

Exploratory mass cytometry analysis reveals immunophenotypes of cancer treatment-related pneumonitis

Reviewed Preprint

Revised by authors after peer review.

About eLife's process

Reviewed preprint version 2

January 25, 2024 (this version)

Reviewed preprint version 1

June 8, 2023

Sent for peer review

March 17, 2023

Posted to preprint server

February 21, 2023

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Abstract

Anti-cancer treatments can result in various adverse effects, including infections due to immune suppression/dysregulation and drug-induced toxicity in the lung. One of the major opportunistic infections is *Pneumocystis jirovecii* pneumonia (PCP), which can cause severe respiratory complications and high mortality rates. Cytotoxic drugs and immune-checkpoint inhibitors (ICIs) can induce interstitial lung diseases (ILDs). Nonetheless, the differentiation of these diseases can be difficult, and the pathogenic mechanisms of such diseases are not yet fully understood. To better comprehend the immunophenotypes, we conducted an exploratory mass cytometry analysis of immune cell subsets in bronchoalveolar lavage fluid from patients with PCP, cytotoxic drug-induced ILD (DI-ILD), and ICI-associated ILD (ICI-ILD) using two panels containing 64 markers. In PCP, we observed an expansion of the CD16⁺ T cell population, with the highest CD16⁺ T proportion in a fatal case. In ICI-ILD, we found an increase in CD57⁺ CD8⁺ T cells expressing immune checkpoints (TIGIT⁺ LAG3⁺ TIM-3⁺ PD-1⁺), FCRL5⁺ B cells, and CCR2⁺ CCR5⁺ CD14⁺ monocytes. These findings uncover the diverse immunophenotypes and possible pathomechanisms of cancer treatment-related pneumonitis.

eLife assessment

This study presents a **useful** inventory of immune signatures that are correlated with cancer treatment-related pneumonitis. The data were collected and analysed using **solid** and validated methodology and can be used as a starting point for further functional studies.

Introduction

The advancements in anti-cancer therapy have revolutionized cancer management and have led to improved patient outcomes. However, these therapies can also lead to various adverse effects, including infections due to immune suppression/dysregulation and drug-induced toxicity, with the lungs being a commonly affected organ (Conte et al., 2022 [DOI](#))(Morelli et al., 2022 [DOI](#))(Skeoch et al., 2018 [DOI](#)). One of the major opportunistic infections in cancer patients or those receiving immunosuppressive treatment is *Pneumocystis jirovecii* pneumonia (PCP), which can cause severe respiratory complications and high mortality rates (Thomas and Limper, 2004 [DOI](#))(Asai et al., 2022 [DOI](#))(Apostolopoulou and Fishman, 2022 [DOI](#)). However, the precise immune reactions during PCP development and mechanisms of lung injury remain largely unknown.

While both cytotoxic drugs and immune-checkpoint inhibitors (ICIs) can induce interstitial lung diseases (ILDs), the mechanisms underlying these complications may differ between the two classes of drugs. In particular, ICI-associated ILDs are considered a type of immune-related adverse events (irAE)(Postow et al., 2018 [DOI](#))(Ando et al., 2021 [DOI](#)). Nonetheless, the precise mechanisms that cause drug induced-ILDs are predominantly unidentified. Further, despite various diagnostic approaches, including radiology and molecular testing, there is a need for useful biomarkers to distinguish PCP, cytotoxic drug-induced (DI)-ILD, and ICI-ILD.

Mass cytometry, also known as cytometry by time-of-flight (CyTOF), is a cutting-edge technology that uses inductively coupled plasma mass spectrometry to detect metal ions tagged to antibodies that bind to specific cellular proteins, allowing for a detailed and multi-dimensional characterization of cellular composition and function (Spitzer and Nolan, 2016 [DOI](#)). This technique provides a unique advantage over traditional flow cytometry, which is limited by spectral overlap and the number of parameters that can be analyzed simultaneously. Mass cytometry has been used to study a variety of biological systems, including immune-mediated diseases, such as cancer and autoimmune conditions (Matsubara et al., 2021 [DOI](#))(Hata et al., 2023 [DOI](#))(Couloume et al., 2021 [DOI](#))(Chedid et al., 2022 [DOI](#)). Through high-throughput analysis of large numbers of single cells, mass cytometry can provide a more comprehensive understanding of the cellular heterogeneity and signaling pathways involved in these diseases, allowing for the identification of potential therapeutic targets and biomarkers.

The purpose of this study is to distinguish pulmonary involvement in patients with malignancy undergoing chemotherapy and to identify potential biomarkers for DI-ILD, ICI-ILD, and PCP by analyzing bronchoalveolar lavage fluid (BALF) samples with mass cytometry. By characterizing the cellular and molecular changes in BALF from patients with these complications, we aim to improve our understanding of their pathogenesis and identify potential therapeutic targets.

Materials and Methods

Patients

In this retrospective study, patients who were newly diagnosed with PCP, DI-ILD, and ICI-ILD and had undergone BALF collection at Kyushu University Hospital from January 2017 to April 2022 were included. The retrospective study was approved by the Ethics Committee of Kyushu University Hospital (reference number 22117-00). Diagnostic criteria for PCP, DI-ILD, and ICI-ILD were in accordance with those previously described (Skeoch et al., 2018 [DOI](#))(Asai et al., 2022 [DOI](#))(Apostolopoulou and Fishman, 2022 [DOI](#))(Delaunay et al., 2019 [DOI](#)). A visual representation of the experimental and analytical workflow can be found in **Figure 1** [DOI](#).

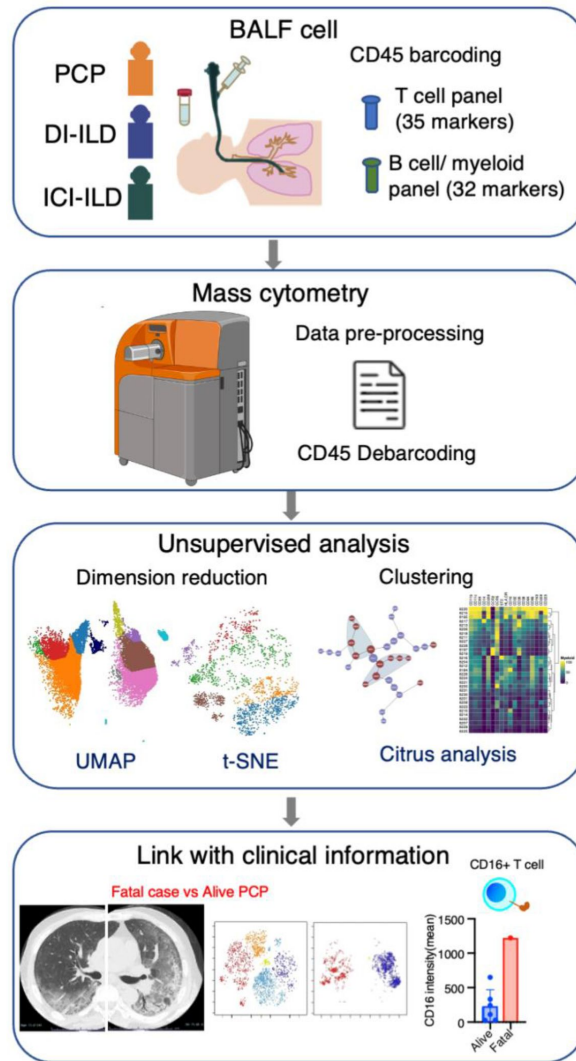


Figure 1.

Graphical abstract of the study.

Bronchoalveolar lavage fluid (BALF) samples were collected from patients with *Pneumocystis jirovecii* pneumonia (PCP), cytotoxic drug-induced interstitial lung disease (DI-ILD), and immune-checkpoint inhibitor-related ILD (ICI-ILD). Subsequently, BALF cells were analyzed using mass cytometry with a T cell panel (35 markers) and B cell/myeloid cell panel (32 markers) following CD45 barcoding for sample identification. The study found that there was a significant increase in the expansion of CD16⁺ T cells in patients with PCP, with the highest CD16 expression observed in a fatal case of PCP.

Mass cytometry

Metal-tagged antibodies were obtained from Standard Biotools or purchased in a purified form (**Supplementary Table 1**) and then labeled with metals using the Maxpar Antibody Labeling Kit (Standard Biotools) as instructed by the manufacturer. The cell labeling was performed as previously described (Hata et al., 2023). Upon collection, BALF samples were immediately centrifuged at 300 g for 5 minutes to pellet the cells. The resultant cell pellets were then resuspended in Cellbanker 1 cryopreservation solution (Takara, catalog #210409). This suspension was aliquoted into cryovials and gradually frozen to -80°C and stored at -80°C until required for experimental analysis. Cryopreserved BALF cells were thawed in PBS and then stained with Cell-ID Cisplatin-198Pt (Standard Biotools #201198, 1:2000 dilution) in PBS before being incubated with FcR blocking reagent (Myltenyi, #130-059-901) and barcoded with each metal-labeled CD45 antibody (**Supplementary Table 1**). After washing, CD45-labeled cells were mixed and stained with APC-conjugated FCRL5 antibodies (for panel #2), followed by staining with the antibody cocktail (Panels #1 and #2, see **Supplementary Table 1**). The antibody amount was determined through preliminary experiments with metal minus one. Cells were then washed, fixed with 1.6% formaldehyde, and resuspended in Cell-ID Intercalator 103Rh (Standard Biotools #201103A) in Fix and Perm buffer (Standard Biotools) at 4°C overnight. For acquisition, cells were resuspended in MaxPar Cell Acquisition Solution (Standard Biotools #201240) containing one-fifth EQ Four Element Calibration Beads (Standard Biotools #201078) and acquired at a rate of 200-300 events/second on a Helios mass cytometer (Standard Biotools). Files were converted to FCS, randomized, and normalized for EQ bead intensity using the Helios software. Concatenating fcs files in the same group into one file was conducted by FlowJo v10.8 (BD Biosciences). Cytobank Premium (Cytobank Inc.) was used to perform manual gating, visualization of t-distributed stochastic neighbor embedding (tSNE), Uniform manifold approximation and projection (UMAP) analysis, and Citrus analysis (Bruggner et al., 2014).

Data analysis

To select live cells, cisplatin-positive cells and doublets were excluded, and CD45^{+} cells were further analyzed. For T cells, $\text{CD2}^{+} \text{CD3}^{+}$ cells were gated and subjected to UMAP and Citrus algorithms. The UMAP analysis included clustering channels for CD4, CD8a, CD27, CD28, CD45RA, CD45RO, Fas, and used the parameters: numbers of neighbors = 15, minimum distance = 0.01. The Citrus algorithm for T cells included clustering channels for CD4, CD5, CD7, CD8a, CD11a, CD16, CD27, CD28, CD44, CD45RA, CD45RO, CD49d, CD57, CD69, CD226, Fas, IL-2R, PD-L1, PD-L2, PD-1, OX40, TIGIT, TIM3, CTLA-4, CD223 (LAG-3), BTLA, ICOS, ST2, CCR7, CXCR3, HLA-DR, and used the parameters: association models = nearest shrunken centroid (PAMR), cluster characterization = abundance, minimum cluster size = 5%, cross validation folds = 5, false discovery rate = 1%. The citrus analysis excluded nivolumab-induced ILD cases due to the apparent loss of PD-1 detection caused by competitive inhibition by nivolumab (**Supplementary Figure 1B**) (Yanagihara et al., 2020). The tSNE analysis for T cells included clustering channels for CD4, CD5, CD7, CD8a, CD11a, CD16, CD27, CD28, CD44, CD45RA, CD45RO, CD49d, CD57, CD69, CD226, Fas, IL-2R, PD-L1, PD-L2, PD-1, OX40, TIGIT, TIM3, CTLA-4, CD223 (LAG-3), BTLA, ICOS, ST2, CCR7, CXCR3, and HLA-DR, and used the parameters: iterations = 1000, perplexity = 30, theta = 0.5.

For myeloid cells, $\text{CD3}^{-} \text{CD11b}^{+} \text{CD11c}^{+}$ cells were gated, and UMAP and Citrus algorithms were used. The UMAP analysis included clustering channels for CD11b, CD11c, CD64, CD14, CD16, CD206, HLA-DR, and CCR2, and used the parameters: numbers of neighbors = 10, minimum distance = 0.01. The Citrus algorithm included clustering channels for CD11b, CD11c, CD64, CD14, CD16, CD32, CD36, CD38, CD84, CD86, CD163, CD206, CD209, CD223, HLA-DR, CCR2, CCR5, and ST2, and used the parameters: association models = nearest shrunken centroid (PAMR), cluster characterization = abundance, minimum cluster size = 5%, cross validation folds = 5, false discovery rate = 1%. One case of PCP was excluded from the Citrus analysis due to low cell numbers.

B cells and Plasma cells were identified using gating of $CD3^+CD64^-$ and $CD19^+$ or $CD138^+$ cells. viSNE analysis was performed to cluster B cells using the following markers: CD19, CD38, CD11c, IgA, IgG, CD138, CD21, ST2, CXCR5, CD24, CD27, TIM-1, IgM, HLA-DR, IgD, and FCRL5. The analysis was performed on both individual and concatenated files using the parameters of 1000 iterations, perplexity of 30, and theta of 0.5.

The selection of the dimensionality reduction technique, UMAP or viSNE, was made based on their ability to retain the relationships between global structures and the distances between cell clusters (where UMAP outperformed viSNE) and their ability to present a distinct and non-overlapping portrayal of cell subpopulations, facilitating the identification of inter-group variations (where viSNE performed better than UMAP).

Statistical analysis

We utilized a PAMR association model with a stringent threshold of 1% FDR, as described in the *Data Analysis* section, for the Citrus algorithm experiment. The Student's two-tailed unpaired t-test was employed to conduct a comparative analysis between the two groups. To determine the significance of serum beta-D-glucan and KL-6 among three groups, a statistical analysis was performed using Kruskal-Wallis tests. We evaluated the manually gated cell proportions by performing a two-way ANOVA, along with Tukey's multiple comparison tests. Data were analyzed using GraphPad Prism 9 software. Statistical significance was considered to be achieved when the P-value was less than 0.05.

