Lymphocytic Alveolitis Is Associated with the Accumulation of Functionally Impaired HIV-Specific T Cells in the Lung of Antiretroviral Therapy–Naive Subjects

C. Preston Neff¹, Jennifer L. Chain¹, Samantha MaWhinney², Allison K. Martin¹, Derek J. Linderman¹, Sonia C. Flores¹, Thomas B. Campbell¹, Brent E. Palmer¹, and Andrew P. Fontenot^{1,3}

¹Department of Medicine, ²Department of Biostatistics and Informatics, and ³Department of Immunology, University of Colorado Anschutz Medical Campus, Aurora, Colorado

Abstract

Rationale: Lymphocytic alveolitis in HIV-1-infected individuals is associated with multiple pulmonary complications and a poor prognosis. Although lymphocytic alveolitis has been associated with viremia and an increased number of CD8⁺ T cells in the lung, its exact cause is unknown.

Objectives: To determine if HIV-1-specific T cells are associated with lymphocytic alveolitis in HIV-1-infected individuals.

Methods: Using blood and bronchoalveolar lavage (BAL) cells from normal control subjects and untreated HIV-1-infected individuals, we examined the frequency and functional capacity of HIV-1-specific T cells.

Measurements and Main Results: We found that HIV-1-specific T cells were significantly elevated in the BAL compared with blood of HIV-1-infected individuals and strongly correlated with T-cell alveolitis. Expression of Ki67, a marker of *in vivo* proliferation, was

significantly reduced on HIV-1-specific T cells in BAL compared with blood, suggesting a diminished proliferative capacity. In addition, HIV-1-specific CD4⁺ and CD8⁺ T cells in BAL had higher expression of programmed death 1 (PD-1) and lower cytotoxic T-lymphocyte antigen 4 (CTLA-4) expression than those in the blood. A strong correlation between PD-1, but not CTLA-4, and HIV-1-specific T-cell proliferation was seen, and blockade of the PD-1/PD-L1 pathway augmented HIV-1-specific T-cell proliferation, suggesting that the PD-1 pathway was the main cause of reduced proliferation in the lung.

Conclusions: These findings suggest that alveolitis associated with HIV-1 infection is caused by the recruitment of HIV-1-specific CD4⁺ and CD8⁺ T cells to the lung. These antigen-specific T cells display an impaired proliferative capacity that is caused by increased expression of PD-1.

Keywords: HIV; T cells; programmed cell death 1 receptor; cytokines; lung

Pulmonary complications, even with the advent of highly active antiretroviral therapy (HAART), are the leading cause of morbidity and mortality in HIV-1-infected individuals (1). These complications include chronic obstructive pulmonary disease; pulmonary hypertension; and

opportunistic lung infections, such as *Pneumocystis pneumonia* and tuberculosis (2). Lymphocytic alveolitis usually accompanies these complications and is associated with a poor prognosis (3–5). Although epidemiologic evidence supports the role of HIV-1 infection in the incidence

of lung disease, it is unclear whether this stems from a paucity of CD4⁺ T cells or from dysfunctional antigen-specific T-cell responses.

Regulation of T-cell function is a delicate balance between costimulatory signals that activate T cells and inhibitory

(Received in original form August 21, 2014; accepted in final form December 22, 2014)

Supported by U.S. Public Health Service Grants HL98996 (A.P.F., S.C.F., and T.B.C.), HL121816 (A.P.F., B.E.P., and T.B.C.), HL102245 (A.P.F.), and T32 AI007405 (C.P.N.) and the Clinical and Translational Sciences Institute (UL1 TR000154) from the National Center for Advancing Translational Sciences.

Author Contributions: C.P.N., J.L.C., S.M., B.E.P., and A.P.F. designed research. C.P.N., J.L.C., S.M., A.K.M., D.J.L., T.B.C., S.C.F., B.E.P., and A.P.F. performed research. C.P.N., J.L.C., S.M., B.E.P., and A.P.F. analyzed data. C.P.N., J.L.C., S.M., B.E.P., and A.P.F. wrote the manuscript.

Correspondence and requests for reprints should be addressed to Brent E. Palmer, Ph.D., University of Colorado Anschutz Medical Campus, Aurora, CO 80045. E-mail: brent.palmer@ucdenver.edu; or Andrew P. Fontenot, M.D., University of Colorado Anschutz Medical Campus, Aurora, CO 80045. E-mail: andrew.fontenot@ucdenver.edu

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 191, Iss 4, pp 464–473, Feb 15, 2015

Copyright © 2015 by the American Thoracic Society

Originally Published in Press as DOI: 10.1164/rccm.201408-1521OC on December 23, 2014

Internet address: www.atsjournals.org

At a Glance Commentary

Scientific Knowledge on the Subject:

Lymphocytic alveolitis is common in HIV-1-infected individuals and is associated with pulmonary complications and a poor prognosis. Although lymphocytic alveolitis has been associated with viremia and an increased number of CD8⁺ T cells in the lung, the exact cause remains unknown.

What This Study Adds to the Field:

This study demonstrates that HIV-1-specific T cells in the lung of HIV-1-infected subjects are associated with the development of lymphocytic alveolitis. These compartmentalized HIV-1-specific T cells are functionally compromised and express high levels of the coinhibitory receptor, programmed death 1, suggesting an inability of these cells to adequately control HIV-1 in the lung.

signals that attenuate harmful inflammatory responses (6–8). Two negative regulators of activated T cells, programmed death 1 (PD-1) and cytotoxic T-lymphocyte antigen 4 (CTLA-4), are elevated on HIV-1-specific T cells in the blood during chronic HIV-1 infection (9–13). T-cell function is enhanced by blockade of these inhibitory pathways, and treatment of simian immunodeficiency virus-infected macaques or HIV-1-infected humanized mice leads to reduced viral loads and increased CD4⁺ T-cell counts (14–16). Although HIV-1-specific T-cell function has been well-characterized in blood, little is known about the functional competence of these T cells in the lung and their role in the development of lymphocytic alveolitis.

