

CXCL11 levels regulate lung Treg responses to deter the onset of HIV-related COPD

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Abstract

Background: Inflammation drives COPD development in people living with HIV (PLwHIV), and the HIV virus impairs T regulatory (Treg) cell responses that deter immune-mediated lung injury. This study sought to determine how cigarette smoke exposure alters lung Treg responses to increase COPD susceptibility in PLwHIV.

Methods: Lung lavage levels of the Treg chemoattractant CXCL11 were quantified in a cohort of 26 HIV-infected subjects and 34 age-matched controls. To ascertain how CXCL11 modifies lung Treg responses, analyses were then conducted using a chimeric HIV (EcoHIV) infection smoke exposure mouse model and elastase treatment of Treg depleted (DEREG) mice.

Results: CXCL11 lung lavage levels increased in HIV+ subjects compared to controls. However, CXCL11 levels were significantly lower in those HIV+ subjects with a reduced diffusing capacity for carbon monoxide compared to HIV+ subjects with normal lung function. Cigarette smoke exposure reduced CXCL11 levels in HIV+ current smokers and decreased lung Cxcl11 levels and Treg frequency in control and EcoHIV-infected mice. Cigarette smoke increased lung c-Src activity in mice and the c-Src inhibitor AZD0530 restored Cxcl11 expression in smoke exposed mice and alveolar macrophages. Direct administration of CXCL11 protein to the airways of EcoHIV infected or smoke exposed mice significantly enhanced lung Treg responses. Treg deficient DEREG mice exhibited increased airway resistance at baseline and had greater lung tissue destruction post elastase treatment.

Conclusions: These findings indicate that cigarette smoke activates c-Src to suppress CXCL11 levels thereby diminishing lung Treg responses that counter airways disease and lung tissue destruction in HIV-infected individuals.

Background

Antiretroviral therapy (ART) has extended the lifespan of people living with the human immunodeficiency virus (HIV) and, as a result, the proportion of deaths due to chronic obstructive pulmonary disease (COPD) in this population has increased[1]. There are estimated to be over 38 million infected with the HIV virus worldwide[2], and respiratory symptoms frequently affect these individuals[3]. Nicotine addiction is common amongst those infected with HIV with surveys showing a smoking prevalence of 24 to 51% in this group[4, 5], which is much higher than the general population. HIV causes immune changes that increase the susceptibility to COPD[6]. Several processes including apoptosis, indolent infection, inflammation and even ART therapy itself, are implicated in the increased susceptibility to COPD in HIV[7]. However, the mechanisms remain poorly defined, limiting strategies to treat and prevent this respiratory disease in those infected with HIV.

T regulatory cells (Tregs) are a subset of CD4 + T-cells with immunosuppressive functions that express high levels of CD25 and forkhead box P3 (Foxp3). Acute HIV infection rapidly increases circulating Tregs[8] while chronic infection depletes Treg responses[9] and diminishes Treg frequency within

specific tissue compartments[10]. Prior studies showed a higher proportion of effector CD4 + or CD8 + T cells in the bronchoalveolar lavage fluid (BALF) in HIV-infected compared to HIV-uninfected adults[11]. This indicates that lung Treg levels are reduced and insufficient to compensate for the inflammatory effects of augmented effector T cell responses. Studies show that Tregs combat acute HIV infection[12] and limit the damaging effects of opportunistic respiratory infections[13]. Deficient lung Treg responses were associated with a rapid decline of lung function in COPD subjects[14]. Thus, impaired lung Treg responses in HIV could play an important role in the pathogenesis of respiratory diseases in HIV-infected individuals.

Chemokine (C-X-C motif) ligand 11 (CXCL11) is highly expressed in both alveolar macrophages[15] and the bronchiolar epithelium of the lung[16]. Upon binding, CXCL11 drives the growth of suppressive Tregs[17] and causes internalization of the CXCR3 receptor. This prevents receptor stimulation by CXCL9 and CXCL10[18], which are chemokines that intensify inflammation by attracting and activating effector T cells[19] while suppressing the inhibitory effects of Tregs[20]. CXCL11 expression by the bronchiolar epithelium suppresses effector T cells by inducing transepithelial migration and T lymphocyte egression from the lung[16] and by controlling the parenchymal distribution of effector and regulatory T cells[17]. Thus, this study hypothesized that altered CXCL11 could play a role in the pathogenesis of HIV-related COPD.

To examine the role of CXCL11 and Tregs in HIV, this study measured CXCL11 lung lavage levels in non-smoking and smoking healthy control and HIV infected subjects and tested the ability of this lavage fluid to degrade CXCL11 *in vitro*. In addition, it utilized EcoHIV, a genetically modified but nearly identical form of HIV that is capable of infecting mice[21]. Smoke exposure of control and EcoHIV infected mice was employed to determine whether cigarette smoke promotes COPD by down regulating CXCL11 and altering Treg biology in the lung. Lastly, the impact of Tregs in COPD was assessed by treating Treg depleted DERE mice with intratracheal elastase.

Methods

Virus construction and preparation

EcoHIV was constructed as previously described[21]. EcoHIV is identical to HIV but glycoprotein 80 (gp80) from mouse leukemia virus replaces gp120 giving the virus tropism for mouse cells. This model reproduces the features of HIV related neurologic and pulmonary disease in EcoHIV infected mice[22, 23]. Virus stocks were prepared by transfecting plasmid DNA into 293T cells and titered for p24 content by using the HIV Antigen kit (Coulter). Chimeric viruses were washed and resuspended in saline for injection.

