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The elevated systemic cytokine levels in HIV patients are not associated with an elevated pulmonary cytokine environment.

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THE ELEVATED SYSTEMIC CYTOKINE RESPONSE IN HIV PATIENTS IS NOT ASSOCIATED WITH AN ELEVATED PULMONARY CYTOKINE RESPONSE

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Sources of funding: National Institutes of Health (NIH) grant NHLBI # 1 U01 HL121807-01**HIGHLIGHTS**

- Plasma levels of several cytokines and chemokines were elevated in HIV-seropositive subjects.

- No significant differences in inflammatory markers (cytokines, chemokines, total protein, cell numbers) were observed in the lungs of HIV-positive *vs.* negative subjects.
- The viral load and BMI may be important factors modulating systemic and lung inflammatory responses in HIV-seropositive individuals.

ABSTRACT

Background: HIV-positive patients on anti-retroviral therapy (ART) are at higher risk of developing many non-AIDS related chronic diseases, including chronic obstructive pulmonary disease (COPD), compared to HIV-negative individuals. While the mechanisms are not clear, a persistent pro-inflammatory state appears to be a key contributing factor. The aims of this study were to investigate whether HIV-positive patients without COPD present evidence of potentially predisposing abnormal pulmonary cytokine/chemokine responses and to explore the relationship between pulmonary and systemic inflammation.

Methods: This study included 39 HIV-seropositive and 34 HIV-seronegative subjects without COPD. All were subjected to outpatient bronchoscopy with bronchoalveolar lavage fluid (BALF) aspiration and blood sample collection. The levels of 21 cytokines and chemokines were measured in plasma and BALF using a bead-based multi-analyte assay.

Results: In plasma, HIV-infected patients showed significantly increased circulating levels of pro-inflammatory (TNFα) and Th1-associated cytokines (IL-12p70) as well as several chemokines (CXCL11 and CX3CL1). However, no statistically significant differences were found in the numbers of cells, the concentrations of protein and urea as well as cytokine levels in the BALF of HIV-positive patients when compared to controls. Correlation analysis indicated a potential effect of the viral load and a modulatory effect of the BMI on proinflammatory markers in HIV-seropositive individuals.

Conclusions: While our results are consistent with the existence of a systemic pro-inflammatory state in HIV-infected patients, they did not detect significant differences in inflammatory markers in the lungs of HIV-positive individuals when compared to HIV-negative controls.

Key words: Chronic lung disease (CLD) , chemokines, cytokines, HIV, inflammation.

1. INTRODUCTION

Thanks to the emergence of ART, the survival of HIV-positive patients has been significantly extended and HIV infection has become a chronic and manageable disease [1-3]. However, when compared to uninfected individuals, HIV patients on ART manifest a higher prevalence of many non-AIDS related chronic diseases, including cardiovascular disease (CVD), diabetes, metabolic disorders, liver steatosis, osteoporosis, obstructive lung diseases, neurocognitive disorders and some types of cancer [4-8]. While the actual mechanisms responsible for these complications are not completely clear, the common denominator appears to be chronic inflammation [6,10]. In fact, while ART is able to suppress viral replication to below detection limits in blood, the immune system of patients with chronic HIV never truly achieves complete quiescence and rather appears to be in a persistently hyper-reactive state [7,11]. In addition, endothelial cells and the coagulation system, which interact closely with the immune system, appear to be chronically-activated as well [9,12,13].

The reasons for the enhanced immune activation in patients with HIV appear to be multifactorial. For example, even low level or even abortive HIV infection, through the presence of viral HIV-1 RNA or DNA, may induce immune activation via pattern recognition receptors (TLR-7 and TLR-9) and activation of caspase-1 [14,15]. Increased intestinal permeability and defects in intestinal immune homeostasis secondary to the loss of mucosal Th17 cells have been thought to lead to enhanced microbial translocation from the gut, resulting in immune activation [6,10,16,17]. In addition, secondary co-infections, $CD4^+$ T cell lymphopenia, immunosenescence and immune dysregulation may also contribute to persistent immune activation [6,10]. Understanding of the mechanisms responsible for the persistent inflammation and immune activation should be key in identifying potential therapeutic targets and designing therapeutic strategies in order to reduce associated comorbidities in HIV patients.