Here, we determined the frequency and function of HIV-1-specific CD4⁺ and CD8⁺ T cells in blood and bronchoalveolar lavage (BAL) of untreated HIV-1-infected subjects and demonstrated that HIV-1-induced lymphocytic alveolitis is associated with the preferential recruitment of HIV-1-specific T cells to the lung. HIV-1-specific T cells in BAL expressed significantly higher levels of PD-1 than their counterparts in blood, and PD-1 expression inversely correlated with *in vivo*

proliferative capacity, suggesting an exhausted T-cell phenotype. Taken together, our data suggest that lymphocytic alveolitis in HIV-1-infected subjects results from an influx of dysfunctional HIV-1-specific T cells into the lung.

Methods

Study Population

BAL cells and peripheral blood mononuclear cells (PBMCs) were obtained from 21 untreated HIV-1-infected subjects and 17 HIV-1-seronegative subjects (see Table E1 in the online supplement). Median viral load in HIV-1-infected subjects was 72,400 copies of HIV-1 RNA per milliliter plasma (range, 418–2,070,000 HIV-1 RNA per milliliter plasma), and the median CD4⁺ T-cell count was 562 cells per microliter (range, 219–1,342 cells per microliter). Informed consent was obtained from each subject, and the protocol was

approved by the Colorado Multiple Institutional Review Board.

Antigen-Specific T-Cell Stimulation, Immunofluorescence Staining, and Flow Cytometry

PBMCs were isolated from heparinized blood, and BAL was obtained as previously described (15, 17, 18). PBMCs and BAL cells were stimulated, washed, incubated with FcR-blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany), stained, and analyzed as detailed in the online supplement. In certain experiments, PBMCs and BAL cells were labeled with Celltrace Violet (Life Technologies, Carlsbad, CA) as previously described (12, 19), and HIV-1 gag-specific T-cell proliferation was determined as detailed in the online supplement.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism (GraphPad, San Diego,

Figure 1. Increased numbers of CD8⁺ T cells in the bronchoalveolar lavage (BAL) of HIV-1-infected subjects. (A) Lymphocytes in the BAL of HIV-1-infected and HIV-1-seronegative subjects were enumerated, and values for each subject are depicted as separate points. Bars represent the median value of each group. Statistical significance of differences between subject groups was determined by Mann-Whitney test. (B) CD4/CD8 T-cell ratios from the blood and BAL of HIV-1-infected and healthy control subjects were determined via flow cytometry. Statistical significance was determined by Wilcoxon matched-pairs signed-rank test. CD4/CD8 T-cell ratio in the blood (C) and BAL (D) was correlated with peripheral viral load. Confidence intervals (CI) are shown. Statistical significance was determined by Spearman correlation test.

CA), SAS version 9.3 (Cary, NC), and R (version 2.13; <http://www.r-project.org/>). Mann-Whitney, paired *t* test, and Spearman correlation analysis were used to determine significance between groups. For regression analyses, which combined blood and BAL outcomes on each subject, the SAS MIXED procedure with a random subject effect was used to model within-subject correlation (20). Sensitivity analyses to adjust for smoking used linear regression and mixed models with log transformations as appropriate. Smoking-adjusted results are mentioned when smoking significantly altered interpretation (*see* Table E2). A *P* value of less than 0.05 was considered statistically significant.

Results

CD8⁺ T Cells Are Recruited to the Lung of HIV-1-Infected Subjects

To assess the severity of lymphocytic alveolitis in HIV-1-infected individuals, we analyzed the absolute number of lymphocytes in BAL of HIV-1-infected and seronegative subjects. The median number of lymphocytes in BAL of HIV-1-infected individuals (20.1×10^6 cells per liter; range, 7.6 – 64×10^6 cells per liter) was significantly higher than in seronegative subjects (4.8×10^6 cells per liter; range, 0.7 – 46×10^6 cells per liter; *P* = 0.0003) (Figure 1A). The median CD4:CD8 T-cell ratio in BAL of HIV-1-infected individuals was significantly decreased compared with blood (median, 0.28 [range, 0.02–1.70] vs. 1.12 [range, 0.33–2.67]; *P* = 0.0002) (Figure 1B). Conversely, no significant difference in the CD4:CD8 T-cell ratio in BAL of HIV-seronegative subjects was observed (*P* = 0.84) (Figure 1B). A strong inverse correlation was found between the CD4:CD8 T-cell ratio in blood and peripheral viral load (*r* = -0.57 ; *P* = 0.009) (Figure 1C), whereas no significant correlation was seen between the CD4:CD8 T-cell ratio in BAL and peripheral viral load (*r* = -0.30 ; *P* = 0.20) (Figure 1D). These findings confirm previous studies (5) and demonstrate that HIV-1-induced lymphocytic alveolitis is primarily caused by CD8⁺ T cells.

