RESEARCH LETTER

Different expression of immune checkpoint markers on bronchoalveolar lavage CD4+ cells: a comparison between hypersensitivity pneumonitis and sarcoidosis

Piotr Kopiński[1,2], Ewa Wypasek[2,3], Tomasz Senderek[3], Ewelina Wędrowska[1], Tomasz Wandtke[1], Grzegorz Przybylski[1]

1 Division of Gene Therapy, Department of Lung Diseases, Cancer and Tuberculosis, Collegium Medicum, Nicolaus Copernicus University, Bydgoszcz, Poland
2 John Paul II Hospital, Kraków, Poland
3 Department of Physiology and Pathophysiology, Andrzej Frycz-Modrzewski Krakow University, Kraków, Poland

Introduction The etiology of hypersensitivity pneumonitis (HP) is specific allergen–driven, while in sarcoidosis it is a subject of research and speculations. However, both diseases share a common pathogenesis, that is, activation of antigen-primed T cells, lymphocytic inflammation of lower airways, T helper 1 and T helper 17 profile domination, and lung granuloma formation.[1,2]

Immune checkpoints (ICPs) regulate the intensity of the immune response and involve an array of molecules expressed on lymphocytes as well as their antigen-presenting cell counterparts. They act either as stimulators of the immune system or, conversely, as its inhibitors (in which case they are called inhibiting ICPs). The latter protect host tissues from damage as they include cytotoxic T-lymphocyte–associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1); the expression of these molecules rises together with lymphocyte activation.[3] Increased expression of CTLA-4 and PD-1 on T cells in lower airways has been described in lung cancer. In this context, it is the result of immunoediting, a process during which tumor cells activate the inhibiting checkpoints to escape immune surveillance.[4] As a consequence, ICP antagonists, that is, monoclonal antibodies directed specifically against PD-1 (eg, nivolumab) and CTLA-4 (eg, ipilimumab), are used increasingly often in therapy of lung cancer.[5,6] The vital role of both ICPs in granulomatous diseases was demonstrated in pulmonary pathology, including HP and sarcoidosis. Thus, experimental HP can be reversed by a CTLA-4 agonist.[7] Cancer treatment with ICP inhibitors may initiate sarcoidosis-like inflammation (so-called biotherapy-induced sarcoidosis) in the lungs.[8] However, to our knowledge, the actual expression of both types of checkpoints in patients with lung granulomatous disease has not been fully established.

A particularly useful method for the examination of interstitial lung diseases in situ is bronchoalveolar lavage (BAL). This technique facilitates diagnosis and helps to assess the local cellular reaction as well as the release of inflammatory mediators.[9]

We aimed to determine the expression of ICPs on bronchoalveolar lavage lymphocytes in HP and sarcoidosis, in association with immunological and clinical data.

Patients and methods We enrolled patients suspected to have interstitial lung diseases. In the diagnostic procedure we followed the European Respiratory Society guidelines; details were described previously.[4] The exclusion criteria comprised: 1) treatment with systemic corticosteroids, methotrexate, azathioprine, biological drugs (eg, infliximab for sarcoidosis), or any other medication known to potentially cause interstitial lung pathology or influence the cytoimmunological BAL profile[8] 2) respiratory failure, heart failure (New York Heart Association class IV), or acute coronary syndromes; 3) poor technical quality of the BAL material (eg, BAL fluid recovery <30% of instilled volume, percentage of contaminating epithelial cells >3% of BAL cells).[9]

Hypersensitivity pneumonitis (n = 14) was diagnosed on the basis of clinical data, including typical symptomatology following allergen

Correspondence to:
Piotr Kopiński, MD, PhD,
Division of Gene Therapy, Department of Lung Diseases, Cancer and Tuberculosis, Collegium Medicum, Nicolaus Copernicus University, ul. M. Skłodowskiej-Curie 9, 85-094 Bydgoszcz, Poland,
phone: +48 52 585 34 88,
email: mpkopins@hotmail.com
Received: May 30, 2021.
Revision accepted: August 13, 2021.
Published online: September 10, 2021.
Pol Arch Intern Med. 2021; 131 (11): 16084
doi:10.20452/pamw.16084

exposure, specific high-resolution computed tomography (HRCT) presentation, lung function test results, and the presence of precipitating antibodies in the serum. In 3 patients with HP (25%), the diagnosis was confirmed by lung biopsy.[2,4]

Sarcoidosis (n = 23) was confirmed by clinical presentation, patient-specific histology (noncaseating granulomas in biopsy) and typical findings on HRCT. Based on chest X-ray, all patients were classified as radiological stage II (mediastinal lymphadenopathy with interstitial lung lesions, n = 13) or III (interstitial lesions only, n = 10); they presented clinical phenotype of chronic progressive (n = 7) or chronic stable (n = 16) sarcoidosis.[4,9]

