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Upregulation of Chitinase 1 in Alveolar Macrophages of HIV-Infected Smokers[1]

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Abstract

Recent studies suggest that HIV infection is an independent risk factor for the development of COPD. We hypothesized that HIV infection and cigarette smoking synergize to alter the function of alveolar macrophages (AMs). To test this hypothesis, global transcriptome analysis was performed on purified AMs from 20 individuals split evenly between HIV-uninfected nonsmokers and smokers and untreated HIV-infected nonsmokers and smokers. Differential expression analysis identified 143 genes significantly altered by the combination of HIV infection and smoking. Of the differentially expressed genes, chitinase 1 (CHIT1) and cytochrome P450 family 1 subfamily B member 1 (CYP1B1), both previously associated with COPD, were among the most upregulated genes (5- and 26-fold, respectively) in the untreated HIV-infected smoker cohort compared to HIV-uninfected nonsmokers. Expression of CHIT1 and CYP1B1 correlated with the expression of genes involved in extracellular matrix organization, oxidative stress, immune response, and cell death. Using CyTOF to characterize AMs, a significantly decreased expression of CD163, an M2 marker, was seen in HIV-infected subjects, and CD163 inversely correlated with CYP1B1 expression in AMs. CHIT1 protein levels were significantly upregulated in bronchoalveolar lavage fluid from HIV-infected smokers, and increased CHIT1 levels negatively correlated with lung function measurements. Overall, these findings raise the possibility that elevated CHIT1 and CYP1B1 are early indicators of COPD development in HIV-infected smokers that may serve as biomarkers for determining this risk.

Keywords

Human; Monocytes/Macrophages; AIDS; Inflammation; Lung

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Introduction

Anti-retroviral therapy (ART)[2] has dramatically decreased the number of opportunistic infections and deaths in HIV-infected patients (1–4). While ART does not eliminate HIV from an infected individual, it dramatically increases life expectancy. Nevertheless, the average lifespan of ART-treated HIV-infected persons remains shorter than HIV-seronegative persons, with the lung being the most frequently involved organ in AIDS autopsy cases (5, 6). Crothers *et al.* (7) showed that COPD, lung cancer, pulmonary hypertension and pulmonary infections were significantly more common among ART-treated, HIV-infected persons compared to uninfected individuals. In this regard, HIV infection has been identified as an independent risk factor for COPD, with HIV infection increasing the odds of a COPD diagnosis by 50–60% (8, 9). Likewise, lung cancer rates were increased in HIV-infected individuals when controlling for smoking rates in the population (10, 11). A higher prevalence of cigarette smoking in the HIV-infected population further adds to the risk of COPD and lung cancer in this susceptible population (12).

Alveolar macrophages (AMs) play an important role in regulating immune responses in the lung, and evidence suggests that these cells also play a key role in the development of COPD (13, 14). A mouse model of COPD demonstrated that the development of air space enlargement was dependent on the presence of AMs (15). In humans, AMs are increased in the lungs of smokers and individuals with COPD (13), accumulating at sites of alveolar wall destruction (16) and correlating with disease severity in COPD patients (17, 18). Both cigarette smoking and HIV infection alter the gene expression profile of AMs. Chronic cigarette smoke exposure reprograms the steady-state AM towards an M1-deactivated, partially M2-activated phenotype with increased extracellular matrix remodeling potential as a result of increased matrix metalloproteinase (MMP) gene expression (19–25). Interestingly, AMs from HIV-infected persons with early emphysema also had increased expression of MMP2, 7, 9, and 12 (26). These studies suggest that both smoking and HIV infection result in a tissue remodeling phenotype in AMs that may contribute to the premature development of COPD. To our knowledge, no study has yet looked at a potential additive or synergistic effect of the combination of smoking and HIV infection in untreated HIV-infected individuals prior to the initiation of ART. Investigation of the early effects of both smoking and HIV infection on AMs could shed light on the mechanisms underlying the increased risk of COPD in the HIV-infected smoker.

To understand how cigarette smoking and HIV infection combine to increase the risk for developing COPD, we performed RNA-seq on AMs isolated from HIV-uninfected nonsmokers and smokers and untreated HIV-infected nonsmokers and smokers. We identified 143 genes that were significantly associated with the combination of smoking and HIV infection. Two of the most upregulated genes in AMs derived from untreated HIV-infected smokers were chitinase 1 (CHIT1) and cytochrome P450 family 1 subfamily B member 1 (CYP1B1), genes with known associations to COPD. CHIT1 and CYP1B1 levels were correlated with genes associated with extracellular matrix organization, oxidative

[2]Abbreviations used in this article: alveolar macrophages, AMs; anti-retroviral therapy, ART; BAL, bronchoalveolar lavage; chitinase1, CHIT1; cytochrome P450 family 1 subfamily B member 1, CYP1B1; matrix metalloproteinase, MMP.

stress, immune response, and cell death. Upregulation of CYP1B1 in AMs from HIV-infected smokers correlated inversely with expression of CD163 on AMs. Importantly, CHIT1 protein levels were increased in bronchoalveolar lavage (BAL) fluid of HIV-infected smokers, indicating tissue damage in the lungs of these patients. Finally, CHIT1 protein levels in the BAL negatively correlated with measurements of lung function. Collectively, these findings suggest that elevated CHIT1 and CYP1B1 levels could serve as biomarkers of the early development of COPD in HIV-infected smokers.

Materials and Methods

Study population

We enrolled 10 HIV-infected participants without a clinical diagnosis of COPD who were equally divided between smokers and nonsmokers (Table 1). Ten aged-matched HIV-seronegative participants with no high-risk HIV exposure in the prior 30 days were recruited as study controls. Informed consent was obtained from each participant, and the study protocol was approved by the Colorado Multiple Institutional Review Board. Entry criteria for HIV-infected participants included: a positive HIV ELISA confirmed by a positive Western Blot or plasma HIV-1 RNA >1,000 copies/ml at any time in the past; ART naive or off ART for > 6 months and intending to start or reinitiate ART; and 18 years and older. Cigarette smoking was defined as an average cigarette use of 6 cigarettes per day in the prior 6 months. Non-smoker is defined as less than 100 lifetime cigarettes and no cigarette use in the past year. Bronchoscopy with BAL was performed as previously described (27). The median plasma HIV-1 RNA in the HIV-infected smokers and nonsmokers was 5740 copies/ml (range: 3000 to 122,000 copies/ml) and 9480 copies/ml (range: 49 to 197,000 copies/ml). The median $CD4^+$ T cell count was 520 cells/μl (range, 219–1342 cells/μl) in HIV-infected smokers and 621 cells/μl (range, 335–829 cells/μl) in HIV-infected nonsmokers.

