

Dysregulation of Alveolar Macrophage PPAR γ , NADPH Oxidases, and TGF β ₁ in Otherwise Healthy HIV-Infected Individuals

Samantha M. Yeligar,^{1,2} Janine M. Ward,² Frank L. Harris,² Lou Ann S. Brown,²
David M. Guidot,^{1,3} and Sushma K. Cribbs^{1,3}

Abstract

Despite antiretroviral therapy (ART), respiratory infections increase mortality in individuals living with chronic human immunodeficiency virus (HIV) infection. In experimental and clinical studies of chronic HIV infection, alveolar macrophages (AMs) exhibit impaired phagocytosis and bacterial clearance. Peroxisome proliferator-activated receptor (PPAR) γ , NADPH oxidase (Nox) isoforms Nox1, Nox2, Nox4, and transforming growth factor-beta 1 (TGF β ₁) are critical mediators of AM oxidative stress and phagocytic dysfunction. Therefore, we hypothesized that HIV alters AM expression of these targets, resulting in chronic lung oxidative stress and subsequent immune dysfunction. A cross-sectional study of HIV-infected ($n=22$) and HIV-uninfected ($n=6$) subjects was conducted. Bronchoalveolar lavage (BAL) was performed, and AMs were isolated. Lung H₂O₂ generation was determined by measuring H₂O₂ in the BAL fluid. In AMs, PPAR γ , Nox1, Nox2, Nox4, and TGF β ₁ mRNA (quantitative real-time polymerase chain reaction) and protein (fluorescent immunomicroscopy) levels were assessed. Compared with HIV-uninfected (control) subjects, HIV-infected subjects were relatively older and the majority were African American; ~86% were on ART, and their median CD4 count was 445, with a median viral load of 0 log copies/ml. HIV infection was associated with increased H₂O₂ in the BAL, decreased AM mRNA and protein levels of PPAR γ , and increased AM mRNA and protein levels of Nox1, Nox2, Nox4, and TGF β ₁. PPAR γ attenuation and increases in Nox1, Nox2, Nox4, and TGF β ₁ contribute to AM oxidative stress and immune dysfunction in the AMs of otherwise healthy HIV-infected subjects. These findings provide novel insights into the molecular mechanisms by which HIV increases susceptibility to pulmonary infections.

Keywords: HIV, immunology, molecular biology

Introduction

ANTIRETROVIRAL THERAPY (ART) has led to a decrease in acquired immune deficiency syndrome (AIDS)-specific opportunistic infections in individuals living with chronic human immunodeficiency virus (HIV) infection.^{1,2} However, despite these advances, individuals living with HIV continue to be susceptible to serious bacterial and viral infections, such as pneumococcus and influenza,³⁻⁶ which lead to greater morbidity and mortality. Increased susceptibility of individuals living with HIV to pulmonary infections is at least partially due to the fact that HIV impairs alveolar macrophage

(AM) immune functions.⁷⁻¹¹ In fact, we have previously shown that the AM harbors HIV, even in otherwise healthy subjects with undetectable plasma viral loads, suggesting that the AM is a potential reservoir for the virus.¹² HIV infection also causes derangements in multiple steps of an immune response, including abnormal activation,¹³⁻¹⁶ diminished oxidative burst,¹⁵ increased anti-inflammatory cytokine secretion,¹³ and impaired phagocytic capacity.^{8,9,12} Most notably, in our previous prospective cross-sectional study of healthy HIV-infected subjects with systemic viral suppression and on ART, AMs from HIV-infected subjects exhibited impaired phagocytic index compared with control subjects.¹²

¹Division of Pulmonary, Allergy, Critical Care and Sleep Medicine, Department of Medicine, Emory University, Atlanta, Georgia.

²Division of Neonatal-Perinatal Medicine, Department of Pediatrics, Emory + Children's Healthcare of Atlanta Center for Developmental Lung Biology, Emory University, Atlanta, Georgia.

³Atlanta VA Medical Center, Decatur, Georgia.

Impaired phagocytosis by AMs in the HIV lung may be due to increased pulmonary oxidative stress. In preclinical^{7,17,18} and clinical studies,^{19,20} HIV infection causes systemic and pulmonary oxidative stress. Oxidative stress can be generated through several mechanisms, including the oxidation of extracellular thiols, such as glutathione (GSH) to glutathione disulfide (GSSG),²¹ but other underlying mechanisms may contribute to oxidative stress. Upregulation of peroxisome proliferator-activated receptor (PPAR)- γ is essential in protecting against pulmonary oxidant injury.²² Therefore, decreases in PPAR γ impair the capacity of the lung to downregulate oxidative stress.²³ In AMs, the expression of NADPH oxidases (Nox) 1 and 2 is required for Nox4 expression²⁴ and increased Nox activity enhances oxidative stress and phagocytic dysfunction.^{24,25} Transforming growth factor β_1 (TGF β_1) is a mediator of reactive oxygen species (ROS) and phagocytic dysfunction in the AM,²⁶ and further, increased signaling through TGF β_1 has been shown to upregulate Nox4.²⁷

Therefore, we hypothesize that individuals living with chronic HIV infection have an increased susceptibility to respiratory infections due to increased alveolar oxidative stress and impaired lung immunity. Specifically, chronic HIV infection is associated with AM oxidative stress through decreases in PPAR γ expression and increases in the expression of Nox1, Nox2, Nox4, and TGF β_1 . Increased oxidative stress is expected to subsequently impair the ability of the AM to phagocytize and clear infectious microbes from the alveolar space in individuals living with HIV, which may contribute to the increased risk for lung infections.

