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Unique cytokine and chemokine patterns in bronchoalveolar lavage are associated with specific causative pathogen among HIV infected patients with pneumonia, in Medellin, Colombia

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ABSTRACT

We wanted to investigate the pro-inflammatory cytokine/chemokine profile associated with the etiological agents identified in HIV patients. Immunosuppressed patients admitted to two hospitals in Medellin, Colombia, with clinical and radiographic diagnosis of pneumonia were enrolled in the study. After consent, bronchoalveolar lavage (BAL) was collected for bacterial, mycobacterial and fungal diagnosis. All patients were followed for a year. A stored BAL sample was used for cytokine/chemokine detection and measurement using commercial, magnetic human cytokine bead-based 19-plex assays. Statistical analysis was performed by assigning cytokine/chemokine concentrations levels into <25 percentile (lower), 25–75 percentile (normal) and >75 percentile (higher). Principal component analysis (PCA) and Kruskal–Wallis analysis were conducted to identify the clustering of cytokines with the various infectious etiologies (fungi, *Mycobacterium tuberculosis* – MTB, and bacteria). Average age of patients was 35, of whom 77% were male, and the median CD4 count of 33 cells/μl. Of the 57 HIV infected patients, in-hospital mortality was 12.3% and 33% died within a year of follow up. The PCA revealed increased IL-10, IL-12, IL-13, IL-17, Eotaxin, GCSF, MIP-1α, and MIP-1β concentrations to be associated with MTB infection. In patients with proven fungal infection, low concentrations of IL-1RA, IL-8, TNF-α and VEGF were identified. Bacterial infections displayed a distinct cytokine pattern and were not misclassified using the MTB or fungi cytokine patterns (p-value < 0.0001). Our results indicate a unique pattern of pro-inflammatory cytokine/chemokine, allowing differentiation between bacterial and non-bacterial pathogens. Moreover, we found distinct, if imperfectly discriminatory, cytokine/chemokine patterns associated with MTB and fungal infections.

1. Introduction

HIV is a risk factor for viral, bacterial, fungal and mycobacterial pneumonia. The risk for developing a lower respiratory tract infections decreases as antiretroviral therapy (ART) is introduced [1,2]. Moreover, even among ART treated HIV infected individuals, pneumonia continues to be a leading cause of hospital admissions; morbidity and mortality [3]. Pulmonary infections caused by bacteria, including *Mycobacterium tuberculosis,* and fungi trigger an inflammatory response, with recruitment of inflammatory cells and release of pro-inflammatory mediators. The role of chronic inflammation and systemic immune activation as a driver of premature ageing of the cardiovascular system and additional end-organs, is supported by mounting evidence [4–11]. In keeping with the role of systemic inflammation, a nested case-control study within the SMART cohort found higher hsCRP and IL-6 concentrations in individuals that developed pneumonia [12], suggesting that inflammation is a predisposing factor and not only a consequence of infection.

A recently published report of the ACTG A5164 trial, found that mycobacterial infection at study entry, number of opportunistic infections, hospitalization, lower CD4, and higher IL-8 and sTNFrII levels and lower interleukin 17 (IL-17) levels were associated with mortality. A combined model incorporating clinical

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and immunologic parameters illustrated that entry mycobacterial infection and higher soluble TNF receptor II levels are significantly associated with death [13].

Pneumocystis jirovecii (PJ) a frequent colonizer of the lung in immunocompetent individuals and a cause of pneumonia among the immunocompromised has been associated with decline of lung function and progression of chronic obstructive pulmonary disease (COPD) [14]. Increased PJ colonization was associated with gene expression of the X-family chemokines CXCL 9-11 and was correlated with greater impediment to airflow, even after adjustment for smoking status. A study of *P. jirovecii* pneumonia (PJP) among non-HIV immunocompromised patients found higher concentrations of IL-8, IL-8/IL-10 ratio, IL-1β/IL-10, IL-1β/TGF-β1 ratio, MCP-1/TGF-β1 ratio and IL-8/TGF-β1 ratio among individuals requiring mechanical ventilation [15]. The susceptibility to PJP among HIV infected individuals is the result of multiple factors playing in concert. Deficient CD4, decreased innate signaling through TLR-4, defective alveolar macrophages activity and Dectin mediated recognition of fungal D-glucans have all been implicated [16–19]. The cytokine and chemokine response in BAL has been extensively studied two in Murine models. In HIV infected individuals with PJP, high IL-8 concentrations in BAL were associated with disease severity and outcome [20]. Another study documented similar TNF-α and IL-6 release in patients with and without PJP, with markedly decreased IL-10 and beta-chemokine (MIP-1α, MIP-1β and RANTES) in the PJP group.