Cytoimmunological BAL examination was performed as previously described.[4] Typing of major BAL (alveolar) lymphocyte subsets included CD3, CD4, CD8, CD19, natural killer (CD3-CD16$^+$56), and CD45 markers, and was completed by examination of ICP expression, that is, CD152 (CTLA-4), CD279 (PD-1), CD274 (programmed cell death protein ligand 1 [PD-L1]), and CD273 (programmed cell death protein ligand 2 [PD-L2]). In brief, samples containing 2–10 $\times 10^5$ of BAL cells were incubated with a saturating amount (usually 10 µl/sample) of murine antihuman monoclonal antibodies or murine isotype controls (Becton-Dickinson Biosciences, San Jose, California, United States and Biolegend, San Diego, California, United States; Supplementary material, *Table S1*). Four-color flow cytometry (BD FACSCanto II, BD Immunocytometry Systems, San Jose, California, United States) with CD4$^+$ (CD8$^+$) or side scatter gating was applied. Flow cytometry analysis was performed with Kaluza Analysis 1.2. software (Beckman Coulter, Brea, California, United States). The representative dot plots are shown in Supplementary material, *Figures S1* and *S2*.

Informed consent was obtained from all enrolled individuals and the study protocol was approved by Bioethics Committee of Nicolaus Copernicus University (approval no. KB107/2017).

Statistical analysis Statistical analysis was performed with Statistica 13 software (StatSoft, Tulsa, Oklahoma, United States). Patient data and the BAL cytoimmunological parameters were presented as median (interquartile range) due to the nonparametric distribution of variables.[4] The Mann–Whitney test was used to compare the studied groups (HP vs sarcoidosis). The correlations between the variables were tested by the Spearman rank correlation coefficient ρ. *P* values of less than 0.05 were considered statistically significant.

Results Baseline patient characteristics and BAL cytoimmunological results, including the expression of ICP markers on alveolar lymphocytes, are shown in TABLE 1. Compared with sarcoidosis, HP was characterized by a significant decrease in the expression of CTLA-4$^+$ BAL lymphocytes as well as CD4$^+$CTLA-4$^+$ cells. The increased percentage of CD4$^+$PD-1$^+$ cells in HP tended to be significant ($P = 0.051$).

A subset of lymphocytes, including CD4$^+$ cells, presented coexpression of PD-1, CTLA-4, and PD-L2, both in HP and sarcoidosis.

The percentage of CD4$^+$ cells carrying ICPs was inversely correlated with both percentages and total counts of lymphocytes in lower airways (eg, CD4$^+$CTLA-4$^+$ cells vs BAL lymphocyte percentage, $\rho = -0.75$, $P < 0.001$; CD4$^+$CTLA-4$^+$ cells vs BAL total T-cell count, $\rho = -0.61$, $P < 0.001$; CD4$^+$PD-1$^+$ cells vs BAL total CD4$^+$ cell count, $\rho = -0.48$, $P < 0.001$; PD-L2$^+$ cells vs BAL total lymphocyte count, $\rho = -0.53$, $P < 0.01$).

The percentage of BAL CD4$^+$CTLA-4$^+$ cells in HP and sarcoidosis was correlated with a predictive value of diffusing capacity of the lungs for carbon monoxide (DLCO, $\rho = 0.33$, $P < 0.05$), but not with a predictive value of vital capacity (TABLE 1).

After exclusion of current cigarette smokers, all differences and correlations remained significant. A suggestion of reference ranges for major BAL (alveolar) lymphocyte subsets in nonsmokers is presented in Supplementary material, *Table S2*.

Discussion The current study is the first to show that the alveolar lymphocyte percentage / count is related to the level of ICP expression in lower airways in HP and sarcoidosis. A subset of CD4$^+$ cells presented a co-expression of both PD-1/CTLA-4 and PD-1/PD-L2. The percentage of BAL CD4$^+$CTLA-4$^+$ cells correlated positively with DLCO.

Of note, in our study the total count of alveolar CD4$^+$ cells in HP, a disease typically associated with a low BAL CD4/CD8 index value, was similar to that in sarcoidosis, which is a condition with a specifically high index value.[9] Recently, Suzuki et al[10] reported extremely low PD-1 values in sarcoidosis; however, they did not provide clinical characteristics of their patients. Braun et al[11] found an increased PD-1 expression in BAL of patients with sarcoidosis together with impaired proliferative capacity of lymphocytes; the defect was experimentally restored by PD-1 blockade. It was pointed out that increased PD-1 expression on BAL lymphocytes may serve as a marker of progressive lung fibrosis.[12]

Suzuki et al[10] demonstrated the PD-1/PD-L1 co-expression on BAL T cells, suggesting a local immune dysregulation in sarcoidosis. Herein, we also found an alternative PD-1 ligand, PD-L2, to be co-expressed with CD4$^+$ in both sarcoidosis and HP. It should be considered whether local PD-1/PD-L2 ligation could drive suicidal T-cell apoptosis.[4]

The expression of CTLA-4 on BAL T cells, similar to PD-1, is controversial. Early studies showed CTLA-4 values ranging from high (approximately 80%) to very low percentage of BAL positive lymphocytes in HP.[13,14] The latter value[14] appeared closer to the current results, and it was confirmed for sarcoidosis by Broos et al.[15] Low

TABLE 1 Baseline characteristics and bronchoalveolar lavage cytoimmunological data of patients with granulomatous diseases

