ORIGINAL ARTICLE

Activation-induced Cell Death Drives Profound Lung CD4$^+$ T-Cell Depletion in HIV-associated Chronic Obstructive Pulmonary Disease

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Abstract

Rationale: As overall survival improves, individuals with HIV infection become susceptible to other chronic diseases, including accelerated chronic obstructive pulmonary disease (COPD).

Objectives: To determine whether individuals with HIV-associated COPD exhibit dysregulated lung mucosal T-cell immunity compared with control subjects.

Methods: Using flow cytometry, we evaluated peripheral blood and lung mucosal T-cell immunity in 14 HIV$^+$COPD$^+$, 13 HIV$^+$COPD$^-$, and 7 HIV$^-$COPD$^+$ individuals.

Measurements and Main Results: HIV$^+$COPD$^+$ individuals demonstrated profound CD4$^+$ T-cell depletion with reduced CD4/CD8 T-cell ratios in bronchoalveolar lavage–derived lung mononuclear cells, not observed in peripheral blood mononuclear cells, and diminished CD4$^+$ T cell absolute numbers, compared with control subjects. Furthermore, HIV$^+$COPD$^+$ individuals demonstrated decreased pulmonary HIV-specific and staphylococcal enterotoxin B–reactive CD4$^+$ memory responses, including loss of multifunctionality, compared with HIV$^+$COPD$^-$ control subjects. In contrast, lung mucosal HIV-specific CD8$^+$ T-cell responses were preserved. Lung CD4$^+$ T cells from HIV$^+$COPD$^+$ individuals expressed increased surface Fas death receptor (CD95) and programmed death-1, but similar bronchoalveolar lavage viral loads as control subjects. However, programmed death-1 expression inversely correlated with HIV-specific lung CD4$^+$IFN-γ^+ T-cell responses, suggesting functional exhaustion. Moreover, lung CD4$^+$ T cells from HIV$^+$COPD$^+$ patients demonstrated increased basal and HIV antigen-induced expression of the early apoptosis marker annexin V compared with control subjects, which was significantly attenuated with anti-Fas blockade. Lastly, lung mucosal, but not blood, CD4$^+$/CD8$^+$ ratios from HIV$^+$ patients significantly correlated with the FEV_1, but not in HIV$^-$COPD$^+$ patients.

Conclusions: Together, our results provide evidence for profound lung mucosal CD4$^+$ T-cell depletion via a Fas-dependent activation-induced cell death mechanism, along with impaired HIV-specific CD4$^+$ immunity as immunologic features of HIV-associated COPD.

Keywords: HIV; T cells; COPD; apoptosis; immune activation

Although advancement of antiretroviral therapies (ART) has led to improved survival in HIV disease, evidence suggests these individuals are susceptible to premature development of other chronic diseases. In this regard, select HIV-infected individuals who smoke cigarettes develop accelerated chronic obstructive pulmonary disease (COPD) through mechanisms that

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At a Glance Commentary

Scientific Knowledge on the Subject: Chronic obstructive pulmonary disease (COPD) is a major cause of death worldwide. Certain HIV-infected individuals are at risk for accelerated development of COPD, though the mechanisms for this are poorly understood. Specifically, little is known regarding lung mucosal T-cell populations and function, and interactions with HIV antigens in HIV-associated COPD. Understanding these mechanisms may lead to targeted therapies in this disease.

What This Study Adds to the Field: Our study provides novel insights into the T-cell immunity features of HIV-associated COPD. We show that HIV^+COPD^+ patients demonstrate profound lung mucosal $CD4^+$ T-cell depletion and impaired HIV-specific $CD4^+$ immunity. Lung $CD4^+$ T cells demonstrated increased expression of Fas death receptor (CD95) and PD-1, and undergo increased activation-induced cell death. We show that the lung $CD4^+$:$CD8^+$ T-cell ratio, but not the blood ratio, significantly correlates with the FEV_1 in our HIV^+ cohort at risk for COPD. Together, our data demonstrate that lung mucosal $CD4^+$ depletion occurs through a Fas-dependent activation-induced cell death mechanism, along with impaired HIV-specific $CD4^+$ T-cell immunity as immune features in HIV-associated COPD.

are incompletely understood (1–4). However, the roles of HIV and host immunity in HIV-associated COPD remain poorly understood. An earlier autopsy study from HIV-infected patients with COPD demonstrated increased HIV RNA in areas of emphysema, whereas only rare HIV-infected cells were evident in normal lung (5). Recently, we demonstrated high HIV plasma viral loads and advanced peripheral $CD4^+$ lymphopenia correlated with accelerated decline in FEV_1 compared with patients without advanced HIV disease or uninfected control subjects at risk (6). Additionally, previous studies reported $CD8^+$ alveolitis in HIV disease but not in association with obstructive lung disease (7–9). Collectively, these data suggest potential roles for HIV and host immunity contributing to lung pathology. Indeed, polyinosinic/polycytidylic acid treatment-induced inflammation, a synthetic analog of viral dsRNA, along with cigarette smoke exposure resulted in increased murine emphysema pathology (10). Furthermore, overexpression of IFN-γ was shown to drive increased emphysema in smoke-exposed mice (11). Thus, the host response to persistent viral infection, along with chronic cigarette smoke exposure, may together be a potent stimulus for HIV-associated COPD.

Profound $CD4^+$ T-cell depletion is the hallmark of progressive HIV infection. Although $CD4^+$ depletion strikingly occurs in the gastrointestinal tract during early HIV-1 infection (12), subsequent studies found $CD4^+$ T-cell depletion in the lung mucosa was proportionate to the periphery (13), with HIV-specific $CD4^+$ memory T cells enriched in the bronchoalveolar space compared with the periphery in healthy HIV-infected individuals. Another cardinal feature of HIV disease progression is immune activation, with elevated levels of proinflammatory cytokines and chemokines produced during the acute and chronic phases of infection (14, 15). A consequence of HIV-induced immune activation is apoptosis of $CD4^+$, $CD8^+$, and B lymphocytes (16). Additionally, evidence suggests proinflammatory cytokines are increased in HIV-uninfected COPD patients and that cigarette smoke is proinflammatory (17, 18). Thus, HIV-infected individuals who smoke may be at higher risk for pulmonary and systemic inflammation that potentiates HIV-associated COPD.

Therefore, we hypothesized increased dysregulation of lung mucosal immunity in HIV-associated COPD. Herein, our studies in an HIV-infected inner-city cohort show profound lung mucosal $CD4^+$ T-cell depletion occurs during HIV-associated COPD and that HIV-specific $CD4^+$, but not $CD8^+$, lung mucosal T-cell immunity is impaired. Additionally, we show higher levels of surface expression of Fas death receptor (CD95) and programed death (PD)-1 in lung mucosal $CD4^+$ T cells from HIV^+COPD^+ patients correlates with impaired $CD4^+$ function and that Fas-dependent activation-induced cell death (AICD) occurs in response to HIV antigen. Moreover, a decreased CD4/CD8 T-cell ratio in the lung mucosa, but not the blood, correlates with the FEV_1, further supporting lung $CD4^+$ depletion is an immune feature of HIV-associated COPD.

