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Interferon-signaling pathways are upregulated in people with HIV with abnormal pulmonary diffusing capacity (DL_{CO})

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Objective: People with HIV (PWH) are at greater risk of developing lung diseases even when they are antiretroviral therapy (ART)-adherent and virally suppressed. The most common pulmonary function abnormality in PWH is that of impaired diffusing capacity of the lungs for carbon monoxide (DL_{CO}), which is an independent risk factor for increased mortality in PWH. Earlier work has identified several plasma biomarkers of inflammation and immune activation to be associated with decreased DL_{CO}. However, the underpinning molecular mechanisms of HIV-associated impaired DL_{CO} are largely unknown.

Design: Cross-sectional pilot study with PWH with normal DL_{CO} (values greater than or equal to the lower limit of normal, $DL_{CO} \geq$ LLN, N = 9) or abnormal DL_{CO} ($DL_{CO} <$ LLN, N = 9).

Methods: We compared the gene expression levels of over 900 inflammation and immune exhaustion genes in PBMCs from PWH with normal vs. abnormal DL_{CO} using the NanoString technology.

Results: We found that 26 genes were differentially expressed in the impaired DL_{CO} group. These genes belong to 4 categories: 1. Nine genes in inflammation and immune activation pathways, 2. seven upregulated genes that are direct targets of the interferon signaling pathway, 3. seven B-cell specific genes that are downregulated, and 4. three miscellaneous genes. These results were corroborated using the bioinformatics tools DAVID (Database for Annotation, Visualization and Integrated Discovery) and GSEA (Gene Sets Enrichment Analysis).

Conclusion: The data provides preliminary evidence for the involvement of sustained interferon signaling as a molecular mechanism for impaired DL_{CO} in PWH.

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Keywords: diffusing capacity, HIV, inflammation, interferon, lung

Introduction

Noninfectious, chronic lung diseases remain prevalent amongst people with HIV (PWH), even when they are antiretroviral therapy (ART)-adherent and virally suppressed [1,2]. The most common pulmonary function abnormality in PWH is that of impaired diffusing capacity of the lungs for carbon monoxide (DL_{CO}), which measures the lungs' ability to transfer gas from inhaled air into the bloodstream. HIV infection is an independent risk factor for decreased DL_{CO} [3–6], which in turn is associated with increased mortality after adjusting for

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other risk factors [7]. Despite the widespread prevalence of diffusing capacity abnormalities in PWH, the mechanisms driving this lung dysfunction phenotype are incompletely understood.

Earlier work has identified HIV-related clinical factors associated with decreased DL_{CO} [1]. Two studies reported that lower $CD4^+$ cell counts and higher viral loads were both associated with decreased DL_{CO} [3,8]. $CD4^+$ T cell count was associated with lower DL_{CO} [5]. A low $CD4^+/CD8^+$ ratio was associated with lower DL_{CO} independent of other risk factors and clinical markers of HIV in another study [9]. The immunological and molecular mechanisms driving this lung dysfunction phenotype are incompletely understood, but one hypothesis posits persistent immune activation and inflammation as potential mechanisms. Chronic immune activation plays a key role in AIDS pathogenesis and is predictive of rapid disease progression and death in uncontrolled HIV infection [10–12]. Although the levels of immune activation and inflammation decrease with successful ART treatment, they remain high compared with HIV-negative patients [13]. In one study, including only PWH, serum IL-6 and C-reaction protein (CRP) were increased in individuals with worse DL_{CO}, suggesting general inflammation [14]. In another study, markers of monocyte activation (sCD163, IL-2 soluble receptor alpha), microbial translocation (lipopolysaccharide), and endothelial dysfunction (endothelin-1) were linked to lower DL_{CO} in PWH [15]. Our group previously reported that in the longitudinal I AM OLD cohort of PWH, higher levels of biomarkers of monocyte activation (sCD14 and sCD163), interferon (IFN) response (IP-10), lymphocyte activation (sCD27) and general inflammation (IL-6, sTNFR-I, and sTNFR-II) and fibrin degradation (D-dimer) were associated with lower DL_{CO}% predicted in multivariate analyses [16].

In the only reported gene expression study of impaired DL_{CO} in PWH, Crothers *et al.* [17] found that HIV infection is associated with different gene expression pathways in normal vs. impaired DL_{CO}, suggesting that HIV-associated immune dysfunctions may be an underlying mechanism for impaired DL_{CO} in PWH. However, when normal vs. impaired DL_{CO} groups were directly compared, two pathways seemingly unrelated to HIV or lung function (the Reactome olfactory signaling pathway and the KEGG olfactory transduction pathways) were found to be enriched. Thus, it was unclear which genes are associated with impaired DL_{CO} within PWH.

One detrimental consequence of chronic immune activation and inflammation is immune exhaustion. Following acute antigen stimulation, immune checkpoint molecules are expressed to terminate the immune response, thereby achieving immune homeostasis. However, in chronic infections such as HIV, persistent viral antigens and subsequent chronic inflammation and immune activation lead to continuous expression of immune checkpoint molecules [18]. Although the expression of these markers correlates with HIV viral load and disease progression [19], whether immune exhaustion contributes to HIV-related lung dysfunction is unknown.

