

## Research Article

# T Cell Senescence is Associated with Development of Chronic Obstructive Pulmonary Disease (COPD) in a Non-Human Primate Model of HIV Infection

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**Abstract**

The life expectancy of successfully-treated HIV-infected individuals has improved; however, patients on long-term anti-retroviral therapy are at increased risk for development of co-morbidities including dementia, cardiovascular disease and chronic obstructive pulmonary disease (COPD). As these latter diseases are also associated with advancing age, it has been postulated that HIV infection results in accelerated immune aging that contributes to disease development, though the underlying pathologic processes are poorly understood. Here we used a cynomolgus macaque (*Macaca fascicularis*) model of HIV infection and COPD to investigate relationships between immune cell senescence and HIV-COPD. We report COPD+ macaques had increased T cell infiltration of airways and evidence of cell proliferation in the lung, compared with COPD- macaques. In the peripheral blood, senescent CD8+ T cells were inversely associated with pulmonary function. These results support the hypothesis that pre-mature T cell senescence is associated with COPD development in the context of chronic HIV infection. We suggest that, while current HIV therapies effectively control virus, chronic immune activation may predispose HIV-infected individuals to comorbidities such as COPD. Combination therapies targeting inflammation and immune senescence could elucidate potential new avenues toward development of improved therapeutics.

**ABBREVIATIONS**

HIV: Human Immunodeficiency Virus; COPD: Chronic Obstructive Pulmonary Disease; Pc: *Pneumocystis jirovecii*; SIV: Simian Immunodeficiency Virus; PFT: Pulmonary Function Test(S); SHIV: Simian Immunodeficiency/HIV Chimeric Virus; BAL: Bronchoalveolar Lavage; BALT: Bronchus-Associated Lymphoid Tissue; FVC: Forced Vital Capacity; PEF: Peak Expiratory Flow; FEV<sub>0.4</sub>: Forced Expiratory Volume in 0.4 Seconds; MMEF: Mid-Maximal Expiratory Flow; Bcl2: B Cell Lymphoma 2 Protein

**INTRODUCTION**

The overall burdens of an aging HIV population coupled with poorly understood HIV-associated immune dysregulation and co-morbidities require new approaches for treatment of this population. In addition to the natural aging of the HIV-infected population, HIV-infected individuals experience a pre-mature immune aging and/or immune exhaustion [1], possibly

a consequence of chronic immune activation and inflammation, though the relationship between a dysregulated immune/inflammatory response and the development of HIV-related co-morbidities is unclear. During normal chronologic aging, there is progressive alteration in the immune system that contributes to increased risk of morbidity and mortality to infections, cancer, and inflammatory diseases among older adults [2,3]. Among these age-associated alterations is the irreversible loss of CD28, the major costimulatory receptor normally required to sustain T cell activation, and shortening of telomeres in immune cells [4,5]. Interestingly, CD28 loss and telomere shortening in an age-disproportionate manner are typical of HIV/AIDS patients [6]. With increased incidence of immune-mediated diseases such chronic obstructive pulmonary disease (COPD) and pulmonary arterial hypertension among HIV-infected persons and in the general aging population [7-12], inflammation and cell senescence may be key components of chronic HIV disease.

COPD is among the most common chronic maladies in adults

and is predicted to become the third most common cause of death by the year 2020 [13]. Although tobacco use is the primary risk factor for COPD development, only approximately twenty percent of smokers develop the disease, suggesting other disease co-factors. Pathogenesis of COPD is characterized by chronic lung inflammation, leading to airway remodeling, lung destruction, and irreversible airflow obstruction [14], likely originating from dysregulated innate and acquired immune responses to inflammatory agents, including cigarette smoke, environmental pollutants and/or infectious pathogens [15-21]. COPD prevalence increases with age [7], and although for some patients this increase could reflect longer periods of exposure to cigarette smoke, age-related changes to the lung and the immune system likely also play a role in pathogenesis. Even in elderly persons without COPD, airspace enlargement in the absence of alveolar wall destruction has been reported [22]. Additionally, systemic changes to the aging immune system, including increased prevalence of inflammatory immune cells, accumulation of pro-inflammatory cytokines, impaired innate immunity, elevated oxidative stress and DNA damage (or reduced repair) and functional/structural changes, are thought to contribute to many chronic diseases associated with advanced age including COPD [22]. The central role of inflammation in COPD pathogenesis has led many researchers to the hypothesis that COPD may be a phenotype of accelerated aging [7].

HIV-infected persons have increased risk for developing COPD, which occurs at a younger age and more frequently in this population compared with HIV-uninfected persons [10,17,23]. Although COPD is more prevalent in HIV-infected smokers than non-smokers, recent studies indicate high frequencies of COPD and respiratory symptoms in never-smokers, suggesting an etiology other than cigarette smoke exposure [24,25]. COPD in non-smokers may be related to the increased frequency of persistent pulmonary pathogens resulting in amplification of pulmonary inflammation. In clinical studies and in experimental animal models, chronic colonization with the fungal opportunistic pathogen, *Pneumocystis jirovecii* (Pc) [20, 26-28] is associated with increased pulmonary inflammation and development of COPD in the context of HIV or simian immunodeficiency virus (SIV) infection [20,27,29-31]. Pc colonization is also associated with COPD and worse pulmonary function in HIV negative individuals [32] and with increased systemic levels of pro-inflammatory cytokines [33]. Thus, persistent Pc colonization is a co-factor in development of COPD.

Infiltration of T cells, particularly CD8+ T cells, in the lungs of COPD patients have been reported [34,35], and there has been increased interest in the role of these cells in COPD pathogenesis because of their ability to produce inflammatory mediators capable of inducing apoptosis in target cells and bystander cells. Additionally, HIV infection is characterized by T cell activation, loss of co-stimulatory molecule (CD28) expression, reduced numbers of naïve T cells and expansion of particular memory T cell subsets, which may have impaired or deregulated functionality [1,36].

Chronic immune activation and T cell senescence are associated with both HIV infection [6,37,38] and COPD [39,40], although the clinical consequences of the accumulation of

senescent T cells are not fully characterized. Senescent T cells are unable to replicate, have shortened telomeres, lose surface expression of CD28 and become apoptotic-resistant, but retain some functional capacity, including production of inflammatory cytokines (IL-6, TNF $\alpha$ ) upon stimulation [6]. Thus the accumulation of these cells in advanced age, HIV infection and other chronic conditions is at least an indicator of, if not a contributor to, dysregulated or impaired immunity.

