testes as they do not contain histological barrier tissues to prevent leukocyte trafficking or antiretroviral penetration. Importantly, in contrast to immune-privileged sites and similar to GALT, the lungs are very active immunological effector sites due to their constant exposure to airborne pathogens. Indeed, the lungs represent potential lymphoid viral reservoirs, which have received less attention than other reservoirs in the ART era, although they have features that make them ideal sanctuaries for HIV persistence. The proximity of millions of alveoli provides a large surface area and fertile ground for cell-to-cell spread of HIV, a proposed mode of HIV persistence [6]. Studies on untreated HIV-positive (HIV^+) individuals demonstrate that HIV can be isolated from cell-free bronchoalveolar lavage (BAL) fluid as well as alveolar macrophages [6,7] and $CD4^+$ T cells [6,8,9]. Notably, the CCR5-tropic strain, HIV-1_{Bal}, was originally isolated from the lungs [10]. Importantly, a recent humanized mice study showed that HIV can persist in tissue macrophages, including in lungs, during suppressive ART [11]. Furthermore, recent rhesus macaque studies have shown that lungs contain high amounts of SIV, even during suppressive ART [12,13]. Rare human studies on the effect of ART on pulmonary HIV have been conducted in individuals on ART for a relatively short time period and showed detectable HIV in the lungs even after 6 months of effective ART [14]. ART appeared to partially restore pulmonary T-cell functions [15].

Studies from the pre-ART era demonstrated that HIV infection results in various changes in pulmonary mucosal immunity including reduced CD4/CD8 ratio, increased alveolar macrophages, neutrophils and eosinophils, polyclonal B-cell activation, and disrupted Th1/Th2 ratios [6]. However, to our knowledge, pulmonary $CD4^+$ T-cell mucosal populations in HIV^+ adults under long-term suppressive ART versus uninfected individuals have not yet been described. Various T-cell subsets are proposed as potential cellular HIV reservoirs in peripheral blood and gut mucosal tissues including central and effector memory $CD4^+$ T cells [4], memory $CD4^+CCR6^+$, Th1Th17, $CD4^+CCR6^+CXCR3^-CCR4^-$ T cells, and more recently $CD4^+CD32a^+$ T cells [16–19]. However, to date, their presence and distributions in pulmonary mucosa have not been documented.

Therefore, we aimed to determine whether HIV could persist within the lung mucosa after long-term suppressive ART in the absence of respiratory symptoms and to describe comprehensively respiratory T-cell immunity in line with HIV persistence.

Materials and methods

Study population and bronchoalveolar lavage, bronchial biopsies, and blood collection

Twenty-four HIV-infected and eight uninfected participants were recruited at the McGill University Health Centre (MUHC, Montreal, Canada). HIV-infected participants were all ART-treated with suppressed plasma viral load for at least 3 years and without any respiratory symptoms or active infections. Prior to bronchoscopies, all participants underwent spirometric testing several weeks earlier to ensure the absence of any undiagnosed obstructive airflow disease. Bronchoscopies were performed to obtain 50–100 ml of BAL fluid. Four to six bronchial biopsies were taken from the subsegmental and segmental carinae of the right middle and right lower lobes. In parallel, 40 ml of matched peripheral blood was also collected.

Ethical considerations

The study was conducted in compliance with the principles included in the Declaration of Helsinki and received approval from the institutional review boards of the MUHC (#15-031), *Université du Québec à Montréal* (UQAM) (#602) and CHUM-Research Centre (#15-180). All study participants signed a written informed consent.

Isolation of pulmonary mucosal cells and PBMCs

Within 1 h following the bronchoscopy, BAL fluid was centrifuged and a portion of BAL cell pellets were collected for phenotyping, fluorescence-activated cell sorting (FACS)-sorting and HIV-DNA/RNA assessments. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll centrifugation.

Flow cytometry cell sorting

A fraction of the BAL cells was used for cell sorting of pulmonary $CD4^+$ T cells and alveolar macrophages. BAL cells were stained with CD3-AlexaFluor700, CD4-FITC, CD8-APC-H7 and CD206-PE. Pulmonary $CD3^+CD4^+CD8^-$ T cells and $CD206^+$ alveolar macrophages [20] were FACS-sorted using a BD-Aria cell-sorter to obtain highly pure populations for HIV-DNA/RNA quantifications. Of note, due to the variable and limited $CD4^+$ T-cell quantities recovered from BAL, these measurements were not performed in all participants (Supplementary 1, http://links.lww.com/QAD/B333).

HIV-DNA/RNA quantifications

We measured the frequency of cells harboring total HIV-DNA (copies per million cells) using a well established assay (sensitivity of 1 copy/PCR reaction) [4,21] with minor modifications to the original protocol. Notably, DNA from PBMCs, matched BAL cell pellets and biopsies was extracted using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) before being subjected to PCR amplification. Cell-associated HIV-RNA was quantified by ultrasensitive RT-PCR, as described previously [22]. Detailed methodology is described in Supplementary 2, http://links.lww.com/QAD/B333.

