RESEARCH ARTICLE

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

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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 ($TIGIT^+$ $LAG3^+$ $TIM\text{-}3^+$ $PD\text{-}1^+$), $FCRL5^+$ B cells, and $CCR2^+$ $CCR5^+$ $CD14^+$ monocytes. These findings uncover the diverse immunophenotypes and possible pathomechanisms of cancer treatment-related pneumonitis.

Keywords

Mass cytometry, cytometry by time-of-flight (CyTOF), *Pneumocystis jirovecii* pneumonia, immune-checkpoint inhibitor, immune-related adverse event, drug-induced interstitial lung disease

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)(Morelli et al., 2022)(Skeoch et al., 2018). 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)(Asai et al., 2022)(Apostolopoulou and Fishman, 2022). 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)(Ando et al., 2021). 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). 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)(Hata et al., 2023)(Couloume et al., 2021)(Chedid et al., 2022). 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 use mass cytometry to analyze bronchoalveolar lavage fluid (BALF) samples to distinguish pulmonary involvement in patients with malignancy undergoing chemotherapy and to identify potential biomarkers for DI-ILD,

ICI-ILD, and PCP. By 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.

Materials and Methods

Patients

Eligible for enrollment in the study were patients newly diagnosed with PCP, DI-ILD, and ICI-ILD who had undergone BALF collection at Kyushu University Hospital between January 2017 and April 2022. The 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)(Asai et al., 2022)(Apostolopoulou and Fishman, 2022)(Delaunay et al., 2019). A visual representation of the experimental and analytical workflow can be found in Figure 1.

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). 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 (viSNE), 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, $CD2^+$ $CD3^+$ 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 viSNE 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, $CD3^-$ $CD11b^+$ $CD11c^+$ 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 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.

Results

Patient characteristics and clinical parameters

We analyzed 7 cases of PCP, 9 of DI-ILD, and 9 of ICI-ILD (Table 1). 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). 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). 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), 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. 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 (FcγRIIIa), 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 3E) 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 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 ± 6.74%, 1.37 ± 2.30%, 2.29 ± 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.

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). 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 3E).

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). 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). 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). 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)(Vlaar et al., 2020), anti-complement treatments may have the potential to treat severe PCP.

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). 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). 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). Similar to ICI-ILD, a recent study identified T cells specific α-myosin drive ICI-related myocarditis (Axelrod et al., 2022). 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)). 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)(Griesenauer and Paczesny, 2017).

Regarding CD57 expression in T cells, it is generally considered a senescent marker (Fehlings et al., 2022). However, $CD57^+$ $CD8^+$ T cells in the periphery show clonal expansion with the overlap of the TCR repatoire of tumor-infiltrating T cells with favorable response to ICI in patients with cancer (Fehlings et al., 2022), 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, which belongs to the immunoglobulin superfamily receptor translocation-associated 2 (Rostamzadeh et al., 2018). In both rheumatoid arthritis (Owczarczyk et al., 2011) and granulomatosis with polyangiitis and microscopic polyangiitis (Owczarczyk et al., 2020), increased expression of FCRL5 was indicative of a positive response to rituximab. Additionally, our recent research revealed an increased FCLR5+ 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, this $CD14^+$ $CCR2^+$ $CCR5^+$ 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.

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Table 1. Characteristics of the study population.

	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.

Figure Legends

Figure 1. Graphical abstract of the study.

Bronchoalveolar lavage fluid (BALF) samples were collected from patients with Pneumocystis jirovecii pneumonia (PCP), cytotoxic drug-related 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.