We enrolled HIV infected individuals admitted to hospitals in Medellin, Colombia, with a clinical and radiographic diagnosis of pneumonia. The main study was designed to improve diagnostic tests for respiratory pathogens. The aim of the current study was to investigate the pro-inflammatory cytokine and chemokine profile associated with the various etiological agents identified and to correlate with clinical outcomes.

2. Materials and methods

2.1. Population

Immunosuppressed patients with pneumonia (respiratory symptoms plus new infiltrates in the chest X rays or chest CT scan). The patients reported in this paper were enrolled in a previous study aimed at etiological diagnosis of pneumonia.

2.2. Settings

Hospital Universitario San Vicente Fundación and Hospital La Maria, Medellin, Colombia.

2.3. Ethics statement

This study was approved by the Ethics Committee from Facultad de Medicina, Universidad de Antioquia, Medellin, Colombia and University of Manitoba. The shipment of samples was approved by the Ministerio de Salud y Protección Social de Colombia.

2.4. Procedures

Per protocol in these hospitals, all patients with HIV and pneumonia undergo bronchoalveolar lavage and the sample is submitted for cytospined stains (Wright, Gram, Toluidine Blue O (TBO) and Ziehl-Neelsen) and for bacterial, mycobacterial and fungal cultures. *P. jirovecii* was diagnosed using direct fluorescent antibody and TBO stain. An aliquot of BAL was stored at −80 °C. Demographic, clinical and laboratory tests were collected from the clinical chart. All patients were followed for a year after their enrollment to the study. Only 3 individuals were on antiretroviral therapy and 10 HIV infected individuals received PJP prophylaxis prior to admission. There were only 3 participants that received 6 or more days of antibiotics before the BAL. One patient received 6 days of antibiotics before the BAL, one received 8 days, and another 12 day. The BAL was performed prior to initiation of antimicrobial therapy in 80% of the patients. Empirical antimicrobial therapy followed ATS/IDSA treatment guidelines for community-acquired pneumonia. The therapy was modified according to culture results once these became available.

2.5. Cytokine/chemokine bead arrays

Supernatants were separated from cell pellets. Cell free supernatants were aliquoted, and frozen at −80 °C, then thawed for cytokine/chemokine testing in duplicate using Human Cytokine Magnetic 19-Plex Panel, Bio-Rad® Bio-Plex Pro™. The assay was performed according to manufacturers' instructions, using 50 µl per sample. The standards were reconstituted and diluted at 7 serial concentrations as per manufacturer's instructions to generate standard curves. Standards included all recombinant cytokines tested and were considered as positive controls for the procedure. Results were reported as mean fluorescence intensity and converted to pictogram/ml concentrations using Bio-Plex® software (Bio-Plex® Manager version 6.0).

2.6. Statistical analysis

All data were analyzed using SPSS® version 21. Frequencies, median and interquartile range were estimated. The percentile 25th and 75th were calculated for all cytokines in all patients, and according to these thresholds each cytokine was classified as higher, lower or normal. Subsequently, we created three groups of microorganisms, fungi (*P. jirovecii, Histoplasma capsulatum* and *Cryptococcus neoformans*), *M. tuberculosis* (MTB) and Bacteria. A Kruskal–Wallis analysis was performed to identify differences between cytokines/chemokines levels among fungi, MTB and bacterial diagnosis. The mean ranks of each group were used to identify if the levels of cytokines between groups were higher, normal or lower. A principal component analysis (PCA) was conducted to identify which cytokines segregate to each group. The principal axis method was used to extract the components, and this was followed by a varimax rotation. We selected the rotated factor pattern that accounts for at least 10% of the total variance. In interpreting the rotated factor pattern, an item was said to load on a given component if the factor loading was 0.6 or greater for that component, and was less than 0.5 for the other. Afterwards, the cytokines that loaded in each rotated factor pattern were compared between fungi, MTB and bacterial microorganisms using the mean ranks and the Kruskal–Wallis analysis to identify lower, normal o higher cytokine levels. The criteria to select a particular cytokine for fungi, MTB or bacterial profile were to have different levels between the three groups, adjusted by CD4 count. Finally, we used the identified cytokine profile of each group to generate pathway analysis highlighting the interconnectedness of the cytokines/chemokines using Cognoscente Biomolecular Interactions online tool.

