RESEARCH ARTICLE

Systemic soluble Programmed Death-Ligand 1 levels in sarcoidosis subjects does not vary with disease progression

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Abstract
Interaction of programmed cell death 1 (PD-1) receptor and its ligand 1 (PD-L1) is well studied in the field of fibrotic lung diseases, supporting its use as a biomarker of progression of interstitial lung disease. Anti PD-L1 therapy has shown effectiveness in improvement of many malignancies and murine models of autoimmune fibrotic lung diseases. Higher PD-1 expression on T cells and PD-L1 expression on human lung fibroblasts are known to contribute towards severity in sarcoidosis and idiopathic pulmonary fibrosis (IPF), respectively. The focus of this investigation was to determine if soluble form of PD-L1 (sPD-L1) serves as predictive biomarker of disease severity in interstitial lung disease (ILD), such as scleroderma, sarcoidosis and IPF. Comparison of local environments, such as bronchoalveolar lavage, revealed significantly higher sPD-L1 levels compared to systemic environments, such as peripheral blood ($p=0.001$, paired two-tailed Student's t test). Investigation of serum samples of healthy control, IPF, scleroderma and sarcoidosis patients reveal significantly higher levels in sarcoidosis and IPF patients, compared to patients with scleroderma ($p=0.001$; $p=0.02$, one-way ANOVA with Tukey's respectively). Comparison of serum levels between sarcoidosis patients and healthy controls revealed no significant differences ($p=0.09$, unpaired two-tailed t test). In addition, comparison of physiologic parameters, such as percent predicated Forced Vital Capacity (FVC) and sPD-L1 levels in sarcoidosis and IPF patients revealed no correlation. These observations suggest that sPD-L1 will not serve as a biomarker of sarcoidosis disease severity. Additional investigation of sPD-L1 in local environments is warranted.

Introduction

Programmed T cell death ligand (PD-L1) is transmembrane protein ligand which is primarily expressed on dendritic cells (DC) and monocytes [1]. PD-L1 binds to programmed cell death 1 (PD-1) receptors, an immunoinhibitory receptor of CD28 family on the surface of T cells [1]. The PD-1/PD-Ls pathway is broadly expressed and exerts a wider range of immunoregulatory roles in T-cell activation and tolerance compared with other B7/CD28 family members [2]. The binding of PD-L1 to PD-1 results in suppression of T cell immune function by physiologically limiting its activation and proliferation [1, 3]. Thus, PD-L1 and PD-1 are immune downregulators or immune checkpoint inhibitors which induce apoptosis of the antigen presenting cell and promotes differentiation of CD4+ T cells [4]. Increased PD-L1 expression has been noted in several malignant and non-malignant diseases [5-8]. Blocking the PD-1/PD-L1 interaction increases antigen-specific T-cell activity whereas decreasing Treg suppressive function [9, 10]. The significance of PD-1 and PD-L1 interactions in immunity against cancer and infectious diseases has been studied extensively and immunotherapy targeting PD-1 and PD-L1 are in the clinic [11]. Antibodies that block either PD-1 or PD-L1 improve tumor-specific immunity, as well as patients' overall survival in many tumor types [12, 13]. Membrane-bound PD-1 and PD-L1 (mPD-1 and mPD-L1) also have soluble forms, the finding contributing to the complexity and multiplicity of the PD-1/PD-L1 (B7-H1/CD274) immunomodulation pathway [14], [15], [16]. Soluble PD-L1 (sPD-L1) in sera is associated with aggressive renal cell carcinoma and shorter survival in multiple myeloma and diffuse large B-cell lymphoma [17, 18]. In gliomas, sPD-L1 is a promising biomarker where elevated levels are associated with progression of the disease [19]. Abundant mousse and human data released recently clearly point that checkpoint regulatory molecules are essential in some autoimmune diseases, not only in cancer [20], [2]. In non-malignant disease such as Dermatomyositis (DM), sPD-L1 level also found to be higher compared to healthy cohort [21]. In 2017 a study proposed, RNA sequencing pointing to CD274 as significantly downregulated gene in human IPF lung tissue obtained by surgical biopsy [22]. Soluble clusters i.e. soluble proteins or molecules present in free form in peripheral system, are encouraging markers of various tumors and auto-immune disease progression, but they need further confirmation in larger studies [23].