Materials and Methods

Study design and protocol

All participants signed the Emory University Institutional Review Board approved consent to participate in the study. Subjects were recruited and enrolled as previously described.¹² In brief, we performed a cross-sectional study of HIV-infected (HIV, $n=22$) and HIV-uninfected (hereafter referred to as control subjects) (Control, $n=6$) individuals within the Grady Health System in Atlanta, GA. Exclusion criteria included active liver disease (known cirrhosis and/or total bilirubin >2.0 mg/dl), heart disease [ejection fraction $<50\%$, history of acute myocardial infarction, New York Heart Association (NYHA) II–IV cardiac symptoms, severe valvular dysfunction], current renal disease (dialysis dependent or creatinine >2.0 mg/dl), current lung disease [spirometry revealing a forced vital capacity or forced expiratory

volume in 1 s $<80\%$ of predicted], diabetes, current pregnancy, malnutrition (body mass index <17), current tobacco use, and age <25 years. All subjects completed a pre-enrollment evaluation, and subjects with exclusions were excluded from further participation.

After completing the pre-enrollment evaluation, subjects presented to the Grady Memorial Hospital Clinical Interaction Site, and demographic data were collected. Flexible fiberoptic bronchoscopy with standardized bronchoalveolar lavage (BAL) was performed by using standard conscious sedation techniques, by installing and withdrawing with low-pressure hand suction, 30 ml aliquots of 0.9% nonbacteriostatic normal saline solution until a total of 180 ml had been administered.

Human AMs cell culture

Human AMs were isolated from the BAL fluid^{24,25} and were determined to be $\sim 90\%$ pure, as measured by Diff-Quik (Dade Behring) staining and cell counting.²⁸ AMs were resuspended in RPMI-1640 medium containing 2% fetal bovine serum and 1% penicillin/streptomycin and cultured for 2 h, allowing AMs to attach to the plastic petri dishes, before experiments. Some AMs were treated with 10 μ M pioglitazone (PIO, United States Biological, Salem, MA) or dimethyl sulfoxide vehicle (0.01%) for 24 h.

Measurement of H₂O₂ generation

H₂O₂ generation in collected BAL fluid was determined by using Amplex Red assay (Invitrogen). In brief, 50 μ l of BAL fluid was incubated with 500 μ l Amplex Red (20 μ M) and horseradish peroxidase (0.1 U/ml) at 37°C for 30 min in the dark. Fluorescence of reaction mixtures was measured in duplicate (excitation 540 nm, emission 590 nm), and H₂O₂ concentrations were calculated by utilizing standard curves that were generated with reagent H₂O₂. BAL total protein concentrations were determined by using BCA assay. H₂O₂ concentrations were normalized to total protein concentrations and expressed as mean \pm standard error of the mean (SEM), relative to average control values.

mRNA studies

Total RNA was extracted from AMs by using TRIzol reagent (Invitrogen, Carlsbad, CA). mRNA levels of PPAR γ , Nox1, Nox2, Nox4, and TGF β_1 were determined and quantified by quantitative real-time polymerase chain reaction using methods previously described,^{23,24} and specific mRNA primers are shown in Table 1. Values for each target are expressed relative to mRNA levels of 9s in the same sample.

TABLE 1. HUMAN mRNA PRIMER SEQUENCES TO MEASURE mRNA EXPRESSION USING QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION

Primer	Forward sequence (5'-3')	Reverse sequence (5'-3')
PPAR γ	GAGTTCATGCTTGCAAGGATGC	CGATATCACTGGAGATCTCGCC
Nox1	CGCTCCCAGCAGAAGGTCGTGATTACCAAG	GGAGTGACCCCAATCCCTGCCCAACCA
Nox2	GTACACCCTTCGCATCCATTCTCAAGTCAGT	CTGAGACTCATCCAGCCAGTGAGGTTAG
Nox4	CTGGAGGAGCTGGCTCGCCAACGAAG	GTGATCATGAGGAATAGCACCACCACCATGCAG
TGF β_1	CAGAAATACAGCAACAATTCCTGG	TTGCAGTGTGTTATCCCTGCTGTC
9s	GAGCTAGCCTCTGCCAGAGG	TCCAAGCCTCAAGACAGGAA

PPAR γ , peroxisome proliferator activated-receptor gamma; Nox, NADPH oxidase; TGF β_1 , transforming growth factor beta-1.