Alveolar macrophage isolation

BAL fluid (BALF) was filtered through a 70 μm mesh cell strainer and centrifuged at 300 g for 10 minutes to pellet the cells. BALF was separated from the pelleted cells, frozen, and stored at −80°C. AMs were purified from the pelleted BAL cells using anti-CD71 magnetic microbeads (Miltenyi Biotec), as previously described (28). Briefly, BAL cells were resuspended in separation buffer (PBS, 0.5% BSA, 2mM EDTA) and incubated with anti-CD71 magnetic microbeads for 15 minutes at 4°C. Labeled cells were washed with separation buffer and isolated using positive selection, according to manufacturer's instructions. A fraction of the positively-selected cells were reserved for differential cell counts, and the post-sort purity averaged >90%. The remaining AMs were pelleted, lysed in 1 mL Trizol, and stored at −80°C. At the time of RNA extraction, purified BAL AM pellets were thawed to room temperature. Chloroform was added, followed by vigorous shaking and centrifugation at 10,000 xg for 18 minutes at 4°C. The RNA-containing aqueous phase was removed, and an equal volume of 100% ethanol was added. This mixture was spun in an RNeasy column (Qiagen, Germantown, MD, USA), and washes were performed using RW1 and RPE buffers, according to the manufacturer's instructions. RNA was eluted in nuclease-free water and stored at −80°C. RNA quantitation was performed using a Nanodrop 2000.

Library construction and RNA sequencing

Library construction and sequencing were performed by the Genomics and Microarray Core Facility at the University of Colorado Anschutz Medical Campus. RNA quality and integrity was first assessed using an Agilent Tape Station 2200. The library was constructed using the Illumina TruSEQ mRNA library construction kit, according to the manufacturer's instructions. Single end sequencing was preformed using an Illumina HiSEQ4000 for 125 cycles. The RNAseq data have been deposited in the Sequence Read Archive (SRA) database, http://www.ncbi.nlm.nih.gov/bioproject/496501.

Data processing

Sequencing data were processed using the applications in the Illumina BaseSpace online platform and first assessed using FASTQC. The FASTQ Toolkit tool was used to filter, adaptor trim, and quality trim reads in the FASTQ files. Alignment to the hg19 reference genome was performed using the TopHat Alignment tool with the subsequent assembly and differential expression analysis performed using the Cufflinks Assembly & DE tool.

Quantitative RT-PCR

Expression levels were determined using the Superscript III Platinum One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. In short, RNA (50–200ng) was mixed with IDT PrimeTime qPCR assay primer/probe mixtures, 2× Reaction Mix, and SuperScript III RT/Platinum Taq mix. The relative quantitation of CHIT1 and CYP1B1 mRNA utilized predesigned primer/probes with Assay IDs Hs.PT. 58.20918243 and Hs.PT.58.25328727.g, respectively. The level of the housekeeping gene PPIA (Assay ID: Hs.PT.58v.38887593.g) was used to determine relative expression levels. All probes used in these assays had Int ZEN 5' 6-FAM 3' Iowa Black® FQ modifications.

Chitinase 1 ELISA

CHIT1 protein levels in BALF were determined using the Human CHIT1 (Chitotriosidase-1) ELISA kit (MyBioSource, San Diego, CA, USA) according to the manufacturer's protocol. Briefly, BALF samples were diluted 2- to 10-fold in Reference Standard & Sample Diluent and added to pre-coated microplate wells. Following incubation of the wells with Biotinylated Detection Antibody, HRP Conjugate, Substrate Reagent, and Stop Solution, the plates were read at 450nm. For consistency between BALs performed in study subjects, BALF CHIT1 levels were normalized to 100 ng total protein in BALF.

Mass cytometry

Cryopreserved BAL cells were thawed in RPMI medium supplemented with fetal bovine serum (FBS), penicillin-streptomycin, and benzonase (Sigma-Aldrich, St. Louis, MO, USA). PBMCs from a healthy donor was included in each assay as a staining control. Briefly, for live-dead cell distinction, cells were stained with 2.5 μM Cisplatin (Fluidigm, San Francisco, CA, USA) in RPMI without serum for 5 min at RT and quenched with RPMI-containing FBS. Cells were re-suspended in barium-free FACS buffer (PBS with 0.1% BSA and 2 mM EDTA) and counted. Approximately 1–2 million live cells were stained. Cells were incubated for 30 min at 4°C with a 30 μl cocktail of metal-conjugated antibodies targeting

surface antigens in an AM panel. Following wash with barium-free FACS buffer and 15 minute fixation using 1% formaldehyde made in PBS (Polysciences Inc., Warminster, PA, USA), nineteen BAL samples and the PBMC staining control were barcoded using the Cell-ID 20-Plex Barcoding Kit following manufacturer's protocol (Fluidigm), and stained samples were combined for analysis. On the day of acquisition (within a week after staining), cells were stained with a DNA intercalator (0.125 μM Iridium-191/193 or MaxPar® Intercalator-Ir; Fluidigm) in 1% formaldehyde made in PBS for 20 min at RT. After multiple washes with CyFACS, PBS, and MilliQ water, cells were filtered through a 35-μm nylon mesh and diluted to 500,000 cells/ml. Cells were acquired at a rate of 300–500 cells/sec using a CyTOF2 mass cytometer (Fluidigm), CyTOF software v.6.0.626 with noise reduction, a lower convolution threshold of 200, event length limits of 10–150 pushes, a sigma value of 3, and a flow rate of 0.045 ml/min. Runs were concatenated using the FCS file concatenation tool from Cytobank and were de-barcoded following manufacturer's protocol. AMs were identified by gating for DNA content, viability and $CD45^+CD3^-CD19^-CD56^-CD11b^+CD71^+$ cells (Supplemental Figure 1). The percentage of $CD163^+$ AMs and mean metal intensity (MMI) were determined using FlowJo.

Statistical analysis

All statistical analyses were performed using GraphPad Prism. Hypothesis testing was performed using ANOVA with Tukey multiple comparison correction. Correlations were determined using Spearman Rank correlation with p value adjustments for multiple comparisons using Benjamini-Hochberg.

Results

Smoking and HIV infection alter gene expression in AMs

RNA-seq was performed using RNA extracted from AMs derived from HIV-uninfected nonsmokers, HIV-uninfected smokers, untreated HIV-infected nonsmokers, and untreated HIV-infected smokers. Demographics of the study population are shown in Table 1. Although HIV-infected subjects who smoked had a greater number of smoking pack years (median) compared to HIV-seronegative smokers (15.6 pack years vs. 4.5 pack years), this difference was not statistically significant ($p = 0.8$). In addition, no statistically significant differences in BAL cell counts or the percentage of BAL macrophages, lymphocytes, or neutrophils were noted (Table 1).

Differential expression analysis was performed by comparing AM gene expression in HIV-uninfected smokers, untreated HIV-infected nonsmokers, or untreated HIV-infected smokers to AM gene expression in HIV-uninfected nonsmokers. Hierarchical clustering demonstrated that patient samples clustered together with samples from their respective cohort and revealed clear patterns of gene expression changes for the three experimental cohort groups (Figure 1). This differential expression analysis identified 353 genes altered by cigarette smoking, 251 genes altered by HIV infection, and an additional 685 genes altered by the combination of smoking and HIV infection.

Consistent with previous work, our data show that smoking reduced the expression of genes involved in the immune function in AMs, including downregulation of the inflammatory chemokines CXCL9 (−2.6 fold reduction; p = 0.004) and CCL15 (−2.8 fold reduction; p = 0.008), as well as other inflammatory genes such as immune-responsive gene 1 (IRG1; −2.7 fold reduction; p 0.004), osteopontin (SPP1; −2.6 fold reduction; p 0.004), and ubiquitin binding domain 9 (UBD; −3.6 fold reduction; p 0.004) (Supplemental Figure 2). Our analysis also demonstrated that HIV infection resulted in the upregulation of the extracellular matrix remodeling gene MMP2 (2.6 fold induction; p = 0.005) (Supplemental Figure 3). These findings are consistent with previous studies showing HIV-induced upregulation of extracellular matrix remodeling genes (26), which may contribute to lung destruction and the development of COPD. Overall, finding similar gene changes in our study validated our experimental approach.