In the current study, we examined the relationship between chronic SIV infection, T cell senescence and the development of COPD in a non-human primate model of HIV-related COPD. We report that development of COPD is associated with increased frequency of airway-associated lymphocytic infiltration and elevated number and frequency of senescent CD8+ T cells in the peripheral blood. Evidence is presented here to support the concept that co-infection with pathogens such as *Pneumocystis* amplify local and systemic inflammation that may contribute to pulmonary tissue destruction and developing of HIV-associated COPD.

## MATERIALS AND METHODS

### Animals

Adult, Chinese-origin cynomolgus (*Macaca fascicularis*) and Rhesus (*Macaca mulatta*) macaques were used in these studies [30,31]. All animals were obtained from National Primate Centers or vendors approved by University of Pittsburgh Department of Laboratory Animal Research. All animals were screened for simian retroviruses prior to purchase, and were housed and maintained in a Biosafety Level 2+ (BSL2+) primate facility at the University of Pittsburgh. Animal research was conducted at the University of Pittsburgh under Assurance number A3187-01 (Office of Lab Animal Welfare of the Public Health Service), following the guidelines described in the *NIH Guide for the Care and Use of Laboratory Animals* [41]. The University of Pittsburgh is accredited by the American Association for the Assessment and Accreditation of Lab Animal Care (AAALAC) and prior to initiation, all studies were approved by University of Pittsburgh Institution Animal Care and Use Committee.

### Virus infection

Cynomolgus macaques were intravenously infected with the simian immunodeficiency/HIV chimeric virus, SHIV<sub>89.6P</sub>, as described [30,31,42]. This virus induces CD4+ T cell decline and AIDS-like disease, including wasting and opportunistic infections in macaques [43,44]. Full characterization of SHIV infection in this cohort has been published elsewhere [30,31].

### Blood and bronchoalveolar lavage (BAL) fluid collection and processing

Peripheral blood and BAL fluid were collected at baseline and at time-points following SHIV-infection, as previously described [30,31,45,46]. Unfractionated BAL fluid was used for detection of Pc by nested PCR [45]. The remaining BAL fluid was passed through a 40um cell strainer and supernatants were used for cytokine analysis [31].

### Immunohistochemistry of lung tissue

At necropsy, the right lung was removed and inflated to 25cm

H<sub>2</sub>O with 10% buffered formalin. Serial mid-sagittal sections from each lobe were paraffin-embedded, then stained with rat anti-CD3 (clone CD3-12, Novocastra, Newcastle upon Tyne, UK) or murine anti-CD20 clone L26, Dako), CD68 (clone KP1, Dako, Carpinteria, CA) and Ki67 (clone NB110-89719ss, Novus Biologicals). IHC procedure was performed as described [47]. Briefly, tissue sections were placed at 60°C for 15 min, and in xylene thrice for 8 min. each, and then in 95% alcohol twice for 8 min each. After tissue sections were dry, they were microwaved in 0.01 M sodium citrate (pH 6.0) for a total of 10 minutes in 2-minute increments. Tissue sections were incubated with primary antibodies at room temperature for 1 hour and detected using the SuperPic-Ture Kit (Zymed Laboratories Inc., South San Francisco, CA), according to the manufacturer's recommendations. Hemotoxylin (Fisher Chemicals, Fairlawn, NJ) was used for counterstaining.

### Flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood as previously described [30,45,46,48]. Cells were preserved in fetal bovine serum (FBS) with 10% DMSO until analysis. Frozen cells were thawed, washed in sterile 1x phosphate buffered saline (PBS), counted and stained for flow cytometry. The following directly conjugated monoclonal antibodies were used to stain cells for analysis by flow cytometry: CD3 (clone SP34-2) – PEcy7, CD8 (clone RPA-T8)-v500, CD28 (clone 28.2) – APCy7, CD95 (clone DX2) – FITC, all from BD Biosciences (San Jose, CA). Anti-human CD127 (clone R34.34)-PE was purchased from Beckman Coulter (Brea, CA). Antibodies to p<sup>53</sup> (clone BP53-12) - FITC and Bcl2 (clone 8C8)-streptavidin, which was detected by secondary staining with biotin-Brilliant Violet 421 (BD Biosciences), were purchased from Thermo Scientific (Rockford, IL). Raw cytometry data were acquired on an LSRII flow cytometer (BD Biosciences) and analyzed using Flow Jo analysis software (Tree Star, Inc.). All cytometry experiments included fluorescence-minus-one (FMO) controls [49], and doublet cells were excluded from analyses based on forward scatter-A and forward scatter-H. For each experiment, the lymphocyte population was gated using forward and side scatter.

### Pulmonary function testing

Pulmonary function tests (PFT) were performed as previously described [31]. Briefly, PFT were performed using whole body plethysmography and forced deflation technique at baseline and every two months following SHIV infection, using a whole-body plethysmograph and BioSystems for Maneuvers Software (Buxco Electronics Inc., Sharon, CT). Three measurements for forced vital capacity (FVC) within 10% of each other were considered valid and used for analysis [31]. Significant pulmonary obstruction was defined by a reduction of at least 12% in peak expiratory flow (PEF), forced expiratory volume in 0.4 seconds (FEV<sub>0.4</sub>) and/or mid-maximal expiratory flow (MMEF) from pre-SHIV infection (baseline) values. COPD in this cohort was also characterized by an increased proportion of emphysematous tissue in the lungs of Pc-colonized animals, compared with Pc-negative macaques [31].

### Statistical analyses

All statistical analyses were performed using Prism software

(GraphPad, La Jolla, CA). Serial pulmonary function data were analyzed in eleven cynomolgus macaques [31] (one animal was removed from analysis due to lack of baseline measurements). Mann-Whitney tests were used to compare percent change between baseline and 10 months post-SHIV infection in *Pneumocystis*-colonized versus *Pneumocystis*-negative animals. For immune histo chemistry data evaluation, 8 SHIV-infected (n=4 COPD+ and 4 COPD-) animals were compared using Mann-Whitney tests and were correlated with lung function parameters using Pearson correlations. Cryo preserved peripheral blood cells were available from 8 uninfected and 7 SHIV-infected animals for T cell phenotyping by flow cytometry, and Pearson correlations were used to evaluate T cell phenotype association with lung function parameters. For proliferation assays, cells from 5 uninfected animals were used and comparisons were made using Student's t-test, Mann-Whitney or Wilcoxon rank-sum tests. A p-value of less than 0.05 was considered significant.