Protein studies

Protein levels of PPAR γ , Nox1, Nox2, Nox4, and TGF β ₁ in AMs were assessed by fluorescent immunomicroscopy. Cultured AMs were fixed to chamber slides with 4% paraformaldehyde. Cells were then incubated with primary antibodies (Santa Cruz Biotechnology) for PPAR γ (1:100), Nox1 (1:100), Nox2 (1:100), Nox4 (1:100), and TGF β ₁ (1:100), followed by incubation with fluorescent TRITC-labeled secondary antibodies.^{24,29} After staining, fluorescent microscopy images were obtained with Fluoview analysis (Olympus, Melville, NY). Fluorescence units of TRITC-target stained cells were normalized to DAPI nuclear stain and are expressed as mean relative fluorescent units (RFU) per cell \pm SEM, relative to control. We previously reported that quantitating protein targets with immunostaining methods correlated well with western blotting of the same targets.^{24,25} RFUs were measured in images that contain at least 10 cells per field for which there are 10 fields per experimental condition. Microscope settings, such as gain and gamma settings, were consistent for each field and experimental condition. The contrast of all provided representative images was increased by 50% to aid in visualization, without causing over-saturation of the fluorescent staining.

In vitro phagocytosis studies

In vitro phagocytic capacity was measured by using pHrodo *Staphylococcus aureus* BioParticles conjugate (Invitrogen).²⁹ Briefly, cells were incubated with 1×10^6 particles of pH-sensitive pHrodo *S. aureus* BioParticles conjugate for 2 h and then fixed with 4% paraformaldehyde. After fixing, cells from 10 fields per experimental condition were assessed for phagocytosis, and fluorescent microscopy images were obtained with Fluoview analysis (Olympus, Melville, NY). Cells were considered positive for phagocytosis if any internalized bacteria were present. Phagocytosis was calculated as the percentage of cells that were positive for phagocytosis multiplied by the relative fluorescence units of *S. aureus* per cell and given as phagocytic index.

Statistical analyses

Univariate comparisons between HIV-infected subjects and control subjects were calculated and evaluated for a significance level of 0.05 by using a chi-squared test for categorical variables and a two-sample *t*-test for continuous variables. The data were log transformed or a Wilcoxon Rank-Sum Test was used when the data were not normally distributed. Data are presented as means \pm SEM. In studies with more than two groups, statistical significance was calculated by using one-way ANOVA followed by Tukey-Kramer *post hoc* tests to detect differences between individual groups using GraphPad Prism version 5 (GraphPad, San Diego, CA). *p* < .05 was considered statistically significant.

Results

Baseline characteristics of HIV-uninfected and HIV-infected subjects

A total of 22 HIV-infected (HIV) subjects and 6 HIV-uninfected (control) subjects were enrolled (Table 2). The HIV-infected subjects were significantly older than control subjects. There were no significant differences in median gender, but the majority of subjects in both groups were African American. Overall, 86.4% of HIV-infected subjects were on ART and their median CD4 count was 445, with a median viral load of 0 log copies/ml.

Chronic HIV infection is associated with H₂O₂ increases in BAL

In previous studies conducted in our laboratory, chronic HIV infection was associated with increased oxidative stress, as defined by decreased glutathione levels and altered glutathione redox balance in the alveolar space, as well as by increased H₂O₂ levels in lung tissue.¹⁷ Measurements of H₂O₂ were performed in the BAL cell-free fluid collected from control and HIV-infected subjects. Compared with controls, BAL from HIV-infected subjects showed a

TABLE 2. CHARACTERISTICS OF HIV-UNINFECTED SUBJECTS (CONTROL, N=6) AND HIV-INFECTED SUBJECTS (HIV, N=22) ENROLLED IN THE STUDY

Variables	HIV uninfected (control)	HIV infected (HIV)	p
Number of subjects (N)	6	22	
Age (years) ^a	38 (28.3–48.3)	51 (46.8–55.5)	.009
Gender (% male)	66.7	59.1	.74
Race n (%)			
White	0 (0)	1 (4.5)	.02
Black	4 (66.7)	21 (95.5)	
Other	2 (33.3)		
BMI ^a	28.5 (26.0–34.5)	30 (26.8–33.5)	.65
SMAST ^a	0 (0–1.3)	0 (0–4)	.33
AUDIT ^a	0.5 (0–4.5)	2 (0–3.3)	.71
CD4 count ^a		445 (317–557.3)	
Viral load (log copies/ml) ^a		0 (0–0)	
Use of ART (%)		86.4	

^aResults are represented as medians with interquartile range.

BMI, body mass index; SMAST, Short Michigan Alcoholism Screening Test; AUDIT, Alcohol Use Disorders Identification Test; ART, antiretroviral therapy.