In the ART era, obstructive lung disease has become a significant cause of morbidity and mortality in HIV-positive individuals. Moreover, several studies have suggested that HIV infection is an independent risk factor for chronic obstructive pulmonary disease (COPD) [18]. In acutely infected patients, HIV is known to promote pulmonary inflammation, particularly in the form of lymphocytic alveolitis, characterized by infiltration with virus-specific CD8+ T lymphocytes and activation of alveolar macrophages [8,19]; while decreasing CD4+ T cell numbers and immune function result in increased susceptibility to AIDS-defining pulmonary infections. Following ART, patients experience a significant reduction in viral titers, normalization of CD4+ T cell numbers and a return to a seemingly normal lung environment, albeit with reported persistent low-level production of IFNγ and induced chemokines, likely as a result of low-level viral persistence [19]. To what extent a systemic pro-inflammatory state contributes to pulmonary inflammation and the increased risk for chronic lung disease (CLD) in ART-treated HIV-patients is unclear. Nevertheless, it is important to point out that the mechanisms responsible for the association of chronic HIV infection and CLD are likely to be multiple, involving not only lung inflammation, but a combination of excess risk behaviors, increased susceptibility to pulmonary infections, lung epithelial cell injury, altered anti-oxidant/oxidant balance and direct effects of ART [8,18].

We undertook the present study in order to investigate whether HIV-positive patients on ART without CLD present evidence of abnormal pulmonary cytokine or chemokine responses and to explore the relationship between those responses in the pulmonary and systemic compartments, using a subgroup of subjects participating in a study of HIV-induced lung disease.

2. MATERIALS AND METHODS

2.1 Study Design

This was a cross-sectional study analyzing the data of a subgroup of subjects included in the study "The Impact of Oxidative Stress on HIV-Induced Lung Disease" performed at the University of Louisville in Louisville, Kentucky, USA [20]. The study enrolled a total of 132 subjects from February 2014 to October 2016, including both HIV-seropositive and seronegative patients with or without CLD (defined as FEV1/FVC ratio of less than 0.70 [21]). Only the subgroup of patients without COPD (n=73), including 39 HIV-seropositive and 34 HIV-seronegative subjects was included in the present study. HIV-seropositive individuals had been a median of eight years under ART and were stable from an immunological standpoint. Patients were excluded if they had a medical history of cardiovascular disease (other than controlled hypertension), acute infection, cancer, or other malignancy-related disorders. Subjects were seen in an outpatient basis and were stable from a respiratory standpoint. During their visit and after providing informed consent, the subjects underwent spirometry, blood sample collection and outpatient bronchoscopy with BALF aspiration. The study was approved by the University of Louisville's Institutional Review Board (IRB), approval # 13.0442. All procedures were in accordance with ethical standards of the University of Louisville IRB and the Helsinki Declaration of 1975, as revised in 2000.

2.2 Samples

Plasma samples: Venous blood (4 mL) was collected using Vacutainer EDTA tubes. Blood samples were transported within 30 minutes of collection to the laboratory for processing. Following centrifugation at 300 x g for 10 min, the plasma was separated by aspiration, aliquoted and stored frozen at -80^{o}C until assayed.

Bronchoalveolar lavage fluid**:** BALF collection was performed as previously described [20]. Briefly, following anesthesia (lidocaine 1% solution) of the vocal cords, a bronchoscope was wedged into a distal segment of a bronchus. Then, a total of 180 ml of sterile saline solution was instilled into a lobe of the lung. After gentle aspiration, the lavage was collected into three 50 mL and two 15 mL centrifuge tubes. The aspirates were then pooled, filtered through sterile gauze and centrifuged at 2,500 x g for 15 minutes. The cell pellets were resuspended in phosphate-buffered saline (PBS) and the total number of nucleated cells determined in a Bio-Rad TC20 automated cell counter (Bio-Rad Laboratories, Hercules, CA). The supernatants were aliquoted and stored at -80^{o}C until assayed.

