Early View

Research letter

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LETTER TO THE EDITOR

Altered macrophage phenotypes in a case of autoimmune pulmonary alveolar proteinosis

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Dear Editor,

Pulmonary alveolar proteinosis (PAP) is an ultra-rare disease characterized 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 analyzing macrophage phenotypes in this condition using mass cytometry.

A 70-year-old male patient underwent surgery for rectal cancer with metastatic liver tumors 10 years earlier and received mFOLFOX therapy. Seven years earlier, the patient underwent right lower lobe resection for a metastatic lung tumor 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
tube of the left upper lobe six months earlier, which subsequently became visible in
both lungs, with a ‘crazy-paving’ appearance observed on chest CT scans (Figure 1A).
Pulmonary function test revealed a restrictive impairment, with a %forced vital capacity
of 76.4%. Following a bronchoscopy, 95 mL of milky aspect of bronchioalveolar 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/mL, normal range <1.7
U/mL, 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 (IPF), connective-tissue disease-related interstitial lung diseases
(CTD-ILD), sarcoidosis, and cytoxic drug-induced ILDs as disease controls. The
procedural details involving cell staining, fixation, acquisition, and analysis are
elaborated, building on previously established methods (2). Shortly, 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-labeled 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 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). Manual gating and UMAP analysis 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 (Figures 1B and 1C). In contrast, the proportion of CD14+ CD64+ monocytes,
CD206- CCR2+ CD64+ macrophages, CD206- CCR2- CD64+ macrophages, and
unidentified myeloid cells were increased (Figures 1C and 1D). We further analyzed 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 utilized as an animal model of PAP (3). Alveolar macrophages in GM-CSF−/− mice exhibited decreased expression of a mannose receptor and cell-surface receptors (TLR2 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-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 (MRC1), 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 internalization and subsequent degradation within cells (5)(6)(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 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-CSF−/− mice, the increased proportion of unidentified myeloid subsets, characterized 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 (PAS)-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 analyzing 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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Take home message
Mass cytometry of BALF cells from a pulmonary alveolar proteinosis patient, positive for anti-GM-CSF antibodies, suggests potential impairment in human alveolar macrophage differentiation.

Figure 1. (A) A chest CT image and (B) Diff-Quik staining of BALF cells of the patient. (C) Uniform manifold approximation and projection (UMAP) plots visualizing cell population, distribution, and intensity of CD206, CD64, CD14, and CCR2 in myeloid cells (gated as CD45+ CD11b+ CD11c+) in BALF from patients with PAP (n = 1), IPF (n = 8), sarcoidosis (n = 11), CTD-ILD (n = 8), 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 characterized by CD64+CD14+CD206+ CD11bhi CD11chai 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 previously described (2).
References