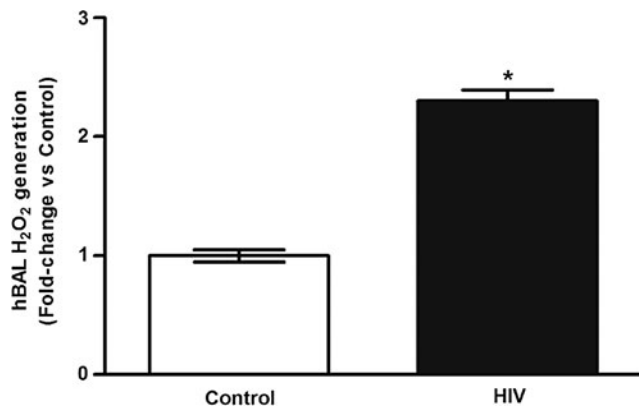


FIG. 1. Chronic HIV infection is associated with increases in BAL oxidative stress. BAL fluid was collected from HIV-uninfected control subjects (Control, $n=6$) and from HIV-infected subjects (HIV, $n=22$). H₂O₂ generation was measured by Amplex Red assay ($n=28$, in duplicate). Values were normalized to total protein and are expressed as mean \pm SEM, relative to control. * $p < .05$ versus control. BAL, bronchoalveolar lavage; SEM, standard error of the mean.

2.3 \pm 0.1-fold increase in H₂O₂ generation (Fig. 1). These data suggest that HIV infection is associated with increased H₂O₂ and oxidative stress in the alveolar space.

Chronic HIV infection is associated with alterations in mRNA and protein levels of PPAR γ , NADPH oxidases, and TGF β ₁ in AMs

Since chronic HIV infection was associated with increased BAL H₂O₂ generation, we determined whether previously established markers of oxidative stress were altered in the AMs,²⁹ particularly PPAR γ , Nox1, Nox2, Nox4, and TGF β ₁.

Compared with controls, AMs from HIV-infected subjects demonstrated a 5.3 \pm 0.1-fold decrease in PPAR γ mRNA (Fig. 2A), a 2.1 \pm 0.03-fold increase in Nox1 mRNA (Fig. 2B), a 2.44 \pm 0.03-fold increase in Nox2 mRNA (Fig. 2C), a 2.5 \pm 0.27-fold increase in Nox4 mRNA (Fig. 2D), and a 2.9 \pm 0.21-fold increase in TGF β ₁ mRNA (Fig. 2E). Although HIV status was not significantly associated with Nox mRNA after a multi-regression analysis with age as a confounder, HIV was still associated with PPAR γ and TGF β ₁ mRNA levels in AMs.

Concomitant with HIV-induced alterations in mRNA levels in AMs, there was attenuation of PPAR γ protein (Fig. 3A) and augmentation of protein levels of Nox1 (Fig. 3B), Nox2 (Fig. 3C), Nox4 (Fig. 3D), and TGF β ₁ (Fig. 3E). These data indicate that HIV infection was associated with decreased mRNA and protein expression of PPAR γ as well as with increased mRNA and protein expression of the Nox isoforms and TGF β ₁, all of which are indicators of exaggerated oxidative stress in the AM.

Ex vivo PIO treatment reverses chronic HIV infection-associated alterations in mRNA levels of PPAR γ , NADPH oxidases, and TGF β ₁ and improves phagocytosis in AMs

Since PPAR γ ligands have been previously shown to downregulate established markers of oxidative stress in AMs,²⁹ we determined whether PIO can reverse alterations in the mRNA levels of PPAR γ , Nox1, Nox2, Nox4, and TGF β ₁ that are associated with chronic HIV infection. Compared with AMs from HIV-infected subjects, PIO treatment *ex vivo* ($n=4$) increased PPAR γ mRNA levels (Fig. 4A) and decreased the mRNA levels of Nox1 (Fig. 4B), Nox2 (Fig. 4C), Nox4 (Fig. 4D), and TGF β ₁ (Fig. 4E). There were no statistically significant differences in raw Ct values between the