Flow cytometry

Multicolor flow cytometry was performed on PBMCs and BAL cells. A viability dye kit (Invitrogen, Life Technologies Corporation, Eugene, Oregon, USA) was used to exclude dead cells from the analysis. Frequency of naive, central memory, effector memory, terminally differentiated, and senescent T cells were measured on live $CD4^+$ T cells by CD28/CD45RA/CD57 expression. Regulatory T cells (Tregs) were defined as $CD4^+CD127^{low}CD25^+FoxP3^+$ and expression of immunosuppressive ectonucleotidases CD39/CD73 was also assessed. T-helper (Th) subsets were determined by CCR4/CCR6/CXCR3. Activated cells were identified as $CD38^+HLA\text{-}DR^+$. HIV co-receptor CCR5 was also assessed. Finally, CD32a and the associated Immunoglobulin G (*IgG*)2b isotype control were used to determine the expression of the low affinity type II Fcγ receptor. Antibodies/fluorochromes used are described in Supplementary 3, http://links.lww.com/QAD/B333. A 3-laser BD Fortessa-X20 was used for acquisition and results were analyzed by FlowJo V10.2 (FlowJo LLC, Ashland, Oregon, USA).

Statistical analyses

GraphPad Prism V6.01 (GraphPad Software, La Jolla, California, USA) was used for statistical analyses. Results are presented as mean ± SEM. Wilcoxon matched-pairs signed-rank test was used to compare paired study variables. Otherwise, a Mann–Whitney *U* test was used for unpaired variables. Spearman's rank correlation coefficient was computed for correlation analyses.

Results

Study population

Twenty-four HIV^+ and eight HIV-negative (HIV^-) adults were enrolled in this study as described in Table 1 and Supplementary 4, http://links.lww.com/QAD/B333. Seven HIV^+ and one HIV^- participants were current tobacco smokers. A minimum of 3 years of HIV suppression was selected since the number of HIV-infected cells, as determined by HIV-DNA levels in $CD4^+$ T cells, typically declines during the initial 1 to 3

Table 1. Patient characteristics at time of bronchoscopy.

	HIV-infected (*N* = 24)	Healthy controls (*N* = 8)
Demographic factors		
Age, years (median, IQR)	52 (47, 58)	62 (51, 65)
Male sex, *n* (%)	19 (79%)	8 (100%)
Ethnicity, *n* (%)		
Caucasian	17 (71%)	8 (100%)
Black/Caribbean	3 (13%)	0 (0%)
Black/African	2 (8%)	0 (0%)
Hispanic	2 (8%)	0 (%)
HIV-related factors		
Duration of HIV infection, years (median, IQR)	15 (12, 25)	–
Duration of time since undetectable plasma viral load[a], years (median, IQR)	9 (4, 10)	–
Antiretroviral regimen, *n* (%)[b]		
Integrase inhibitor	16 (67%)	–
NNRTI	4 (17%)	
PI	6 (25%)	
$CD4^+$ cell count (cells/μl), median (IQR)	558 (430,876)	536 (305,610)
CD4 percentage, median (IQR)	32 (27, 37)	41 (37, 46)
CD4/CD8 ratio	0.7 (0.60, 0.97)	2.35 (2.13, 3.23)
Nadir $CD4^+$ cell count (cells/μl), median (IQR)	232 (136, 288)	–
Nadir CD4 percentage, median (IQR)	17 (12, 27)	–
Comorbidities		
Hypertension	7 (29%)	2 (25%)
Dyslipidemia	7 (29%)	0 (0%)
Diabetes	8 (33%)	1 (13%)
Previous pulmonary tuberculosis	0 (0%)	0 (0%)
Previous *Pneumocystis jirovecii* pneumonia	1 (4%)	0 (0%)
Lifestyle factors		
Tobacco smoker, *n* (%)		
Current	7 (29%)	1 (13%)
Ever	12 (50%)	2 (25%)
Never	12 (50%)	6 (75%)
Cannabis smoker, *n* (%)		
Current	2 (8%)	2 (25%)

IQR, interquartile range; NNRTI, nonnucleoside reverse transcriptase inhibitor; PI, protease inhibitor.
[a]undetectable viral load defined as blow 40 HIV RNA copies/ml.
[b]One patient was on a regimen containing both an integrase inhibitor and protease inhibitor; 1 patient was on a regimen containing both an integrase inhibitor and NNRTI.

years of ART then reaches a stable level that does not decline further during subsequent treatment [23].

HIV persists in the lungs of antiretroviral therapy-treated individuals

Ultrasensitive real-time PCR was performed to quantify the frequency of infected cells in matched BAL cells, bronchial biopsies and PBMCs (Supplementary 5, http://links.lww.com/QAD/B333). The levels of HIV-DNA (copies/10^6 cells) were significantly higher in total BAL cells compared to PBMCs and to bronchial biopsies (mean ± SEM 3910 ± 2396 versus 296.9 ± 68.68, $P = 0.009$; and 47 ± 12.27, $P = 0.001$, Fig. 1a). Numbers of HIV-DNA copies in BAL and PBMCs were positively correlated (Fig. 1b) while, in contrast to the peripheral blood [24], no association was found between CD4 nadir and pulmonary HIV-DNA (not shown). HIV-DNA was then quantified in FACS-sorted $CD4^+$ T cells from BAL and peripheral blood as well as in alveolar macrophages. $CD4^+$ T cells from the BAL harbored higher levels of total HIV-DNA compared to peripheral $CD4^+$ T cells (Fig. 1c). In addition, pulmonary $CD4^+$ T cells were infected at higher frequencies than total BAL cells and PBMCs (not shown). Of note, no significant difference in pulmonary HIV-DNA was found between smokers and nonsmokers (not shown). Importantly, in a subset of study participants in whom we had sufficient numbers of BAL cells ($n = 7$), cell-associated HIV-RNA was detected in BAL from four participants, with donor-to-donor variations (Fig. 1d). Donor-to-donor variations were observed in the relative HIV-RNA loads in blood versus lungs, with higher T-cell-associated HIV-RNA levels in the lungs of two of seven donors. This finding supports preferential active HIV replication in the lungs of certain

