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Review article

Stimulation of Lung Immunity by Vitamin D in People Living With HIV, Stratified By Cigarette Smoking Status

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ABSTRACT

Purpose: Cigarette smoking is three times more prevalent in people living with HIV (PLWH) compared HIV uninfected individuals.. Smoking and HIV infection have a synergistic detrimental effect on lung immunity. Vitamin D has unique beneficial involvement in lung immunity.

Methodology: Alveolar Macrophages (AM) were isolated from 18 subjects stratified by cigarette smoking and HIV status and treated with 100nM and 200nM doses of $25(OH)D_3$ [cholecalciferol] and $1,25(OH)_2D_3$ [calcitriol] and then challenged with *Staphylococcus aureus* (pneumonia model). The primary outcome was the change from baseline in phagocytosis with vitamin D treatment. Secondary analysis measured bronchial alveolar lavage fluid (BALF) free 25-hydroxyvitamin D [25(OH)D] and LL-37 levels.

Findings: PLHIV who smoked cigarettes showed improvement in phagocytic activity across all vitamin D dosing groups, although not statistically significant. This study shows that there is greater variability in plasma compared to BALF, and measuring BALF more accurately reflects lung immunity instead of plasma.

Research limitations/implications: This pilot, feasibility study is hypothesis generating and highlights potential improvement in alveolar phagocytic function with vitamin D in PLWH who smoke.

Practical implications: Investigating the molecular and clinical benefits of vitamin D supplementation on lung immunity is a low cost and high yield endeavor, which can have a significant impact on lung disease that has yet to be elucidated.

Original Value of paper: This a hypothesis generating study that reports level of LL-37 and free 25(OH)D in BALF in PLWH and smokers. Previous vitamin D treatment

trials in lung immunity have been inconclusive due lack of clarity on vitamin D pathway in the lung. This study elucidates the lung immune pathway effect of vitamin D through LL-37 and free 25 (OH)D in BALF compared to plasma, which has not been previously reported in this patient population.

INTRODUCTION

Vitamin D, a steroid hormone, influences many organ systems, classically the skeleton but also the cells of the immune system such as the lungs [1,2]. There is exciting, emerging literature highlighting the beneficial biochemical effects of vitamin D [2,3] and its unique involvement in lung immunity, specifically stimulating anti-microbial peptides, namely the cathelicidin family of antimicrobial peptides (LL-37). Vitamin D stimulates protein expression of LL-37, which aids in immune cell phagocytosis of microorganisms (Figure 1) [4-6].

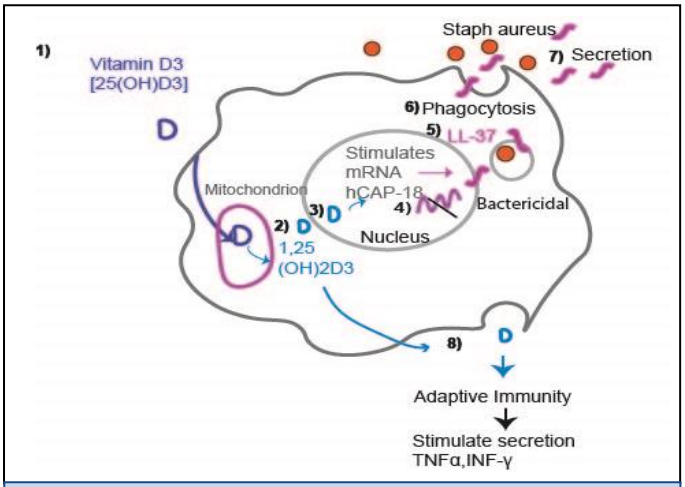

Figure 1: Vitamin D Pathway in Alveolar Macrophages.
Vitamin D [25 (OH)D3] enters alveolar macrophages [1]. It is then converted into active form 1,25-dihydroxyvitamin D [1,25 (OH)D2D3; calcitriol] in mitochondria [2]. Calcitriol, upregulates mRNA expression of human cationic antimicrobial protein (hCAP-18) [3]. hCAP-18 is cleaved to produce LL-37 [4]. LL-37, an antimicrobial peptide, is bactericidal [5]. LL-37 stimulates alveolar macrophage phagocytosis of microorganisms. [6]. LL-37 is also secreted into bronchial alveolar fluid [7]. In addition, calcitriol is secreted and stimulates adaptive immunity secretion of inflammatory cytokines, such as TNF-α, and INF-γ to help fight infection [8].