2.3 Cytokine measurements

Plasma and BALF samples were thawed and centrifuged at 10,000 x g for 5 minutes prior to use in the assays. The concentrations of twenty-one different cytokines and chemokines in serum and BAL samples were measured using Milliplex MAP High Sensitivity Human T cell panel kits (HSCYTMAG-28SK, EMD Millipore, Billerica, MA) according to the manufacturer's instructions. The measured cytokines and chemokines included: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, IL-17A, IL-21, IL-23, IFNγ, TNFα, GM-CSF, CCL3 (MIP-1α), CCL4 (MIP-1β), CCL20 (MIP-3α), CXCL11 (I-TAC) and CX3CL1 (Fractalkine). Cytokine/chemokine levels in plasma and BALF were reported in picogram per milliliter (pg/mL). Additionally, cytokine levels in BALF were expressed as pg/mg of protein or pg/ng urea after adjusting for protein or urea concentration, respectively. The minimum detectable concentrations of cytokines were (all in pg/mL): IL-1β (0.14), IL-2 (0.19), IL-4 (1.12), IL-5 (0.12), IL-6 (0.11), IL-7 (0.42), IL-8 (0.13), IL-10 (0.56), IL-12p70 (0.15), IL-13 (0.23), IL-17A (0.33), IL-21 (0.14), IL-23 (3.25), IFNγ (0.48), TNFα (0.16), GM-CSF (0.35), CCL3 (0.94), CCL4 (0.67), CCL20 (0.83), CXCL11 (1.25) and CX3CL1 (8.17).

2.4 Protein and Urea measurements

The concentration of total protein in BALF supernatants was measured colorimetrically using the Bio-Rad Protein Assay dye reagent and a bovine serum albumin protein standard according to the manufacturer's instructions (Bio-Rad, Hercules, CA). The concentration of urea in BALF was measured using a colorimetric Urea Assay kit (Cat. No. MAK006, Sigma-Aldrich, St. Louis, MO) and reading at 570 nm in a Beckman Coulter AD340C Absorbance detector (Beckman Couler, Inidianapolis, IN).

2.5 Statistics.

Categorical variables summarized with frequencies and percentages and differences between groups were tested using Fisher's exact test. Continuous variables (cytokine data) are summarized as means and standard deviations or as medians and intraquartile ranges. Data distribution was analyzed using the D'Agostino and Pearson omnibus normality test. Statistical comparisons between the groups were performed using the Mann Whitney U-test. Spearman's correlations were calculated between plasma cytokines, BALF cytokines, viral load, CD4 count, and body mass index (BMI). The two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli was used to control for false discovery rate (FDR, Q=5%) [22]. *p*-values of <0.05 were considered statistically significant. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA) and R version 3.3.2 (www.r-project.org).

3. RESULTS

3.1 Patient Characteristics

The demographics and clinical characteristics of the subjects are shown in **Table 1**. HIV-seropositive subjects were overall older than HIV-seronegative ones (mean age 49.5±8.5 *vs.* 40.5±11.6 years old [*p*=0.004]; range 21.3-64.3 *vs.* 24.6-61.0 years old, respectively). Twenty-six (69%) subjects from the HIV-seropositive group and 16 (47%) from the HIV-seronegative group were male (*p*=0.103). Fourteen

(41%) subjects from the HIV-seropositive group and eight (24%) from the HIV-seronegative group identified themselves as White; while 22 (59%) and 26 (74%) identified themselves as African American in the HIV- seropositive and seronegative groups, respectively. The two groups did not differ statistically in terms of BMI (average BMI of 28.1 *vs.* 30.0 for HIV-seropositive and seronegative patients, respectively; p=0.151). With the exception of a higher prevalence of hepatitis C in the HIV-seropositive group (n=13 *vs.* 1; p=0.001), the two groups did not differ statistically with respect to history of comorbidities nor in terms of smoking, alcohol or drug abuse history. The HIV-seropositive group had a median (IQR) of 8 (12.5) years on ART and 13 patients had previous history of AIDS-defining illness. The median $CD4^+$ T cell count in the HIV-positive group was 550/mm^3 (IQR: 362/mm^3) and HIV viral loads ranged from undetectable to 35,500/mL (mean 2846/mL).