## RESULTS

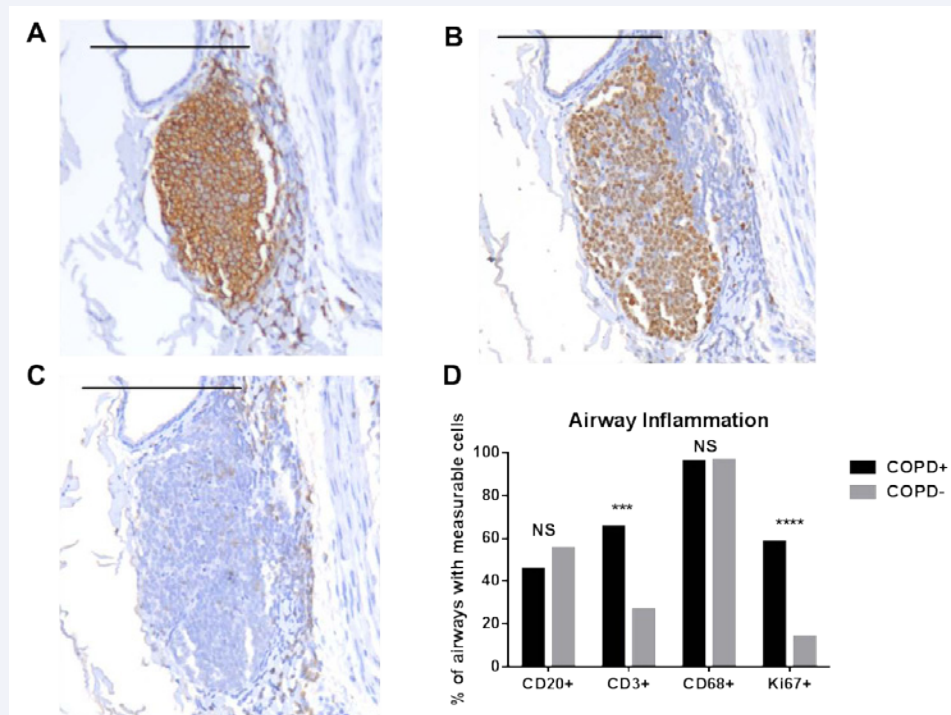
### Development of COPD in SHIV-infected macaques

We previously reported that SHIV-infected cynomolgus macaques develop irreversible pulmonary obstruction and emphysema following colonization with *Pneumocystis* [31]. We demonstrated that Pc colonization was a co-factor in development of COPD in SHIV-infected macaques. Macaques infected with SHIV alone did not develop evidence of COPD; these animals maintained pulmonary function consistent with baseline values and did not develop emphysema [31]. In the current study, we examined the relationship between pulmonary function, circulating T cell activation and airway-associated immune responses in this cohort. Pc-colonized, SHIV-infected animals showed significant decreases in peak expiratory flow (PEF), forced expiratory volume in 0.4 seconds (FEV<sub>0.4</sub>), and mid-maximal expiratory flow (MMEF) co-incident with detection of Pc in BAL fluid [31]. At study termination (58 weeks post-SHIV infection), macaques that were persistently colonized with *Pneumocystis* (n=7) exhibited an average decline of 14.5% in FEV<sub>0.4</sub>, compared with 0.73% change in the Pc-negative group (n=4) (p=0.042). PEF declined by mean of 16.1% in the Pc-colonized animals while PEF in Pc-negative animals changed by 0.88% (p=0.024) (Figure 1). Full spirometry and emphysema quantitation in this cohort has been reported elsewhere [31].

### Cell proliferation and T cell infiltration of airways are associated with COPD in SHIV-infected macaques

Consistent with the reported role of the acquired immunity in the pathogenesis of COPD [34,35,50-54], we have shown previously that SHIV-infected, Pc-colonized animals developed significantly more bronchus-associated lymphoid tissue (BALT), compared with SHIV-infected macaques without evidence of concurrent Pc colonization [31]. To characterize the BALT cellular components that may be contributing to the development of COPD in this model, lung tissue sections were evaluated for the presence of airway-associated CD3 (T cells), CD20 (B cells), CD68 (macrophages) and Ki67 (proliferation marker) by immune histo chemistry. Representative images of BALT are shown in (Figure 1) (A-C). In total, 83 airways from COPD+ animals and 72 airways from COPD- animals were examined, which included airways





**Figure 1** T cell infiltration of airways is associated with development of COPD in SHIV-infected macaques.

Serial lung tissue sections were stained for, CD20 (A), Ki67 (B), CD3 (C) and CD68 (not shown). Representative images of lymphoid follicles in COPD+ macaques are shown in panels A-C. Airway-associated follicles were typically comprised primarily of CD20+ cells (A), which also stained positive for Ki67 (B), a marker of proliferation. CD3+ T cells were also detected, primarily scattered around the edge of the follicle closest to the airway (C). Frequencies of airways with stained cells were compared between COPD+ and COPD- animals (D). Frequency of airways with CD3+ cells (D;  $p=0.0002$ ) and Ki67+ cells (D,  $p=0.0001$ ) were significantly higher in the COPD+ compared with the COPD- animals (Mann-Whitney tests). There was no difference between COPD+ and COPD- animals in percentage of airways associated with macrophages (CD68+ cells) or B (CD20+) cells. Scale bar = 200 $\mu$ m.