HIV-1-Specific IFN- γ -Producing CD4⁺ and CD8⁺ T Cells Are Compartmentalized to the Lung

To evaluate the frequency of virus-specific T cells in the lung of HIV-1-infected

Figure 2. Frequencies of HIV-1 gag-specific IFN- γ -producing CD4⁺ and CD8⁺ T cells in the blood and bronchoalveolar lavage (BAL) of HIV-1-infected subjects. (A) Representative dot plots showing the frequency of HIV-1 gag-specific IFN- γ -producing CD4⁺ and CD8⁺ T cells in blood and BAL. The frequency of IFN- γ -producing T cells is shown in the upper right quadrant of each dot plot. (B) Frequency of HIV-1 gag-specific IFN- γ -producing CD4⁺ and CD8⁺ T cells in blood and BAL of 21 HIV-infected subjects is shown. Paired values for each subject are depicted as separate points and connected by a solid line. (C) Frequency of cytomegalovirus (CMV)-specific IFN- γ -producing CD4⁺ and CD8⁺ T cells in blood and BAL of HIV-1-infected subjects (n = 14) is shown. Statistical significance of differences between subject groups in B and C was determined by Wilcoxon matched-pairs signed-rank test.

individuals, BAL cells were stimulated with overlapping HIV-1 gag peptides, and IFN- γ -producing T cells were detected using intracellular cytokine staining. Representative dot plots depicting the frequency of HIV-1-specific IFN- γ -producing CD4⁺ and CD8⁺ T cells from blood and BAL of an HIV-1-infected subject are shown in Figure 2A. HIV-1-specific IFN- γ -producing CD4⁺ T cells were increased 18.2-fold in BAL compared with blood in 21 subjects (median, 0.14 vs. 2.55%; $P < 0.0001$) (Figure 2B). HIV-1-specific IFN- γ -producing CD8⁺ T cells were also compartmentalized to the lung, representing a 4.5-fold increase in lung compared with blood (median, 0.47 vs. 2.08; $P < 0.0001$) (Figure 2B). Although HIV-1-specific IL-2-producing CD4⁺ and CD8⁺ T cells were detected in BAL of HIV-1-infected individuals, the frequency was low compared with IFN- γ -producing T cells and was not statistically different between blood and BAL (see Figure E1). Cytomegalovirus (CMV)-specific IFN- γ -producing CD4⁺ and CD8⁺ T cells were also observed; however, the frequencies did not differ between blood and BAL (Figure 2C).

To determine if HIV-1-specific T cells contribute to lymphocytic alveolitis in HIV-1-infected subjects, we examined the association between the frequency of HIV-1-specific IFN- γ -producing T cells in BAL and absolute number of BAL lymphocytes. A strong correlation between the frequency of total HIV-1-specific IFN- γ -producing T cells and absolute number of BAL lymphocytes was observed ($r = 0.77$; $P < 0.0001$; confidence interval [CI], 0.47–0.91) (Figure 3A, left). Similarly, we examined the relationship between the number of BAL lymphocytes and HIV-1-specific IFN- γ -producing CD4⁺ and CD8⁺ T cells. A weak positive correlation was seen between the frequency of HIV-1-specific IFN- γ -producing CD4⁺ T cells and absolute number of BAL lymphocytes ($r = 0.43$; $P = 0.08$; CI, –0.05 to 0.75) (Figure 3B, left), whereas a strong positive correlation was seen between the frequency of HIV-1-specific IFN- γ -producing CD8⁺ T cells and absolute number of BAL lymphocytes ($r = 0.62$; $P = 0.006$; CI, 0.20–0.85) (Figure 3B, right).

These data suggest that although both HIV-1-specific CD4⁺ and CD8⁺ T cells contribute to alveolitis, HIV-1-specific

Figure 3. Correlation between the frequency of HIV-1 gag-specific IFN- γ -producing CD4⁺ and CD8⁺ T cells in bronchoalveolar lavage (BAL) and lymphocytic alveolitis. (A) Correlation between the combined frequency of HIV-1 gag-specific IFN- γ -producing CD4⁺ and CD8⁺ T cells (left) and cytomegalovirus (CMV)-specific IFN- γ -producing CD4⁺ and CD8⁺ T cells (right) and the absolute number of BAL lymphocytes is shown. (B) Correlation between the frequency of HIV-1 gag-specific IFN- γ -producing CD4⁺ (left) and CD8⁺ (right) T cells and the absolute number of BAL lymphocytes is shown. (C) The correlation between the frequency of CMV-specific IFN- γ -producing CD4⁺ (left) and CD8⁺ (right) T cells and the absolute number of BAL lymphocytes is shown. Confidence intervals (CI) are shown for each correlation.

CTL are the primary drivers. Similar findings were observed when using percentage of BAL lymphocytes, although a stronger correlation was seen with HIV-1-specific IFN- γ -producing CD4⁺ T cells compared with HIV-1-specific IFN- γ -producing CD8⁺ T cells (see Figure E2). No significant correlations were seen between the frequency of CMV-specific IFN- γ -producing CD4⁺ or CD8⁺ T cells

and number of BAL lymphocytes (Figure 3C) or combined frequencies of CMV-specific T cells and BAL lymphocytes (Figure 3A, right). Taken together, these findings suggest that HIV-1-specific IFN- γ -producing T cells are compartmentalized to the lung and are in part responsible for the lymphocytic alveolitis observed in HIV-1-infected subjects.

Proliferation of HIV-1-Specific CD4⁺ and CD8⁺ T Cells Is Decreased in the Lungs of HIV-1-Infected Individuals

HIV-1-specific T cells in blood have a decreased proliferative capacity (21–23). To evaluate if HIV-1-specific IFN- γ -producing T cells in BAL have similarly decreased proliferation, we examined Ki67 expression, a nuclear protein that demarks recent proliferation, in a subset of 13 subjects. Representative dot plots depicting frequency of Ki67-expressing HIV-1-specific IFN- γ -producing CD4⁺ and CD8⁺ T cells from blood and BAL of an HIV-1-infected subject are shown in Figure 4A. Ki67 expression on total CD4⁺ T cells was not significantly different between blood (median, 2.2; range, 1.1–3.4) and BAL (median, 2.6; range, 0.5–6.2; $P = 0.17$) in HIV-1-infected individuals or in seronegative subjects ($P = 0.19$) (Figure 4B, left). A significantly lower Ki67 expression was seen in total CD8⁺ T cells in BAL compared with blood in HIV-1-infected subjects (median, 4.5 vs. 2.8; $P = 0.001$) (Figure 4A, right) but not in seronegative individuals ($P = 0.92$) (Figure 4C, left). HIV-1-specific IFN- γ -producing CD4⁺ (median, 5.8; range, 0.4–12.5) and CD8⁺ (median, 6.6; range, 0.8–27) T cells in BAL had significantly lower Ki67 expression compared with their counterparts in blood (CD4 median, 5.8; range, 0–23; $P = 0.03$) (CD8 median, 20; range, 3.3–37; $P = 0.03$) (Figures 4B and 4C, right).