Methods

Subjects and Tissue Samples

Participants were recruited from two potential sources: the AIDS Linked to the IntraVenous Experience (ALIVE) study and the Johns Hopkins HIV Care Clinic. The ALIVE study, a longitudinal cohort of persons with a history of injecting drugs with or at risk for HIV infection followed in Baltimore, Maryland since 1988 (19), collects spirometric, clinical, and laboratory data at regular 6-month intervals. The Johns Hopkins HIV Clinic provides primary care for approximately 3,500 patients with HIV in the Baltimore area. Participants were screened at scheduled study or clinic visits, and considered eligible if they were willing to undergo spirometry and bronchoscopy, and had no history of pneumonia or worsening of breathing status requiring steroids or antibiotics in the prior 6 weeks. COPD was defined as a post-bronchodilator FEV_1/FVC ratio less than 0.70 (20). Subjects underwent bronchoalveolar lavage (BAL) using a standard protocol with instillation of 180 ml sterile normal saline in the right middle lobe. Study subjects had blood drawn on the same day.

Cell Preparation, Culture, and Antigen Stimulation

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by density gradient centrifugation using Ficoll-Paque (GE Healthcare, Piscataway, NJ). Lung mononuclear cells (LMNC) were obtained via BAL centrifugation. Cells were cryopreserved in liquid nitrogen storage. Pooled overlapping 15-mer peptides for Gag and Pol were obtained from the National Institutes of Health AIDS Reagent Program. Cells were cultured in round-bottom tissue culture tubes (Sarstedt) with and without HIV peptides or control staphylococcal enterotoxin B (SEB; Toxin Technology) at 1 μg/ml for 6 hours at 37°C.

Flow Cytometry

Stimulations for intracellular cytokine staining (ICS) were performed for 6 hours at 37°C and brefeldin-A (10 μg/ml; Sigma) added for the final 4 hours of culture, followed by Cytofix/Cytoperm (BD Biosciences). For CD107a ICS (anti–CD107a-Pacific Blue), monensin (5 μg/ml), and brefeldin-A were added at the beginning of culture. Following restimulation cells were surface-stained with anti–CD3-AlexaFluor700, anti–CD4-allophycocyanin-Cy7, anti–CD8-violet 500, anti-CD95 fluorescein isothiocyanate, and anti–PD-1 phycoerythrin. 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–MIP-1β-phycoerythrin, anti–IL-2–fluorescein isothiocyanate, and anti-CD107a Pacific Blue. For apoptosis studies cells were stained with anti–annexin V-Brilliant Violet-450 using either anti-Fas IgG1 (clone-ZB4; neutralizing) or anti-Fas IgM (clone- CH11 activating) at 10 μg/ml plate-bound for 12 hours at 37°C or isotype control Abs (Millipore). Antibodies were purchased from BD Biosciences unless otherwise noted. Cell fluorescence was analyzed using a FACS LSR Fortessa cytometer (BD Biosciences) and FlowJo software (Tree Star).

Statistical Analysis

Distributions of all measured variables were performed using nonparametric testing. Mean cytokine production was compared using Mann-Whitney and Wilcoxon signed rank analysis using GraphPad Prism (La Jolla, CA). The multifunctional analysis and presentation of distributions was performed using SPICE software (version 5.1), downloaded from http://exon.niaid.nih.gov/spice (21). A P value of less than 0.05 was used to determine statistical significance. For additional details, *see* the online supplement.

Results

Markedly Decreased $CD4^+/CD8^+$ Lung T-Cell Ratio and Lung Mucosal $CD4^+$ T-Cell Numbers in HIV-associated COPD

We evaluated T-cell immunity in an inner-city cohort of 27 HIV-infected individuals comprised of 14 HIV^+COPD^+, 13 HIV^+COPD^-, and 7 HIV^-COPD^+ subjects, whose clinical characteristics are shown (Table 1). Despite similar cigarette smoke exposure, HIV^+COPD^+ individuals demonstrated significantly reduced FEV_1% predicted values and FEV_1/FVC ratios compared with HIV^+COPD^- control subjects. Notably, there were no significant differences between plasma HIV viral loads, antiretroviral usage, or peripheral $CD4^+$ T-cell counts between the HIV^+ groups.

We evaluated BAL-derived LMNC and PBMC $CD4^+/CD8^+$ T-cell ratios from HIV^+COPD^+, HIV^+COPD^-, or HIV^-COPD^+ patients using flow cytometry. LMNC $CD4^+/CD8^+$ ratios were significantly decreased in HIV^+COPD^+ versus HIV^+COPD^- and HIV^-COPD^+ individuals (Figures 1A and 1B). In contrast, there was no difference in PBMC $CD4^+/CD8^+$ ratios between HIV^+ groups (Figures 1D and 1E).

Next, we determined whether the absolute number of lung mucosal $CD4^+$ T cells differed. We observed a significant reduction in LMNC $CD4^+$ T-cell absolute numbers in HIV^+COPD^+ patients, but no difference in absolute $CD8^+$ T cells (Figure 1C). Interestingly, decreased absolute BAL $CD4^+$ T-cell numbers/volume were detected despite significantly lower total BAL volume yields in HIV^+COPD^+ subjects (*see* Figures E1A and E1B in the online supplement). Together, these data demonstrate a marked decrease in BAL $CD4^+/CD8^+$ ratios, and a quantitative reduction of lung $CD4^+$ T cells during HIV-associated COPD, not seen in HIV-negative COPD.

Diminished Lung Mucosal HIV-Specific $CD4^+$ T-Cell Memory in HIV-associated COPD

Functional viral-specific T-cell memory is critical for viral control at mucosal sites. We

Table 1. Clinical Characteristics of Study Cohort

	HIV^+COPD^- (*n = 13*)	HIV^+COPD^+ (*n = 14*)	HIV^-COPD^+ (*n = 7*)
Age	48.2 (10.5)	51.2 (7.8)	50.6 (7.3)
African American, n (%)	11 (85)	14 (100)	7 (100)
Male, n (%)	10 (77)	12 (86)	4 (57)
Current smoker, n (%)	9 (69)	9 (64)	9 (100)
Pack-years smoked, median (IQR)	8.45 (2.2–22.5)	13.4 (7.2–30)	18.7 (13.6–35.9)
FEV_1/FVC ratio	0.78 (0.04)	0.56 (0.10)*	0.63 (0.05)*
FEV_1			
Absolute, L	2.84 (0.63)	1.92 (0.76)†	2.00 (0.63)†
% Predicted	89.5 (13)	62.6 (20)*	78.0 (20)
Plasma viral load			
Undetectable, n (%)	9 (69)	10 (71)	
Copies/ml, median (IQR)‡	30,779 (1,717–58,122)	18,492 (7,552–40,252)	—
Antiretroviral use, n (%)	9 (69)	11 (79)	—
Blood CD4			
Less than 200, n (%)	2 (15)	3 (21)	—
Cells/cm^3, median (IQR)	485 (427–551)	352 (293–609)	

Definition of abbreviations: COPD = chronic obstructive pulmonary disease; IQR = interquartile range.
All values mean (SD) unless otherwise indicated.
P value versus HIV^+COPD^- participants: *$P < 0.001$, †$P < 0.01$.
‡Among detectable viral load.