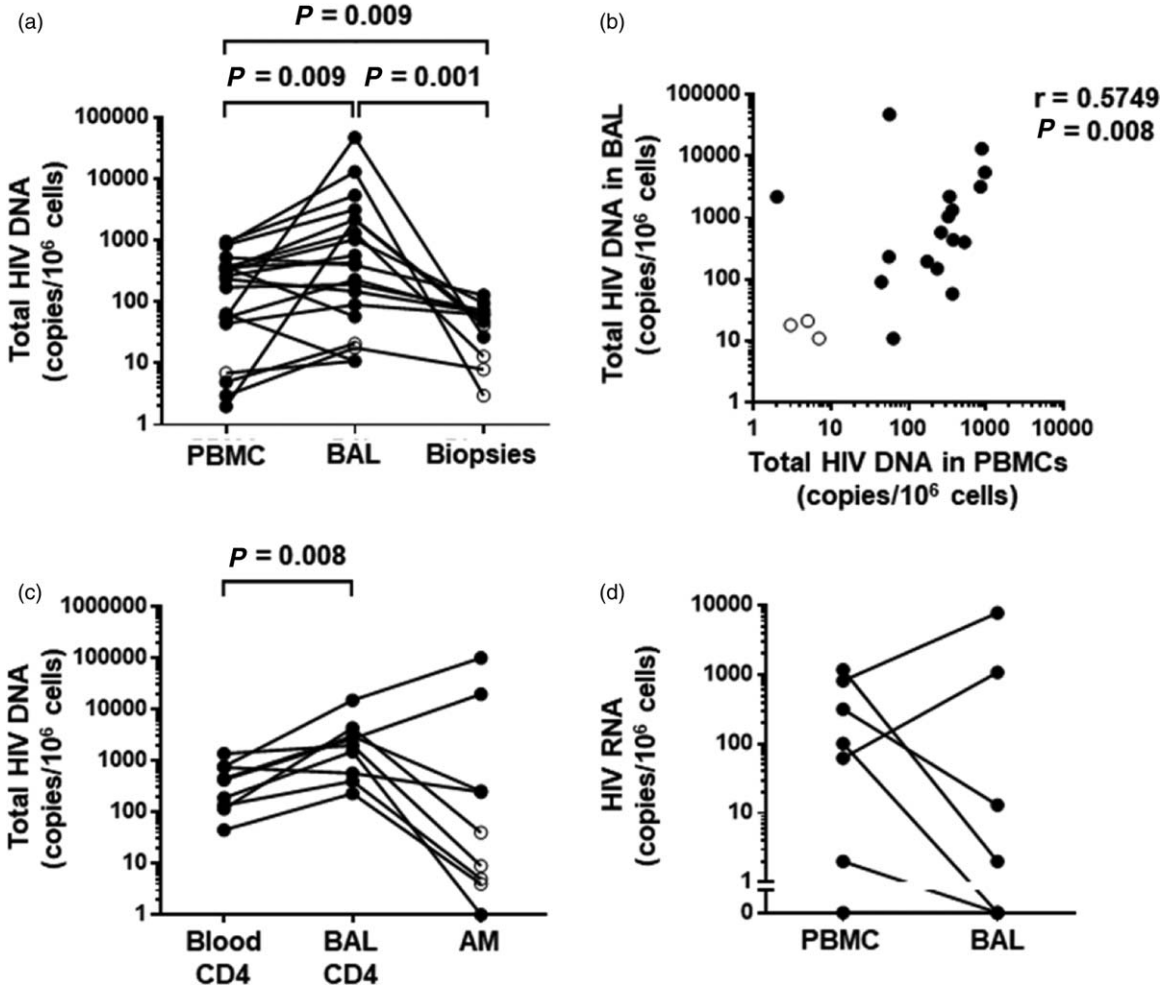

Fig. 1. Quantification of HIV DNA in cells isolated from lungs and peripheral blood. (a) HIV DNA in PBMCs, BAL cells and pulmonary biopsies (copies/10^6 cells) ($n = 20$ for PBMCs and BAL cells, $n = 12$ for biopsies). (b) Correlation analysis of HIV DNA in BAL cells versus PBMCs ($n = 20$). (c) HIV DNA in $CD4^+$ T cells purified by FACS-sorting from PBMCs and $CD4^+$ T cells purified from BAL cells and alveolar macrophages (copies/10^6 cells) ($n = 10$). (d) Cell-associated HIV RNA in BAL cells versus PBMCs ($n = 7$). *Nota bene*: Samples with undetectable HIV DNA are plotted as open symbols and the limit of detection based on cell input is represented. BAL, bronchoalveolar lavage; PBMCs, peripheral blood mononuclear cells.

HIV-infected individuals during suppressive ART. Altogether, these observations demonstrated clearly that the lung is enriched in CD4$^+$ T cells harboring high levels of HIV-DNA despite long-term ART.