Concurrently, consequence of low vitamin D and LL-37 results in persistently exaggerated dysregulated immune activation, which is associated with increased mortality in AIDS and non-AIDS defining illnesses [7,8]. Vitamin D deficiency is very common in PLWH and is associated with increased morbidity [7]. PLWH have much higher prevalence of pneumonia cases compared to HIV unaffected individuals. With the SARS Coronavirus-19 (COVID-19) outbreak, vitamin D deficiency has been associated with increased risk of COVID-19 infection and increased mortality across 20 European countries [9]. Smoking is three times more prevalent in PLWH compared to HIV uninfected people [10]. The innate immune response involves several signaling pathways that trigger cytokines and antibodies, which stimulate immune cells to kill pathogens. Smoking tobacco, which contains Lipopolysaccharides (LPS), suppresses the innate immune response [11], impairs Alveolar Macrophages(AM) phagocytosis and efferocytosis which leads to further inflammation (Figure 2) [12,13]. All of these dysregulated innate immune pathways increase susceptibility to pulmonary infections [14]. Therefore, investigating the molecular and clinical benefits of vitamin D on lung immunity is a worthwhile, low cost and high yield endeavor and could yield more fruitful research in vast areas of lung disease that has yet to be elucidated. This background provided the scientific premise for our research group to measure the impact of vitamin D on AM phagocytosis and analyze the combined effect of HIV plus smoking on lung immunity.

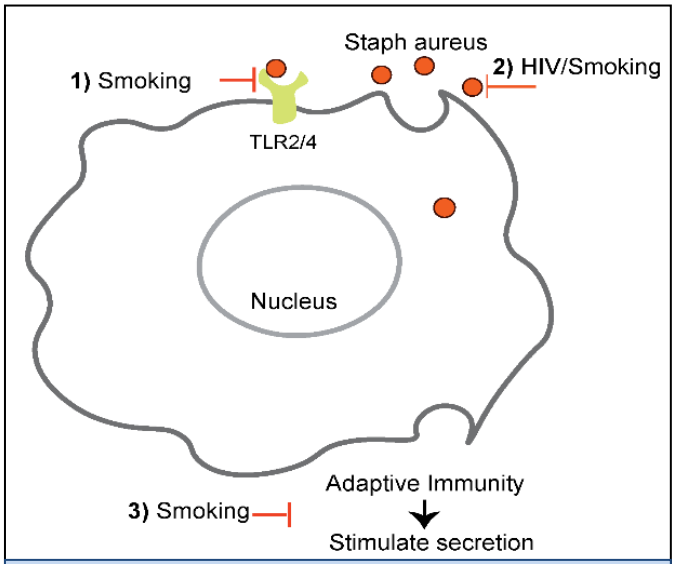

Figure 2: Synergistic Deleterious Effects of Smoking and HIV on Alveolar Macrophages.

1) Smoking decreases toll-like-receptor (TLR)2 and TLR 4.
2) Smoking + HIV decreases alveolar macrophage phagocytosis and antimicrobial activity.
3) Smoking decreases adaptive immunity function.

Materials and Methods

Study population

The study was approved by the Emory University Institutional Review Board and Grady Memorial Hospital Research Oversight Committee and written informed consent was obtained from study participants prior to study enrollment. Eighteen subjects were included in this study.

Inclusion 1) Men and women without HIV-1 or PLWH who have been on Antiretroviral Therapy (ART) for a minimum of 12 months and are followed longitudinally for their healthcare requirements at Grady Memorial Hospital; 2) ability to give consent to bronchoscopy; 3) current smokers were defined as those smoking more than one cigarette a day for at least 30 consecutive days, which can include any form of tobacco product that is inhaled such as vaping and e-cigarettes; non-smokers were defined as those that never smoked as verified by urine cotinine testing.

Exclusion: 1) Age <21 years old; 2) known or possible pregnancy or breastfeeding; 3) documented history of cirrhosis or a direct bilirubin ≥ 2.0 mg/dL; 4) documentation of left ventricular ejection fraction < 40% or myocardial infarction within the past 6 months; 5) end-stage renal disease requiring dialysis or a serum creatinine ≥ 2 mg/d; 6) spirometry with forced vital capacity (FVC) or forced expiratory volume in 1 sec (FEV1)<70% of predicted value; 7) bleeding disorders such as thrombocytopenia or significant gastrointestinal bleeding within the past year; 8) inability to undergo bronchoscopy safely; 9) unknown HIV status; 10) current hypercalcemia (albumin-corrected serum calcium > 10.8 mg/dL or ionized calcium > 5.2 mg/dL); 11) history of therapy with high-dose vitamin D_3 (greater than or equal to 50,000 IU a week) to treat vitamin D deficiency within previous 6 months; 12) history of disorders associated with hypercalcemia (history of cancer with history of hypercalcemia within the past 1 year, hyperparathyroidism, sarcoidosis, nephrolithiasis). All medical history were verified by electronic medical records.