Much of the literature examining mechanisms underlying diffusing capacity abnormalities utilizes plasma biomarkers as a measure of inflammation and immune activation, but few studies have investigated the role of gene expression within these pathways as an upstream factor. Thus, we conducted a cross-sectional pilot study among PWH with abnormal vs. normal DL_{CO} to compare gene expression levels in peripheral mononuclear blood cells (PBMCs). Given the critical roles that inflammation and immune exhaustion play in HIV-associated diseases, we focused on two gene sets: the inflammation panel and the immune exhaustion panel, using NanoString technology [20], which simultaneously measures the mRNA expression levels of hundreds of genes. We analyzed the data using three approaches: the differential expression analysis with NanoString nSolver's Advanced Analysis Module; Database for Annotation; Visualization and Integrated Discovery (DAVID) analysis [21,22]; and Gene Set Enrichment Analysis (GSEA) [23,24]. These three approaches converge to the same finding: IFN signaling is upregulated in PWH with impaired DL_{CO}.

IFNs are pro-inflammatory cytokines with pleiotropic functions, including direct antiviral effects as well as roles in cell proliferation, survival, and differentiation [25]. The binding of IFNs to their cell surface receptors leads to induction of IFN-stimulated genes (ISGs). Type I IFNs (IFN-I) are one of three types of IFNs and are critical to controlling HIV-1 infection in the acute phase; a robust IFN-I response is required to limit HIV reservoir size and disease progression [26]. Despite its importance in the initial phases of infection, sustained IFN-I response during the chronic infection phase is detrimental, as it contributes to increased systemic inflammation, impaired effector-to-memory cell differentiation of heterologous T cell responses, and apoptosis of T cells. IFN signaling also promotes immunosuppressive responses and $CD8^+$ T cell exhaustion [27]. Thus, our finding of increased IFN-stimulated gene expression suggests that sustained IFN signaling is a potential molecular link between inflammation and immune exhaustion and impaired DL_{CO} in PWH.

Materials and methods

Study cohort

The Inflammation, Aging, Microbes, and Obstructive Lung Disease (I AM OLD) Study is a prospective, longitudinal cohort study of PWH in the United States

and Uganda assessed for incidence and progression of lung function abnormalities over time. In the United States, I AM OLD is comprised of adult PWH (age ≥18 years) recruited at San Francisco General Hospital and Seattle's Harborview Medical Center. Patients in this project are from the San Francisco cohort.

Study procedures

The I AM OLD study and its procedures have been described previously [16,27]. Participants provided written informed consent. The study protocol was approved by the Institutional Review Board of the University of California San Francisco (IRB#13-11328).

Clinical data collection

Participants were enrolled during routine clinic visits or upon admission for acute pneumonia and then followed through recovery. During study visits, participants underwent blood draw to measure $CD4^+$ and $CD8^+$ counts and HIV RNA and completed structured questionnaires regarding demographic and clinical characteristics. On the same day, most participants also underwent pulmonary function testing (PFTs) consisting of prebronchodilator and postbronchodilator spirometry and single-breath diffusing capacity of the lung for carbon monoxide (DL_{CO}) using the ndd EasyOne Pro (ndd Medizintechnik AG, Zurich, Switzerland). Participants enrolled during hospitalization completed PFTs 3 months after the resolution of pneumonia. They underwent PFTs only if they denied symptoms of acute respiratory illness and were without new or worsening respiratory symptoms. DL_{CO} values were corrected for same-day hemoglobin and carboxyhemoglobin values. PFTs were performed by and overread by trained personnel in accordance with the 2022 American Thoracic Society/European Respiratory Society (ATS/ERS) guidelines for quality and repeatability [28]. DL_{CO} tests with grades A through D were included in this analysis.

Percentage predicted values for DL_{CO} were calculated using the Neas formulas – adjusted for age, sex at birth, race/ethnicity, and height [29]. Consecutive pilot study participants were subsequently categorized as either normal DL_{CO} (DL_{CO} values greater than or equal to the lower limit of normal, $DL_{CO} \geq$ LLN) or abnormal DL_{CO} ($DL_{CO} <$ LLN). For individuals whose PFTs on the day of the blood draw did not meet ATS/ERS standards, an alternate PFT was used. Nonpassing PFTs were included if there was PFT data from before and after their blood draw date and their lung phenotype ($DL_{CO} \geq$ LLN vs. $DL_{CO} <$ LLN group) was consistent between the preblood and postblood draw PFT.

Measures of gene expression using nanostring

PBMCs were purified from EDTA whole blood using Ficoll gradient, cryopreserved in freezing medium (90% fetal bovine serum S + 10% DMSO), and stored in liquid nitrogen. Total RNA from 5 to 10 million PBMCs was extracted, using the Qiagen RNeasy Plus Mini kit. RNA concentration was measured using a Nanodrop2000 and the integrity of the RNA samples was confirmed by running all samples on a Bioanalyzer (Agilent Technologies, Santa Clara, California, USA) with an Agilent Technologies Agilent RNA 6000 Nano Kit. The RNA Integrity Number (RIN) for each sample is shown in Supplemental Table 1, http://links.lww.com/QAD/D237. Student's *t* test was performed to compare RIN of $DL_{CO} \geq$ LLN vs. $DL_{CO} >$ LLN, and the results were not statistically different.