Phenotypic characterization of lung mucosal CD4$^+$ T cells subsets

The frequencies of naive and terminally differentiated CD4$^+$ T cells were significantly lower in BAL cells versus PBMCs in both study arms (mean ± SEM HIV$^+$: 0.95 ± 0.29 versus 32.4 ± 3.36%, $P < 0.0001$; 0.65 ± 0.13 versus 5.1 ± 0.84%, $P < 0.0001$; HIV$^-$: 4.08 ± 3.48 versus 32 ± 5.93%, $P = 0.008$; 0.79 ± 0.32 versus 9.46 ± 5%, $P = 0.02$; Fig. 2a). Conversely, effector memory CD4$^+$ T cells, a known cellular reservoir for intact HIV genomes [4,25–27], were present at significantly higher frequencies in BAL *versus* PBMCs in both groups (HIV$^+$: 52.7 ± 4.8 versus 6.79 ± 11.3%, $P < 0.0001$; HIV$^-$: 55.25 ± 4.5 versus 1.14 ± 0.57%, $P = 0.008$; Fig. 2a). Frequencies of central memory CD4$^+$ T cells did not differ between the two compartments in HIV-infected participants but were lower in the BAL of uninfected participants (HIV$^-$: 39.5 ± 4.64 versus 57.15 ± 4.84%, $P = 0.02$; Fig. 2a). Higher frequencies of CD4$^+$ T cells expressing immune activation markers (HLA-DR$^+$: 27 ± 2.73 versus 5.22 ± 0.6%, $P < 0.0001$; HLA-DR$^+$CD38$^+$: 3.68 ± 0.95 versus 1.38 ± 0.16%, $P = 0.002$) and immuno-senescence markers (CD28$^-$CD57$^+$: 22.35 ± 3.71 versus 6.15 ± 3.58%, $P = 0.002$) were observed in BAL versus PBMCs of HIV-infected participants (Fig. 2b and c). Meanwhile, activated HLA-DR$^+$ (32.5 ± 9.14 versus 5 ± 1.73%, $P = 0.008$) and senescent (16.6 ± 5.02 versus 0.43 ± 0.14%, $P = 0.02$) pulmonary CD4$^+$ T cells were also enriched versus blood of uninfected participants (Fig. 2b and c). Importantly, BAL CD4$^+$ T cells from HIV-infected individuals expressed higher levels of activated HLA-DR$^+$CD38$^+$ compared to uninfected individuals (3.68 ± 0.95 versus 1.55 ± 0.7%, $P = 0.04$; Fig. 2b). Altogether, these results pointed to an important enrichment in the proportion of highly differentiated, activated, and senescent T cells in the lungs when compared to blood in both study arms.

Robust infiltration of CCR6$^+$ T-cell subsets into the lung mucosa

We also assessed the presence of various CD4$^+$ Th-cell subsets previously proposed as preferential cellular reservoirs of HIV [16,17,28]. Compared to PBMCs, the BAL of HIV$^+$ participants was enriched in memory CCR6$^+$Th17 (CD4$^+$CD45RA$^-$CCR6$^+$) and Th1Th17 (CD4$^+$CD45RA$^-$CCR6$^+$CCR4$^-$CXCR3$^+$) T cells (mean ± SEM 47.1 ± 6.14 versus 28.2 ± 4.25%, $P = 0.002$;