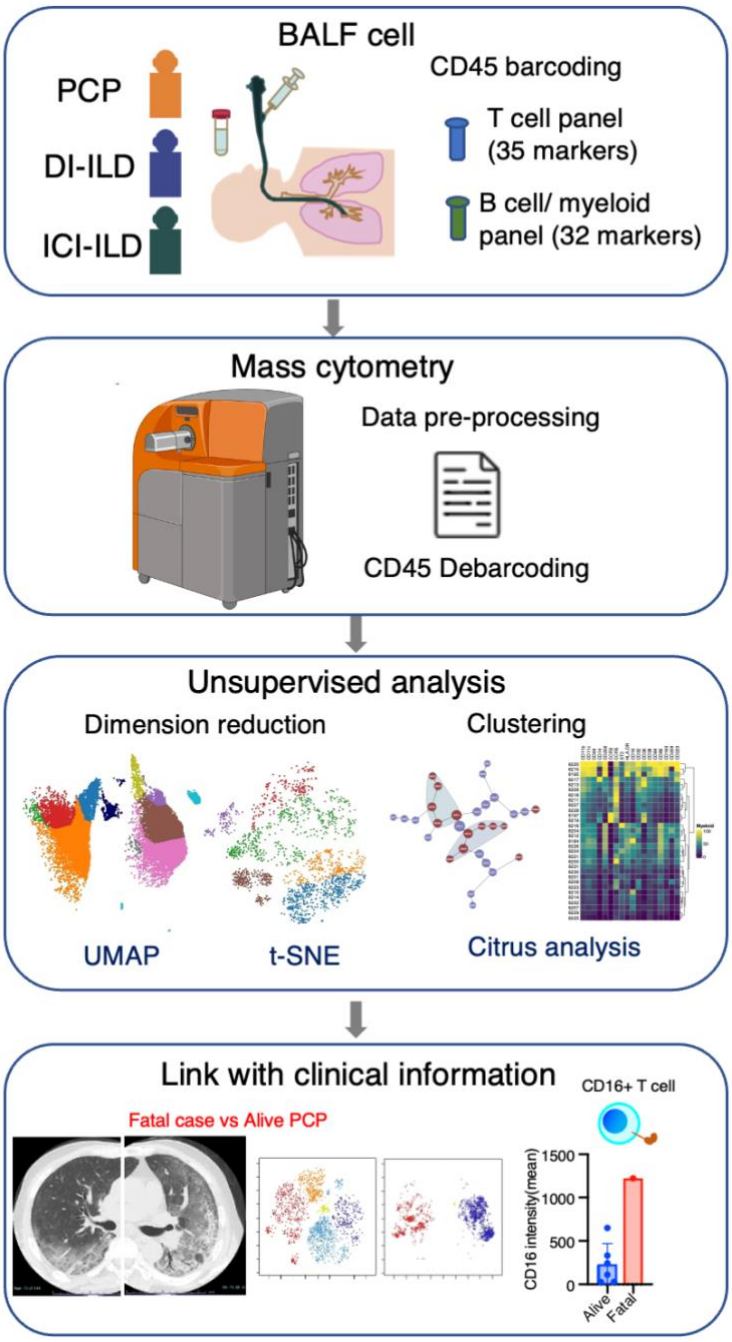


Figure 2. Serum levels of beta-D-glucan and KL-6 from patients with PCP, DI-ILD, and ICI-ILD.

(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.

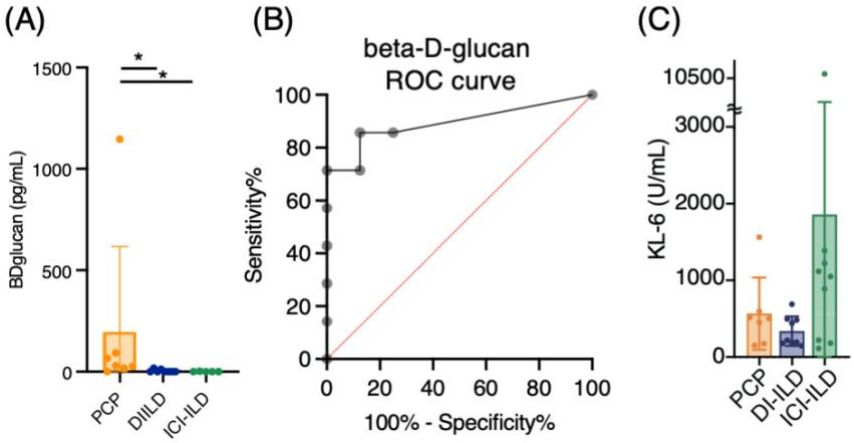


Figure 3. Characterization of T cell subsets in BALF from patients with PCP, DI-ILD, and ICI-ILD.

(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 and intensity 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) Heatmap demonstrates the expression of various markers in different clusters of T cells, as identified through the Citrus analysis. (F) Citrus-generated violin plots for six representative and differentially regulated populations. Each cluster number (#) corresponds to the number shown in panel (D). All differences in abundance were significant at a false discovery rate < 0.01.