Gene expression levels of the Inflammation Panel and Immune Exhaustion Panel Plus were measured using NanoString's nCounter SPRINT Profiler. The samples were run in two batches (up to 12 samples each) and for the two panels separately with 100 ng total RNA each. Background was subtracted using the geometric mean of the negative controls, and the data was normalized using the geometric means of the built-in positive controls and housekeeping genes. The processed datasets were analyzed using differential gene expression analysis by nSolver's Advanced Analysis Module ($P < 0.05$, Benjamini–Yekutieli correction).

Database for Annotation, Visualization, and Integrated Discovery analysis

We performed gene set analysis using the bioinformatic tool Database for Annotation, Visualization, and Integrated Discovery (DAVID). Enriched gene sets between $DL_{CO} <$ LLN and $DL_{CO} \geq$ LLN groups represent their relative difference in expression levels. A positive enrichment represents over-expression in the $DL_{CO} <$ LLN group compared with the $DL_{CO} \geq$ LLN group, whereas a negative enrichment represents relative under-expression. DAVID analysis provided a more agnostic approach to understanding the gene sets and pathways altered in the $DL_{CO} <$ LLN group.

The normalized mRNA count values were first processed by nSolver Advanced Analysis module to obtain the differentially expressed genes (DEG) between the $DL_{CO} <$ LLN and $DL_{CO} \geq$ LLN groups with a Benjamini–Yekutieli adjusted *P* value threshold of 0.15, which is higher than the typical value of 0.05, to increase search range. We used these DEGs as input sets against four pathway-related gene set collections from DAVID (GOTERM_BP_DIRECT, KEGG_PATHWAY, REACTOME_PATHWAY, WIKIPATHWAYS) [30–34], with genes in the Exhaustion and Inflammation datasets as respective background sets.

Gene sets enrichment analysis

Gene sets enrichment analysis (GSEA) takes the entire gene expression data and compares them to pathways in the GSEA database. As multiple mRNA variants exist for a single gene, we converted the NM accession number that is associated with each mRNA probe into its

corresponding unique Gene Symbol and Entrez ID. We then removed all duplicate genes as well as two viral genes (*EBV LMP2* and *CMV UL83*) from the Exhaustion Panel. We performed GSEA for the two cleaned-up datasets (817 genes in Exhaustion Panel and 249 genes in Inflammation Panel) using the MsigDB C2 Canonical pathways (version v2022.1.Hs) [35]. Due to our limited data size, we chose a P value of 0.15 as our cutoff for the false discovery rate (FDR), wherein a lower value represents a higher probability of actual enrichment.

Results

Cohort and participant characteristics

Eighteen individuals with treated HIV were enrolled in this pilot study, with nine patients each in the $DL_{CO} \geq$ LLN and $DL_{CO} <$ LLN groups (Table 1). Three individuals had nonpassing DL_{CO} tests, for which their DL_{CO} categorization was inferred from passing PFTs conducted at adjacent timepoints. Four participants had their blood drawn before the day of their PFTs (ranging from within 24 h to within 6 months), and three participants' blood draw was after their PFT date (ranging from within 1 month to within 6 months). All patients were on ART therapy at the time of PFTs and blood draw. The two groups did not differ in demographic and clinical features, although the $DL_{CO} <$ LLN group had a shorter time since HIV diagnosis (22.2 ± 8.8 vs. 29.6 ± 3.9 years, $P = 0.04$) and fewer total years on ART (15.7 ± 7.0 vs. 23.8 ± 7.6, $P = 0.03$), despite no differences in viral suppression as measured by viral load. The $DL_{CO} <$ LLN group also had lower post-BD FEV1%predicted (69.0 ± 19.8 vs. 84.9 ± 8.4, $P = 0.04$) but no other differences in spirometry readouts.

Differentially expressed genes

The Inflammation Panel measure 249 genes involved in a broad range of pathways related to inflammation. The Immune Exhaustion Panel measures 773 genes involved in immune cell activation; immune cell suppression; immune cell status; immune checkpoints; epigenetics; and metabolism and microenvironment. As such, the Immune Exhaustion Panel covers a broad range of genes involved in key immune functions. Additionally, because of the long-standing interest of the lab and unpublished results linking impaired DL_{CO} to short telomere length [36], we added probes for 50 telomere maintenance genes to the Immune Exhaustion Panel (Supplement Table 2, http://links.lww.com/QAD/D237). The total number of unique genes was 984 after considering a small set of genes that overlap between the two panels.

From the two panels, 26 total unique genes were found to be differentially expressed (Benjamini–Yekutieli correction adjusted $P < 0.05$). Of these, 12 were upregulated

Table 1. Baseline participant characteristics.

