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Research Article

RESEARCH ARTICLE

Highly active antiretroviral therapy reduces pulmonary IL-8 in HIV-positive women smokers

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One sentence summary: Treatment of HIV infection in women who smoke reduces markers of inflammation in the lungs.
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ABSTRACT

Increased levels of the proinflammatory cytokine IL-8 are detected in the sputum of patients with chronic obstructive pulmonary disease (COPD) and during the pathological pulmonary manifestations of HIV infection. To explore a potential interrelationship between smoking, highly active antiretroviral therapy (HAART) and HIV immune status, we collected sputum samples, along with complete pulmonary function tests from groups of HIV-infected women smokers who were either on or off HAART. Analysis of the patient's sputum for cell count along with quantitative measures of IL-8 was performed and correlated with concurrent assessment of pulmonary function test (PFT). We found that HIV-positive smokers had decreased measurements on PFT of the diffusing capacity of the lung for carbon monoxide (D_{LCO}) compared to standard reference values that did not differ with HAART usage. HAART, when controlled for CD4, showed a suppressive effect on the levels of pro inflammatory cytokine IL-8 in sputum. We conclude that in the era of HAART, HIV along with concurrent tobacco smoking is associated with declines in PFT in HIV-infected women. The use of HAART in patients appears to mitigate the increases in IL-8 levels in relation to immune status based on CD4 count.

Keywords: HIV; IL-8; antiretroviral therapy; pulmonary function; smoking; tobacco

INTRODUCTION

The negative effects of cigarette smoking have long been recognized as comorbid conditions in patients with HIV infection, and the prevalence of smoking among US adults with HIV receiving medical care is nearly twice that of the general US adult population (Mdodo *et al.* 2015). Pathologic changes within the pulmonary system have been directly observed in those with HIV infection and many of these individuals suffer from concurrent tobacco-related disease processes such as COPD. The interaction of tobacco and HIV infection and how it contributes to pulmonary pathology is not well understood.

Before the advent of HAART (highly active antiretroviral therapy), studies of pulmonary pathology in cohorts of majority male subjects demonstrated incremental and longitudinal declines in pulmonary function as measured by pulmonary function tests (PFTs), including diffusion lung capacity (D_{LCO}). This decline in

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D_{LCO} directly paralleled the progression of HIV infection as measured by the progressive stages of HIV disease and decline in CD4 count (Mitchell *et al.* 1992). In addition, emphysematous changes were documented on pulmonary CT scans in subjects who were current cigarette smokers.

Explanation of this phenomenon suggested a concurrent effect of HIV-induced inflammation with smoking. Studies revealed elevated levels of the proinflammatory cytokine IL-8 in pulmonary lavage samples from various populations of HIV patients, including those infected with pneumocystis pneumonia (PCP), bacterial pneumonia and even in asymptomatic patients (Lipschik *et al.* 1993; Denis and Ghadirian 1994a; Benfield *et al.* 1995; Krarup *et al.* 1997). It had been postulated that alveolar macrophages contributed to the increased IL-8 levels because when macrophages were stimulated from HIV-1 subjects with various inducers they released IL-8 in levels dependent on HIV disease state and the type of inducer (Denis and Ghadirian 1994b; Gordon *et al.* 2005). COPD likewise is a disease state that is characterized by local and systemic inflammation and is in turn associated with elevated levels of the circulating and pulmonary cytokines such as IL-8 (Hacievliyagil *et al.* 2006; Parr *et al.* 2006; Damia Ade *et al.* 2011; Paone *et al.* 2011).

With the advent of HAART, HIV-positive individuals may experience a partial recovery of portions of their immune system which have been damaged by infection with HIV. This study was designed to investigate, in HIV-positive women who smoke, the relationship between levels of the proinflammatory cytokine IL-8 and pathologic changes in pulmonary function, with HIV disease state as measured by CD4 count, and explore the hypothesis that HAART affects this interplay.