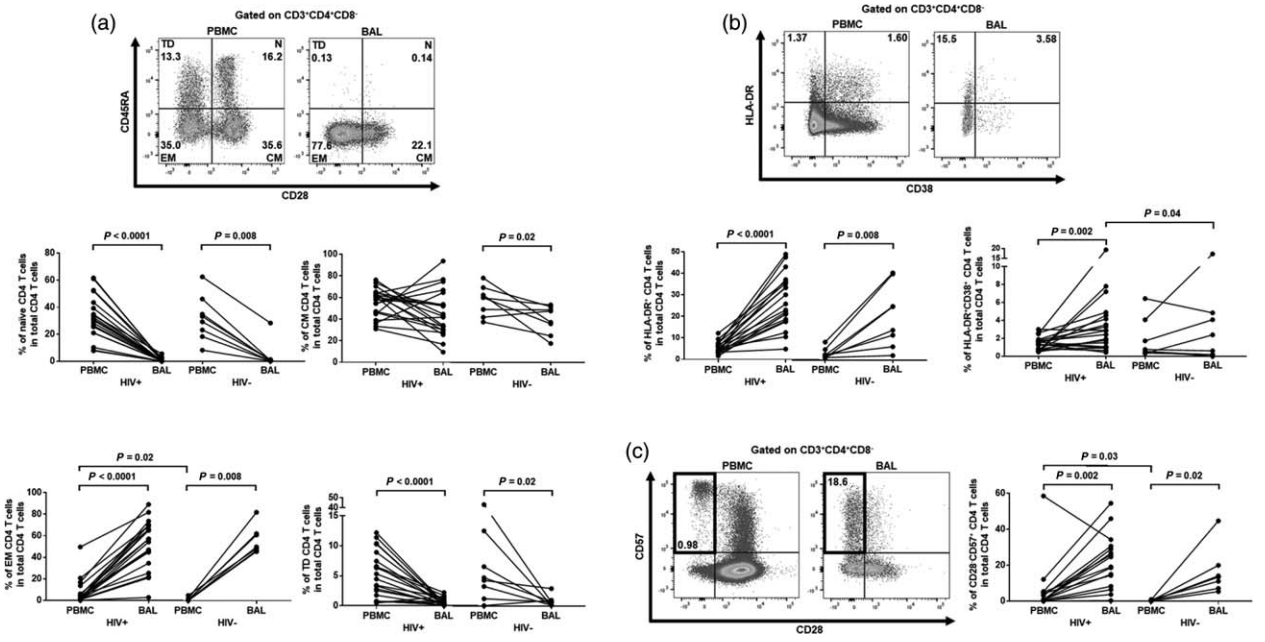

Fig. 2. Distribution and phenotypic characterization of CD4$^+$ T-cell subsets in the lungs compared to peripheral blood. (a) Gating strategy used in flow cytometry to define CD4$^+$ T-cell subsets. Naive (N), central memory (CM), effector memory (EM), and terminally differentiated (TD) CD4$^+$ T cells were defined as CD45RA$^+$CD28$^+$, CD45RA$^-$CD28$^+$, CD45RA$^-$CD28$^-$, and CD45RA$^+$CD28$^-$, respectively. Percentages of naive CD4$^+$ T cells, central memory CD4$^+$ T cells, effector memory CD4$^+$ T cells and terminally differentiated CD4$^+$ T cells among total CD4$^+$ T cells in BAL compared to blood (HIV$^+$: $n = 21$; HIV$^-$: $n = 8$). (b) Percentage of activated HLA-DR$^+$CD4$^+$ T cells and activated HLA-DR$^+$CD38$^+$ CD4$^+$ T cells among total CD4$^+$ T cells in BAL compared to blood (HIV$^+$: $n = 20$; HIV$^-$: $n = 8$). (c) Senescent CD4$^+$ T cells were characterized as CD57$^+$CD28$^-$. Percentage of senescent CD4$^+$ T cells among total CD4$^+$ T cells in BAL compared to blood (HIV$^+$: $n = 16$; HIV$^-$: $n = 7$).

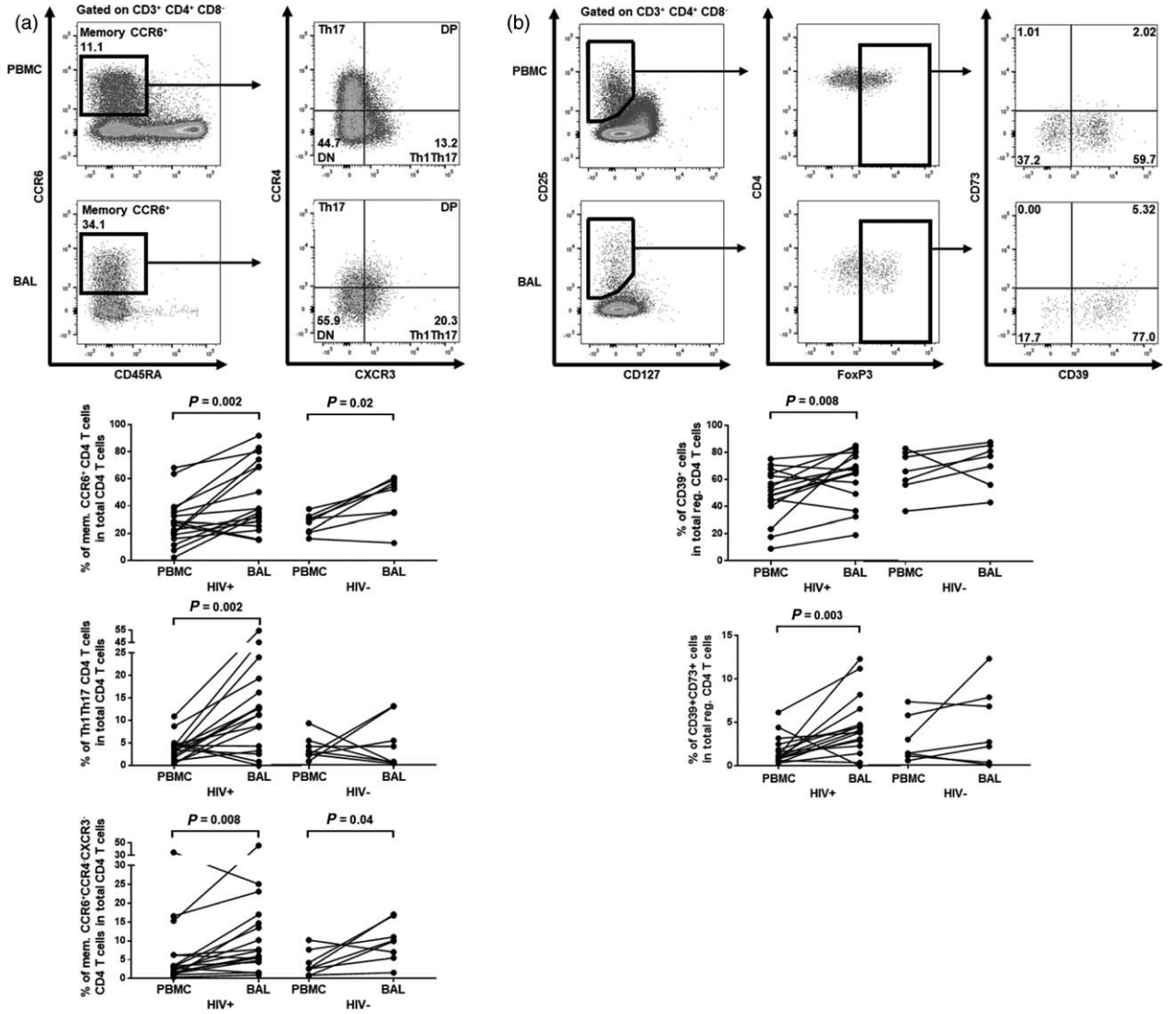

Fig. 3. Distribution of CD4$^+$ Th cells and Tregs in the lungs mucosa compared to peripheral blood. (a) Gating strategy used in flow cytometry to isolate memory CCR6$^+$, Th1Th17 and memory CCR6$^+$CCR4$^-$CXCR3$^-$ CD4$^+$ T cells. Percentage of memory CCR6$^+$, Th1Th17 and memory CCR6$^+$CCR4$^-$CXCR3$^-$ CD4$^+$ T cells among total CD4$^+$ T cells in BAL compared to blood (HIV$^+$: $n = 17$; HIV$^-$: $n = 8$). (b) Regulatory CD4$^+$ T cells were characterized as CD127^{low}CD25$^+$FoxP3$^+$. Percentage of CD39$^+$ Tregs and CD39$^+$CD73$^+$ Tregs among total regulatory CD4$^+$ T cells in BAL compared to blood (HIV$^+$: $n = 16$; HIV$^-$: $n = 7$).