CHIT1 and CYP1B1 were upregulated in AMs from HIV-infected smokers

To understand how HIV infection and cigarette smoking interact to affect gene expression in AMs, differential expression analysis was performed comparing gene expression in AMs from HIV-infected smokers to expression in HIV-seronegative nonsmokers (685 genes), HIV-infected nonsmokers (444 genes), and HIV-seronegative smokers (320 genes). Comparing the overlap between these differential expression lists identified 143 genes that were present in all three differential expression lists and therefore were significantly altered in HIV-infected smokers when compared to the other patient groups (Figure 2A). Many of these genes were also identified as having dysregulated expression due to either HIV infection or smoking, but displayed further dysregulation due to the combination of smoking and HIV infection. Therefore, these 143 genes represent genes with expression significantly altered by the combination of smoking and HIV infection.

Figure 2 shows a selection of differentially expressed genes in AMs derived from HIV-infected smokers compared to HIV-seronegative smokers and nonsmokers as well as HIV-infected nonsmokers. The genes that were most upregulated by the combination of HIV infection and smoking include genes involved in oxidative stress, extracellular matrix remodeling and inflammation (Figure 2B). In particular, two genes, cytochrome P450 family 1 subfamily B member 1 (CYP1B1) and chitinase 1 (CHIT1), were of particular interests since these genes had been previously linked to COPD (29–32). For example, CYP1B1 expression in AMs of HIV-infected smokers was significantly increased compared to that seen in HIV-seronegative smokers and nonsmokers and HIV-infected nonsmokers ($p < 0.05$) (Figure 2C). CHIT1 expression was also significantly increased in AMs of HIV-infected smokers compared to expression in HIV-seronegative smokers (Figure 2C). We confirmed the gene expression levels of CYP1B1 and CHIT1 observed with RNA-seq using qRT-PCR (Figure 2D). A significant positive correlation was noted between the expression of CYP1B1 and CHIT1, respectively, as measured by RNA-seq and qRT-PCR (Figure 2D).

CHIT1 and CYP1B1 were correlated with a list of 33 genes associated with AM activation, matrix reorganization, oxidative stress and DNA damage/apoptosis (Tables 2 and 3). CYP1B1 expression was significantly correlated with 28 of 33 genes (bolded in Table 2), with 24 of these genes having correlation coefficients greater than 0.5. CHIT1 showed

slightly less correlation with significant correlations for 20 of 33 genes (bolded in Table 3), with 16 genes having correlation coefficients greater than 0.5. Therefore, CYP1B1 and CHIT1 were chosen for further analysis due to their correlation with other genes defining the stress-activated AM profile and their prior association with COPD and cancer (29–33).

Expression of CYP1B1 and CHIT1 was determined in a larger sampling of patients from each cohort using qRT-PCR. In this larger cohort, the expression of CYP1B1 was significantly increased in AMs derived from HIV-infected smokers relative to AMs obtained from HIV-uninfected nonsmokers ($p < 0.0001$) and smokers ($p= 0.004$), with a trend towards significance in HIV-infected smokers ($p = 0.06$) (Figure 3A). Removal of the single outlier in HIV-uninfected smokers and in HIV-infected nonsmokers and smokers improved the significance of the differences in CYP1B1 expression between HIV^- nonsmokers vs HIV^+ smokers ($p <0.0001$), HIV^- smokers vs HIV^+ smokers ($p = 0.01$), and HIV^+ nonsmokers vs HIV^+ smokers ($p = 0.03$). The expression of CHIT1 was also significantly increased in AMs from HIV-infected smokers relative to AMs from HIV-uninfected nonsmokers ($p = 0.02$) and HIV-uninfected smokers ($p = 0.02$) (Figure 3B). There was also a trend toward increased CHIT1 expression AMs from HIV-infected smokers compared to HIV-infected nonsmokers; however, this did not reach statistical significance. Removal of outliers in CHIT1 expression had no effect on the significance of differences between the groups. These expression data using a larger patient cohort confirm the altered gene expression of CYP1B1 and CHIT1 in AMs derived from HIV-infected smokers.

CYP1B1 expression was inversely associated with the frequency of CD163-expressing alveolar macrophages

To determine if reduced CYP1B1 expression associated with changes in the phenotype of AMs, we examined the relationship between the expression of CYP1B1 as determined by RNAseq and AM phenotype ($CD45^+CD3^-CD19^-CD56^-CD11b^+CD71^+$) determined by Time of Flight Mass Cytometry (CyTOF). Since CyTOF relies on the detection of the ionization spectrum of stable metal isotopes rather than fluorochrome emissions, autofluorescence, which has plagued flow cytometry-based analysis of AM phenotype, was eliminated as an issue. We examined the expression of CD163, a marker that has been used to define M2 AMs. We examined the expression of CD163 on BAL cells from 18 HIV seronegative and 18 HIV-infected ART-naive subjects with matching for smoking status. Figure 4A shows a significantly lower percentage of $CD163^+$ AMs in the BAL of HIV-infected compared to HIV-seronegative individuals, regardless of smoking. Similar findings were also seen for CD206 expression on AMs (data not shown). Interestingly, in the HIV-infected cohort, the percentage and mean metal intensity (MMI), which is analogous to mean fluorescence intensity (MFI) in flow cytometry, inversely correlated with CYP1B1 gene expression determine by RNAseq, suggesting an association between the HIV-induced loss of M2 AMs and genes associated with COPD.

CHIT1 levels are elevated in the BALF of HIV-infected smokers

To further investigate the altered expression of CHIT1, we measured CHIT1 protein levels in BALF obtained from the larger cohort using a specific CHIT1 ELISA. The normalized CHIT1 levels in the BALF of HIV-infected smokers were significantly increased when

compared to BALF CHIT1 levels measured in HIV-infected nonsmokers (p = 0.001), HIV-uninfected nonsmokers (p = 0.0004), and HIV-uninfected smokers (p = 0.03) (Figure 5A). BALF CHIT1 levels in HIV-uninfected smokers was similar to that seen in the BAL of HIV-uninfected nonsmokers (p = 0.35) (Figure 5A). Thus, the greatest concentrations of BALF CHIT1 were noted in the HIV-infected smokers (Figure 5A), suggesting that both smoking and HIV infection play a role in CHIT1 gene expression and protein secretion.

Six months after initiating ART, follow-up bronchoscopies were conducted for 11 HIV-infected patients. Measuring CHIT levels in the BALF from these follow-up visits demonstrated that there was no statistically significant change in CHIT1 levels in BALF following ART treatment (p = 0.43) (Figure 5B). Together, these data suggest that measuring CHIT1 levels in the lung may be a useful approach to monitor stress-induced activation of AMs in the lung of HIV-infected patients and that CHIT1 expression and protein secretion are not significantly altered after 6 months of ART and full viral suppression.