2.7. Definitions

Higher cytokine concentration: A value equal or higher than percentile 75 (upper quartile).

Lower cytokine concentration: A value equal or lower than percentile 25 (lower quartile).

Normal cytokine concentration: A value between percentile 25th and 75th. Similar use of clustering pattern of cytokines has

been used to describe mucosal inflammation as a predictor of HIV transmission in the CAPRISA 002 and 004 studies [21,22]. Another study applied quartiles of multiple cytokines in cervical lavage [23].

According to the PCA and Kruskal–Wallis analysis, we defined the following cytokine profiles:

Fungi cytokine profile: 1. Patients with lower concentrations of 3 or more cytokines (IL-1RA, IL-8, TNF-α, vascular endothelial growth factor – VEGF), or 2. Patients with lower concentration of 2 cytokines (IL-1RA, IL-8, TNF-α, VEGF) plus two or more cytokines (IL-12, IL-13, IL-17, MIP-1β) with normal levels, or 3. Patients with one cytokine (IL-1RA, IL-8, TNF-α, VEGF) with lower level plus higher concentrations of IL-7, and without elevation of any other proinflammatory cytokine.

MTB cytokine profile: Patients with higher concentrations of three or more cytokines (IL-10, IL-12, IL-13, IL-17, Eotaxin, GCSF, MIP-1α and MIP-1β).

Bacterial cytokine profile: Patients with higher concentrations of two or more cytokines (IL-1RA, IL-8 or VEGF).

Negative cytokine profile: People who did not meet the previous criteria.

Severe pneumonia: we created a composite outcome defined as alveolar-arterial gradient (A-a O2) > 45 mmHg and/or in hospital mortality and/or ICU admission.

3. Results

Among 158 immunocompromised patients, we enrolled 73 individuals meeting the diagnostic criteria of pneumonia with an identified pathogen by conventional techniques (direct microscopy, culture or histopathology of lung tissue) and available BAL sample. Fifty-seven individuals had HIV as the immunocompromising condition. Table 1 highlights the baseline characteristics of the 57 HIV infected individuals diagnosed with lung infection.

Over three quarters were male, with a mean age of 35 years. Symptoms duration was 15–60 days with a mean of 30 days. The mean CD4 count was 33 cells/μl, and an interquartile range of 16–104, indicating advanced HIV disease. More than 15% required admission to an intensive care unit and 12.3% succumbed to the infection during hospital admission, and 19/57 (33.3%) died within a year of follow-up. Thirty seven percent met the composite outcome of severe disease (either ICU admission, A-a gradient >45 mmHg or mortality). The most common pathogens were *M. tuberculosis* (47.4%), *P. jirovecii* (33.3%), *C. neoformans* (17.5%) and bacterial etiology was identified in 12.3%.

Table 2 shows the cytokine/chemokine concentrations according to lower and higher (percentile 25th and 75th).

Of the 19 measured chemokines/cytokines only 3 were not significantly different between the three groups (MTB, fungi, Bacterial) on bivariate analysis, namely IFN-γ, IL-7 and MCP-1. The 16 remaining cytokines/chemokines were statistically different between the three groups ($p < 0.05$) (Table 3).