Enrollment

Eleven plasma and Bronchial Lavage Fluid (BALF) samples were taken from previous NIH R01HL125042-01 and NIH K23AI134182 studies enrolling PLWH with the same inclusion and exclusion criteria as stated above. Seven subjects were recruited from the Infectious Disease Clinic on Ponce de Leon Avenue, and the Grady Medicine Clinic through flyer advertisements in the general medicine, pulmonary and infectious disease clinics from July 1, 2018 through December 31, 2018. Total participants 18 subjects.

Bronchoscopy

All bronchoscopy research is done by a collaborative group of investigators who have collectively standardized the approach and follow standard conscious sedation protocol procedures [15,16]. Bronchial Alveolar Lavage Fluid (BALF) samples were collected in 45ml sterile containers and transported to the lab on ice for processing immediately following the bronchoscopy. These were performed at Grady Memorial Hospital from April, 2015-October, 2018.

Alveolar macrophage isolation and function

The BALF was centrifuged at 400g for 10 minutes and the cell pellet re-suspended at a concentration of 1 million AM per mL in RPMI-1640 media containing 2% fetal bovine serum, 100IU/mL penicillin, 100µg/mL streptomycin and 40 µg/mL gentamicin. This technique isolates a cell population that is 95% AM as determined by MAC1 staining and Diff Quik analysis with cell viability >95% [17]. There's one pellet per BALF, and the pellet is resuspended at a concentration of 1 million AM per mL, so each BAL yields 2-4 mL of cell suspension. Typically, 2-4 million AM are isolated by this technique. Alveolar macrophages were cultured in chamber slides for 1 hour at 37°C and 10% CO_2 to allow adherence, then treated with 100nM or 200nM of $1,25(OH)_2D_3$ [calcitriol] or 100nM or 200nM of $25(OH)D_3$ [cholecalciferol] for 24 hours. Higher doses were used to mirror high oral dosing strategies in previous research [18,19]. For each BAL, the AMs were plated on a 16-well chamber slide. There were 2 chambers per treatment group (total 4 treatment groups per sample); the remaining 8 chambers were untreated. We chose both the active and in-active form of vitamin D_3 to investigate if the type of vitamin D would have different alveolar macrophage phagocytic treatment effects. Alveolar macrophage phagocytic function was determined by evaluating internalization of inactive fluorescent *Staphylococcus aureus* and assessing by fluorescent microscopy [20]. Alveolar phagocytic index is represented as the mean RFUs/cell x % positive between groups (Figure 3).

Alveolar macrophage LL-37 expression and free vitamin D levels

LL-37 protein expression was measured by Enzyme-Linked Two-Step Immunosorbent Assay (ELISA), (Hycult Biotech; Uden, The Netherlands). Free 25 (OH)D is more biologically active and correlates with mRNA expression of gene product LL-37, as previously studied [19]; therefore was analyzed. Free concentrations of 25(OH)D samples were measured with ELISA, and calibrated against a symmetric dialysis method (DIA source Immuno Assays, Louvain-la-Neuve, Belgium).We corrected BALF LL-37 and free 25(OH)D to BALF urea concentrations. BALF was centrifuged (1000 rpm; 15 min) and cells washed in phosphate buffered saline (PBS). For Immunohistochemistry (IHC) studies, cytospin preparations were made and cells fixed on glass slides with 4% paraformaldehyde, washed with PBS and stored in 70% ethanol prior to experiments, using standard IHC methods with LL-37 antibody.