To determine whether the decreased proliferative capacity was restricted to HIV-1-specific T cells in BAL, we analyzed Ki67 expression on CMV-specific T cells in blood and BAL in 10 HIV-1-infected subjects. Although a trend toward decreased Ki67 expression in CMV-specific IFN- γ -producing CD4⁺ and CD8⁺ T cells in BAL compared with blood was seen, statistical significance was not achieved (Figures 4B and 4C, right). Overall, these data suggest that HIV-1-specific T cells are proliferation-incompetent and preferentially recruited to the lung.

Coinhibitory Receptor Expression Is Altered on HIV-1-Specific CD4⁺ and CD8⁺ T Cells in BAL

Coinhibitory molecule expression is elevated on HIV-1-specific T cells in blood and associated with diminished T-cell function and reduced control of HIV-1 replication (10–12). To evaluate whether inhibitory receptors are associated with reduced HIV-1-specific T-cell proliferation

Figure 4. Proliferation of HIV-1 gag-specific IFN- γ -producing T cells in blood and bronchoalveolar lavage (BAL) of HIV-1-infected individuals. (A) Representative dot plots showing the frequency of HIV-1 gag-specific IFN- γ -producing CD4⁺ and CD8⁺ T cells expressing Ki67 in blood and BAL are shown. (B) Frequency of total (left) and virus-specific (right) CD4⁺ T cells from the blood and BAL of 10 seronegative and 13 HIV-1-infected subjects expressing Ki67 is shown. (C) Ki67 expression in total (left) and virus-specific (right) CD8⁺ T cells from the blood and BAL of seronegative and HIV-1-infected subjects is shown. Statistical significance of differences between blood and BAL was determined by a paired t test. CMV = cytomegalovirus.

in the lung, we examined PD-1 expression on T cells in BAL from HIV-1-infected individuals. Representative dot plots depicting PD-1 expression on total and

HIV-1-specific CD4⁺ and CD8⁺ T cells from blood and BAL of an HIV-1-infected subject are shown in Figure 5A. Mean fluorescence intensity of PD-1 on total

CD4⁺ (2,038; range, 728–4,398) and CD8⁺ (1,944; range, 874–6,889) T cells in BAL was significantly elevated compared with its expression on CD4⁺ (1,019; range, 676–1,683; $P < 0.0001$) and CD8⁺ (1,483; range, 518–4,793; $P < 0.0001$) T cells in blood (Figure 5B). However, after adjusting for smoking, PD-1 expression on CD4⁺ T cells in BAL was no longer significantly different compared with blood ($P = 0.24$) because of a 23.4% (0.36–49.8%) higher PD-1 expression on CD4⁺ T cells in BAL for smokers compared with nonsmokers (see Figure E3A). PD-1 expression on BAL CD8⁺ T cells was not different between smokers and nonsmokers (see Figure E3C). Percentage of T cells expressing PD-1 was also examined, and a similar expression pattern was seen (see Figure E4). Because of increased PD-1 expression on BAL T cells, no significant differences were seen in PD-1 expression on total CD4⁺ and CD8⁺ T cells in BAL from normal subjects and HIV-1-infected individuals (see Figure E5).

PD-1 expression on BAL HIV-1-specific IFN- γ -producing CD4⁺ (median, 2,526; range, 1,099–8,000) and CD8⁺ (median, 2,361; range, 1,126–6,069) T cells was even higher compared with HIV-1-specific T cells in blood (CD4⁺ median, 1,398; range 728–5,111; $P = 0.0005$) (CD8⁺ median, 1,773; range, 942–4,038; $P = 0.0012$) (Figure 5C). After adjusting for smoking, PD-1 expression on HIV-1-specific IFN- γ -producing CD4⁺ T cells in BAL compared with blood remained statistically significant ($P = 0.0004$). In addition, PD-1 expression on HIV-1-specific IFN- γ -producing CD4⁺ and CD8⁺ T cells was not different in smokers and nonsmokers (see Figures E3B and E3D). PD-1 expression on CMV-specific IFN- γ -producing T cells in blood and BAL was not significantly differentially expressed in these two compartments (see Figure E6).

Although PD-1 is expressed by both CD4⁺ and CD8⁺ T cells, CTLA-4, another coinhibitory receptor, preferentially plays a role in CD4⁺ T cells (10). Therefore, we examined CTLA-4 expression on CD4⁺ T cells in blood and BAL from 13 subjects. Expression of CTLA-4 was significantly increased on total CD4⁺ T cells in BAL compared with blood ($P < 0.0001$) (Figure 6A). Conversely, HIV-1-specific IFN- γ -producing CD4⁺ T cells in BAL lose CTLA-4 expression compared with blood ($P = 0.006$) (Figure 6B), in contrast

Figure 5. Programmed death 1 (PD-1) expression on total and HIV-1 gag-specific IFN- γ -producing T cells in the blood and bronchoalveolar lavage (BAL) of HIV-1-infected subjects. (A) Representative histograms of PD-1 expression on total and HIV-specific IFN- γ -producing CD4⁺ T cells in blood and BAL (top) and on total and HIV-specific IFN- γ -producing CD8⁺ T cells in blood and BAL (bottom) is depicted. (B) Mean fluorescence intensity (MFI) of PD-1 expression on total CD4⁺ (left) and CD8⁺ (right) T cells in the blood and BAL of HIV-1-infected subjects is shown. Because of the identified influence of smoking, the smoking-adjusted P value is shown for A. (C) PD-1 expression (MFI) on HIV-1 gag-specific IFN- γ -producing CD4⁺ (left) and CD8⁺ (right) T cells in blood and BAL is depicted. Paired values for each subject shown in B and C are depicted as separate points and connected by a solid line. Statistical significance of differences between blood and BAL was determined by a Wilcoxon matched-pairs signed-rank test. FMO = fluorescence minus one.