14.6 ± 3.61 versus $3.83 \pm 0.66\%$, $P = 0.002$, Fig. 3a) while the BAL of uninfected participants was enriched to a lesser extent only in CCR6$^+$Th17 cells (45.1 ± 6.21 versus $26.85 \pm 2.49\%$, $P = 0.02$). Moreover, the memory CCR6$^+$CCR4$^-$CXCR3$^-$CD4$^+$ T-cell subset, another recently proposed HIV reservoir [17], was also enriched in the BAL in both groups (HIV$^+$: 11.4 ± 2.76 versus $6.08 \pm 2.12\%$, $P = 0.008$; HIV$^-$: 9.83 ± 1.87 versus $3.92 \pm 1.18\%$, $P = 0.04$; Fig. 3a).

CD39$^+$CD73$^+$Tregs are enriched in the bronchoalveolar lavage of HIV-infected individuals receiving antiretroviral therapy

In other immunological effector tissues such as GALT, high frequencies of immunosuppressive Tregs and Tregs expressing CD39/CD73 were described during chronic HIV/SIV infection as these cells inhibit anti-HIV specific responses and contribute to disease progression [29–31]. CD39/CD73 are ectonucleotidases involved in hydrolyzing inflammatory ATP into immunosuppressive adenosine. In the HIV-infected arm, total Tregs frequencies did not differ between the lungs and blood (not shown). However, we observed significantly higher proportions of CD39$^+$Tregs and CD39$^+$CD73$^+$Tregs in the BAL compared to PBMCs (mean $\pm$ SEM 62.6 ± 4.82 versus $48.7 \pm 4.73\%$, $P = 0.008$; 4.6 ± 0.86 versus $1.7 \pm 0.4\%$, $P = 0.003$, respectively, Fig. 3b), suggesting their potential role in reducing anti-HIV responses. No changes in CD39$^+$Tregs and CD39$^+$CD73$^+$Tregs frequencies were observed in uninfected individuals between BAL and blood.

CD32a expression on lung mucosal CD4+ T cells

CD32a is a low affinity Fcγ type II receptor recently proposed as a marker of quiescent CD4+ T cells enriched in HIV reservoirs in the blood of virally suppressed individuals [19]. To explore the potential role of CD32a+ T cells in pulmonary HIV persistence during ART, the expression of CD32a was assessed on CD4 T-cell subsets. To properly assess CD32a expression, an isotype control IgG2b-APC was used to eliminate nonspecific binding signals of antibodies (Fig. 4a). The frequency of CD32a+ cells was significantly higher in CD4+ T cells from BAL compared to PBMCs (mean ± SEM 1.89 ± 0.7 versus $0.17 \pm 0.05\%$, $P = 0.005$; Fig. 4a). Interestingly, higher levels of CD32a+CD4+ T cells expressing the HIV co-receptor CCR5 were observed in the BAL compared to peripheral blood (0.41 ± 0.17 versus $0.054 \pm 0.03\%$, $P = 0.04$) while the proportion of CD4+CCR5+CD32a+ T cells were similar in BAL and blood (Fig. 4b). In addition, CD32a+CD4+ T cells expressing the activation marker HLA-DR were highly enriched in the BAL versus PBMCs ($P = 0.02$; Fig. 4c). Indeed, the majority of pulmonary CD32a+CD4+ T cells were HLA-DR+ (mean ± SEM $92.5 \pm 3.43\%$ versus $37.9 \pm 13\%$ in the blood). CD32a was also assessed in four uninfected volunteers. Although statistical significance could not be achieved due to the small sample size, increased frequencies were observed in total, CCR5+ and HLA-DR+ CD32a+CD4+ T cells in the BAL versus PBMCs.

Discussion

Herein, we provided evidence that the lungs may contribute to HIV persistence during long-term ART by performing extensive phenotypic characterization of lung mucosal CD4+ T-cell subsets. Previous studies performed in the pre-ART era revealed the role of alveolar macrophages in HIV replication [6]. This is consistent with the fact that pulmonary opportunistic infections were historically associated with HIV-infection [1,6,15]. However, the role of pulmonary mucosal CD4+ T cells in HIV persistence within the lungs of individuals under long-term suppressive ART remained unclear. Our study is one of the few that addresses these important questions of HIV persistence within the lungs. Indeed, our findings reveal the infiltration of various CD4+ T-cell subsets previously proposed in the blood as HIV reservoirs into the lung mucosa, pointing to the necessity of considering the pulmonary HIV burden in eradication strategies.