CHIT1 association with decreased lung function

Based on previously published reports in HIV-seronegative COPD patients (30–32), we hypothesized that CHIT1 functions as an indicator of decreasing lung function in HIV-infected smokers. To investigate this hypothesis, we performed lung function testing on a cohort of individuals and correlated BALF CHIT1 protein with markers of lung function. We noted a significant negative correlation between BALF CHIT1 protein and the percent predicted forced vital capacity (FVC %) (p = 0.02) (Figure 6A). Although trends towards significance were noted for the relationship between BALF CHIT1 and percent predicted forced expiratory volume in 1 sec (FEV1 %; p = 0.08) and corrected DCLO (p = 0.07) (Figure 6A), these negative correlations did not reach statistical significance. Due to the small sample size, we included samples from HIV-infected and -uninfected individuals with a known diagnosis of COPD in the analysis. With the addition of the COPD patients (n = 11), a significant negative correlation was seen between BALF CHIT1 protein levels and %FEV1 (p = 0.004), %FVC (p = 0.04), and DLCO (p = 0.01) (Figure 6B). Collectively, these data suggest a link between the altered expression of CHIT1 in AMs and in BALF and the loss of lung function observed in smokers infected with HIV.

Discussion

HIV-infected smokers develop emphysema at an earlier age and with higher prevalence compared to uninfected smokers, suggesting an interaction between HIV infection and cigarette smoking to increase disease risk (7–9, 26). Due to the critical role of AMs in the pathogenesis of COPD, we analyzed the transcriptomes of AMs derived from HIV-infected and -uninfected smokers and nonsmokers and showed differential expression of 143 genes, including CHIT1 and CYP1B1, in HIV-infected smokers. These two genes were of particular interest because of their association with COPD in previous studies of HIV-seronegative subjects (30, 34) and their correlation with the expression of a number of genes that are indicative of tissue damage and extracellular matrix remodeling. Phenotyping of AMs from HIV-infected and -uninfected subjects matched for smoking showed an HIV-induced loss of M2 AMs and an inverse correlation between CD163 expression on AMs and

expression of CYP1B1. The increased expression of CHIT1 in AMs corresponded to a similar increase in CHIT1 protein in BALF, and CHIT1 expression was not changed six months after initiation of ART. We further demonstrated that the CHIT1 level in BALF was negatively correlated with lung function measurements. Collectively, our findings suggest that CHIT1 and CYP1B1 may be useful biomarkers to track in the early development of COPD in HIV-infected smokers.

Our findings confirmed previous results showing altered gene expression in AMs from smokers and HIV-infected individuals. Shaykhiev et al. (19) reported transcriptional suppression of M1-related inflammatory genes in AMs from smokers, including CXCL9, CXCL10, and IRG1. Our experiments showed similar downregulation of these genes as well as other inflammation and/or immune-related genes. These downregulated genes included the pro-inflammatory genes IDO1, CCL15, and UBD9, and the suppression of such genes may explain the decrease in lung immune responses for smokers. Woodruff et al. (32) reported the upregulation of MMP12, SPP1, CYP1B1, FLT1, and TGFBR1 in AMs from smokers. All of these genes are involved in extracellular matrix remodeling; however, our analysis did not identify any of these genes as significantly upregulated in AMs derived from HIV-uninfected smokers. One possibility for this discrepancy is that our samples were from individuals who were significantly younger and with less pack years as compared the Woodruff study. Interestingly, upregulation of these genes, including CYP1B1 and MMP12, was seen in AMs derived from HIV-infected smokers despite being of a younger age and with less smoking history. These findings suggest that the combination of HIV infection and smoking could potentially accelerate the deleterious changes in AMs, which are normally only seen in individuals with longer smoking histories and may lead to a more rapid development of COPD.

The upregulation of MMP12, MMP2, MMP9, and other extracellular matrix remodeling proteins prior to COPD development in HIV-infected smokers suggests that tissue remodeling is initiated early in the development of COPD. MMP12 has a strong association with COPD development, and a recent study of AMs from HIV-infected smokers with early emphysema identified MMP12 upregulation in AMs and in BALF in these individuals (26). Our analysis corroborates these findings and suggests that MMP12 upregulation may begin prior to the development of emphysema in HIV-infected smokers. In addition, the upregulation of both CHIT1 and CYP1B1 strongly correlated with the expression of MMP12 in AMs derived from HIV-infected smokers. Overall, these data indicate that deleterious tissue remodeling may occur early in HIV-infected smokers relative to the other cohorts and begin the process of alveolar destruction that ultimately leads to COPD development.

Our findings also suggest that CHIT1 is a key indicator of a deleterious AM activation state that exists in HIV-infected smokers. Its expression correlated with the expression of a number of genes involved in tissue remodeling, apoptosis, and oxidative stress. The level of AM-expressed chitinases, CHIT1 and CHI3L, were previously correlated with COPD severity (29–31). CHIT1 levels in smokers with and without COPD have been associated with the presence of airflow obstruction (30, 31). Here, we also show a similar association between BALF CHIT1 and decreased lung function even prior to the development of COPD.

These chitinases may function to directly cause lung injury or as markers for a deleterious AM activation state. Regardless, the cascade of events occurring as a consequence of smoking and HIV infection sets the stage for an accelerated breakdown of the lung extracellular matrix. In any case, increased CHIT1 levels may be an important biomarker for lung damage occurring in HIV-infected smokers and a key indicator of increased risk of COPD development.

The cytochrome p450 protein, CYP1B1, was one of the most highly upregulated genes in HIV-infected smokers and may also play an important role in the development of COPD and lung cancer in this susceptible cohort of HIV-infected smokers. This gene is generally considered a detoxifying enzyme, which is upregulated in response to cigarette smoking (35). This upregulation of CYP1B1 was noted in AMs from long-term smokers (32), and based on our data this appears to be accelerated in AMs from HIV-infected smokers. The detoxification process for polycyclic aromatic hydrocarbons in cigarette smoke by CYP1B1 can itself lead to the formation of toxic byproducts (36). These toxic byproducts can lead to the formation of carcinogenic DNA adducts and oxygen free radicals (37). The correlation of CYP1B1 expression with oxidative stress-related genes (e.g., TXNRD1, CYGB, GSR) and apoptosis associated genes (e.g., OSGIN1, FANCE, BCL2A1) suggests that these toxic byproducts are in fact being produced in the lungs of these HIV-infected smokers. AMs from smokers and COPD patients demonstrate impaired efferocytosis of apoptotic cells (13, 38–42), which may also contribute to increased inflammation and tissue damage within the lung when combined with an increase in apoptotic cells due to CYP1B1-related toxicity. Furthermore, the inverse association between CYP1B1 and the percentage of CD163-expressing M2 AMs, which are known to be anti-inflammatory and play a role in tissue repair (43), may also contribute to tissue damage in the lung of HIV-infected subjects (14). These findings raise the possibility that some of the soluble CD163 found in plasma and used to track macrophage activation and HIV disease progression (44) may be derived from AMs, which follows since it has also been shown to predict chronic lung disease in HIV (45).