3.2 Plasma cytokine profiles in chronic HIV-infected patients show significant differences with those of HIV-negative controls

The plasma cytokine profiles for both groups are shown in **Table 2**. Generally, patients in the HIV-seropositive group showed a pattern consistent with enhanced systemic inflammation and/or immune activation. Even after controlling for the false discovery rate, the HIV-seropositive group had statistically significant higher levels of pro-inflammatory (TNFα), Th1-associated cytokines (IL-12p70) and two chemokines (CXCL11, an IFNγ-induced chemokine, and CX3CL1) compared with HIV-seronegative controls.

3.3 BALF cytokine profiles in chronic HIV-infected patients do not show significant differences with those of HIV-negative controls

As depicted in **Table 3 (upper panel)**, the composition of the BALF recovered from the HIV-seropositive group did not differ significantly from that of the HIV-seronegative group in terms of total numbers of nucleated cells, protein and urea concentrations. The rate of detectability of the 21

measured cytokines in BALF samples varied widely. While some cytokines and chemokines were detectable in the majority of subjects, some were measurable in only a few. **Table 3** shows the number of patients in which each of the measured cytokines/chemokines was detectable (above the minimum detectable concentration [MDC]) (lower left panel) as well as the median levels, expressed in pg/mL, for those cytokines with detectable levels in at least 5 or more patients (lower right panel). Overall, the two groups did not significantly differ from each other in terms of detectability nor in the levels of cytokines and chemokines measured. Even after normalizing BALF cytokine and chemokine levels to the BALF protein or urea concentrations, no significant differences were observed between the two groups **(Supplemmentary Tables 1 and 2**, respectively). While there seemed to be higher levels of IL-13 and IL-7 in the HIV-seropositive group when adjusted for protein (p<0.05, Mann-Whitney), these differences were not significant after controlling for FDR.

3.4 Correlations of Systemic and BALF cytokine levels

Results of a correlation analysis among plasma and BALF cytokines as well as other important variables are shown in **Table 4**. For all cytokines measured in both the HIV-seropositive and seronegative groups, no significant correlation was found between their levels in plasma and those in BALF. When the correlations between cytokines levels in plasma and BALF of HIV-seropositive patients with infection parameters (CD4+ T cell count and viral load) were analyzed, no significant correlations were found. However, there appeared to be suggestive correlation tendencies (defined here as p<0.1) of the viral load with TNFα levels in both plasma and BALF as well as the total BALF cell count. Inclusion of the BMI in the correltions analyses showed a statistically significantly negative correlation with the BALF levels of two chemokines, IL-8 and CCL20, the total cell counts in BALF, as well as with the viral load in the HIV-group.

4. DISCUSSION

The aim of this study was to investigate the lung and systemic cytokine patterns, as measured in BALF and plasma respectively, in HIV-infected subjects without CLD and other chronic lung disorders participating in a larger study of HIV-induced lung disease. The HIV-positive group was compared to a control, HIV- negative group, also without chronic lung disease. HIV-positive patients had significantly increased circulating levels of several cytokines, including pro-inflammatory (TNFα), Th1-associated cytokines (IL-12p70) as well as several chemokines (CXCL11, and CX3CL1). In contrast, our study did not find statistically significant differences in BALF cytokine levels between HIV-positive and negative subjects, either when expessed as pg/mL or when normalized to both protein or urea concentrations. No significant correlations were found between the levels of cytokines in plasma and those in BALF and between plasma or BALF cytokines and the $CD4^{+}$T cell count in HIV-positive patients. Although correlations between the viral load and cytokines did not reach statistical significance (p<0.05), there were tendencies (p<0.1) for moderate correlations with both, plasma and BALF TNFα levels, as well as with the BALF total cell count.