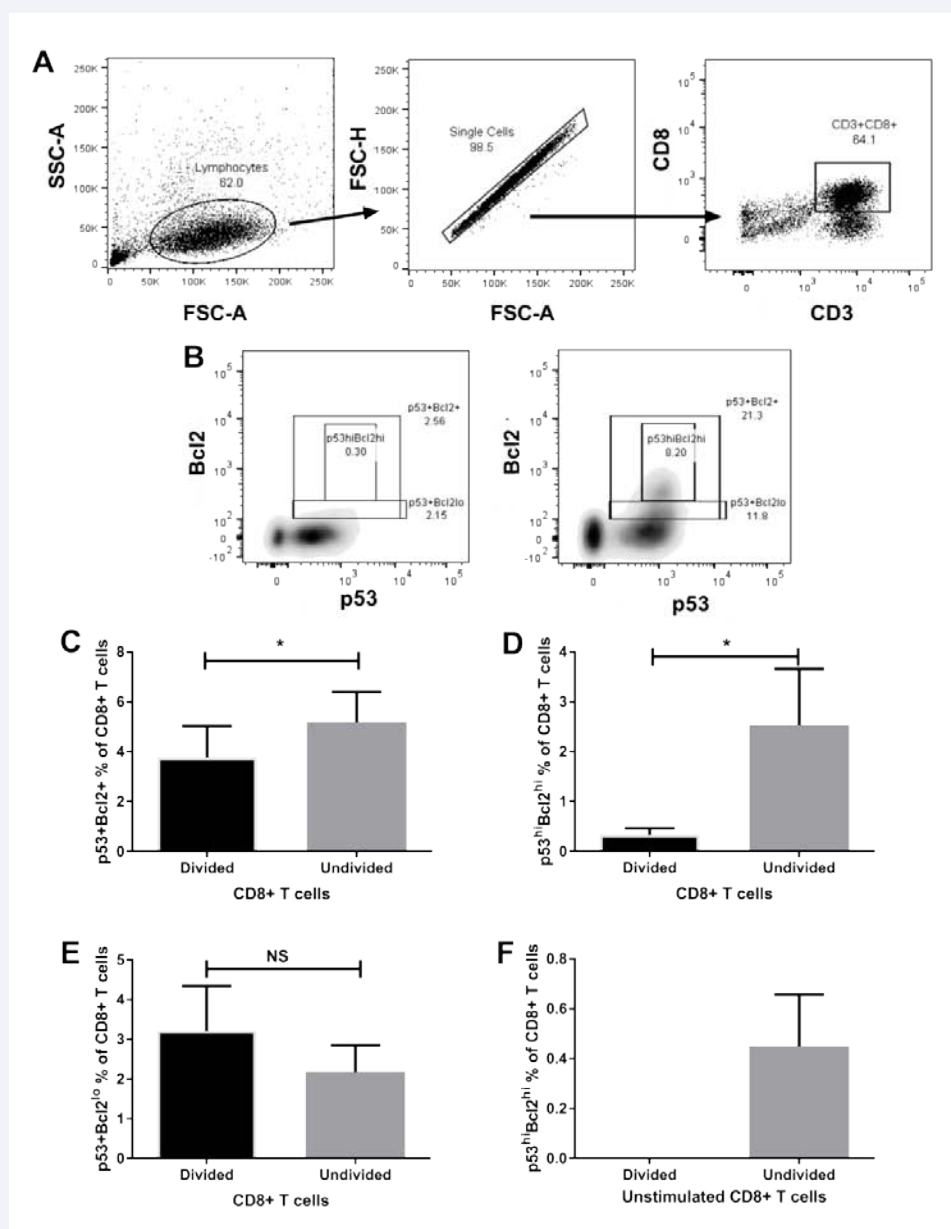
from upper, middle and lower lung lobes. We found that airway-associated follicles were typically comprised primarily of CD20+ cells (Figure 1A), and also stained positive for Ki67 (Figure 1B), a marker of proliferation. CD3+ T cells were also detected, and were primarily scattered around the edge of the follicle closest to the airway (Figure 1C). Follicles varied in size and organization among animals and within the same tissue section, ranging from small clusters of cells to large, well-organized structures, consistent with reports of inducible BALF by others [55]. Frequency of airways with CD3+ cells was significantly higher in animals with COPD compared with those without COPD (Figure 1D), ( $p=0.0002$ ). Additionally, the percentage of airways with cells staining positive for Ki67 was also increased in COPD+ compared with COPD- macaques (Figure 1D), ( $p=0.0001$ ).

### Frequency of CD8+ T cells expressing markers associated with cellular senescence is increased in SHIV infection

Because T cell infiltration of airways was associated with COPD in SHIV-infected macaques, we evaluated peripheral blood T cells for evidence of dysregulation that may contribute to development or pathogenesis of COPD. During HIV infection, it has been reported that chronic immune stimulation may contribute to T cell senescence [1,6]. We also observed that Ki67 expression, a marker of cell proliferation, in the airways

was increased in COPD+ animals, compared with those without evidence of obstruction. Because hyper proliferation of somatic cells can result in replicative senescence, we examined T cells in the periphery for evidence of senescence in association with COPD development.

Tumor suppressor protein, p53, and B cell lymphoma 2 protein, Bcl2, have been shown to be associated with cellular senescence [56-59]. P53 indirectly induces cell cycle arrest, and Bcl2 suppresses apoptosis, which leads to accumulation of senescent cells [58,59]. We examined peripheral blood p53+ Bcl2+ cells and the associated subpopulations within the CD3+CD8+ T cell compartment of macaques for evidence of replicative senescence. The gating strategy for this population is shown in (Figure 2A), and FMO controls are shown in (Figure 2A). We observed that a subpopulation of p53+ Bcl2+ cells, p53<sup>hi</sup>Bcl2<sup>hi</sup>, was expanded in chronic SHIV infection, compared with the same population in healthy animals (Figure 2B). Thus, we examined this and other subsets of p53+Bcl2+ cells for reduced proliferation in response to *in vitro* stimulation, an indicator of replicative senescence. The p53<sup>hi</sup>Bcl2<sup>hi</sup> population is significantly more frequent in non-dividing CD8+ T cells, compared with CD8+ T cells that divided following *in vitro* stimulation (Figure 2D), indicating reduced proliferative capacity, as would be expected for senescent somatic cells. This is in contrast to frequencies of p53+Bcl2<sup>lo</sup> cells, which are not significantly different between



**Figure 2** P53<sup>hi</sup>Bcl2<sup>hi</sup> CD8+ T cells have poor proliferative capacity following *ex vivo* simulation and are increased in chronic SHIV infection

Flow cytometry analysis gating strategy is shown in A. Lymphocytes were gated on forward and side scatter, and CD3+CD8+ T cells were gated from single cells. P53 and Bcl2 expressing cells (B) were gated from CD3+CD8+ T cells. Expression of p53 and Bcl2 in the peripheral blood CD8+ T cell compartment is shown prior to infection (B, left panel) and in late (>10 months post-) SHIV infection (B, right panel) in the same animal. To examine proliferation capacity of p53 and Bcl2 populations, peripheral blood cells from healthy macaques (n=5) were labeled with carboxyfluorescein succinimidyl ester (CFSE) and stimulated with phytohemagglutinin (PHA) *ex vivo* for 6 days prior to assessment by flow cytometry. Panel C shows the percentages of divided versus undivided (p=0.044, log-transformed) CD8+ T cells that are p53+Bcl2+ see also panel A of (Figure 1). When CD8+ T cells are examined for p53<sup>hi</sup>Bcl2<sup>hi</sup> expression (D), this population is significantly more frequent in the undivided cells, compared with the divided cells (p=0.013, log-transformed). This is in contrast to frequencies of p53+Bcl2<sup>lo</sup> cells, which are not different between dividing and non-dividing CD8+ T cells (E, p=0.354). Furthermore, in unstimulated peripheral blood T cells, p53<sup>hi</sup>Bcl2<sup>hi</sup> CD8+ T cells are absent from the dividing cell population (F). Frequencies were log-transformed prior to analysis to correct for large spread and non-normal distribution of the data.