The purpose of this investigation is to delineate if sPD-L1 serves as biomarker for interstitial lung disease severity in patients with sarcoidosis. We included, as controls, patients with systemic sclerosis (SSc) and Idiopathic Pulmonary Fibrosis (IPF), as well as healthy controls with no lung disease. Our lab has shown that PD-1 is upregulated on CD4+ T cells in pulmonary fibrosis patients [24, 25]. We also showed that there is a longitudinal increase in PD-1+CD4+ T cells in sarcoidosis subjects experiencing disease progression compared to healthy controls or sarcoidosis subjects resolving their disease [25]. Independent laboratories support the role of the PD-1 pathway in pulmonary fibrosis [24, 26]. Here, we would like to examine that if sPDL1 levels are indicative of disease progression in chronic lung diseases such as sarcoidosis, IPF and SSc.

Method

Study Population

To participate in this study, all subjects signed a written informed consent form, and patients were enrolled from Cleveland Clinic and Vanderbilt University Medical Center. The appropriate Institutional Review Boards approved all protocols. For inclusion

in this study, the clinical and radiographic criteria used to define sarcoidosis were applied [27]. IPF subjects were defined according to recent American Thoracic Society (ATS) guidelines [28] and systemic sclerosis patients were defined according to the 2013 American College of Rheumatology criteria [29]. There were four human cohorts in this study: patients with sarcoidosis patients (n=37), idiopathic pulmonary fibrosis (IPF) patients (n=28), scleroderma Patients (n=10) and healthy controls (n=14). Information on study subject demographics is provided in Table 1.

Table 1: Demographic of Healthy controls, Sarcoidosis, Idiopathic Pulmonary Fibrosis and Systemic Sclerosis patients used in this study

	Number (Gender)	**Age in years Median (Minimum, Maximum)**	**Race**
Healthy Control	**24 (11M, 13F)**	**44 (23,65)**	**11AA, 13C**
Sarcoidosis	**37 (21M, 16F)**	**50 (27,72)**	**23AA, 14C**
Systemic Sclerosis	**10 (6M, 4F)**	**60 (49, 86)**	**2AA, 1AsA, 7C**
Idiopathic Pulmonary Fibrosis	**28 (20M, 8F)**	**68 (51, 83)**	**28C**
C: Caucasian, AA: African American; AsA: Asian American			

Enzyme-linked immunosorbent assay (ELISA)

Serum samples obtained from 26 sarcoidosis patients, 10 systemic sclerosis patients, 28 IPF patients and 14 health controls were assayed by a commercially available ELISA Kit for sPD-L1 measurement (Invitrogen), following the manufacturer's protocol. Briefly, 96-well plates were incubated with standards at different concentrations, and serum samples were incubated for 2 h at 37 ℃. After covering biotinylated antibodies and several aspiration/wash processes, horseradish peroxidase (HRP)-conjugated streptavidin was prepared at 37 ℃ for 30 min and protected from light-colormetric responses were measured following completion of the enzymatic reactions. The absorbance was measured at 450 nm (A_{450}) in Microplate Reader immediately. Protein levels were calculated against the standard using four parametric logistic (4-PL) regression standard curves. The measured protein values were multiplied with the respective dilution factor to obtain the absolute quantification. For a correct evaluation of the results, parallel investigations were made in healthy controls. All samples were run in triplicates and average concentration was compared to make them statically relevant.

Flow Cytometry

Bronchoalveolar lavage fluid (BAL) and peripheral blood mononuclear cells (PBMC) from 11 other sarcoidosis patients were analyzed by flow cytometry. All flow cytometry experiments were acquired with 4-Laser Fortessa flow cytometer (BD Biosciences), and all antibodies used in this study were obtained from BD Biosciences (San Jose, CA). Live cells were gated based on forward and side scatter properties, and surface staining of cells was performed as

previously described [30]. Cells were gated on singlets, live, CD3+ and CD4+ cells. Data Analysis was performed using FlowJo software (Tree Star, Ashland, OR). A minimum of 1,000,000 events were acquired per sample. For cell staining, we used the following antibodies; Anti- CD3 Alexa Fluor 700, CD4 PE-Cy5, PD-1 PerCP-Cy5.5, obtained from BD Biosciences.