Variable		HP (n = 15)	Sarcoidosis (n = 23)	*P* value[a]
Baseline patient information				
Age, y		54 (36–67)	49 (37–60)	0.31
Sex	Male	8 (53)	13 (57)	0.88
	Female	6 (47)	10 (43)	
Current smoker		2 (16.7)	4 (17.3)	0.43
Disease duration, mo, mean (SD)		9.5 (5.6)	9.0 (3.1)	0.89
Inhaled corticosteroid therapy		1 (7.1)	2 (8.7)	0.29
Lung function tests				
VC, %		95 (83–113)	92 (87–106)	0.76
DLCO, %		69.5 (52–81)	79 (73–86)	0.044
BAL cytological and immunological results				
Recovery, %		50 (41–53)	50.5 (43–57)	0.89
Total cell count, × 10^3/ml		281 (150–340)	270 (145–452)	0.99
Macrophages, %		52 (35–69)	61 (46–73)	0.07
Lymphocytes, %		44 (27–57)	34 (20–47)	0.67
Neutrophils, %		2.0 (1.3–5.0)	2.4 (0.6–5)	0.66
Eosinophils, %		0.1 (0–2.0)	1.1 (0–1.9)	0.52
Total lymphocyte count, × 10^3/ml		77 (55–211)	83 (31–149)	0.07
Total T cell count, × 10^3/ml		68 (53–203)	68 (25–138)	0.31
Total $CD4^+$ cell count, × 10^3/ml		43 (17–118)	60 (20–116)	0.34
Total $CD8^+$ cell count, × 10^3/ml		32 (17–74)	12 (5.8–25)	<0.001
CD4/CD8		1.40 (0.6–2.2)	4.5 (3.0–6.6)	<0.001
Lymphocytes CTLA-4^+, %		1.9 (1.1–6.2)	2.6 (1–6.5)	0.54
Lymphocytes PD-1^+, %		33 (11–37)	29 (20–43)	0.50
Lymphocytes PD-$L1^+$, %		3.9 (1.8–4.8)	2.9 (0.5–5.6)	0.92
Lymphocytes PD-$L2^+$, %		7.3 (2.4–11)	5.0 (4.4–18)	0.85
$CD4^+$PD-1^+ cells, %		35 (29–54)	22 (16–30)	0.051
$CD4^+$CTLA-4^+ cells, %		3.0 (1.0–3.7)	4.6 (2.7–6.8)	0.016
$CD4^+$PD-1^+CTLA-4^+ cells, %		1.5 (0–2.5)	3.4 (2.1–4.1)	0.015
$CD4^+$PD-$L1^+$ cells, %		3.9 (1.8–4.8)	2.9 (0.5–5.6)	0.92
$CD4^+$PD-$L2^+$ cells, %		7.3 (2.4–11)	5.0 (4.4–18)	0.85
$CD4^+$PD-1^+PD-$L2^+$ cells, %		1.9 (0.5–7.6)	4.8 (3.8–7.4)	0.19

Data are presented as number (percentage) or median (interquartile range) unless otherwise indicated. All results of ICP marker expression are presented as a proportion of BAL lymphocytes.

a Categorical variables were compared by the Mann–Whitney test.

Abbreviations: BAL, bronchoalveolar lavage; CTLA-4, cytotoxic-T–lymphocyte associated protein 4; DLCO, diffusing capacity of the lungs for carbon monoxide; HP, hypersensitivity pneumonitis; PD-1, programmed cell death protein 1; PD-L1 or 2, programmed cell death protein ligand 1 or 2; VC, vital capacity

CTLA-4 expression on T cells may lead to pulmonary fibrosis and can be reversed with the use of a CTLA-4 agonist (eg, abatacept) in both HP and in sarcoidosis.[7] Of note, our results suggested that it was rather CTLA-4, but not PD-1 expression, that showed a significant inverse correlation with DLCO, a useful marker of progressive pulmonary fibrosis in the course of active interstitial disease.[2]

The study has several limitations. First, the number of participants with HP was low, though representative of patients with both diseases. Second, we did not explore ICP markers shed from the BAL cell surface into the extracellular microenvironment. Third, the BAL procedure serves mainly diagnostic purposes and it is rarely performed in patients with acute sarcoidosis, including Lofgren syndrome. Therefore, our results cannot be easily extrapolated to such patients.

Overall, the results of the present study suggest that detection of ICP markers on alveolar T cells in patients with chronic granulomatous diseases might facilitate the assessment of local inflammatory process activity and risk of lung fibrosis. The study should be repeated in a larger group of patients and complemented with the examination of soluble ICPs in BAL supernatants.

SUPPLEMENTARY MATERIAL

Supplementary material is available at www.mp.pl/paim.

ARTICLE INFORMATION

CONFLICT OF INTEREST None declared.

HOW TO CITE Kopiński P, Wypasek E, Senderek T, et al. Different expression of immune checkpoint markers on bronchoalveolar lavage $CD4^+$ cells: a comparison between hypersensitivity pneumonitis and sarcoidosis. Pol Arch Intern Med. 2021; 131: 16084. doi:10.20452/pamw.16084

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