Animal exposure model

EcoHIV infection and cigarette smoke exposure of mice were conducted on male and female mice as previously described[23]. For the AZD0530 studies, male A/J mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained in a specific pathogen-free facility at Mount Sinai Medical Center as previously described[23, 24]. Mice (8-week-old) were used at the initiation point for all experiments, and each experimental parameter had 10 animals/group. For the EcoHIV studies, our initial analyses and Treg characterizations were conducted in the A/J background. However, the CXCL11 protein rescue studies were conducted in C57Bl/6 mice, which lack endogenous Cxcl11 production. To infect the mice with EcoHIV, they were anesthetized by intraperitoneal injection of a mixture of ketamine and xylazine (100:10 mg/kg ip). Animals were tail vein inoculated with 10^5 pg of EcoHIV in PBS. Controls were injected with PBS alone. Later (1-month), mice were exposed to cigarette smoke in a chamber (Teague Enterprises, Davis, CA) for 4 hours/day 5 days/week at a total particulate matter concentration of 80 mg/m^3 as per standard laboratory protocol[24], which equates to a mixture of passive and side-stream smoke from two cigarettes delivered to the smoking chamber every 9 min. Approximately 53 cigarettes were burned over this time interval per day. We strictly adhered to guidelines for the care and use of laboratory animals from the National Institutes of Health and Mt. Sinai's Institutional Animal Care and Use Committee (IACUC), which approved the protocol.

Mouse physiology, lung morphometry and histology

Negative pressure forced expiratory measurements, airway hyper responsiveness and lung compliance were determined in accordance with established protocols using a Flexivent apparatus system (Scireq, Montreal, Canada)[25, 26]. Lung morphometry was determined as per established protocols[27].

Preparation of lung single cell suspensions for flow cytometry

We infused the lungs with 1 mL Dispase (BD Bioscience, Franklin Lakes, New Jersey, USA) and elastase 3 U/mL (Worthington Biochemical Corp) prior to adding 1% (w/v) low melting agarose (Invitrogen, Waltham, MA, USA) infusion similar to previous descriptions[28]. The lungs were minced and incubated at 37°C in an enzyme cocktail containing 2.4 mg/mL collagenase I (Invitrogen) and 20 mg/mL DNase I (Sigma, St. Louis, MO, USA) for 30 minutes. We prepared and filtered single cell suspensions as previously described[29, 30]. Total cell number counted with a hemocytometer after staining with trypan blue.

Flow cytometry

To quantify lung Tregs, lung single cell suspensions were resuspended in buffer (PBS with 3% [w/v] bovine serum albumin) and stained ($1-2 \times 10^6$ cells) in the presence of (CD4, CD8, Foxp3 antibodies, gd T cell antibodies, LS Bio, Shirley, Massachusetts, USA). Flow cytometry was performed with the Guava easyCyte flow cytometer (Millipore, Burlington, Massachusetts, USA) and the data was analyzed with the GuavaSoft software (version 3.3).

EcoHIV detection

Lungs of EcoHIV infected mice were excised and homogenized using a mechanical homogenizer (Kinematica, Bohemia, NY, USA). RNA was isolated with RNeasy kits (Qiagen). EcoHIV RNA present in lung tissue was determined by real-time PCR (RT-PCR) as previously described[21]. Murine β -actin was amplified in parallel to standardize RNA input.

CXCL11 protein analysis

Mouse bronchoalveolar lavage fluid (BALF), human BAL and cell media CXCL11 protein levels were determined using a commercially available assay (RayBiotech, Norcross, GA, USA) as per the manufacturers' instructions and using standard immunoblotting techniques.

Elastase mouse model and mouse cell lines

To induce Treg depletion, (DEREG) BAC transgenic mice (Jackson Labs, Bar Harbor, ME, USA) received a 1- μ g Diphtheria toxin (DT) injection intraperitoneally on three consecutive days. These mice are in the C57BL/6NCrl background. Then, 1.2-U of porcine pancreatic elastase (Sigma) dissolved in 100- μ l of sterile saline was injected into the airway using a microsyringe (Penn Century, Philadelphia, PA) and mice were euthanized three weeks later.

Murine alveolar macrophages in the BALB/c background from cell line MH-S (CRL-2019; American Type Culture Collection (ATCC), Manassas, VA, USA) were maintained as per the manufacturers' instruction and were treated with EcoHIV infection and/or 5% cigarette smoke extract (CSE), which was prepared as previously outlined[24].

Human samples and cell lines

This study was approved by the University of Miami's Institutional Review Board. Human BALF was collected from age-matched HIV-infected and uninfected individuals as per protocol[31]. Informed consent was obtained from all subjects and/or their legal guardian(s). All methods were performed in accordance with the relevant guidelines and regulations. Primary human small airway epithelial cells (Lonza, Walkersville, Maryland, USA) were cultured and treated with 100-ng/ml actinomycin D (Sigma Aldrich, St. Louis, Missouri, USA) or control siRNA, *HuR* siRNA or *c-Src* siRNA.

Preparation of cigarette smoke extract

To prepare cigarette smoke extract, the smoke of one cigarette (3R4F, University of Kentucky) is passed through 25-ml of phosphate buffered saline (PBS). This fluid is then pH balanced (pH 7.4) and then passed through a 0.23- μ filter to make it sterile.

Gene expression analysis

Real-time polymerase chain reaction (RT-PCR) for *CXCL11* was conducted on lung tissue and cell pellets as per established protocols[32]. *CXCL11* values were normalized for b-actin.

Statistics. All analysis was performed using GraphPad Prism Software (Version 6 for Mac OS X). Normality testing (D'Agostino & Pearson omnibus normality test) was performed on all data sets. A comparison of groups was performed by Student's t-test (two-tailed) when data passed the normality test and by Mann Whitney test if data did not pass normality testing. A P-value < 0.05 was regarded as statistically significant. The study is reported in accordance with ARRIVE guidelines

Data availability

The data that support the findings of this study are available from the corresponding author, [RF], upon reasonable request.

Results

CXCL11 lavage levels correlate with disease severity in HIV and HIV lavage fluid degrades CXCL11 protein. To determine how HIV status and cigarette smoke exposure influenced CXCL11 lung expression, CXCL11 levels were measured in the bronchoalveolar lavage fluid (BALF) of HIV + and HIV- subjects. Table 1 presents the characteristics of the HIV + and HIV- cohorts. Both cohorts were well matched by age but a higher proportion of the HIV + subjects were African American while the majority of the HIV- subjects were Hispanic. The proportion of subjects with COPD did not substantially differ between races. CXCL11 protein levels were significantly higher in the HIV + study cohort (Fig. 1A). CXCL11 levels were at the lower limit of detection in HIV- subjects and ongoing cigarette smoke did not further alter these low levels (Fig. 1B). In contrast, ongoing smoke exposure significantly reduced CXCL11 levels in HIV + subjects (Fig. 1C). Of note, incubating recombinant CXCL11 protein with COPD BALF or HIV BALF led to the degradation of this chemokine (Fig. 1D, **left panel**). In comparison, incubating CXCL11 with normal BALF or BALF from non-diseased smokers did not degrade the protein (Fig. 1D, **left panel**). Incubating HIV + BALF with the matrix metalloproteinase (MMP) inhibitor EDTA prevented CXCL11 degradation (Fig. 1D, **right panel**).