Statistical Analysis

Unpaired two-tailed Student t test was used for comparison of two groups. Multiple-group comparisons were performed using a one-way analysis of variance with Tukey's post hoc test. Pearson coefficient was used for correlating sPD-L1 concentration with disease severity data and percentage PD1+CD4+T cells. Statistical analysis for all Figures were carried out using Prism version 7.02 (GraphPad Software). For a result to be considered statistically significant, a p value of less than 0.05 was used.

Results

Serum PDL1 levels are significantly different between fibrotic lung diseases and cutaneous disease.

Soluble PDL1 (sPDL1) expression has positive correlation with disease progression in several tumor types [7, 8]. To investigate the role of sPDL1 in severity of various chronic lung diseases, we measured sPDL1 by ELISA in serum samples from sarcoidosis (S) patients, idiopathic pulmonary fibrosis (IPF) patients, systemic sclerosis (SSc) patients and healthy controls (HC). SSc patients had significantly lower sPDL1 levels than patients from sarcoidosis and IPF (For S, $p = 0.001$; For IPF, $p = 0.02$ one-way ANOVA with Tukey's post hoc test Figure 1A) indicating difference between fibrotic lung diseases. Level of sPD-L1 in healthy control serum was not significantly different than the serum of sarcoidosis patients ($p=0.0945$ using unpaired two-tailed t test Figure 1B). Soluble PD-L1 level in healthy control serum in this investigation has comparable values (0-0.5 ng/ml) to previously published data by Liu et al. [19]. This finding confirms earlier reports of the lack of distinctions in the percentages of PD-L1+CD4+ T cells among sarcoidosis and healthy control subjects, even though these are distinct cohorts [25].

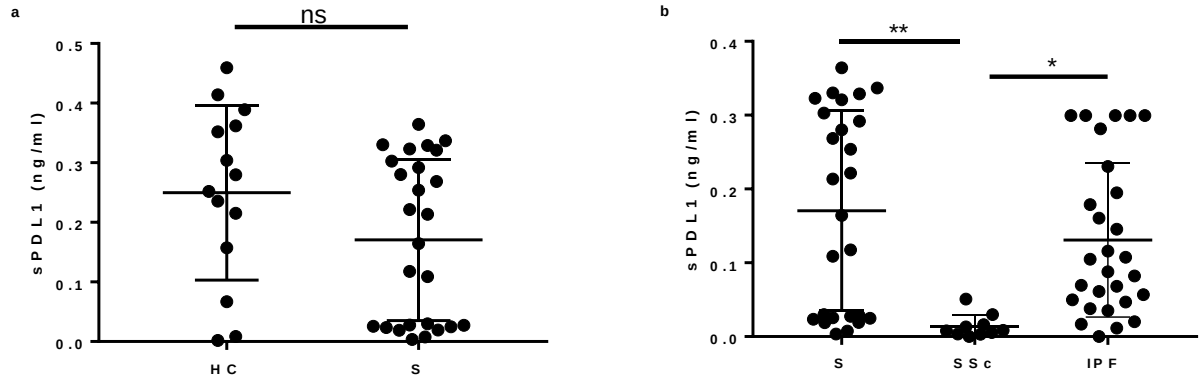


Figure 1: Serum PDL1 levels in various chronic lung diseases and healthy controls. (a) Serum PDL1 in sarcoidosis patients, systemic sclerosis patients and Idiopathic Pulmonary Fibrosis Patients (S= 26, SSc=10, IPF=28) (b) Serum PDL1 in healthy controls and sarcoidosis patients (HC=14, S=26). Comparisons between cohorts were performed using an unpaired two-tailed Student's t test for two groups and ANOVA Tukey's post-hoc test for three or more groups. Bars indicate SEM. *P < 0.05, **P < 0.01, ns=no significance. HC: Healthy Control, S: Sarcoidosis, SSc: Systemic Sclerosis, IPF: Idiopathic Pulmonary Fibrosis

PD-L1 expression on T cells and APCs are significantly higher in local environment than periphery

The lack of distinction in the systemic environment prompted us to investigate for distinctions in PD-L1 expression between local and systemic environments. We analyzed samples from sarcoidosis patients on whom we possessed both BAL (bronchoalveolar lavage) and peripheral blood mononuclear cells (PBMC) to assess for distinctions local and systemic environments. We did not possess both BAL and PBMC on IPF, scleroderma or healthy controls.