We found that the mean HIV-DNA copies/10^6 cells was 13-fold higher in BAL versus PBMCs of participants on long-term suppressive ART (median 9 years). Importantly, the levels of HIV-DNA were correlated between both compartments. Interestingly, we found higher HIV-DNA levels in BAL versus pulmonary biopsies, suggesting that HIV-DNA is seeded mainly within the mucosa as it is likely that biopsies contained mostly nonimmune cells

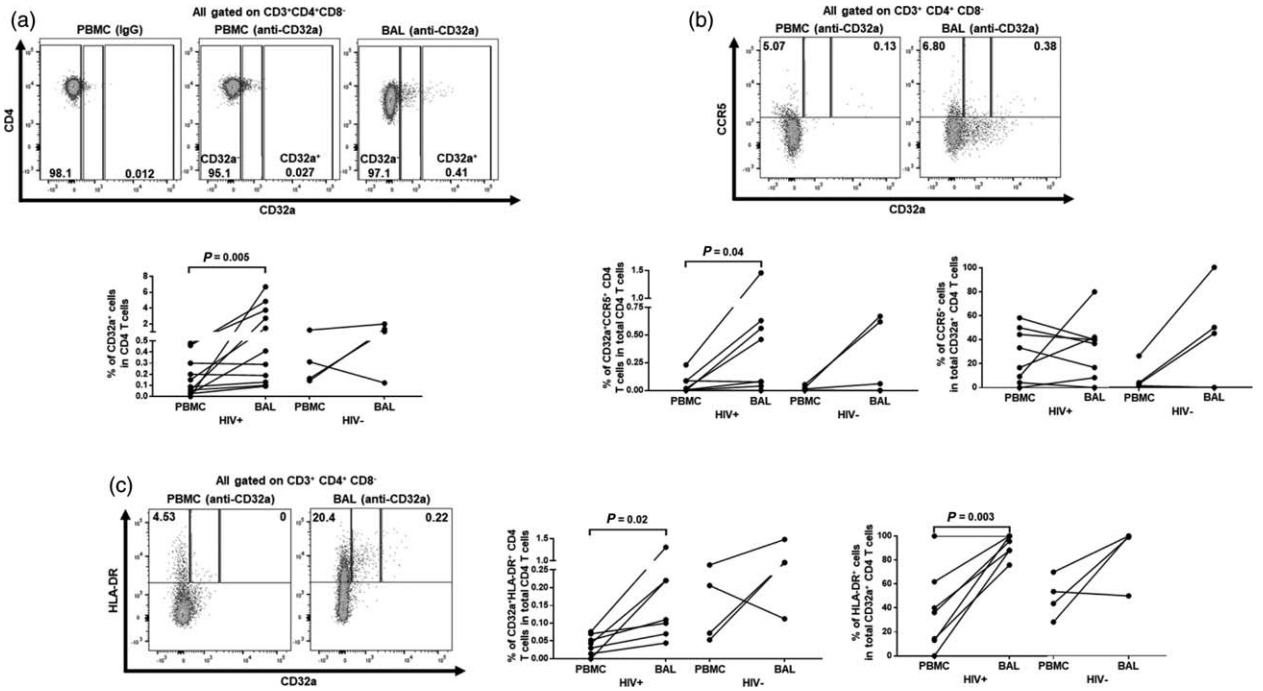

Fig. 4. CD32a expression in CD4+ T cells subsets in the lungs compared to peripheral blood. (a) Gating strategy used in flow cytometry to quantify CD32a+ T cells using IgG2bκ isotype controls. Percentage of CD32a+ cells among total CD4+ T cells in BAL compared to blood (HIV+: $n = 11$; HIV−: $n = 4$). (b) Representative figure showing the co-expression of CD32a and CCR5 on pulmonary mucosal CD4+ T cells, and expression of CCR5 on CD32a+ CD4+ T cells in BAL compared to peripheral blood (HIV+: $n = 8$; HIV−: $n = 4$). (c) Representative figure showing the co-expression of CD32a and HLA-DR on pulmonary mucosal CD4+ T cells, and expression of HLA-DR on CD32a+ CD4+ T cells in BAL compared to peripheral blood (HIV+: $n = 7$; HIV−: $n = 4$).

compared to the mucosal surface. As alveolar macrophages were reported to be infected by HIV [20], we assessed whether alveolar macrophages or $CD4^+$ T cells are enriched in HIV-DNA in FACS-sorted matched ultra-pure lung mucosal $CD4^+$ T cells, alveolar macrophages and peripheral $CD4^+$ T cells. We found that both mucosal $CD4^+$ T cells and alveolar macrophages may contain HIV-DNA. The measurement of HIV-DNA does not reflect the size of the replication-competent HIV reservoir given that most viral DNA is defective [32]. Although it will be important to confirm our observation by using functional assays, several studies have found that total HIV-DNA can be used as a surrogate to determine the frequency of cells capable of producing viral proteins [27] and even infectious viruses [33]. Importantly, we were able to detect cell-associated HIV-RNA in BAL cells from four of seven participants, indicating that HIV transcription persists in the lungs of some individuals during long-term ART. These findings are consistent with the fact that antiretrovirals do not act on viral transcription [34], thus allowing residual viral transcription to occur during ART. Such residual viral transcription happens mainly at barrier surfaces where T cells are activated by the interaction with microbial flora. This was clearly reported for GALT by other groups [35] and by our current results for the lungs.

We observed an enrichment in effector memory $CD4^+$ T cells in BAL cells versus PMBCs. The immune system is organized in primary and secondary lymphoid tissues and peripheral effector sites including the lungs [36,37]. This finding is expected given that lung mucosal tissues are immunological effector sites constantly exposed to infectious and environmental agents and undergo chronic antigenic stimulation. effector memory cells were previously identified as the main subset supporting HIV transcription in both peripheral blood and intestinal tissues in ART-treated adults [38]. Importantly, in peripheral blood of individuals on ART, effector memory T cells may encompass the majority of the intact and replication competent HIV-DNA among other memory T cells [4,26,27,33]. These cells promote HIV persistence via their ability to undergo homeostatic proliferation, which maintains and may increase the number of infected cells independently of de-novo infection after prolonged ART [22].

