The Journal of Infectious Diseases

MAJOR ARTICLE

HIV Suppression Restores the Lung Mucosal CD4$^+$ T-Cell Viral Immune Response and Resolves CD8$^+$ T-Cell Alveolitis in Patients at Risk for HIV-Associated Chronic Obstructive Pulmonary Disease

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Background. Lung CD4$^+$ T-cell depletion and dysfunction, CD8$^+$ T-cell alveolitis, smoking, and poor control of human immunodeficiency virus (HIV) are features of HIV-associated chronic obstructive pulmonary disease (COPD), but these changes have not been evaluated in smokers at risk for COPD. We evaluated the impact of viral suppression following initiation of antiretroviral therapy (ART) on HIV-specific immunity and the balance of the CD4$^+$ T-cell to CD8$^+$ T-cell ratio in the lung.

Methods. Using flow cytometry, we assessed the T-cell immune response in lung and blood specimens obtained from 12 actively smoking HIV-positive patients before ART initiation and after ART-associated viral suppression.

Results. HIV suppression resulted in enhanced lung and systemic HIV-specific CD4$^+$ T-cell immune responses without significant changes in CD8$^+$ T-cell responses. We observed an increase in lung ratios of CD4$^+$ T cells to CD8$^+$ T cells and CD4$^+$ T-cell frequencies, decreased CD8$^+$ T-cell numbers, and resolution of CD8$^+$ T-cell alveolitis after ART in 9 of 12 individuals. Viral suppression reduced Fas receptor and programmed death 1 expression in lung CD4$^+$ T cells, correlating with enhanced effector function and reduced susceptibility to apoptosis. HIV suppression rescued peripheral but not lung HIV-specific CD4$^+$ T-cell proliferation, resulting in augmented effector multifunction.

Discussion. Together, our results demonstrate that HIV suppression restores lung mucosal HIV-specific CD4$^+$ T-cell multifunctional immunity and balance in the ratio of CD4$^+$ T cells to CD8$^+$ T cells, often resolving CD8$^+$ T-cell alveolitis in active smokers. Peripheral expansion and redistribution of CD4$^+$ T cells and increased resistance to apoptosis are 2 mechanisms contributing to immunologic improvement following viral suppression in patients at risk for HIV-associated COPD.

Keywords. HIV; COPD; CD4$^+$ T cells; CD8$^+$ alveolitis; antiretroviral therapy.

From early in the human immunodeficiency virus (HIV) epidemic, studies of bronchoalveolar lavage (BAL)–derived lung mononuclear cells (LMNCs) demonstrated that HIV-infected individuals frequently develop CD8$^+$ T-cell alveolitis [1–3]. These studies showed that CD8$^+$ T-cell lymphocytic alveolitis often occurred in asymptomatic individuals and in the absence of opportunistic infection. Additionally, lung mucosal CD8$^+$ T cells from HIV-infected individuals demonstrated the capacity to kill autologous HIV-infected macrophages and produce high levels of the effector cytokine interferon γ (IFN-γ) [1, 4]. However, CD8$^+$ T-cell alveolitis has also been associated with respiratory symptoms and worse outcomes in HIV-infected individuals, particularly in those with HIV progression and decreased survival [2, 5]. The advancement of antiretroviral therapy (ART) has led to improved survival among HIV-infected individuals [6], with an estimated 1.2 million people living with HIV/AIDS in the United States in 2008. However, a recent study evaluating the use of ART in the United States surprisingly found that only 37% of 1.2 million HIV-infected individuals were prescribed ART and that only 30% achieved effective HIV suppression [7]. Thus, suboptimal ART use and viral suppression remain significant challenges in the HIV-infected population, as poor HIV control is known to drive chronic immune activation, HIV progression and shortened survival [8].

Evolving data support an increased likelihood of chronic obstructive pulmonary disease (COPD) development among HIV-infected individuals [9, 10]. Indeed, CD8$^+$ lymphocytic alveolitis was postulated early on to play an important role in the pathogenesis of HIV-associated COPD, although the

Received 6 June 2016; accepted 1 September 2016; published online 9 September 2016.

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The Journal of Infectious Diseases® 2016;214:1520–30

DOI: 10.1093/infdis/jiw422

mechanisms were undefined [11, 12]. We previously showed that increased levels of markers of poor HIV control were associated with accelerated lung decline in HIV-infected smokers, compared with HIV-negative controls [13]. Additionally, in an analysis of lung autopsy specimens, areas of histologically confirmed emphysema displayed a strong presence of HIV RNA, whereas only rare HIV-infected cells were evident in normal lungs [14]. Further, we recently demonstrated that individuals with HIV-associated COPD demonstrate a significant $CD8^+$ T-cell alveolitis immunophenotype, as well as depletion and dysfunction of lung mucosal $CD4^+$ T cells, compared with HIV-infected controls without COPD [15]. Interestingly, we observed that the HIV-specific $CD8^+$ T-cell immune response in the lung is preserved in subjects with HIV-associated COPD, in contrast to $CD4^+$ T-cell dysfunction. However, it remains unclear what the contributory roles of both HIV control and smoking have in driving $CD8^+$ T-cell alveolitis and T-cell function among individuals at risk for COPD, particularly with smoking being more prevalent in the HIV-infected population. We hypothesized that HIV replication would have a substantial impact on the presence or absence of $CD8^+$ T-cell alveolitis and, in particular, lung $CD4^+$ T-cell numbers and the HIV-specific $CD4^+$ T-cell immune response among smokers without spirometric abnormalities consistent with COPD.

To test this, we studied paired samples from the LMNC and peripheral blood mononuclear cell (PBMC) tissue compartments following effective HIV suppression after initiation of ART in individuals at risk for HIV-associated COPD, defined as current smokers with no evidence of airflow obstruction revealed by spirometry. We evaluated lung mucosal and peripheral lymphocyte subsets and phenotype and effector function at time points before and after ART initiation and found that effective HIV suppression with ART resulted in resolution of $CD8^+$ T-cell alveolitis in the majority of subjects, significant enhancement in lung and peripheral $CD4^+$ T-cell numbers, and a striking enhancement of lung mucosal and systemic HIV-specific $CD4^+$ T-cell effector memory multifunction (ie, cells producing >1 effector cytokine or molecule).

MATERIALS AND METHODS

Subjects and Tissue Samples

Participants were recruited from the Johns Hopkins HIV Care Clinic after being screened at scheduled clinic visits. They were considered eligible for this study if they provided informed consent to undergo bronchoscopy before and following initiation of ART and, in the prior 6 weeks, had no history of pneumonia or worsening of breathing status requiring steroids or antibiotics. Prebronchodilator spirometry testing was performed in 10 of 12 participants to assess for the presence of obstructive lung disease (defined as a forced expiratory volume in 1 second [FEV1]/forced vital capacity [FVC] ratio of < 0.70). Prebronchodilator spirometry to determine FEV1 and FVC was performed using KOKO pneumotachometers (nSpire Health, Longmont, CO). All spirograms were assessed automatically and reviewed by a pulmonary physician in accordance with American Thoracic Society guidelines [16]. Percentages of predicted values were calculated using standard formulas [17]. COPD was defined as an FEV1/FVC ratio of <0.70 [18]. Only individuals free of COPD were eligible for this analysis. All study procedures were approved by the Johns Hopkins Institutional Review Board, and all participants provided informed consent. Study participants underwent bronchoalveolar lavage (BAL) by use of a standard protocol with instillation of 180 mL of sterile normal saline in the right middle lobe of the lung. A blood specimen was obtained on the same day for PBMC collection.