	$DL_{CO} <$ LLN ($n = 9$)	$DL_{CO} \geq$ LLN ($n = 9$)	P value
Age	56.9 ± 10.4	58.8 ± 7.4	0.66
BMI	32.3 ± 8.2	32.6 ± 6.9	0.94
Gender (male %)	6 (67%)	8 (89%)	0.58
Race/ethnicity (%)			>0.99
Caucasian	5 (56%)	5 (56%)	
African American	3 (33%)	2 (22%)	
Native Hawaiian or other Pacific Islander	1 (11%)	0	
American Indian/Alaska native	0	1 (11%)	
Mixed	0	1 (11%)	
Time since HIV diagnosis (years)	22.2 ± 8.8	29.6 ± 3.9	0.03
$CD4^+$ cell counts (cells/μl)	874.2 ± 1308.5	694.8 ± 286.0	0.69
$CD4^+/CD8^+$ ratio	1.15 ± 1.47	0.87 ± 0.45	0.59
HIV viral RNA (not detected/detected)	100%	89%/11%[a]	>0.99
Length of continuous ART adherence (years)	14.5 ± 8.6	17.6 ± 9.8	0.46
Total estimate of ART (years)	15.7 ± 7.0	23.8 ± 7.6	0.04
Cigarette smoking, ever (Y/N)	89%/11%	78%/22%	>0.99
Bacterial pneumonia (Y/N)	67%/33%	33%/67%	0.35
Pneumocystis pneumonia (Y/N)	44%/56%	44%/56%	>0.99
Post-BD FEV1/FVC	0.73 ± 0.09	0.78 ± 0.07	0.23
Post-BD FVC% predicted	72.0 ± 18.7	83.3 ± 6.2	0.11
Post-BD FEV1% predicted	69.0 ± 19.8	84.9 ± 8.4	0.04
Post-BD FEV1/FVC <0.70 (Y/N)	33%/67%	22%/78%	>0.99
Post-BD FEV1/FVC <LLN (Y/N)	33%/67%	0%/100%	0.21
DL_{CO} corr %Pred	50.1 ± 19.9	85.8 ± 12.0	0.00029
DL_{CO} <80% predicted (Y/N)	100%/0%	67%/33%	0.009

All numerical categories are expressed as mean ± SD. Student's t tests were used to compare group differences for normally distributed continuous variables and Mann–Whitney U tests were used to compare group differences for continuous variables that did not pass normality tests. Fisher's exact tests were used to compare group differences for categorical variables. Analyses were done with GraphPad Prism version 9.5.1. ART, antiretroviral therapy; BD, bronchodilator; DL_{CO}, diffusing capacity of the lungs for carbon monoxide; FVC, forced vital capacity; FEV1, forced expiratory volume in one second; LLN, lower limit of normal

[a]One patient has a viral RNA load of 47 copies/ml.

and 14 downregulated in the DL_{CO} < LLN group. Seven genes were from the inflammation panel and 19 from the immune exhaustion panel (Supplemental Table 3, http://links.lww.com/QAD/D237). Box plots for each gene are shown in Supplemental Figure 1, http://links.lww.com/QAD/D237. Upon closer examination, these genes can be categorized into four groups: genes involved in inflammation/immune activation ($N=9$), IFN pathway genes ($N=7$), B-cell proliferation and function genes ($N=7$), and miscellaneous genes ($N=3$) (Fig. 1a).

Overall, the differential gene expression involved in inflammation and immune activation shows that among the DL_{CO} < LLN group, there is an increase in inflammation and immune activation (Fig. 1b): proinflammatory genes *CXCL5*, *SOCS3*, and *FCGR1A/B* [37–39] are upregulated in the DL_{CO} < LLN group, whereas genes that inhibit and suppress inflammation and immune activation are downregulated [40]. These differential gene expression findings are consistent with existing literature that has identified plasma biomarkers of immune activation and inflammation to be associated with decreased DL_{CO} [16].

Of the DEGs associated with IFN signaling, all seven are upregulated in the DL_{CO} < LLN group (Fig. 1c). Although IFN pathways are integral to the inflammation responses and critical to HIV disease progression, we grouped these seven genes separately to highlight this important finding. As discussed later, DAVID and GSEA analyses also suggest the positive enrichment of IFN-signaling pathways. Notably, all seven genes are type I ISG [41] with *MX1*, *STAT1*, and *C2* being core type I ISGs shared by multiple vertebrae species [42]. Specifically, STAT1 is the transcriptional factor shared by all three types of IFNs to activate the transcription of ISG. IDO1 catalyzes the initial, rate-limiting step of tryptophan (Trp) catabolism along the kynurenine (KYN) pathway and is induced by cytokines in immune cells [43]. Expression of IDO1 is induced by both type I and II IFNs. A high ratio of kynurenine and tryptophan (K/T) in plasma, seen in states with high IDO1 activity, has been reported in PWH and is associated with impaired T-cell function [44–46].