The flow cytometry analysis on PBMC and BAL showed that PD-L1 expression is significantly ($p = 0.0008$) higher on local CD4+ T cells than peripheral in same sarcoidosis patients (Figure 2A). Dendritic cell (DC) and macrophage populations also showed significantly (For, CD11c [DC]: $p = 0.0002$ and CD14 [Macrophage in BAL, monocyte in periphery]: $p = 0.0016$) higher expression of PD-L1 in local environment than periphery (Figure 2B) while B cell population (CD19) had similar expression ($p = 0.4626$) of PD-L1 in BAL and PBMC (Figure 2B). However, in peripheral environment, systemic PD-L1 expression on these APC subsets has no significant difference between healthy controls and sarcoidosis patients (CD11c: $p = 0.6895$; CD14: $p = 0.7372$ and CD19: $p = 0.8765$) (Figure 2C). These data suggest that in patients with fibrotic lung disease, distinctions in PD-L1 expression are present based on location. Distinctions between disease cohorts, such as sarcoidosis, and healthy controls are noted in local environments.

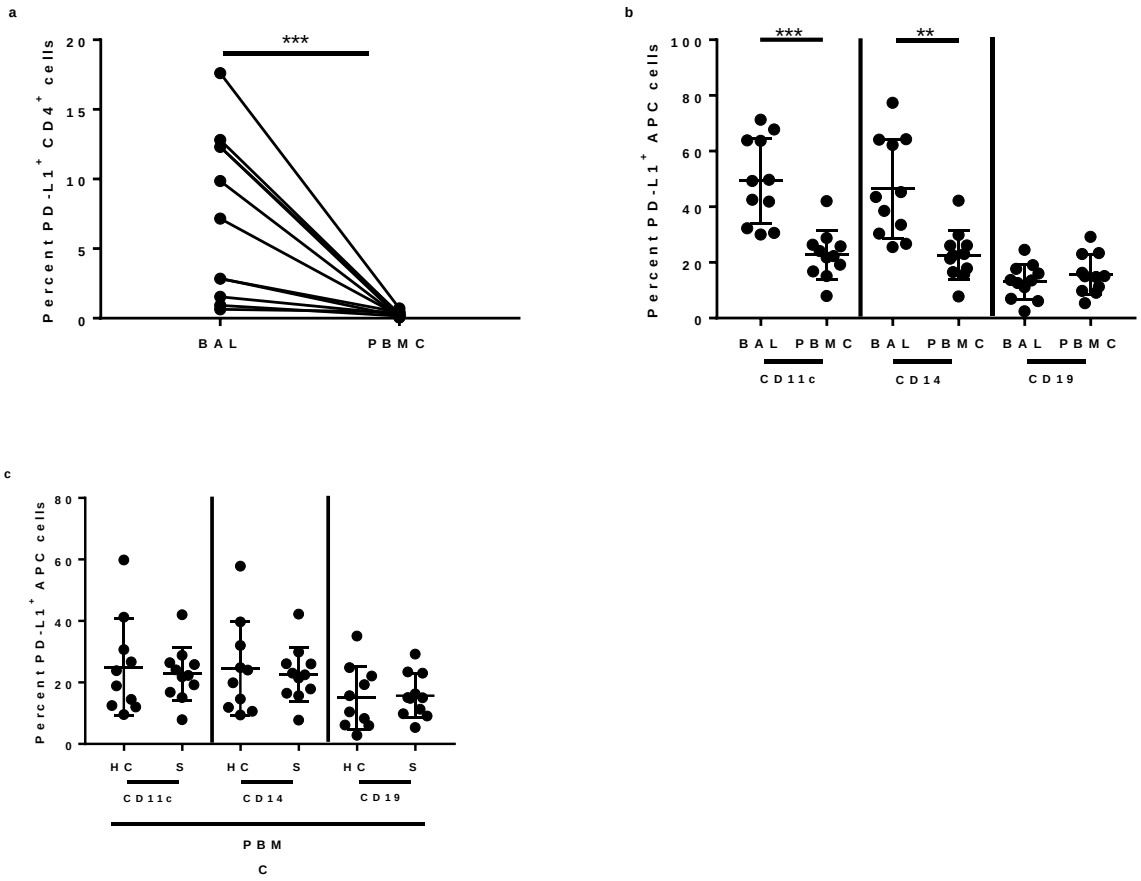
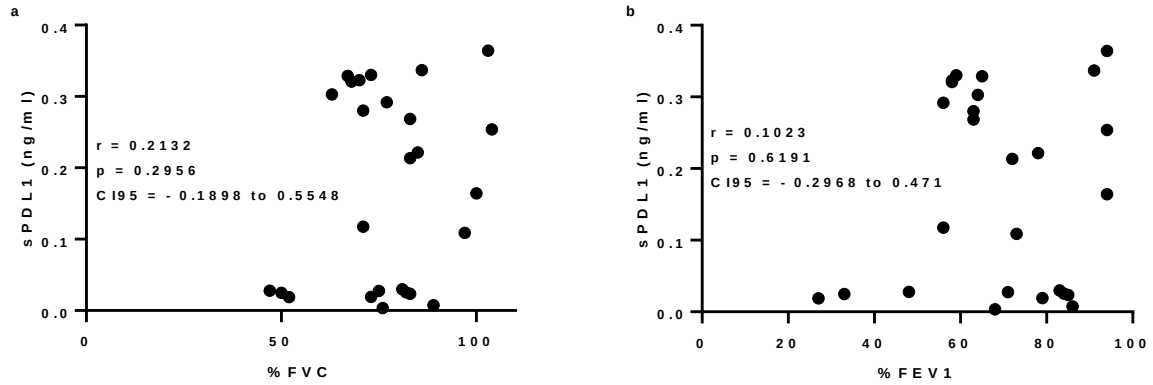