Cell Preparation, Culture, and Antigen Stimulation

PBMCs were isolated from heparinized blood by density gradient centrifugation, using Ficoll-Paque (GE Healthcare). LMNCs were obtained via centrifugation of BAL fluid. Cells were cryopreserved in liquid nitrogen storage. Pooled overlapping 15-mer peptides for HIV Gag and Pol were obtained from the National Institutes of Health AIDS Reagent Program. Cells were cultured in round-bottomed tissue culture tubes (Sarstedt) with or without HIV peptides or anti-CD3/anti-CD28 antibodies at 1 μg/mL each for 6 hours at 37°C. All stimulations for intracellular cytokine production were performed using 10^6 cells/condition for 6 hours at 37°C, with brefeldin A (10 μg/mL) added for the final 4 hours of culture. Monensin (5 μg/mL) along with brefeldin A and anti-CD107a-Pacific Blue (PacBlue) were added when CD107a was measured. In proliferation assays, cells were labeled with CFSE (0.2 μM; Invitrogen) and cultured for 6 days. Cell cultures were harvested on day 6, washed, and rested overnight in medium alone. Secondary restimulation was performed in the presence or absence of HIV peptides (ie, Pol or Gag) or anti-CD3/anti-CD28 for 6 hours, and proliferation was assessed by CFSE dilution and intracellular cytokine staining (ICS).

Flow Cytometry

Following restimulation, cells underwent surface staining with anti–CD3-AlexaFluor700, anti–CD4-allophycocyanin-Cy7, anti–CD8-violet500, anti–CD95-FITC, and anti–programmed death 1 (PD-1)-PE. Live/Dead Fixable Blue Dead Cell stain was used for gating on viable cells (Invitrogen). ICS was performed using anti–IFN-γ-allophycocyanin, anti–tumor necrosis factor (TNF-α)-PE-cyanine7, anti–interleukin 2 (IL-2)-BV650, and anti-CD107a-PacBlue (BD Biosciences). All cells were collected for flow cytometry, with a range of 0.5×10^6 to 1×10^6 events collected per condition. All gates for cytokine frequencies were set using the medium-alone control and subtracted from peptide-restimulated sample frequencies. For apoptosis studies cells, were stained with anti–annexin V-Brilliant Violet 450, using 10 μg/mL concentrations of either anti-Fas immunoglobulin G1 (IgG1; clone-ZB4; neutralizing) or anti-Fas immunoglobulin M (IgM; clone-CH11; activating), and were bound

to plates for 12 hours at 37°C, or they were stained with isotype control antibodies (Millipore). LMNCs were cultured with or without plate-bound anti-Fas antibodies (ZB4) or activating antibodies (CH11) at 10 μg/mL, or they were cultured with an isotype control (IgG1) with or without Pol peptides. Cell fluorescence was analyzed using a custom LSR Fortessa cytometer with a UV laser (BD Biosciences). Flow cytometry data were analyzed using FlowJo software (Tree Star).

Statistical Analysis

Distributions of all measured variables were performed using nonparametric testing. Mean ICS frequencies were compared by means of Mann–Whitney and Wilcoxon signed rank analysis, using GraphPad Prism. Multifunctional analysis and presentation of distributions was performed using SPICE software, version 5.1 (available at: http://exon.niaid.nih.gov/spice) [19]. A P value of $< .05$ was used to determine statistical significance.

RESULTS

HIV Suppression With ART Results in Increased Lung Mucosal and Systemic CD4$^+$ T-Cell Frequencies, Increased CD4$^+$ T-Cell to CD8$^+$ T-Cell Ratios, and Diminished CD8$^+$T-Cell Numbers

We assessed the impact of initiating ART on the balance of T-cell subsets in LMNCs in 12 HIV-infected individuals. The cohort characteristics are described in Table 1. At study enrollment, the median age of the cohort was 40 years (interquartile range [IQR], 29–48 years). Nearly two thirds of the cohort was African American, and 82% were male. All participants were active smokers at study enrollment, and none had ceased smoking at the time of the second bronchoscopy. The median FEV1/FVC ratio was 0.80 (IQR, 0.77–0.84; range, 0.70–0.86). The median percentage of the predicted FEV1 value was 96.5% (IQR, 89%–103%; range, 72%–113%). The median number of months between the first and second bronchoscopy was 8.6 months (IQR, 6.4–13.8 months; range, 6.2–17.7 months). As seen in Table 1, at the time of the pre-ART bronchoscopy, the median viral load was 19 646 copies/mL (IQR, 5114–48 363 copies/mL), which decreased to a median of <20 copies/mL at the time of the second bronchoscopy. The median CD4$^+$ T-cell count at time of the pre-ART bronchoscopy was 387 cells/mm^3 (IQR, 331–532 cells/mm^3), which significantly increased to 518 cells/mm^3 after ART initiation, whereas the absolute CD8$^+$ T-cell counts did not significantly decrease. As shown in Figure 1*A*–*D*, ART resulted in increased lung and blood CD4$^+$ T-cell frequencies, along with a significant increase in the ratio of CD4$^+$ T cells to CD8$^+$ T cells, compared with pre-ART values (the gating strategy is available in Supplementary Figure 1). In contrast, CD8$^+$ T-cell frequencies were reduced in the LMNC and PBMC compartments following HIV suppression (Figure 1*D*). In addition, absolute numbers of CD4$^+$ LMNCs were significantly increased and CD8$^+$ LMNCs were significantly decreased per milliliter of BAL obtained (Supplementary Figure 1*B* and 1*C*). Further, ART resulted in an increase in the BAL ratio of CD4$^+$ T cells to CD8$^+$ T cells in all patients and in resolution of CD8$^+$ T-cell alveolitis in 9 of 12 patients, based on a BAL CD4$^+$ T-cell to CD8$^+$ T-cell ratio of >1.0. Similar increases in CD4$^+$ T-cell frequencies and ratios of CD4$^+$ T cells to CD8$^+$ T cells were observed in PBMCs following ART initiation, along with diminished CD8$^+$ T-cell frequencies. Together, these data demonstrate that HIV control significantly increases lung mucosal and peripheral CD4$^+$ T-cell numbers while diminishing HIV-associated CD8$^+$ T-cell alveolitis, despite ongoing cigarette smoking.

Table 1. Clinical and Demographic Characteristics of 12 Human Immunodeficiency Virus (HIV)–Positive Study Participants

Characteristic	Value
Age, y, mean ± SD	39.6 ± 8.7
African American	8 (66)
Male sex	9 (75)
Current smoker	12 (100)
FEV1/FVC ratio	0.80 (0.70–0.86)
FEV1, % of predicted value	96.5 (72–113)
Time between bronchoscopies, mo	8.6 (6.2–17.7)
ART use	12 (100)
T-cell count and viral load	
Before ART initiation	
CD4$^+$ T-cell count, cells/mm^3	387 (6–668)
CD8$^+$ T-cell count, cells/mm^3	628 (185–1036)
Viral load, copies/mL	19 646 (1576–1 511 420)
After ART initiation	
CD4$^+$ T-cell count, cells/mm^3	518[a] (158–853)
CD8$^+$ T-cell count, cells/mm^3	588 (95–1018)
Viral load, copies/mL	<20[a] (<20–81)

Data are no. (%) of participants or median value (range), unless otherwise indicated.

Abbreviations: ART, antiretroviral therapy; FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity.

[a] $P < .001$, compared with before ART initiation.

HIV Suppression With ART Leads to Strikingly Increased Lung Mucosal HIV-Specific CD4$^+$ T-Cell Effector Frequencies and Multifunction but No Change in CD8$^+$ T-Cell Effector Capacity

Mucosal virus-specific T-cell responses play an important role in HIV control. Therefore, we next assessed HIV-specific effector function in lung mucosal T cells at time points before and after ART initiation. Systemic HIV suppression resulted in significant increases in HIV-specific lung mucosal production of IFN-γ, TNF-α, IL-2, and cytotoxic CD107a (degranulation marker) by CD4$^+$ T cells in response to pooled peptides of major HIV antigens (Pol [Figure 2*A*–2*C*] and Gag) and anti-CD3/CD28 (Supplementary Figure 2*A*–2*F*). HIV-specific CD4$^+$ T-cell responses in the periphery were mixed, with enhanced Pol-specific responses and a trend toward IFN-γ–secreting, Gag-specific CD4$^+$ T-cell responses in PBMCs after ART initiation, compared with before ART initiation (Supplementary Figure 2). In contrast, we did not observe significant