We observed lower expression levels of seven genes related to B-cell proliferation and function genes in the DL_{CO} < LLN group (Fig. 1d). Of these, *CD19*, *FCRL2*, *IGHM*, and *MS4A1* are exclusively expressed in naive and mature B cells (https://proteinatlas.org), whereas TLC1A is also expressed in plasmacytoid dendritic cells (pDC) and TCF4 is also expressed in pDC, myeloid dendritic cells (mDC), and monocytes [47,48]. The lower expression of multiple B-cell-specific genes suggests that relative B-cell abundance is lower in the DL_{CO} < LLN

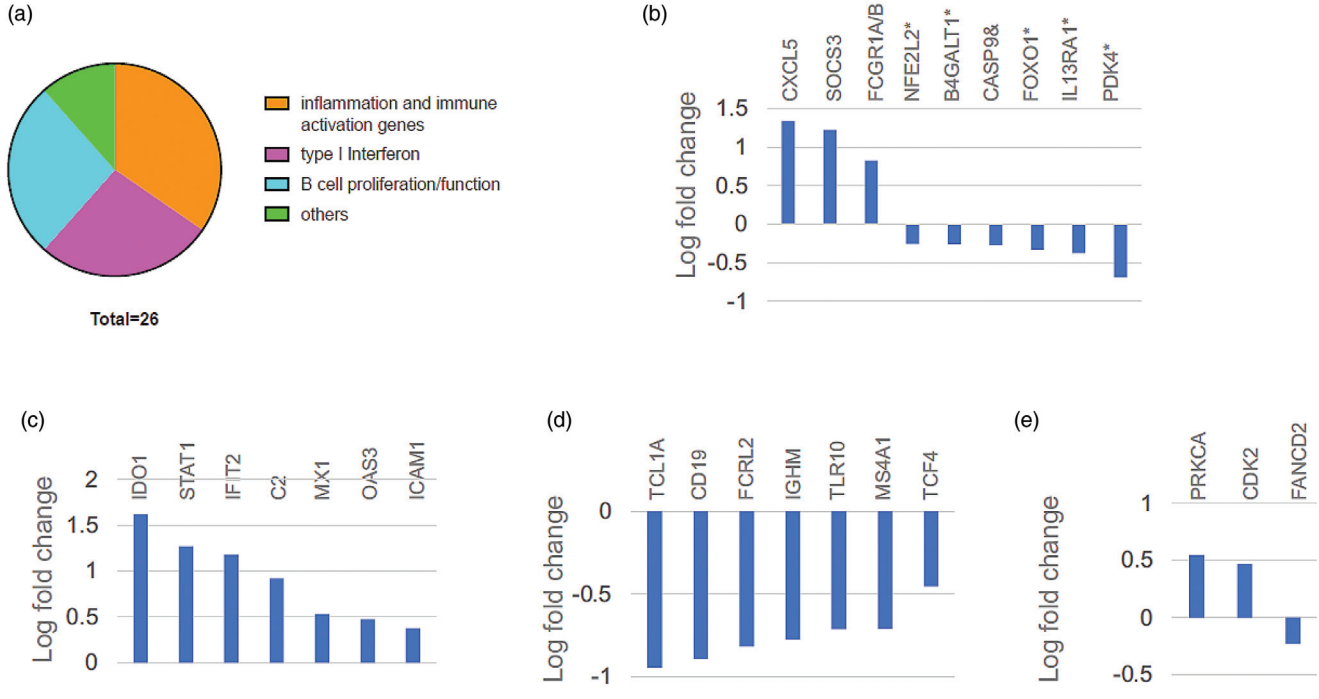

Fig. 1. Differentially expressed genes between DL_{CO} < LLN and DL_{CO} ≥ LLN patients. Differentially expressed genes comparing DL_{CO} (diffusing capacity of the lungs for carbon monoxide) <LLN vs. DL_{CO} ≥ LLN. The difference between DL_{CO} < LLN vs. DL_{CO} ≥ LLN is statistically significant for all genes. (a) Summary of 26 differentially expressed genes (DEG). (b) Genes in inflammation immune activation pathways. Genes with the '*' sign are in inflammation and immune activation pathways, but they act to inhibit inflammation and immune activation and prevent induction of interferon. (c) Genes in interferon pathways. (d) Genes involved in B-cell proliferation and function. (e) Three other DEGs. Log (2)-fold changes are shown in b–e. LLN, lower limit of normal.

group. We performed an immune profiling experiment using full spectral flow cytometry with a 5-laser Cytek Aurora Flow Cytometer and a modified Cytek 25-Color Immunoprofiling Assay panel with additional seven surface markers (*CCR2*, *CCR4*, *CCR6*, *CD21*, *CX3CR1*, *CXCR3*, and *CXCR5*) and Zombie UV (Biolegend, San Diego, CA, USA) for viability dye. Analysis of the frequencies of $CD4^+$ T cell, $CD8^+$ T cell, B cell, monocyte, and NK cell shows that the frequency of B cells within the single, live cell population is lower in the DLco < LLN group (Supplemental Table 4 http://links.lww.com/QAD/D237, Supplemental Figure 2, http://links.lww.com/QAD/D237 for gating strategies and Supplemental Materials and Methods, http://links.lww.com/QAD/D237). Although this data does not rule out the possibility of decreased expression levels of B-cell-specific genes (Fig. 1d) at single-cell levels, it is consistent with the interpretation that the decreased expression levels seen in total PBMCs is at least partially because of a lower percentage of B cells. Correlational analysis showing strong, positive correlations between the B-cell frequency and the expression levels of the 7 B-cell-specific genes (Supplemental Figure 3, http://links.lww.com/QAD/D237) supports this interpretation. Analysis of the full immune profiling data will be reported in a separate manuscript.