Analysis of single cell RNA sequencing data of human BALF cells

For our investigation, we utilized the publicly available dataset GSE145926, which contains single-cell RNA sequencing data from BALF cells of both healthy controls (n=3) and patients with severe COVID-19 (n=6) (Liao et al., 2020 [\[link\]](#)). We initiated our analysis by normalizing the filtered gene-barcode matrix using the 'NormalizeData' function in Seurat version 4, applying the default settings. Subsequently, we identified the top 2,000 variable genes utilizing the 'vst' method available in the 'FindVariableFeatures' function of Seurat. Principal Component Analysis (PCA) and UMAP were then conducted to reduce dimensionality and visualize the data. To isolate T cells for further analysis, we defined them as cells expressing *CD2* and *CD3E* with expression values greater than 1. We specifically examined the expression of *FCGR3A* (CD16) by setting an expression threshold of 0.5 to identify relevant cell populations. The resulting expression patterns were illustrated using violin plots and bar plots, which were generated using the 'ggplot' function in R.

Results

Patient characteristics and clinical parameters

We analyzed 7 cases of PCP, 9 of DI-ILD, and 9 of ICI-ILD (Table 1 [\[link\]](#)). One of the PCP cases was fatal. Differential cell counts for BALF revealed lymphocytosis in 7 out of 7 cases (100%) of PCP as well as in 8 out of 9 cases (88.9%) of DI-ILD and 7 out of 9 cases (63.6%) of ICI-ILD when the cut-off for the percentage of lymphocytes was set to >20%. Serum levels of beta-D-glucan were significantly higher in patients with PCP than those with DI-ILD or ICI-ILD (196.5 ± 419.8 , 3.7 ± 7.1 , 0.6 ± 1.4 pg/mL, respectively) (Figure 2A [\[link\]](#)). The receiver operating characteristic (ROC) curve was constructed to evaluate the diagnostic utility of beta-D-glucan for PCP, with values from the DI-ILD group used as a reference (Figure 2B [\[link\]](#)). The area under the curve was determined to be 0.8929. When the cut-off value was set to 14.70 pg/mL, the sensitivity and specificity of PCP were 85.71% and 87.50%, and the positive likelihood ratio was 6.86. The diagnostic value of beta-D-glucan was consistent with the previous study (Tasaka et al., 2007 [\[link\]](#)), where the positive likelihood ratio was 6.57, with a cut-off value of 31.0. The serum levels of KL-6, an indicator of various types of

interstitial pneumonitis (Ishikawa et al., 2012), tended to be higher in patients with ICI-ILD than PCP or DI-ILD, though insignificant (**Figure 2C**). Four out of 7 (57.1%) in PCP, 1 out of 9 (11.1%) in DI-ILD, and 6 out of 9 (66.7%) in ICI-ILD were positive for KL-6 with a cut-off value of 500 U/mL.

Expansion of CD16⁺T cells in BALF from patients with PCP

First, we investigated whether subsets of T cells (gated CD2⁺ CD3⁺) differentially existed in PCP, DI-ILD, and ICI-ILD with mass cytometry. T-cell lymphocytosis was observed in all groups (PCP: 62.9 ± 17.5%, DI-ILD: 58.3 ± 24.5%, ICI-ILD: 50.4 ± 29.9%) with a higher tendency of CD4/CD8 ratio observed in DI-ILD compared to PCP and ICI-ILD (PCP: 1.13 ± 0.86, DI-ILD: 6.08 ± 6.06, ICI-ILD: 1.9 ± 1.40)(**Figure 3A**). To visualize T cell differentiation within the affected lungs, we generated UMAP plots (**Figure 3B**). The majority of T cells in the BALF displayed either memory or effector phenotypes, with a limited population of naïve T cells (**Figures 3B** and **3C**). Specifically, we observed a tendency of a higher abundance of transient memory/ effector memory CD4 T cells and a lower abundance of transient memory/ effector memory CD45RA (EMRA) CD8 T cells in DI-ILD compared to the other two groups (**Figure 3C**).

We then utilized the Citrus algorithm to classify T cell subpopulations with varying degrees of abundance in an unsupervised manner by analyzing 31 parameters (**Figure 3D**, **Supplementary Figures 1C** and **1D**). Our analysis identified 30 clusters of T cells, of which 13 were significantly differentiated between the groups. Interestingly, clusters #26299, #26293, and #26278, characterized by CD8⁺ T cells expressing CD16 (FcyRIIIa), and cluster #26286, characterized by CD4⁺ T cells expressing CD16, were abundant in PCP compared to DI-ILD and ICI-ILD (**Figures 3E** and **3F**). These CD8⁺ T cells expressing CD16 were also positive for HLA-DR and CXCR3 (**Figure 3F**) but negative for CD14, a marker for monocytes (median CD14 expression of these clusters: 0.0–0.1 vs. 7.4–9.5 in monocyte populations). Cluster #26303, prevalent in ICI-ILD, comprised CD8⁺ CD57⁺ TIGIT⁺ LAG3⁺ but CD16[−] subpopulation (**Figures 3E** and **3F**). These cells also expressed PD-1 and TIM-3, although the expression was lower than clusters #26299, #26293, and #26278. These CD8⁺ T cells expressed CD45RA but no expression of CD45RO and CCR7, indicating EMRA phenotype. Cluster #26281, prevalent in DI-ILD, was marked by CD4⁺ with low immune checkpoint expression (PD-1, TIM-3, TIGIT, LAG3, PD-L1, PD-L2, and OX40; **Figures 3E** and **3F**).

Myeloid cell subpopulations in the lungs of PCP, DI-ILD, and ICI-ILD

Next, we investigated myeloid cell populations (identified as CD3[−] CD11b⁺ CD11c⁺). We first conducted UMAP to see the major myeloid cell populations. The UMAP plot categorized four major subtypes: monocytes, CCR2⁺ macrophages, alveolar macrophages, and dendritic cells, with no significant difference in PCP, DI-ILD, and ICI-ILD (**Figures 4A** and **4B**). We next utilized the Citrus algorithm to further investigate differently abundant myeloid cell subpopulations by analyzing 18 parameters. Our analysis identified 31 clusters of myeloid cells, of which 17 were significantly differentiated between the groups (**Figure 4C**, **Supplementary Figure 2**). Clusters #8220, #8215, and #8195, prevalent in PCP, were comprised of CD11b^{hi} CD11c^{hi} CD64⁺ CD206⁺ alveolar macrophages with HLA-DR^{hi} (**Figures 4D** and **4E**). Clusters #8219 and #8197, prevalent in ICI-ILD, were characterized by CD14⁺ CCR2⁺ CCR5⁺ monocyte subpopulations (**Figures 4D** and **4E**). Clusters #8227, #8223, and #8208, prevalent in DI-ILD, were marked by CD11b^{lo} CD11c^{lo} CD64^{lo} CCR5⁺ subpopulations (**Figures 4D** and **4E**).

B cell subpopulations in the lungs of ILDs

In addition to T cells and myeloid cells, we sought to investigate whether there were differential representations of B cells in BALF from PCP, DI-ILD, and ICI-ILD. By utilizing CD45⁺CD3[−]CD64[−] and CD19⁺ or CD138⁺ as gating parameters, we were able to detect the presence of B cells and plasma cells. The frequency of B cells/ plasma cells was found to be relatively higher in patients with PCP

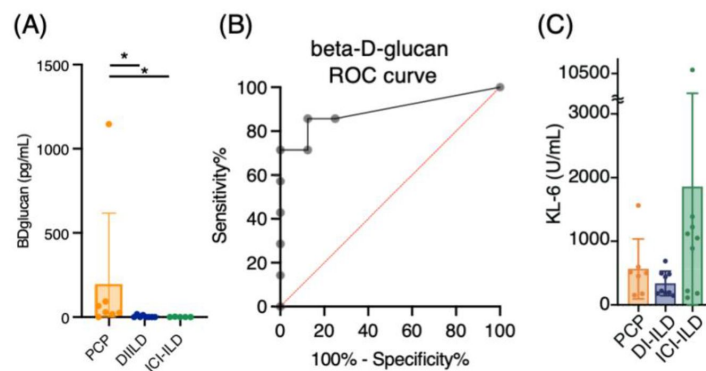


Figure 2.

Serum levels of beta-D-glucan and KL-6 from patients with PCP, DI-ILD, and ICI-ILD.

(A) Serum levels of beta-D-glucan from patients with *Pneumocystis jirovecii* pneumonia (PCP), cytotoxic drug-related interstitial lung disease (DI-ILD), and immune-checkpoint inhibitor-related ILD (ICI-ILD). (B) A receiver operating characteristic (ROC) curve was constructed to evaluate the diagnostic utility of beta-D-glucan for PCP, with values from the DI-ILD group used as a reference. The area under the curve was determined to be 0.8929. (C) Serum levels of KL-6.

	PCP	DI-ILD	ICI-ILD
Number	7	9	9
Age	66.9±4.6	58.8±19.3	71.9±4.5
Male %	42.8(3)	77.8(7)	100(9)
Suspected drug/regimen		VAC (2), FOLFIRI(2), DTX(1), AC(1), GEM(1), TS- 1(1), BEP(1)	Nivolumab (3), Pembrolizumab (3), Durvalmab (2), Atezolizumab (1)
CTCAE grade	G2(5), G3(1), G5(1)	G1(2), G2(7)	G2(7), G3(2)
BALF cell differentiation (%)			
Macrophage	25±8.9	40.2±23.5	44.4±26.6
Neutrophil	13.6±29.7	1.9±1.3	14.4±26.7
Lymphocyte	58.8±30.5	56.2±24.8	35.6±23.7
Eosinophil	0.9±1.1	1.7±1.9	5.6±8.5

Data for age and BALF analysis are presented as means ± SD. PCP: *Pneumocystis jirovecii* pneumonia, DI-ILD: cytotoxic drug-related interstitial lung disease, ICI-ILD: immune-checkpoint inhibitor-related ILD, BALF: bronchoalveolar lavage fluid. VAC: vincristine, actinomycin D, and cyclophosphamide; FOLFIRI: folinic acid, fluorouracil, and irinotecan hydrochloride; DTX: docetaxel, AC: Adriamycin and cyclophosphamide; GEM: gemcitabine; TS-1: Tegafur Gimeracil Oteracil Potassium; BEP: bleomycin, etoposide, and platinum. Common Terminology Criteria evaluated the severity of the disease for Adverse Events (CTCAE) grading. Grade 1: asymptomatic, grade 2: symptomatic, grade 3: severe symptoms, requiring oxygen therapy, grade 4: life-threatening respiratory failure, grade 5: death.

Table 1.

Characteristics of the study population.

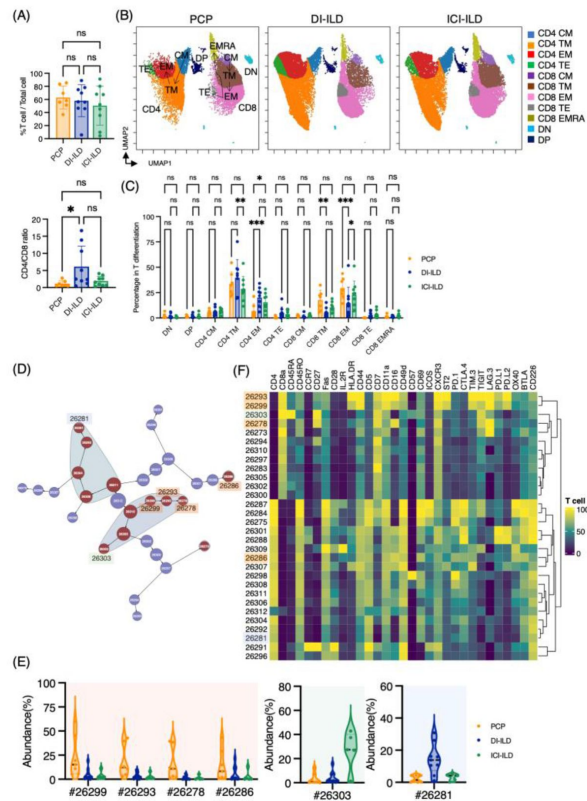


Figure 3.

Characterization of T cell subsets in BALF from patients with PCP, DI-ILD, and ICI-ILD.

(A) Percentage of T cells (defined as $CD2^+CD3^+$) in $CD45^+$ BALF cells and CD4/CD8 ratio in T cells from patients with *Pneumocystis jirovecii* pneumonia (PCP), cytotoxic drug-related interstitial lung disease (DI-ILD), and immune-checkpoint inhibitor-related ILD (ICI-ILD). (B) Uniform manifold approximation and projection (UMAP) of concatenated samples visualizing the distribution of T cell subpopulations. Central memory (CM) T cells were defined by $CCR7^+ CD45RO^+ CD28^+ Fas^+$, Transitional memory (TM) by $CCR7^- CD45RO^+ CD28^+ Fas^+$, Effector memory (EM) by $CCR7^- CD45RO^+ CD28^- Fas^+$, Terminal effector (TE) by $CCR7^- CD45RO^{+/-} Fas^-$, Effector memory RA (EMRA) by $CCR7^- CD45RO^- CD45RA^+ Fas^{+/-}$. Arrows indicate the trajectory of T-cell differentiation. DN: $CD4^- CD8^-$ double negative, DP: $CD4^+ CD8^+$ double positive. (C) Percentage of T cell subpopulations. (D) Citrus network tree visualizing the hierarchical relationship of each marker between identified T cell populations gated by $CD45^+CD2^+ CD3^+$ from PCP ($n = 7$), DI-ILD ($n = 9$), and ICI-ILD ($n = 5$). Clusters with significant differences were represented in red, and those without significant differences in blue. Circle size reflects the number of cells within a given cluster. (E) Citrus-generated violin plots for six representative and differentially regulated populations. Each cluster number (#) corresponds to the number shown in panel (D). (F) Heatmap demonstrates the expression of various markers in different clusters of T cells, as identified through the Citrus analysis. All differences in abundance were significant at a false discovery rate < 0.01 .