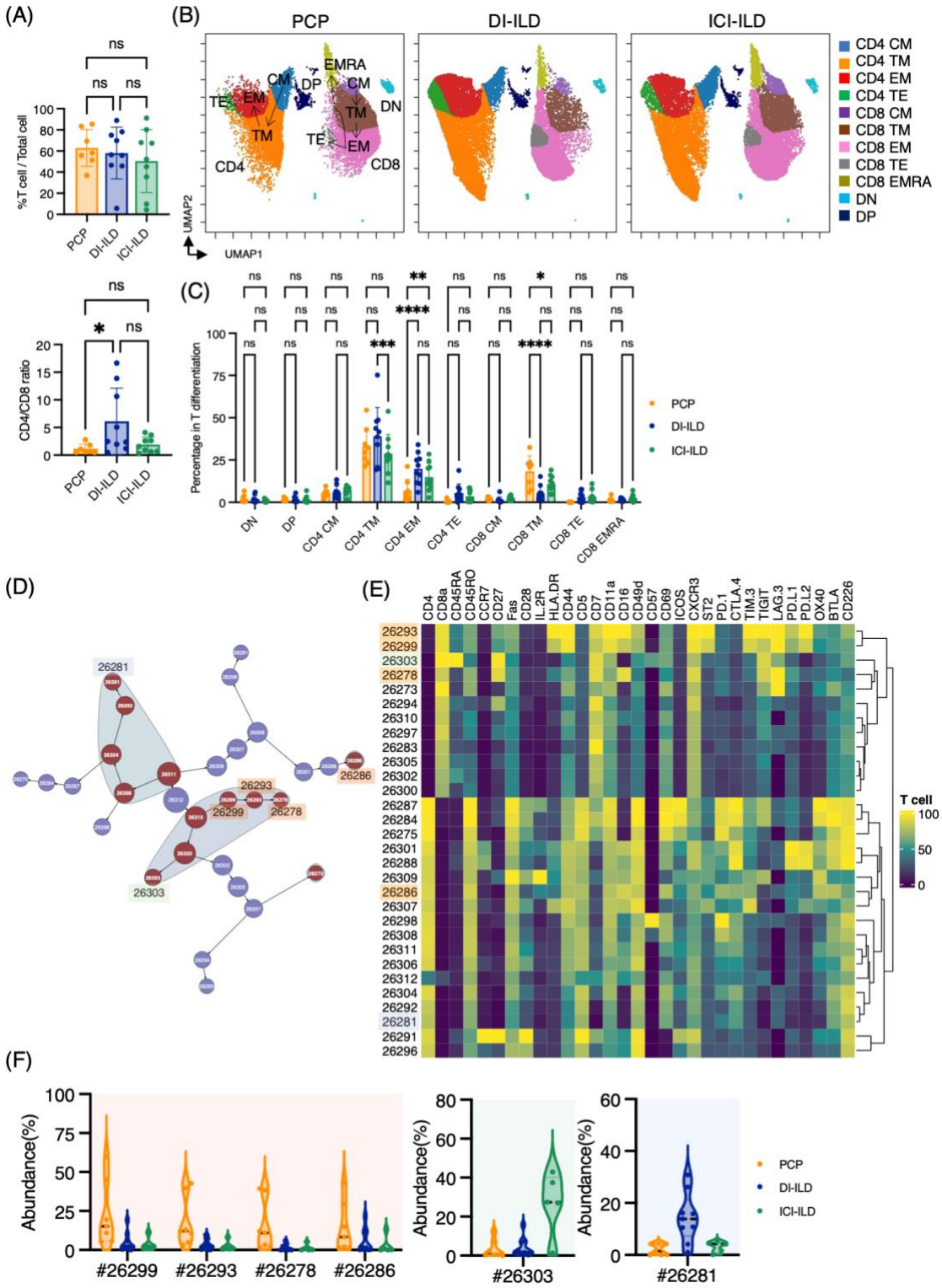

(A)
(B)
PCP
DI-ILD
ICI-ILD
%T cell / Total cell
CD4/CD8 ratio
UMAP1
UMAP2
CD4
CD8
CD4 CM
CD4 TM
CD4 EM
CD4 TE
CD8 CM
CD8 TM
CD8 EM
CD8 TE
CD8 EMRA
DN
DP
(C)
Percentage in T differentiation
(D)
(E)
T cell
(F)
Abundance(%)
#26299
#26293
#26278
#26286
#26303
#26281

Figure 4. Characterization of myeloid cell subsets in BALF from patients with PCP, DI-ILD, and ICI-ILD.