Overall, our findings indicate that the combination of smoking and HIV infection induces a unique AM activation state. The hallmark of this activation state is the upregulation of MMP12, CYP1B1, and CHIT1. The expression of these genes were correlated with multiple genes involved in oxidative stress response, cell death, and extracellular matrix remodeling, which all indicate a destructive state in the lung. Our data indicate that the CHIT1 level in lung is a particularly attractive biomarker for monitoring lung damage and determining risk for developing COPD in HIV-infected smokers. Likewise, CYP1B1 may be an attractive target for inhibition as it may be driving cell death and mutagenesis in the lung, which could be key drivers of the destructive AM state in the lung. Such a biomarker and therapeutic target could be beneficial not only for HIV-infected smokers but for any individual at an increased risk of developing COPD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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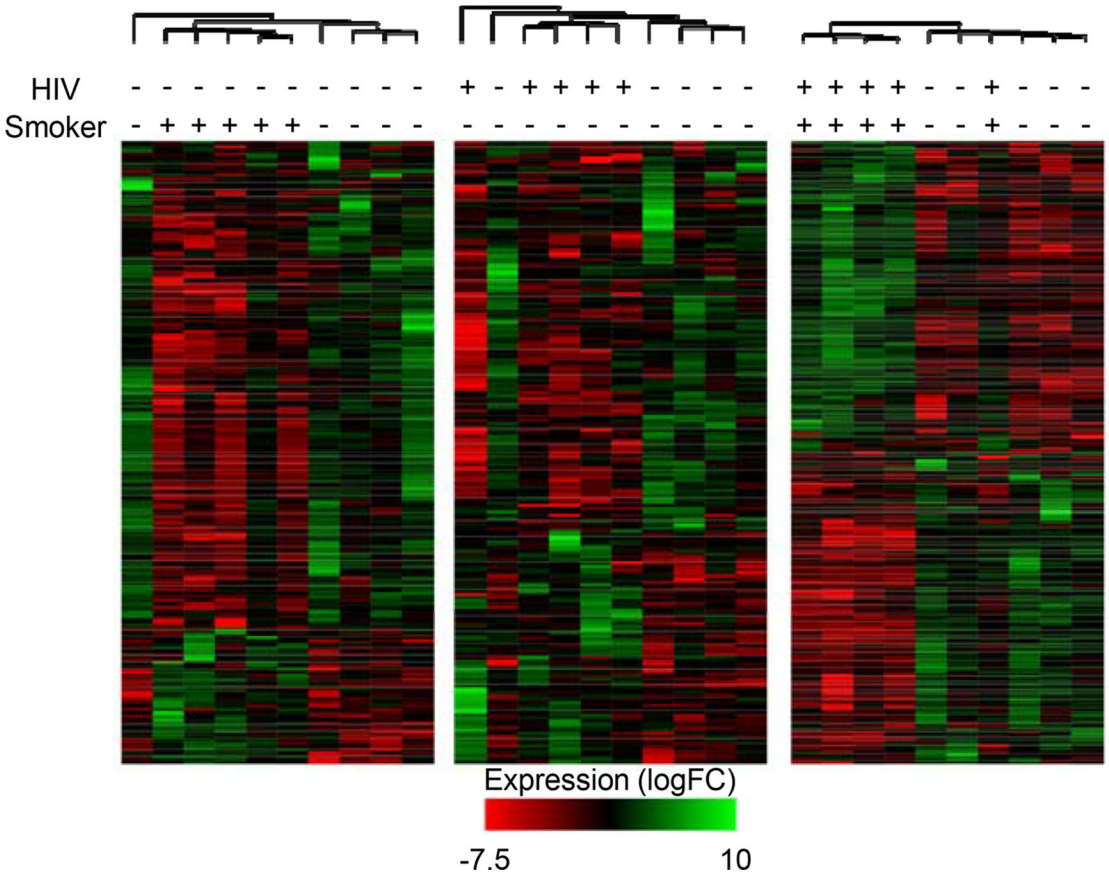

Figure 1. Smoking and HIV infection result in the differential expression of genes in alveolar macrophages.

Differential expression analysis identified expression changes in alveolar macrophages (AMs) resulting from smoking (left), HIV-infection (middle), or the combination of the two (right) when compared to HIV- nonsmoker AMs. Heatmaps demonstrate gene expression changes where patient samples and genes were subject to hierarchical clustering.

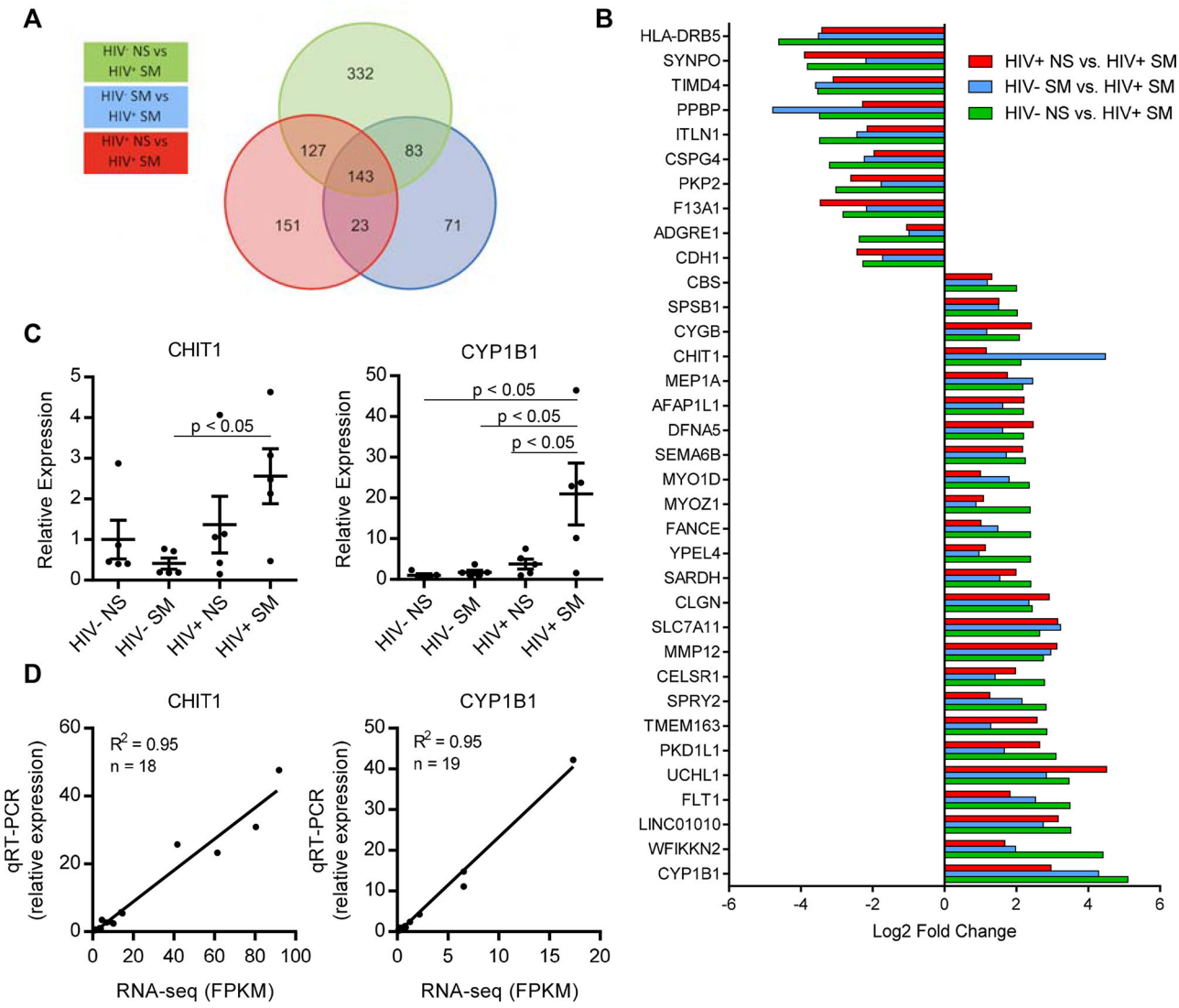

Figure 2. Dysregulation of genes due to an interaction between HIV infection and cigarette smoking.