dividing and non-dividing CD8+ T cells (Figure 2E). Furthermore, p53<sup>hi</sup>Bcl2<sup>hi</sup> cells are absent from the dividing cell population in unstimulated peripheral blood T cells (Figure 2F). We also examined this population for expression of cell surface markers typically associated with senescence and/or reduced proliferative capacity. Consistent with human studies of normal aging and

HIV [4,6], the majority of cells in this population lack surface expression of CD28 (Figure 2C), a co-stimulatory molecule, and the loss of which is associated with senescence[4]. In addition, the majority of these cells lack surface expression of IL-7Rα (CD127), a survival and proliferation signal [60] that is reported to be reduced in HIV infection[36, 61-63] and in aging [64], but

express Fas (CD95), which is associated with apoptosis [65-68]. Compared with  $p53^{\text{hi}}Bcl2^{\text{lo}}$ ,  $p53^{\text{hi}}Bcl2^{\text{hi}}$  cells have significantly greater frequency of cells that are CD28-negative ( $p=0.016$ ) or CD127-negative ( $p=0.016$ ) and cells that are double-negative for CD28 and CD127 ( $p=0.016$ ) (Figure 2). Taken together, these data suggest that  $p53^{\text{hi}}Bcl2^{\text{hi}}$  cells exhibit a senescent phenotype.

We then examined frequencies of senescent cells (defined as  $p53^{\text{hi}}Bcl2^{\text{hi}}$ ) in subpopulations of CD8+ T cells in healthy and SHIV-infected macaques, defined by expression of CD28, CD95 and CD127. In healthy animals, frequency of  $p53^{\text{hi}}Bcl2^{\text{hi}}$  cells was significantly reduced in CD28+CD95- (naïve) CD8 T cells compared with CD28+CD95+ (central memory) ( $p=0.016$ ), CD95+CD28- (effector memory [69]) ( $p=0.016$ ), CD127- ( $p=0.031$ ), CD28-CD127- ( $p=0.031$ ) and CD95+CD28-CD127- ( $p=0.031$ ) CD8+ T cells (Figure 3A). Frequency of  $p53^{\text{hi}}Bcl2^{\text{hi}}$  cells were increased in chronic SHIV infection in all subsets; however, frequencies of this population within CD28+CD95- and CD28+CD95+ subsets were significantly lower than in the other subsets examined (Figure 3B). These results suggest that frequency of senescent cells increases with progressive cell differentiation; however, changes in this population may be affected by different factors in healthy animals, compared with SHIV-infected animals.

Numbers of peripheral blood  $p53^{\text{hi}}Bcl2^{\text{hi}}$  CD8+ T cells are negligible in healthy animals, but are expanded in chronic SHIV infection (10-12mpi;  $p=0.0003$ ; Figure 4). Notably, this increase occurs despite the fact that numbers of total CD8+ T cells are not increased in chronic SHIV infection ( $p=0.63$ ). Numbers of  $p53^{\text{hi}}Bcl2^{\text{hi}}$  CD8+ T cell subsets were also significantly higher in chronically-SHIV-infected macaques compared with healthy animals (CD28+CD95+ [ $p=0.027$ ], CD95+CD28- [ $p=0.0003$ ], CD127- ( $p=0.0003$ ), and CD95+CD28-CD127- [ $p=0.0003$ ]) (Figure 4). Percent of  $p53^{\text{hi}}Bcl2^{\text{hi}}$  cells was also increased within CD8+ T cell subsets in chronic SHIV infection compared with healthy animals (Figure 3).

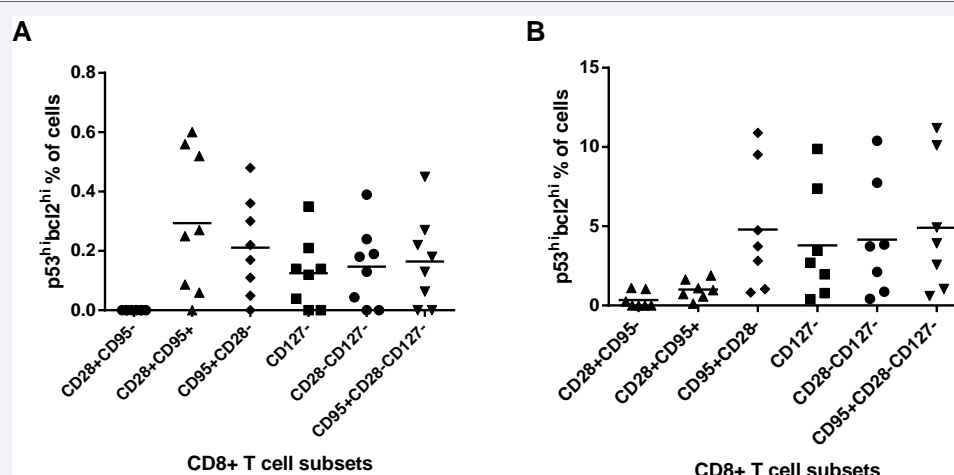
Taken together, these data describe a subset of senescent CD8+ T cells that are  $p53^{\text{hi}}$  and  $Bcl2^{\text{hi}}$  in macaques. In addition, the data indicate that the size of the senescent CD8+ T cell population was larger in chronic SHIV infection than in healthy animals. The total number of CD8+ T cells was not significantly increased in chronic SHIV infection, suggesting that the increase in senescent CD8+ T cells was not due to passive increased frequency of total CD8+ T cells with the loss of CD4+ T cells during SHIV infection, but rather to active CD8+ T cell differentiation.