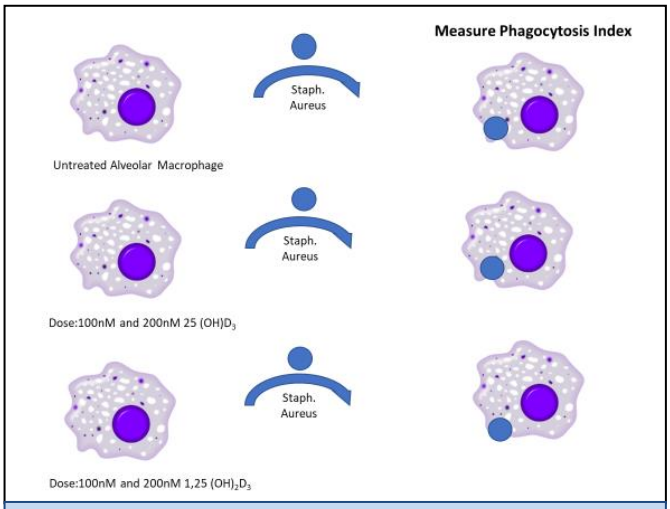

Figure 3: Alveolar macrophage untreated cells compared to vitamin D_3 treated cells and measurements ofalveolar phagocytosis index.

AM were isolated and phagocytic function was determined by evaluating internalization of inactive fluorescent *Staph. Aureus* by confocal microscopy. Phagocytic index is represented as the mean RFUs/cell x % positive cells. AM phagocytic index was compared between untreated AM to 100nM and 200nM 25(OH)D_3 [cholecalciferol] and 100nM and 200nM 1,25$(OH)_2D_3$ [calcitriol] treated AM cells.

Statistical analysis

Demographic variables were analyzed using descriptive analysis. Medians and inter-quartile ranges, and frequencies and percentages were calculated for each of four groups, categorized by tobacco smoking and HIV status. Tests for differences across groups were obtained from Kruskal-Wallis tests, or Fischer's Exact Test. The primary outcome was the change of phagocytosis index from baseline of four different vitamin D treatment groups (as compared to no treatment) with the four treatment groups including combinations of two different doses 100nM and 200nM of 1,25$(OH)_2D_3$ [calcitriol] levels and two different doses 100nM and 200nM of 25(OH)D_3 [cholecalciferol] in AM untreated cells.

The primary statistical analysis compared the difference between change in phagocytosis index from baseline by these four combinations of smoking (+/-) and HIV (+/-) status. The Wilcoxon sign rank test was used to test for change from baseline within each of the four HIV/smoking status groups and a Kruskal Wallis test was used to test for differences in change from baseline across the four groups. Based on previous preliminary data performed on HIV infected AM dosed with 100nM 1,25$(OH)_2D_3$, we anticipated an increase of 50% in phagocytosis index with standard deviation of 10, power of 80% and alpha of 0.05, this required total of 10 patients. All analysis was performed using SAS software version 9.4.

RESULTS

Characteristics of the study population

There were a total of 18 samples. Eleven plasma and BALF samples were taken from previous NIH R01HL125042-01 and NIH K23AI134182 study enrolling PLWH. Seven subjects were enrolled stratified by smoking and HIV status. Three subjects were healthy controls; five subjects were PLWH and non-smokers; four subjects were smokers without HIV; and six subjects were PLWH who smoke (Table 1).

Table 1. Baseline Demographics by HIV and Smoking Status.Medians (interquartile ranges) and frequencies (percentages) were calculated for each group.

Variable	HIV - /Non-Smoker (n=3)	HIV + / Non-Smoker (n=5)	HIV - /Smoker (n=4)	HIV +/ Smoker (n=6)	p-value[1]
Age yr (IQR)	29.0 (29.0 – 55.0)	55.0 (54.0 – 66.0)	56.0 (40.0 – 60.0)	50.0 (42.0 – 53.0)	0.65
Race (African-American)	1/3 (33.3%)	3/5 (60%)	2/4 (50%)	5/6 (83.3%)	0.55
Gender (Male)	0/3 (0%)	3/5 (60%)	2/4 (50%)	4/6 (66.7%)	0.39
BMI (IQR)	31.4 (26.4 – 31.6)	38.4 (31.3 – 44.4)	26.9 (24.3 – 28.9)	25.7 (22.4 – 38.6)	0.68
CD4 (IQR)	-	552.0 (237.0 – 713.0)	-	545.5 (456.0 – 613.0)	0.83

Medians were compared using Kruskal-Wallis tests, and frequencies were compared using Fisher's Exact test.