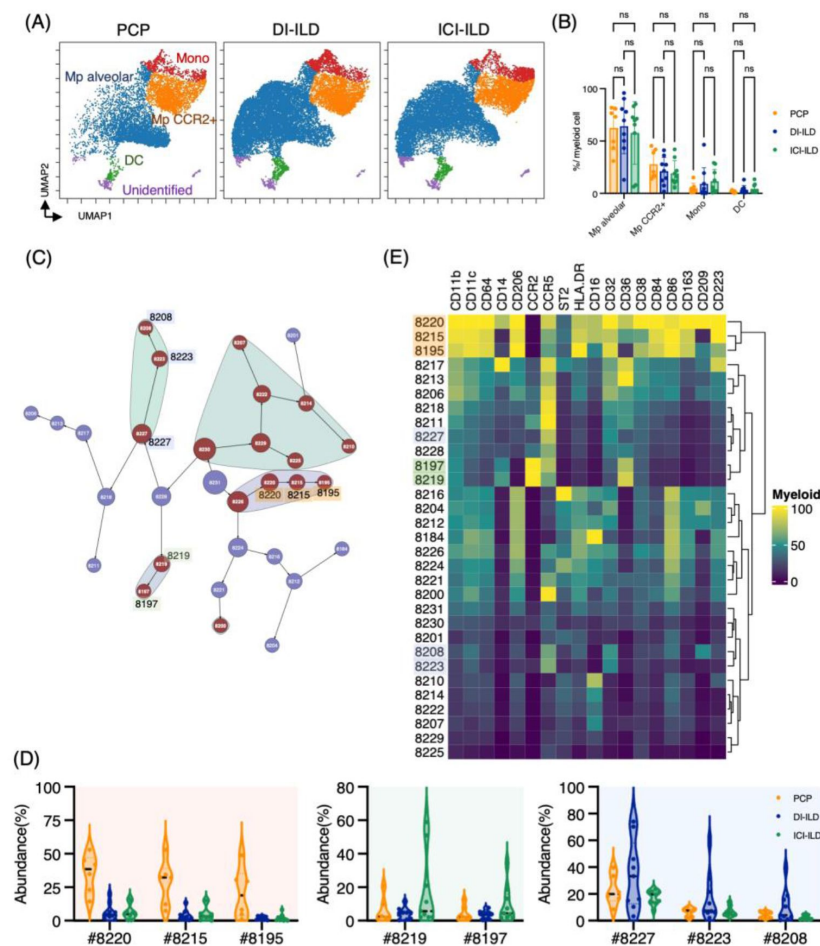


Figure 4.

Characterization of myeloid cell subsets in BALF from patients with PCP, DI-ILD, and ICI-ILD.

(A) UMAP of concatenated samples visualizing the distribution of myeloid cell sub-populations in CD3⁻ CD11b⁺ CD11c⁺ gated myeloid cells in BALF from patients with *Pneumocystis jirovecii* pneumonia (PCP), cytotoxic drug-related interstitial lung disease (DI-ILD), and immune-checkpoint inhibitor-related ILD (ICI-ILD). Monocytes were defined by CD64⁺ CD14⁺, CCR2⁺ macrophages (Mp) by CD64⁺ CD14⁻ CCR2⁺, Alveolar Mp by CD64⁺ CD14⁻ CCR2⁻ CD206⁺, dendritic cells (DC) by CD64⁻ CD14⁻ CD206⁻ CD11c⁺ HLA-DR⁺, Unidentified subsets by CD64⁻ CD11b⁺ CD11c⁺ CD14⁻ CD206⁻. (B) Percentage of myeloid cell sub-populations in PCP (n = 7), DI-ILD (n = 9), and ICI-ILD (n = 9). Dot plots represent individual samples. (C) Citrus network tree visualizing the hierarchical relationship of each marker between identified myeloid cell populations gated by CD45⁺ CD3⁻ CD11b⁺ CD11c⁺ from PCP (n = 6), DI-ILD (n = 9), and ICI-ILD (n = 9). Clusters with significant differences were represented in red, and those without significant differences in blue. Circle size reflects the number of cells within a given cluster. (D) Citrus-generated violin plots for six representative and differentially regulated populations. Each cluster number (#) corresponds to the number shown in panel (C). All differences in abundance were significant at a false discovery rate < 0.01. (E) Heatmap demonstrates the expression of various markers in different clusters of myeloid cells, as identified through the Citrus analysis.

compared to the other two groups, although the proportion of B cells and plasma cells remained low in all groups. (Figure 5A). A t-SNE analysis of 17 parameters among B cells/plasma cells revealed the presence of various B cell subpopulations, including IgD⁺ naïve B cells, IgM⁺ B cells, IgG⁺ B cells, IgA⁺ B cells, plasmablasts, and plasma cells (Figure 5B). Although not significant, higher levels of IgG⁺ B cells were observed in patients with PCP, while individuals with ICI-ILD exhibited a greater presence of plasma cells (Figure 5C). Previous research has suggested that FCRL5⁺ B cells contribute to the pathogenesis of autoimmune disorders (Owczarczyk et al., 2020) (Dement-Brown et al., 2012). Therefore, we investigated the frequency of these cells in our study and found that FCRL5⁺ B cells were more abundant in patients with ICI-ILD compared to those with PCP and DI-ILD, suggesting these FCRL5⁺ B cells may have a role in irAE ($8.37 \pm 6.74\%$, $1.37 \pm 2.30\%$, $2.29 \pm 3.81\%$, respectively) (Figure 5D).

Marked CD16⁺ T cell expansion in BALF from a fatal case of PCP

One case of PCP that developed during the use of immunosuppressive drugs after living donor liver transplantation had a fatal course despite intensive treatment. Upon admission, chest CT images revealed the emergence of diffuse ground glass opacities and infiltration in the bilateral lungs (Figure 6A). The serum levels of beta-D-glucan and KL-6 were highly elevated (1146 pg/mL and 1561 U/mL, respectively). The T cell percentage was 51.3%, and the CD4/CD8 ratio was 0.58, which was not particularly different from other PCP cases (Figure 6B). Strikingly, 97.5% of T cells expressed CD16 in the BALF from the fatal case (Figures 6C and 6D). Further, not only the proportion but the CD16 intensity was the highest in the fatal case among PCP cases (Figure 6E). On the contrary, there did not appear to be any significant differences in myeloid cell fractions, and CD16 expression was not particularly high in the fatal case (Figure 6F). The correlation matrix of clinical parameters and mass cytometry parameters revealed that CD16 intensity in T cells may be correlated with disease severity ($r = 0.748$, $p = 0.053$) and beta-D-glucan ($r = 0.868$, $p = 0.011$) (Figure 6G). Therefore, CD16 expression in T cells is not only a characteristic of PCP but also a potential indicator of disease severity. For reference, we investigated CD16 expression in BALF T cells from healthy controls along with severe COVID-19 patients using the publicly available single-cell RNA sequencing data (GSE145926). *FCGR3A* (CD16) was expressed by a minority of T cells in healthy BALF—1.0% of CD4⁺ T cells and 1.6% of CD8⁺ T cells, while 16.3% of CD4⁺ T cells and 17.1% of CD8⁺ T cells expressed *FCGR3A* (CD16) in severe COVID-19 patients (Figures 6H and 6I).

Discussion

We have here demonstrated the characteristic immune cell subpopulations present in BALF from patients with PCP, DI-ILD, and ICI-ILD. Our analysis revealed an expansion of CD16⁺ T cells in patients with PCP, an increase in CD57⁺ CD8⁺ T cells expressing immune checkpoints, and FCRL5⁺ B cells in ICI-ILD.

CD16 is a low-affinity Fc receptor (FcγRIIIa) for IgG, commonly expressed on NK cells as well as on neutrophils and monocytes (Ravetch and Bolland, 2001), with only a small fraction of T cells expressing CD16 in healthy peripheral blood (Sandor and Lynch, 1993) and BALF (Figures 6H and 6I). On the contrary, CD16⁺ T cells are induced in specific conditions, such as chronic hepatitis C infection (Björkström et al., 2008) and COVID-19 (Georg et al., 2022). In chronic hepatitis C infection, CD16⁺ CD8 T cells displayed a late-stage effector phenotype with high levels of perforin with a restricted TCR profile (Björkström et al., 2008). Stimulation of CD16 on CD8 T cells evokes a vigorous response such as degranulation and cytokine production (IFN-γ and TNF-α) similar to that of CD16 stimulation in NK cells (Björkström et al., 2008). These CD16⁺ CD8⁺ T cells also express a senescent marker, CD57, which differs from our findings that expanding CD16⁺ CD8 T cells in PCP did not express CD57 (Figure 3F).

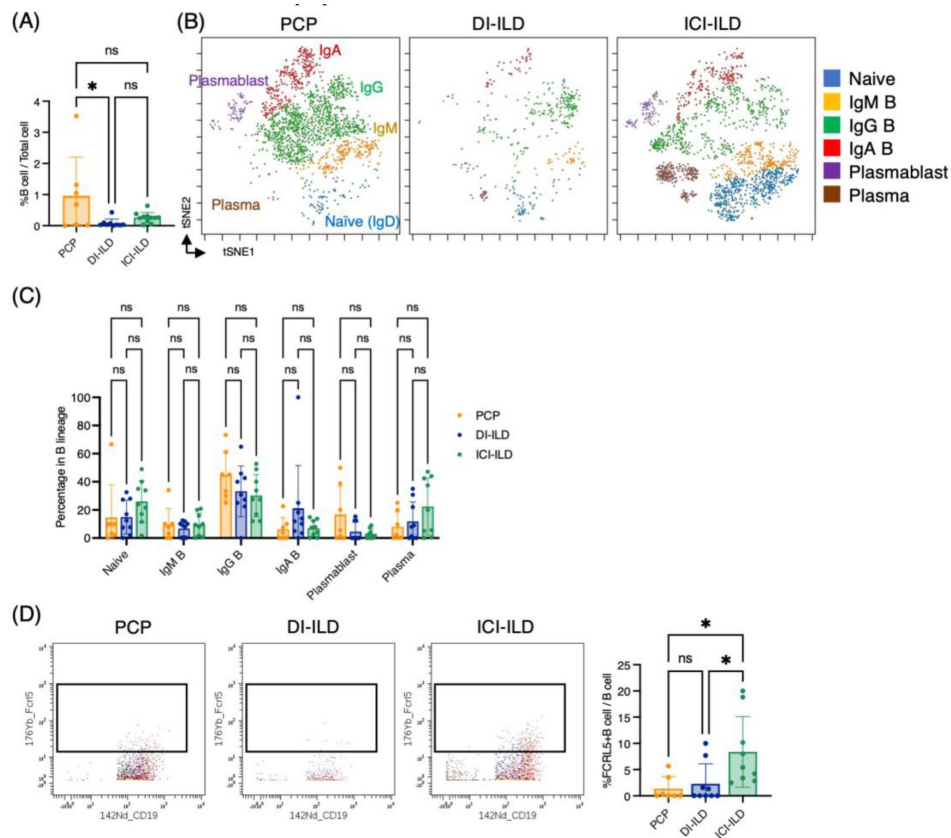


Figure 5.

Characterization of B cell subsets in BALF from patients with PCP, DI-ILD, and ICI-ILD.

(A) Percentages of B cells and plasma cells in CD45⁺ BALF cells. (B) t-stochastic neighborhood embedding (t-SNE) plots of concatenated samples visualizing the distribution of B cell subpopulations in CD64⁻CD3⁻ and CD19⁺ or CD138⁺ gated B cells in BALF from patients with PCP, DI-ILD, and ICI-ILD. Naive B cells are defined by CD19⁺IgD⁺, IgM B cells: CD19⁺ IgM⁺, IgG B cells: CD19⁺ IgG⁺, IgA B cells: CD19⁺ IgA⁺, plasmablasts: CD19⁺ CD27⁺ CD38⁺ CD138⁻, plasma cells: CD19⁻ CD138⁺ and IgG⁺ or IgA⁺. (C) Percentages of B cell subpopulations in PCP (n = 7), DI-ILD (n = 9), and ICI-ILD (n = 9). Dot plots represent individual samples. (D) Two-dimensional dot plots depicting FCRL5-expressing B cells within a gated population of B cells defined as CD64⁻CD3⁻ and CD19⁺ or CD138⁺. Percentages of FCRL5-expressing B cells within the total B cell population are also shown.

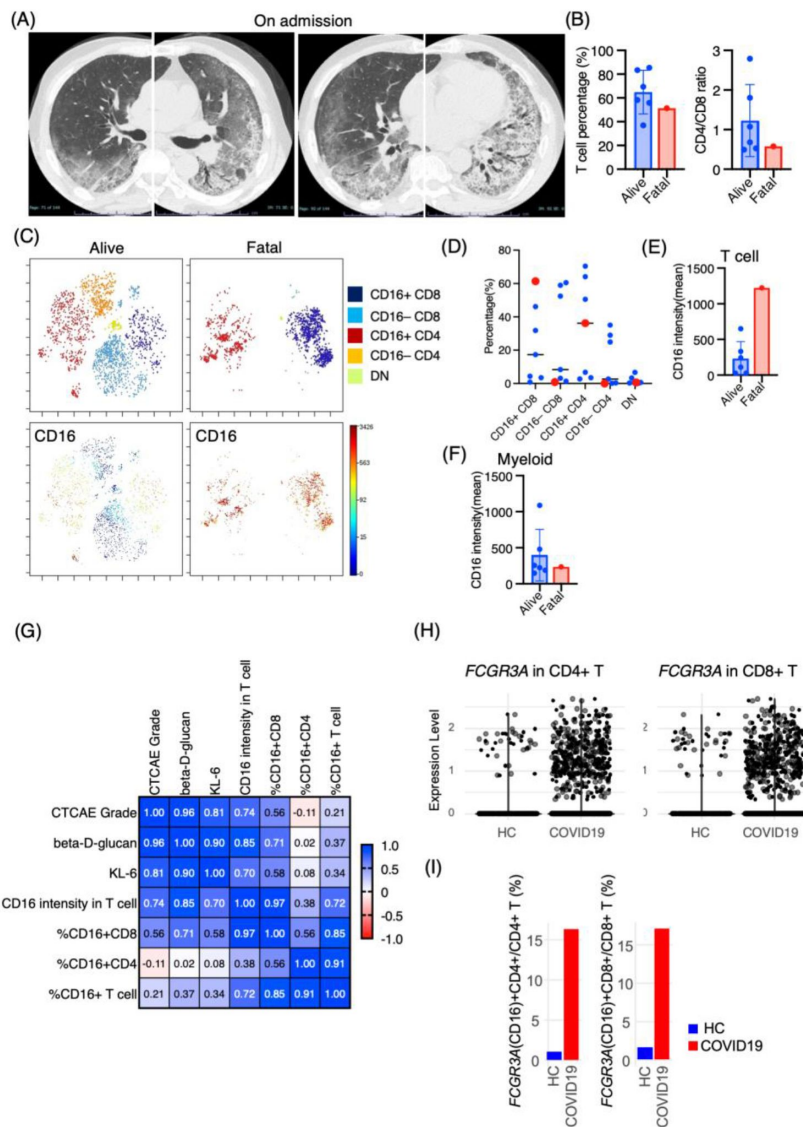


Figure 6.