The main contribution of our study to the literature was the finding that despite increased plasma levels of pro-inflammatory cytokines and chemokines, cytokine profiles in the lungs of HIV-positive patients without CLD do not differ substantially from HIV-seronegative controls. The higher systemic levels of cytokines in the HIV-infected group were consistent with the persistent inflammation and immune hyperreactivity that has been reported in chronically-infected HIV patients [6,7,11]. This immune activation is considered to multifactorial, including a low-level or abortive HIV infection, microbial translocation from the gut, co-infections by other microbes, immunosenescence and dysregulation of immunoregulatory pathways [6,7]. The elevated levels of TNFα and the chemokines, signs of chronic inflammation, may arise from macrophage activation due to bacterial translocation from

the gut and/or to other causes including pyroptosis of $CD4^+$ T cells [7,23]. The finding of increased levels of Th1-associated cytokines (IL-12p70) may be influenced by lingering viral infection and/or by the general hyper-responsive state of the immune system. In this respect, although we did not find a significant correlation between the viral load and IFNγ, there was a tendency for a modest correlation with plasma levels of CXCL11, itself an IFNγ-induced chemokine.

Notwithstanding our results demonstrating systemic inflammation in HIV-infected patients, we were unable to detect a significant inflammatory response in the lungs of HIV-positive patients. In fact, our study did not find statistically significant differences in BALF cytokine or chemokine levels between HIV-infected and control groups. Moreover, the lack of correlation between the levels of serum and BALF cytokines suggest that at least in HIV-positive subjects who do not present with pulmonary symptoms, the lung remains separate from the systemic compartment in terms of cytokines and is probably not the source of the increased systemic cytokine levels.

It is interesting to point out that, while not statistically significant in our study ($p>0.05$), there was a tendency ($p<0.1$) for a positive correlation between the viral load with the levels of TNFα in both plasma and BALF, suggesting that perhaps low-level viremia may be an important factor driving a pro-inflammatory environment, mainly systemically, but potentially also in the lung. Thus, while HIV-infected patients may not have an overt inflammatory response in the lung, persistent viremia, even if low-level, might subtly contribute along with other risk factors to the risk of obstructive lung disease. These findings reagarding the potential role of the viral load in the lung environment need to be further substantiated.

The BMI seems to be an important factor, not only in HIV infection, but also the development of CLD. In fact, BMI has been reported to be negatively correlated with COPD in HIV-infected patients [24]. Moreover, the BMI has been indirectly correlated with both morbidity and mortality in COPD patients

[25-27]. These observations have suggested that adiposity may have some moderating effects on the diseease. Our results showing a negative correlation between BMI and the levels of two pro-inflammatory chemokines (IL-8 and CCL20) and the total number of nucleated cells in the BALF as well as the viral load in the HIV-positive subjects suggest that the BMI may potentially have a "protective" or modulatory effect on the HIV infection and on the risk of lung complications, potentially by modulating inflammation. The mechanistic bases of such phenomenon remain to be better understood.

4.1 Limitations of the Study

There are several limitations to our study . First, there were significant differences in age between the two study groups, with HIV-seropositive patients being older (average age 49.5±8.5 vs. 40.5±11.6 years old) than the HIV-seronegative controls. Although not consistent, there is evidence that circulating levels of pro-inflammatory, T-cell derived cytokines and chemokines generally increase with age, albeit most studies have compared children to adults [28] or a wider age range than the difference in the two groups in our study (e.g, <30 to >60 years old) [29-31]. Nonetheless, it is still plausible that the age difference may have also contributed to the differences observed in our study. Second, although there were no significant differences in terms of smoking history between the two groups, the fact that most of the patients included in the study were smokers or ex-smokers, may have led to increased cytokine levels in the lung, preventing the detection of baseline differences. Finally, the relatively low numbers of subjects in this study is a limitation and further studies are necessary to establish the validity of these results.