Table 1
Demographic and biological parameters of study subjects

Factor	HIV-	HIV+	p value
% African American	3.2	76.67	< 0.005
% Caucasian	6.5	16.67	NS
% Females	47.8	6.7	NS
% Hispanic	91.3	6.67	< 0.005
Age	53.4 ± 1.7	53.5 ± 1.3	NS
% Undetectable viral load	N/A	52	N/A
CD4 (#/mm ³)	N/A	477 ± 45	N/A
CD4 (%)	N/A	26 ± 2	N/A
CD8 (#/mm ³)	N/A	830 ± 79	N/A
CD8 (%)	N/A	44 ± 3	N/A
CD4/CD8 Ratio	N/A	0.70 ± 0.08	N/A
Smokers %	82.6	71.4	NS
Pack years in smokers	23 ± 3	13 ± 2.7	< 0.03
FEV1	2.94 ± 0.11	2.81 ± 0.11	NS
FEV1 (% predicted)	97 ± 3	76 ± 3	< 3.0x10⁻⁶
FVC	3.75 ± 0.15	3.69 ± 0.15	NS
FVC (% predicted)	96 ± 3	79 ± 3	< 0.02
FEV1/FVC	0.86 ± 0.02	0.77 ± 0.01	NS
TLC (L)	5.5 ± 0.2	5.3 ± 0.2	NS
DLCO (% predicted)	99.5 ± 4	65 ± 4	< 2x10⁻⁷

A reduced diffusing capacity of the lungs for carbon monoxide (DLCO) is the most common pulmonary function abnormality in HIV + subjects[33] and those HIV + subjects with a DLCO less than 75% of predicted had significantly lower CXCL11 levels (Fig. 2A). There also was a trend positive correlation ($p = 0.07$ by Spearman Rank Correlation) between CXCL11 levels and DLCO in the HIV + subjects (Fig. 2B). Absolute CD4 cells counts were not altered in the cohort with a reduced DLCO (Fig. 2C).

EcoHIV infection diminishes lung Treg responses. To better understand how cigarette smoke and HIV infection influence CXCL11 expression, an EcoHIV smoke exposure mouse model was utilized. EcoHIV infection in A/J mice reproduced CD4 + T cell responses that occurred during acute HIV infection in

humans. Four weeks post-infection, the EcoHIV infected mice showed a reduction in their CD4/CD8 ratio; but this ratio recovered once the virus established latency at 32 weeks (Fig. 3A). EcoHIV infection by itself diminished lung Treg cells and cigarette smoke reduced lung Tregs both in infected and uninfected mice (Fig. 3B). The loss of Tregs post smoke exposure was associated with the development of emphysema-like physiology with decreased tissue damping (Fig. 3C) and tissue elastance (Fig. 3D) in both control and EcoHIV infected mice.

EcoHIV and cigarette smoke down regulate lung CXCL11 expression. RT-PCR analyses for *Cxcl11* were conducted on the lung tissue and alveolar macrophages isolated from control and EcoHIV infected A/J mice. *Cxcl11* expression trended higher within the lung parenchyma (Fig. 4A) and alveolar macrophages (Fig. 4B) of the EcoHIV-infected mice but these changes did not reach statistical significance. However, cigarette smoke exposure significantly reduced Cxcl11 protein levels within the BALF of control (PBS) and EcoHIV-infected mice (Fig. 4C).

HIV acts via c-Src to regulate CXCL11 in the lung. The Src family of kinases regulate Treg proliferation and function [34] so we assessed how the smoke-mediated activation of c-Src influenced Cxcl11 responses. The bronchiolar epithelium is a key site of CXCL11 production in the lung[35]. Half-life analyses determined that *CXCL11* mRNA is highly unstable and degrades within 2 hours in human airway epithelial cells (Fig. 5A). Src can alter the effects of RNA binding proteins like HuR that regulate mRNA stability[36]. Importantly, silencing *HuR* or *c-Src* up regulated *CXCL11* within airway epithelial cells (Fig. 5B). This suggests that c-Src may cooperate with HuR to regulate *CXCL11* mRNA stability in the lung. Next, we measured the *in vivo* effects of the Src inhibitor AZD0530 on Cxcl11 lung expression in A/J mice. Src inhibition prevented the reduction in BAL Cxcl11 levels in response to one week of cigarette smoke exposure (Fig. 5C). To determine if Src regulated Cxcl11 in macrophages, we treated the mouse alveolar macrophage cell line (CRL2019) with AZD0530. The CRL2019 cell line is derived from BALB/c mice. Administration of this Src inhibitor significantly enhanced *Cxcl11* mRNA expression within these macrophages following CSE treatment (Fig. 5D).

Both HIV infection and cigarette smoke exposure suppress lung Tregs. To better determine if enhancing CXCL11 levels could overcome this suppression, EcoHIV infected and cigarette smoke exposed mice in the C57Bl/6 background were intranasally treated with CXCL11 protein one day prior to euthanasia. C57Bl/6 mice have a 2bp insertion close to the start codon of *Cxcl11* that creates a frameshift mutation and a premature stop codon[37]. As a result, C57Bl/6 mice can express *Cxcl11* mRNA but do not produce any functional Cxcl11 protein. Thus, all the effects observed would be due to the provision of exogenous human CXCL11 protein. Administration of CXCL11 significantly up regulated lung Tregs in EcoHIV-infected (Fig. 6A) and cigarette smoke-exposed mice (Fig. 6B). In contrast, CXCL11 protein did not restore gd T cells after cigarette smoke exposure showing that CXCL11 did not have a generalized effect on all T cell levels (Fig. 6C).