Immunological phenotypes in a fatal case of PCP.

(A) Chest computed tomography images of the patient upon admission reveal the emergence of bilateral diffuse ground glass opacities and infiltration in both lungs. (B) Comparison of T cell percentage and CD4/CD8 ratio between a fatal case and surviving cases of PCP. (C) t-SNE plots illustrating the distribution of T cell subpopulations in BALF T cells (gated as CD45⁺CD2⁺CD3⁺) from a fatal case and surviving cases of PCP. Double negative (DN) T cells were defined as CD4⁻CD8⁻ T cells. (D) Percentages of each T-cell subpopulation. Red dots represent the value of the fatal case. (E) Mean CD16 intensity in the T cell population. (F) Mean CD16 intensity in the myeloid cell population. (G) The correlation matrix in PCP cases. Pearson *r* values are shown in each square. (H) Violin plots and (I) percentage representation of FCGR3A (CD16) on CD4⁺ and CD8⁺ T cells in BALF from healthy controls (HC) and COVID-19 patients, derived from single-cell RNA-seq dataset GSE145926.

Georg *et al.* recently discovered that COVID-19 severity was associated with highly activated CD16⁺ T cells, which exhibit increased cytotoxic functions (Georg *et al.*, 2022 [\[1\]](#)). This increased population of CD16⁺ T cells in BALF from severe COVID-19 patients was further confirmed by data mining of scRNA-seq (Figures 6H [\[1\]](#) and 6I [\[1\]](#)). CD16 expression allows T cells to degranulate and exert cytotoxicity in an immune-complex-mediated, TCR-independent manner. Moreover, CD16⁺ T cells from COVID-19 patients caused injury to microvascular endothelial cells and induced the release of neutrophil and monocyte chemoattractants (Georg *et al.*, 2022 [\[1\]](#)). Georg *et al.* also discovered that severe COVID-19 induced increased generation of C3a, which further activates CD16⁺ cytotoxic T cells. The proportions of activated CD16⁺ T cells and plasma levels of complement proteins upstream of C3a were found to be associated with fatal outcomes of COVID-19, proving support for the pathological role of exacerbated cytotoxicity and complement activation in COVID-19 (Georg *et al.*, 2022 [\[1\]](#)). Given these findings and the observation of the highest proportion of CD16⁺ T cells in the fatal case of PCP, we hypothesize that these CD16⁺ T cells may possess excessive cytotoxicity toward pulmonary microvascular endothelial cells, contributing to lung injury in PCP, in addition to targeting *Pneumocystis jirovecii*. Based on the therapeutic efficacy of an anti-complement therapy (anti-C5a antibody) for severe COVID-19 (Campbell, 2020 [\[2\]](#))(Vlaar *et al.*, 2020 [\[3\]](#)), anti-complement treatments may have the potential to treat severe PCP. One of the other T cell markers correlated with the severity of PCP was CCR7 (Supplementary Figure 4 [\[1\]](#)), although the precise biological significance of the correlation remains to be elucidated.

We discovered an increase of CD57⁺ CD8⁺ T cells expressing immune checkpoints, PD-1⁺ TIGIT⁺ LAG3⁺ TIM-3⁺ in ICI-ILDs, which supports our previous findings of increased proportions of CD8⁺ T cells positive for both PD-1 and TIM-3 or TIGIT in ICI-ILD (Suzuki *et al.*, 2020 [\[4\]](#)). In the methods section, the low detection of PD-1 expression on T cells in patients treated with nivolumab was noted; this was due to the competitive nature of the PD-1 detection antibody EH12.2 with nivolumab (Yanagihara *et al.*, 2020 [\[5\]](#)). The absence of a metal-conjugated PD-1 antibody with the MIH4 clone presented a limitation in our study. Ideally, we would have conjugated the MIH4 antibody with 155Gd for our analysis, which is a refinement we aim to incorporate in future research. A population of PD-1⁺ TIM-3⁺ CD8⁺ cells was detected in the peritumoral pleural effusion and ILD lesions of a cancer patient undergoing ICI treatment (Yanagihara *et al.*, 2017 [\[6\]](#)). Through the application of next-generation sequencing technology to the DNA encoding the complementarity-determining region of the T-cell receptor (TCR), identical T-cell clones were identified in both peritumoral pleural effusion and ILD lesions (Tanaka *et al.*, 2018 [\[7\]](#)). Similar to ICI-ILD, a recent study identified T cells specific to α -myosin drive ICI-related myocarditis (Axelrod *et al.*, 2022 [\[8\]](#)). These results indicate that ICI-activated CD8⁺ T cells, with a phenotype of PD-1⁺ TIGIT⁺ LAG3⁺ TIM-3⁺, could potentially trigger ILD by recognizing self-peptides or shared epitopes between tumors and lungs. CD16⁺ CD8⁺ T cells in PCP exhibit high CXCR3 and ST2 expression, and CD57⁺ CD8⁺ T cells expressing immune checkpoints in ICI-ILD express high CXCR3 (consistent with the previous study (Kim *et al.*, 2021 [\[9\]](#))). Therefore, these CD8⁺ T cells could potentially migrate into the lungs via chemokines, such as CXCL4, CXCL9, CXCL10, and IL-33 (Van Raemdonck *et al.*, 2015 [\[10\]](#))(Griesenauer and Paczesny, 2017 [\[11\]](#)).

Regarding CD57 expression in T cells, it is generally considered a senescent marker (Fehlings *et al.*, 2022 [\[12\]](#)). However, CD57⁺ CD8⁺ T cells in the periphery show clonal expansion with the overlap of the TCR repertoire of tumor-infiltrating T cells with favorable response to ICI in patients with cancer (Fehlings *et al.*, 2022 [\[12\]](#)), indicating these CD57⁺ CD8⁺ T cells may have a highly active phenotype in ICI-treated individuals.

FCRL5 is a B cell-restricted member of the Fc receptor-like family encoded by the *IRTA2* gene (Rostamzadeh *et al.*, 2018 [\[13\]](#)). In both rheumatoid arthritis (Owczarczyk *et al.*, 2011 [\[14\]](#)) and granulomatosis with polyangiitis and microscopic polyangiitis (Owczarczyk *et al.*, 2020 [\[15\]](#)), increased expression of FCRL5 was indicative of a positive response to rituximab. Additionally, our recent research revealed an increased FCRL5⁺ B cells in connective-tissue disease-related ILD

(Hata et al., 2023 [↗](#)). These findings suggest a potential pathological role of FCRL5 in autoimmunity. Given that ICI-ILD is considered a type of irAE, it is reasonable to hypothesize that FCRL5⁺ B cells may be one of the immune cells associated with autoimmunity.

Regarding myeloid cells, we found that CD14⁺ CCR2⁺ CCR5⁺ monocyte subpopulations were prevalent in ICI-ILD. Franken *et al.* revealed a decrease in anti-inflammatory resident alveolar macrophages and an increase in pro-inflammatory ‘M1-like’ monocytes (expressing TNF, IL-1B, IL-6, IL-23A, and GM-CSF receptor CSF2RA, CSF2RB) in BALF from ICI-ILD compared with controls (Franken et al., 2022 [↗](#)). Taken together with this report, the CD14⁺ CCR2⁺ CCR5⁺ monocytes we found may be an M1-like monocyte.

Our study has several limitations, including the absence of data from healthy individuals, a relatively small sample size, and a retrospective design that resulted in missing clinical data for certain cases. Selection bias may also be a factor, as only patients who underwent bronchoalveolar lavage were eligible for enrollment in this study. It is important to note that these findings demonstrate a correlation rather than proof of causation.

In summary, our study has shown distinct immune cell phenotypes in PCP, DI-ILD, and ICI-ILD. Specifically, we have identified an expansion of CD16⁺ T cells in PCP, as well as an increase in CD57⁺ CD8⁺ T cells expressing immune checkpoints, FCRL5⁺ B cells, and CCR2⁺ CCR5⁺ monocytes in ICI-ILD, which may play a pathogenic role. Based on these findings, further confirmatory research may lead to the development of diagnostic methods and novel strategies that target these specific cell populations to treat chemotherapy-induced pneumonitis.

Author contributions

Conceptualization: TY; Methodology: KH, TY, KM, KK, and YB; Investigation: KH, TY, KM, KK, KS, KT, DE, HA, and MU; Visualization: KH, TY, KM, KK, KS, KT, DE, HA, MU, SI, and YB; Funding acquisition: TY and YF; Supervision: SI, YB, YF, and IO; Writing-original draft: KH and TY; Writing-review and editing: SI, YB, YF, and IO. All authors contributed to the article and approved the submitted version.

Acknowledgements

We thank Ms. Sanae Sekihara, PhD. from Standard Biotools, for her technical assistance. We also extend our appreciation to the Medical Research Center Initiative for High Depth Omics at Kyushu University.

Funding

This research was supported by the Kakihara Foundation and Boehringer Ingelheim (T.Y.), and the Japan Agency for Medical Research and Development (Y.F.).

For CD45 barcoding

Label	Target	product_id	clone	Amount (uL)
89Y	CD45	3089003B	HI30	1
106Cd	CD45	3106001C	HI30	1
110Cd	CD45	3110001C	HI30	1
112Cd	CD45	3112001C	HI30	1
116Cd	CD45	3116001C	HI30	1
196Pt	CD45	3196001C	HI30	1

For antibody cocktail #1 (T cell panel)

Label	Target	Product ID	Clone	Amount (uL)
141Pr	CD3	3141019B	UCHT1	1
142Nd	CD11a	3142006B	HI111	0.5
143Nd	CD5	3143007B	UCHT2	1
145Nd	CD4	3145001B	RPA-T4	1
146Nd	CD8a	3146001B	RPA-T8	1
147Sm	CD7	3147006B	CD7-6B7	1
148Nd	CD274 (PD-L1)	3148017B	29E.2A3	1
149Sm	CD25 (IL-2R)	3149010B	2A3	1
150Nd	CD134 (OX40)	3150023B	ACT35	1
151Eu	CD2	3151003B	TS1/8	1
152Sm	CD95/Fas	3152017B	DX2	1
153Eu	TIM-3	3153008B	F38-2E2	1
154Sm	TIGIT	3154016B	MBSA43	1
155Gd	CD279 (PD-1)	3155009B	EH12.2H7	1
156Gd	CD183 (CXCR3)	3156004B	G025H7	1
158Gd	ST2 (R&D AF523)	Labeling (201158A)		1
159Tb	CD197 (CCR7)	3159003A	G043H7	1
160Gd	CD28	3160003B	CD28.2	1
161Dy	CD152 (CTLA-4)	3161004B	14D3	1
162Dy	CD69	3162001B	FN50	1
163Dy	CD272 (BTLA)	3163009B	MIH26	1
164Dy	CD45RO	3164007B	UCHL1	1

Supplemental Table 1.

Mass cytometry antibody panels.