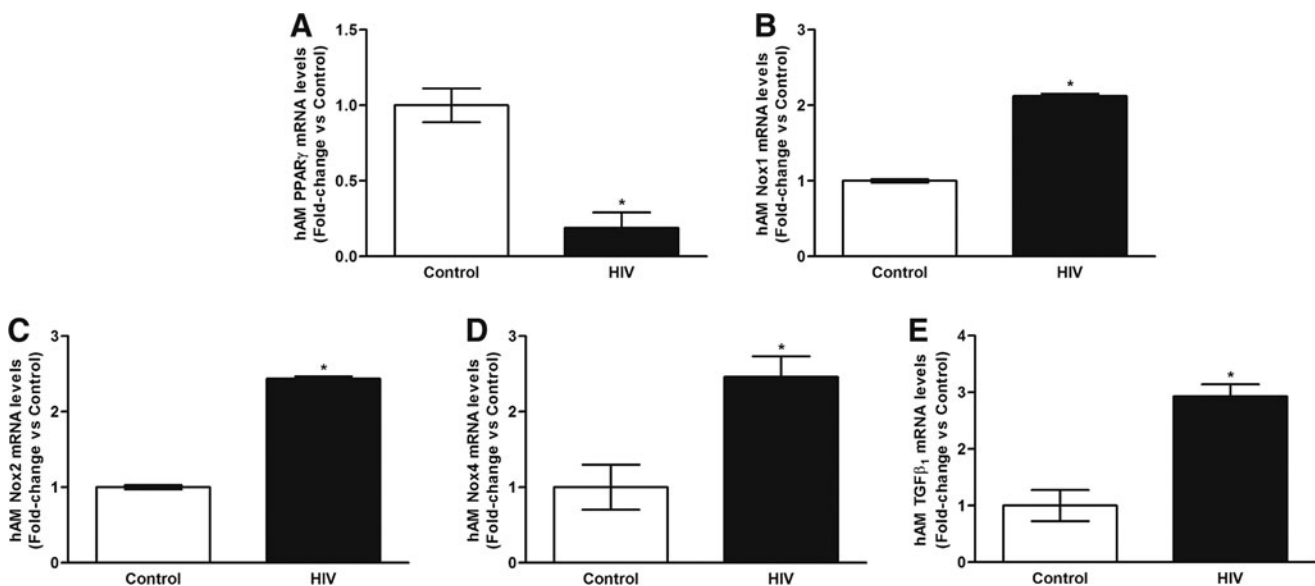


FIG. 2. Chronic HIV infection is associated with alterations in mRNA levels of PPAR γ , Nox1, Nox2, Nox4, and TGF β ₁ in AMs. AMs were collected from HIV-uninfected control subjects (Control, $n=6$) and from HIV-infected subjects (HIV, $n=22$). mRNA levels of PPAR γ (A), Nox1 (B), Nox2 (C), Nox4 (D), and TGF β ₁ (E) were measured by quantitative real-time polymerase chain reaction ($n=28$, in duplicate), normalized to 9s mRNA, and expressed as mean \pm SEM, relative to control. * $p < .05$ versus control. AMs, alveolar macrophages; PPAR γ , peroxisome proliferator-activated receptor gamma; TGF β ₁, transforming growth factor beta-1.