## Greater numbers of senescent CD8+ T cells are associated with reduced pulmonary function

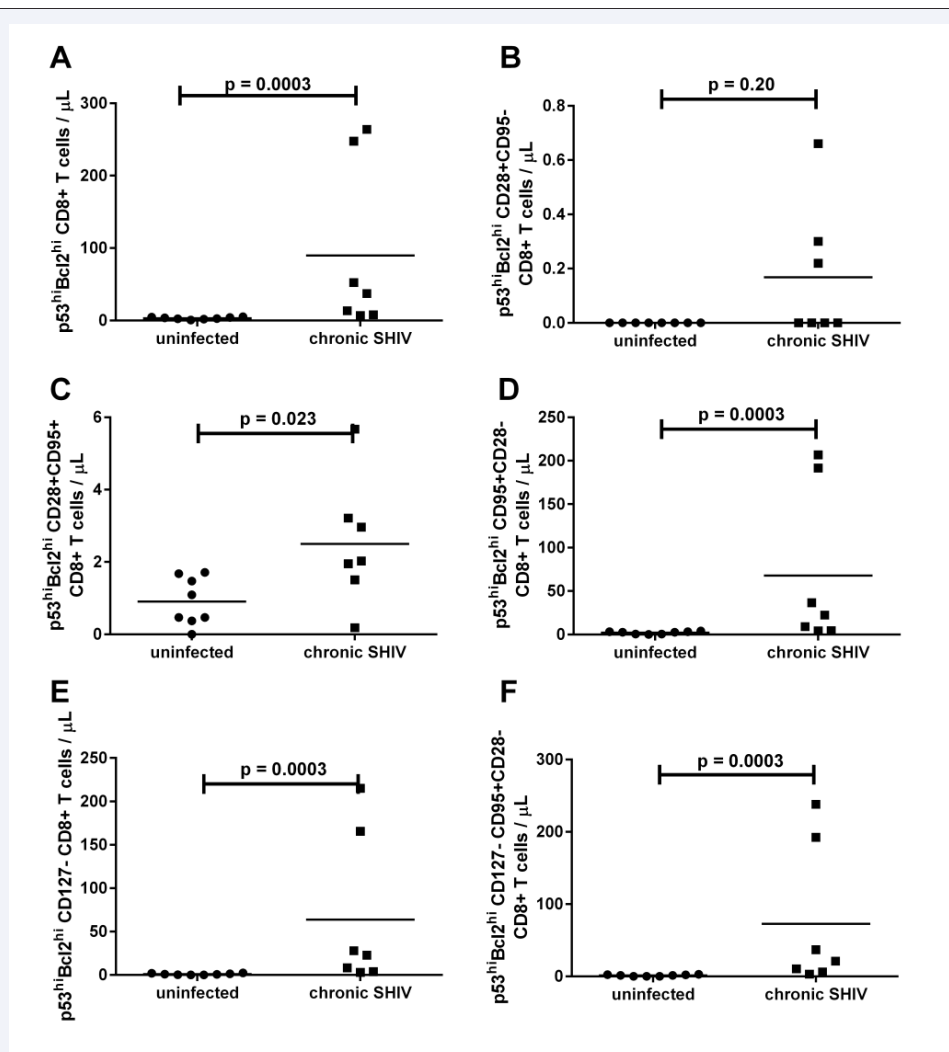
Numbers of circulating  $p53^{\text{hi}}Bcl2^{\text{hi}}$  CD8+ T cells (Figure 5A) ( $p=0.011$ ; Pearson  $r=-0.656$ ), as well as percent of  $p53^{\text{hi}}Bcl2^{\text{hi}}$  CD8+ cells (Figure 5B) ( $p=0.002$ ; Pearson  $r=-0.747$ ) were inversely correlated with  $FEV_{0.4}$ . Subsets of senescent CD8+ T cells (Figure 5C), including CD28+CD95+ (central memory,  $p=0.029$ , Pearson  $r=-0.603$ ) and CD127- senescent CD8+ T cells ( $p=0.045$ , Pearson  $r=-0.586$ ) were also associated with reduced pulmonary function. Similar trends, though not statistically significant, were observed for CD95+CD28- (effector memory,  $p=0.107$ ; Pearson  $r=-0.467$ ) and CD127-CD28-CD95+ ( $p=0.070$ , Pearson  $r=-0.541$ ) senescent CD8+ T cells (Figure 5D,5F). Together, these results support an association between T cell senescence and development of COPD in a nonhuman primate model of chronic HIV infection.

## DISCUSSION

The present study shows that the development of airway obstruction in the context of a chronic SHIV and Pc co-infection is associated with T cell infiltration of airways, evidence of hyperproliferation of airway cellular infiltrate and a larger population of peripheral blood CD8+ T cells co-expressing the classic somatic cell senescence marker p53 and the anti-apoptotic protein Bcl2.  $p53^{\text{hi}}Bcl2^{\text{hi}}$  macaque CD8+ T cells are predominantly lacking



**Figure 3** Frequency of  $p53^{\text{hi}}Bcl2^{\text{hi}}$  cells in CD8+ T cell subsets in uninfected animals (A) and in chronic SHIV infection (B). CD8+ T cell subsets were examined for proportion of cells expressing p53 and Bcl2. Frequency of  $p53^{\text{hi}}Bcl2^{\text{hi}}$  cells was significantly lower in CD28+CD95- CD8+ T cells compared with CD28+CD95+ ( $p=0.016$ ), CD95+CD28- ( $p=0.016$ ), CD127- ( $p=0.031$ ), CD28-CD127- ( $p=0.031$ ) and CD95+CD28-CD127- ( $p=0.031$ ) CD8+ T cells in uninfected ( $n=8$ ) animals (A; Wilcoxon signed rank tests). Frequencies of  $p53^{\text{hi}}Bcl2^{\text{hi}}$  cells increased in all CD8+ T cell subsets in SHIV-infected ( $n=7$ ) macaques; however, the frequency of these cells within the CD28+CD95- and CD28+CD95+ CD8+ T cell populations were significantly lower than in all other subsets examined (B; Wilcoxon signed rank tests).