4.2 Conclusions

In summary, while the results of this study are consistent with previous reports of the chronic systemic inflammation and immune hyperresponsiveness of HIV-positive patients, our results were unable to establish that these patients have significant levels of pulmonary inflammation. However,

there might still be subtle influences related to chronic HIV infection (e.g., viral load and TNFα production) or the BMI of the patient, that might modulate the risk of CLD in HIV-positive patients.

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Table 1. Demographic Characteristics

	Control Subjects	HIV+ patients	*p*-value
n	34	39	
Age (years)	40.5±11.6	49.5±8.5	0.004**
Sex (male)	16	26	>0.05
(female)	18	13	
Ethnicity;			
Caucasian	8	14	
African American	26	22	
Native American	0	1	
BMI	30.0±8.5	28.1±9.5	>0.05
CD4 count [median (IQR)]		550 (362)	
CD4% [median (IQR)]		26.3 (16.5)	
HIV Viral load [median (IQR)]		20 (280)	
Years on ART [median (IQR)]		8 (12.5)	
Asthma history	11	8	>0.05
Bacterial pneumonia history	5	11	>0.05
Hypertryglyceridemia	1	4	>0.05
Lipodystrophy	0	1	>0.05
Hypercholesterolemia	5	5	>0.05
Diabetes	5	2	>0.05
Hypertension	10	20	>0.05
Hepatitis C	1	13	0.001**
Hepatitis B	0	2	>0.05
Alcohol history	1	5	>0.05
Drugs history	11	21	>0.05

** $p<0.01$

Table 2. Plasma Cytokine Analysis

Cytokine	Control (pg/mL)	HIV+ (pg/mL)	*p*-value
Innate			
IL-1β	1.1 (0.7)	1.4 (1.7)	0.041*[&]
IL-6	0.9 (1.3)	1.6 (1.7)	0.072
TNFα	3.7 (2.7)	5.8 (4.4)	0.010*[$]
IL-10	5.3 (3.9)	7.9 (12.0)	0.039*[&]
Th1			
IL-12p70	0.9 (1.8)	1.5 (3.5)	0.004**[$]
IFNγ	9.5 (7.1)	12.5 (18.4)	0.039*[&]
Th2			
IL-4	1.8 (4.1)	10.0 (19.2)	0.020*[&]
IL-5	0.7 (1.8)	1.4 (1.8)	0.057
IL-13	1.9 (2.9)	1.7 (3.0)	0.432
Th17			
IL-17A	2.5 (4.9)	2.2 (7.9)	0.298
IL-23	16.7 (33.6)	28.5 (133.9)	0.061
Other			
IL-2	0.9 (0.9)	0.8 (1.6)	0.746
IL-7	4.3 (3.2)	4.9 (8.2)	0.296
IL-21	0.5 (0.7)	0.7 (1.8)	0.068
GM-CSF	24.3 (47.0)	35.1 (33.6)	0.220
Chemokines			
CXCL8 (IL-8)	2.9 (2.2)	3.6 (3.5)	0.263
CXCL11 (ITAC)	15.2 (27.2)	35.1 (41.6)	0.007**[$]
CX3CL1 (Fractalkine)	35.7 (47.9)	71.9 (99.5)	0.004**[$]
CCL3 (MIP-1α)	14.1 (7.4)	14.6 (9.2)	0.956
CCL4 (MIP-1β)	9.3 (6.1)	11.5 (5.0)	0.022*[&]
CCL20 (MIP-3α)	18.4 (17.8)	22.9 (24.7)	0.147

Cytokine concentration values represent medians (IQR).
*p<0.05, ** p<0.01, [&]q>0.05, [$]q<0.05