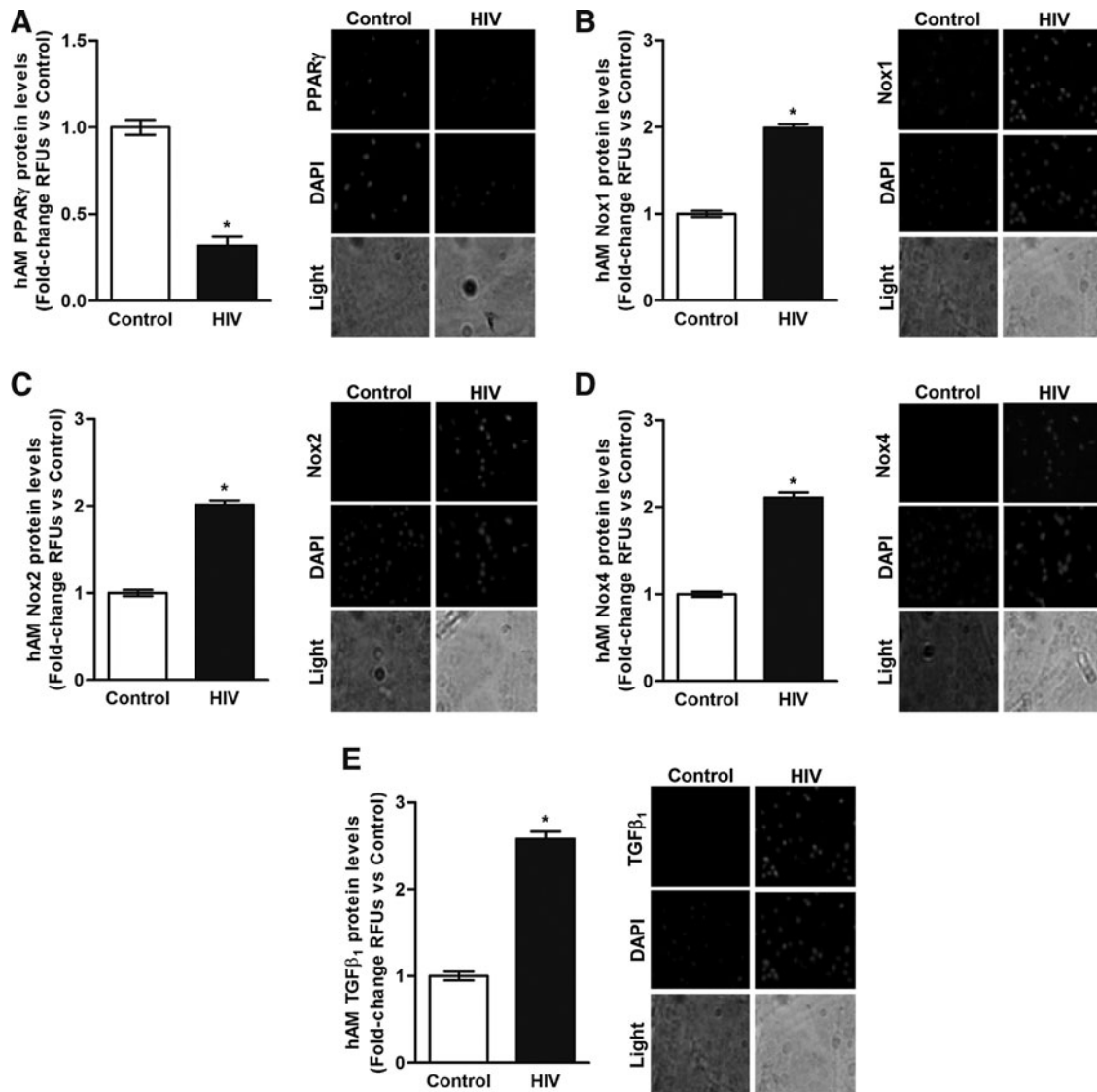


FIG. 3. Chronic HIV infection is associated with alterations in protein levels of PPAR γ , Nox1, Nox2, Nox4, and TGF β_1 in AMs. AMs were collected from HIV-uninfected control subjects (Control, $n=6$) and from HIV-infected subjects (HIV, $n=22$). Protein levels of PPAR γ (A), Nox1 (B), Nox2 (C), Nox4 (D), and TGF β_1 (E) were determined in AMs ($n=28$, 10 fields per experimental condition) by using confocal fluorescence microscopy. Representative images are provided. Quantification of fluorescence values was normalized to DAPI nuclear stain and expressed as mean relative fluorescence units (RFU) per cell \pm SEM, relative to control. * $p < .05$ versus control.

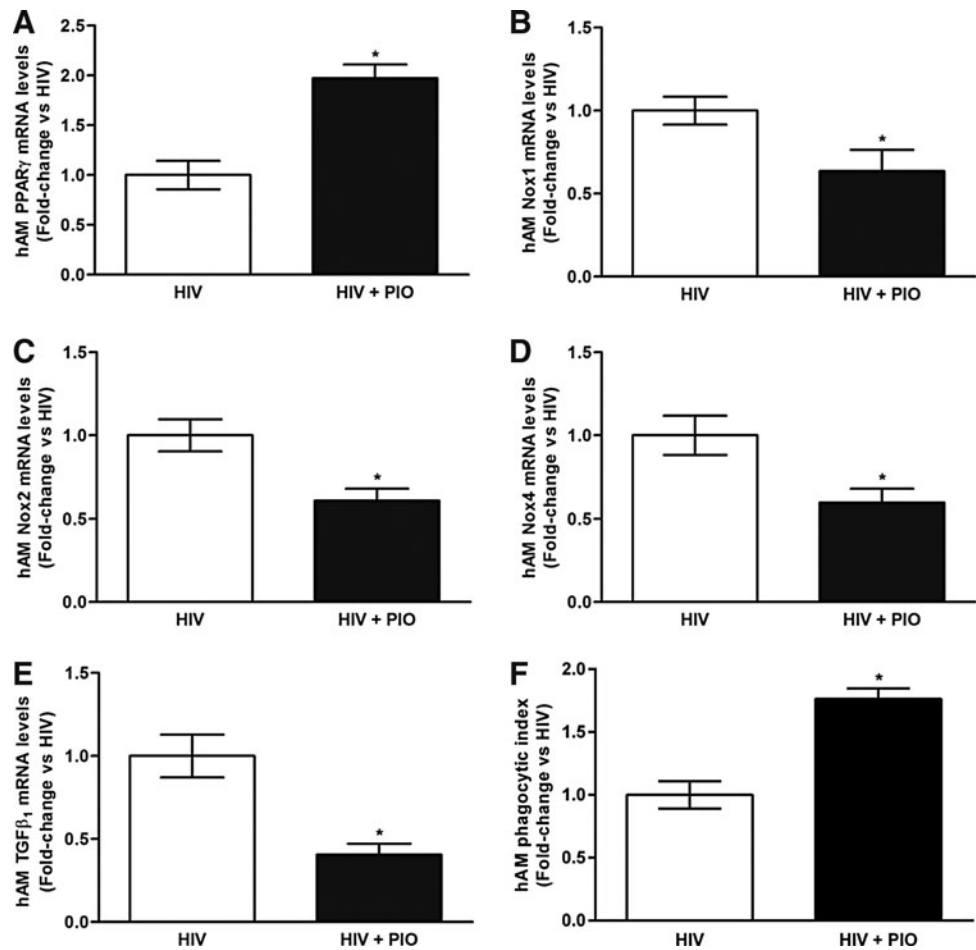
dimethyl sulfoxide vehicle and PIO treatment groups, indicating that 0.01% dimethyl sulfoxide is not responsible for the expression of the targeted genes. PIO treatment *ex vivo* also improved phagocytosis in AMs from HIV-infected individuals (Fig. 4F). These data suggest that the exaggerated AM oxidative stress response and impaired phagocytic function associated with chronic HIV infection can be improved with PIO treatment.