A) A Venn diagram displays overlap between differentially expressed genes for alveolar macrophages (AMs) from HIV-infected smokers vs. AMs from HIV-uninfected nonsmokers, HIV-uninfected smokers, and HIV-uninfected nonsmokers. **B)** Bar graph shows fold change differences for the genes most dysregulated due to the combination of HIV infection and smoking. **C)** Relative expression of CHIT1 (left) and CYP1B1 (right) are shown. Mean and SEM for relative CHIT1 and CYP1B1 expression are shown. A one-way analysis of variance was used to determine differences between groups. **D)** Expression levels measured by RNA-seq were compared to expression levels measured by qRT-PCR for CYP1B1 and CHIT1. A linear model was fitted to each scatter plot with the corresponding R^2 value displayed for the model.

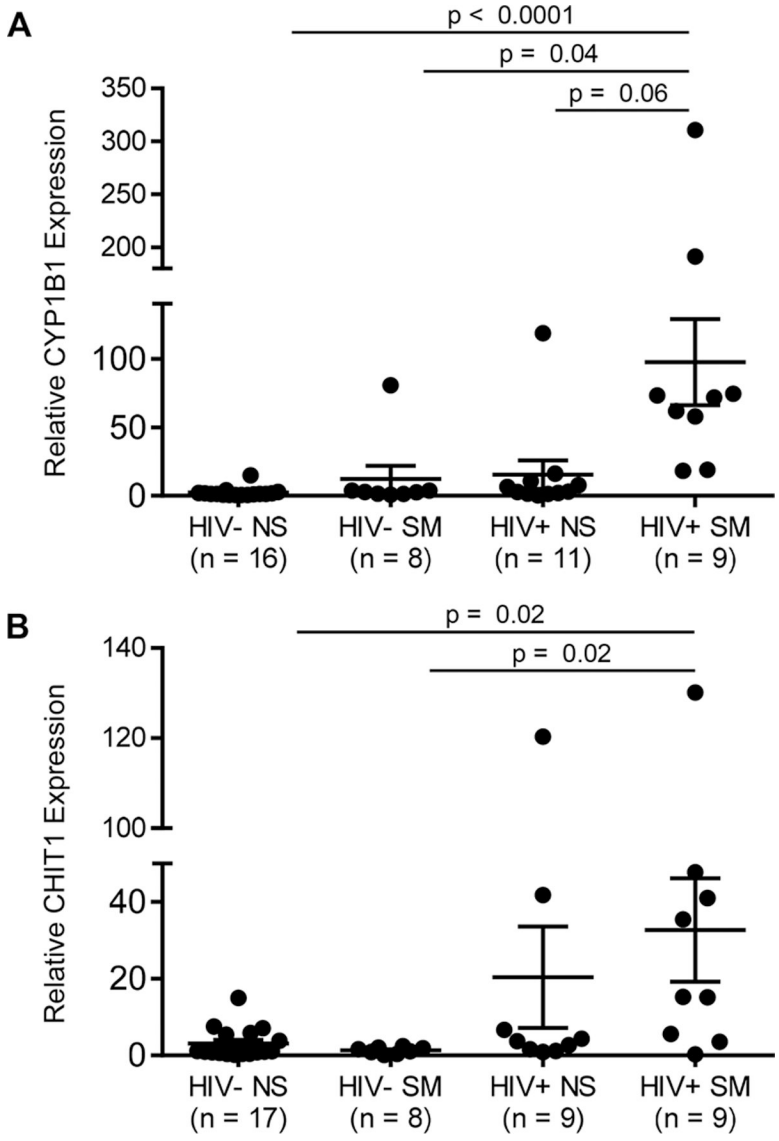

Figure 3. CHIT1 and CYP1B1 upregulation in HIV-infected smokers confirmed by qRT-PCR.

A) CHIT1 and **B)** CYP1B1 expression were determined in alveolar macrophages (AMs) using qRT-PCR. The graphs display relative gene expression, with solid lines indicating the mean and SEM. Hypothesis testing was performed using ANOVA with Tukey multiple comparison correction. The p values < 0.05 are displayed on the graph.

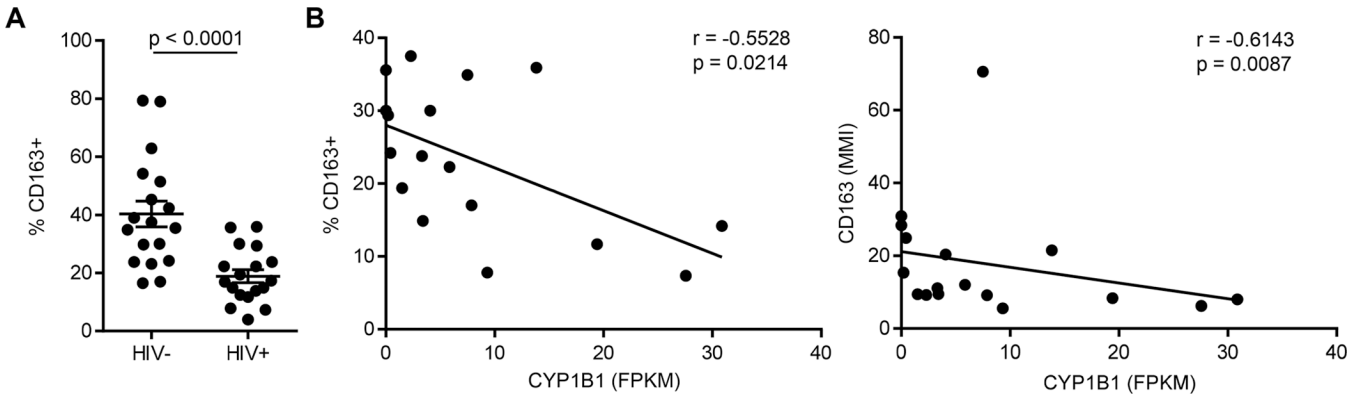

Figure 4. Alveolar macrophages from HIV-infected subjects lose the M2 phenotype.

A) CD163 expression on alveolar macrophages (AMs) from HIV-uninfected and HIV-infected subjects is shown, with solid lines indicated mean and SEM. Statistical significance was determined by a Mann-Whitney test. **B)** Correlation of CD163 (% positive, left panel) and CD163 (mean metal intensity, MMI, right panel) with CYP1B1 expression (RNA-seq FPKM) in AMs is shown. Graphs display Spearman correlation coefficients (r) and the one-sided p values for these correlation coefficients.