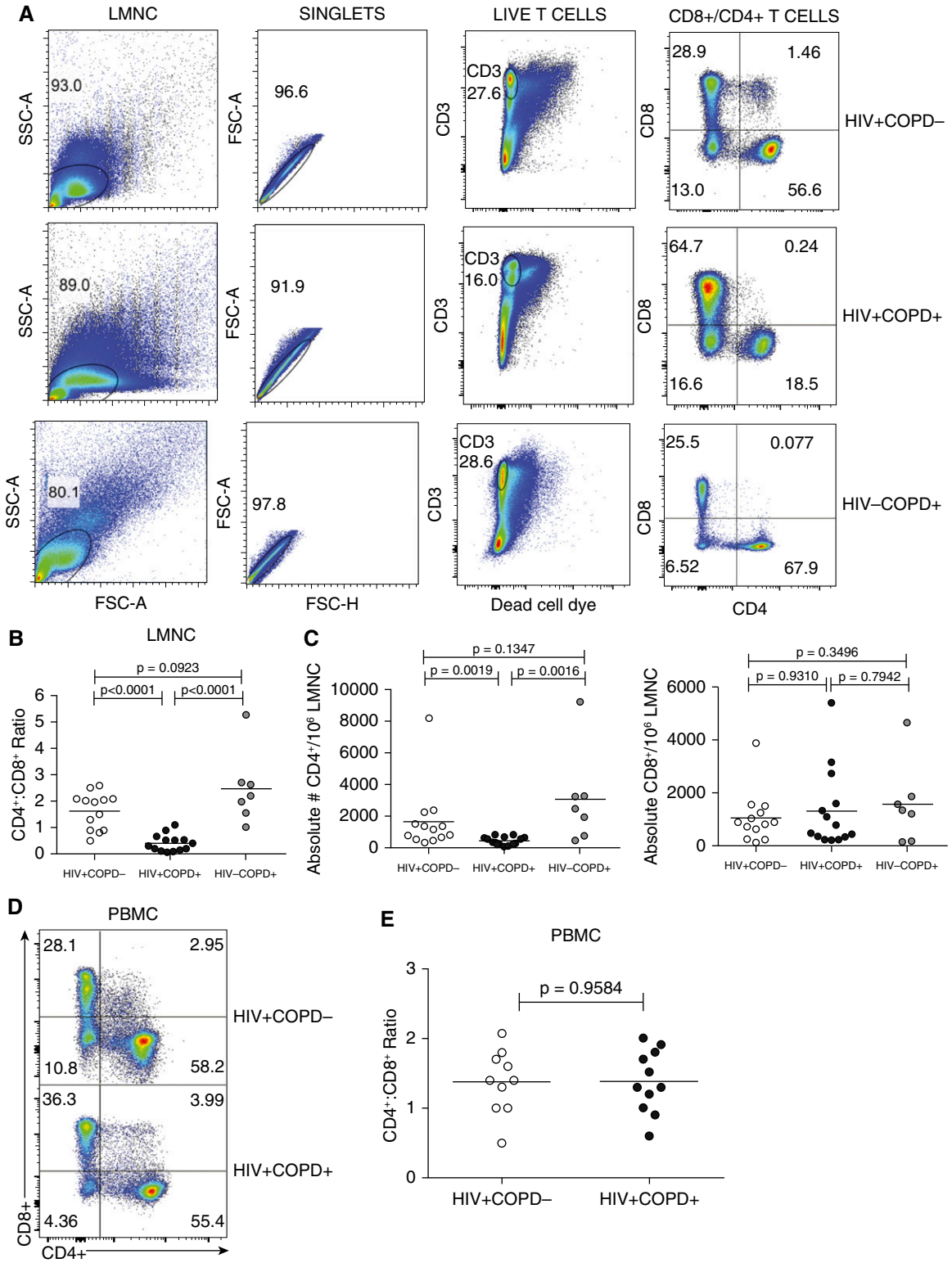

Figure 1. Marked depletion of lung mucosal $CD4^+$ T cells in HIV-associated chronic obstructive pulmonary disease (COPD). (*A*) Representative flow cytometric plots of gating strategy to analyze the numbers of lung mononuclear cells (LMNC) $CD8^+$ or $CD4^+$ T cells from HIV^+COPD^-, HIV^+COPD^+, and HIV^-COPD^+ patients. Gating was on LMNC cells with doublet exclusion, live/$CD3^+$ T cells, and *flow plot numbers* indicate frequencies of $CD4^+$ or $CD8^+$ T-cell populations. Plots are representative of 34 LMNC analyzed from individuals during chronic HIV infection (14 $COPD^+$, 13 $COPD^-$, and 7 HIV^-COPD^+). (*B*) Pooled data showing the $CD4^+/CD8^+$ ratio of LMNC from 13 HIV^+COPD^- (*white circles*), 14 HIV^+COPD^+ (*black circles*), and 7 HIV^-COPD^+ (*gray circles*) patients. (*C*) Pooled data showing the absolute numbers of $CD4^+/10^6$ LMNC T cells from 13 HIV^+COPD^- (*white circles*), 14 HIV^+COPD^+ (*black circles*), and