Tregs counter elastase-induced emphysema in mice. Tregs were depleted in a mouse model to assess if this altered the effect of elastase on lung physiology and structural integrity. The Treg depleted mice that

were not treated with elastase exhibited increased respiratory resistance at baseline (Fig. 7A) and normal lung morphometry (Fig. 7B and 7C). In addition, the loss of Tregs in the DEREg C57Bl/6 mice exacerbated the development of emphysematous changes post elastase treatment (Fig. 7B and 7C).

Discussion

CXCL11 BAL levels were significantly higher in HIV + subjects compared to age-matched HIV- subjects. Since HIV preferentially infects and reduces Tregs, we suspect that CXCL11 induction is a compensatory reaction to offset viral mediated Treg depletion. Chronic cigarette smoke, however, decreased CXCL11 levels in HIV + subjects and increased CXCL11 proteolytic degradation in both HIV- and HIV + subjects. This diminishes Treg responses thereby enhancing immune-mediated lung injury. Indeed, those HIV + subjects with a reduced DLCO had significantly lower CXCL11 levels compared to HIV + subjects with preserved lung function. Similar to our human findings, EcoHIV increased and cigarette smoke exposure diminished lung Cxcl11 levels in A/J mice. Decreased Cxcl11 levels correlated with reduced lung Treg frequency and human CXCL11 protein administration directly up regulated lung Treg responses in C57Bl/6 EcoHIV infected and cigarette smoke exposed mice, which lack endogenous Cxcl11 protein. The effects of CXCL11 on lung Tregs is important as the loss of Tregs in DEREg mice increased respiratory resistance and augmented elastase mediated emphysema. Further studies showed that the smoke-induced activation of c-Src suppresses Cxcl11 production in mouse lavage fluid of A/J mice *in vivo* and murine macrophages of BALB/c mice *in vitro* by increasing its mRNA decay intracellularly and its proteolytic cleavage extracellularly. Together, these findings indicate that the regulation of Treg responses by c-Src and CXCL11 affects the development of HIV-related COPD.

One mechanism by which c-Src may have influenced CXCL11 levels was through the post-transcriptional regulation of *CXCL11* mRNA stability. The RNA-decay 'machinery' regulates the half-life of pro-inflammatory proteins to alter immune responses. AU rich elements (AREs) within a gene make the mRNA susceptible to decay[38]. Some unstable mRNAs, such as *CXCL11*, have AU rich 3' regions and undergo spontaneous 3'-5' decay. The AU-rich areas of these mRNAs bind RNA binding proteins (RBPs) like HuR that regulate mRNA stability. c-Src modulates mRNA decay by controlling the binding of these proteins to the ARE of the 3' untranslated region (UTR) region of these target mRNAs[39]. In this way, c-Src controls gene expression and subsequent biological responses. Our group previously published that cigarette smoke activates c-Src in primary human airway epithelial cells and mouse lung[24]. These studies showed that cigarette smoke acts via c-Src to promote lung inflammation and tissue destruction[24] and here we show that c-Src mediates lung inflammatory responses by decreasing *CXCL11* mRNA stability and translation.

Incubating recombinant CXCL11 protein with bronchoalveolar lavage fluid from COPD subjects or HIV-infected subjects caused proteolytic breakdown of CXCL11. Tregs normalize lung protease responses post injury[40] and the loss of Tregs in both control and EcoHIV smoke exposed mice likely enhanced proteolytic responses and Cxcl11 degradation. We previously showed that EcoHIV infection enhanced proteolytic activity post smoke exposure with smoke-exposed EcoHIV infected A/J mice exhibiting

increased airway neutrophil elastase, cathepsin G and MMP-9 activity and protein levels in their BALF[23]. Besides affecting *CXCL11* mRNA decay, it is conceivable that c-Src inhibition boosts CXCL11 responses by deterring the smoke-induced expression of lung proteases. We previously demonstrated that targeting c-Src prevented the smoke-mediated induction of MMP-9 in mouse lung *in vivo* and human airway epithelial cells *in vitro*[24]. Thus, inhibiting c-Src could be a therapeutic approach to prevent CXCL11 degradation and preserve lung Treg responses in smokers with and without HIV infection.

Both EcoHIV infection and cigarette smoke exposure reduced lung Treg frequency in C57Bl/6 mice. As discussed, mice in this background do not produce endogenous Cxcl11 protein. However, they retained the ability to boost Treg levels in response to treatment with human CXCL11 protein. The reduction in Treg levels in our murine HIV-related COPD model is consistent with a previous study, which found that prolonged smoke exposure in C57Bl/6 mice similarly diminished lung Tregs[41]. We conducted Treg analyses in smoke exposed EcoHIV infected mice in both the A/J background and C57Bl/6 background and prolonged smoke exposure similarly reduced lung Tregs in both strains. As we showed, CXCL11 can rescue lung Treg responses in EcoHIV-infected or smoke exposed mice, so the loss of CXCL11 in HIV + smokers could impair Treg responses in this clinical cohort. Tregs play a protective role in COPD by countering inflammation and recruiting other anti-inflammatory cells to the lung[42]. Indeed, we found that depleting Tregs in DERE mice increased respiratory resistance at baseline and exacerbated elastase induced emphysema. However, the effects of Tregs in the lung are complex and it remains to be determined whether enhancing Treg responses would be a viable therapeutic approach in smokers with or without HIV.

Studies on the presence of Tregs in COPD have yielded conflicting results. Barcelo et al. found that BALF Treg levels in COPD subjects were comparable to non-smokers while healthy smokers exhibited increased levels[43]. In a more recent study, Strom et al. similarly found that stable COPD subjects had normal BALF Treg levels. However, those COPD subjects with a rapid decline in lung function, defined as a forced expiratory volume in 1 second (FEV1) decline ≥ 60 ml/year, had significantly lower BALF Treg levels[14]. Likewise, another BALF study showed that even though CD4 + CD25 + cell numbers increased in COPD, the percentage of these cells expressing Foxp3 (Forkhead Box P3) decreased suggesting that this CD4 + CD25 + cell population lacked immunosuppressive capacity[44]. Together, these BALF studies suggest that Tregs protect against lung damage in healthy smokers and their loss in cell numbers or functionality promotes more unstable disease.