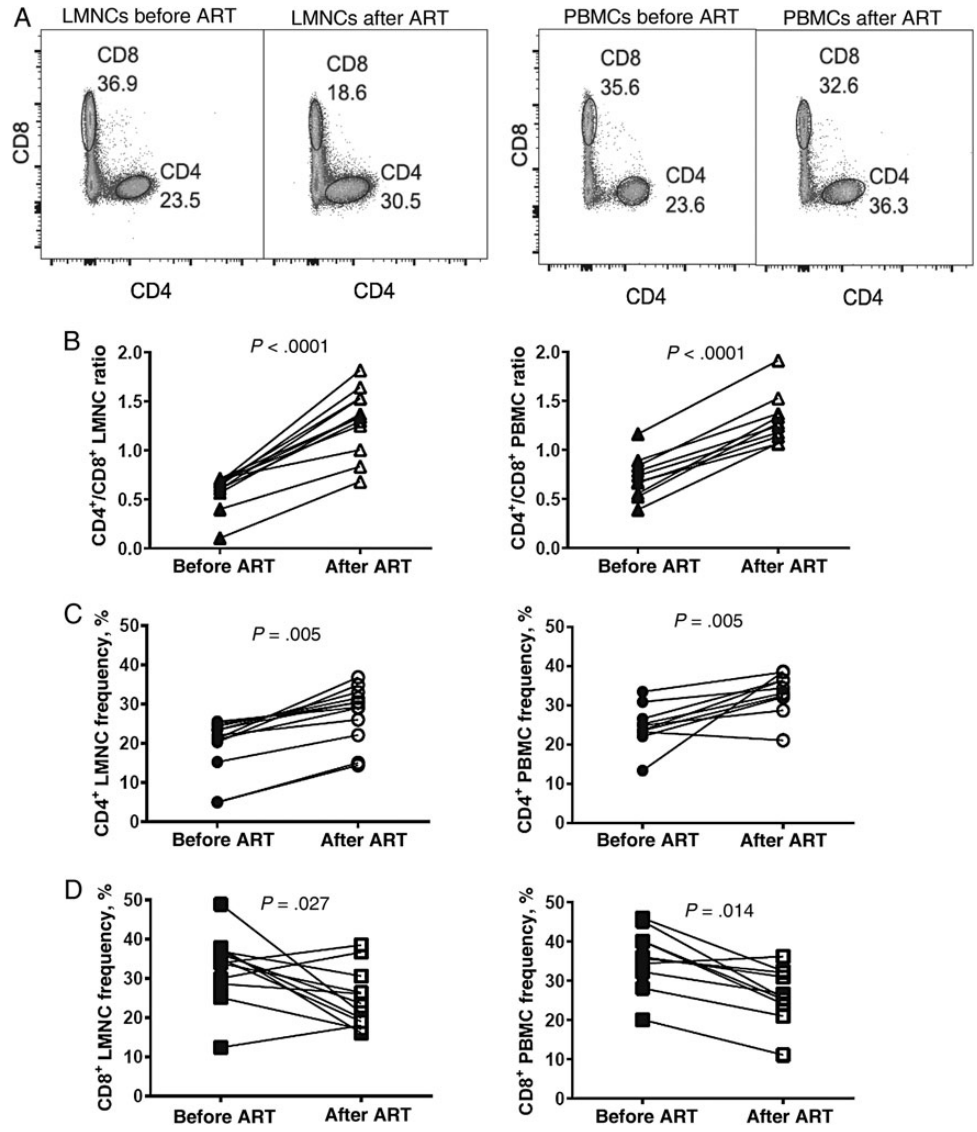

Figure 1. Human immunodeficiency virus (HIV) suppression with antiretroviral therapy (ART) results in increased lung mucosal and systemic $CD4^+$ T-cell frequencies, increased $CD4^+$ T-cell to $CD8^+$ T-cell ratios, and diminished $CD8^+$ T-cell frequencies in active smokers. *A*, Representative flow cytometry plots of $CD4^+$ and $CD8^+$ T-cell frequencies of lung mononuclear cells (LMNCs; left panels) and peripheral blood mononuclear cells (PBMCs; right panels) obtained from individuals during chronic HIV infection (before ART initiation), compared with post-ART values. *B*, Pooled data showing the ratio of $CD4^+$ T cells to $CD8^+$ T cells among LMNCs from 12 individuals (left panel) and PBMCs from 10 individuals (right panel) from HIV-positive patients before (filled triangles) and after (empty triangles) ART initiation. *C*, Pooled data showing the frequencies of $CD4^+$ T cells among LMNCs from 12 HIV-positive patients (left panel) and among PBMCs from 10 patients (right panel) after ART initiation (empty dots), compared with pre-ART values (filled dots). *D*, Pooled data showing that $CD8^+$ T-cell frequencies decreased in the LMNC (left panel) and PBMC (right panel) compartments before ART initiation (filled squares) and after HIV suppression (empty squares). *P* values were calculated using the Wilcoxon signed rank test.

changes in HIV-specific or polyclonal effector $CD8^+$ T-cell frequencies in the lung mucosa or PBMC compartment following ART initiation in our cohort (Supplementary Figures 3 and 4). We next evaluated the quality of HIV-specific $CD4^+$ T-cell immune responses in the lung mucosa and PBMC compartment and assessed effector multifunction (Figure 2*C*). $CD4^+$ HIV Pol–specific effector multifunction was significantly enhanced in both LMNCs and PBMCs after ART initiation. Together, these data show significant quantitative and qualitative improvements in lung mucosal HIV-specific $CD4^+$ T-cell immune response with HIV suppression following ART, compared with less substantial changes in the HIV-specific $CD8^+$ T-cell immune response.

HIV Suppression Significantly Reduces Expression of the Fas Receptor (CD95) and Coinhibitory Molecule PD-1 in Lung Mucosal $CD4^+$ T Cells and Correlates With Enhanced Effector Function and Reduced Susceptibility to HIV Antigen–Induced Cell Death Via the Fas Pathway

We previously demonstrated increased surface expression of CD95/PD-1 and susceptibility to activation-induced cell death (AICD) via the Fas pathway in $CD4^+$ T cells in the LMNC