Table 3. BALF Composition and Cytokine Analysis

Component	Control Subjects	HIV+ patients	*p*-value
Total cells (x 10^6)	74.9 (55.1)	56.8 (58.2)	0.241
Total protein (μg/mL)	113.3 (78.3)	101.9 (98.7)	0.407
Urea (ng/mL)	3.1 (5.2)	5.3 (3.9)	0.160

Cytokine	Control (>MDC) n=34	HIV+ (>MDC) n=39	*p*-value	Control (pg/mL)	HIV+ (pg/mL)	*p*-value
Innate						
IL-1β	10	10	0.796	0.4 (0.8)	0.3 (0.4)	0.274
IL-6	31	35	1.000	2.2 (3.3)	2.2 (4.1)	0.857
TNFα	32	34	0.438	0.7 (0.5)	0. 8 (0.7)	0.324
IL-10	9	7	0.410	1.5 (4.1)	2.1 (11.2)	0.295
Th1						
IL-12p70	4	1	0.177	-	-	-
IFNγ	7	6	0.760	1.0 (2.1)	2.3 (4.1)	0.394
Th2						
IL-4	11	15	0.631	3.2 (1.4)	2.8 (1.3)	0.721
IL-5	3	2	0.659	-	-	-
IL-13	7	5	0.529	0.4 (0.3)	0.3 (0.1)	0.751
Th17						
IL-17A	3	1	0.333	-	-	-
IL-23	3	1	0.333	-	-	-
Other						
IL-2	5	4	0.725	0.2 (2.4)	0.4 (1.4)	0.556
IL-7	17	11	0.091	1.0 (5.2)	4.6 (12.6)	0.182
IL-21	11	17	0.347	1.0 (4.5)	0.3 (1.5)	0.447
GM-CSF	28	26	0.182	0.7 (0.5)	0.7 (0.4)	0.551
Chemokines						
CXCL8 (IL-8)	34	39	1.000	22.9 (33.6)	30.2 (35.8)	0.285
CXCL11 (ITAC)	18	22	0.817	2.3 (2.0)	2.8 (2.9)	0.424
CX3CL1 (Fractalkine)	15	16	0.816	17.7 (58.3)	11.8 (67.2)	0.429
CCL3 (MIP-1α)	27	26	0.295	2.9 (2.1)	2.7 (3.2)	0.351
CCL4 (MIP-1β)	19	23	0.816	1.9 (1.4)	1.7 (2.0)	0.713
CCL20 (MIP-3α)	21	29	0.315	5.3 (14.1)	4.6 (12.4)	0.667

MDC : Minimum detectable concentration

Cytokine concentration and other values represent medians (IQR). Results shown only for those cytokines with at least 5 values above the MDC.

Table 4. Summary of correlations in HIV-seropositive patients

	BALF Cytokines	CD4+ T Cells	Viral load	BMI
Plasma cytokines	None	None	TNFα (r: 0.318)* CXCL11 (r: 0.316)*	None
BALF cytokines	-	None	TNFα (r: 0.331)*	IL-8 (r: -0.377)** CCL20 (r: -0.478)**
BALF cell number	-	None	(r: 0.301)*	(r: -0.416)**
Viral load	-	None	-	(r: -0.434)**

*p<0.1, **p<0.05

Supplementary Table 1. BALF Cytokine Analysis – Values Normalized to Protein Concentration