Figure 2: Programmed death-1 ligand (PD-L1) expression in peripheral and local environment in Sarcoidosis patients (a) Percentage of PD-L1$^+$ CD4$^+$ T cells from bronchoalveolar lavage (BAL) and the periphery (peripheral blood mononuclear cells (PBMC)) of same sarcoidosis patients (n= 11) (b) Percentage of PD-L1$^+$ antigen presenting cells (APC) in BAL and PBMCs of same sarcoidosis patients (n=11) (c) Percentage of PD-L1$^+$ antigen presenting cells (APC) in PBMCs of healthy controls (n=10) and sarcoidosis patients (n=11) Comparisons between cohorts were performed using an paired two-tailed Student's t test. Bars indicate SEM. **P < 0.01, ***P < 0.001, ns=no significance.



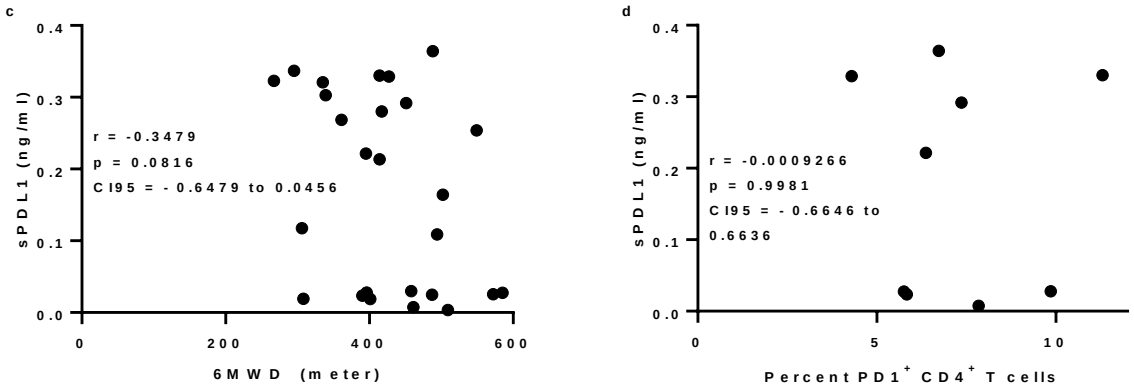


Figure 3: Correlation between disease severity and sPDL1 level in sarcoidosis patients (a) correlation between sPDL1 and percent Force Vital Capacity (%FVC) of patients' lung (n=26) (b) correlation between sPDL1 and percent Force Expiratory Volume (%FEV1) of patients' lung (n=26) (c) correlation between sPDL1 and six minute walk distance (6MWD) by patients with sarcoidosis (n=26) (d) correlation between sPDL1 and percent PD-1+ CD4+ T cells in patients with sarcoidosis (n=9). Correlation analysis was assessed by Pearson correlation coefficient (r). CI95: 95% Confidence interval.