Figure 6. Cytotoxic T-lymphocyte antigen 4 (CTLA-4) expression in total and HIV-1-specific CD4⁺ T cells in blood and bronchoalveolar lavage (BAL) of HIV-1-infected subjects. Mean fluorescence intensity (MFI) of CTLA-4 expression on total (A) and HIV-1 gag-specific IFN- γ -producing (B) CD4⁺ T cells from the blood and BAL of HIV-1-infected subjects is shown. Statistical significance of differences between blood and BAL was determined by a paired *t* test.

to increased PD-1 expression on HIV-1-specific T cells in BAL. This differential expression of PD-1 and CTLA-4 on HIV-1-specific CD4⁺ T cells in BAL suggest that each coinhibitory receptor has a distinct role in regulating immune responses in the lung.

PD-1 Mediates Proliferation of HIV-1-Specific CD4⁺ and CD8⁺ T Cells in the Lungs

Coinhibitory receptor pathways mediate T-cell exhaustion and limit HIV-1-specific T-cell proliferation in blood during chronic HIV infection (7, 11, 24). To determine if reduced proliferation in lung was correlated with PD-1 and/or CTLA-4 expression, we compared receptor expression on

HIV-1-specific IFN- γ -producing T cells with Ki67 expression using nine subjects from whom PD-1, CTLA-4, and Ki67 expression was assessed. Consistent with previous reports (11), Ki67 expression on antigen-specific T cells in blood was inversely correlated to PD-1 expression ($r = -0.70$; $P = 0.034$) (data not shown). Conversely, PD-1 and Ki67 expression on T cells in BAL were not statistically correlated ($r = -0.24$; $P = 0.49$) because of uniform high PD-1 and low Ki67 expression. However, combining PD-1 and Ki67 expression from blood and BAL T cells and using a statistic model that accommodates within-subject correlation, a negative relationship was seen between PD-1 and Ki67 expression on HIV-1-specific

IFN- γ -producing CD4⁺ T cells ($P = 0.02$) (Figure 7A). Similar findings were observed when we modeled the relationship between the expression of PD-1 and Ki67 on HIV-1-specific IFN- γ -producing CD8⁺ T cells in blood and BAL ($P = 0.04$) (Figure 7B). Conversely, CTLA-4 expression was not significantly correlated to Ki67 expression in blood ($P = 0.52$) or in lung ($P = 0.86$) and did not show a trend when blood and BAL cells were analyzed together ($P = 0.61$) (Figure 7C). These combined data suggest that PD-1 expression is driving the diminished T-cell proliferation in the lung, whereas CTLA-4 plays a minimal role in HIV-1-specific T-cell proliferation.

To determine if PD-1 expression was a marker of activation or exhaustion, we performed proliferation studies with blockade of the PD-1/PD-L1 pathway using PBMCs and BAL cells from eight HIV-1-infected subjects. Compared with expansion following stimulation with HIV-1 gag peptides, addition of PD-L1 blockade resulted in a significantly greater expansion of HIV-1-specific CD4⁺ T cells in blood ($P = 0.02$) and BAL ($P = 0.01$) (Figure 8A). Similar findings were seen for blood and BAL CD8⁺ T cells (Figure 8B). The greater percentage of proliferating HIV-1-specific CD4⁺ and CD8⁺ T cells with gag peptides alone in BAL compared with blood reflects the higher percentage of HIV-1-specific IFN- γ -producing T cells in BAL (Figure 2B). Interestingly, when comparing

Figure 7. Prediction of Ki67 on HIV-1-specific CD4⁺ and CD8⁺ T cells in blood and bronchoalveolar lavage (BAL) of HIV-1-infected subjects with programmed death 1 (PD-1) and cytotoxic T-lymphocyte antigen 4 (CTLA-4) expression. The expression of Ki67 is predicted by PD-1 on HIV-1 gag-specific IFN- γ -producing CD4⁺ (A) and CD8⁺ (B) T cells in the blood (black circles) and BAL (red circles) of HIV-1-infected subjects is shown. (C) The expression of Ki67 predicted by CTLA-4 in HIV-1 gag-specific IFN- γ -producing CD4⁺ T cells in the blood (black circles) and BAL (red circles) of HIV-1-infected subjects is shown. A mixed effects model was used to determine significance while accommodating for within-subject correlation. For each increase of 1,000 PD-1 mean fluorescence intensity (MFI) we saw a decrease of 2.32% (confidence interval [CI], 0.6–4.45%) and 7.58% (CI, 0.46–14.7%) HIV-1 gag-specific IFN- γ -producing CD4⁺ and CD8⁺ T cells expressing Ki67, respectively. For each increase of 1,000 CTLA-4 MFI, a 0.37% (CI, –1.24 to 2.0%) increase in HIV-1 gag-specific IFN- γ -producing CD4⁺ T cells expressing Ki67 was observed. PBMC = peripheral blood mononuclear cell.