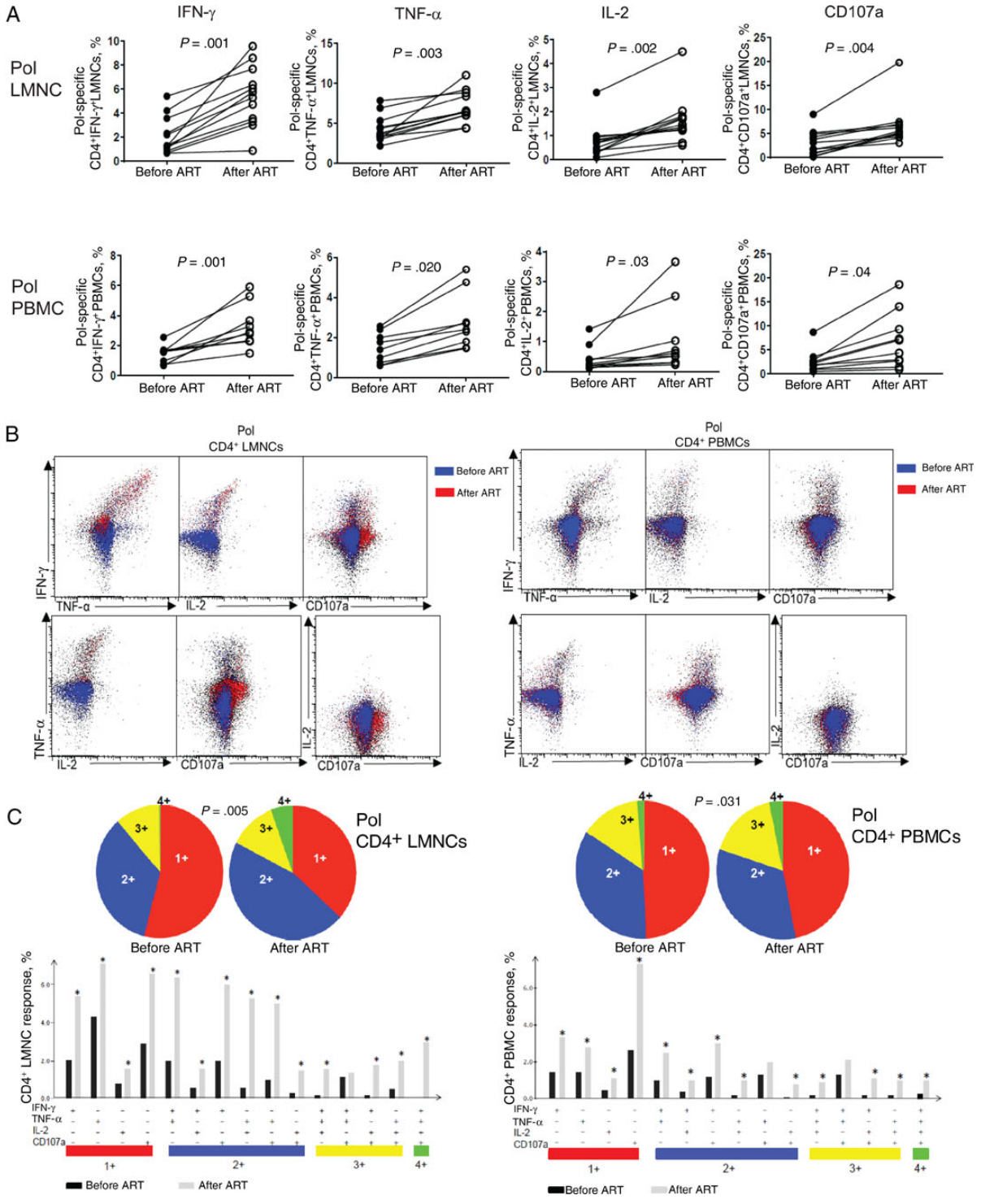

Figure 2. Human immunodeficiency virus (HIV) suppression results in strikingly increased lung mucosal HIV-specific CD4$^+$ effector T-cell frequencies and enhanced CD4$^+$ T-cell immune response and effector multifunction in lung mononuclear cells (LMNCs) and peripheral blood mononuclear cells (PBMCs). *A*, Pooled data showing frequencies of HIV-specific CD4$^+$ effector T cells expressing interferon γ (IFN-γ), tumor necrosis factor α (TNF-α), interleukin 2 (IL-2), and cytotoxic CD107a (degranulation marker) at time points before (filled dots) and after (empty dots) antiretroviral therapy (ART) initiation in lung mucosal (upper panels) and PBMC (lower panels) compartments in response to pooled peptides of the major HIV antigen Pol. P values were calculated using the Wilcoxon signed rank test. *B*, Representative flow cytometric plots of CD4$^+$ T-cell frequencies among LMNCs (left panels) and PBMC (right panels) from individuals during chronic HIV infection (before ART initiation; red dot plots), compared with after ART initiation (blue dot plots). Analyzed plots from effector CD4$^+$ T cells recovered before and after ART initiation were then overlayed (FlowJo) and compared. *C*, Pooled data showing individual pie charts of multifunctional effector responses of HIV Pol–specific lung mucosal CD4$^+$ T cells (left panels) and peripheral blood mononuclear CD4$^+$ T cells (right panels) after ART initiation (right pie charts), compared with before ART initiation (left pie charts). Using Boolean analysis, the percentage of total, individual multifunctional subset responses before (black bars) and after (gray bars) ART initiation are shown in the bar graph for the multifunctional subsets. *$P \leq .05$ by the Kruskal–Wallis 1-way analysis of variance and Wilcoxon signed rank test (SPICE) for comparison of mean frequencies of Pol-specific single and multifunctional responses.

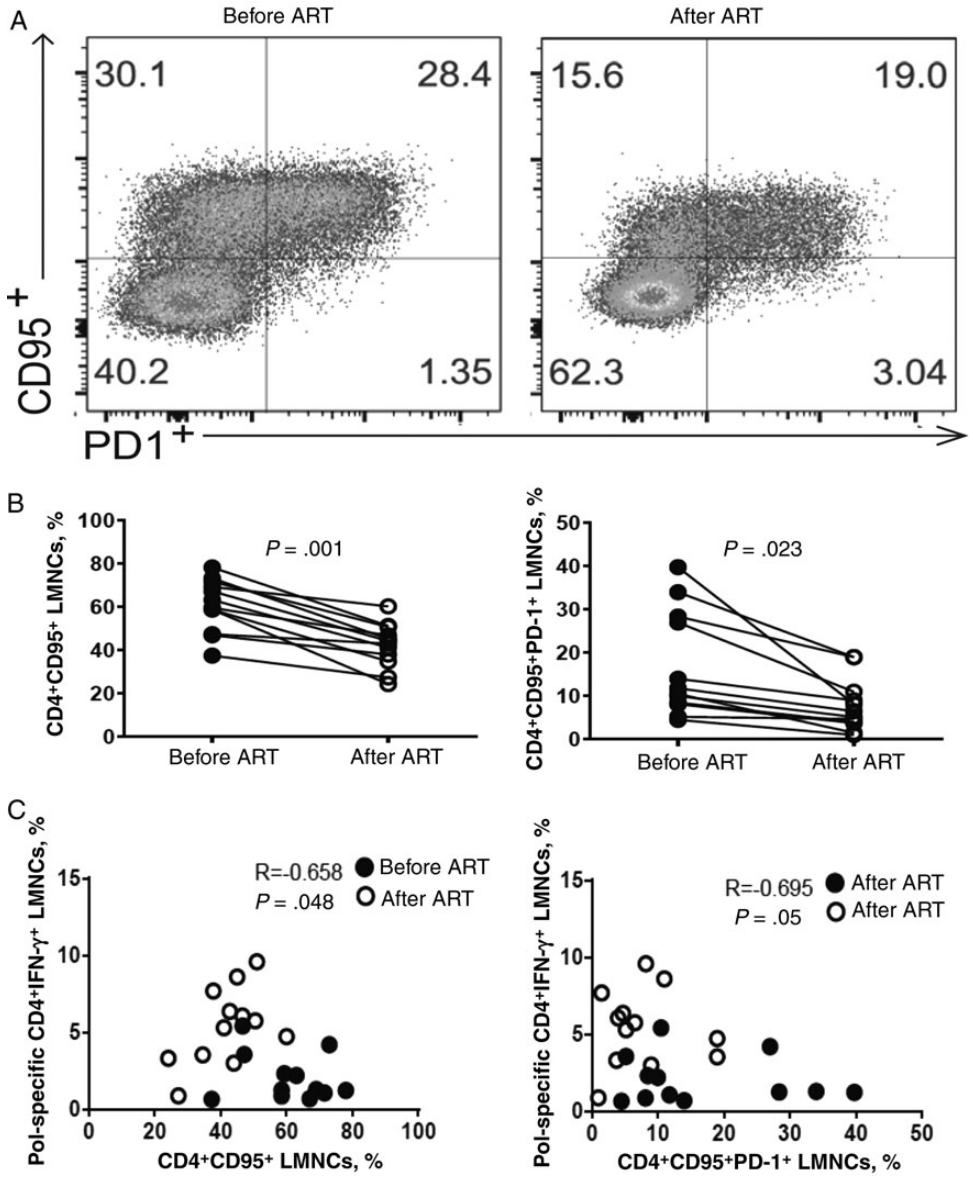

Figure 3. Human immunodeficiency virus (HIV) suppression reduces CD95/programmed death 1 (PD-1) surface expression on lung mucosal $CD4^+$ T cells and correlates with enhanced effector function during antiretroviral therapy (ART). *A*, Representative flow cytometric plots of $CD4^+$ T-cell frequencies of 12 lung mononuclear cells (LMNCs) analyzed from individuals during chronic HIV infection (before ART initiation; left dot plots), compared with after ART initiation (right dot plots). *B*, Pooled data showing the expression of $CD95^+$ in $CD4^+$ LMNCs (left panel) and $CD95^+PD1^+$ in $CD4^+$ LMNCs (right panel) at time points before (filled dots) and after (empty dots) ART initiation. *C*, Correlation of frequencies of lung HIV Pol–specific, interferon γ (IFN-γ)–expressing $CD4^+$ LMNCs and $CD4^+CD95^+$ (left panel) or $CD4^+CD95^+PD\text{-}1^+$ (right panel) LMNCs after ART initiation (empty dots), compared with before ART initiation (filled dots). Correlation coefficients (R) were calculated by Spearman rho analysis, and *P* values were calculated using the Wilcoxon signed rank test.