Discussion

Despite advances in HIV therapeutic strategies such as ART, individuals living with chronic HIV infection remain susceptible to serious bacterial and viral infections,³⁻⁶ leading to greater morbidity and mortality. Pulmonary infections,

particularly bacterial pneumonia, are significantly more likely among individuals living with HIV.⁶ However, the mechanisms by which HIV increases the risk of developing respiratory infections remain poorly understood. For the first time, the results presented here describe that, in a healthy cohort of HIV-infected subjects who are nonsmokers and without lung disease, HIV infection is associated with: (a) increased H₂O₂ levels in BAL; (b) decreased PPAR γ mRNA and protein levels in AMs; and (c) increased mRNA and protein levels of Nox1, Nox2, Nox4, and TGF β_1 in AMs. These results suggest that HIV infection is associated with the promotion of H₂O₂ levels in the alveolar space and the expression of oxidative stress markers in AMs, which may contribute to impaired lung immune functions and increased susceptibility to respiratory infections.

FIG. 4. *Ex vivo* PIO treatment reverses chronic HIV infection-associated alterations in mRNA and protein levels of PPAR γ , Nox1, Nox2, Nox4, and TGF β ₁ and improves phagocytosis in AMs. AMs were collected from HIV-infected subjects and were treated with PIO (HIV + PIO) or dimethyl sulfoxide vehicle (HIV) ($n=4$). mRNA levels of PPAR γ (A), Nox1 (B), Nox2 (C), Nox4 (D), and TGF β ₁ (E) were measured by qRT-PCR ($n=8$, in duplicate), normalized to 9s mRNA, and expressed as mean \pm SEM, relative to HIV. AM phagocytic ability (F) was assessed by phagocytosis assay and fluorescent microscopy imaging (10 fields per experimental condition). Phagocytic index was calculated as the percentage of phagocytic cells multiplied by the relative fluorescence units of *S. aureus* per cell. All values were expressed as mean \pm SEM, relative to HIV. * $p < .05$ versus HIV. PIO, pioglitazone.



In the alveolar space, the AM is the first line of lung immune defense. HIV impairs AM immune functions^{7–11} through dysregulated activation,^{13–16} impaired oxidative burst,¹⁵ and enhanced anti-inflammatory cytokine secretion.¹³ In an experimental model using HIV transgenic rats, AM phagocytosis was significantly decreased in HIV-infected rats, compared with phagocytosis in AMs from wild-type rats.^{8,9} The HIV-infected subjects enrolled in our study were healthy individuals, as evidenced by their lack of underlying medical problems (exclusion criteria), nonsmoking status, and a majority of them being on ART where their median CD4 count was 445 and median viral load was 0 log copies/ml (Table 2). However, in our previous prospective cross-sectional study of otherwise healthy HIV-infected subjects on ART with systemic viral suppression, AMs from HIV-infected subjects exhibited a significantly lower median phagocytic index compared with control subjects.¹² These observations suggest that individuals living with HIV have diminished immune responses in AMs, which may contribute to their increased susceptibility to respiratory infections.

Impaired AM phagocytic ability may be linked to increased oxidative stress within the AM. HIV has been shown to induce pulmonary oxidative stress in preclinical^{7,17,18} and clinical studies.^{19,20} Our data confirmed these studies and showed that HIV infection was associated with increased H₂O₂ levels in the BAL (Fig. 1). Lung oxidative stress can be generated through several mechanisms. In previous studies

conducted by our laboratory, HIV infection was associated with altered alveolar redox state by decreasing levels of the primary alveolar antioxidant GSH and increasing the oxidized form GSSG.¹⁷ In addition, the loss of PPAR γ in AMs has been shown to promote oxidative stress and phagocytic dysfunction.²⁹ Upregulation of PPAR γ has been shown to be essential in protecting against pulmonary oxidant injury via activation of NF-E2 related factor 2 (Nrf2) and binding to the antioxidant response element (ARE).²² Further, PPAR γ activation by its ligand rosiglitazone decreased alcohol-induced lung oxidative stress through downregulation of eNOS²³ and protected against AM phagocytic dysfunction by attenuating the expression of Nox proteins and TGF β ₁.²⁹ To our knowledge, this study is the first to show that AMs from a healthy cohort of HIV-infected subjects exhibit attenuated PPAR γ mRNA (Fig. 2) and protein (Fig. 3) levels, compared with control subjects. These studies suggest a role for decreased PPAR γ in HIV-associated AM oxidative stress and phagocytic dysfunction.