MATERIALS AND METHODS

We performed a study of IL8 levels and pulmonary function studies in HIV-infected women who were current smokers with at least a 10-pack-year history of smoking. The length of smoking was chosen as a minimum time for the manifestation of COPD to appear (Lokke *et al.* 2006). The women were recruited from a primary infectious disease clinic at the University of Maryland Hospital through brochures and provider referral. Participants were stratified into two groups: women who were being treated with HAART and had an HIV viral load <400 copies ml^{-1} for at least 3 months, and women not on HAART with an HIV viral load >400 copies ml^{-1}. In order to minimize the chance of underlying pulmonary opportunistic infection (i.e. PCP, cytomegalovirus or Mycobacterium avium complex) on results, inclusion criteria required a negative PPD or chest x-ray and a CD4 count above 200 counts mm^{-3}. Other exclusion criteria included current use of systemic or inhaled corticosteroids or other immune modifying agents, a past history of an opportunistic infection, a respiratory infection within the previous 6 weeks, a history of asthma or a history of any other chronic pulmonary diseases besides COPD.

Data collection pertained to clinical history, demographics, CD4 count, HIV viral RNA, total number of years smoked and pack-years smoked. Sputum was collected in a reproducible and an easily performed method in the outpatient setting and was processed in the manner of Efthimiadis *et al.* (2002). Sputum cell counts were quantified using a cell cytometer, and Gram stains were performed; at least 400 squamous cells were counted to determine the differential. Only those sputum samples with a >50% ratio of leukocytes to squamous cell were deemed adequate for inclusion. Quantitative ELISA assays that measure the presence of IL-8 were performed using the sputum specimen, according to the manufacturer's recommendations (Quantikine; R&D Systems, Minneapolis, MN). In order to minimize errors in IL-8 detection due to differences in viscosity, sputum samples were serially diluted, and tested at different concentrations, obtaining values that, once adjusted for the dilution factor, were comparable and within the dynamic range of the test.

Within 1 month of sputum collection, participants returned to the study site and underwent PFT with diffusion capacity adjusted for race and hemoglobin. COPD status was defined by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria as 'airway limitation that is not fully reversible with a FEV1/FVC ratio <70% and FEV1 >80% predicted for mild COPD, and a FEV1/FVC ratio <70% and FEV1 <80% for moderate COPD' (Vestbo, Hurd and Rodriguez-Roisin 2012). This study was approved by the IRB at University Maryland at Baltimore. Data analyses were performed using SPSS for Windows (Chicago, IL) version 21.

Table 1. Patient demographics.

	On HAART N = 13 Mean(SD)	Off HAART N = 12 Mean(SD)	P-value
Age	42.8(5.5)	42.8 (7.3)	0.980
% African American	92.3	83.3	0.511
Smoking history			
Age started smoking	14.9(2.2)	14.0(2.2)	0.347
Packs per day	0.94(0.48)	0.83(0.30)	0.510
Years smoked	29.3(6.6)	28.8(6.8)	0.838
Pack-years	28.0(16.0)	24.0(10.6)	0.475
Clinical status			
CD4 count	554.9(476.1)	659.8(534.7)	0.609
FEV1/FVC post measure %	77.5(10.3)	80.0(6.5)	0.473
COPD	0.23(.44)	0.08(.29)	0.329
IL 8 ng ml^{-1}	3855.1(5108.9)	4187.1(6604.6)	0.889
HIV viral load copies ml^{-1}	4.4 (15.8)	23444 (34172.7)	0.037*
FEF25-75%PostRef	72.7 (31.3)	74.3 (23.8)	0.885
DCLO% Ref	63.5 (13.2)	61.6 (14.6)	0.728
DLCO/VA %Ref	88.1 (17.1)	90.5 (12.4)	0.691
TLC %Ref	89.5 (14.8)	88.5 (18.4)	0.886

RESULTS

A total of 25 HIV-positive women, 13 (52%) on HAART and 12 (48%) not on HAART, met inclusion criteria and produced adequate sputum samples. All returned for full PFT. There were no significant differences between groups on background variables, smoking history or measures of clinical status other than HIV viral load, which was related to being on HAART therapy (see Table 1).