One limitation of our study is that we did not further characterize the Tregs with additional markers to outline their immunosuppressive capacity. For example, we did not assess their production of IL-10 or suppressor of cytokine synthesis-1 or -3 (SOCS-1 or -3), which Tregs express to suppress local cytokine production. Additionally, we did not test the effect of Treg depletion in the cigarette smoke exposure model. We opted for the elastase model since it requires only three weeks for the development of emphysematous changes. Emphysema appears only after several months of cigarette smoke exposure, and this would have allowed time for Treg recovery in the DERE mice. Thus, we cannot definitively state that the Treg changes cause cigarette smoke-induced emphysema. Nevertheless, the EcoHIV mice

exhibit increased susceptibility to cigarette smoke-induced emphysema[23]. Thus, the Treg changes we report correlate with exaggerated emphysema in the smoke exposure model. Lastly, though our HIV- and HIV + cohorts were age-matched, there were significant racial differences between these groups that could potentially confound our results.

Conclusions

In summary, our findings show that HIV and cigarette smoke alter lung Treg responses by modulating *CXCL11* mRNA and protein stability within the lung. Furthermore, we demonstrate that Treg deficiency mediated by HIV infection and cigarette smoke exposure reproduce the pathophysiology of COPD. Our results delineate a novel mechanism by which cigarette smoke exposure acts via c-Src to suppress CXCL11 production and Treg responses. Future studies will need to address whether modulating Treg levels by targeting c-Src or CXCL11 could deter the onset and progression of COPD in smokers with and without HIV.

Abbreviations

People living with HIV (PLwHIV), Treg depleted (DEREG), Antiretroviral therapy (ART), human immunodeficiency virus (HIV), chronic obstructive pulmonary disease (COPD), T regulatory cells (Tregs), forkhead box P3 (Foxp3), bronchoalveolar lavage fluid (BALF), Chemokine (C-X-C motif) ligand 11 (CXCL11), glycoprotein 80 (gp80), intraperitoneal (IP), phosphate buffered saline (PBS), cigarette smoke extract (CSE), Animal Research: Reporting of In Vivo Experiments (ARRIVE), mean linear intercept (MLI), RNA binding proteins (RBPs), suppressor of cytokine synthesis (SOCS), Real-time polymerase chain reaction (RT-PCR), AU rich elements (AREs), untranslated region (UTR)

Declarations

Ethics approval and consent to participate

We strictly adhered to guidelines for the care and use of laboratory animals from the National Institutes of Health and Mt. Sinai's Institutional Animal Care and Use Committee (IACUC), which approved the protocol. The human study was approved by This study was approved by the University of Miami's Institutional Review Board and conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from all participants.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Author Contribution

A.J.D., P.G., M.C., E.H., B.-H.K., and R.F.F. performed experiments. P.G. and R.F.F. conceived and designed the study. A.J.D., P.G., and R.F.F. conducted data analysis and interpretation. R.F.F. drafted the manuscript. A.J.D., P.G., O.E., M.C., E.H., B.-H.K., M.J.P., D.J.V., and R.F.F. reviewed and edited the manuscript.

Data Availability

The data that support the findings of this study are available from the corresponding author, [RF], upon reasonable request.