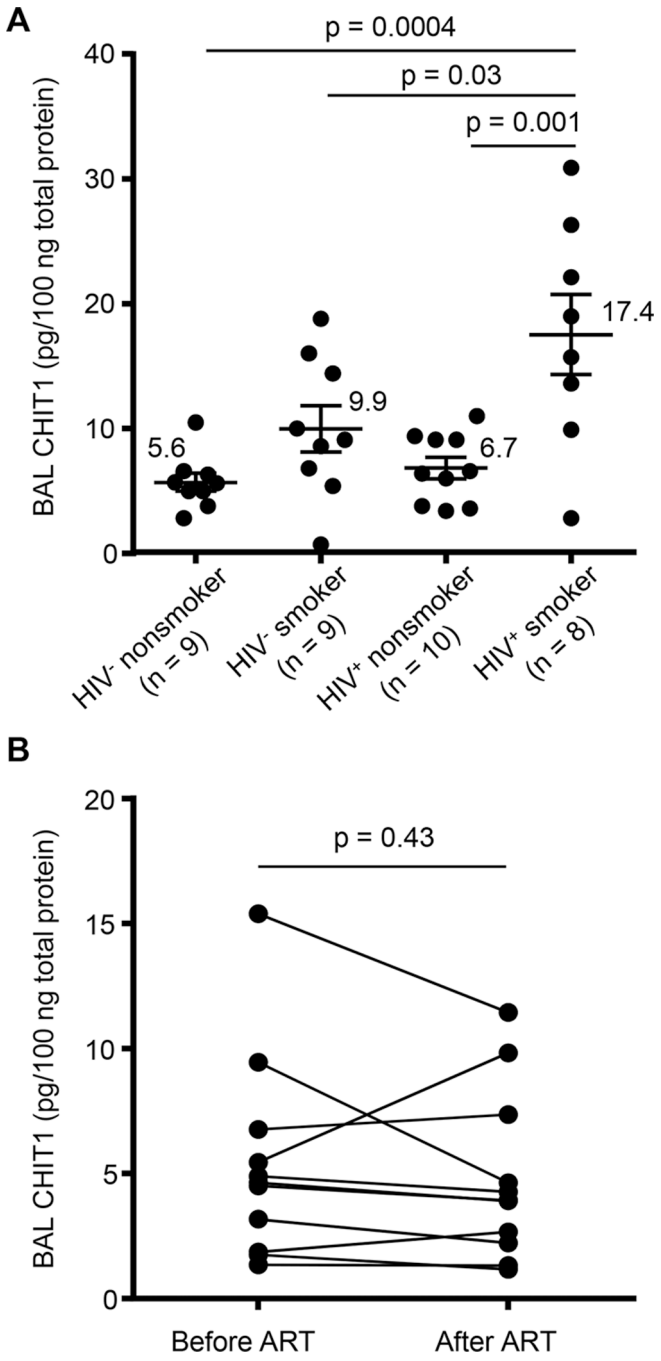

Figure 5. CHIT1 concentrations in BALF are increased in HIV-infected smokers.

A) CHIT1 concentration in BALF from smokers and nonsmokers with and without HIV infection were determined using an ELISA. BALF CHIT1 levels (pg) were normalized to 100 ng of total protein in the BALF. The graph displays the mean CHIT1 concentration and SEM as solid lines. Hypothesis testing was performed using ANOVA with Tukey multiple comparison correction. **B)** CHIT1 concentrations in BALF were determined for HIV-infected patients before and after > 6 months of anti-retroviral therapy. Points represent

concentrations with paired samples from before and after therapy connected by a line. Lack of significance was determined using a paired t-test.

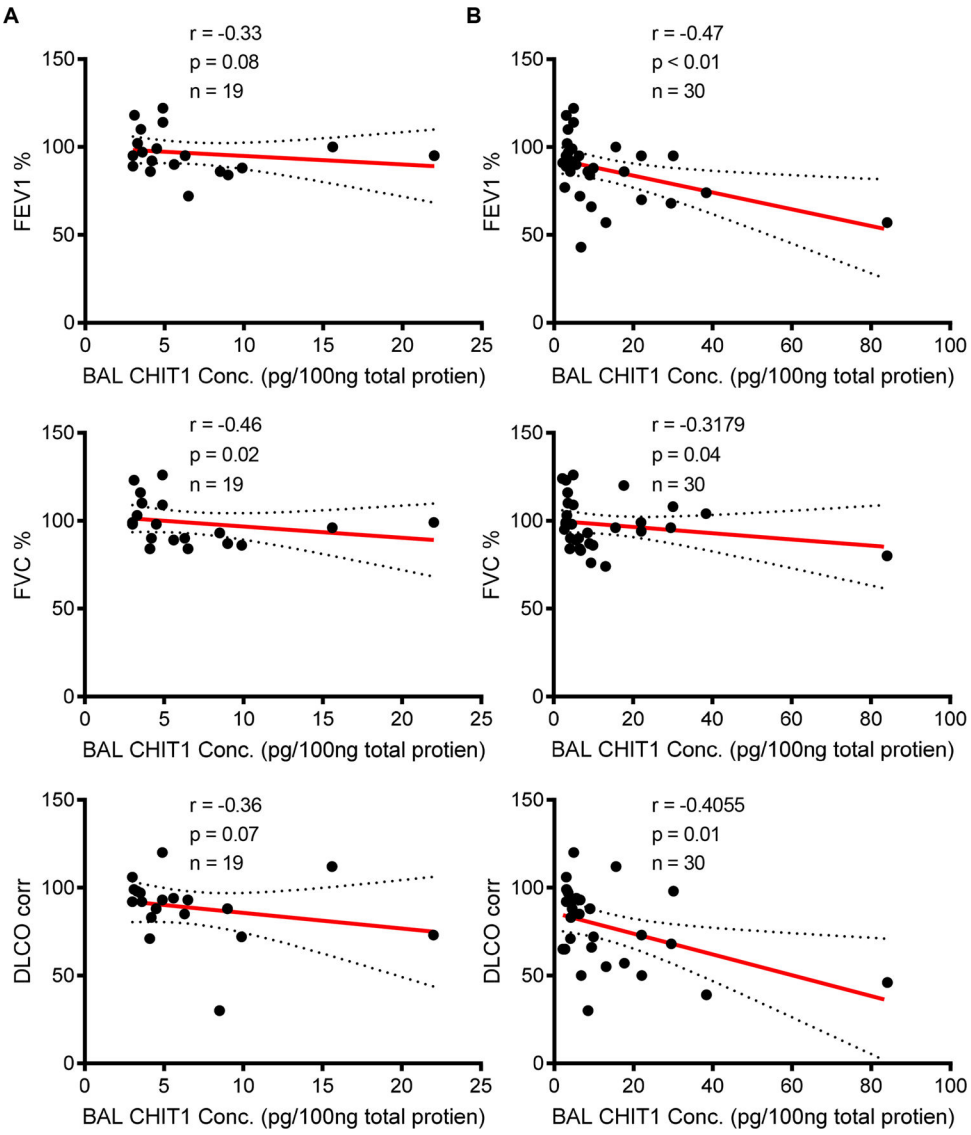

Figure 6. BALF CHIT1 concentrations correlate with a decrease in lung function.