Disease severity and PD-1 expression on T cells in sarcoidosis and IPF patients does not correlate with sPDL1 levels

It has been shown that in sarcoidosis and IPF patients disease severity could be expressed by percent Force Vital Capacity (%FVC) and Six Minute Walk Distance (6MWD). To further assess if sPD-L1 is a biomarker of disease severity, we correlated sPD-L1 levels with above-mentioned parameter, as well as Forced Expiratory Volume in one minute (FEV1). In our dataset, we did not find any correlation of sPDL1 levels on CD4+ T cells with %FVC, %FEV1 or 6MWD in sarcoidosis patients (For %FVC, p=0.2956; For %FEV1 p=0.6191; For 6MWD, p=0.0816; Figure 3A-C). Correlations with IPF disease severity was also lacking as well as in IPF patients (For %FVC, p=0.1289; For %FEV1 p=0.4528; Figure 4A-B). In previous study we have shown that systemic PD-1 expression on CD4+ T cells is increased in sarcoidosis and IPF patients, compared to healthy controls [30]. PD-1+CD4+ T cells in both sarcoidosis (p=0.9981 Figure 3D) and IPF (p=0.3199 Figure 4C) patients had no significant correlation with sPDL1 levels.

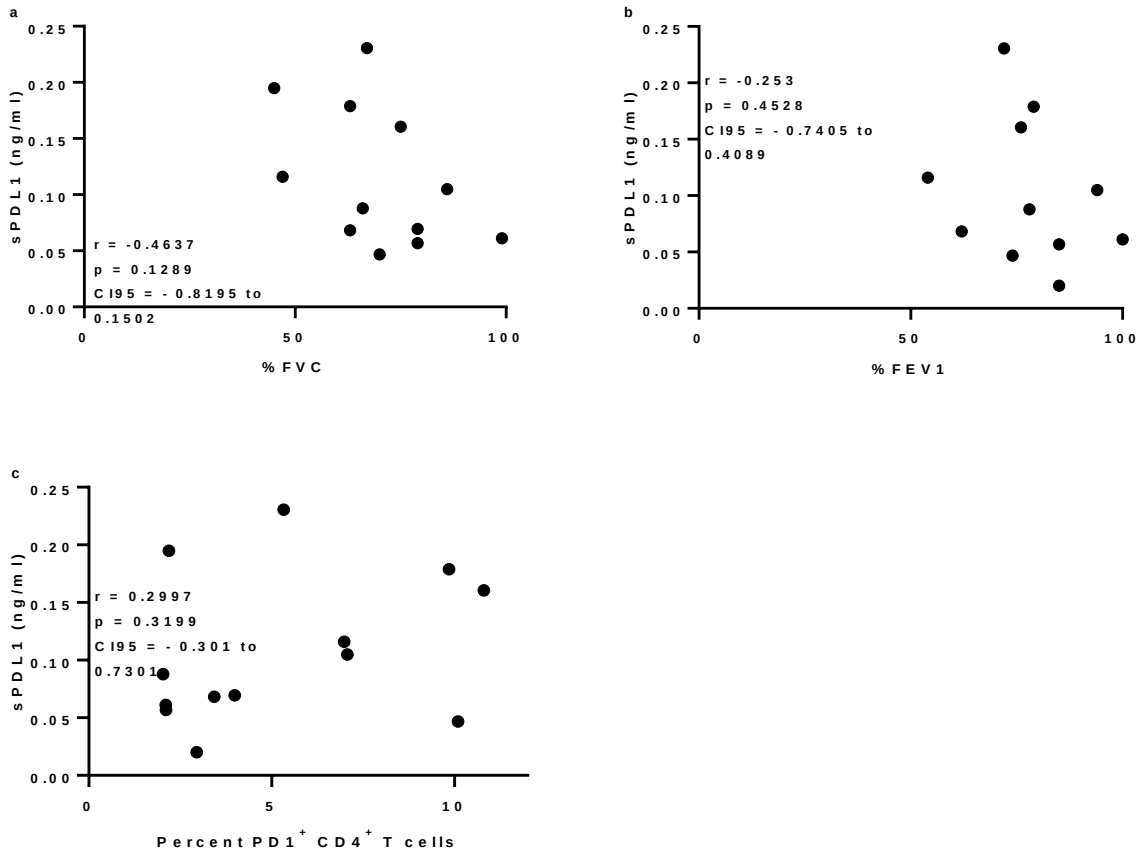


Figure 4: Correlation between disease severity and sPDL1 level in IPF patients (a) correlation between sPDL1 and percent Force Vital Capacity (%FVC) of patients' lung (n=12) (b) correlation between sPDL1 and percent Force Expiratory Volume (%FEV1) of patients' lung (n=11) (c) correlation between sPDL1 and percent PD-1+ CD4+ T cells in patients with IPF (n=13). Correlation analysis was assessed by Pearson correlation coefficient (r). CI95: 95% Confidence interval.

Discussion

Sarcoidosis is a granulomatous disease that progresses to pulmonary fibrosis in 10-20% of patients[31, 32]. The ability to identify a reliable biomarkers of disease progression is paramount. Soluble PD-L1 from serum and cerebral spinal fluid (CSF) is well reported as a biomarker of disease severity in various malignancies and autoimmune diseases[17-19, 21]. Our lab previously reported increased percentages of PD-1+CD4+ T cells sarcoidosis and IPF patients experiencing disease progression [25, 30]. sPD-L1 has been reported as a possible biomarker of disease progression [11-13]. Considering the aforementioned reports, we presumed that free PD-L1 levels could be significantly different between cohorts in chronic lung diseases like sarcoidosis, SSc and IPF based upon disease severity. The ELISA measurements of serum samples and correlation analysis with disease severity parameters showed no significance for sPD-L1 as biomarker in sarcoidosis or IPF patients (Figures 1, 3-4). It is interesting to note that distinctions in sPD-L1 expression were detected between BAL-derived antigen-presenting cells, such as macrophages and dendritic cells, but not on B cells; whereas systemic antigen-presenting