(A) Uniform manifold approximation and projection (UMAP) of concatenated samples visualizing the distribution of myeloid cell sub-populations in $CD3^{-}$ $CD11b^{+}$ $CD11c^{+}$ gated myeloid cells in BALF from patients with IPF, CTD-ILD, sarcoidosis. Monocytes were defined by $CD64^{+}$ $CD14^{+}$, $CCR2^{+}$ macrophages (Mp) by $CD64^{+}$ $CD14^{-}$ $CCR2^{+}$, Alveolar Mp by $CD64^{+}$ $CD14^{-}CCR2^{-}$ $CD206^{+}$, dendritic cells (DC) by $CD64^{-}CD14^{-}$ $CD206^{-}CD11c^{+}$ $HLA\text{-}DR^{+}$, 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). Graphical plots represent individual samples. (C) Citrus network tree visualizing the hierarchical relationship and intensity 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) Heatmap demonstrates the expression of various markers in different clusters of myeloid cells, as identified through the Citrus analysis. (E) 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.

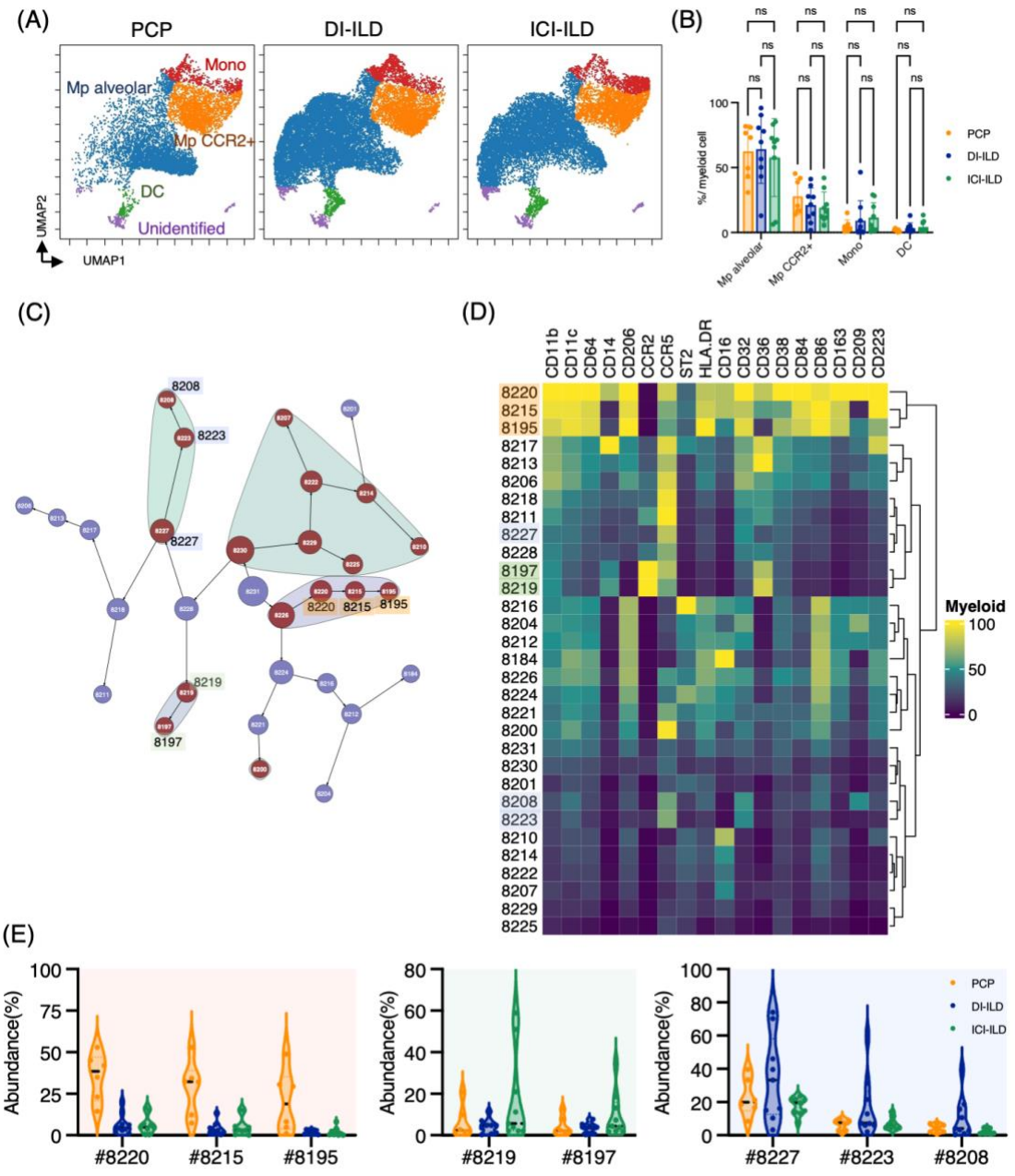

(A)
PCP
DI-ILD
ICI-ILD
Mono
Mp alveolar
Mp CCR2+
DC
Unidentified
UMAP2
UMAP1
(B)
ns
%/ myeloid cell
100
50
0
Mp alveolar
Mp CCR2+
Mono
DC
PCP
DI-ILD
ICI-ILD
(C)
8208
8223
8227
8220
8215
8195
8219
8197
(D)
CD11b
CD11c
CD64
CD14
CD206
CCR2
CCR5
ST2
HLA.DR
CD16
CD32
CD36
CD38
CD84
CD86
CD163
CD209
CD223
8220
8215
8195
8217
8213
8206
8218
8211
8227
8228
8197
8219
8216
8204
8212
8184
8226
8224
8221
8200
8231
8230
8201
8208
8223
8210
8214
8222
8207
8229
8225
Myeloid
100
50
0
(E)
Abundance(%)
#8220
#8215
#8195
#8219
#8197
#8227
#8223
#8208
PCP
DI-ILD
ICI-ILD

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). Graphical 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.

