Altered macrophage phenotypes in a case of autoimmune pulmonary alveolar proteinosis

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To the Editor:

Pulmonary alveolar proteinosis (PAP) is an ultra-rare disease characterised by abnormal accumulation of surfactant components in the alveoli [1]. The majority of cases are of an autoimmune nature (previously named primary or idiopathic) and are linked to the presence of an autoantibody targeting granulocyte–macrophage colony-stimulating factor (GM-CSF) [1]. This anti-GM-CSF antibody impedes the ability of alveolar macrophages to remove pulmonary surfactant. As a consequence, a significant proportion of patients experience progressive respiratory failure and immune deficiency [1]. While considerable progress has been made in understanding the pathophysiology of PAP over the past two decades, there has yet to be an in-depth analysis of macrophage phenotypes in human lungs with PAP. Our study reports on a case of PAP and presents a novel approach to analysing macrophage phenotypes in this condition using mass cytometry.

A 70-year-old male patient underwent surgery for rectal cancer with metastatic liver tumours 10 years earlier and received mFOLFOX therapy. 7 years earlier, the patient underwent right lower lobe resection for a metastatic lung tumour and had been under observation since then. The patient was referred to our hospital due to the presence and gradual enlargement of ground-glass opacities around the bronchovascular bundle of the left upper lobe 6 months earlier, which subsequently became visible in both lungs, with a "crazy-paving" appearance observed on chest computed tomography scans (figure 1a). Pulmonary function testing revealed a restrictive impairment, with a forced vital capacity of 76.4%. Following a bronchoscopy, 95 mL of milky aspect of bronchoalveolar lavage fluid (BALF) was retrieved after injecting 150 mL of saline solution into the right upper lobe B3. PAP was then suspected based on the macroscopic appearance of the BALF. The cytology of BALF revealed no malignancy. Diff-Quik staining demonstrated the cloudy extracellular proteinaceous materials (arrows), as well as many cell fragments (arrowheads) (figure 1b). The patient was later diagnosed with autoimmune PAP based on the positivity of serum anti-GM-CSF antibodies (33.8 $U \cdot mL^{-1}$, normal range <1.7 $U \cdot mL^{-1}$; commercially measured by SRL, Inc. in Japan).

Mass cytometry analysis of BALF cells from the patient revealed distinct patterns of myeloid cell populations compared to other lung diseases, such as idiopathic pulmonary fibrosis, connective tissue disease related interstitial lung disease (ILD), sarcoidosis and cytotoxic drug-induced ILD as disease controls. The procedural details involving cell staining, fixation, acquisition and analysis are elaborated, building on previously established methods [2]. Briefly, cryopreserved BALF cells were thawed and stained with Cell-ID Cisplatin-198Pt (Standard Biotools), followed by incubation with Fc receptor blocking reagent (Takara #210409) and metal-labelled CD45 antibodies (Standard Biotools). After washing, CD45-labelled cells were mixed and stained with antibody cocktail. After staining, cells were fixed with 1.6% formaldehyde, and resuspended in Cell-ID Intercalator 103Rh in Fix and Perm buffer at 4°C overnight. For acquisition, cells were resuspended in MaxPar Cell Acquisition Solution containing one-fifth EQ Four Element Calibration Beads and were acquired on a Helios mass cytometer (Standard Biotools). Files were converted to flow cytometry standard (FCS), randomised and normalised for EQ bead intensity using the Helios software. Concatenating FCS files in the same group into one file was conducted using FlowJo v10.8 (BD Biosciences). Manual gating and Uniform manifold approximation and projection analyses were performed using Cytobank Premium (Cytobank Inc.). We observed a significantly decreased proportion of $CD206^+$ $CD64^+$ alveolar macrophages in the BALF of our PAP patient compared to these other diseases (figure 1b and c). In contrast, the proportion of $CD14^+$ $CD64^+$ monocytes, $CD206^-$ $CCR2^+$ $CD64^+$ macrophages,