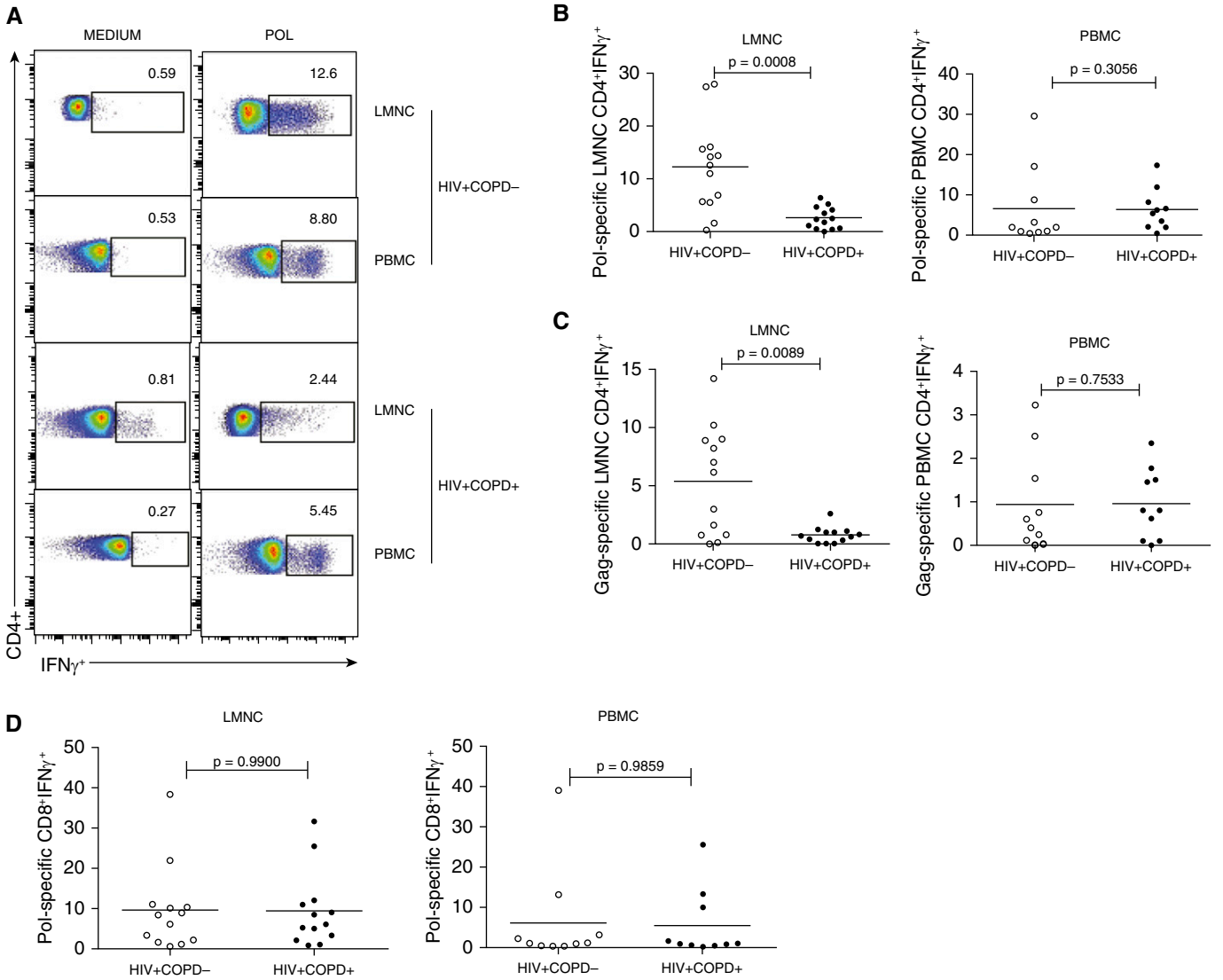

Figure 2. Lung mucosal HIV-specific $CD4^+IFN\gamma^+$ T-cell memory responses are reduced in HIV-associated chronic obstructive pulmonary disease (COPD). (*A*) Representative flow cytometric plots of the $CD4^+IFN\text{-}\gamma^+$ lung mononuclear cells (LMNC) and peripheral blood mononuclear cells (PBMC) from HIV^+COPD^- and HIV^+COPD^+ patients cultured in the presence or absence of Pol HIV peptides, followed by intracellular cytokine staining as detailed in the METHODS section. *Flow plot numbers* indicate HIV-specific $IFN\gamma^+$ frequencies of populations, with gating on $CD3^+CD4^+$ T cells. Plots are representative of LMNC analyzed from 26 subjects, 13 $COPD^-$ and 13 $COPD^+$ patients during chronic HIV chronic infection, following 6 hours *in vitro* restimulation with pooled overlapping 15-mer Pol peptides. (*B*) Pooled data showing cumulative frequencies of Pol-specific $CD3^+CD4^+IFN\text{-}\gamma^+$ in LMNC (*left*) versus PBMC (*right*). (*C*) Cumulative frequencies of Gag-specific LMNC $CD3^+CD4^{+\ +}$ (*left*) and Gag-specific PBMC $CD3^+CD4^+IFN\text{-}\gamma^+$ (*right*), following *in vitro* restimulation with pooled overlapping 15-mer Gag peptides. (*D*) Pooled data showing cumulative frequencies of lung Pol-specific $CD3^+CD8^+IFN\gamma^+$ in LMNC (*left*) and blood PBMC (*right*). *Lines* represent mean values. *P* values were calculated using the Wilcoxon signed rank test or the Mann-Whitney *t* test.

hypothesized HIV-specific functional T-cell responses from LMNC or PBMC were dysregulated in HIV^+COPD^+ versus HIV^+COPD^- individuals. For this, cells from each tissue compartment were cultured in the presence or absence of pooled peptides for the major HIV antigens Pol or Gag, and $IFN\text{-}\gamma^+$ T-cell responses determined using ICS and flow cytometry (22, 23). Pol- and Gag-specific $CD4^+IFN\text{-}\gamma^+$ T-cell frequencies in LMNC were diminished in HIV^+COPD^+ patients compared with HIV^+COPD^- patients (Figures 2A–2C), whereas HIV-specific $CD4^+IFN\text{-}\gamma^+$ T-cell responses in PBMC

Figure 1. (Continued). 7 HIV^-COPD^+ (*gray circles*) patients. (*D*) Representative flow cytometric plots of peripheral blood mononuclear cells (PBMC) $CD8^+$ or $CD4^+$ T cells from cohort. (*E*) Pooled data showing the $CD4^+/CD8^+$ ratio of PBMC T cells from 11 HIV^+COPD^+ (*black circles*) and 10 $COPD^-$ (*white circles*) patients. Values represent mean ± SEM. *P* values were calculated using the Wilcoxon signed rank test or the Mann-Whitney *t* test.