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Figures

Figure 1

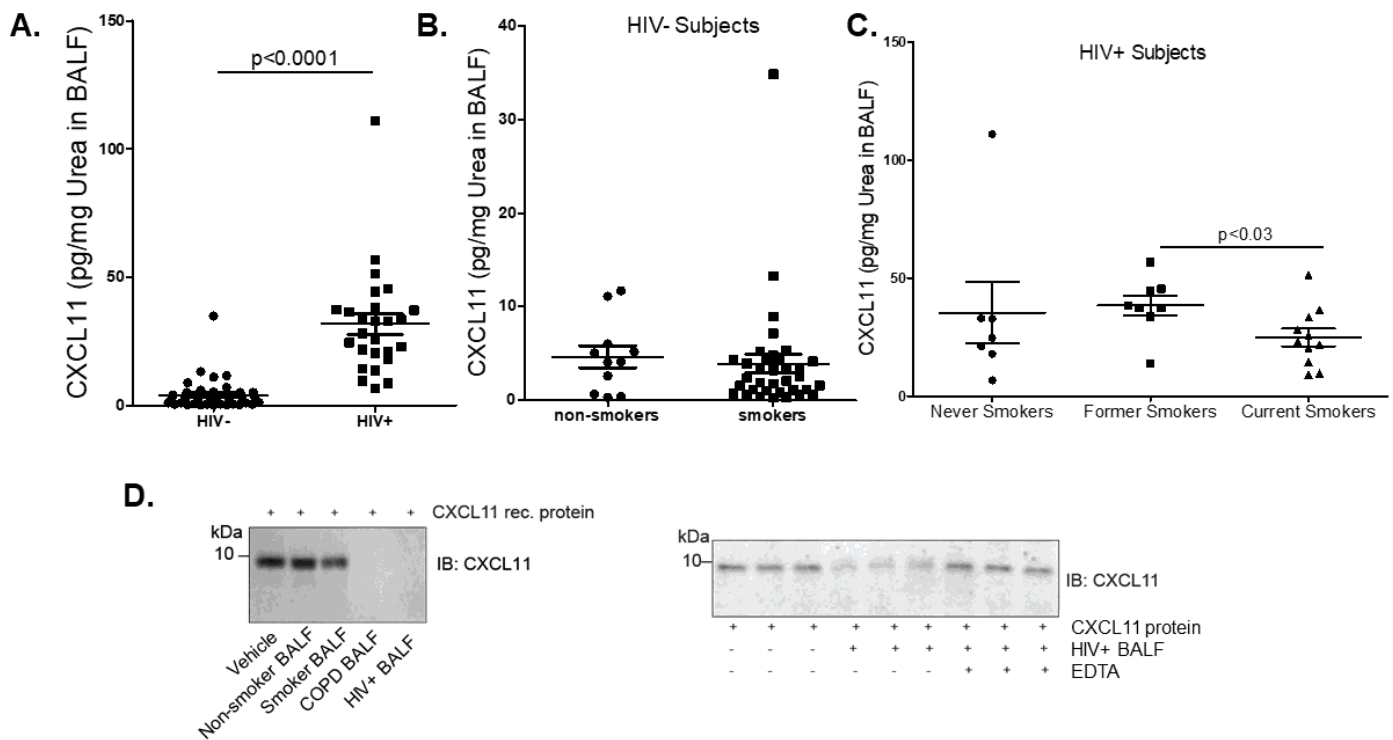


Figure 1

The effect of HIV and cigarette smoke on BALF CXCL11 levels. **A.** CXCL11 BALF levels were determined in age-matched healthy control subjects (N=34) and HIV+ subjects (N=26). **B.** CXCL11 BALF levels were compared between HIV- non-smokers (N=11) and HIV- smokers (N=23). **C.** CXCL11 BALF levels were compared between HIV+ never-smokers (N=7), HIV+ former smokers (N=8) and HIV+ smokers (N=11). P values were determined by Student's t-test when comparing two groups or one-way ANOVA when comparing more than two groups. **D. (Left Panel)** BALF from healthy subjects, normal smokers, COPD subjects, and HIV+ subjects was incubated with recombinant CXCL11 for 24 hours, and CXCL11 immunoblots were performed. **(Right Panel)** Immunoblots for CXCL11 were conducted after recombinant CXCL11 was added to HIV+ BALF pretreated with EDTA to inhibit MMP activity.

Figure 2

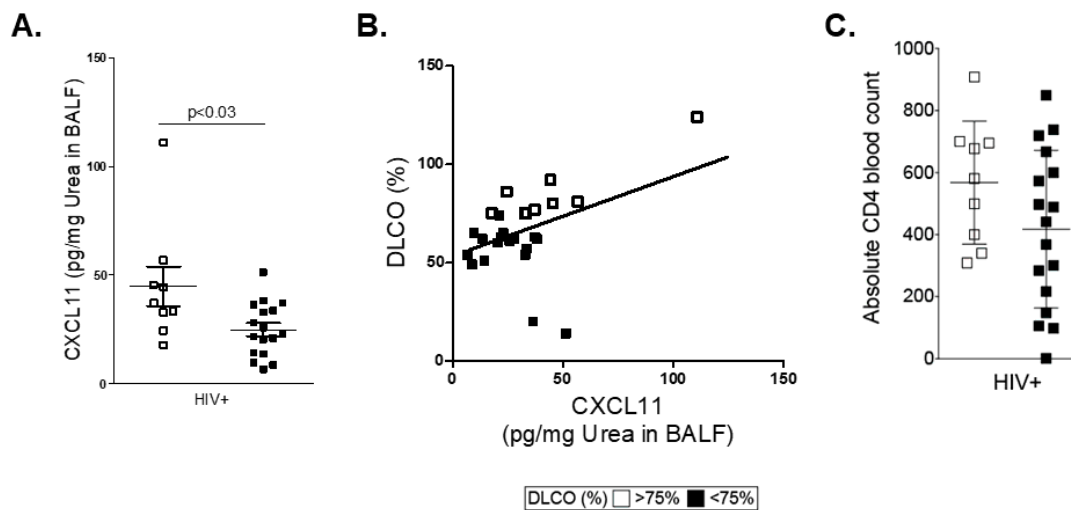


Figure 2

Reduced CXCL11 BALF levels in HIV were associated with reduced DLCO. **A.** CXCL11 BALF levels were determined in HIV+ subjects and were grouped into >75% (N=10) or < 75% DLCO % predicted (N=16). CXCL11 levels were standardized to BALF urea levels. **B.** DLCO (% predicted) had a trend correlation with CXCL11 levels in HIV+ patients (where $R^2 = 0.4$ and $p = 0.08$). **C.** Blood CD4 counts (cells/mm³) were also determined in this cohort. Graphs represent mean $\pm$ S.E.M.

Figure 3

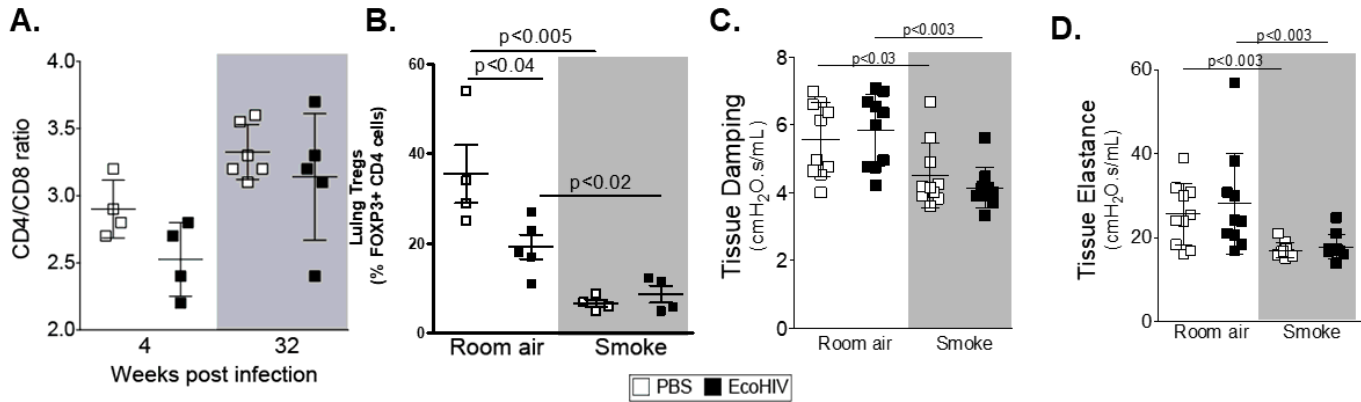


Figure 3

EcoHIV lowers lung CD4+ cells and cigarette smoke reduce lung Tregs. **A.** Blood ratio of CD4/CD8 T cells in mice, 4 or 32-weeks post EcoHIV infection. **B.** Foxp3+CD3+CD4+ lung cells quantified from control room air, EcoHIV-infected, smoke-exposed and EcoHIV-infected and smoke exposed mice (N≥4 per group). Smoke exposure caused significant reductions in **C.** Tissue Damping and **D.** Elastance in both the EcoHIV-infected and control smoke exposed mice.