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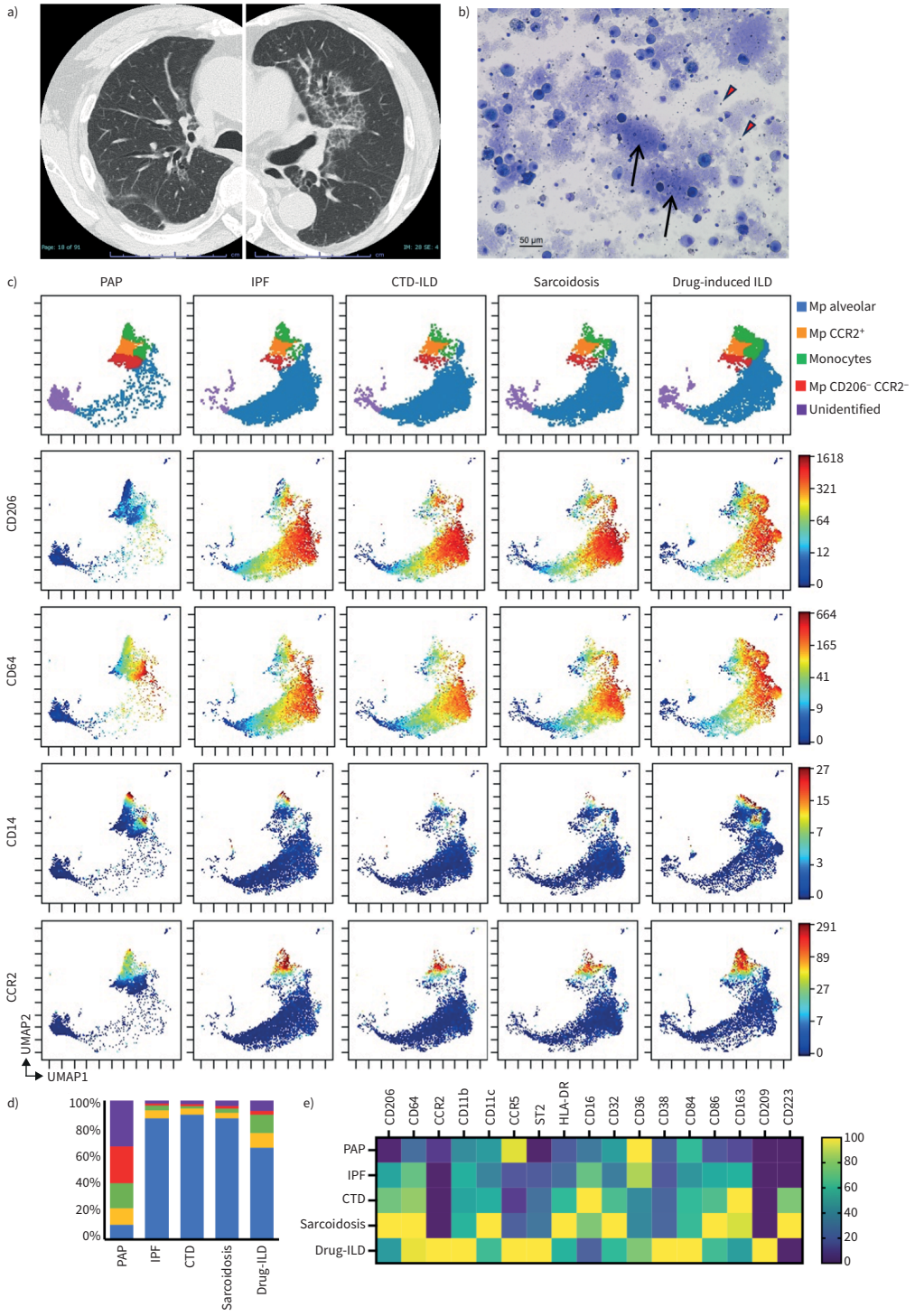