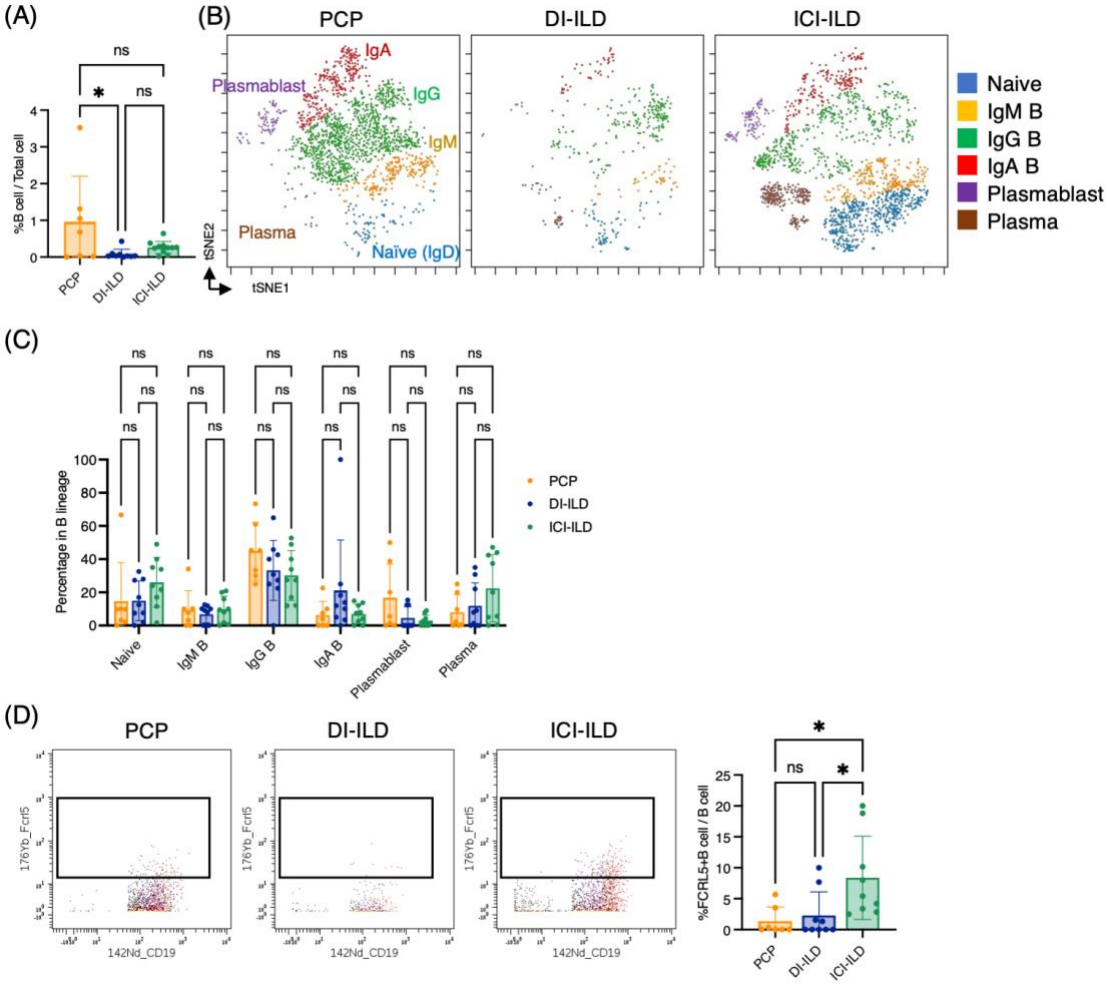
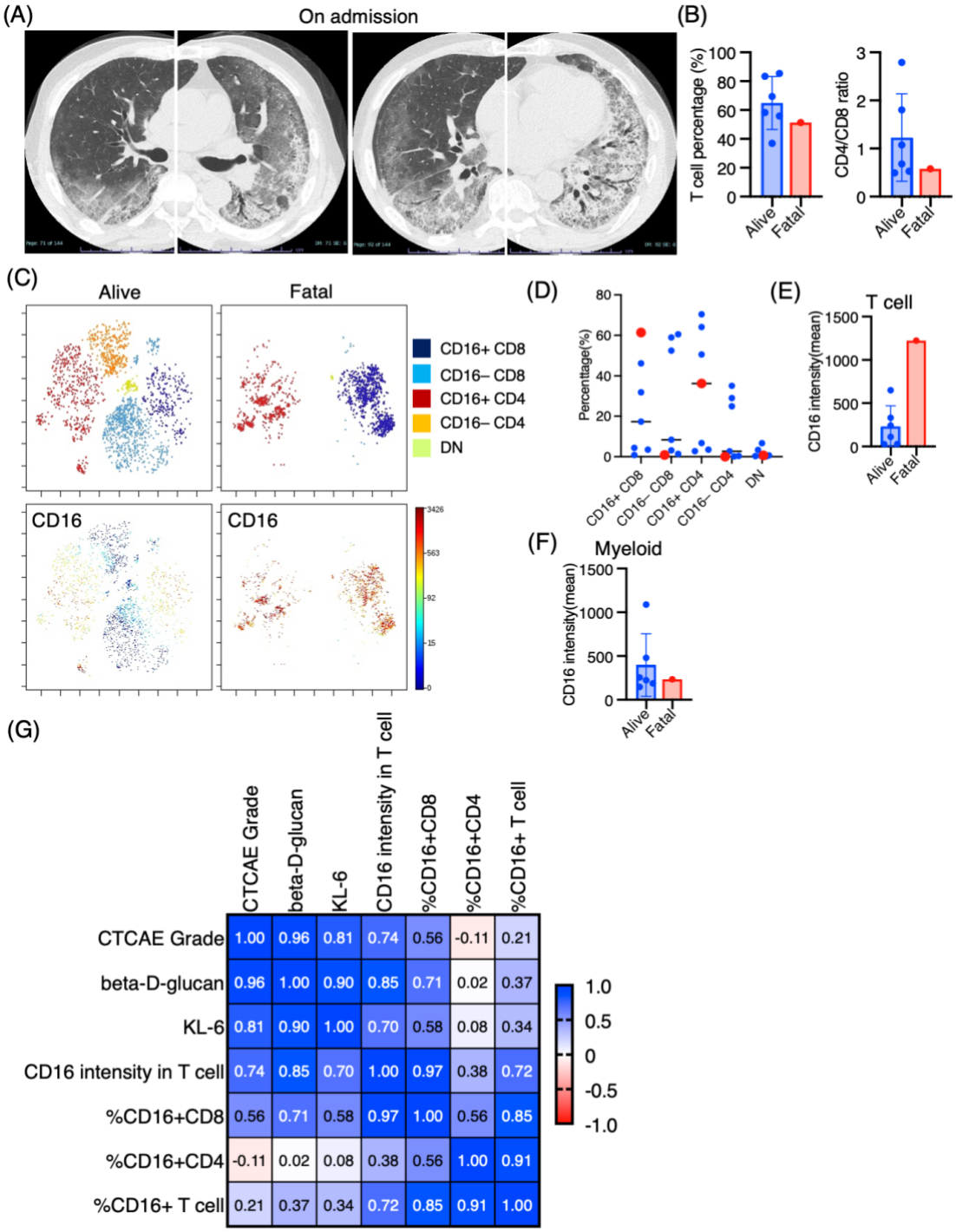


Figure 6. Immunological phenotypes in a fatal case of PCP.
(A) Chest computed tomography images of the patient upon admission reveal the emergence of bilateral diffuse ground glass opacities and infiltration in both lungs. (B) Comparison of T cell percentage and CD4/CD8 ratio between a fatal case and surviving cases of PCP. (C) t-stochastic neighborhood embedding (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.