Finally, the upregulation of protein kinase C alpha (PRKCA) and cyclin-dependent kinase 2 (CDK2) in the DL_{CO} < LLN group (Fig. 1e) is consistent with a higher level of cell turnover. FANCD2, a DNA damage protein involved in homology-directed DNA repair, is down-regulated in DL_{CO} < LLN (Fig. 1e).

Database for Annotation, Visualization and Integrated Discovery analysis

DAVID analysis revealed significantly enriched pathways in the Exhaustion dataset, but not in the Inflammation dataset. We found significant positive enrichment of the IFN-alpha/beta pathway in the DL_{CO} < LLN group (fold change 8.02, FDR $P = 2.25E-04$, Fig. 2, and Supplemental Figure 4, http://links.lww.com/QAD/D237).

Gene sets enrichment analysis

GSEA analysis similarly revealed significantly enriched gene sets in the Exhaustion dataset but not in the Inflammation dataset. We saw positive enrichment of genes involved in IFN alpha beta signaling, TGF-beta receptor signaling, and IL2/STAT5 pathway in the DL_{CO} < LLN group (Fig. 3a and Supplemental Figures 5 and 7, http://links.lww.com/QAD/D237), with the gene set corresponding to IFN-alpha/beta pathway having the highest enrichment score (normalized enrichment Score 2.02, FDR adjusted $P = 0.088$). Among the negatively enriched gene sets, the pathway with the highest enrichment score is 'antigen activation of B-cell receptor that leads to the generation of secondary messengers' (normalized enrichment score -2.18, FDR-adjusted $P = 0.0028$, Fig. 3b and Supplemental Figures 6 and 8, http://links.lww.com/QAD/D237).

Discussion

Gene expression studies of HIV-associated impaired DL_{co}

Chronic lung disease is a key contributor to morbidity and mortality in HIV patients in the ART era, and

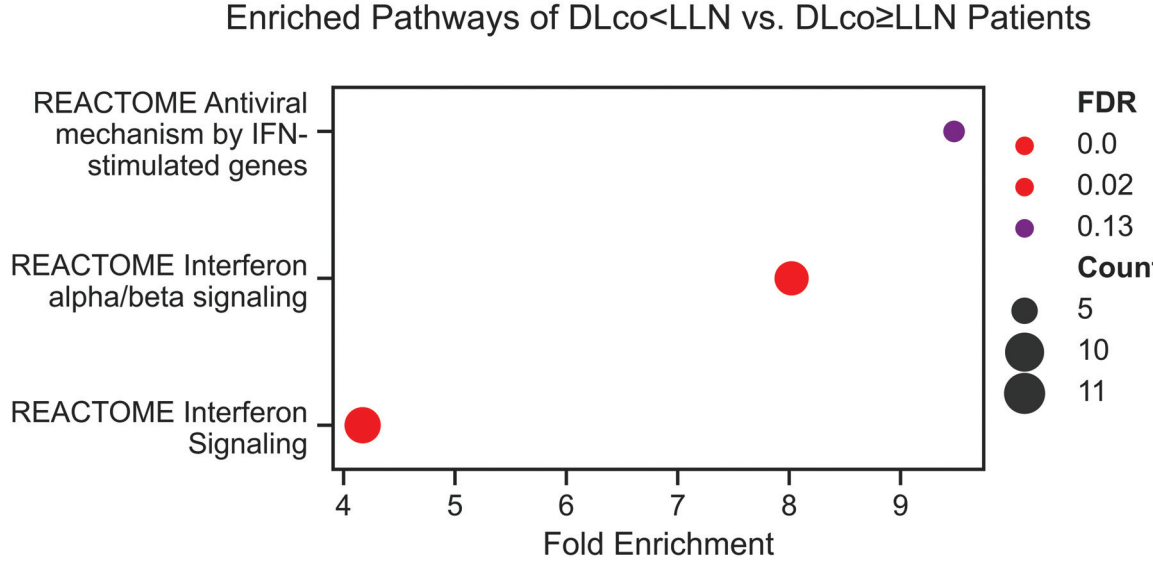

Fig. 2. Enriched pathways between DL_{CO} < LLN and DL_{CO} ≥ LLN patients. Dot plot of enriched pathways of up-regulated genes in the Exhaustion data using DAVID when comparing the DL_{CO} < LLN group to the DL_{CO} ≥ LLN group. The top three enriched pathways with FDR less than 0.15 are shown and ranked by their fold enrichment. DL_{CO}, diffusing capacity of the lungs for carbon monoxide; LLN, lower limit of normal.