compartment in patients with HIV-associated COPD [15]. Therefore, we assessed expression of these molecules at time points before and after ART initiation in lung $CD4^+$ T cells. Effective HIV suppression with ART resulted in reduced expression of CD95 and PD-1 in $CD4^+$ T cells in the LMNC compartment (Figure 3*A* and 3*B*). In addition, we observed reduced frequencies of $CD4^+CD95^+$ and $CD4^+CD95^+PD\text{-}1^+$ T cells among LMNCs after ART initiation as compared to before ART initiation, which directly correlated with increased HIV-specific, IFN-γ–secreting effector function among $CD4^+$ T cells in the lung (Figure 3*C*). Next, we investigated whether a reduction in CD95 surface expression following HIV suppression influenced AICD in $CD4^+$ T cells in the LMNC compartment. For this, we measured the early apoptotic marker annexin V and compared $CD4^+$ T cells in the LMNC compartment in medium alone and following in vitro restimulation with HIV antigen or anti–Fas-activating antibody at time points before and after ART initiation. We found that AICD was significantly

reduced following stimulation after ART initiation, as compared to time points before ART initiation (Figure 4*A* and 4*B*). We also evaluated blockade of Fas/Fas-ligand interactions, using an anti–Fas-neutralizing antibody, and found that HIV antigen–induced AICD via the Fas pathway was significantly reduced among $CD4^+$ T cells in the LMNC compartment after ART initiation, compared with controls (Figure 4*A* and 4*B*). Collectively, our data indicate that HIV control reduces CD95/PD-1 surface expression on lung mucosal $CD4^+$ T cells and correlates with enhanced effector function and reduced susceptibility of LMNC-associated $CD4^+$ T cells to AICD via the Fas pathway.

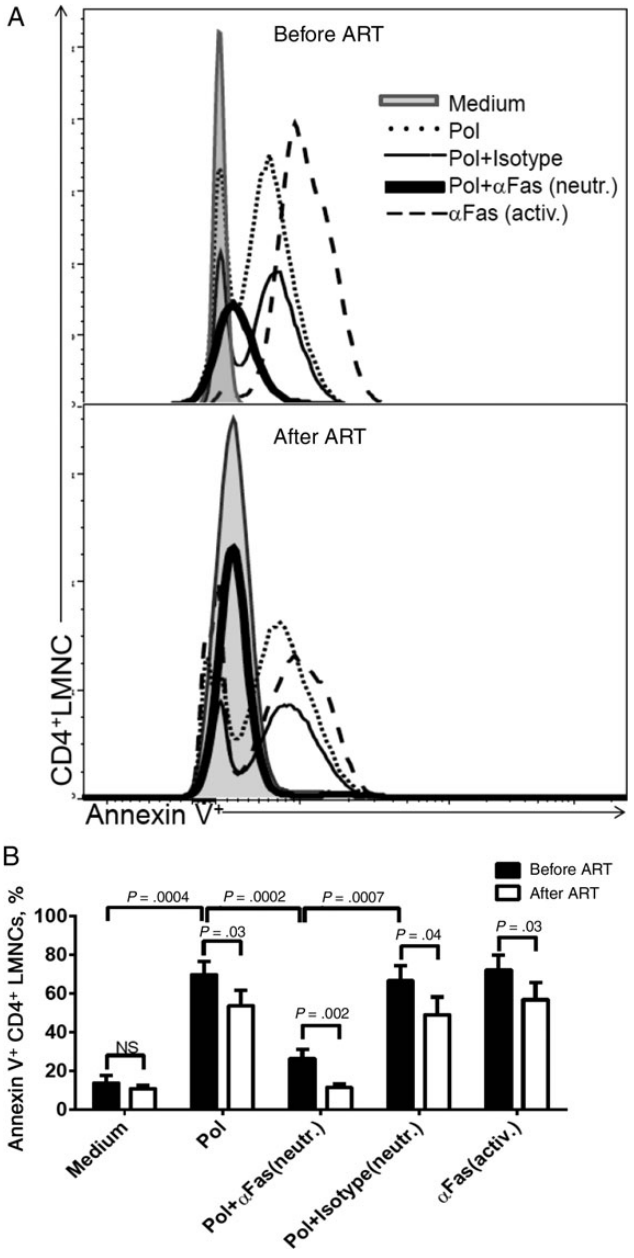

Figure 4. Human immunodeficiency virus (HIV) suppression reduces the susceptibility of lung mononuclear $CD4^+$ T cells to activation-induced cell death via the Fas pathway. *A*, Representative histograms depicting annexin V positivity among lung mononuclear $CD4^+$ T cells before (upper panel) and after (lower panel) antiretroviral therapy (ART) initiation. Lung mononuclear cells (LMNCs) were cultured in the presence or absence of 10 μg/mL plate-bound anti-Fas antibodies that are either neutralizing (ZB4) or activating (CH11), or they were cultured in the presence of an isotype control (immunoglobulin G1 for neutralizing clone shown) with or without stimulation with HIV-specific Pol peptides, as described in Methods. *B*, Pooled data showing annexin V–positive LMNC subsets from gating on live $CD3^+CD4^+$ T cells from 12 HIV-positive patients. Data are cumulative mean frequencies ± standard errors of the mean of annexin V–positive lung mononuclear $CD4^+$ T cells obtained before (filled columns) and after (empty columns) ART initiation. *P* values were calculated using the Wilcoxon signed rank test. Abbreviation: NS, not significant.

HIV Suppression Rescues Peripheral but Not Lung Mucosal HIV-Specific $CD4^+$ T-Cell Proliferation as a Mechanism for Enhanced Effector Function

The capacity of virus-specific T cells to proliferate is critical for the expansion of effector memory T cells [20]. Therefore, we next measured HIV-specific proliferative responses from T-cell subsets among LMNCs versus PBMCs at time points before and after ART initiation, using the CFSE dilution method. Lung mucosal $CD4^+$ and $CD8^+$ T cells demonstrated very poor to absent proliferative capacity in response to HIV antigen at day 6 (Figure 5*A*–5*C*). In striking contrast, the suppression of HIV viremia with ART resulted in strikingly (ie, 8–10-fold) increased HIV-specific $CD4^+$ T-cell proliferative responses at day 6 among PBMCs. Proliferative responses among the $CD8^+$ T-cell subset in PBMCs only increased by approximately 2-fold following ART initiation (Figure 4*B* and 4*C*). We then performed secondary restimulation of cultures of PBMCs obtained before and after ART initiation at day 6, and evaluated effector frequencies. The restoration of HIV-specific $CD4^+$ T-cell proliferative responses with suppression of HIV viremia resulted in significantly enhanced HIV-specific multifunctional responses at day 6 after ART initiation, compared with those on day 6 before ART initiation (Figure 4*C*). Collectively, our results support enhanced peripheral but not local lung expansion of HIV-specific $CD4^+$ T cells as a mechanism for increased $CD4^+$ T-cell numbers and HIV-specific effector multifunction in the lung mucosa following HIV suppression with ART.