Our population of HIV-infected female smokers demonstrated a moderate decline in D_{LCO}% compared to population reference standards with a mean decline of 63% (range 43–99% SD:13.6) with 92% of the patients having at least a mild decrease in D_{LCO}% (≤80%), and 62% having a moderate decrease in D_{LCO}% (≤60%). Another study has examined the effects of HIV infection on DCLO% and spirometry in a cross-sectional analysis of 300 HIV-infected men. Comparing our population of women to these men in which the rate of D_{LCO} ≤60% was seen in 30% of

Table 2. Comparison of HIV+ women to men.

Characteristics	Women (from current study)	Men (from Crothers *et al.* 2013)	*P*-value
N	25	300	
Age, mean years (SD)	43 (6)	54 (8)	<0.001
% African American	88	47	<0.001
On antiretroviral therapy, %	52	89	0.001
Smoking pack-years among ever smokers, median (IQR)	21 (15–38)	26 (13–40)	[a]
Ever smokers, %	100	74	[a]
DLCO<60%, %	64	30	0.002
DLCO% predicted, mean (SD)	63 (14)	69 (19)	0.027
FEV1 postRef, mean (SD)	84 (13)	95 (19)	<0.001
FVC post, mean (SD)	83 (14)	97 (15)	<0.001
FEV1/FVC post %, mean (SD)	79 (9)	76 (10)	0.132
FEV1/FVC <70%,	16	18	0.792
CD4 count < 200 cells μL^{-1}, %	0	8	[a]
HIV viral load ≤ 500 copies ml^{-1}, %	64	84	0.052

[a]Statistical comparisons could not be run on these characteristics due to lack of variance in the sample or information provided for the comparison group.

the men, our patient population of HIV-infected women demonstrated a prevalence of D_{LCO} ≤60% more than twice that at 64% ($P = 0.002$).In addition, our population of women had a mean D_{LCO}% of 62.6% vs this male cohort D_{LCO}% of 69% (Crothers *et al.* 2013). Besides sex, there were differences in other demographics. In our study, the majority of patients were African American, of a younger age, had a higher percentage of smokers, were from a single clinic, were from the same geographic region and all had a CD4 count >200 (Table 2). This may make the differences in DLCO% more pronounced as CD4 <200 is associated with a greater decrease in DLCO%. Whether the differences in DLCO% reflect a gender specific difference or manifestations of other pathologies such as pulmonary hypertension, interstitial lung disease or injection drug use is beyond the scope of our study.

There was no significant correlation between HAART usage and CD4 count, possibly due to the fact that in our study even subjects not on ARVs had a relatively high CD4 count. Only four of the patients met the GOLD criteria for COPD with an average age of 44.5 yr and mean tobacco smoking history of 43 pack-years. There were no significant differences in CD4, IL-8, D_{LCO}% or age based on COPD status. There was a marked increase in pack-years smoked among those with COPD compared to those without COPD 43 vs 22.8 ($P = 0.004$). Due to the low overall number of patients, we were unable to determine any statistical significance of HAART on COPD incidence.

We found that sputum IL-8 levels and CD4 count were significantly positively correlated ($r = 0.44$, $P = 0.026$). This correlation was largely due to the participants who were not on HAART, who showed a strong correlation between CD4 count and IL-8 ($r = 0.92$, $P < 0.001$), compared to patients who were on HAART, who did not show a significant correlation between CD4 count and IL-8 ($r = -0.18$, $P = 0.554$) (see Fig. 1). This suggests that being on HAART may suppress the relationship of increasing levels of IL-8 with increasing CD4 counts. While the sample size was too small to perform regression analyses to formally test interaction effects without violating assumptions, additional correlations were run to ensure that the observed correlations were not due solely to outliers. Findings were consistent when outliers were removed, in that CD4 and IL-8 were not significantly correlated for participants who were on HAART, but were correlated for those who were not on HAART (see Table 3).