The PCA identified five rotated factor patterns for fungi and three factors for MTB with eigenvalues greater than 1, and the results of a screening test also suggested that those factors were meaningful. Therefore, only those components were retained for rotation. Combined, the 5 factors associated with fungi in Table 4 accounted for 78.7% of the total variance (factor 1, 2, 3, 4 and 5: 20.9%, 16.6%, 15.5%, 13% and 12.7%), and the 3 factors for MTB account for 74.8% of total variance (factor 1, 2 and 3: 29.7%, 25.9%, 19.2%). In interpreting the rotated factor pattern, a cytokine

Table 1
Baseline characteristics of 57 immunocompromised HIV patients.

Variables	Value
	n (%)
Male	44 (77.2)
ICU admission	9 (15.8)
Pulmonary lung infection	
Pneumocystis jirovecii	19 (33.3)
Mycobacterium tuberculosis	27 (47.4)
Histoplasma capsulatum	1 (1.8)
Cryptococcus neoformans	10 (17.5)
Bacteria	7 (12.3)
Severity of pneumonia	
A-a O2 gradient >45 mmHg*	18 (50)
Composite outcome**	21 (36.8)
In hospital mortality	7 (12.3)
	Median (IQR)
Age in years	35 (30–44)
Days of symptoms	30 (15–60)
Leucocytes, cells/μl	5720 (4400–7800)
Neutrophils, cells/μl	4318 (2893–6170)
Lymphocytes, cells/μl	770 (572–1519)
Alveolar-arterial gradient	41.8 (14.7–174.3)
CD4 count, cells/μl	33 (16–104)

* 36 patients had data available.
** Composite outcome: A-a O2 gradient >45 mmHg and/or in hospital mortality and/or ICU admission. IQR: Interquartile range.

Table 2
Cytokine/chemokine concentrations of all 73 immunocompromised patients (including 57 infected with HIV).

Cytokines	Percentile 25th	Percentile 75th
IL-1RA	28.85	169.34
IL-6	1.16	16.61
IL-7	0.24	0.53
IL-8	33.47	428.33
IL-10	0.99	1.92
IL-12	2.47	7.14
IL-13	1.03	3.33
IL-17	2.09	4.56
Eotaxin	2.54	5.01
GCSF	9.66	54.83
IFN-γ	1.10	4.09
IP-10	65.92	2558.83
MCP-1	55.89	277.49
MIP-1α	1.20	3.95
PDGF-BB	1.60	6.39
MIP-1β	8.75	53.40
RANTES	20.80	297.12
TNF-α	1.43	3.40
VEGF	20.70	116.47

Table 3
Cytokine/chemokine concentrations of 57 HIV infected individuals.

Cytokines	Mean ranks			*P*-value (Kruskal–Wallis test)
	Fungi *N* = 25	MTB *N* = 27	Bacterial *N* = 5	
IL-1RA	22.30	31.80	47.40	0.004
IL-6	26.48	34.59	11.40	0.01
IL-7	31.08	29.30	17.00	0.154
IL-8	20.18	34.28	44.60	0.001
IL-10	21.60	36.48	25.60	0.005
IL-12	24.24	36.00	15.00	0.005
IL-13	23.36	36.80	15.10	0.002
IL-17	22.34	36.86	19.80	0.003
Eotaxin	27.28	34.63	7.20	0.002
GCSF	21.70	37.43	20.00	0.001
IFN-γ	28.86	30.89	19.50	0.243
IP-10	25.72	35.44	10.60	0.004
MCP-1	32.28	28.85	13.40	0.067
MIP-1α	25.30	34.87	15.80	0.02
PDGF-BB	24.62	35.17	17.60	0.02
MIP-1β	23.72	36.37	15.60	0.004
RANTES	27.56	33.15	13.80	0.048
TNF-α	18.86	38.31	29.40	<0.0001
VEGF	20.24	32.85	52.00	<0.0001

IQR: Interquartile range.

Table 4
Principal component analysis of 19 cytokines in fungi and *M. tuberculosis*.