DISCUSSION

Our results provide new insights into lung mucosal HIV-specific immunity, particularly underscoring impaired $CD4^+$ T-cell viral immunity that was quantitatively and qualitatively restored with viral suppression. Our studies also demonstrate resolution of $CD8^+$ T-cell alveolitis in the majority of patients with HIV suppression, but there was no significant change in lung HIV-specific effector frequencies, in contrast to $CD4^+$ T cells. Prior work had found either increased BAL-associated $CD4^+$ T-cell frequencies or decreased $CD8^+$ T-cell alveolitis after initiation of ART, although HIV-specific immunity was not directly assessed [21]. Importantly, we have identified 2 mechanisms that contribute to our observations of restored HIV-specific effector function among $CD4^+$ T cells: (1) an enhanced proliferative

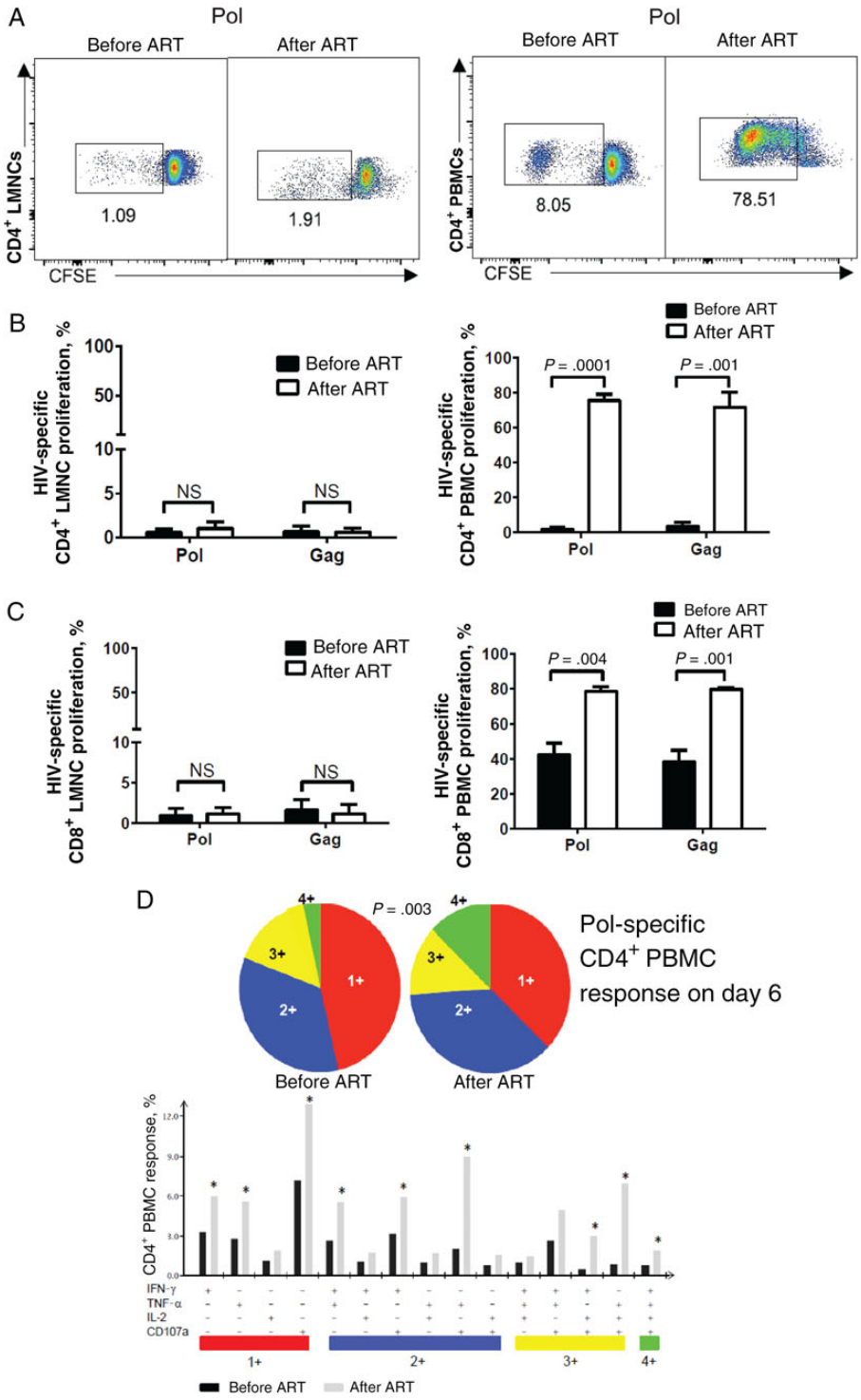

Figure 5. Human immunodeficiency virus (HIV) suppression rescues peripheral but not lung mucosal HIV-specific CD4$^+$ T-cell proliferation as a mechanism for enhanced effector multifunction. *A*, Representative flow cytometry plots showing Pol-specific lung mononuclear CD4$^+$ T-cell proliferative responses (left panels) and peripheral blood mononuclear CD4$^+$ T-cell responses (right panels) at day 6, determined by CFSE dilution, in HIV-positive patients before antiretroviral therapy (ART) initiation (left panel), compared with after ART initiation (right panel). Numbers indicate frequencies (percentages) of CFSE-gated diluted events. *B*, Pooled data showing the frequencies of HIV-specific CD4$^+$ T cells with proliferative responses in lung mononuclear cell (LMNC; left panels) and peripheral blood mononuclear cell (PBMC; right panels) compartments before (filled columns) and after (empty columns) ART initiation. *C*, Pooled data showing the frequencies of HIV-specific CD8$^+$ LMNCs (left panels) and PBMCs (right panels) before (filled columns) and after (empty columns) ART initiation. *D*, Individual pie charts of multifunctional responses at day 6 following restimulation with HIV Pol peptides and lung mononuclear CD4$^+$ T-cell effector multifunction after ART initiation (right pie charts), compared with before ART initiation (left pie charts). Using Boolean analysis, the percentage of individual multifunctional subset responses for pre-ART (black bars) and post-ART (gray bars) time points are shown in the bar graph for the multifunctional subsets. *$P \leq .05$ by Kruskal–Wallis 1-way analysis of variance or the Wilcoxon signed rank test for comparison of mean frequencies of Pol-specific single and multifunctional responses. Abbreviations: IFN-γ, interferon γ; IL-2, interleukin 2; NS, not significant; TNF-α, tumor necrosis factor α.

capacity of HIV-specific $CD4^+$ T cells in the periphery but not in the lung mucosa following viral suppression and (2) reduced susceptibility of lung $CD4^+$ T cells to AICD. Further, we have shown that the enhanced capacity of peripheral HIV-specific memory $CD4^+$ T cells to proliferate results in enhanced effector multifunction, consistent with our observations in the lung and periphery. In contrast, these antigen-specific mechanisms might not impact the total $CD4^+$ T-cell pool in these compartments, and they explain why polyclonal responses were impacted to a lesser degree. Notably, in addition to increases in IFN-γ, TNF-α, and CD107a secretion among $CD4^+$ T cells with viral suppression, HIV-specific IL-2 responses significantly increased in the lung and periphery. Earlier studies have demonstrated that loss of multifunctional $CD4^+$ T cells, and particularly IL-2 production by HIV-specific $CD4^+$ T cells, correlated with HIV disease progression [22–24]. Unexpectedly, our studies found that viral suppression did not significantly impact lung mucosal and peripheral HIV-specific $CD8^+$ effector T-cell frequencies and multifunction, although some responses trended toward improvement. Taken together, our findings support the concept that effective HIV control has a more substantial impact on HIV-specific $CD4^+$ effector T-cell frequencies and multifunction in the lung and periphery.

$CD8^+$ T-cell alveolitis is often present in asymptomatic individuals but is also found in patients with respiratory symptoms and HIV disease progression. Our study shows a significant increase in the ratio of $CD4^+$ T cells to $CD8^+$ T cells among LMNCs due to increased $CD4^+$ T-cell frequencies/absolute numbers and reduced $CD8^+$ T-cell frequencies/absolute numbers, with similar findings in the blood as previously described [25]. Therefore, these dynamic changes in the lung mucosal T-cell pool following ART initiation result in significant resolution of $CD8^+$ T-cell alveolitis. Thus, while immune reconstitution pulmonary syndromes have been well described after initiation of ART, the overall restoration to a more physiologic ratio of $CD4^+$ T cells to $CD8^+$ T cells among LMNCs appears beneficial in regard to long-term pulmonary symptoms and disease [26]. For example, we recently showed that patients with HIV-associated COPD demonstrated profound $CD4^+$ T-cell depletion and $CD8^+$ T-cell alveolitis, compared with HIV-infected controls without COPD [15]. Moreover, the patients in our current study remained active smokers, underscoring the critical importance of viral control in maintaining lung mucosal homeostasis in the setting of HIV infection. However, ongoing smoking may synergize with chronic inflammation that is known to occur during chronic HIV infection even after viral control and may contribute to persistent inflammation that may be deleterious to lung function [8, 27–29].