165Ho	CD223/LAG-3	3165037B	11C3C65	1
166Er	CD44	3166001B	BJ18	1
167Er	CD27	3167002B	O323	1
168Er	CD278/ICOS	3168024B	C398.4A	1
169Tm	CD19	3169011B	HIB19	1
170Er	CD45RA	3170010B	HI100	1
171Yb	CD226	3171013C	DX11	1
172Yb	CD273 (PD-L2)	3172014B	24F.10C12	1
173Yb	HLA-DR	3173005B	L243	0.5
174Yb	CD49d	3174018B	9F10	1
175Lu	CD14	3175015B	M5E2	2
176Yb	CD57	3176019B	HCD57	1
209Bi	CD16	3209002B	3G8	1

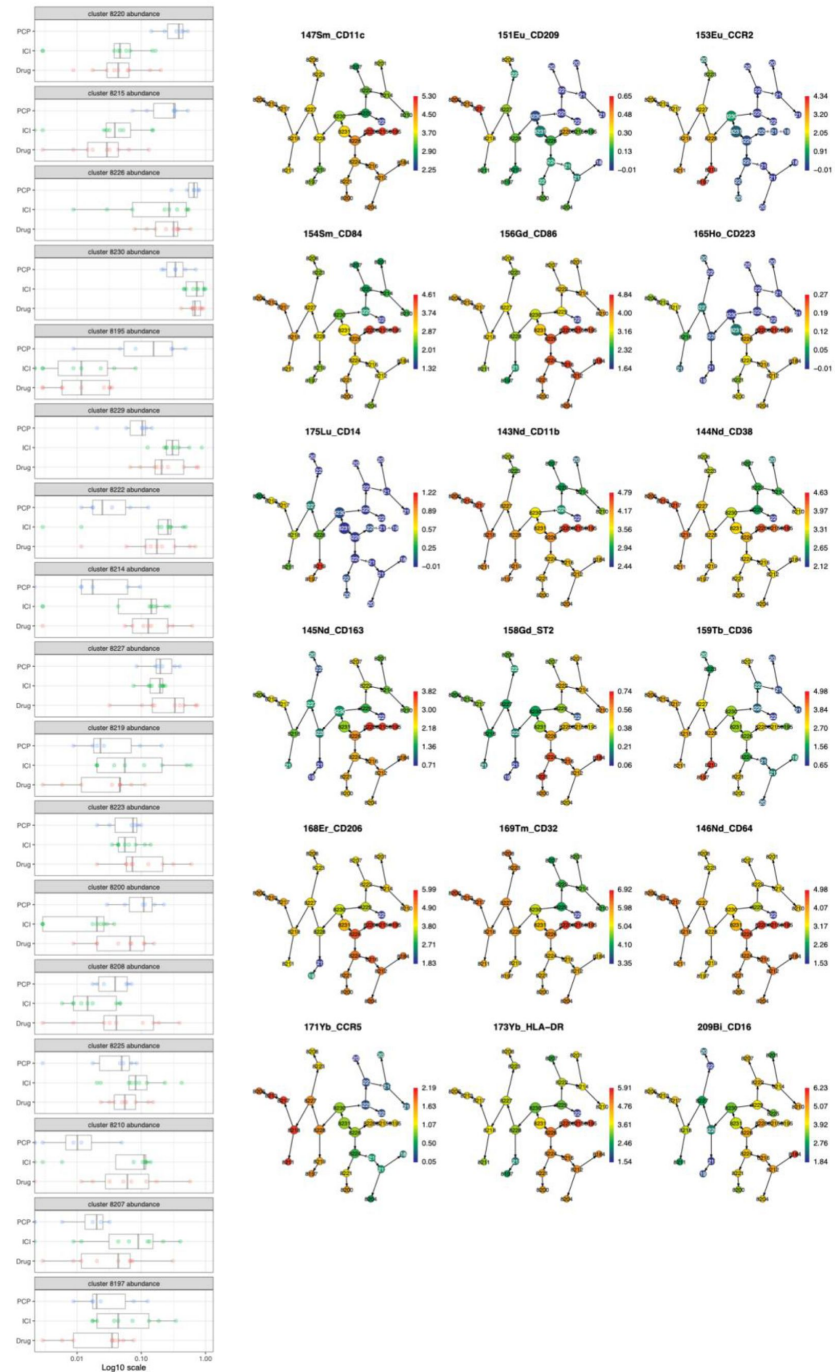
For antibody cocktail #2 (B cell/ myeloid cell panel)

Label	Target	Product ID	Clone	Amount (uL)
141Pr	CD3	3141019B	UCHT1	1
142Nd	CD19	3142001B	HIB19	0.5
143Nd	CD11b (Biolegend #301302)	Labeling(201143A)	ICRF44	0.5
144Nd	CD38	3144014B	HIT2	1
145Nd	CD163	3145010B	GHI/61	1
146Nd	CD64	3146006B	10.1	1
147Sm	CD11c	3147008B	Bu15	0.5
148Nd	IgA	3148007B	Polyclonal	1
149Sm	IgG (Biolegend #410701)	Labeling(201149A)		1
150Nd	CD138	3150012B	DL-101	0.5
151Eu	CD209 (Biolegend #343002)	Labeling(201209A)		1
152Sm	CD21	3152010B	BL13	1
153Eu	CD192 (CCR2)	3153023B	K036C2	1
154Sm	CD84	3154013B	CD84.1.21	1
156Gd	CD86	3156008B	IT2.2	1
158Gd	ST2 (R&D AF523)	Labeling (201158A)		1
159Tb	CD36 (Biolegend #336215)	Labeling(201159A)	5-271	1
160Gd	CD28	3160003B	CD28.2	0.5

Supplemental Table 1. (continued)

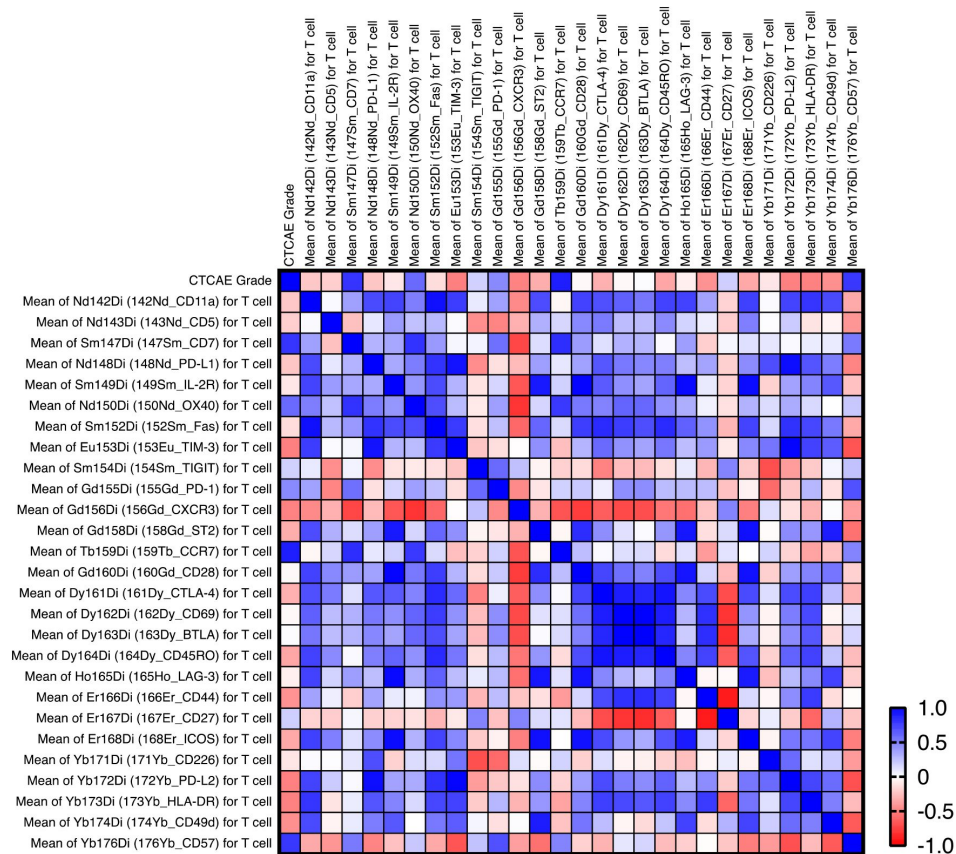
164Dy	CD185/CXCR5	3164029B	RF8B2	1
165Ho	CD223/LAG-3	3165037B	11C3C65	1
166Er	CD24	3166007B	ML5	1
167Er	CD27	3167002B	O323	1
168Er	CD206 (MMR)	3168008B	44607	1
169Tm	CD32	3169020B	FUN-2	1
170Er	TIM-1 (Biolegend #353902)	Labeling(201170A)		1
171Yb	CD195 (CCR5)	3171017A	NP-6G4	1
172Yb	IgM	3172004B	MHM-88	1
173Yb	HLA-DR	3173005B	L243	0.5
174Yb	IgD (Biolegend #348235)	Labeling(201174A)	IA6-2	1
175Lu	CD14	3175015B	M5E2	2
176Yb	antiAPC-176Yb	3176007B	APC003	1
209Bi	CD16	3209002B	3G8	1

Supplemental Table 1. (continued)



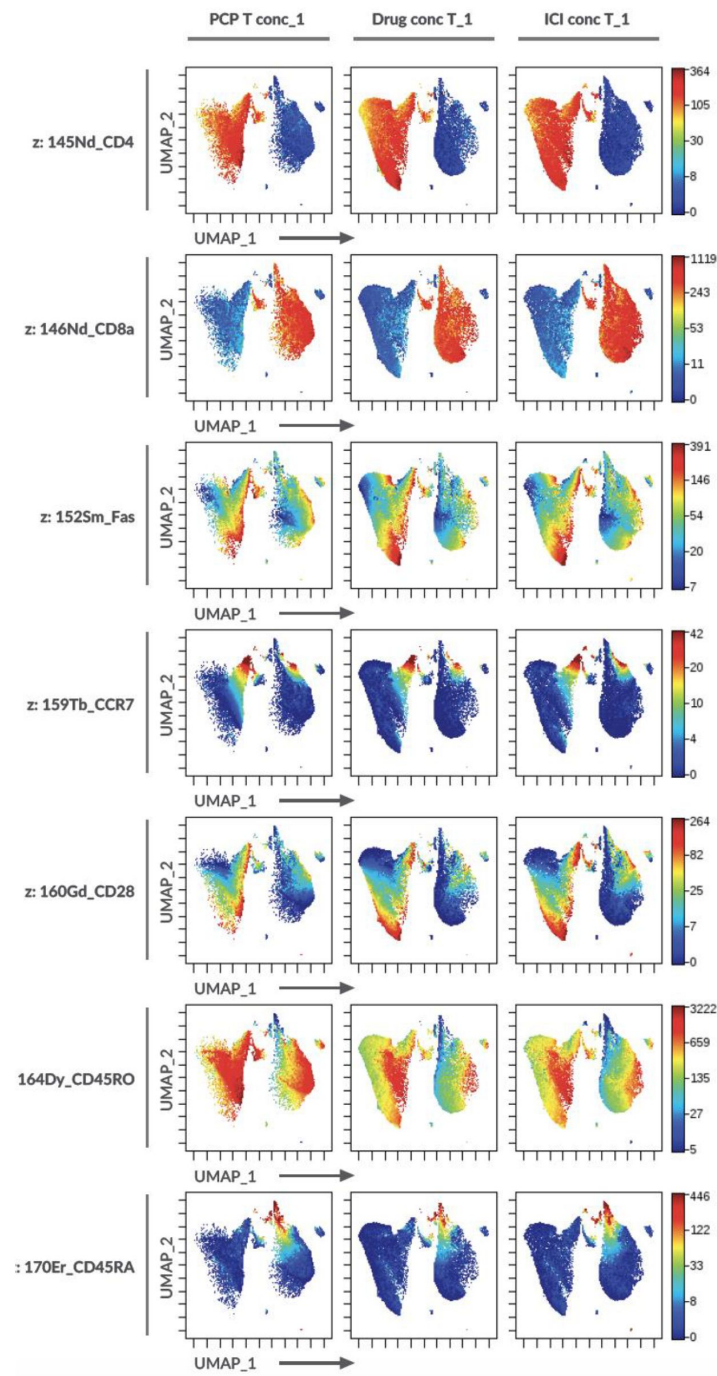
Supplementary Figure 2.

The Citrus analysis of myeloid cell populations in BALF cells from PCP, DI-ILD, and ICI-ILD.



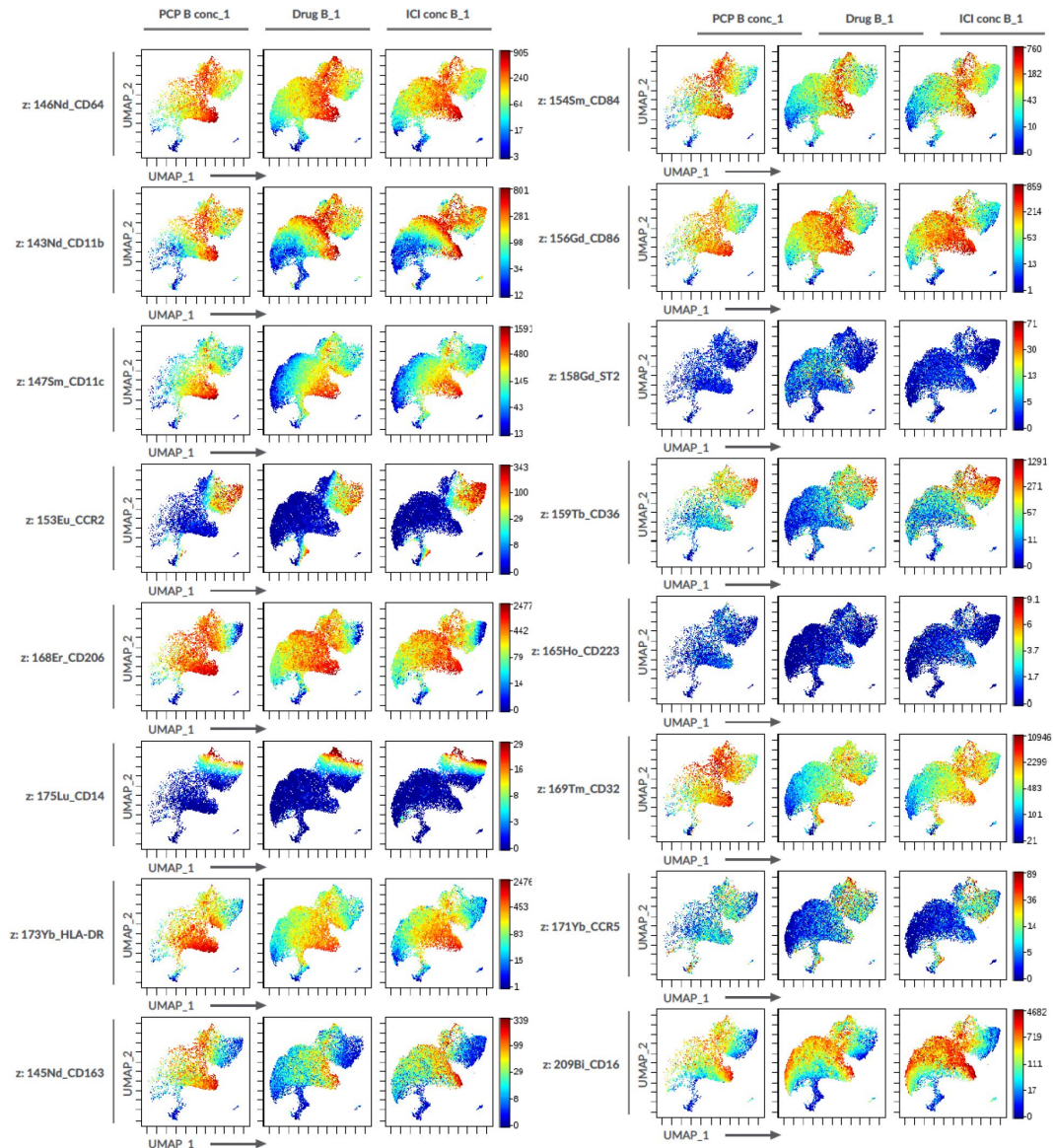
Supplementary Figure 3.