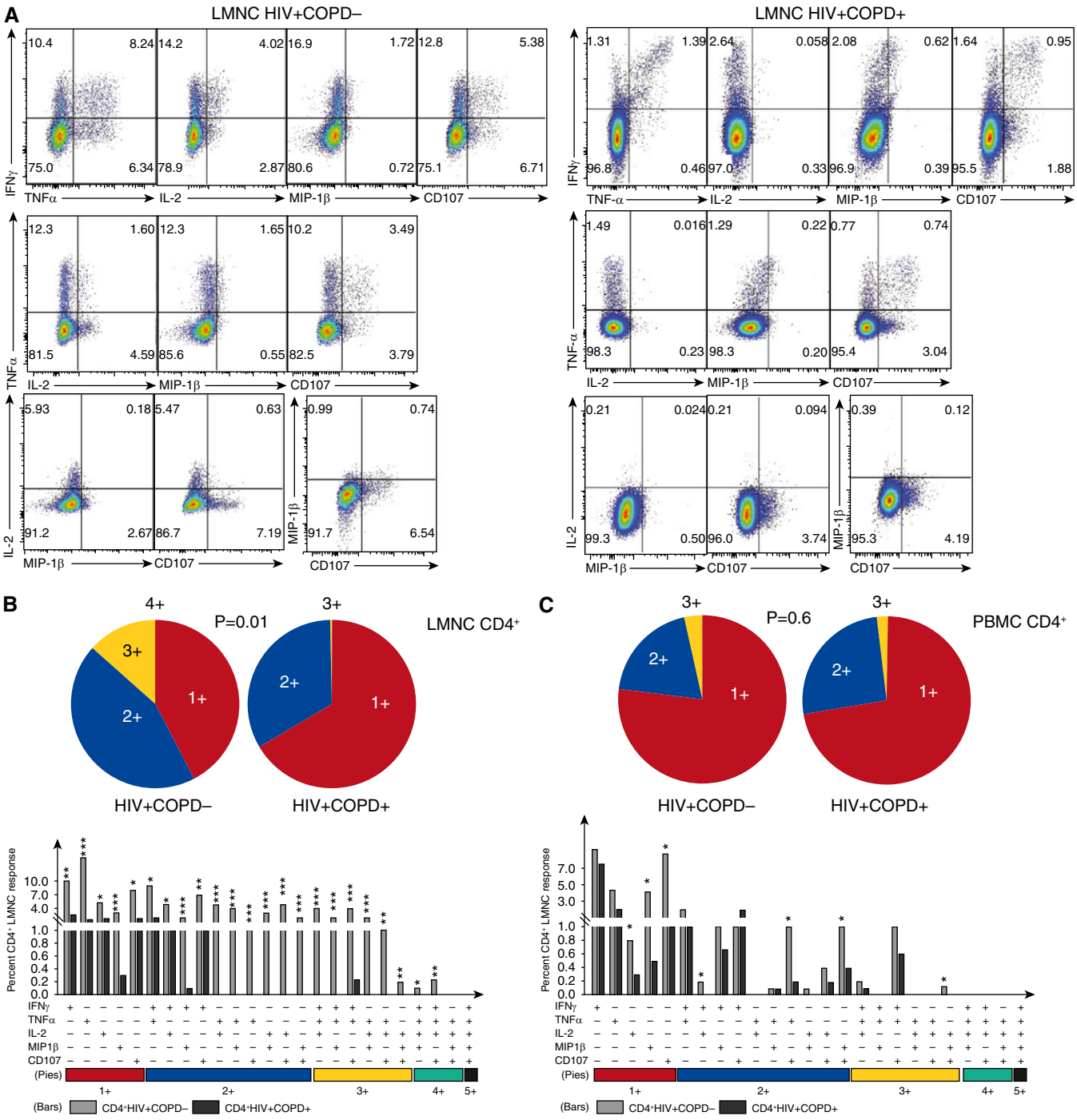

Figure 3. Loss of multifunctional HIV-specific $CD4^+$ T-cell memory in the lung mucosa in HIV-associated chronic obstructive pulmonary disease (COPD). (*A*) Representative multifunctional flow cytometric plots of lung mononuclear cells (LMNC) from HIV^+COPD^- (*left*) versus HIV^+COPD^+ (*right*), following restimulation with Pol peptides showing multifunctional subset frequencies of $CD4^+$ T cells producing IFN-γ, tumor necrosis factor (TNF)-α, IL-2, MIP-1β, and CD107 during chronic HIV infection. (*B*) Individual pie charts of LMNC $CD4^+$ T cell reflecting the percentage of Pol-specific $CD4^+$ T cells from HIV^+COPD^- (*left pie chart*) versus HIV^+COPD^+ (*right pie chart*) patients producing one, two, three, or four functional responses. (*C*) Individual pie charts of peripheral blood mononuclear cells (PBMC) $CD4^+$ T cells, reflecting the same cohort responses as in *B*. Using Boolean analysis, the percentage of total for individual multifunctional subset responses for HIV^+COPD^- (*gray bars*) and HIV^+COPD^+ (*black bars*) are shown in the bar graph for each of the multifunctional subsets (*B* and *C*). Significant differences when comparing mean frequencies of Pol-specific single and multifunctional responses are indicated by $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. All *P* values were determined by the Kruskal-Wallis one-way analysis of variance or Wilcoxon signed rank test.

did not significantly differ. In contrast, HIV-specific $CD8^+IFN\text{-}\gamma^+$ T-cell responses were similar in both compartments (Figure 2D). Together, our results demonstrate impaired HIV-specific $CD4^+IFN\text{-}\gamma^+$ T-cell responses in LMNC from HIV^+COPD^+ individuals versus control subjects.

Next, we evaluated the multifunctional capacity of effector function in HIV-specific $CD4^+$ memory T cells in LMNC and PBMC for IFN-γ, TNF-α, IL-2, CD107 mobilization, and MIP-1β. Lung HIV-specific $CD4^+$ T cells from a representative HIV^+COPD^+ subject exhibit diminished multifunctional capacity compared with an HIV^+COPD^- individual (Figure 3A). Using Boolean analysis, we determined the percentage of individual multifunctional responses between HIV^+COPD^+ and HIV^+COPD^- individuals for LMNC and PBMC and found that LMNC from HIV^+COPD^+ demonstrated decreased frequencies of Pol- and Gag-specific ($P = 0.03$; data not shown) $CD4^+$ T cells producing 2+, 3+, or 4+ cytokines and chemokines in contrast to $CD4^+$ T cells from HIV^+COPD^- individuals (Figure 3B).

As a control, we compared $IFN\text{-}\gamma^+$ and multifunctional SEB-reactive lung $CD4^+$ T-cell responses between the groups, and observed significantly reduced responses in HIV^+COPD^+ patients compared with control subjects (*see* Figures E2A and E2B). In contrast, HIV-specific multifunctional $CD4^+$ memory was similar in PBMC between both groups for Pol-specific responses (Figure 2C) and Gag-specific responses ($P = 0.98$; data not shown). Additionally, HIV-specific multifunctional $CD8^+$ memory from LMNC (*see* Figure E3) and PBMC (data not shown) were similar between the groups. An intercompartmental comparison of HIV-specific $CD4^+$ memory showed increased multifunction in the LMNC more than PBMC from the HIV^+COPD^- ($P = 0.03$), but not the HIV^+COPD^+ group ($P = 0.8$; data not shown). As an additional control for the cytokine milieu, we measured neat BAL supernatants for TNF-α and the antiinflammatory cytokine IL-10 using ELISA. However, TNF-α was not detected and IL-10 levels in only 3 of 21 HIV^+COPD^+ subjects and there were no significant differences between groups (data not shown). Together, these data show both quantitative and qualitative impairment in HIV-specific/SEB-reactive multifunctional $CD4^+$ T-cell memory restricted to the lung mucosa during HIV-associated COPD.

Lung Mucosal $CD4^+$ T Cells Express Increased Surface Fas (CD95) and PD-1, and Undergo Increased Fas-Dependent AICD in HIV-associated COPD

To understand the mechanisms regulating lung mucosal $CD4^+$ T-cell depletion in HIV^+COPD^+ individuals, we evaluated surface expression of CD95 (Fas) and PD-1. We detected increased surface expression of PD-1 and/or CD95 in LMNC $CD4^+$ T cells from HIV^+COPD^+ compared with HIV^+COPD^- individuals (Figures 4A–4C). Unexpectedly, most $CD4^+PD\text{-}1^+$ T cells coexpressed CD95, with a higher percentage of double-positive cells in lung $CD4^+$ T cells from HIV^+COPD^+ patients. Notably, lung $CD4^+$ T cells from HIV^-COPD^+ patients demonstrated significantly reduced frequencies of $CD95^+$ and $CD95^+PD\text{-}1^+$ double-positive cells compared with HIV-infected groups. Additionally, we observed more than 98% of lung $CD4^+CD95^+$ T cells were $CD28^-$ and $CTLA4^-$ and did not differ between the HIV^+ groups ($P = 0.35$ data not shown). We also compared BAL HIV viral loads with $CD4^+CD95^+PD\text{-}1^+$ expression and did not find a relationship ($P = 0.4$; data not shown). However, comparing LMNC HIV-specific $CD4^+IFN\text{-}\gamma^+$ responses with $CD95^+PD\text{-}1^+$ expression revealed a significant inverse correlation (Figure 4D).

We next determined whether increased Fas expression resulted in apoptosis using the early apoptotic marker annexin V in LMNC $CD4^+$ T cells. Lung $CD4^+$ T cells from HIV^+COPD^+ patients demonstrated increased basal levels of annexin V in medium alone compared with control subjects, which increased significantly higher following *in vitro* restimulation with HIV peptides or anti-Fas activating Ab compared with HIV^+COPD^- subjects (Figures 5A and 5B). Blockade of Fas/Fas ligand (FasL) interactions using an anti-Fas neutralizing Ab significantly reduced annexin V expression in antigen-activated LMNC $CD4^+$ T cells from HIV^+COPD^+ and HIV^+COPD^- individuals compared with isotype control Ab. As expected, $CD4^+$ cells from HIV^-COPD^+ patients did not express annexin V in response to HIV antigens, and all three groups showed similar induction in response to SEB. Collectively, these data show increased expression of Fas/PD-1 in lung mucosal $CD4^+$ T cells in HIV-associated COPD along with increased Fas-mediated AICD, as one mechanism for reduced lung mucosal $CD4^+$ T-cell numbers in HIV-associated COPD.

Next we asked whether differences in HIV lung viral burden might contribute to differences in LMNC $CD4^+$ T cells between HIV^+ patients. Although most (71%) patients in our cohort were receiving ART (Table 1), three subjects in both the HIV^+COPD^+ and HIV^+COPD^- groups were not receiving ART, and measurement of their HIV RNA viral loads in BAL supernatants did not reveal differences ($P = 0.7$; *see* Figure E4). Furthermore, BAL HIV viral loads were similar between HIV^+COPD^+ and HIV^+COPD^- patients receiving ART ($P = 0.44$; *see* Figure E4), whereas comparison of BAL HIV viral loads between HIV^+COPD^+ patients either receiving or not receiving ART showed a reduction in viral loads, although this did not reach statistical significance ($P = 0.096$; *see* Figure E4). Thus, our data do not indicate significant differences in BAL HIV viral load between HIV^+COPD^+ and HIV^+COPD^- patients in our cohort to account for differences in lung mucosal $CD4^+$ T cells. Nonetheless, these data do suggest ART can effectively reduce lung HIV viral loads in individuals with HIV-associated COPD.