Table 3. Correlations between IL-8 and CD4 adjusting for outliers.

	On HAART N = 13 Correlation r (*P*-value)	Off HAART N = 12 Correlation r (*P*-value)
All participants	**−0.181 (P = 0.554)**	**0.915 (P < 0.001)***
Excluding CD4 outliers[a]	**−0.208 (P = 0.516)**	**0.629 (P = 0.038)***
Excluding IL-8 outliers	**−0.144 (P = 0.656)**	**0.676 (P = 0.032)***
Excluding CD4 and IL-8 outliers	**−0.059 (P = 0.863)**	**0.676 (P = 0.032)***

[a]Outliers defined using the outlier labeling rule (Hoaglin and Iglewicz 1987).

DISCUSSION

In this study of HIV-positive women smokers, we found a correlation between levels of IL-8 and CD4 counts for patients not on HAART. In addition, we observed a marked decline in the D_{LCO}% in all patients regardless of HAART which is similar to, but more severe than, data in HIV-infected men. The mechanisms of pulmonic pathology in HIV and COPD both share many characteristics. Smokers with or without COPD have higher percentages of $CD8^+$ T cells and NKT-like cells than never smokers (Tzanakis *et al.* 2004; Forsslund *et al.* 2014). Similarly CD8 cells in an activated state are found in increased amounts in the alveolar spaces of HIV-infected individuals (Guillon *et al.* 1988; Bofill *et al.* 1998).

Elevations in the levels of IL-8 from the sputum of smokers correlate directly with inflammation and act as a viable biomarker for the occurrence of COPD (Tanino *et al.* 2002; Schulz *et al.* 2003). In turn, elevated levels of IL-8 in sputum have been detected in various processes involving pulmonary inflammation during HIV infection from the pre-HAART era (Lipschik *et al.* 1993; Krarup *et al.* 1997).

The basis of pulmonary inflammation occurring with HIV infection has not been well delineated. One underlying mechanism may involve the HIV protein Tat which has the ability to induce expression of IL-8 in cells via activation of NF-kappaB in the nucleus of HIV Tat-expressing cells (Ott *et al.* 1998). This HIV-induced upregulation of IL-8 expression, which is linked to the cell cycle, is thought to protect the cells from death and thus accelerate continued HIV production (Mahieux *et al.* 2001).