Previous studies also demonstrated that increased expression of Nox1, Nox2, and Nox4²⁴ and TGF β ₁²⁶ promote AM oxidative stress and phagocytic dysfunction. In the AM, expression of Nox1 or Nox2 is required for expression of Nox4, a constitutively active Nox protein that is a primary source of H₂O₂.²⁴ Increased Nox1, Nox2, and Nox4 expression in AMs is associated with increased oxidative stress and phagocytic dysfunction.^{24,25} TGF β ₁, another mediator of alcohol-

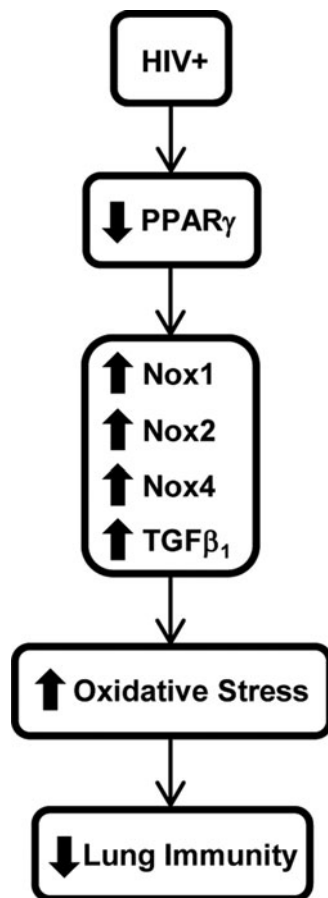


FIG. 5. Hypothetical schema of the molecular mechanisms involved in HIV-associated lung immune dysfunction. Chronic HIV infection is associated with impaired lung immunity through decreases in AMs PPAR γ expression. Attenuation of PPAR γ increases AMs expression of Nox isoforms, and TGF β_1 , which, in turn, enhances lung oxidative stress. This increase in lung oxidative stress causes the lung immune dysfunction observed in HIV-infected individuals.

induced ROS and phagocytic dysfunction in the AM,²⁶ has also been shown to upregulate Nox4 via activation of Smad2/3²⁷ and to suppress lung Nrf2-mediated activation of the ARE.³⁰ For the first time, our data show that, in an otherwise healthy cohort of HIV-infected subjects, AMs exhibit increased expression of Nox1, Nox2, Nox4, and TGF β_1 mRNA (Fig. 2) and protein (Fig. 3). Since *ex vivo* treatments with PPAR γ ligands reversed the expression of Nox1, Nox2, Nox4, and TGF β_1 and improved AM phagocytic ability in other model systems²⁹ as well as in HIV-infected AMs (Fig. 4), we hypothesize that the immune dysfunction and risk of respiratory infections in HIV subjects is mediated by decreased PPAR γ , resulting in increased Nox1, Nox2, Nox4, and TGF β_1 in the AM, and subsequent enhanced pulmonary oxidative stress (schema Fig. 5).

Studies have shown impaired phagocytic capacity in small AMs of HIV-infected individuals³¹ and impaired AM phagosomal proteolysis function in HIV-infected individuals.^{31,32} These studies focused on whether the change in AM phenotype correlates with the HIV infection status of the AM

or the volunteer, whereas our study focuses on elucidating the molecular mechanisms by which chronic HIV infection is associated with increased oxidative stress in the alveolar space and AM, whereby the lungs of HIV-infected individuals are potentially primed for an increased risk of developing respiratory infections. NADPH oxidase expression, specifically Nox2, has been shown to negatively regulate phagosomal proteolysis in macrophages,³³ and our data show that chronic HIV infection is associated with increased NADPH oxidase expression in AM (Fig. 2B–D and Fig. 3B–D). Further studies are warranted to explore whether NADPH oxidases impair phagosomal proteolysis in AM in the context of chronic HIV infection.

Similar to the aforementioned studies, our data also implicate HIV-associated AM dysregulation in the potential susceptibility of individuals living with HIV to developing respiratory infections; however, there are several limitations to our study. Subjects enrolled in the study represent a cross-sectional cohort, and the sample size of subjects enrolled ($n=28$) and *ex vivo* treatment with PIO ($n=4$) were relatively small. Further, HIV-infected subjects in this study are older; although oxidative stress increases with age,³⁴ the effects of aging on the lung and lung immune function have not been well characterized. Multi-regression analysis with age as a confounder showed that HIV was not significantly associated with Nox mRNA, but HIV status was still significantly associated with PPAR γ and TGF β_1 mRNA levels in AMs (Fig. 2). Since both age and HIV may have contributed to the Nox-mediated increase in oxidative stress in the lung, this is an important area for future investigations. Age-matched controls and HIV-infected subjects will be included in future studies.

In spite of these limitations, our results show that even individuals living with HIV, without any evidence of other medical problems, may be at risk for lung infections due to impaired immune responses of their AMs. In a longitudinal prospective study, the relative expression of these mediators could potentially be correlated with risk and severity of respiratory infections, morbidity, and mortality. Also, in a larger study, subjects could be further stratified into different groups according to age, smoking status, chronic obstructive pulmonary disease status, etc. This will allow for further clarification on how these AM mediators could be used in the future. Our data demonstrate the critical importance in evaluating BAL H₂O₂ levels and AM expression of PPAR γ , Nox1, Nox2, Nox4, and TGF β_1 as potential biomarkers that might be predictive of poor outcomes and increased susceptibility to respiratory infections in the future of individuals living with chronic HIV infection.

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Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

Samantha M. Yeligar

Division of Pulmonary, Allergy, Critical Care

and Sleep Medicine

Department of Medicine

Emory University

1670 Clairmont Road, 12C-104

Decatur, GA 30033

E-mail: syeliga@emory.edu