cells did not demonstrate these distinctions (Figure 2). It is possible that the limited number of macrophages and dendritic cells in the periphery, along with greater percentage of B cells, explains the lack of distinction when the systemic environment is assessed. Unfortunately, we possessed only a limited amount of BAL and thus were not able to fully delineate if sPD-L1 in BAL can serve as a biomarker. Also, the side effects associated with repeated assessments of BAL makes this test is not clinically feasible.

We also have observed previously that PD-L1 expression on CD4+ T cells in periphery is not significantly different between sarcoidosis patients and healthy cohorts[25]. The SSc cohort had primarily either mild (FVC>65% predicted) or moderate (FVC=50-64% predicted) lung disease. While most of the Ssc patients had mild disease, the three with moderately severe lung disease did not possess elevated sPD-L1 levels (data not shown). Analysis of immune subsets noted no distinctions between FVC and sPD-L1, suggesting that it does not correlate well with IPF disease severity. Considering the fact, sPD-L1 may not be a suitable biomarker in chronic lung diseases such as sarcoidosis, SSc and IPF.

Bound PD-L1 has different expression levels in local vs peripheral environment in same sarcoidosis patients (Figure 2). BAL samples show much higher expression of PD-L1 than PBMC on both CD4 + T cells and macrophages. Soluble PD-L1 levels are higher in local environments. However, serial acquisition of BAL would be too invasive to be used for routine clinical care. Peripheral analysis being less invasive would be more beneficial over local environment analysis. Future studies to compare longitudinal serum PD-1 level on CD4+ T cells instead of one-point in time serum PD-L1 should be considered.

There were limitations to this investigation. The potential limitation should be considered that the cohort is small, and we should enlarge the sample size to confirm the results. We did not have longitudinal serum or BAL fluid on all study participants which would have allowed a more in-depth investigation of the changes in sPD-L1 over the clinical course. We also did not have healthy control BAL fluid, which would have been a more optimal negative control.

Conclusion

This study observed the circulating levels of PD-L1 expression in ILD. Briefly, this investigation demonstrates that serum PD-L1 does not correlate with ILD severity. It does reveal that comparison of local and systemic environments demonstrates a significant difference; acquisition from local environment may be more indicative of lung progression. Future investigations for alternative biomarkers, such as sPD-1, should also be considered.

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