Cytokines	Rotated factor pattern (Fungi)					Rotated factor pattern (MTB)		
	1	2	3	4	5	1	2	3
IL-1RA	−0.004	−0.002	−0.057	−0.018	**0.962**	0.058	0.036	0.003
IL-6	**0.911**	0.055	0.062	0.330	0.012	**0.976**	−0.015	−0.091
IL-7	0.001	**0.879**	−0.222	−0.106	−0.009	−0.052	**0.925**	0.190
IL-8	0.175	−0.041	−0.115	0.460	**0.691**	0.188	−0.047	0.575
IL-10	0.234	**0.838**	0.267	0.254	0.215	0.477	**0.787**	0.157
IL-12	−0.111	**0.893**	0.229	−0.078	0.115	0.135	**0.936**	0.044
IL-13	0.057	0.184	**0.849**	0.078	−0.053	0.237	**0.663**	0.290
IL-17	0.570	−0.199	0.572	0.273	0.297	0.486	0.299	**0.763**
Eotaxin	0.638	0.695	−0.008	0.115	−0.034	**0.656**	0.214	0.028
GCSF	0.088	0.205	0.099	−0.025	0.307	**0.948**	0.008	−0.003
IFN-γ	**0.951**	0.026	0.127	0.187	−0.017	**0.935**	0.008	0.282
IP-10	**0.875**	0.071	0.366	0.164	−0.026	**0.730**	0.085	0.403
MCP-1	0.314	0.077	0.056	**0.864**	0.001	**0.658**	0.352	0.166
MIP-1α	0.436	0.145	0.334	0.520	−0.010	0.039	0.156	**0.940**
PDGF-BB	0.105	0.034	**0.659**	−0.057	−0.141	0.046	0.666	0.680
MIP-1β	0.247	−0.042	**0.789**	0.319	0.057	0.084	0.273	**0.908**
RANTES	0.354	0.140	0.571	−0.440	−0.175	−0.062	**0.754**	0.347
TNF-α	0.370	−0.063	0.199	**0.754**	−0.062	**0.958**	0.008	0.100
VEGF	−0.113	0.461	0.027	−0.172	**0.835**	−0.012	**0.867**	0.050

Individual cytokines/chemokines that are associated with specific pathogen (Fungi or MTB) are highlighted in bold.

Table 5
Profile patterns for Fungi, *M. tuberculosis* and Bacteria based on PCA and Kruskal–Wallis analysis.

Cytokines patterns	Pulmonary infections		
	Fungi	MTB	Bacteria
Negative profile	6	6	0
Fungi profile	13	3	0
MTB profile	4	16	0
Bacterial profile	1	0	4

p-value: <0.0001. There were four patients that met the criteria of two cytokine profiles (for example MTB and Fungi) and are therefore included in both categories.

with a value of 0.6 or greater was said to load on a given component. For bacterial infections, there were three factors that account for 74% of the total variance, however, due to the small sample size these were not included in the table.

Those cytokines that have a unique pattern (higher, normal or lower) were selected for each group to create fungi, MTB or bacterial cytokine profile. Based on the aforementioned, fungi infections were associated with low IL-1RA, IL-8, TNF-α and VEGF. MTB infection was associated with high concentrations of IL-10, IL-12, IL-13, IL-17, Eotaxin, MIP-1α, MIP-1β and GCSF. Bacterial infections were associated with high concentrations of IL-1RA, IL-8 and VEGF. Bacterial infections were not misclassified using the MTB or fungi cytokine patterns. However, the fungi profile misclassified 3 MTB patients and the MTB profile misclassified 4 patients with proven fungal infection (Table 5). We adjusted for CD4 in order to determine whether the observed patterns are unique to the pathogen or the level of immune deficiency. We found that the patterns held true even after the adjustment to CD4 count suggesting they are correlated with the infecting organism. The positive predictive values for fungi, MTB and bacterial cytokine profile were 82% (61–100), 82% (63–100), 81% (63–100), and the negative predictive values were 73% (57–88), 71% (55–88), 71 (55–88).

The identified factors accounting for the variance in the cytokine pattern, for each group of pathogens was used to generate a pathway image. The generated pathway is used to examine the inter-connectedness of the cytokine/chemokines. Fig. 1. depicts the cytokines/chemokines that were elevated in those patients with the MTB infections. The pathway analysis highlights the interactions between Eotaxin, MIP-1α and MIP-1β. It also illustrates some potential interactions with HIV proteins depicted in grey.