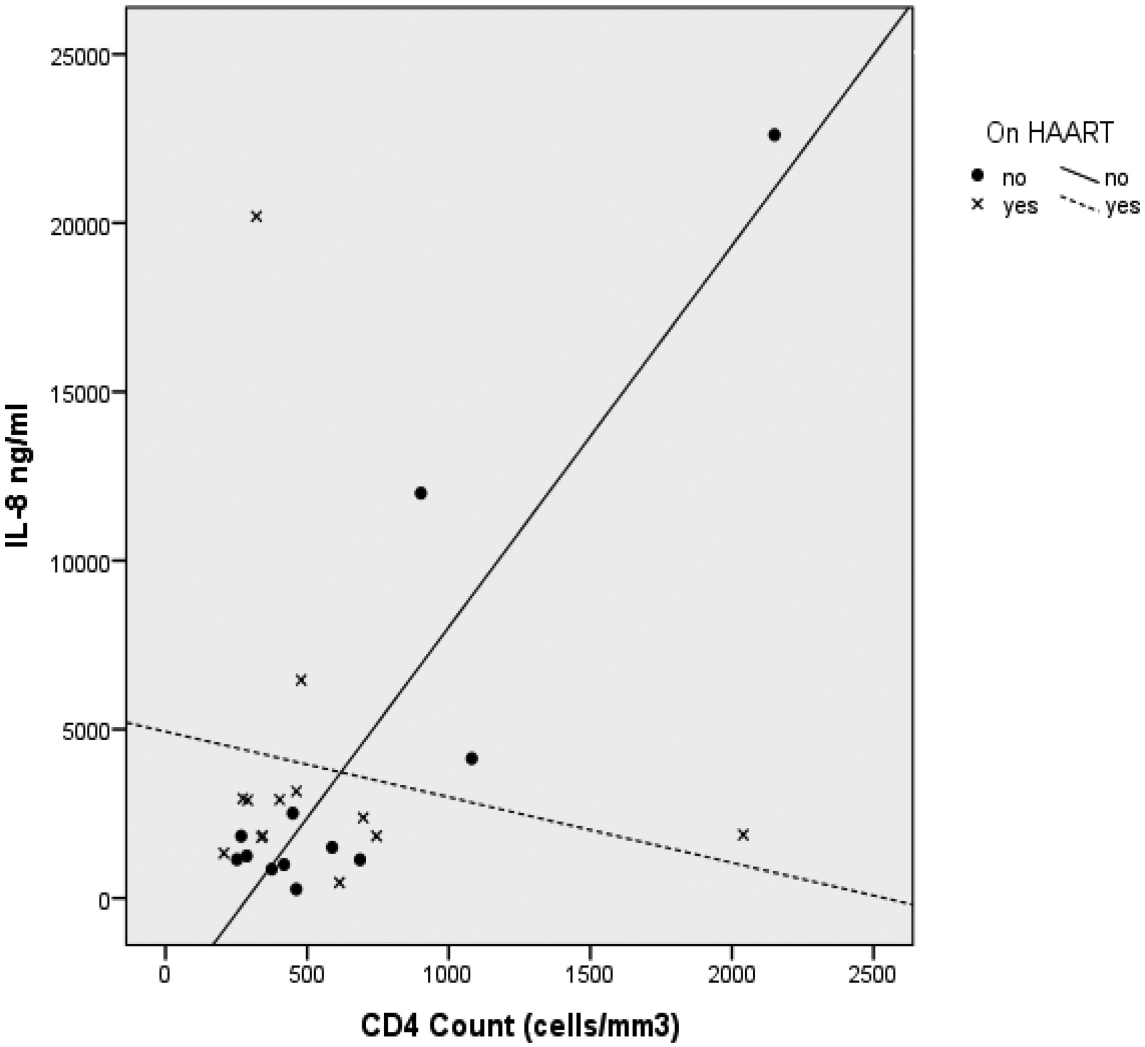

Figure 1. Correlations of IL-8 levels and CD4 count for those on and off HAART.

In contrast, during the stages of HIV disease, production and the expression of IL-8 may fluctuate and even decrease depending on state of host immunity. Bronchial alveolar lavage samples from more advanced HIV-infected patients with lower CD4 counts (mean CD4 263) reveal reduced interleukin-8 production with resultant decreased neutrophil recruitment, and increased susceptibility to pneumococcal infection (Gordon *et al.* 2005).

We surmised that this may have been the case in our patients who were not on HAART as those with a higher CD4 count also demonstrated higher levels of sputum IL-8. This may represent a direct effect of HIV on a more intact immune system by acting to induce further viral replication. In other studies, a similar elevation of the cytokine MIP-1-α in the lungs with higher CD4 counts has been documented (Aung *et al.* 2001). In theory, HAART may functionally suppresses pulmonary viremia and return the balance of alveolar lymphocytes to a near normal ratio thus acting to decrease IL-8 levels (Twigg Iii *et al.* 2008; Twigg *et al.* 2010). This phenomenon may not be unique to the pulmonary compartment, as studies have shown that HAART has reduced IL-8 levels in other compartments, such as the salvia and peripheral serum (Haissman *et al.* 2009; Nittayananta *et al.* 2014).