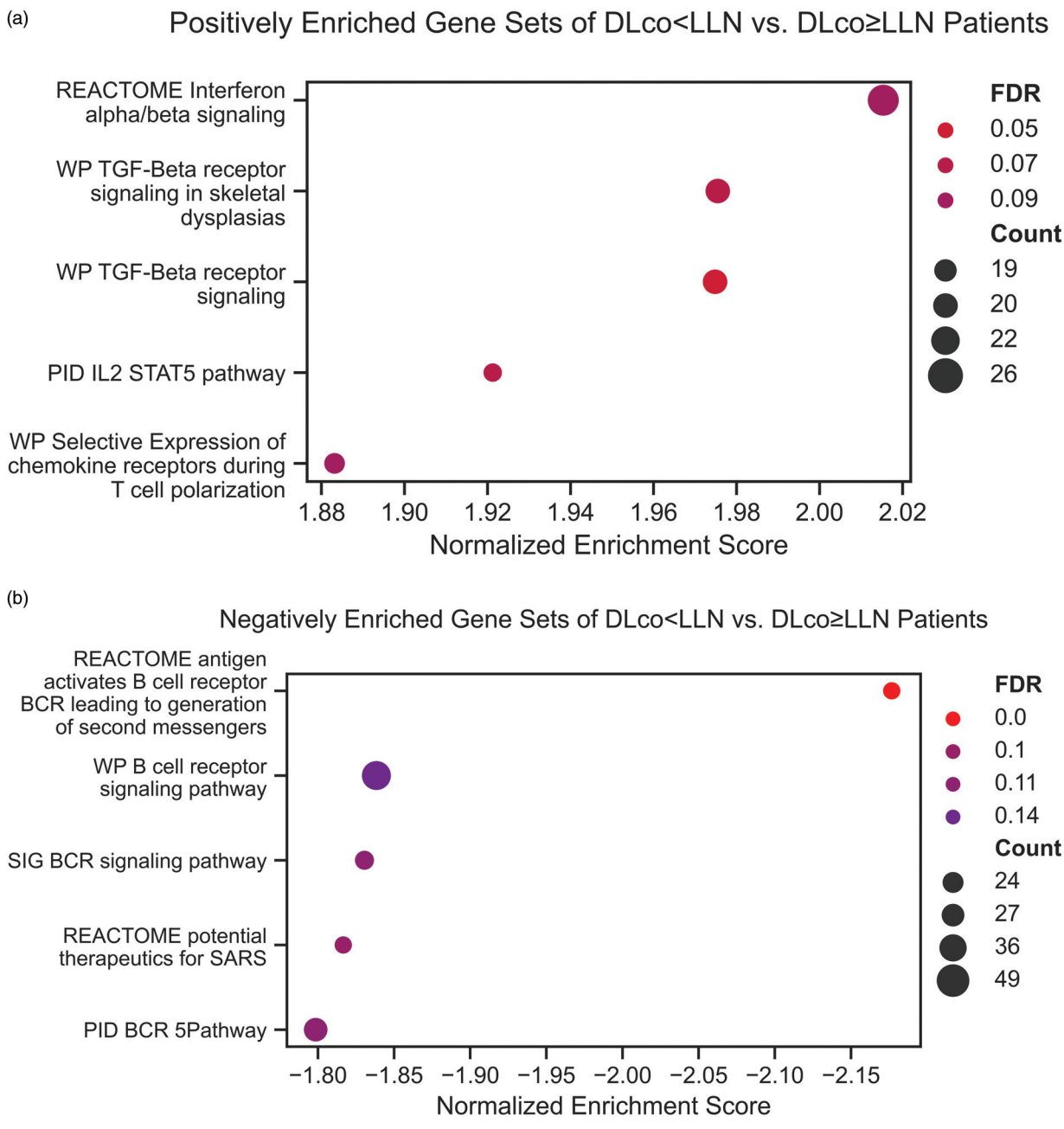

Fig. 3. Gene Sets Enrichment Analysis results of positively and negatively enriched gene sets. Dot plot of positively (a) and negatively (b) enriched gene sets of the Exhaustion data using GSEA when comparing the DL_{CO} < LLN group with the DL_{CO} ≥ LLN group. The top five enriched gene sets with FDR less than 0.15 are shown, as ranked by their normalized positive enrichment score. The dot size is determined by the gene count in its particular gene set. The gene count represents the number of genes that are in both the inputs and a gene set. DL_{CO}, diffusing capacity of the lungs for carbon monoxide; LLN, lower limit of normal.

diffusion capacity impairment is a prominent feature of HIV-associated lung dysfunction. The molecular and cellular mechanisms of HIV-associated DL_{CO} impairment is poorly understood. The only published gene expression study on low DL_{CO} in HIV patients we are aware of was by Crothers *et al.* [17]. Using the microarray platform, they compared gene expression levels of 20 000 genes in whole blood of HIV+ and HIV− patients with preserved or low DL_{CO}. In patients with preserved DL_{CO}, they found that HIV infection is associated with the activation of processes involved in immunity, cell cycle, and apoptosis, whereas in their low DL_{CO} counterparts, they found a much broader repertoire of pro-inflammatory and immune-related pathways in HIV+ patients relative to HIV- participants. Their results suggest that impaired DL_{CO} in HIV− and HIV+ are associated with different pathways and that HIV-associated immune dysfunctions may be an

underlying mechanism for impaired DL_{CO} in HIV+ patients. However, when preserved vs. impaired DL_{CO} groups were compared, two pathways seemingly unrelated to HIV or lung function were found to be enriched: the Reactome olfactory signaling pathway and the KEGG olfactory transduction pathway. Thus, it was unclear from their study which genes are associated with lung dysfunction within HIV+ patients.