We did not find a significant association between cytokine profile and any of the severity outcomes (A-a gradient >45, severe pneumonia, in-hospital mortality and one year mortality), but the number of patients with these evaluated outcomes was limited in each group. Patients infected with PJP had significantly higher A-a gradient ($p = 0.046$).

4. Discussion

This study was a sub-study of a cohort of immunocompromised patients with lung infections. We identified 73 patients with documented pneumonia, 57 of whom had HIV infection as the predisposing condition. HIV disease was advanced in this cohort with a very low median CD4 count of 33 cell/μl. Using the discriminatory cytokines/chemokines identified using the PCA we could ascribe a pattern to each of the groups. Fungal infections were associated with low IL-7, IL-8, TNFα and VEGF. A plethora of studies in murine models documented the cytokine/chemokine response in BAL, with abundant TNF-α, IL-1 and IL-6 release [24]. In humans with PJP, a study of 23 HIV infected individuals documented elevated IL-1 concentrations only [25]. A more contemporaneous study of 64 immunocompromised patients with PJP compared the BAL cytokine release to non-immunocompromised and to immunocompromised without PJP. IL-6 concentrations were found to be increased in the immunocompromised patients with PJP, while IL-7, IL-10, TNF-α and TGFβ did not differ between the groups [26]. Another study compared cytokine concentrations in BAL from patients with PJP in the setting of immunocompromising conditions, including malignancies, autoimmune disease and HIV. The BAL concentrations of MCP-1, IL-8, and IL-6 were higher in patients with autoimmune diseases and lower in those with HIV and AIDS, although the number of patients in the HIV groups was limited to 8 [27]. The pulmonary HIV viral load was significantly higher in the presence of PJP and correlated negatively with levels of MIP-1α, RANTES and IL-10 in BAL [28], in another study. These factors are known to enhance HIV replication and may underlie the enhanced disease progression in the context of PJP. Our results are in keeping with the lack of increase in IL-7 and TNF-α seen in the study by Iriart et al. [26], and may represent the degree of immunocompromised induced by the underlying advanced HIV rather than response to the organism. The results are also in agreement with the recent observation by Chou et al., of elevated IL-8, TNF-α and IL-1β