FIGURE 1 a) A chest computed tomography image and b) Diff-Quik staining of bronchoalveolar lavage fluid (BALF) cells of the patient. c) Uniform manifold approximation and projection (UMAP) plots visualising cell population, distribution and intensity of CD206, CD64, CD14 and CCR2 in myeloid cells (gated as $CD45^+$ $CD11b^+$ $CD11c^+$) in BALF from patients with pulmonary alveolar proteinosis (PAP) (n=1), idiopathic pulmonary fibrosis (IPF) (n=8), connective tissue disease (CTD)-associated interstitial lung disease (ILD) (n=8), sarcoidosis (n=11) and cytotoxic drug-ILD (n=9). For IPF, sarcoidosis, CTD-ILD and cytotoxic drug-ILD, individual data were concatenated into one file per disease group. Alveolar macrophages (Mp) are characterised by $CD64^+$ $CD14^-$ $CD206^+$ $CD11b^{hi}$ $CD11c^{hi}$ expression, $CCR2^+$ macrophages by $CCR2^+$ $CD206^-$ $CD64^+$, monocytes by $CD14^+$ $CD64^+$, $CCR2^-$ $CD206^-$ macrophages by $CCR2^-$ $CD206^-$ $CD64^+$, unidentified population by a lower expression of most surface markers ($CD11b^{+/-}$ $CD11c^{+/-}$ $CD64^-$ $CD206^-$ $CD14^-$ $CCR2^-$ $CD16^{+/-}$). d) The proportion of cell populations in myeloid cells (gated as $CD45^+$ $CD11b^+$ $CD11c^+$) as determined by the UMAP analysis. e) The heatmap illustrates the median expression markers in myeloid cells (gated as $CD45^+$ $CD11b^+$ $CD11c^+$). The methodology has been described previously [2].

$CD206^{-}$ $CCR2^{-}$ $CD64^{+}$ macrophages and unidentified myeloid cells were increased (figure 1c and d). We further analysed the intensity of specific markers expressed by these myeloid cell populations and observed lower expression of HLA-DR, CD163 and CD64, along with higher CCR5 expression in the myeloid cell lineage from PAP (figure 1e).

GM-CSF knockout mice displayed alveolar proteinosis and have been utilised as an animal model of PAP [3]. Alveolar macrophages in $GM\text{-}CSF^{-/-}$ mice exhibited decreased expression of a mannose receptor and cell-surface receptors (Toll-like receptor (TLR)2 and TLR4), as well as impaired phagocytosis and surfactant catabolism [3]. These features are considered as maturation impairment of alveolar macrophages. Exogenous GM-CSF expression in the alveoli was able to rescue these impairments in alveolar macrophages in $GM\text{-}CSF^{-/-}$ mice, indicating that GM-CSF promotes the terminal differentiation and maturation of alveolar macrophages [3]. These findings later led to the development of therapeutic inhaled GM-CSF in patients with PAP [4].

CD206, also known as mannose receptor C type 1, is a protein expressed on the surface of macrophages and dendritic cells that plays a vital role in the recognition and clearance of foreign substances such as bacteria and viruses by facilitating their internalisation and subsequent degradation within cells [5–7]. CD206 also contributes to immune regulation by modulating the production of cytokines and the activation of T-cells [6]. Alveolar macrophages are known to express high levels of CD206, making it a valuable marker for these cells [5, 8, 9].

Our exploratory analysis has revealed a significant decrease in CD206-expressing alveolar macrophages in the case of PAP, thus implying impairment in the differentiation of these macrophages. Furthermore, the reduced expression of human leukocyte antigen (HLA)-DR in myeloid gates may also suggest a burden in their differentiation. We assume that the proportional rise in monocytes could be a compensatory effect of the impaired maturation of alveolar macrophages. Given the decreased surface markers in alveolar macrophages from $GM\text{-}CSF^{-/-}$ mice, the increased proportion of unidentified myeloid subsets, characterised by a decreased expression of most surface markers ($CD11b^{+/-}$ $CD11c^{+/-}$ $CD64^{-}$ $CD206^{-}$ $CD14^{-}$ $CCR2^{-}$ $CD16^{+/-}$) in PAP may consist of immature alveolar macrophages due to maturation impairment.

In PAP, the strong autofluorescence exhibited by periodic acid–Schiff-positive eosinophilic material and macrophages has made conventional analyses, such as flow cytometry, challenging. By employing heavy metal ion tags, mass cytometry effectively addresses the issue of autofluorescence, enabling an in-depth analysis of alveolar macrophages in PAP. Despite the limitations posed by analysing a single case with a past cancer history, our approach has revealed altered macrophage immunophenotypes, indicative of macrophage differentiation impairment within the alveoli. Investigation of macrophages in BALF from patients with PAP before and after inhaled GM-CSF therapy may elucidate the maturation process of human alveolar macrophages, approaching human physiology. We believe this methodology will offer further insights into the immunophenotypic attributes of the rare disease.

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