***Ex-vivo* effect of vitamin D on AM phagocytosis in smokers and PLWH**

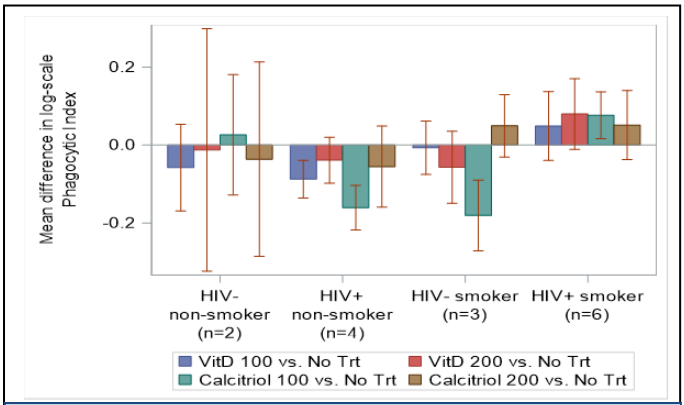

Figure 4. Difference in Log-scale Phagocytic Index of untreated cells compared to vitamin D_3treated cells.

AMs were challenged ex *vivo* with *Staph. aureus* and their AM phagocytic index was measured first untreated with vitamin D and then with varying doses of inactive (blue and red bars)100nM or 200nM cholecalciferol [25(OH)D_3]and active (green and brown bars) calcitriol [1,25(OH)$_2D_3$] 100nM and 200nM, p=0.16. The Wilcoxon sign rank test was used to test for change in mean difference in log-scale phagocytic index from baseline within each group. A Kruskal Wallis test was used to test for differences in change from baseline across the four groups.

PLWH who are smokers showed increase in AM phagocytic index after100nM or 200nM of 1,25(OH)$_2D_3$ [calcitriol] or 100nM or 200nM of 25(OH)D_3 [cholecalciferol] dosing as reflected by a positive improvement from baseline (untreated cells) but was not found to be statistically significant (p = 0.17) (Figure 4). Comparing across groups, there was no statistically significance difference in AM phagocytosis across the four HIV/smoking status groups (p = 0.09)

Secondary outcome analysis consisted of baseline LL-37 and free 25(OH)D in BALF and plasma across the four groups (Figure 5). We performed Kruskal-Wallis test for additional analysis to determine whether there was a difference in medians between PLWH vs. non-HIV subjects in relation to LL-37 and free 25(OH)D. (Figure 6) shows that LL-37 in plasma is higher in PLWH compared non-HIV subjects, (p.0.01), but there is no difference in LL-37 in BALF, (p.0.11). Also,PLWH have higherfree 25(OH)D levels in BALF compared to non-HIV subjects in, (p=0.01), but no change in plasma free 25 (OH)D. There was no significant correlation between plasma LL-37 and BALF LL-37 and plasma 25 (OH)D_3 and BALF 25 (OH)D. These pilot results are only hypothesis generating, and shows feasiblity to measure the effects of vitamin D treatment on these biological signatures.

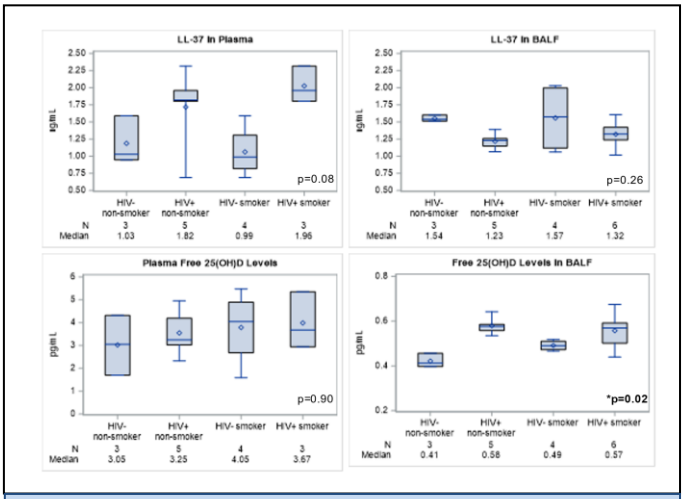

Figure 5: Comparision of LL-37 and Free 25(OH)D levels in plasma and bronchial alveolar lavage fluid.

Secondary outcome analysis consisted of baseline dilution LL-37 and free 25(OH)D in BALF and plasma across the four groups.LL-37 in plasma, p=0.08, LL-37 in BALF, p=0.26. Free 25(OH)D in plasma, p=0.90. Free 25(OH)D in BALF, *p=0.02, signifiyingat least one of the four groups is different. There is no significant difference in plasma and BALF across groups except for free 25(OH)D in the lungs. Kruskal Wallis tests were use to compare median values across the four groups.