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Supplemental Table 1. Mass cytometry antibody panels.

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

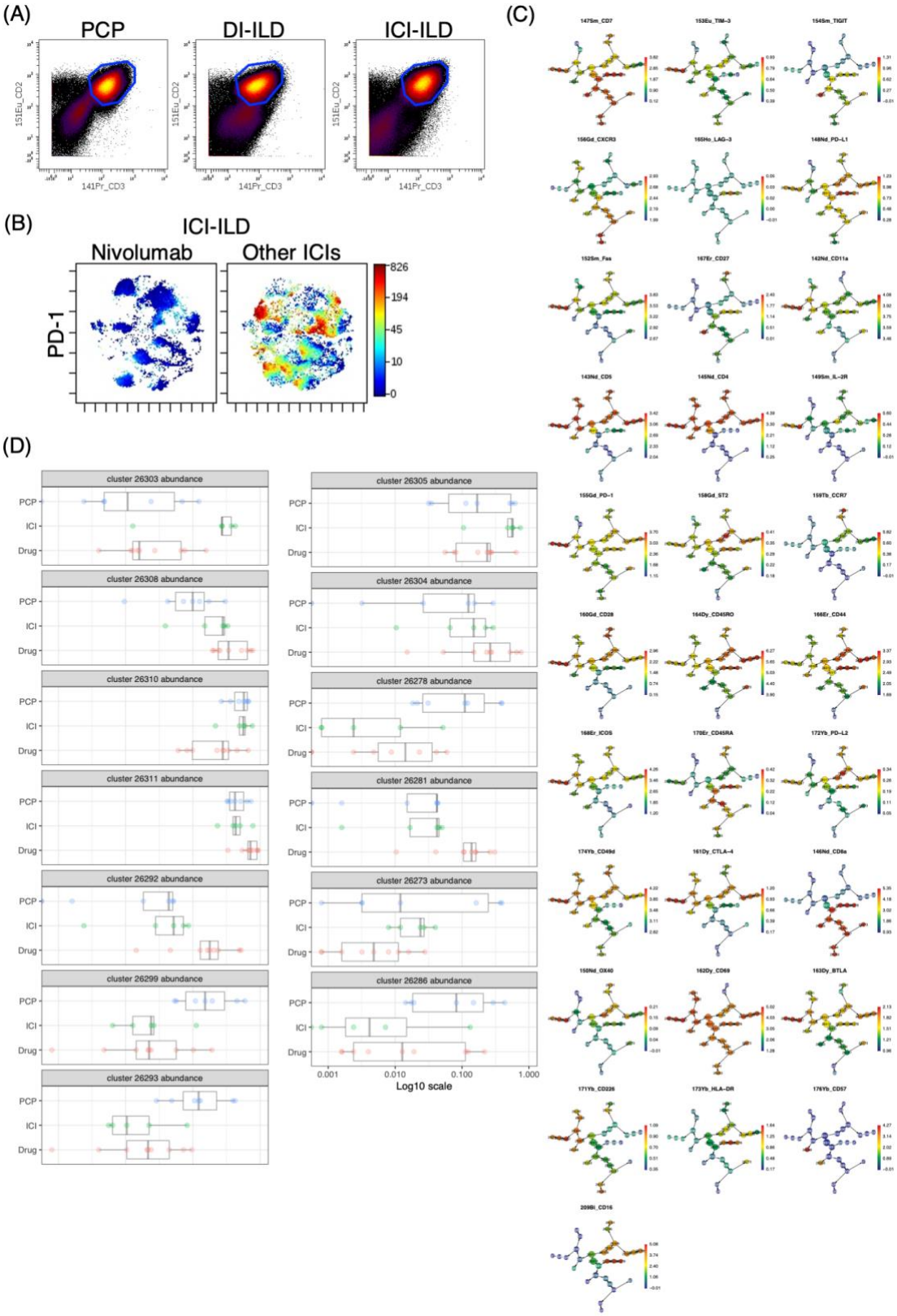
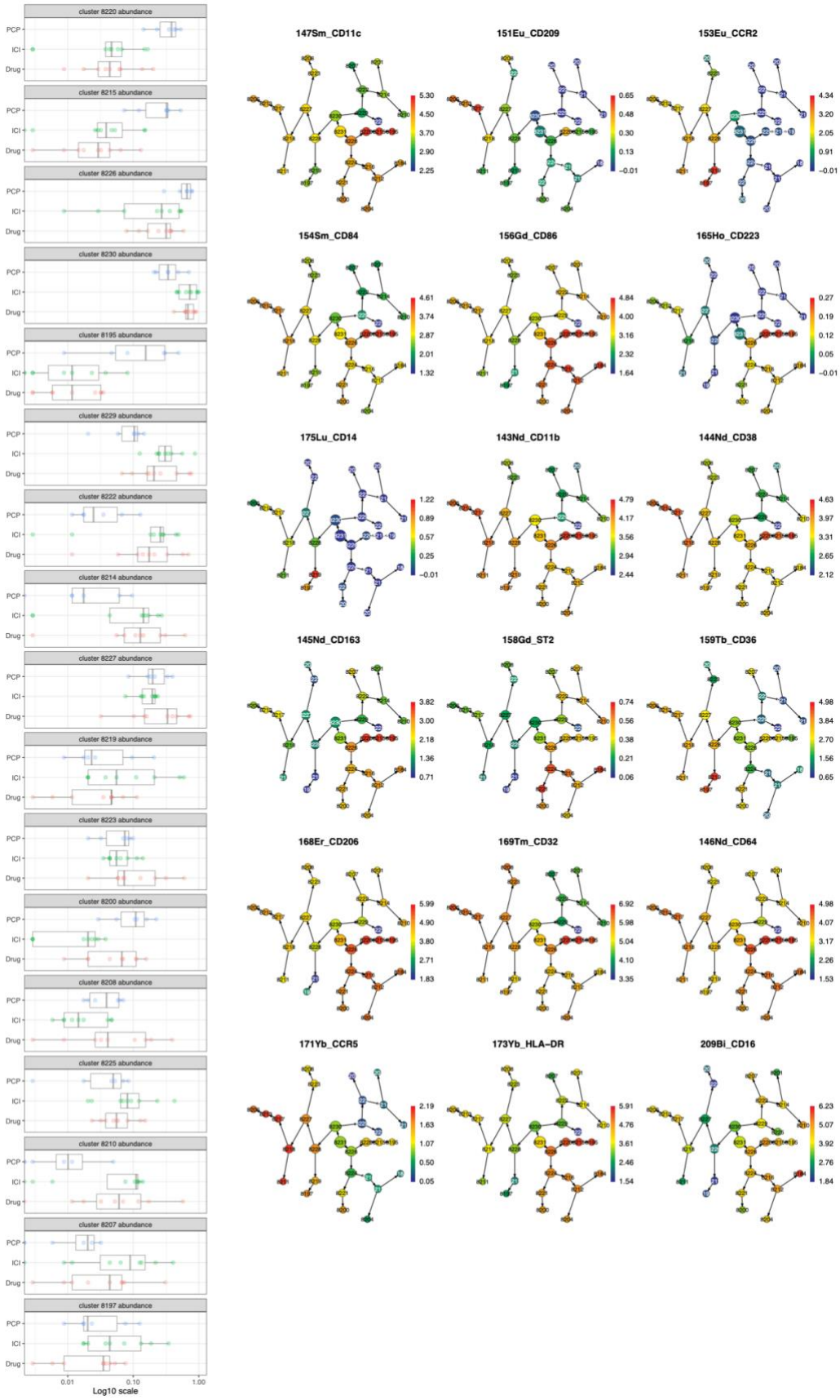
165Ho	CD223/LAG-3	3165037B	11C3C65	1
166Er	CD44	3166001B	BJ18	1
167Er	CD27	3167002B	O323	1
168Er	CD278/ICOS	3168024B	C398.4A	1
169Tm	CD19	3169011B	HIB19	1
170Er	CD45RA	3170010B	HI100	1
171Yb	CD226	3171013C	DX11	1
172Yb	CD273 (PD-L2)	3172014B	24F.10C12	1
173Yb	HLA-DR	3173005B	L243	0.5
174Yb	CD49d	3174018B	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

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

Supplementary Figure 1. A T cell gate ($CD2^{+}CD3^{+}$) and Citrus analysis of T cell populations in BALF cells from PCP, DI-ILD, and ICI-ILD.



Supplementary Figure 2. The Citrus analysis of myeloid cell populations in BALF cells from PCP, DI-ILD, and ICI-ILD.