Decreased Lung Mucosal $CD4^+/CD8^+$ T-Cell Ratio Correlates with HIV-associated COPD and the FEV_1 in HIV-associated COPD

We hypothesized that the LMNC $CD4^+/CD8^+$ T-cell ratio, being reduced in HIV-associated COPD, would exhibit a relationship with the FEV_1. To test this, we performed a scatterplot analysis of the LMNC $CD4^+/CD8^+$ T-cell ratio and the FEV_1% predicted, and found that these parameters were significantly correlated (Figure 6A). Similar results were found plotting the LMNC $CD4^+/CD8^+$ T-cell ratio and FEV_1 absolute values ($P = 0.046$; $r = 0.44$) from our cohort patients. In contrast, we did not find a relationship between PBMC $CD4^+/CD8^+$ T-cell ratio and the FEV_1% predicted (Figure 6B), or the LMNC $CD4^+/CD8^+$ ratio and FEV_1% predicted in HIV^-COPD^+ patients ($P = 0.18$ and $r = -0.56$; data not shown). Thus, the lung mucosal $CD4^+/CD8^+$ T-cell ratio correlates with the FEV_1 in HIV-associated COPD but not HIV-negative COPD.

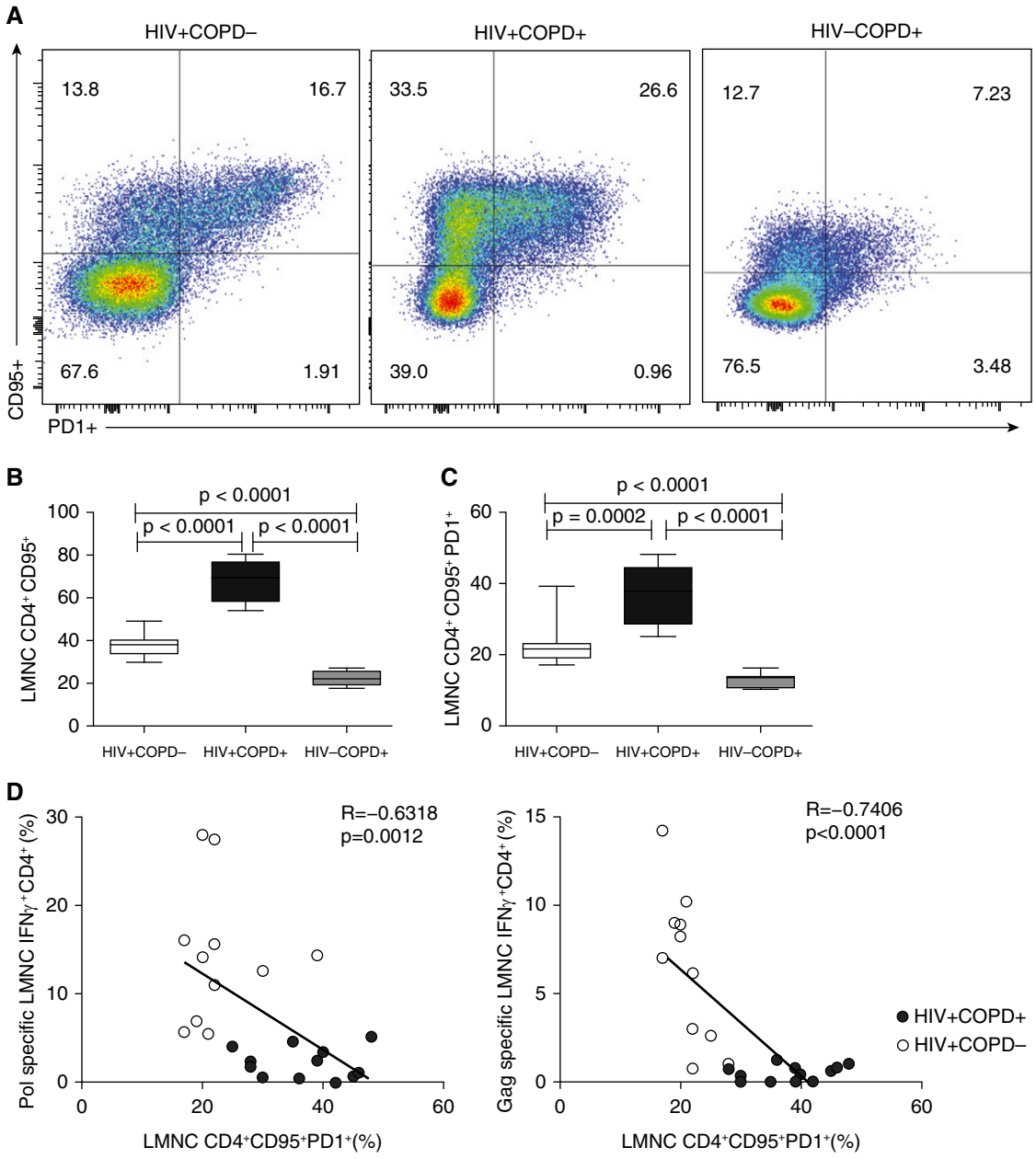

Figure 4. HIV$^+$COPD$^+$ patients have a significantly higher surface expression of CD95$^+$ and/or programed death (PD)-1$^+$ on lung mucosal CD4$^+$ T cells. (*A*) Representative flow cytometric plots of the lung mononuclear cells (LMNC) CD4$^+$CD95$^+$PD-1$^+$ for HIV$^+$COPD$^-$ (*left*) versus HIV$^+$COPD$^+$ (*middle*), and HIV$^-$COPD$^+$ (*right*) patient. (*B*) Pooled data showing cumulative frequencies of LMNC CD4$^+$CD95$^+$ for 11 HIV$^+$COPD$^-$, 12 HIV$^+$COPD$^+$, and 7 HIV$^-$COPD$^+$ patients. (*C*) Pooled data showing cumulative frequencies of LMNC CD4$^+$CD95$^+$PD-1$^+$ for 11 HIV$^+$COPD$^-$ patients (*white bars*), 12 HIV$^+$COPD$^+$ (*black bars*), and 7 HIV$^-$COPD$^+$ (*gray bars*) patients. Values represent mean ± SEM, and *P* values were calculated using the Mann-Whitney *t* test. (*D*) Scatterplot analysis of LMNC Pol- and Gag-specific CD4$^+$IFNγ^+ responses and CD4$^+$CD95$^+$PD-1$^+$ frequencies. Values are for 12 HIV$^+$COPD$^+$ (*black circles*) and 11 HIV$^+$COPD$^-$ (*white circles*) individuals in the cohort. Analysis was performed using the Spearman rho test.