*signifies statistical significance.

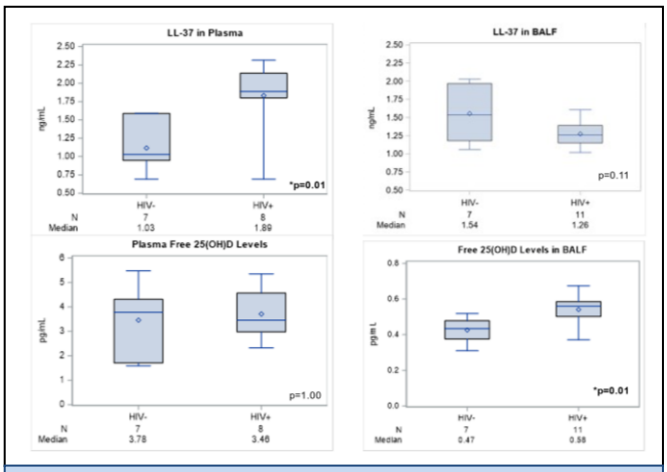

Figure 6: Comparision of LL-37 and Free 25(OH)D levels in plasma and bronchial alveolar lavage fluidPLWH compared to non-HIV infected subjects.

LL-37 (ng/ml) and free 25(OH)D (pg/ml) in BALF and plasma across two groups stratified by HIV status.LL-37 in plasma, *p=0.01. LL-37 in BALF, p=0.11. Free 25(OH)D in plasma, p>0.99. Free 25(OH)D in BALF, *p=0.004.Figure shows that LL-37 in plasma of PLWH is higher than non-HIV infected, although that change is not seen in the lungs. Free 25(OH)D levels in plasma are similar in both groups.But free 25(OH)D levels in BALF is higher in PLWH compared to non-infected HIV subjects.Measuring BALF LL-37 and 25(OH)D in HIV subjects more accurately reflect the local lung immune response compared to plasma.Kruskal Wallis tests were use to compare median values across the two groups.

* signifies statistical significance.

DISCUSSION

This is the first study to investigate possible direct mechanism to improve lung dysregulation caused by HIV and smoking. This study is novel in several ways. One, this study shows that lung immunity is different from systemic immunity (Figure 5) shows that there is greater variability in plasma compared to BALF, and measuring BALF more accurately reflects lung immunity instead of plasma. Two, this study measured LL-37 and free 25(OH)D in BALF that has not been previously investigated and has only been reported by our research group [19]. The clinical implication of this is that we will be able to measure these biological signatures after in-vivo vitamin D treatment, and measure the effect of vitamin D on lung versus systemic immune pathways. Third, we measured free 25(OH)D instead of total 25(OH)D. Free 25 (OH)D is more biologically active and correlates with mRNA LL-37 [19]. 25 (OH)D is bound to Vitamin D Binding Protein (VDBP). The rest is bound to albumin (10%-15%) and even smaller portion (<1%) is circulating freely [21]. In a previously published study, after high dose vitamin D administration, there were positive correlations in plasma level of free and total 25(OH)D, ($p<0.001$) and a positive association with mRNA expression of hCAP18 and free 25 (OH)D, ($p=0.04$)[19] . The significance of these results shows that we can measure LL-37 and free 25(OH)D in plasma and BALF and these biological markers will allow us to directly characterize the lung immune response to vitamin D therapy compared to systemic immune response, which has been lacking in previous vitamin D related research. With direct biological pathway elucidated, future research can investigate the direct impact of vitamin D on AM lung immunity during infections.

A series of studies provide strong evidence to support the role of vitamin D in improving AM lung immunity. In a study by Heulens *et al*, AMs acquired through bronchoscopy were obtained from 10 non-smoking and smoking adults. After the AMs were stimulated with 10 nM 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$) the researcher reported significantly improved mRNA expression of hCAP-18 (LL-37), which aids in phagocytosis [22]. In another study, human HIV-infected AM exposed to mycobacterium tuberculosis revealed very low TNF-α release. TNF-α was restored with vitamin D treatment, which showed a dose-dependent dramatic increase in macrophage TNF-α release in response to mycobacterium tuberculosis through the TLR ligand pathway improving phagocytic function [23]. In limited clinical trials, findings suggest that administering vitamin D can improve the inflammatory response. Coussen *et al* demonstrated that treating pulmonary TB+/HIV+ patients with vitamin D_3 improved the dysregulated inflammatory response [24]. In another study of teenagers and young adults infected with HIV, supplementing with vitamin D_3 7,000 IU orally/day for 12 weeks, increased serum vitamin D levels, improved immune system function by decreasing RNA viral load and activating TLR 2 [25,26]. Another study by Gerke *et al* showed that subjects treated with vitamin D_3 1,000 IU/day for three months had improved gene expression of AMs [27]. Also, in a randomized double blinded trial, vitamin D_3 supplementation 1,000IU/day for 90 days increased airway epithelial antimicrobial activity [28]. The conclusion drawn from these trials demonstrate that vitamin D supplements can directly