There are many reasons that could explain why our results differ. First, whole blood was used by Crothers *et al.* whereas we used PBMCs only. In addition to PBMCs, whole blood contains a large percentage of granulocytes, which might mask gene expression differences that are only present in subsets of cells. Also, there were differences in the immune cell compositions between the groups in the HIV+ and HIV− groups of the Crothers *et al.* study. Finally, we took a targeted gene approach, which in principle is more susceptible to false discovery.

Elevated interferon signaling pathway in HIV-associated impaired DL_{CO}

IFNs have many antiviral functions, and a rapid IFN response during the acute HIV infection phase is critical to infection control. However, prolonged IFN signaling has multiple detrimental downstream effects. In addition to inflammation and immune activation, IFN-stimulated gene expression is correlated also with increased apoptosis and T cell immune exhaustion. IFNα promotes the expression of the immune exhaustion marker PD1 and leads to a weakened T cell response [49]. This effect is not restricted to virus-specific $CD8^+$ T cells, as the transition from effector to memory $CD8^+$ T cells in the nonvirus-specific bystander $CD8^+$ T cells is also impaired [50,51]. These earlier findings of chronic HIV-associated inflammation and immune exhaustion prompted us to ask if the gene expression levels of these pathways contribute to HIV-associated impaired DL_{CO}. Although we did not find differences in immune exhaustion markers, our data showed increased inflammation and immune activation in the $DL_{CO} <$ LLN group, which is consistent with findings using plasma biomarkers [16]. More importantly, the upregulation of multiple ISGs suggests that sustained IFN signaling upstream of inflammation and immune exhaustion pathways contributes to the molecular mechanism, driving impaired DL_{CO} in PWH. Nevertheless, how IFN-induced inflammatory and immune exhaustion pathways might translate into pulmonary diffusion defects remains to be elucidated.

B-cell perturbations in DL_{CO}

B cells are not directly infected by HIV, but abnormalities in B-cell phenotypes and functions have been reported in PWH. Peripheral B-cell numbers are lower in chronic HIV patients and are only partially restored with ART [52]. In addition, the frequencies of subsets of B cells are altered [53,54]. Our results of lower expression levels of genes exclusively expressed in B cells and lower frequency of B cells in $DL_{CO} <$ LLN HIV+ patients are consistent with the idea that B-cell perturbation caused by HIV is associated with diffusion capacity abnormality.

Strengths, limitations, and future directions

Our study is the first to report upregulation of the IFN pathway in PWH with abnormal DL_{CO}, providing preliminary evidence for a potential therapeutic target to treat this prevalent HIV-associated lung dysfunction. Our study has several strengths. By design, all participants are HIV+, which allowed us to directly investigate underlying biological causes for why some HIV+ patients have impaired diffusing capacity compared with others with normal DL_{CO}. Additionally, the normal and abnormal DL_{CO} groups largely do not differ demographically or clinically. Notably, $CD4^+$ count, $CD4^+/CD8^+$ ratio, and the percentage of patients with detectable viral load – all important indicators of immune reconstitution and viral control – are not significantly different between the groups (Table 1). Thus, our findings of upregulation of the IFN pathway shed light on its potential role in driving HIV-associated diffusing abnormalities, independent of other clinical factors. Lastly, in addition to the NanoString's data analysis module, we added more in-depth analyses using DAVID and GSEA. The convergence of these approaches onto the finding that IFN pathways are upregulated in abnormal DL_{CO} further strengthens our conclusion.

Our study has a few limitations as well. As a pilot study with a small sample size, we were unable to look at differences in DL_{CO} in the context of spirometric measures. Although the post-BD FEV1/FVC ratio did not significantly differ between the normal and abnormal DL_{CO} groups, the mean post-BD FEV1% predicted was slightly decreased in the impaired DL_{CO} group. A larger sample size would allow us to examine different combinations of spirometric phenotypes with DL_{CO} measurements, specifically analyzing patients with normal spirometry and an isolated reduction in DL_{CO} (iso↓ DL_{CO}).

Another limitation is that we only included HIV+ patients in this study. Although this study design allowed us to investigate gene expression patterns associated with DL_{CO} impairment within the HIV+ population, it prevents us from investigating whether the molecular underpinnings of diffusion abnormalities differ between those with and without HIV. Future, larger, studies with both HIV+ and HIV− patients and a range of lung dysfunction will help further elucidate the molecular mechanisms driving lung dysfunction.

Finally, we used PBMCs for the gene expression measurement because they can be easily obtained. However, PBMCs contain multiple cell types, and we do not know which cell types are responsible for the

observed IFN pathway upregulation nor the functional consequences of this regulation within the specific cell types. Our main conclusion of upregulation of IFN pathway in DLco < LLN group is based on the NanoString technology, which measures the abundance of RNA without PCR amplification. Ideally, the upregulation of each gene should be validated through another technology, for example, RT-qPCR. Elevated expression of multiple ISGs collectively and not individually, supports our conclusion of upregulation of the IFN pathway. In the future, single-cell analyses will be critical to reveal granular pictures of immunological, cellular, and molecular mechanisms. Finally, a finer understanding of how gene expression changes in the peripheral blood are mechanistically connected to lung function abnormality will require a deeper investigation of both the blood and lung environment.

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Conflicts of interest

There are no conflicts of interest.

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