The negative impact of HIV viremia on the capacity for peripheral HIV-specific $CD4^+$ T-cell proliferation was first reported in a seminal study by McNeil et al [30] Importantly, this effect on $CD4^+$ T-cell proliferation has been documented in other viral infections, such as measles [31], hepatitis B [32], dengue [33], and acute CMV infection [34, 35]. Using the sensitive CFSE dilution method, we did not detect significant T-cell proliferation in LMNCs even following effective viral suppression, in striking contrast to the observed restoration of HIV-specific $CD4^+$ grater than $CD8^+$ PBMC proliferative responses. Our results are consistent with those of earlier murine studies that demonstrated an impaired ability of BAL-associated T cells to proliferate yet retain a high capacity for effector cytokine secretion and continual systemic recruitment to the airways [36, 37]. Whether this represents an intrinsic defect in lung mucosal T cells or whether it is due to reduced accessory cell function capable of supporting robust proliferation, as suggested by earlier human studies, remains unclear [38, 39]. Nevertheless, our results support a model for peripheral redistribution of $CD4^+$ T cells leading to restoration of lung mucosal $CD4^+$ T-cell numbers, rather than local expansion. In addition to restoration of robust peripheral HIV-specific $CD4^+$ T-cell proliferation with ART, our studies show that viral suppression significantly reduces lung $CD4^+$ T-cell susceptibility to apoptosis via the Fas pathway [40, 41], providing a mechanism for enhanced survival. However, while our data support the concept that reduced chronic immune activation in aviremic individuals attenuates susceptibility to apoptosis, other factors, such as ongoing immune activation and persistent latent viral reservoirs, may also contribute to AICD susceptibility in $CD4^+$ T cells. Further, our studies found that reduced surface expression of CD95 and PD-1 following viral control correlated with improved lung $CD4^+$ effector T-cell function in addition to survival fitness. These results are consistent with those of a recent study that also reported lung T-cell dysfunction in the setting of chronic HIV replication, as well as those of our previous study showing a functional defect in lung mucosal $CD4^+CD95^+PD\text{-}1^+$ T cells in patients with HIV-associated COPD [15, 42]. Collectively, our findings support restoration of peripheral but not local $CD4^+$ T-cell proliferation and reduced susceptibility to AICD, resulting in enhanced HIV-specific $CD4^+$ lung mucosal/systemic effector T-cell function following effective HIV suppression.

There are several caveats to our studies. First, we recognize the small number of patients in our study, as the bronchoscopies were not clinically indicated, but rather were performed for research purposes. Nonetheless, we were able to detect significant differences in lung/systemic HIV-specific immunity, particularly in $CD4^+$ T cells. However, the clinical relevance of these findings for HIV host defense remains unclear, as these immune findings may be a function of control of viremia, given that patients relapse after stopping ART. Our findings strongly support that initiation of ART restores the lung mucosal environment to a more physiologic balance in the ratio of $CD4^+$ T cells to $CD8^+$ T cells despite ongoing smoking in patients. This is important because 2 major risk factors for the development of COPD, smoking and lower socioeconomic

status, are overrepresented among HIV-infected individuals [43, 44]. Further, recent evidence indicates that ART remains significantly underprescribed in the United States and, surprisingly, that a minority of patients have achieved optimal HIV suppression [7]. Moreover, resolution of $CD8^+$ T-cell alveolitis may reduce the risk for development of HIV-associated COPD, as we recently demonstrated that these patients have profound lung $CD4^+$ T-cell depletion and $CD8^+$ T-cell alveolitis, which may exacerbate the ongoing inflammation associated with smoking. Importantly, several studies have implicated $CD8^+$ T cells in the pathogenesis of non–HIV-associated COPD, with enhanced cytotoxic potential correlating with disease severity [45–48]. However, while our BAL findings show decreased lung mucosal $CD8^+$ T cells following ART initiation, without histopathologic analysis we cannot be absolutely certain that lung parenchymal $CD8^+$ T cells are decreased. A second limitation to our study was that we were not able to fully discern the inflammatory effect of smoking in this population, as all subjects were actively smoking during the study. Nevertheless, we argue that our immunologic findings underscore the importance of effective viral suppression in this population at risk for developing HIV-associated COPD. Indeed, our findings provide plausible mechanisms that support our previous findings of an accelerated decline in subjects with markers of advanced HIV disease in a predominantly smoking population [49, 50]. However, further studies are needed in HIV-infected individuals with effective viral suppression to elucidate the proinflammatory effects of smoking, compared to HIV+ non smokers.

In summary, we report that effective HIV suppression with ART restores lung mucosal HIV-specific $CD4^+$ T-cell immune responses and resolves $CD8^+$ lymphocytic alveolitis in active smokers at risk for HIV-associated COPD. We have found that lung mucosal HIV-specific $CD4^+$ immune responses were quantitatively and qualitatively enhanced following ART initiation. These numerical and functional increases in lung $CD4^+$ T cells were observed along with strikingly increased peripheral but not local $CD4^+$ T-cell proliferative responses to HIV antigen and reduced susceptibility to AICD. Our findings support the concept that initiating ART in HIV-infected smokers is an effective and conservative clinical management strategy for restoring more physiologic balance in the lung mucosal ratio of $CD4^+$ T cells to $CD8^+$ T cells, notwithstanding continued efforts at smoking cessation. An improved understanding of the underlying pulmonary immune mechanisms in patients at risk for developing HIV-associated COPD may lead to a more comprehensive strategy and uncover novel targets to prevent this important complication of chronic HIV infection.

Supplementary Data

Supplementary materials are available at http://jid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes

Acknowledgments. We thank Dr Mark Connors and Dr Stephen Migueles for careful review of this manuscript.

Financial support. This work was supported by the National Institutes of Health (grants HL090483 and HL121814).

Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Plata F, Autran B, Martins LP, et al. AIDS virus-specific cytotoxic T lymphocytes in lung disorders. Nature **1987**; 328:348–51.
2. Guillon JM, Autran B, Denis M, et al. Human immunodeficiency virus-related lymphocytic alveolitis. Chest **1988**; 94:1264–70.
3. Ognibene FP, Masur H, Rogers P, et al. Nonspecific interstitial pneumonitis without evidence of Pneumocystis carinii in asymptomatic patients infected with human immunodeficiency virus (HIV). Ann Intern Med **1988**; 109:874–9.
4. Twigg HL III, Spain BA, Soliman DM, et al. Production of interferon-gamma by lung lymphocytes in HIV-infected individuals. Am J Physiol **1999**; 276:L256–62.
5. Agostini C, Zambello R, Trentin L, et al. Prognostic significance of the evaluation of bronchoalveolar lavage cell populations in patients with HIV-1 infection and pulmonary involvement. Chest **1991**; 100:1601–6.
6. Walensky RP, Paltiel AD, Losina E, et al. The survival benefits of AIDS treatment in the United States. J Infect Dis **2006**; 194:11–9.
7. Bradley H, Hall HI, Wolitski RJ, et al. Vital Signs: HIV diagnosis, care, and treatment among persons living with HIV–United States, 2011. MMWR Morb Mortal Wkly Rep **2014**; 63:1113–7.
8. Moir S, Chun TW, Fauci AS. Pathogenic mechanisms of HIV disease. Annu Rev Pathol **2011**; 6:223–48.
9. Diaz PT, King MA, Pacht ER, et al. Increased susceptibility to pulmonary emphysema among HIV-seropositive smokers. Ann Intern Med **2000**; 132:369–72.
10. Crothers K, Butt AA, Gibert CL, Rodriguez-Barradas MC, Crystal S, Justice AC. Increased COPD among HIV-positive compared to HIV-negative veterans. Chest **2006**; 130:1326–33.
11. Diaz PT, Clanton TL, Pacht ER. Emphysema-like pulmonary disease associated with human immunodeficiency virus infection. Ann Intern Med **1992**; 116:124–8.
12. Gingo MR, Morris A, Crothers K. Human immunodeficiency virus-associated obstructive lung diseases. Clin Chest Med **2013**; 34:273–82.
13. Drummond MB, Merlo CA, Astemborski J, et al. The effect of HIV infection on longitudinal lung function decline among injection drug users: a prospective cohort. AIDS **2013**; 27:1303–11.
14. Yearsley MM, Diaz PT, Knoell D, Nuovo GJ. Correlation of HIV-1 detection and histology in AIDS-associated emphysema. Diagn Mol Pathol **2005**; 14:48–52.
15. Popescu I, Drummond MB, Gama L, et al. Activation-induced cell death drives profound lung CD4(+) T-cell depletion in HIV-associated chronic obstructive pulmonary disease. Am J Respir Crit Care Med **2014**; 190:744–55.
16. Miller MR, Hankinson J, Brusasco V, et al. Standardisation of spirometry. Eur Respir J **2005**; 26:319–38.
17. Hankinson JL, Odencrantz JR, Fedan KB. Spirometric reference values from a sample of the general U.S. population. Am J Respir Crit Care Med **1999**; 159:179–87.
18. Youngblood B, Wherry EJ, Ahmed R. Acquired transcriptional programming in functional and exhausted virus-specific CD8T cells. Curr Opin HIV AIDS **2012**; 7:50–7.
19. Roederer M, Nozzi JL, Nason MC. SPICE: exploration and analysis of post-cytometric complex multivariate datasets. Cytometry A **2011**; 79:167–74.
20. Seder RA, Darrah PA, Roederer M. T-cell quality in memory and protection: implications for vaccine design. Nat Rev Immunol **2008**; 8:247–58.
21. Twigg Iii HL, Weiden M, Valentine F, et al. Effect of highly active antiretroviral therapy on viral burden in the lungs of HIV-infected subjects. J Infect Dis **2008**; 197:109–16.
22. Younes SA, Yassine-Diab B, Dumont AR, et al. HIV-1 viremia prevents the establishment of interleukin 2-producing HIV-specific memory CD4+ T cells endowed with proliferative capacity. J Exp Med **2003**; 198:1909–22.
23. Tilton JC, Luskin MR, Johnson AJ, et al. Changes in paracrine interleukin-2 requirement, CCR7 expression, frequency, and cytokine secretion of human immunodeficiency virus-specific CD4+ T cells are a consequence of antigen load. J Virol **2007**; 81:2713–25.