Discussion

Herein, we report for the first time a quantitative depletion of lung mucosal CD4$^+$ T cells, along with qualitative impairment in lung HIV-specific CD4$^+$ T-cell immunity, occurs in HIV-associated COPD. Moreover, we show that LMNC CD4$^+$/CD8$^+$ T-cell ratios correlate with FEV_1 values in our cohort of HIV$^+$COPD$^+$ and HIV$^+$COPD$^-$ individuals, showing CD4$^+$ T-cell dysregulation as an immune feature in HIV-associated COPD. Notably, the HIV$^+$COPD$^-$ and the HIV$^-$COPD$^+$ individuals in our cohort had preserved, normal LMNC CD4$^+$/CD8$^+$ T-cell ratios of approximately 1–2.5:1 (24, 25), in striking contrast to reduced ratios observed in HIV$^+$COPD$^+$ individuals. These findings are consistent with a previous study showing a relative

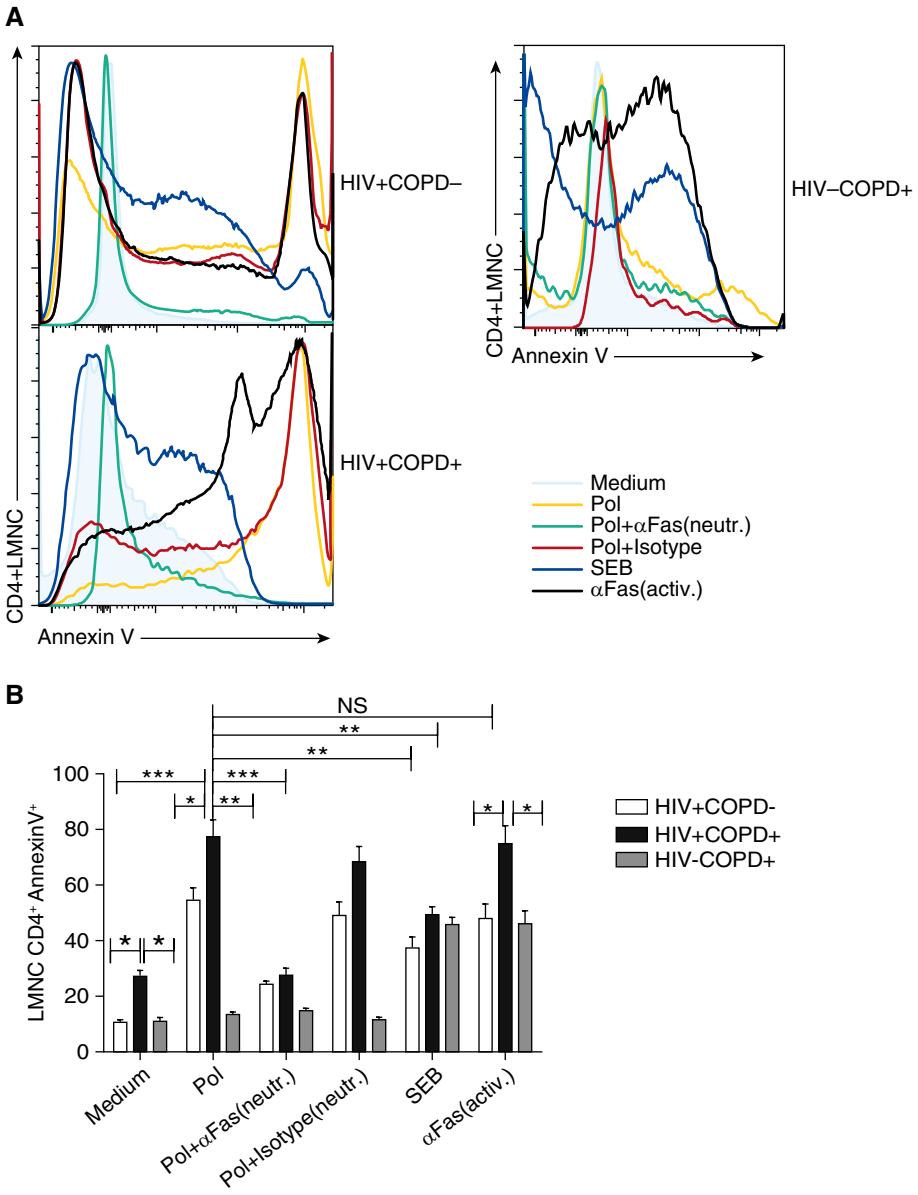

Figure 5. CD95/Fas regulate increased activation-induced cell death in lung mucosal HIV-specific $CD4^+$ T cells in HIV-associated chronic obstructive pulmonary disease (COPD). (*A*) Representative histograms depicting the annexin V positivity on lung mononuclear cells (LMNC) $CD4^+$ from HIV^+COPD^- (*upper left*), HIV^+COPD^+ (*lower left*), and HIV^-COPD^+ (*right*) donors. LMNCs were cultured in the presence or absence of plate-bound anti-Fas antibodies that are either neutralizing (ZB4) or activating (CH11) at 10 μg/ml, or an isotype control (IgG for neutralizing clone shown), with/without restimulation with Pol peptides or staphylococcal enterotoxin B (SEB), as described in the METHODS section. (*B*) Pooled data showing LMNC annexin V^+ subsets from gating on live $CD3^+CD4^+$ T cells from four HIV^+COPD^-, five HIV^+COPD^+, and seven HIV^-COPD^+ patients. Cumulative mean frequencies ± SEM of LMNC $CD4^+$ annexinV^+ cells from HIV^+COPD^- (*white bars*), HIV^+COPD^+ (*black bars*), and HIV^-COPD^+ (*gray bars*) patients. $^*P < 0.05$, $^{**}P < 0.003$, $^{***}P < 0.0002$, calculated using the Wilcoxon signed rank test or the Mann-Whitney *t* test.

preservation of $CD4^+$ T cells in the lung mucosa of HIV-infected individuals compared with the massive $CD4^+$ T-cell depletion that occurs in the gut mucosa during the acute phase of HIV infection (13). However, our data demonstrate certain individuals are at increased risk for progressive lung mucosal $CD4^+$ T-cell depletion disproportionately higher compared with peripheral $CD4^+$ T-cell depletion and this correlates with the presence of HIV-associated COPD. Finally, our data from HIV-negative $COPD^+$ control subjects indicate $CD4^+$ T-cell depletion in HIV-associated COPD is not caused by the COPD itself.

$CD4^+$ T cells demonstrate significant heterogeneity in function (26–28). In addition to depletion of lung mucosal $CD4^+$ T cells, we observed a quantitative and qualitative impairment in lung HIV-specific $CD4^+$ T-cell responses in HIV-associated COPD. Our findings of diminished frequencies of lung mucosa Pol- and Gag-specific $CD4^+IFN\text{-}\gamma^+$ T-cell memory responses in HIV^+COPD^+ individuals, but not in the PBMC, was somewhat surprising and not predicted by $CD4^+$ T-cell depletion alone, because we anticipated reduced HIV-specific $CD4^+$ T-cell immunity commensurate with $CD4^+$ depletion. However, our findings may reflect preferential HIV-specific $CD4^+$ T-cell depletion previously demonstrated (29). Additionally, our findings of increased PD-1 expression on $CD4^+$ T cells inversely correlating with HIV-specific $CD4^+IFN\text{-}\gamma^+$ responses are consistent with lung $CD4^+$ T-cell exhaustion, although this did not correlate with BAL viral loads (30). In contrast, HIV^+COPD^- individuals demonstrated increased lung mucosal HIV-specific $CD4^+$ memory responses relative to PBMC as previously reported (13). Furthermore, we found impairment extended to $CD4^+$ multifunctional memory, with increased single-positive $CD4^+IFN\text{-}\gamma^+$ T cells in HIV-associated COPD. Indeed, previous studies have shown loss of $CD4^+$ multifunctional cells is correlated with HIV progression (31, 32). Although our data did not show increased HIV RNA viral loads in the BAL fluid from HIV^+COPD^+ subjects, it is possible that low levels of viral replication in the lung are caused by impaired $CD4^+$ HIV-specific immunity. To this end, we recently demonstrated an important role for $CD4^+$ T cells in lung mucosal viral control during chronic cytomegalovirus infection (28). Moreover, we found impaired $CD4^+$ SEB-reactive T-cell responses in HIV^+COPD^+ patients compared with control subjects indicating more global effector dysfunction. Collectively, our data show quantitative and qualitative impairment of lung mucosal $CD4^+$ T-cell immunity