improve the host immune response and enhance pathogen clearance.

There are several limitations to this study. First, the small sample size only allows this study to be hypothesis generating as it signals a possible improvement in AM phagocytic function *ex-vivo* after vitamin D dosing in PLWH smokers, as all six subjects showed improvement in function from baseline (untreated cells). Second, the limited plasma samples do not allow for correlation between plasma and BALF of LL-37 and free 25(OH)D and measurement of VDBP, as some study subjects did not have enough BAL fluid return to complete the analysis. Due to limited samples and funding, the authors chose not to analyze total 25(OH) D on plasma and BAL due to previously reported results that there was no meaningful correlation between plasma and BAL fluid of total 25(OH)D [29].Third, with limited cells, we were not able to perform additional vitamin D dosing strategies. Fourth, we were unable to enroll same number of subjects per group; however, we did enroll the most patients in the highest risk group. Lastly, three subjects did not have enough alveolar macrophages isolated during their bronchoscopy to analyze their phagocytic index and three subjects where there wasn't enough plasma to analyze.

Our study provides the pilot data and rationale for a larger and more robust clinical trial. We were enrolling subjects in a clinical trial stratified by smoking and HIV status. Subjects were given 450,000 IU vitamin D_3 over three days and were undergoing repeat bronchoscopy seven days later to measure the impact of high dose vitamin D on AM immune function when challenged *ex-vivo* to *S. aureus*. Alveolar macrophage immune function analysis were going to include AM phagocytic function, mRNA and protein expression of LL-37; Toll Like Receptor (TLR) 4; and secretion of LL-37, free and total 25(OH)D, VDBP, TNF-α and IFNγ and alveolar oxidative stress (NCT03270709). However, due to the COVID pandemic this study is on hold and uncertain when it will proceed. There has been increased interest in vitamin D administration and its potential effects in COVID patients [30-37]. This pilot study can provide novel useful information to help create and design future research trials.

Due to the specific nature of vitamin D impact on lung immunity on AM function, focusing on the highest risk patients for lung infection such as PLWH and smokers are likely the optimal target population to show improvement in lung immunity with vitamin D treatment. Smoking and HIV independently alter lung immunity particularly AM immune function and synergistically alters oxidative stress, cytokine pathway regulation and phagocytic function [11-14,38,39]. Even in well controlled PLWH with undetectable viral loads, AMs were found to harbor the virus and impair phagocytosis and immune function [40], and with the increased prevalence of smokers in PLWH the risk for lung infection increases significantly [15]. Smoking cessation in HIV+ patients reduces the risk of pulmonary infections [41], unfortunately smoking relapse rates are as high as 40% [41]. We need to better understand why smoking exerts such a profound risk of pneumonia on PLHIV. While working towards tobacco cessation, focusing on alternative ways to reduce the harmful effects of smoking and to prevent pneumonia requiring hospitalization is an important objective to reduce the overall care burden of PLWH, especially in the era of emerging viral diseases such as COVID-19.

CONCLUSION

This pilot feasibility study is hypothesis generating and highlights compelling evidence to investigate vitamin D immune pathway function in PLWH who smoke. We report for the first-time measurable values of free 25(OH)D and LL-37 in BALF of PLWH who smoke. This observation should be confirmed in larger studies. This study reveals that we can quantify and measure direct effects of vitamin D therapy in lung immunity. Therefore, investigating the molecular and clinical benefits of vitamin D on lung immunity is a low cost and high yield endeavor, which can have a significant impact on lung disease that has yet to be elucidated.