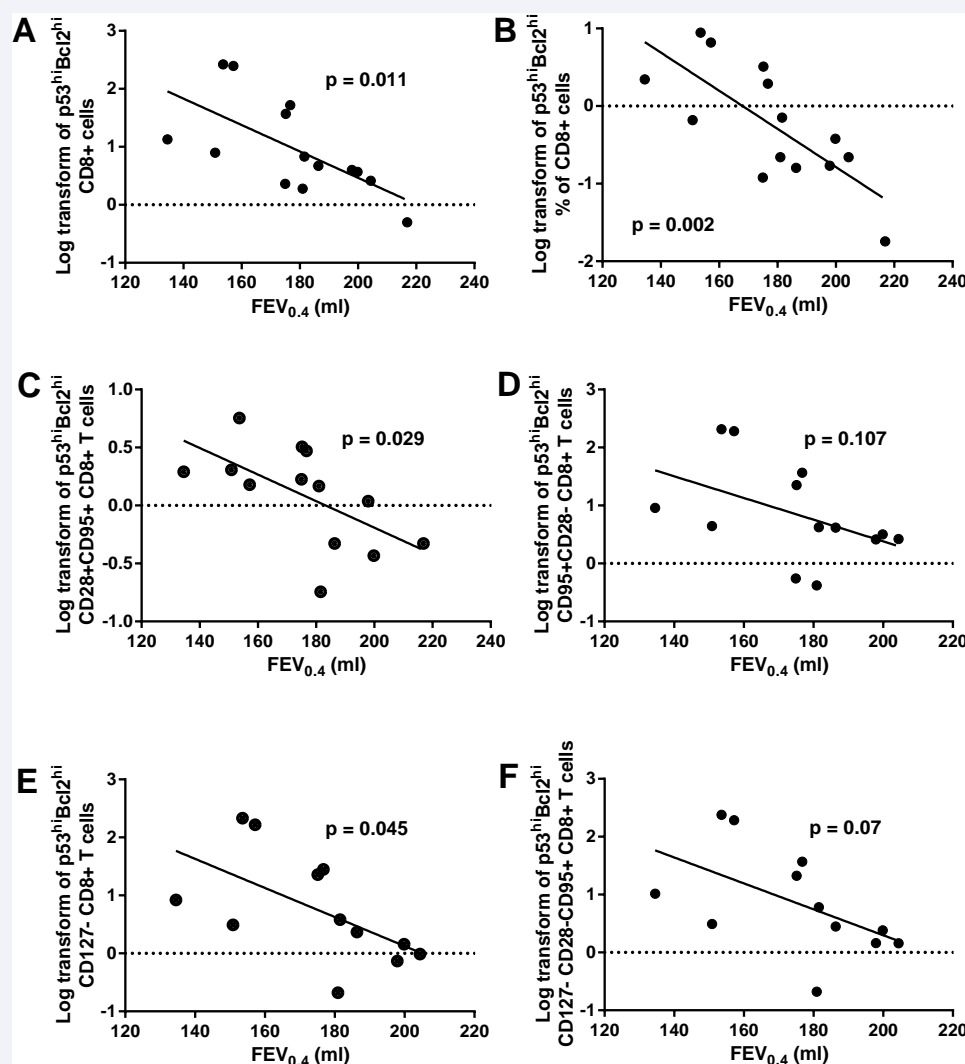


**Figure 4** Numbers of senescence CD8+ T cells and subsets in chronically SHIV-infected compared with uninfected macaques  
Peripheral blood cells were examined for numbers of p53<sup>hi</sup>Bcl2<sup>hi</sup> CD8+ T cells in uninfected (n=8) and SHIV-infected (n=7) macaques. Numbers of p53<sup>hi</sup>Bcl2<sup>hi</sup> CD8+ T cells were significantly greater in SHIV-infected, compared with uninfected, animals in the total CD8+ T cells (A, p=0.0003), as were numbers of p53<sup>hi</sup>Bcl2<sup>hi</sup> CD28+CD95- (naïve) CD8+ T cells (B, p=0.02), CD28+CD95+ (central memory) CD8+ T cells (C, p=0.023), CD95+CD28- (effector memory) CD8+ T cells (D, p=0.0003), CD127- CD8+ T cells (E, p=0.0003) and CD127- CD95+CD28- CD8+ T cells (F, p=0.0003) Comparisons were made using Mann-Whitney tests.

surface expression of CD28, observations consistent with studies of normal chronologic aging in humans [56]. In addition, we found increased frequency of this senescent population correlated with pulmonary obstruction. Together, these results demonstrate for the first time concordance between T cell aging and pulmonary obstruction in an age-disproportionate fashion in a non-human primate model of chronic HIV infection.

Immuno histo chemical staining of tertiary/ectopic lymphoid structures in the lung tissue of SHIV-infected macaques supports the concept of immune cell involvement in pathogenesis and, in particular, suggests T cell involvement in development of COPD. Chronic airway inflammation from activation of lung epithelial cells and alveolar macrophages likely leads to infiltration of inflammatory cells, including T cells, further exacerbating ongoing inflammation. We have previously reported that frequency of BALT was significantly higher in Pc+/COPD+ macaques

compared to those infected with SHIV alone [31]. The current study expands on this to investigate immune cell populations comprising the BALT structures. Our findings are consistent with reports elsewhere describing pathological findings in the lungs of COPD patients, indicating that follicles are composed primarily of B cells, with T cells scattered around the edge of the follicle [35]. However, we found that the frequency of CD20+ follicles did not correlate with COPD status, while the frequency of CD3+ follicles was associated with pulmonary obstruction. Lung T cells may be activated by a variety of antigens, and in turn, may release proteolytic enzymes [51,70] that promote apoptotic or necrotic tissue damage. We also report that the frequency of Ki67+ airways is significantly greater in COPD+ animals, indicating increased frequency of proliferating cells, suggestive of an active disease process. This also indicates increased replicative pressure that may lead to senescence, reminiscent of well-characterized cell senescence systems [71,72]. Resistance to apoptosis in



**Figure 5** Numbers and percent of senescent CD8+ T cells are inversely correlated with pulmonary function in SHIV-infected macaques. Numbers (A,  $p=0.011$ ) and percentage (B,  $p=0.002$ ) of peripheral blood  $p53^{hi}Bcl2^{hi}$  CD8+ T cells were inversely associated with pulmonary function. Additionally, numbers of  $p53^{hi}Bcl2^{hi}$  CD28+CD95+ (C,  $p=0.029$ ) and  $p53^{hi}Bcl2^{hi}$  CD127- CD8+ (E,  $p=0.045$ ) were also inversely correlated with pulmonary function. Numbers of  $p53^{hi}Bcl2^{hi}$  CD95+CD28- (D) and CD127-CD95+CD28- (F) cells were trending toward an inverse associated with pulmonary function, but did not reach statistical significance. Cell numbers were log-transformed to account for the large spread of values between uninfected and SHIV-infected animals. Pearson correlations were used for all comparisons.

infiltrating CD8+ T cells in the lungs of COPD patients may result in increased accumulation of this cell type in airways resulting in progressive tissue destruction [73].