Figure 4

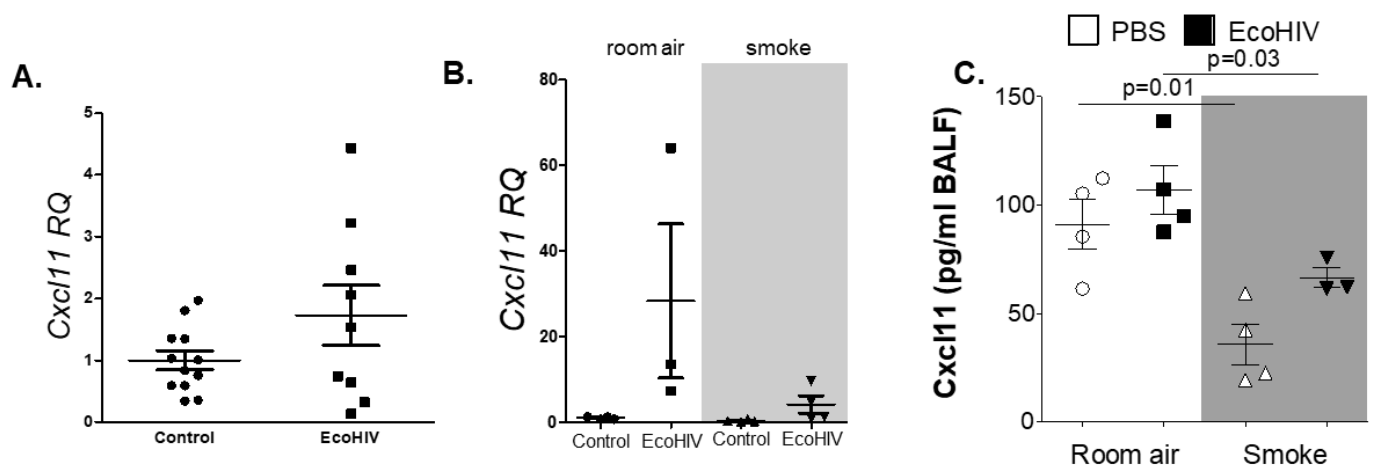


Figure 4

The effect of EcoHIV infection and cigarette smoke exposure on lung *Cxcl11* expression. **A.** *Cxcl11* RT-PCR analysis was conducted on the lungs of EcoHIV infected A/J mice. Data is represented on the y-axis as relative expression compared to PBS treated air-exposed mice. Data is presented as mean $\pm$ SEM. (N $\geq$ 9 per group). **B.** *Cxcl11* RT-PCR was performed on air and smoke exposed control and EcoHIV infected alveolar macrophages from A/J mice. Data is represented on the y-axis as relative expression compared to PBS treated air-exposed mice. Data is presented as mean $\pm$ SEM. (N $\geq$ 3 per group). **C.** A/J mice were inoculated once with PBS or EcoHIV and exposed to 2 months of room air (RA) or cigarette smoke. *Cxcl11* levels in BALF of mice was quantified by ELISA. Data is expressed as mean $\pm$ SEM (N $\geq$ 3/group).

Figure 5

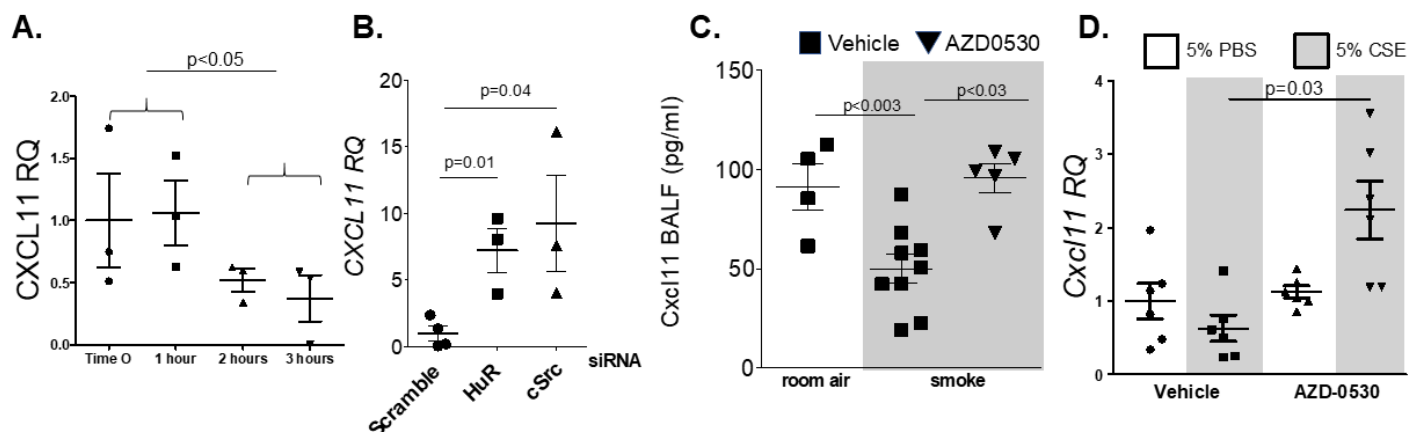


Figure 5

HuR and c-Src regulate CXCL11 expression in the lung. **A.** *CXCL11* mRNA expression was determined via RT-PCR in normal human airway epithelial cells that were treated with 100-ng/ml of actinomycin for a three-hour time period. $p < 0.05$ comparing the 0 and 1-hour timepoints with the 2 and 3-hour timepoints. **B.** RT-PCR for *CXCL11* was conducted as above on human airway epithelial cells treated with control, *c-Src* or *HuR* siRNA for 24 hours. **C.** Cxcl11 protein levels were measured by ELISA from the BALF of A/J mice treated orally with vehicle or 10 mg/kg AZD0530 while they were exposed to room air or cigarette smoke for one week. Data is presented as mean $\pm$ SEM. ($N \geq 4$ per group). **D.** Cxcl11 expression was measured by RT-PCR in mouse alveolar macrophages (ATCC CRL2019) treated with vehicle or 1-mM AZD0530 with or without 5% CSE for 24 hours ($N \geq 6$ per group). Data is expressed as relative quantification (RQ) compared to PBS/vehicle treated cells.