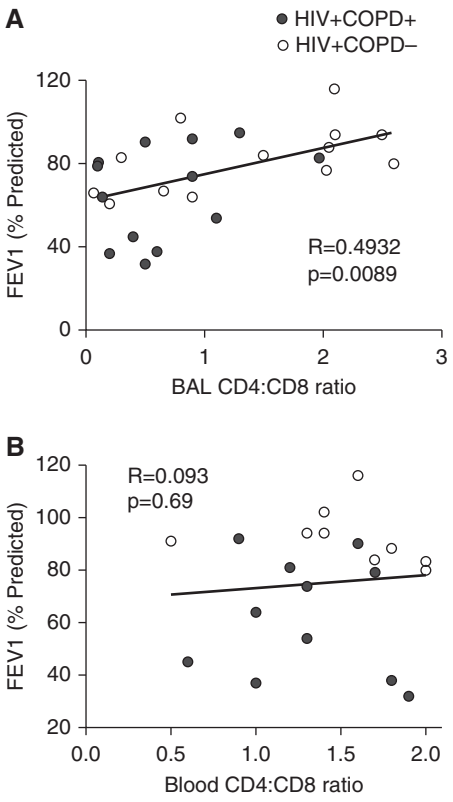

Figure 6. The bronchoalveolar lavage (BAL) but not peripheral blood mononuclear cells $CD4^+/CD8^+$ ratio correlates with the FEV_1% predicted. Scatterplot analysis of the (*A*) lung mononuclear cells $CD4^+/CD8^+$ ratio and the FEV_1% predicted values for 14 HIV^+COPD^+ (*black circles*) and 13 HIV^+COPD^- (*white circles*) individuals in the cohort and (*B*) peripheral blood mononuclear cells (n = 21 total). Analysis was performed using the Spearman rho test. COPD = chronic obstructive pulmonary disease.

that occurs during HIV-associated COPD.

$CD8^+$ T cells have been implicated in the pathogenesis of non-HIV COPD through unclear mechanisms (33–35), although recent evidence suggests increased cytotoxic potential with disease severity (36). Additionally, earlier reports of $CD8^+$ T-cell alveolitis in HIV disease led investigators to speculate whether $CD8^+$ T cells play a pathogenic role in HIV-associated COPD (1, 2). Indeed, enhanced IFN-γ in combination with cigarette smoke exposure (11), or in the presence of viral pathogen-associated molecular patterns, such as polyinosinic/polycytidylic acid or influenza infection, result in increased pulmonary inflammation and emphysema in mice, suggesting viral pathogen-associated molecular patterns and the host response are integral in the emphysema development (10). Our studies unexpectedly showed lung HIV-specific $CD8^+$ responses are similar in HIV^+COPD^+ versus HIV^+COPD^- individuals, apparently contradicting the $CD8^+$ alveolitis hypothesis for HIV-associated COPD. However, loss of lung mucosal $CD4^+$ T cells might result in a differential impact of preserved $CD8^+$ T-cell responses in HIV^+COPD^+ individuals. For example, progressive $CD4^+$ regulatory T-cell loss might lead to unopposed $CD8^+$ T-cell inflammation resulting in pulmonary pathology. Indeed, increased $CD8^+$ T-cell activation was previously associated with HIV disease progression and shortened survival (37). Therefore, further studies are needed to determine whether $CD8^+$ T cells play an important role in HIV-associated COPD.

Immune activation is a hallmark of chronic HIV infection, with apoptosis being a physiologic consequence of this process (16). We find lung mucosal $CD4^+$ T cells express increased levels of surface PD-1 and/or CD95/Fas from HIV^+COPD^+ individuals with increased spontaneous apoptosis by higher basal annexin V expression compared with control subjects. Our data are consistent with a study showing PD-1 and CD95/Fas coexpression in blood $CD8^+$ T cells in HIV infection, further supporting PD-1 as a preapoptotic factor in T cells (38). Furthermore, our studies show anti-Fas neutralization blocks enhanced apoptosis following *in vitro* restimulation with HIV antigen, demonstrating Fas-dependent apoptosis as a mechanistic pathway for lung $CD4^+$ T-cell apoptosis in HIV-associated COPD. However, although the Fas/FasL-pathway was previously shown to be important in HIV infection (39, 40), it is plausible other pathways play a role in lung $CD4^+$ T-cell depletion (41). Furthermore, although we detected similar HIV viral load levels in the BAL between groups, low levels of HIV antigen may be an important factor driving AICD in lung $CD4^+$ T cells, because earlier studies in blood and lymph node showed $CD4^+$ apoptosis correlated with lymphocyte immune activation, although interestingly, not with viral burden (42, 43). Taken together, our data demonstrate that Fas-dependent AICD in response to HIV is an important mechanism driving lung mucosal $CD4^+$ T-cell depletion in HIV-associated COPD and not a consequence of COPD.

There are several caveats to our studies. First, because the entire cohort of patients was actively smoking cigarettes, it is possible this factor differentially contributed to immune activation and dysregulation among patients. However, this characteristic of the ALIVE cohort does allow for isolation of the effects of HIV infection independent of smoking. Although the characteristics of this cohort may not represent the "standard" COPD patient, the presence of unique characteristics in the HIV-infected and HIV-uninfected groups removes these traits as potential confounders in our comparisons. As well, this cohort is highly representative of the population at risk for COPD and HIV: lower-income African Americans with high smoking and injection drug use history. Second, although we detected similar BAL HIV viral loads between groups, our data suggest efficacy of ART in reducing lung viral loads in HIV^+COPD^+. Although these findings need to be validated in more patients, one could argue implementing ART therapy in current HIV-infected smokers is the most conservative clinical management strategy, in addition to smoking cessation, because our data suggest lung viral replication would exacerbate AICD in $CD4^+$ T cells. Third, although our data indicate lung mucosal $CD4^+$ correlates with the FEV_1, we do not yet know whether this is causal in driving disease, because this was a cross-sectional study. Nonetheless, it is plausible given our previous study correlating markers of advanced HIV disease with accelerated decline in pulmonary function in a larger prospective cohort (6). We did not collect data on inhaled corticosteroid use, which may impact the observed associations. Furthermore, although the diffusing capacity was not measured in this study, its impairment has been shown in HIV disease (44). Alternatively, other opportunistic infections beyond HIV itself may contribute to lung disease in the setting of lung mucosal $CD4^+$ depletion in these patients (45). Therefore, longitudinal studies are needed to further elucidate the role of $CD4^+$ T-cell depletion in the pathogenesis of HIV-associated COPD.

In conclusion, we report profound lung mucosal $CD4^+$ T-cell depletion, with reduction in absolute $CD4^+$ numbers and the BAL $CD4^+/CD8^+$ ratio, along with impaired $CD4^+$ T-cell mucosal immunity in HIV-associated COPD. We also find increased expression of the CD95/Fas and PD-1, and apoptosis in mucosal $CD4^+$ T cells from HIV^+COPD^+ patients, and demonstrate Fas-dependent AICD in response to HIV antigen as a mechanism for lung mucosal $CD4^+$ apoptosis. Finally, we show the lung $CD4^+/CD8^+$ ratio correlates with the FEV_1 in HIV-infected smokers. An improved understanding of the underlying mechanisms that contribute to HIV-associated COPD may lead to targeted interventions to prevent this important complication of chronic HIV infection. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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