Figure 8. Effect of blockade of programmed death 1/programmed death-ligand 1 (PD-1/PD-L1) pathway on HIV-specific CD4⁺ and CD8⁺ T cell proliferation. (A) Percentage of Celltrace Violet^{low} CD4⁺ T cells in blood and bronchoalveolar lavage (BAL) after 6 days of stimulation with HIV-1 gag peptides in the presence or absence of anti-PD-L1 mAb is shown. (B) Percentage of proliferating CD8⁺ T cells in blood and BAL after 6 days of stimulation with HIV-1 gag peptides in the presence or absence of anti-PD-L1 mAb. Data shown in A and B are minus background proliferation. (C) The fold change of CD4⁺ and CD8⁺ T-cell proliferation induced by PD-1/PD-L1 pathway blockade in blood and BAL is shown. Statistical significance of differences between blood and BAL was determined by a paired *t* test.

the median fold change of gag-specific CD8⁺ T-cell proliferation induced by PD-1/PD-L1 blockade in blood with BAL, a significant increase was seen ($P = 0.02$) (Figure 8C). Conversely, the fold change of proliferating gag-specific CD4⁺ T cells induced by PD-1/PD-L1 pathway blockade was not statistically different ($P = 0.38$). These data suggest that HIV-1-specific T cells, especially HIV-1-specific CD8⁺ T cells, are functionally impaired and that function can be restored with PD-1/PD-L1 pathway blockade.

Discussion

The aim of this study was to determine the underlying mechanism of lymphocytic alveolitis, which has been associated with a poor prognosis in HIV-1-infected individuals. As previously described (3–5), our HAART-naïve HIV-1-infected subjects had increased numbers of lymphocytes in BAL. We hypothesized that HIV-1-associated lymphocytic alveolitis is caused by an influx of HIV-1-specific T cells. We found that HIV-1-specific T cells in the lung are significantly increased compared with blood, and a strong positive correlation between the frequency of HIV-1-specific IFN- γ -producing CD4⁺ and CD8⁺ T cells and absolute number of lymphocytes in BAL was seen. Furthermore, phenotypic and functional analysis of these cells in BAL showed an inverse correlation between PD-1 expression and *in vivo* T-cell proliferation. Taken together, these data

suggest that lymphocytic alveolitis of HIV-1-infected subjects is associated with recruitment of dysfunctional HIV-1-specific T cells to the lung.

The prevalence of lymphocytic alveolitis is increased in individuals with chronic HIV-1 infection and is associated with pulmonary complications (5). Consistent with these reports, lymphocytic alveolitis with an increase in CD8⁺ T cells was observed in our HIV-1-infected cohort. Preferential recruitment of CD8⁺ T cells to the lung is in part responsible for the imbalance in the CD4:CD8 T-cell ratio that has been associated with HIV-1-specific CTLs and disease progression (5, 25). Early studies hypothesized that CTL activity was directed against HIV-1-infected alveolar macrophages that coexpress HIV-1 antigens and CD4 (26). Recent studies have demonstrated that alveolar macrophages in HIV-1-infected patients with alveolitis secrete CXCR3 ligands that are capable of inducing T-cell chemotaxis (27). Furthermore, a significant positive correlation between macrophage inflammatory protein-1 α , a cytokine produced by alveolar macrophages and chemotactic for CD8⁺ T cells, and CD8⁺ T-cell alveolitis has been shown (28).

Although these studies suggest that the influx of virus-specific T cells, and more specifically CD8⁺ T cells, causes lymphocytic alveolitis, an association between HIV-1-specific T cells and alveolitis had not been demonstrated until now. A correlative analysis revealed a strong relationship between the frequency of total HIV-1-specific T cells and absolute

number of BAL lymphocytes, whereas no significant correlation with CMV-specific T cells was observed. These data suggest that the influx of HIV-1-specific T cells, and more specifically CD8⁺ T cells, is closely linked to lymphocytic alveolitis in HIV-1-infected individuals.

The influx of HIV-1-specific T cells into the lung is presumably caused by the continued presence of HIV-1. This is supported by the finding that HIV-1 viral copies per milliliter BAL directly correlates with the percentage of BAL lymphocytes, especially when peripheral CD4⁺ T-cell count is less than 200 cells per microliter (5). After initiation of HAART, HIV-1 viral load and BAL CD8⁺ lymphocytes decrease, suggesting that viral burden is a direct cause of increased CD8⁺ lymphocytes in the lung (29). It has been shown that alveolar macrophages, CD4⁺ and CD8⁺ T cells, and fibroblasts (30, 31) in the lung can harbor HIV-1. Although the frequency of infected CD4⁺ T cells in BAL is not higher than in blood (32), alveolar macrophages, particularly small ones, are preferentially infected, providing a focus of HIV-1 infection in the lung that drives the influx of HIV-1-specific T cells (33). Alveolar macrophages are long-lived and resistant to apoptosis so it is plausible that they can serve as cellular reservoirs of HIV-1 (34). Furthermore, in ART-treated macaques, the second largest burden of simian immunodeficiency virus RNA in the body after lymphatic tissues was the lung (35). Taken together, our findings suggest that elevated levels of HIV-1-specific T cells in the lung are likely caused by an active focus of HIV-1 infection.

HIV-1-specific T cells in the blood are functionally impaired during chronic HIV-1 infection (21–23, 36), which is in part caused by exhaustion and overexpression of inhibitory receptors (11–13). These exhausted T cells lose effector functions and exhibit antigen-independent homeostatic proliferation (37). The severity of T-cell dysfunction directly correlates with expression of inhibitory receptors, particularly PD-1. However, it is unknown if these findings from circulating T cells translate to those residing in the lung. For example, circulating hepatitis C virus-specific CD8⁺ T cells responded to PD-1 pathway blockade *in vitro*, whereas those from the liver were unresponsive (38). We have shown that HIV-1-specific T cells in lymph nodes express higher levels

of PD-1 compared with blood (12). These findings, although from liver and lymph node, illustrate that differences in immune regulation of T cells exist in different body compartments. Beryllium-specific CD4⁺ T cells in BAL of patients with chronic beryllium disease have significantly higher PD-1 expression than their counterparts in blood, and blockade of the PD-1 pathway augmented beryllium-induced T-cell proliferation (19).