In a study of 63 HIV-infected patients (48% smokers, 97% on ARVs) who had longitudinal lung function test repeated within a median follow-up period of 4.4 yr, the carbon monoxide diffusion capacity was reduced over time in both smokers and nonsmokers, and FEV1/FVC was decreased in both groups but especially in smokers (Kristoffersen *et al.* 2012). Despite the fact that the patients in our study were pre-screened to rule out any underlying pulmonary infection such as PCP or tuberculosis, the majority still showed impairments of D_{LCO}% on measures of their pulmonary function similar to those changes seen in HIV patients from the pre HAART era, suggesting that therapies to protect or restore lung function are needed (Mitchell *et al.* 1992; Rosen *et al.* 1995; McCabe *et al.* 1997; Diaz *et al.* 2000). This is of concern clinically as the unexpected high prevalence of reduced D_{LCO}% in our cohort of female patients may represent a gender difference in pathology in female smokers. Our female patients, when directly compared to a cohort of HIV-infected men despite being over a decade younger, manifested a significantly lower mean DLCO%, DLCO <60%, FEV1 and FVC. Interestingly, gender differences in the prevalence of COPD in non-HIV populations of women have been noted in other studies

(Mcafee and Burnette 2014). As the trend of more women smoking increased, so did the COPD prevalence increase and when compared to men, women have seen a higher age-adjusted prevalence of COPD in the 1–9, 20–29 and ≥30 yr smoking duration periods (Liu *et al.* 2015). To delineate the separate or synergistic contributions of smoking and HIV to pulmonary pathology in women would require larger studies with controlled groups.

The multifaceted interplay of HIV infection, smoking, substance abuse and other infections as a causation of COPD makes it difficult to identify the role of each of the various interacting and concurrent factors in COPD pathogenesis (Crothers 2007; Drummond *et al.* 2012; Samperiz *et al.* 2014). Other studies exploring the direct effects of HAART on COPD have given conflicting conclusions possibly due to varied patient characteristics and study methods (Kunisaki 2014). In one large cohort of men and women HIV infected vs HIV-uninfected participants, COPD was more common pre-HAART era but not after the introduction of HAART (Crothers *et al.* 2011; Gingo *et al.* 2013). Limitations of our study were that our cohort study was cross-sectional and relatively small in size, and we were unable to make a direct correlation of HIV and COPD. Although some variations may occur in PFTs, we attempted to control for the modifiable variables by using a lab operated per ATS procedural standards with all test preformed after 9 AM to account for the diurnal variations in DLCO, corrected results for HgB, performed two test of DLCO that had to be within 10% of each other and had all results interpreted by a board certified pulmonologist (Medarov, Pavlov and Rossoff 2008).

Even though we demonstrated elevated levels of IL-8 correlating with CD4 count, we did not observe an expected increase in IL-8 within the patients with COPD; however, the influence and duration of smoking while HIV positive and other parameters of their immune status could not be factored into our calculations. Interestingly, regulatory T cells (Tregs), that are known to be depleted in the periphery in HIV+ subjects (Kinter *et al.* 2004; Oswald-Richter *et al.* 2004; Apoil *et al.* 2005; Eggena *et al.* 2005; Tsunemi *et al.* 2005; Baker *et al.* 2007; Chase *et al.* 2007; Ndhlovu *et al.* 2008), but augmented in lymphoid organs (Kinter *et al.* 2004; Andersson *et al.* 2005; Estes *et al.* 2006; Nilsson *et al.* 2006; Boasso *et al.* 2007), are among the cell types that express IL8 (Himmel *et al.* 2011). HAART restores Treg function, which is preserved in patients that control virus (Chase *et al.* 2008; Presicce *et al.* 2011). Future studies should address whether Tregs are involved in COPD, in HIV seronegative and seropositive subjects, and whether the restoration observed during HAART could account for the increased levels of IL-8. Also we could not ascertain any direct influences of HAART on PFTs due to the non-interventional single measure design of the study. Nonetheless, it is conceivable that HAART usage could reduce the risk of developing COPD during HIV infection and the levels of pulmonic IL-8 acting as a biomarker for immune activation could predict future pulmonary pathology. To that end, larger studies to verify an association of HAART with decreased levels of IL-8 in both genders of HIV-positive smokers are needed. If HAART does demonstrate longitudinally a positive influence on pulmonary function, this would further support current CDC guidelines to start ARVs at all stages of HIV infections and reinforce that smoking cessation is a paramount clinical intervention for all HIV patients (CDC 2015).

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