Figure 6

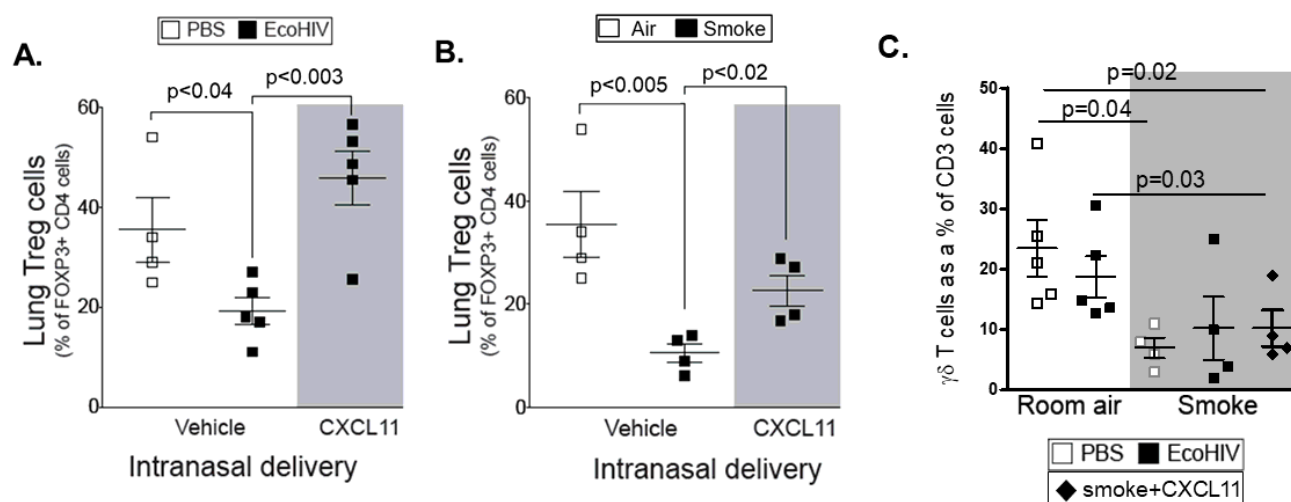


Figure 6

CXCL11 increases lung Tregs in EcoHIV-infected and cigarette smoke exposed mice. **A.** 10-week-old C57Bl/6J mice were infected with EcoHIV and then followed for six weeks. One day prior to euthanasia they were treated via the nares with 4-μg of CXCL11 protein and euthanized the next day. Flow cytometry for Foxp3+CD4+ cells was conducted on lung cells isolated from the mice (N≥4 per group). **B.** 10-week-old mice were smoke exposed for 4 hours/day, 5 days/week for 2 weeks at a total particulate matter of 100-μg/m³. Immediately after the last exposure the mice were treated via the nares with 4-μg of CXCL11 protein or albumin as a control. Data in both cohorts is expressed as FOXP3+ cells as a percentage of CD4+ cells on the y-axis (N=5 per group). **C.** Flow cytometry for γδ T cells was conducted on lung cells isolated from the above treated mice (N≥4 per group).

Figure 7

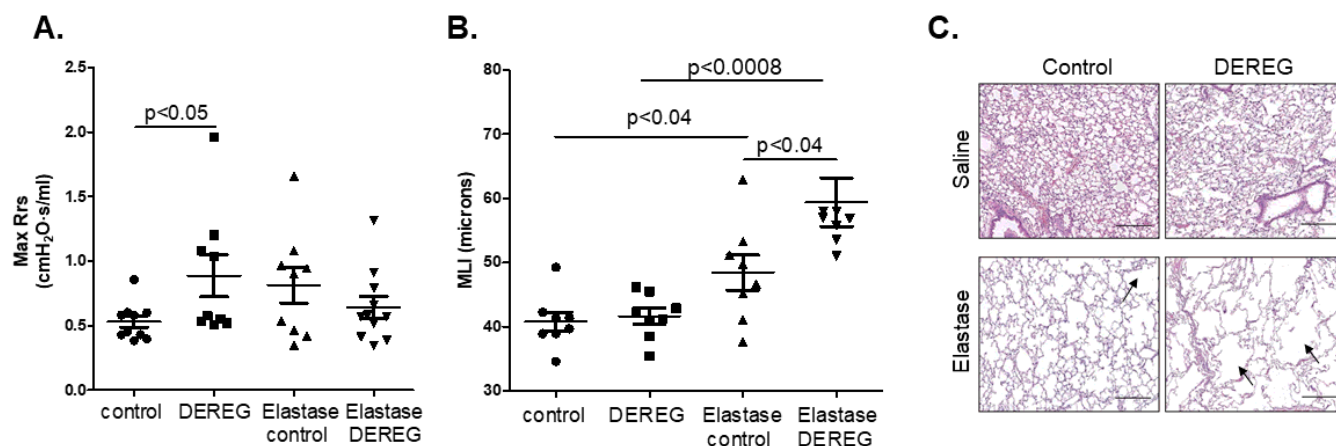


Figure 7

Treg depletion increases respiratory resistance and elastase mediated emphysema formation.

Respiratory resistance was measured in control and elastase treated C57/Bl6J and DEREg mice using a Flexivent apparatus (Scireq, Montreal, Canada). Measurements in the elastase group were taken three weeks following elastase exposure. **A.** Respiratory resistance (cmH₂O·s/ml) is reported on the y-axis. **B.** Lung morphometry and mean linear Intercept (MLI) were determined for all mice at the time of euthanasia. Data is represented as mean linear intercept (MLI) measured in microns on the y-axis. **C.** Comparative histology images of the four mouse groups are presented (bar, 100-μM). Arrows indicates areas of significant airspace enlargement.

Supplementary Files

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