Here, we found a similar PD-1 expression pattern on HIV-1-specific T cells, and blockade of the PD-1 pathway also augmented proliferative capacity. This was especially true for HIV-1-specific CD8⁺ T cells in BAL. These findings show that PD-1 is a marker of exhaustion as

opposed to activation. We also found that when incubated with gag peptides alone there was a higher percentage of proliferating HIV-1-specific CD4⁺ and CD8⁺ T cells in BAL compared with blood, which is a direct reflection of the 18.2- and 4.5-fold higher frequencies of HIV-1-specific CD4⁺ and CD8⁺ T cells, respectively, in the lung compared with blood. In addition, an inverse correlation between PD-1 and Ki67 on HIV-1-specific T cells was seen, suggesting that the PD-1 pathway plays the predominant role in regulating proliferation in the lung.

Before development of advanced HIV-1-induced CD4⁺ T-cell depletion, our data indicate that HIV-1-induced lymphocytic alveolitis is driven by accumulation of

dysfunctional HIV-1-specific T cells in the lung, suggesting that these cells are recruited to the lung rather than undergoing *in vivo* expansion. Although our cohort consisted of untreated HIV-1-infected subjects, which may not translate with treated patients, these findings could shed light into other lung complications that persist with treatment. Finally, our findings provide insight into mucosal T-cell responses to HIV-1 infection, the role of T-cell exhaustion in lung disease, and their implications for therapeutic coinhibitory blockade during chronic infection. ■

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

References

- Gingo MR, Morris A. Pathogenesis of HIV and the lung. *Curr HIV/AIDS Rep* 2013;10:42–50.
- Crothers K, Huang L, Goulet JL, Goetz MB, Brown ST, Rodriguez-Barradas MC, Oursler KK, Rimland D, Gibert CL, Butt AA, *et al*. HIV infection and risk for incident pulmonary diseases in the combination antiretroviral therapy era. *Am J Respir Crit Care Med* 2011;183:388–395.
- Benito N, Moreno A, Filella X, Miró JM, González J, Pumarola T, Valls ME, Luna M, García F, Rañó A, *et al*. Inflammatory responses in blood samples of human immunodeficiency virus-infected patients with pulmonary infections. *Clin Diagn Lab Immunol* 2004;11:608–614.
- Twigg HL III, Spain BA, Soliman DM, Knox K, Sidner RA, Schnitzlein-Bick C, Wilkes DS, Iwamoto GK. Production of interferon-gamma by lung lymphocytes in HIV-infected individuals. *Am J Physiol* 1999;276:L256–L262.
- Twigg HL, Soliman DM, Day RB, Knox KS, Anderson RJ, Wilkes DS, Schnitzlein-Bick CT. Lymphocytic alveolitis, bronchoalveolar lavage viral load, and outcome in human immunodeficiency virus infection. *Am J Respir Crit Care Med* 1999;159:1439–1444.
- Chen L. Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity. *Nat Rev Immunol* 2004;4:336–347.
- Crawford A, Wherry EJ. The diversity of costimulatory and inhibitory receptor pathways and the regulation of antiviral T cell responses. *Curr Opin Immunol* 2009;21:179–186.
- Watts TH. TNF/TNFR family members in costimulation of T cell responses. *Annu Rev Immunol* 2005;23:23–68.
- Kaufmann DE, Walker BD. Programmed death-1 as a factor in immune exhaustion and activation in HIV infection. *Curr Opin HIV/AIDS* 2008;3:362–367.
- Kaufmann DE, Kavanagh DG, Pereyra F, Zaunders JJ, Mackey EW, Miura T, Palmer S, Brockman M, Rathod A, Piechocka-Trocha A, *et al*. Upregulation of CTLA-4 by HIV-specific CD4⁺ T cells correlates with disease progression and defines a reversible immune dysfunction. *Nat Immunol* 2007;8:1246–1254.
- Day CL, Kaufmann DE, Kiepiela P, Brown JA, Moodley ES, Reddy S, Mackey EW, Miller JD, Leslie AJ, DePierres C, *et al*. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* 2006;443:350–354.
- D'Souza M, Fontenot AP, Mack DG, Lozupone C, Dillon S, Meditz A, Wilson CC, Connick E, Palmer BE. Programmed death 1 expression on HIV-specific CD4⁺ T cells is driven by viral replication and associated with T cell dysfunction. *J Immunol* 2007;179:1979–1987.
- Trautmann L, Janbazian L, Chomont N, Said EA, Gimmig S, Bessette B, Boulassel MR, Delwart E, Sepulveda H, Balderas RS, *et al*. Upregulation of PD-1 expression on HIV-specific CD8⁺ T cells leads to reversible immune dysfunction. *Nat Med* 2006;12:1198–1202.
- Finnefrock AC, Tang A, Li F, Freed DC, Feng M, Cox KS, Sykes KJ, Guare JP, Miller MD, Olsen DB, *et al*. PD-1 blockade in rhesus macaques: impact on chronic infection and prophylactic vaccination. *J Immunol* 2009;182:980–987.
- Chain JL, Martin AK, Mack DG, Maier LA, Palmer BE, Fontenot AP. Impaired function of CTLA-4 in the lungs of patients with chronic beryllium disease contributes to persistent inflammation. *J Immunol* 2013;191:1648–1656.
- Seung E, Dudek TE, Allen TM, Freeman GJ, Luster AD, Tager AM. PD-1 blockade in chronically HIV-1-infected humanized mice suppresses viral loads. *PLoS ONE* 2013;8:e77780.
- Kassu A, Marcus RA, D'Souza MB, Kelly-McKnight EA, Golden-Mason L, Akkina R, Fontenot AP, Wilson CC, Palmer BE. Regulation of virus-specific CD4⁺ T cell function by multiple costimulatory receptors during chronic HIV infection. *J Immunol* 2010;185:3007–3018.
- Mack DG, Lanham AM, Falta MT, Palmer BE, Maier LA, Fontenot AP. Deficient and dysfunctional regulatory T cells in the lungs of chronic beryllium disease subjects. *Am J Respir Crit Care Med* 2010;181:1241–1249.
- Palmer BE, Mack DG, Martin AK, Gillespie M, Mroz MM, Maier LA, Fontenot AP. Up-regulation of programmed death-1 expression on beryllium-specific CD4⁺ T cells in chronic beryllium disease. *J Immunol* 2008;180:2704–2712.
- Laird NM, Ware JH. Random-effects models for longitudinal data. *Biometrics* 1982;38:963–974.
- Palmer BE, Boritz E, Blyveis N, Wilson CC. Discordance between frequency of human immunodeficiency virus type 1 (HIV-1)-specific gamma interferon-producing CD4⁺ T cells and HIV-1-specific lymphoproliferation in HIV-1-infected subjects with active viral replication. *J Virol* 2002;76:5925–5936.
- Younes SA, Yassine-Diab B, Dumont AR, Boulassel MR, Grossman Z, Routy JP, Sekaly RP. HIV-1 viremia prevents the establishment of interleukin 2-producing HIV-specific memory CD4⁺ T cells endowed with proliferative capacity. *J Exp Med* 2003;198:1909–1922.
- McNeil AC, Shupert WL, Iyasere CA, Hallahan CW, Mican JA, Davey RT Jr, Connors M. High-level HIV-1 viremia suppresses viral antigen-specific CD4⁽⁺⁾ T cell proliferation. *Proc Natl Acad Sci USA* 2001;98:13878–13883.
- El-Far M, Halwani R, Said E, Trautmann L, Doroudchi M, Janbazian L, Fonseca S, van Grevenynghe J, Yassine-Diab B, Sékaly RP, *et al*. T-cell exhaustion in HIV infection. *Curr HIV/AIDS Rep* 2008;5:13–19.