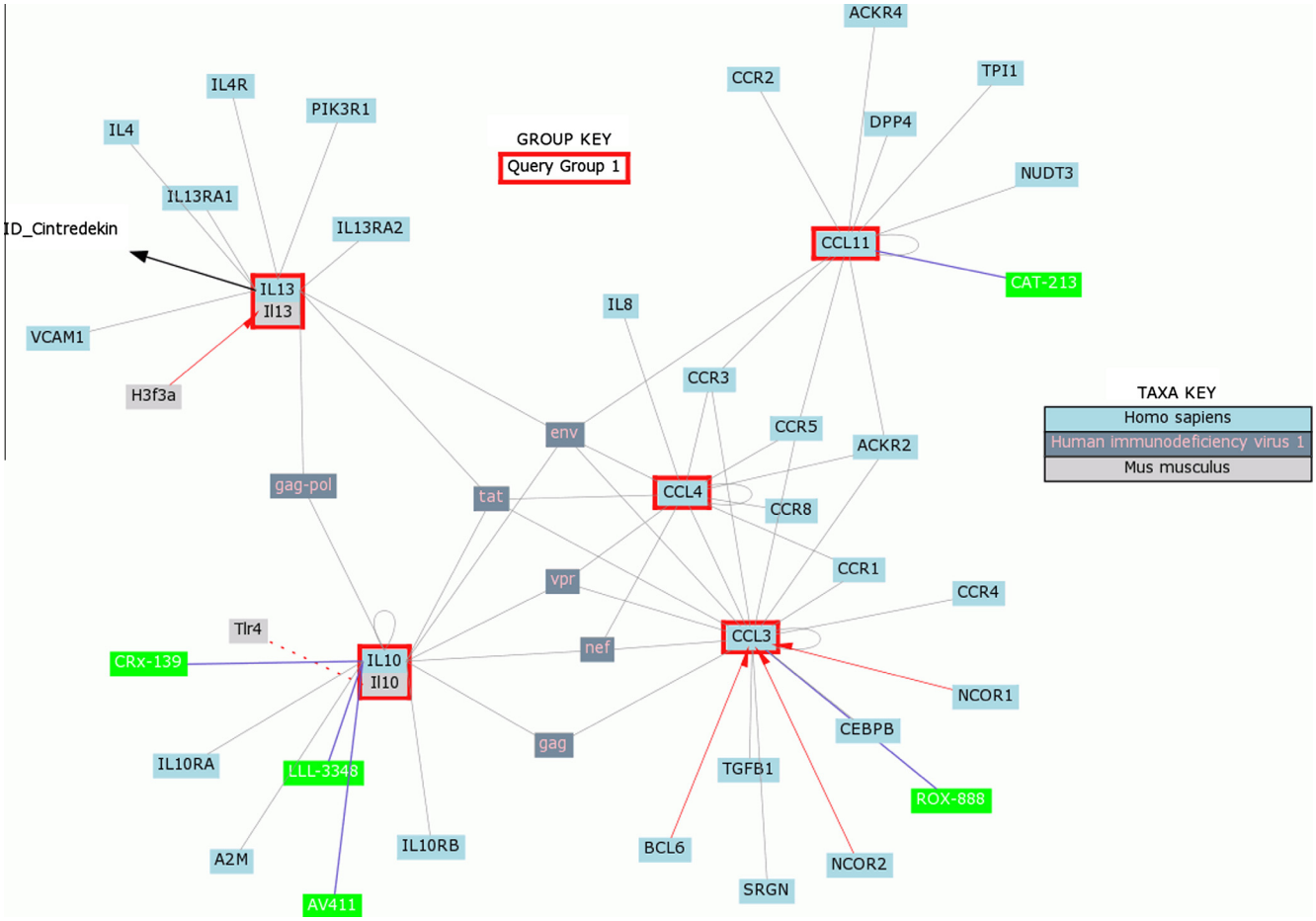

Fig. 1. High cytokine concentrations in *Mycobacterium tuberculosis* infection, presented with interaction pathway. CCL3 (MIP-1α); CCL4 (MIP-1β); CCL11 (Eotaxin). GCSF and IL-17 did not appear on the network using the specific database. Graphic Image of Cognoscente Biomolecular Interactions by [27–30] is licensed under a Creative Commons Attribution 3.0 Unported License. Based on a work at http://vanburenlab.tamhsc.edu/cognoscente.html.

only when PJP was coinfecting with another pathogen [29]. Immune-mediated inflammatory responses play an important role in the pathogenesis of PjP, and even more significant in determining the outcome of PjP than direct damage due to the organism itself [30]. Several studies have shown the pulmonary dysfunction in HIV patients with respiratory infections. Shaw et al. [31] found in 169 HIV-patients that transfer factor for carbon monoxide (TLCO) were lower in patients during the acute (13 patients) and recovery (17 patients) phases of PjP (50% and 63%). The authors could not explain the observed reductions in lung function in those with the AIDS related complex and non-pulmonary AIDS, and concluded that prognostic and predictive value of these changes needs to be evaluated. Another study by Pothoff et al. [32], showed that PjP acute episode in 18 patients compromised lung function and gas exchange impairment persisted for 1–3 months after the PjP. These studies of pulmonary infections in HIV infected individuals document a significant impact on the pulmonary function however, they did not include characterization of the inflammation in those patients. Improved understanding of the lung inflammation and the contribution of lung infections to the inflammatory pathways that are operating in the lungs carries potential implications to targeting of specific tissue damaging proinflammatory mediators, tailored to the specific infection.