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SUPPLEMENT DATA

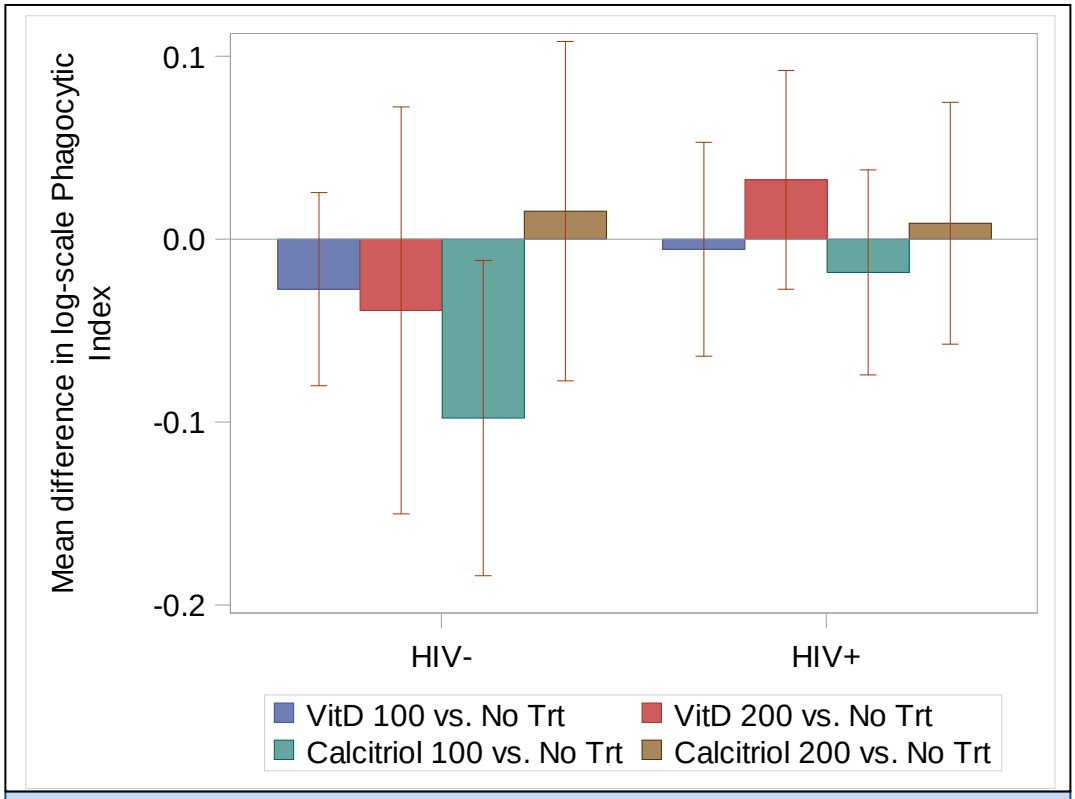

Figure 7: Mean Difference in Log-scale Phagocytic Index Comparing HIV- and HIV+ Subjects

AMs were challenged *ex vivo* with *Staph. aureus* and their AM phagocytic index was measured first untreated with vitamin D and then with varying doses of inactive (blue and red bars) cholecalciferol [25(OH)D_3]and active (green and brown bars) calcitriol [1,25$(OH)_2D_3$], p=0.16.

VitD 100 vs. No Trt
VitD 200 vs. No Trt
Calcitriol 100 vs. No Trt
Calcitriol 200 vs. No Trt

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Table 2. Free 25(OH)D Plasma and BALF levels.

Patient	Smoker	HIV Status	Plasma Free 25(OH)D (pg/mL)	BALF Free 25(OH)D (pg/mL)
01	Yes	HIV+	67.953	0.674
02	Yes	HIV+	.	0.592
03	Yes	HIV+	2.94	0.50
04	Yes	HIV+	.	0.56
05	Yes	HIV+	5.35	5.35
06	Yes	HIV+	3.67	0.44
07	Yes	HIV-	5.47	0.48
08	Yes	HIV-	1.59	0.50
09	Yes	HIV-	3.78	0.47
10	Yes	HIV-	4.31	0.52
11	No	HIV+	3.251	0.642
12	No	HIV+	2.32	0.59
13	No	HIV+	4.2	0.58
14	No	HIV+	3.02	0.54
15	No	HIV+	4.95	0.56
16	No	HIV-	1.70	0.40
17	No	HIV-	4.312	0.457
18	No	HIV-	3.048	0.412

*Patient 01 plasma was not included in analysis due to being outlier.
*Patient 02 and 04 plasma level were not detectable and not included in analysis.
*Patient 05 BALF was an outlier and not included in analysis.