25. Guillon JM, Autran B, Denis M, Fouret P, Plata F, Mayaud CM, Akoun GM. Human immunodeficiency virus-related lymphocytic alveolitis. *Chest* 1988;94:1264–1270.
26. Autran B, Mayaud CM, Raphael M, Plata F, Denis M, Bourguin A, Guillon JM, Debre P, Akoun G. Evidence for a cytotoxic T-lymphocyte alveolitis in human immunodeficiency virus-infected patients. *AIDS* 1988;2:179–183.
27. Agostini C, Facco M, Siviero M, Carollo D, Galvan S, Cattelan AM, Zambello R, Trentin L, Semenzato G. CXC chemokines IP-10 and mig expression and direct migration of pulmonary CD8+/CXCR3+ T cells in the lungs of patients with HIV infection and T-cell alveolitis. *Am J Respir Crit Care Med* 2000;162:1466–1473.
28. Denis M, Ghadirian E. Alveolar macrophages from subjects infected with HIV-1 express macrophage inflammatory protein-1 alpha (MIP-1 alpha): contribution to the CD8+ alveolitis. *Clin Exp Immunol* 1994; 96:187–192.
29. Twigg HL III, Knox KS. Impact of antiretroviral therapy on lung immunology and inflammation. *Clin Chest Med* 2013;34:155–164.
30. Semenzato G, Agostini C, Chieco-Bianchi L, De Rossi A. HIV load in highly purified CD8+ T cells retrieved from pulmonary and blood compartments. *J Leukoc Biol* 1998;64:298–301.
31. Costiniuk CT, Jenabian MA. The lungs as anatomical reservoirs of HIV infection. *Rev Med Virol* 2014;24:35–54.
32. Brechley JM, Knox KS, Asher AI, Price DA, Kohli LM, Gostick E, Hill BJ, Hage CA, Brahmi Z, Khoruts A, et al. High frequencies of polyfunctional HIV-specific T cells are associated with preservation of mucosal CD4 T cells in bronchoalveolar lavage. *Mucosal Immunol* 2008;1:49–58.
33. Jambo KC, Banda DH, Kankwatira AM, Sukumar N, Allain TJ, Heyderman RS, Russell DG, Mwandumba HC. Small alveolar macrophages are infected preferentially by HIV and exhibit impaired phagocytic function. *Mucosal Immunol* 2014;7: 1116–1126.
34. Tan J, Sattentau QJ. The HIV-1-containing macrophage compartment: a perfect cellular niche? *Trends Microbiol* 2013;21: 405–412.
35. Horiike M, Iwami S, Kodama M, Sato A, Watanabe Y, Yasui M, Ishida Y, Kobayashi T, Miura T, Igarashi T. Lymph nodes harbor viral reservoirs that cause rebound of plasma viremia in SIV-infected macaques upon cessation of combined antiretroviral therapy. *Virology* 2012;423:107–118.
36. Migueles SA, Weeks KA, Nou E, Berkley AM, Rood JE, Osborne CM, Hallahan CW, Coglian-Shutta NA, Metcalf JA, McLaughlin M, et al. Defective human immunodeficiency virus-specific CD8+ T-cell polyfunctionality, proliferation, and cytotoxicity are not restored by antiretroviral therapy. *J Virol* 2009;83:11876–11889.
37. Wherry EJ, Ha SJ, Kaech SM, Haining WN, Sarkar S, Kalia V, Subramaniam S, Blattman JN, Barber DL, Ahmed R. Molecular signature of CD8+ T cell exhaustion during chronic viral infection. *Immunity* 2007;27:670–684.
38. Nakamoto N, Kaplan DE, Coleclough J, Li Y, Valiga ME, Kaminski M, Shaked A, Olthoff K, Gostick E, Price DA, et al. Functional restoration of HCV-specific CD8 T cells by PD-1 blockade is defined by PD-1 expression and compartmentalization. *Gastroenterology* 2008;134: 1927–1937, e1–e2.