MTB infection was associated with high concentrations of IL-10, IL-12, IL-13, IL-17, Eotaxin, MIP-1α, MIP-1β and GCSF. A clinical study found an association between higher levels of IL-10 in peripheral blood and the presence of HIV-TB co-infection [33], however, the elevated levels of a single cytokine could be the result of a IL-10 polymorphism. One study reports that in HIV patients with GG genotype at IL-10 (−1082) position had significantly high stimulated levels of IL-10 compared to AG and AA, and conclude that this mutation and high levels of IL-10 may increase the risk of developing TB co-infection [34]. Variability in cytokine concentrations in HIV-TB patients depends on several factors. Conesa-Botella et al. investigated the relationship between cytokine/chemokine profiles, corticosteroid use, and vitamin D deficiency in TB-immune reconstitution inflammatory syndrome (IRIS) patients receiving corticosteroid therapy pre-ART for severe tuberculosis. They found that in patients with HIV-TB co-infection that developed IRIS, IL-6, IL-8, IL-12p40, IL-18, IP-10 and TNF increased during 2 weeks of antiretroviral therapy, whereas non-IRIS patients showed increased MIP-1α and MIP-1β. Conversely, patients on corticosteroid who developed TB-IRIS showed no significant cytokine increase during the 2 weeks of ART, whereas non-IRIS patients showed significantly increased MIP-1β and decreased IL-18 [35]. The pattern identified reflects the balance between the innate cells (neutrophils, invariant NKT cells) mediated release of chemotactic factors [36,37] (Eotaxin, MIP-1α, MIP-1β and GCSF), the presence of IL-12 associated with migration of dendritic cells [38] and the signatures of loss of containment of TB infection [39]. The increased IL-13 has been shown to correlate with lung damage in post-primary tuberculosis in an animal model [40]. Despite small number of patients, the study shows the potential clinical implication of modifying the immune response in MTB. Understanding the cytokine profile associated with MTB and the clustering and interactions between the associated cytokines may allow for targeted

immunomodulation in order to attenuate the severity of the disease associated with IRIS. Recently Mayer-Barber et al. [41], published that interleukin-1 confers host resistance through the induction of eicosanoids that limit excessive type I interferon production and foster bacterial containment. Thus, IL-1 and type I IFNs represent two major counter-regulatory classes of inflammatory cytokines that control the outcome of *M. tuberculosis* infection and are functionally linked via eicosanoids [41]. They suggest that these are feasible alternatives to conventional chemotherapy.

Bacterial infections were associated with high concentrations of IL-1RA, IL-8 and VEGF. The cytokine concentrations in patients with bacterial infections did not overlap with the MTB and fungi groups. However, the fungi profile misclassified 3 MTB patients and the MTB profile misclassified 4 patients with proven fungal infection. The reason for this misclassification may be related to the measurement of only 19 cytokines. Using the same approach with a more comprehensive coverage of inflammatory mediators may improve the ability to distinguish between fungal and MTB infection, and should be further studied.

Our results indicate that there is a unique pattern of pro-inflammatory cytokine/chemokine associated with the distinct groups of infecting organisms, and that these signature combinations of cytokines are discriminatory between bacterial and non-bacterial pathogens. Moreover, we found distinct, if imperfectly discriminatory, cytokine/chemokine patterns associated with MTB and fungal infections. We did not find a significant association between cytokine profile and any of the severity outcomes (A-a gradient >45, severe pneumonia, in-hospital mortality and one year mortality), but the number of patients with these evaluated outcomes was small for each group. PJP infection was associated with significantly higher A-a gradient that could not be explained solely by the cytokine profile. This study combines several cytokines/chemokines in order to ascribe patterns to pathogens, the advantage of this approach is the ability to identify an inflammatory signature with pathophysiological consequences. The potential implications of the identified patterns are that they can be used to assist with etiological diagnosis and in presenting targets for adjuvant anti-inflammatory interventions to accentuate the tissue damaging inflammation.

There are several limitations to the study: the small number of participants does not allow for assessment of the impact of the inflammatory profile on clinical outcomes (such as length of stay, ICU admission, A-a difference or mortality); The advanced HIV disease with very low CD4 counts, adds multiple comorbid conditions and potentially other unidentified infections that may influence the observed cytokine patterns; In this study viral pathogens were not recovered and nucleic acid-based testing was not performed, the presence of such viral co-infections may affect the observed mediator concentrations.

4.1. Conclusions

The study illustrates the utility of measuring multiple cytokines/chemokines and performing PCA analysis to identify distinct inflammatory patterns associated with respiratory pathogens among HIV infected individuals with pneumonia. It adds to the understanding of the inflammatory signatures of the various pathogens and may forge the way to future adjuvant anti-inflammatory interventions.

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