Another important observation we made was the increased frequency of activated $CD4^+$ T cells in lung mucosa compared to blood. Cellular immune activation may contribute to HIV persistence through both promotion of HIV replication and enhanced susceptibility of bystander cells to infection [39]. Immune activation drives cycles of immune cell expansion until these cells eventually reach a replicative limit, characterized by the loss of co-stimulatory receptor CD28 in senescent T cells which are in cell-cycle arrest [40]. Importantly, we observed significantly higher levels of pulmonary CD4 T-cell activation in the HIV^+ group versus HIV^- participants. Senescent T cells are dysfunctional and less efficient at clearing infections. They also contribute to persistent upregulation of the pro-inflammatory responses [40]. Repeated exposures to tobacco, environmental toxins, and infectious agents induce repeated stress on the lungs and may result in immune activation and senescence. The accumulation of senescent T cells could impair the cell maintenance and repair capabilities of the lungs and promote lung tissue destruction in the long-term [40]. In HIV^+ adults, chronic obstructive pulmonary disease and lung cancers are reported much earlier than in general population and are associated with high mortality independent of smoking [1,2]. Therefore, the high $CD4^+$ T-cell differentiation, activation and senescence could contribute to greater HIV burden and mucosal pro-inflammatory milieu [1].

$CCR6^+$ Th17 cells trafficking into the gut and vaginal mucosae are preferentially infected very early following infection and harbor higher levels of replication-competent HIV-DNA than $CCR6^-$ T cells despite suppressed viral load by ART [16,41,42]. In addition, Th1Th17 cells express molecular signatures associated with HIV permissiveness [43] and contained high levels of HIV-DNA under suppressive ART [17,44]. Furthermore, $CCR6^+CXCR3^-CCR4^-$ Th cells have been proposed as another preferential reservoir for HIV [16]. Importantly, we found higher frequencies of memory $CCR6^+$, Th1Th17, and $CCR6^+CXCR3^-CCR4^-$ $CD4^+$ T cells in the pulmonary mucosa versus blood, highlighting the potential contribution of various lymphoid subsets to higher HIV burden within the lungs. Of note, Th1Th17 cells were enriched within the lungs only in HIV^+ individuals. In line with our data, Brenchley *et al*. observed that, in contrast to the gut, Th17 cells were not preferentially lost from BAL of HIV-infected individuals [45]. Considering the limitations in performing HIV reservoir measurement on rare cell subsets from the lungs, whether lung-infiltrating Th17 cells comprise HIV reservoirs in the lungs remains an open question.

We previously showed that higher levels of immunosuppressive Tregs and imbalance of effector T cells and Tregs in blood and gut mucosa are associated with suppression of anti-HIV T-cell responses [31,46]. Here, we observed a higher frequency of Tregs expressing ectonucleotidases CD39/CD73 in lungs versus blood of HIV^+ individuals. These Tregs are involved in HIV disease progression and suppression of anti-HIV/SIV T-cell responses via the hydrolysis of inflammatory ATP into immunosuppressive adenosine [30,31,47]. The increase in ectonucleotidases-expressing Tregs could be a consequence of the presence of highly activated effector cells in the lungs' mucosa and/or proinflammatory senescent T-cells as observed in this study. Tregs could be either beneficial, suppressing T cells

activation, or harmful, decreasing HIV-specific T-cell responses, cytokine production, and T-cell proliferation, thus contributing to viral persistence [31,46].

We finally investigated the expression of the newly proposed marker of HIV latency in quiescent infected cells, CD32a, a low-affinity IgG Fc fragment receptor [19]. We found higher expression of CD32a on pulmonary CD4$^+$ T cells versus blood. Importantly, these cells expressed high levels of activation marker HLA-DR and CCR5 within the lungs, which is in line with another recent study [48]. Furthermore, following HIV infection of unstimulated PBMCs *ex vivo*, infected cells concomitantly upregulated the expression of both CD32 and HLA-DR [25]. Indeed, productively infected cells expressing HIV-RNA and Gag protein upregulated these markers by two-folds [25]. Although we assessed CD32a as a potential marker of HIV infection, it is not known whether CD32$^+$ pulmonary cells are indeed HIV-infected. Due to their very low proportion within pulmonary CD4$^+$ T cells we were not able to address this question by FACS-sorting.

Our study has some limitations. The lungs are complex organs with various cells that synthesize an abundance of fluids, antimicrobial proteins, and mucins. It is unknown whether HIV may persist in these cells and how their products may contribute to viral persistence. We attempted to examine FACS-sorted CD4$^+$ T-cell subtypes proposed to be HIV reservoirs, but we were limited by cell numbers required for the sorting since macrophages represent more than 90% of cells, while lymphocytes represent less than 10% of cells in BAL [49]. We also did not perform genetic sequencing of HIV variants found in BAL versus blood. Studies from the ART era showed a phylogenetic separation of HIV lineages versus blood [7,50].

In summary, we provide evidence supporting the lungs as anatomical sites of HIV persistence during long-term ART. Cells that contribute to HIV persistence in the lungs include CD4$^+$ T-cell subtypes proposed as HIV reservoirs in the blood. This knowledge on the composition of immune cells infiltrating or residing within the lung mucosa is a necessary step toward elucidating the factors contributing to HIV persistence and, ultimately, for bringing us closer toward an HIV cure.

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Conflicts of interest

There are no conflicts of interest.

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