24. Iyasere C, Tilton JC, Johnson AJ, et al. Diminished proliferation of human immunodeficiency virus-specific CD4+ T cells is associated with diminished interleukin-2 (IL-2) production and is recovered by exogenous IL-2. J Virol **2003**; 77:10900–9.
25. Chun TW, Justement JS, Pandya P, et al. Relationship between the size of the human immunodeficiency virus type 1 (HIV-1) reservoir in peripheral blood CD4+ T cells and CD4+:CD8+ T cell ratios in aviremic HIV-1-infected individuals receiving long-term highly active antiretroviral therapy. J Infect Dis **2002**; 185:1672–6.
26. Calligaro G, Meintjes G, Mendelson M. Pulmonary manifestations of the immune reconstitution inflammatory syndrome. Curr Opin Pulm Med **2011**; 17:180–8.
27. Ortiz AM, Silvestri G. Immunopathogenesis of AIDS. Curr Infect Dis Rep **2009**; 11:239–45.
28. Appay V, Sauce D. Immune activation and inflammation in HIV-1 infection: causes and consequences. J Pathol **2008**; 214:231–41.
29. Bauer CM, Morissette MC, Stampfli MR. The influence of cigarette smoking on viral infections: translating bench science to impact COPD pathogenesis and acute exacerbations of COPD clinically. Chest **2013**; 143:196–206.
30. McNeil AC, Shupert WL, Iyasere CA, et al. High-level HIV-1 viremia suppresses viral antigen-specific CD4(+) T cell proliferation. Proc Natl Acad Sci U S A **2001**; 98:13878–83.
31. Whittle HC, Dossetor J, Oduloju A, Bryceson AD, Greenwood BM. Cell-mediated immunity during natural measles infection. J Clin Invest **1978**; 62:678–84.
32. Boni C, Bertoletti A, Penna A, et al. Lamivudine treatment can restore T cell responsiveness in chronic hepatitis B. J Clin Invest **1998**; 102:968–75.
33. Mathew A, Kurane I, Green S, et al. Impaired T cell proliferation in acute dengue infection. J Immunol **1999**; 162:5609–15.
34. Carney WP, Hirsch MS. Mechanisms of immunosuppression in cytomegalovirus mononucleosis. II. Virus-monocyte interactions. J Infect Dis **1981**; 144:47–54.
35. Popescu I, Pipeling MR, Mannem H, et al. IL-12-Dependent Cytomegalovirus-Specific CD4+ T Cell Proliferation, T-bet Induction, and Effector Multifunction during Primary Infection Are Key Determinants for Early Immune Control. J Immunol **2016**; 196:877–90.
36. Harris NL, Watt V, Ronchese F, Le Gros G. Differential T cell function and fate in lymph node and nonlymphoid tissues. J Exp Med **2002**; 195:317–26.
37. Ely KH, Cookenham T, Roberts AD, Woodland DL. Memory T cell populations in the lung airways are maintained by continual recruitment. J Immunol **2006**; 176:537–43.
38. Toews GB, Vial WC, Dunn MM, et al. The accessory cell function of human alveolar macrophages in specific T cell proliferation. J Immunol **1984**; 132:181–6.
39. Mayernik DG, Ul-Haq A, Rinehart JJ. Differentiation-associated alteration in human monocyte-macrophage accessory cell function. J Immunol **1983**; 130:2156–60.
40. Gougeon ML, Lecoeur H, Boudet F, et al. Lack of chronic immune activation in HIV-infected chimpanzees correlates with the resistance of T cells to Fas/Apo-1 (CD95)-induced apoptosis and preservation of a T helper 1 phenotype. J Immunol **1997**; 158:2964–76.
41. Petrovas C, Casazza JP, Brenchley JM, et al. PD-1 is a regulator of virus-specific CD8+ T cell survival in HIV infection. J Exp Med **2006**; 203:2281–92.
42. Neff CP, Chain JL, MaWhinney S, et al. Lymphocytic alveolitis is associated with the accumulation of functionally impaired HIV-specific T cells in the lung of antiretroviral therapy-naive subjects. Am J Respir Crit Care Med **2015**; 191:464–73.
43. Burns DN, Hillman D, Neaton JD, et al. Cigarette smoking, bacterial pneumonia, and other clinical outcomes in HIV-1 infection. Terry Beirn Community Programs for Clinical Research on AIDS. J Acquir Immune Defic Syndr Hum Retrovirol **1996**; 13:374–83.
44. Crothers K, Griffith TA, McGinnis KA, et al. The impact of cigarette smoking on mortality, quality of life, and comorbid illness among HIV-positive veterans. J Gen Intern Med **2005**; 20:1142–5.
45. O'Shaughnessy TC, Ansari TW, Barnes NC, Jeffery PK. Inflammation in bronchial biopsies of subjects with chronic bronchitis: inverse relationship of CD8+ T lymphocytes with FEV1. Am J Respir Crit Care Med **1997**; 155:852–7.
46. Saetta M, Di Stefano A, Turato G, et al. CD8+ T-lymphocytes in peripheral airways of smokers with chronic obstructive pulmonary disease. Am J Respir Crit Care Med **1998**; 157:822–6.
47. Hogg JC, Chu F, Utokaparch S, et al. The nature of small-airway obstruction in chronic obstructive pulmonary disease. N Engl J Med **2004**; 350:2645–53.
48. Freeman CM, Han MK, Martinez FJ, et al. Cytotoxic potential of lung CD8(+) T cells increases with chronic obstructive pulmonary disease severity and with in vitro stimulation by IL-18 or IL-15. J Immunol **2010**; 184:6504–13.
49. Drummond MB, Kirk GD, Astemborski J, et al. Association between obstructive lung disease and markers of HIV infection in a high-risk cohort. Thorax **2012**; 67:309–14.
50. Drummond MB, Merlo CA, Astemborski J, et al. The effect of HIV infection on longitudinal lung function decline among IDUs: a prospective cohort. AIDS **2013**; 27:1303–11.