The correlation matrix with the CTCAE and intensity of each T cell markers in PCP cases.



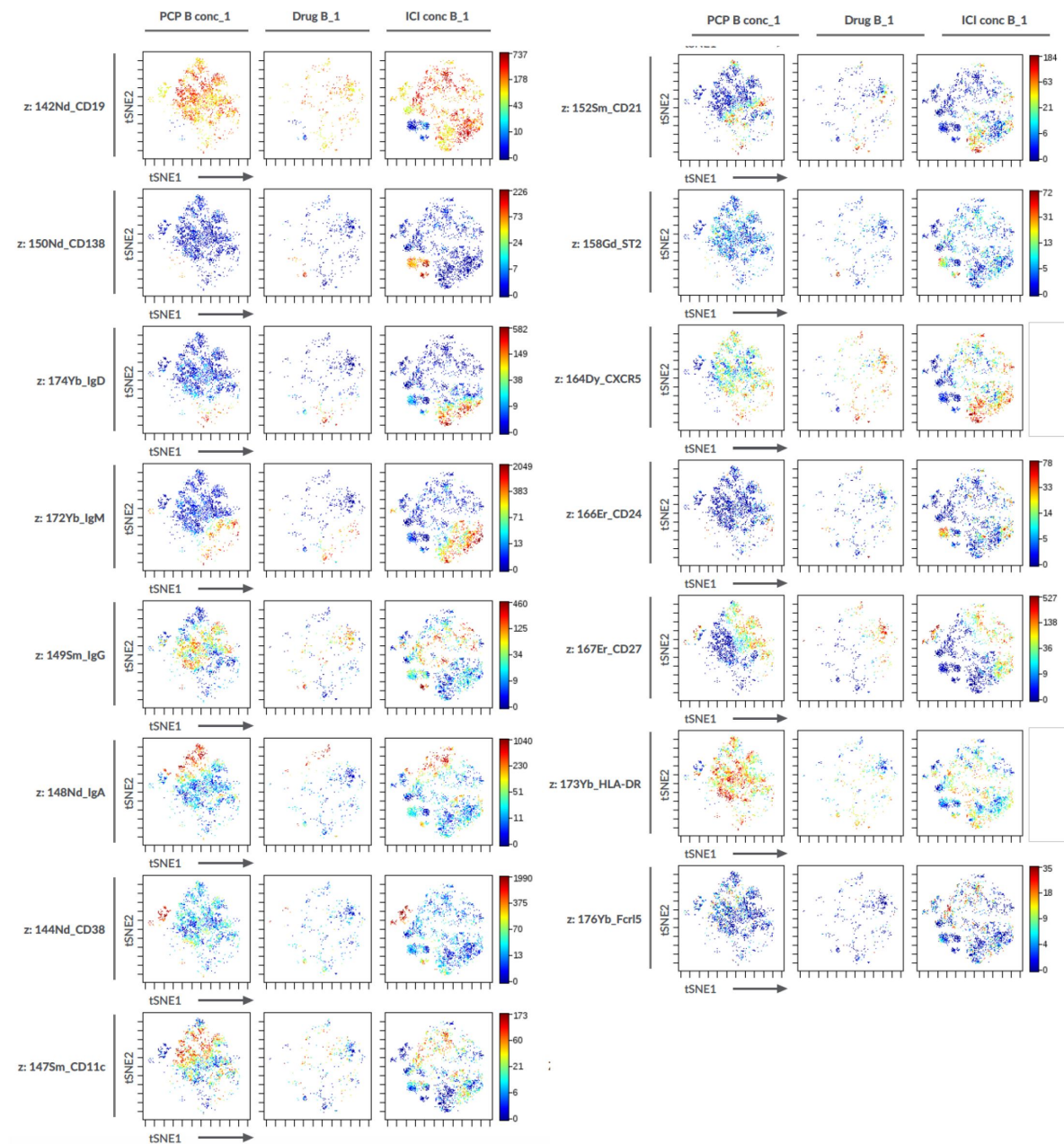
Supplementary Figure 4.

T-cell marker expression on the UMAP projection in Figure 3B [↗](#).



Supplementary Figure 5.

Myeloid-cell marker expression on the UMAP projection in Figure 4A [↗](#).



Supplementary Figure 6.

B-cell marker expression on the tSNE projection in Figure 5B [↗](#).

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38. Yanagihara T, Tanaka K, Ota K, Kashiwagi E, Takeuchi A, Tatsugami K, Eto M, Nakanishi Y, Okamoto I (2017) **Tumor-infiltrating lymphocyte-mediated pleuritis followed by marked shrinkage of metastatic kidney cancer of the chest wall during nivolumab treatment** *Ann Oncol* **28**:2038–2039 <https://doi.org/10.1093/annonc/mdx214>

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Reviewer #2 (Public Review):

Yanagihara and colleagues investigated the immune cell composition of bronchoalveolar lavage fluid (BALF) samples in a cohort of patients with malignancy undergoing chemotherapy and with lung adverse reactions including *Pneumocystis jirovecii* pneumonia (PCP) and immune-checkpoint inhibitors (ICIs) or cytotoxic drug induced interstitial lung diseases (ILDs). Using mass cytometry, their aim was to characterize the cellular and molecular changes in BAL to improve our understanding of their pathogenesis and identify potential biomarkers and therapeutic targets. In this regard, the authors identify a correlation between CD16 expression in T cells and the severity of PCP and an increased infiltration of CD57⁺ CD8⁺ T cells expressing immune checkpoints and FCRL5⁺ B cells in ICI-ILD patients.

The conclusions of this paper are mostly well supported by data, but some aspects of the data analysis need to be clarified and extended.

1. The authors should elaborate on why different set of markers were selected for each analysis step. E.g., Different set of markers were used for UMAP, CITRUS and viSNE in the T cell and myeloid analysis.
2. The authors should state if a normality test for the distribution of the data was performed. If not, non-parametric tests should be used.
3. The authors should explore the correlation between CD16 intensity and the CTCAE grade in T cell subsets such as EMRA CD8 T cells, effector memory CD4, etc as identified in Figure 1B.
4. The authors could use CITRUS to better assess the B cell compartment.

<https://doi.org/10.7554/eLife.87288.2.sa1>

Reviewer #3 (Public Review):

The authors collected BALF samples from lung cancer patients newly diagnosed with PCP, DI-ILD or ICI-ILD. CyTOF was performed on these samples, using two different panels (T-cell and B-cell/myeloid cell panels). Results were collected, cleaned-up, manually gated and pre-processed prior to visualisation with manifold learning approaches t-SNE (in the form of viSNE) or UMAP, and analysed by CITRUS (hierarchical clustering followed by feature selection and regression) for population identification - all using Cytobank implementation - in an attempt to identify possible biomarkers for these disease states. By comparing cell abundances from CITRUS results and qualitative inspection of a small number of marker expressions, the authors claimed to have identified an expansion of CD16⁺ T-cell population in PCP cases and an increase in CD57⁺ CD8⁺ T-cells, FCRL5⁺ B-cells and CCR2⁺ CCR5⁺ CD14⁺ monocytes in ICI-ILD cases.

By the authors' own admission, there is an absence of healthy donor samples and, perhaps as a result of retrospective experimental design and practical clinical reasons, also an absence of pre-treatment samples. The entire analysis effectively compares three yet-established disease states with no common baseline - what really constitutes a "biomarker" in such cases? These are very limited comparisons among three, and only these three, states.

By including a new scRNA-Seq analysis using publicly available dataset, the authors addressed this fundamental problem. Though more thorough and numerical analysis would be appreciated for a deeper and more impactful analysis, this is adequate for the intended objectives of the study.

<https://doi.org/10.7554/eLife.87288.2.sa0>

Author Response

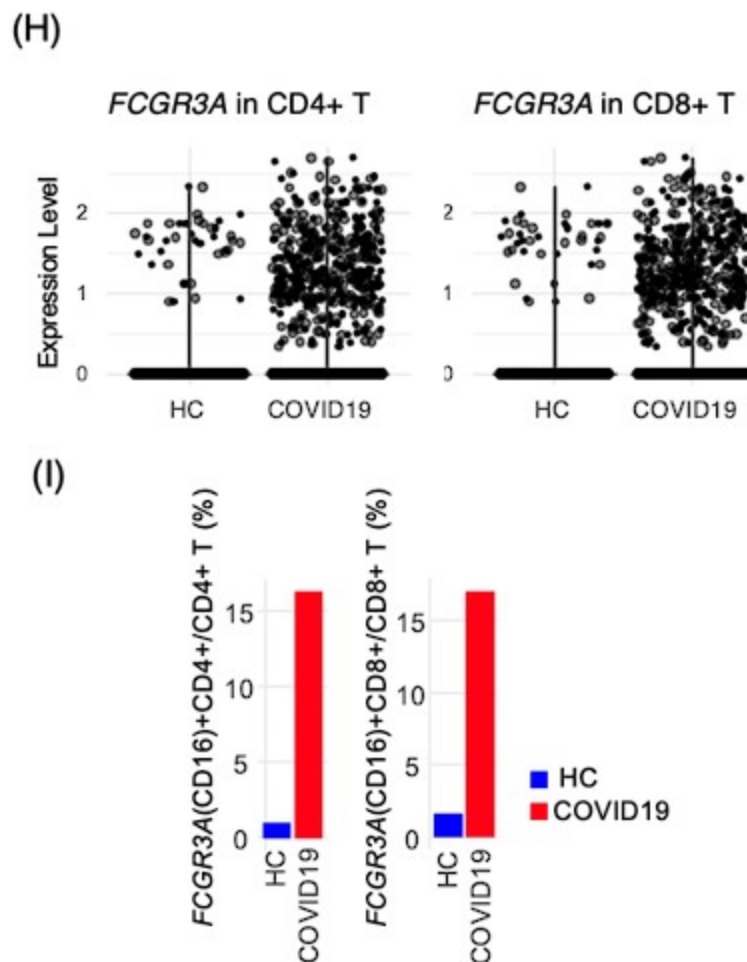
The following is the authors' response to the original reviews.

eLife assessment

This study presents a valuable finding on the immunophenotypes of cancer treatment-related pneumonitis. The evidence supporting the claims of the authors is solid, although the inclusion of controls, as suggested by one of the reviewers, strengthened the study. The work will be of interest to cancer immunologists.

Response: We are thankful for the editor's recognition of the contribution our study makes to understanding the immunophenotypes associated with cancer treatment-related pneumonitis. We agree that the inclusion of control data is pivotal for benchmarking biomarkers. While our initial study design was constrained by the availability of BALF from healthy individuals within clinical settings, we addressed this limitation by incorporating scRNA-seq data from healthy control and COVID-19 BALF cells sourced from the GSE145926 dataset. This additional analysis has provided a baseline for comparison, revealing that CD16 is expressed in a minority of T cells in healthy BALF, specifically 1.0% of CD4⁺ T cells and 1.6% of CD8⁺ T cells. The inclusion of this data as Figures 6H and 6I in our manuscript offers a robust context for the significant increase in CD16-expressing T cells observed in patients with PCP, thus enhancing the robustness of our study's conclusions.

Author response image 1.



Reviewer #1 (Recommendations For The Authors):

Many thanks for giving me the opportunity to review your paper. I really enjoyed the way you carried out this work - for example, your use of a wide panel of markers and the use of two analytical methods - you have clearly given great thought to bias avoidance. I also greatly appreciated your paragraph on the limitations, as there are several, but you do not 'over-sell' your conclusions so there is no issue here for me.

To improve the piece, there are a few typos (eg 318 - specific to alpha-myosin) and I was briefly confused about the highlighted clusters in Figure 4. Perhaps mention why they are highlighted when they first appear in 4D instead of E?

Response: We have corrected the typos, and we have rearranged the sequence of Figures 3E and 3F, as well as 4D and 4E, to ensure a logical flow. Citrus-generated violin plots are now presented prior to the heatmap of the clusters, which better illustrates the progression of our analysis and the derivation of the clusters.

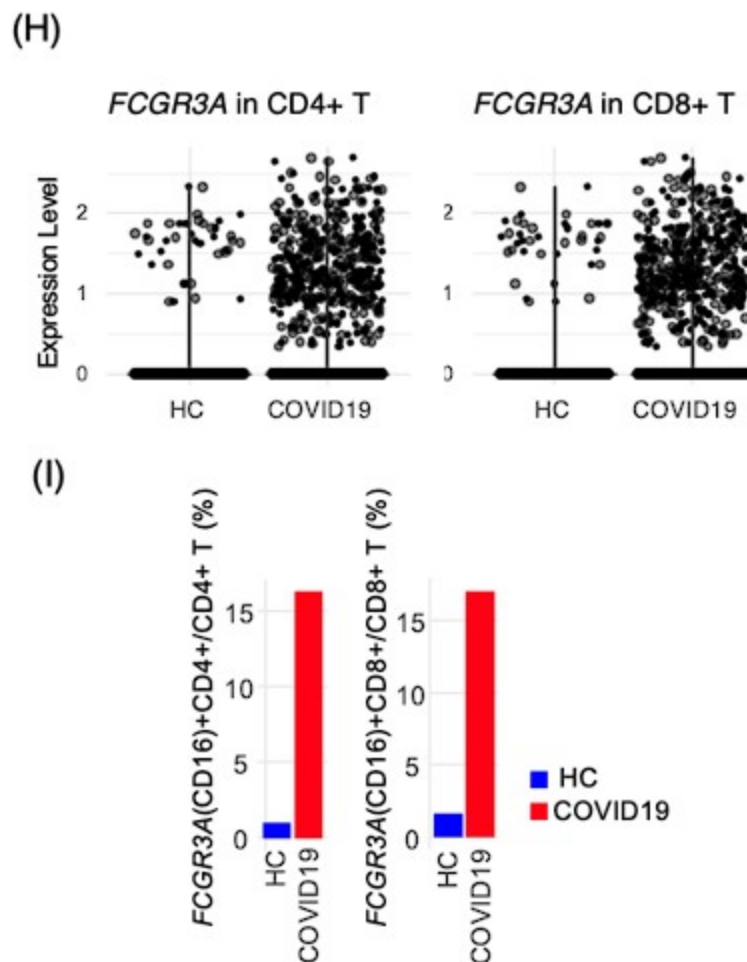
In terms of improvements to the data, obviously it would have been ideal if you had had some sort of healthy control as a point of reference for all cohorts, but working in the field I understand the difficulties in getting healthy BAL. It would be worth your while

however trying to find more supportive data in the literature in general. There are studies which assess various immune markers in healthy BAL eg <https://journal-inflammation.biomedcentral.com/articles/10.1186/1476-9255-11-9>. and so I think it is worth looking wrt the main findings. For example, are CD16+ T cells seen in healthy BAL or any other conditions (at present the COVID study is being over-relied on)? Could these cells be gamma deltas? (gamma deltas frequently express CD8 and CD16, and can switch to APC like phenotypes).