CHIT1 concentrations in BAL were compared to the following lung function measurements: FEV1 %, FVC %, and DLCO corrected. Comparisons were made using samples from individuals **(A)** without COPD only and from individuals **(B)** both with or without COPD. The graphs display data points with linear models (red lines) fitted to these data and 95% confidence intervals for these models (dashed lines). The graphs also display Spearman correlation coefficients (r) and the one-sided p values for these correlation coefficients.

Table 1.

Demographics of the Study Population[A]

Cohort	HIV-Infected Smokers	HIV-Infected Nonsmokers	HIV-Uninfected Smokers	HIV-Uninfected Nonsmokers
Number	5	5	5	5
Gender (M/F)	4/1	5/0	3/2	4/1
Race (C/AF/H)[B]	4/1/0	3/1/1	3/0/2	3/1/1
Age (years)	31.3 (23.7 – 51.6)	35.2 (24.4 – 50.6)	34.3 (24.9 – 44.7)	29.7 (27.8 – 53.7)
Body Mass Index	21.3 (18 – 49.8)	25.4 (23.3 – 34.3)	27.3 (23.4 – 28.2)	26.5 (19.7 – 27.6)
Cigarette use (pack years)[C]	15.6 (1.5 – 17)	0	4.5 (2.0 – 31)	0
Viral load	5740 (3000 – 122000)	9480 (49 – 197000)	N/A	N/A
CD4 T cell count	520 (103 – 745)	621 (335 – 829)	N/A	N/A
BAL cells[C]				
WBC count ($\times 0^6$)	50.7 (17.5 – 62)	36.0 (2.5 – 58.8)	12.5 (9.3 – 39)	22.7 (2.0 – 30.0)
Macrophages (%)	95 (64 – 100)	77 (60 – 92)	90 (87 – 99)	88 (58 – 96)
Lymphocytes (%)	4 (0 – 32)	23 (7 – 38)	5 (0 – 13)	11 (2 −36)
Neutrophils (%)	1 (0 – 8)	0 (0 – 2)	0	0

[A] Data expressed as median (range).

[B] C = Caucasian; AF = African-American; H = Hispanic.

[C] No statistically significant differences were seen.

Table 2.

Correlation of Gene Expression with CYP1B1

Gene	r	p (two-tailed)	GO Tag
SLC7A11	0.50	0.024	Response to oxidative stress
CYGB	0.65	0.0019	Response to oxidative stress
TXNRD1	0.76	0.0001	Response to reactive oxygen species
NQO1	0.46	0.0435	Response to oxidative stress
GSR	0.68	0.0009	Response to reactive oxygen species
NFE2L3	−0.48	0.0342	Regulation of transcription, DNA-templated
OSGIN1	0.62	0.0035	Positive regulation of apoptotic process; oxidation-reduction process
BCL2A1	0.66	0.0015	Intrinsic apoptotic signaling pathway in response to DNA damage
FANCE	0.73	0.0003	Cellular response to DNA damage stimulus
TREM2	0.77	<0.0001	Regulation of immune response
IGF1R	0.71	0.0004	Immune response
ATP6V0D2	0.65	0.0021	Phagosome acidification
DNASE2B	0.84	<0.0001	Apoptotic DNA fragmentation
MFGE8	0.22	0.3626	Positive regulation of apoptotic cell clearance
GDF15	0.62	0.0039	TGF-β receptor signaling pathway
THBS1	−0.51	0.0221	Extracellular matrix organization; inflammatory response
TGFBR1	0.82	<0.0001	Extracellular structure organization
MMP12	0.53	0.0164	Extracellular matrix disassembly
A2M	0.62	0.0039	Extracellular matrix disassembly
PAPSS2	0.57	0.0081	Metabolic process
CELSR1	0.76	0.0001	Cell adhesion; lateral sprouting involved in lung morphogenesis
MEP1A	0.70	0.0006	Proteolysis; metalloprotease activity
MRC2	0.57	0.0087	Collagen catabolic process
COL6A1	0.43	0.0584	Collagen catabolic process
COL6A2	0.48	0.033	Collagen catabolic process
CHIT1	0.63	0.0027	Immune response
ADORA2B	0.67	0.0013	Cellular defense response
PLA2G7	0.74	0.0002	Positive regulation of inflammatory response
SEMA7A	0.53	0.0171	Inflammatory response; immune response
IL17RA	0.65	0.0018	Positive regulation of inflammatory response
IL17RB	0.03	0.8949	Positive regulation of inflammatory response
IL1A	0.38	0.0951	Inflammatory response; immune response
IL1B	0.04	0.875	Inflammatory response; immune response

Table 3.

Correlation of Gene Expression with CHIT1

Gene	r	p (two-tailed)	GO Tag
SLC7A11	0.6541	0.0018	Response to oxidative stress
CYGB	0.3895	0.0896	Response to oxidative stress
TXNRD1	0.6737	0.0011	Response to reactive oxygen species
NQO1	0.8090	<0.0001	Response to oxidative stress
GSR	0.5729	0.0083	Response to reactive oxygen species
NFE2L3	−0.4256	0.0614	Regulation of transcription, DNA-templated
CYP1B1	0.6346	0.0027	Intrinsic apoptotic signaling pathway in response to oxidative stress
OSGIN1	0.7789	<0.0001	Positive regulation of apoptotic process; oxidation-reduction process
BCL2A1	0.5414	0.0137	Intrinsic apoptotic signaling pathway in response to DNA damage
FANCE	0.2812	0.2297	Cellular response to DNA damage stimulus
TREM2	0.5789	0.0075	Regulation of immune response; phagocytosis; engulfment
IGF1R	0.4316	0.0574	Immune response
ATP6V0D2	0.4586	0.0420	Phagosome acidification
DNASE2B	0.4797	0.0323	Apoptotic DNA fragmentation
MFGE8	0.3744	0.1038	Positive regulation of apoptotic cell clearance
GDF15	0.4632	0.0397	TGF-β receptor signaling pathway
THBS1	−0.3669	0.1115	Extracellular matrix organization; inflammatory response
TGFBR1	0.5504	0.0119	Extracellular structure organization
MMP12	0.6707	0.0012	Extracellular matrix disassembly
A2M	0.6000	0.0052	Extracellular matrix disassembly
PAPSS2	0.5128	0.0208	Metabolic process
CELSR1	0.3609	0.1180	Cell adhesion; lateral sprouting involved in lung morphogenesis
MEP1A	0.6752	0.0011	Proteolysis; metalloprotease activity
MRC2	0.3714	0.1069	Collagen catabolic process
COL6A1	0.6165	0.0038	Collagen catabolic process
COL6A2	0.5128	0.0208	Collagen catabolic process
ADORA2B	0.3639	0.1147	Cellular defense response
PLA2G7	0.8015	<0.0001	Positive regulation of inflammatory response
SEMA7A	0.4241	0.0624	Inflammatory response; immune response
IL17RA	0.2436	0.3007	Positive regulation of inflammatory response
IL17RB	0.3609	0.1180	Positive regulation of inflammatory response
IL1A	0.3729	0.1053	Inflammatory response; immune response
IL1B	0.3714	0.1069	Inflammatory response; immune response