Cytokine	Control (>MDC) n=34	HIV+ (>MDC) n=39	*p*-value	Control (pg/mg prot)	HIV+ (pg/mg prot)	*p*-value
Innate						
IL-1β	10	10	0.796	2.9 (2.3)	2.3 (4.2)	0.962
IL-6	31	35	1.000	24.3 (25.3)	22.3 (41.3)	0.995
TNFα	32	34	0.438	4.9 (4.6)	7.7 (7.7)	0.100
IL-10	9	7	0.410	9.7 (23.6)	44.0 (66.7)	0.128
Th1						
IL-12p70	4	1	0.177	-	-	-
IFNγ	7	6	0.760	3.8 (12.0)	17.8 (30.4)	0.126
Th2						
IL-4	11	15	0.631	23.5 (25.9)	26.5 (25.8)	0.233
IL-5	3	2	0.659	-	-	-
IL-13	7	5	0.529	1.1 (1.2)	2.0 (3.6)	0.032*
Th17						
IL-17A	3	1	0.333	-	-	-
IL-23	3	1	0.333	-	-	-
Other						
IL-2	5	4	0.725	-	-	-
IL-7	17	11	0.091	9.7 (22.8)	53.8 (73.5)	0.036*
IL-21	11	17	0.347	1.8 (4.5)	2.0 (12.5)	0.182
GM-CSF	28	26	0.182	6.0 (6.5)	6.0 (6.9)	0.922
Chemokines						
CXCL8 (IL-8)	34	39	1.000	207.3 (369.2)	267.3 (311.5)	0.386
CXCL11 (ITAC)	18	22	0.817	21.4 (19.5)	27.9 (26.2)	0.354
CX3CL1 (Fractalkine)	15	16	0.816	89.3 (142.6)	88.5 (345.6)	0.717
CCL3 (MIP-1α)	27	26	0.295	22.4 (29.3)	19.5 (28.2)	0.893
CCL4 (MIP-1β)	19	23	0.816	16.7 (9.5)	15.6 (28.4)	0.563
CCL20 (MIP-3α)	21	29	0.315	32.2 (106.9)	53.9 (163.6)	0.671

MDC : Minimum detectable concentration

Cytokine concentration values represent medians (IQR). Results shown only for those cytokines with at least 5 values above the MDC.

*$p<0.05$, q=0.302

Supplementary Table 2. BALF Cytokine Analysis – Values Normalized to Urea Concentration

Cytokine	Control (>MDC) n=34	HIV+ (>MDC) n=39	*p*-value	Control (pg/ng urea)	HIV+ (pg/ng urea)	*p*-value
Innate						
IL-1β	10	10	0.796	0.16 (0.29)	0.08 (0.12)	0.274
IL-6	31	35	1.000	0.65 (1.28)	0.43 (0.79)	0.348
TNFα	32	34	0.438	0.17 (0.20)	0.18 (0.22)	0.686
IL-10	9	7	0.410	0.62 (1.75)	0.63 (4.01)	0.456
Th1						
IL-12p70	4	1	0.177	-	-	-
IFNγ	7	6	0.760	0.14 (1.20)	0.43 (1.53)	0.931
Th2						
IL-4	11	15	0.631	0.79 (0.76)	0.49 (0.12)	0.892
IL-5	3	2	0.659	-	-	-
IL-13	7	5	0.529	0.12 (2.49)	1.96 (3.64)	0.149
Th17						
IL-17A	3	1	0.333	-	-	-
IL-23	3	1	0.333	-	-	-
Other						
IL-2	5	4	0.725	-	-	-
IL-7	17	11	0.091	0.34 (0.58)	1.05 (3.13)	0.259
IL-21	11	17	0.347	0.17 (0.31)	0.08 (0.26)	0.853
GM-CSF	28	26	0.182	0.18 (0.28)	0.13 (0.36)	0.751
Chemokines						
CXCL8 (IL-8)	34	39	1.000	5.23 (13.02)	5.45 (9.77)	0.841
CXCL11 (ITAC)	18	22	0.817	0.64 (0.59)	0.66 (0.67)	0.849
CX3CL1 (Fractalkine)	15	16	0.816	4.64 (5.44)	3.27 (12.67)	0.779
CCL3 (MIP-1α)	27	26	0.295	0.99 (1.09)	0.52 (01.16)	0.296
CCL4 (MIP-1β)	18	21	1.000	0.48 (0.88)	0.38 (0.9.0)	0.954
CCL20 (MIP-3α)	21	29	0.315	0.86 (6.37)	0.88 (2.35)	0.427

MDC : Minimum detectable concentration

Cytokine concentration values represent medians (IQR). Results shown only for those cytokines with at least 5 values above the MDC.