Because the immune histo chemistry data obtained from lung tissue suggests an association between T cell infiltration and COPD, we sought to examine T cells in the peripheral blood for differences between healthy and SHIV-infected macaques. Further, because T cell senescence is reported in chronic HIV infection [1,6], we examined T cells for evidence of replicative senescence and determine whether there was an association with development of COPD in the context of SHIV infection. Here, we report an increase in CD8+ T cells that are bright for p53, a mitotic inhibitor, and Bcl2, shown to confer resistance to apoptosis, which others have shown to be bioindicators of human cell senescence [56-58]. We demonstrate that  $p53^{hi}Bcl2^{hi}$

cells have poor proliferative capacity when stimulated *ex vivo*, and are predominantly negative for surface expression of CD28 and CD127, but express CD95. Irreversible loss of surface CD28 on T cells is typical of chronologic aging [4] as well as in the setting of chronic HIV/SIV infection, disproportionate with age [1,6,74], and is associated with increased cytotoxicity, reduced proliferative capacity and shortened telomeres. CD28 is the classic co-stimulatory molecule, required to sustain T cell activation, proliferation and survival, and is down-regulated during differentiation from memory to effector cells and irreversibly suppressed during chronic activation [71]. CD8+CD127- T cells have been reported to express higher levels of the negative regulator programmed death-1 (PD-1) receptor and the adhesion molecule CD57, both of which have been associated with T cell senescence and normal aging in humans [62,75]. Such T cells produce high levels of IFN $\gamma$ , but low levels of IL-2, and are also



expanded in HIV infection [36].

Frequency and numbers of p53<sup>hi</sup>Bcl2<sup>hi</sup> cells are increased significantly during chronic SHIV infection, compared with healthy animals. Notably, this increase occurs despite the lack of significant increase in total CD8<sup>+</sup> T cells in chronic SHIV infection compared with pre-infection values. Thus, the data suggest that the increase in senescent CD8<sup>+</sup> T cells results from active differentiation induced by chronic viral infection, rather than passive increased frequency of total CD8<sup>+</sup> T cells coincident with reduced CD4<sup>+</sup> T cells. We also examined the relative proportions of senescent cells in CD8<sup>+</sup> T cell subsets, and found that frequency of these cells is affected by loss of CD28 and/or CD127 surface expression, which is consistent with reported progressive differentiation of T cells [60,76]. Interestingly, we found that the association of senescence frequency with CD8<sup>+</sup> T cell surface markers contrasted between healthy and SHIV-infected macaques. In healthy macaques, surface expression of CD95 and CD127, but not CD28, determined the frequency of senescent cells within the CD8 T cell compartment. In SHIV-infected macaques, however, surface expression of CD28 was more predictive of cellular senescence, which is consistent with studies in HIV infection [1,6] and aging [4]. Together these results suggest that changes in the senescent T cell population may be affected by different factors in chronically SHIV-infected macaques, compared with healthy animals. Cellular senescence can be induced by many different stimuli, and can occur even without significant telomere erosion [77]; thus, it is reasonable that T cell senescence induced under varying conditions may result in differential expression of surface markers associated with survival and proliferation.

We further demonstrate that frequency and numbers of senescent (p53<sup>hi</sup>Bcl2<sup>hi</sup>) CD8<sup>+</sup> cells are associated with pulmonary obstruction. This relationship is supported by a recent study in HIV-infected persons that reported an association between elevated senescent T cells (CD57<sup>+</sup>CD28<sup>-</sup>) in the peripheral blood and airflow obstruction [78]. We also examined correlations between pulmonary obstruction and some subsets of senescent CD8<sup>+</sup> T cells.

It is likely that chronic antigenic exposure and stimulation contribute to T cell and senescence through multiple pathways. We previously demonstrated that macaques infected with SHIV alone maintained pulmonary function, whereas SHIV-infected macaques developed COPD following persistent *Pc* colonization [31]. In the setting of a chronic viral infection, with the additional insult of persistent pulmonary colonization with an opportunistic pathogen, as in the current study, immune cells are exposed to chronic stimulation, leading to ongoing inflammation, cell exhaustion and senescence. Frequencies and numbers of senescent CD8<sup>+</sup> T cells were associated with worse pulmonary obstruction in chronic SHIV infection, independent of changes to CD4/CD8 T cell ratios resulting from chronic SHIV infection, suggesting an association between immune senescence and COPD.

A limitation of the current study is the lack of cell samples from all animals pre- and post SHIV infection, which limited our capacity to perform paired analyses on some of the data sets. Additionally, we were unable to evaluate these T cell subsets in

the lungs (BAL fluid) of COPD+ animals, although lung histological data demonstrate T cell infiltration and increased proliferation (indicated by Ki67 expression), which can lead to replicative senescence, in airways of COPD+ macaques, suggesting T cell involvement in lung pathology associated with COPD. Current studies are underway to evaluate T and B cell populations in the BAL fluid of SHIV-infected macaques in a longitudinal study, to better characterize immune cell infiltration and/or expansion in the setting of COPD associated with chronic SHIV infection.

COPD is a complex, multifactorial disease process, which is accelerated in the context of HIV infection, and improved understanding of the underlying mechanisms involved in disease progression is needed for development of effective therapies. Furthermore, given the high levels of immune activation and premature immune aging/senescence in HIV-infected individuals [38,79,80], including those on ART [81], “inflammaging” and the association between persistent immune stimulation and immune senescence may be especially important in HIV-related COPD. In aggregate, our model of HIV-COPD suggests that local inflammation, driven by persistent *Pneumocystis* colonization of the lung, and amplification of systemic inflammation, leading to cellular senescence, may drive the development of progressive pulmonary obstruction in the context of chronic SHIV infection. In contrast, SHIV infection alone does not result in similar pulmonary disease progression or pathology. The non-human primate model of HIV-related COPD is a highly relevant system in which to interrogate the potential pathways whereby prematurely senescent T cells contribute to COPD pathogenesis and test treatment strategies that target these pathways. Furthermore, given the commonalities among immune cell aging, chronic infection and chronic inflammation, it is likely that delineation of such pathways will provide insight into alternative therapies, not only for the HIV-infected population, but also in non-HIV-associated COPD.

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