Response: We are grateful for the reviewer's consideration of the practical challenges associated with collecting BALF from healthy individuals. Alternatively, we have supplemented our analysis with single-cell RNA sequencing data from BALF cells of healthy controls, as found in existing literature (Nature Medicine 2020; 26: 842-844). We have accessed to GSE145926 and downloaded data of BALF cells from healthy control (n=3) and severe COVID19 (n=6). The filtered gene-barcode matrix was first normalized using 'NormalizeData' methods in Seurat v.4 with default parameters. The top 2,000 variable genes were then identified using the 'vst' method in Seurat FindVariableFeatures function. Then PCA and UMAP was performed. T cells were identified as CD2 >1 and CD3E >1, and FCGR3A expression was explored using an expression threshold of 0.5. Violin plots and bar plots were generated by ggplot function.

Regarding the pivotal finding of increased CD16-expressing T cells in patients with PCP, the scRNA-seq data mining indicates that CD16 is expressed by a minority of T cells in healthy BALF—1.0% of CD4+ T cells and 1.6% of CD8+ T cells. These figures, now incorporated into our revised manuscript as Figures 6H and 6I, substantiate our findings. These cells could be gamma delta T cells, but we could not confirm it with the limited data. We will investigate in the future study. The main text has been updated to reflect these findings.

Author response image 2.



I would agree with your approach of not going down the transcript route, so just focus on protein expression.

I think you need to mention more about the impact of ICI on PD1 expression - in the methods you lose one approach owing to low T cell expression (132) but in the discussion you mention ICI induced high expression (311) as previously reported. This apparent contradiction needs an explanation.

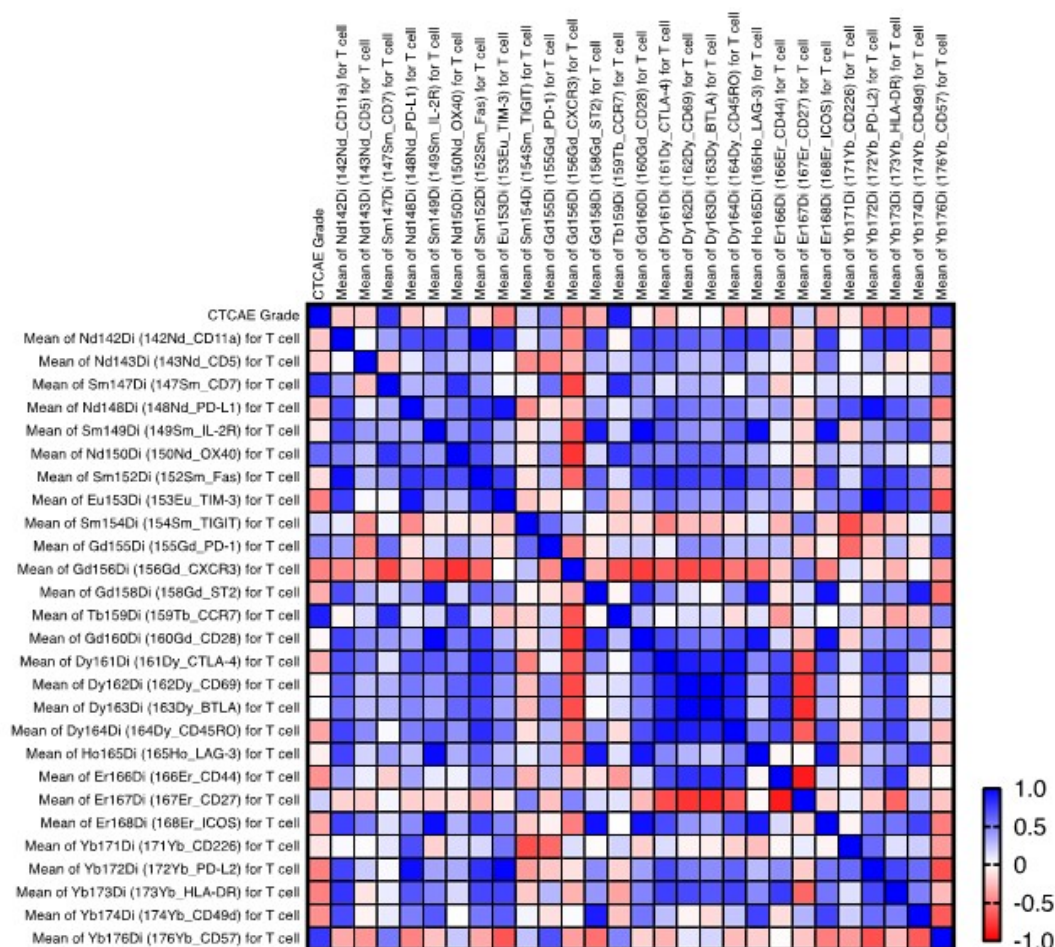
Response: We acknowledge the need for clarification regarding the impact of ICIs on PD-1 expression. In the methods section, the low detection of PD-1 expression on T cells in patients treated with nivolumab was indeed noted; this was due to the competitive nature of the PD-1 detection antibody EH12.2 with nivolumab. As reported by Suzuki et al. (International Immunology 2020; 32: 547-557), T cells from patients with ICI-induced ILD, including those treated with nivolumab, exhibit upregulated PD-1 expression, where the PD-1 detection antibody (clone: MIH4). Conversely, as outlined by Yanagihara et al. (BBRC 2020; 527: 213-217), the PD-1 detection antibody clone EH12.2 conjugated with 155Gd (#3155009B) used in our study is unable to detect PD-1 when patients are under nivolumab treatment due to competitive inhibition. The absence of a metal-conjugated PD-1 antibody with the MIH4 clone presented a limitation in our study. Ideally, we would have conjugated the MIH4 antibody with 155Gd for our analysis, which is a refinement we aim to incorporate in future research.

We have now included this discussion in our manuscript to clarify the contradiction between the methodological limitations and the high PD-1 expression induced by ICIs, as reported in the literature. This addition will guide readers through the nuances of antibody selection and its implications for detecting PD-1 expression in the context of ICI treatment.

Finally, since you have the severity data, it would be good to assess all the significantly different clusters against this metric, as you have done for CD16+ T cells. Not only may this reveal more wrt the impact of other immune populations, but it'll also give a point of reference for the CD16+ T cell data.

Response: Thank you for the suggestion to assess all significantly different clusters against the disease severity metric. We have expanded our analysis to include a thorough correlation study between the disease severity and intensity of various T-cell markers. Notably, we observed that intensity of CCR7 expression correlates with the disease severity. Although the precise biological significance of this correlation remains to be elucidated, it may suggest a role for CCR7+ T cells in the pathogenesis or progression of the disease. We have considered the potential implications of this finding and included it as Supplementary Figure 5. We have also discussed this observation in the discussion section.

Author response image 3.



Overall though I think this is a really nice study, with a potentially very significant finding in linking CD16+ T cells with severity. Congratulations.

Response: We would like to thank the reviewer's heartfelt comments on our manuscript.

Reviewer #2 (Recommendations For The Authors):

General:

1. *The fact that this is a retrospective study should be indicated earlier in the paper.*

Response: Now we have mentioned the retrospective nature of the study in the method section as follows: In this retrospective study, patients who were newly diagnosed with PCP, DI-ILD, and ICI-ILD and had undergone BALF collection at Kyushu University Hospital from January 2017 to April 2022 were included. The retrospective study was approved by the Ethics Committee of Kyushu University Hospital (reference number 22117-00).

1. *tSNE and UMAP are dimensionality reduction techniques that don't cluster the cells, the authors should specify what clustering algorithm was used subsequently (e.g FlowSOM)*

Response: The cluster was determined manually by their expression pattern.

1. *With regards to the role of CD16 in a potential exacerbated cytotoxicity in the fatal PCP case, the authors could measure the levels of C3a related proteins in patient serum to link to a common immunopathogenic pathway with COVID.*

Response: We did not collect serum from the patients in this study as our research protocol was approved by the Ethics committee for the use of BALF only. However, we agree with your assessment that the measurement of serum C3a levels would be informative. In future studies, we will incorporate the measurement of serum C3a levels to provide more comprehensive insights into the impact of C3a on immune function. Thank you for your valuable feedback and for helping us to improve the quality of our research.

Line-specific:

- 101 *The authors should provide some information on how the cryopreservation of the BALF was carried out.*

Response: Upon collection, BALF samples were immediately centrifuged at 300 g for 5 minutes to pellet the cells. The resultant cell pellets were then resuspended in Cellbanker 1 cryopreservation solution (Takara, catalog #210409). This suspension was aliquoted into cryovials and gradually frozen to -80°C using a controlled rate freezing method to ensure cell viability. The samples were stored at -80°C until required for experimental analysis. We have added the information in the method section.

- Fig 3B: It would be very helpful if the authors could add a supplementary figure with marker expression on the UMAP projection.*

Response: We have added Supplementary Figure 4 with marker expression on the UMAP projection in Figure 3B.

- Fig 4A: Same as Fig 3B*

Response: We have added Supplementary Figure 5 with marker expression on the UMAP projection in Figure 4A.

| Fig 5B: Same as Fig 3B

Response: We have added Supplementary Figure 6 with marker expression on the tSNE projection in Figure 5B.

| 266 Authors should state if the data is not shown with regards to differences in myeloid cell fractions

| 430 Marker intensity is not shown in panel D

Re: Corrected as follows: “Citrus network tree visualizing the hierarchical relationship of each marker between identified T cell ~”

| 446 The legend says patients have IPF, CTD-ILD, sarcoidosis but the figure shows PCP, DI-ILD, ICI-ILD.

Re: Corrected.

| 451 What do the authors mean in "Graphical plots represent individual samples"? Panel B is a dot plot of all samples.

Response: Corrected as “Dot plots represent ~”.

| 472 What do the authors mean in "Graphical plots represent individual samples"? Panel C is a dot plot of all samples.

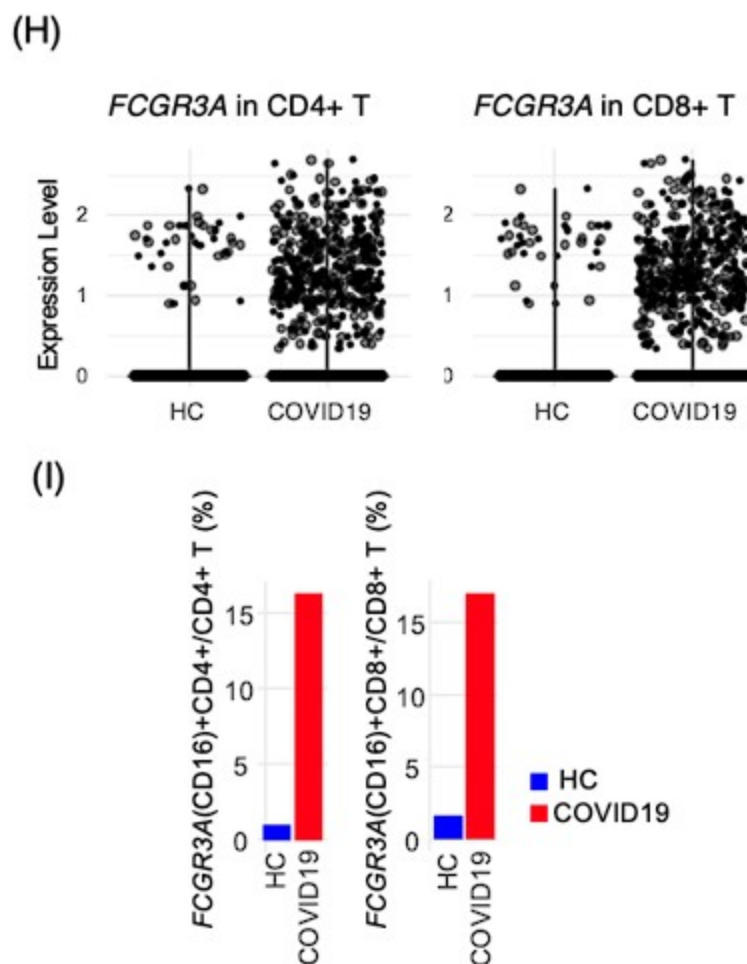
Response: Corrected as “Dot plots represent ~”.

Reviewer #3 (Recommendations For The Authors):

An important thing is to add comparisons against healthy donors, at least. A common baseline is needed to firmly establish any biomarkers.

Response: We acknowledge the reviewer's concern regarding the comparison with healthy donors. Although our study did not initially include BALF collection from healthy controls due to the constraints of clinical practice, we recognize the importance of a control baseline to validate biomarkers. To address this, we have integrated scRNA-seq data from healthy control BALF cells available in public datasets (Nature Medicine 2020; 26: 842-844), accessed from GSE145926. This dataset includes BALF cells from healthy controls (n=3) alongside severe COVID-19 patients (n=6). Data mining confirmed that CD16 expression is in a minority of T cells in healthy BALF—1.0% of CD4+ T cells and 1.6% of CD8+ T cells. We have included this comparative data in our manuscript as Figures 6H and 6I to provide context for the observed increase in CD16-expressing T cells in PCP patients, which substantiates our findings.

Author response image 4.

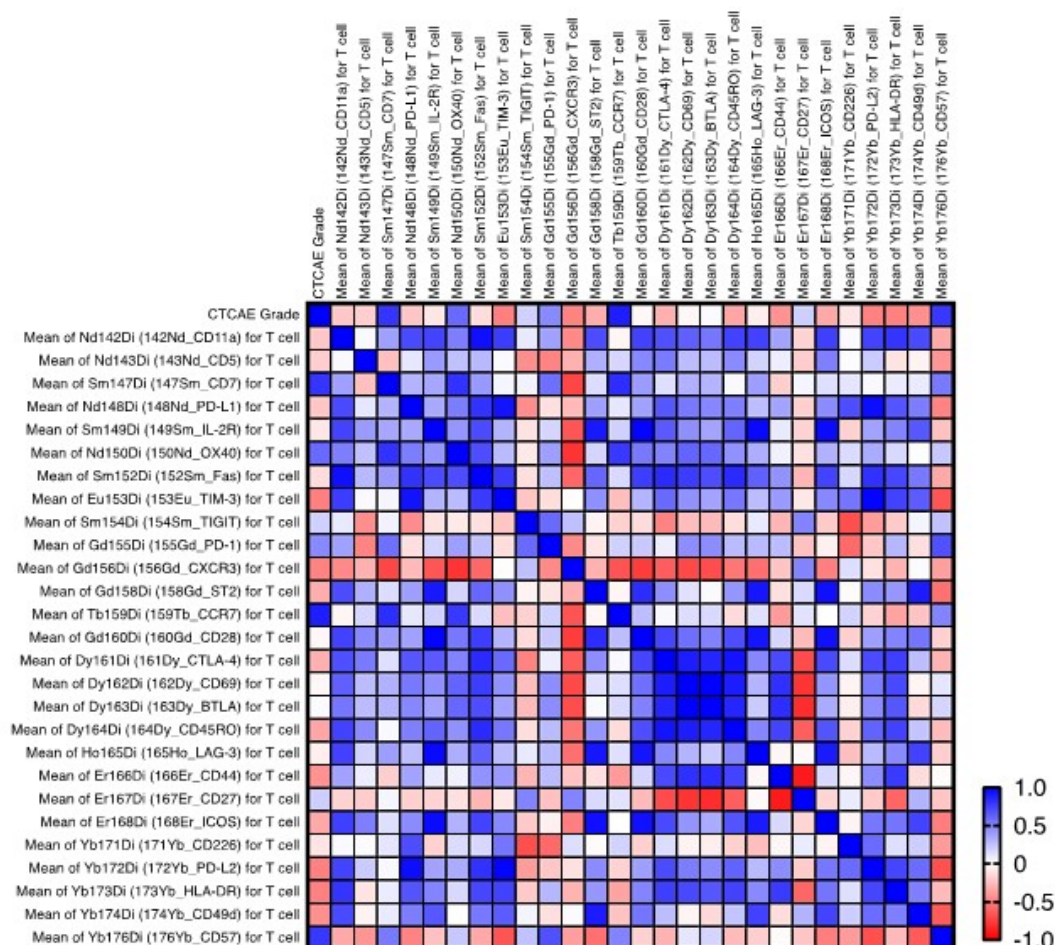


Data analysis needs to go deeper. There are several other tools on Cytobank alone that would allow a more quantitative analysis of the data. Fold changes in marker expressions would be very important as measurements of phenotypic changes.

Response: We thank the reviewer for their constructive feedback on the depth of our data analysis. We acknowledge the value of a more quantitative approach, including the use of fold change measurements to assess phenotypic alterations, and recognize the potential insights such tools on Cytobank could provide. Due to the scope and limited space of the current study, we have focused our analysis on the most pertinent findings relevant to our research questions. We believe the present analysis serves the immediate objectives of this study. However, we agree that further quantitative analysis would enhance the understanding of the data. We have expanded our analysis to include a thorough correlation study between the disease severity of PCP and intensity of various T-cell markers. Notably, we observed that intensity of CCR7 expression correlates with the disease severity of PCP. Although the precise biological significance of this correlation remains to be elucidated, it may suggest a role for CCR7+ T cells in the pathogenesis or progression of the disease. We have considered the potential implications of this finding and included it as Supplementary Figure 5. We have also discussed this observation in the discussion section. We aim to consider these approaches in future work to build upon the foundation laid by this study.

Your suggestions are invaluable and will be kept at the forefront as we plan subsequent research phases.

Author response image 5.



Reviewer #1 (Public Review):

Cytotoxic agents and immune checkpoint inhibitors are the most commonly used and efficacious treatments for lung cancers. However their use brings two significant pulmonary side-effects; namely *Pneumocystis jirovecii* infection and resultant pneumonia (PCP), and interstitial lung disease (ILD). To observe the potential immunological drivers of these adverse events, Yanagihara et al. analysed and compared cells present in the bronchoalveolar lavage of three patient groups (PCP, cytotoxic drug-induced ILD [DI-ILD], and ICI-associated ILD [ICI-ILD]) using mass cytometry (64 markers). In PCP, they observed an expansion of the CD16+ T cell population, with the highest CD16+ T proportion (97.5%) in a fatal case, whilst in ICI-ILD, they found an increase in CD57+ CD8+ T cells expressing immune checkpoints (TIGIT+ LAG3+ TIM-3+ PD-1+), FCRL5+ B cells, and CCR2+ CCR5+ CD14+ monocytes. Given the fatal case, the authors also assessed for, and found, a correlation between CD16+ T cells and disease severity in PCP, postulating that this may be owing to endothelial destruction. Although n numbers are relatively small (n=7-9 in each cohort; common numbers for CyTOF papers), the authors use a wide panel (n=65) and two clustering methodologies giving greater strength to the conclusions. The differential populations discovered using one or two of the analytical methods are robust: whole population shifts with clear and significant clustering. These

data are an excellent resource for clinical disease specialists and pan-disease immunologists, with a broad and engaging contextual discussion about what they could mean.

Strengths:

- The differences in immune cells in BAL in these specific patient subgroups is relatively unexplored.*
- This is an observational study, with no starting hypothesis being tested.*
- Two analytical methods are used to cluster the data.*
- A relatively wide panel was used (64 markers), with particular strength in the alpha beta T cells and B cells.*
- Relevant biomarkers, beta-D-glucan and KL-6 were also analysed*
- Appropriate statistics were used throughout.*
- Numbers are low (7 cases of PCP, 9 of DI-ILD, and 9 of ICI-ILD) but these are difficult samples to collect and so in relative terms, and considering the use of CyTOF, these are good numbers.*
- Beta-D-glucan shows potential as a biomarker for PCP (as previously reported) whilst KL-6 shows potential as a biomarker for ICI-ILD (not reported before). Interestingly, KL-6 was not seen to be increased in DI-ILD patients.*
- Despite the relatively low n numbers and lack of matching there are some clear differentials. The CD4/CD8+CD16+HLA-DR+CXCR3+CD14- T cell result is striking - up in PCP (with EM CD4s significantly down) - whilst the CD8 EMRA population is clear in ICI-ILD and 'non-exhausted' CD4s, with lower numbers of EMRA CD8s in DI-ILD.*
- The authors identify 17/31 significantly differentiated clusters of myeloid cells, eg CD11bhi CD11chi CD64+ CD206+ alveolar macrophages with HLA-DRhi in PCP.*
- With respect to B cells, the authors found that FCRL5+ B cells were more abundant in patients with ICI-ILD compared to those with PCP and DI-ILD, suggesting these FCRL5+ B cells may have a role in irAE.*
- One patient's extreme CD16+ T cell (97.5% positive) and death, led the authors to consider CD16+ T cells as an indicator of disease severity in PCP. This was then tested and found to be correct.*
- Authors discuss results in context of literature leading them to suggest that CD16+ T cells may target endothelial cells and wonder if anti-complement therapy may be efficacious in PCP.*
- Great discussion on auto-reactive T cell clones where the authors suggest that in ICI-ILD CD8s may react against healthy lung, driving ILD.*
- An observation of CXCR3 in different CD8 populations in ICI-ILD and PCP lead the authors to hypothesise on the chemoattractants in the microenvironment.*
- Excellent point suggesting CD57 may not always be a marker of senescence on T cells - reflective of growing change within the community.*
- Well considered suggestion that FCRL5+ B cells may be involved in ICI-ILD driven autoimmunity.*

- The authors discuss the main weaknesses in the discussion and stress that the findings detailed in the paper "demonstrate a correlation rather than proof of causation".

- Figures and legends are clear and pleasing to the eye.

Weaknesses:

- This is an observational study, with no starting hypothesis being tested.

- Only patients who were able to have a lavage taken have been recruited.

- One set of analysis wasn't carried out for one subgroup (ICI-ILD) as PD1 expression was negative owing to the use of nivolumab.

- Some immune cell subsets wouldn't be picked up with the markers and gating strategies used; e.g. NK cells.

- Some immune cells would be disproportionately damaged by the storage, thawing and preparation of the samples; e.g. granulocytes.

- Numbers are low (7 cases of PCP, 9 of DI-ILD, and 9 of ICI-ILD), sex, age and adverse event matching wasn't performed, and treatment regimen are varied and 'suspected' (suggesting incomplete clinical data) - but these are difficult samples to collect. These numbers drop further for some analyses e.g. T cell clustering owing to factors such as low cell number.

- The disease comparisons are with each other, there is no healthy control.

- Samples are taken at one time point.

- The discussion on probably the stand out result - the CD16+ T cells in PCP - relies on two papers - leading to a slightly skewed emphasis on one paper on CD16+ cells in COVID. There are other papers out there that have observed CD16+ T cells in other conditions. It is also worth being in mind that given the markers used, these CD16+ T cell may be gamma deltas.

- The discussion on ICI patient consistently showing increased PD1, could have been greater, as given the ICI is targeting PD1, one would expect the opposite as commented on, and observed, in the methods section.

Reviewer #2 (Public Review):

Yanagihara and colleagues investigated the immune cell composition of bronchoalveolar lavage fluid (BALF) samples in a cohort of patients with malignancy undergoing chemotherapy and with lung adverse reactions including *Pneumocystis jirovecii* pneumonia (PCP) and immune-checkpoint inhibitors (ICIs) or cytotoxic drug induced interstitial lung diseases (ILDs). Using mass cytometry, their aim was to characterize the cellular and molecular changes in BAL to improve our understanding of their pathogenesis and identify potential biomarkers and therapeutic targets. In this regard, the authors identify a correlation between CD16 expression in T cells and the severity of PCP and an increased infiltration of CD57+ CD8+ T cells expressing immune checkpoints and FCLR5+ B cells in ICI-ILD patients.

The conclusions of this paper are mostly well supported by data, but some aspects of the data analysis need to be clarified and extended.

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1. The authors should state if a normality test for the distribution of the data was performed. If not, non-parametric tests should be used.

1. The authors should explore the correlation between CD16 intensity and the CTCAE grade in T cell subsets such as EMRA CD8 T cells, effector memory CD4, etc as identified in Figure 1B.

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The authors collected BALF samples from lung cancer patients newly diagnosed with PCP, DI-ILD or ICI-ILD. CyTOF was performed on these samples, using two different panels (T-cell and B-cell/myeloid cell panels). Results were collected, cleaned-up, manually gated and pre-processed prior to visualisation with manifold learning approaches t-SNE (in the form of viSNE) or UMAP, and analysed by CITRUS (hierarchical clustering followed by feature selection and regression) for population identification - all using Cytobank implementation - in an attempt to identify possible biomarkers for these disease states. By comparing cell abundances from CITRUS results and qualitative inspection of a small number of marker expressions, the authors claimed to have identified an expansion of CD16+ T-cell population in PCP cases and an increase in CD57+ CD8+ T-cells, FCRL5+ B-cells and CCR2+ CCR5+ CD14+ monocytes in ICI-ILD cases.

By the authors' own admission, there is an absence of healthy donor samples and, perhaps as a result of retrospective experimental design, also an absence of pre-treatment samples. The entire analysis effectively compares three yet-established disease states with no common baseline - what really constitutes a "biomarker" in such cases? The introduction asserts that "y characterizing the cellular and molecular changes in BAL from patients with these complications, we aim to improve our understanding of their pathogenesis and identify potential therapeutic targets" (lines 82-84). Given these obvious omissions, no real "changes" have been studied in the paper. These are very limited comparisons among three, and only these three, states.

Even assuming more thorough experimental design, the data analysis is unfortunately too shallow and has not managed to explore the wealth of information that could potentially be extracted from the results. CITRUS is accessible and convenient, but also make a couple of big assumptions which could affect data analysis - 1) Is it justified to concatenate all FCS files to analyse the data in one batch / small batches? Could there be batch effects or otherwise other biological events that could confuse the algorithm? 2) With a relatively small number of samples, and after internal feature selection of CITRUS, is the regression model suitable for population identification or would it be too crude and miss out rare populations? There are plenty of other established methods that could be used instead. Have those methods been considered?

Colouring t-SNE or UMAP (e.g. Figure 6C) plots by marker expression is useful for quick identification of cell populations but it is not a quantitative analysis. In a CyTOF analysis like this, it is common to work out fold changes of marker expressions between conditions. It is inadequate to judge expression levels and infer differences simply by looking at colours.

The relatively small number of samples also mean that most results presented in the paper are not statistical significant. Whilst it is understandable that it is not always possible to collect a large number of patient samples for studies like this, having several entire major figures showing "n.s." (e.g. Figures 3A, 4B and 5C), together with limitations in the comparisons themselves and inadequate analysis, make the observations difficult to be convincing, and even less so for the single fatal PCP case where $N = 1$.

It would also be good scientific practice to show evidence of sample data quality control. Were individual FCS files examined? Did the staining work? Some indication of QC would also be great.

This dataset generated and studied by the authors have the potential to address the question they set out to answer and thus potentially be useful for the field. However, in the current state of presentation, more evidence and more thorough data analysis are needed to draw any conclusions, or correlations, as the authors would like to frame them.