Supplementary material

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

Table S1 Murine anti-human monoclonal antibodies and murine isotype controls (manufacturer, cat. number) used in the study

Flow cytometer fluorescence channel	**FL1**	**FL2**	**FL3**	**FL4**	**Notes**
Fluorescent dye	FITC	PE	PerCP / PE Cy5.5	APC	
Sample 1	Isotype IgG (Biolegend 400109)	Isotype IgG (Biolegend 400111)	Isotype IgG (Biolegend 400149)	CD4, T helper (Biolegend 317416)	Obligatory control for sample 5 and 6
Sample 2	CD14 (BD Biosciences 555397)	CD125, IL5-R (BD Biosciences 555902)	CD45, LCA (BD Biosciences 345809)	Isotype IgG (Biolegend 401209)	Detection of non-lymphocyte cells in BAL lymphocyte gate
Sample 3	CD3. T cells	CD8, T cytotoxic cells	CD45, LCA	CD4, T helper cells	MULTI 1 set (BD Biosciences 342417)
Sample 4	CD3, T cells	CD 16+56, NK cells	CD45, LCA	CD19, B cells	MULTI 2 set (BD Biosciences 342416)
Sample 5	CD279, PD-1	CD152, CTLA-4	CD273, PD-L2	CD4, T helper	

	(Biolegend 329904)	(Biolegend 349906)	(BD Biosciences 564256)	(Biolegend 317416)
Sample 6	CD8, T cytotoxic (BD Biosciences 345772)	CD274, PD-L1 (Biolegend 393608)	-	CD4, T helper (Biolegend 317416)
Sample 7	Unstained sample			

APC, allophycocyanin; BD, Becton-Dickinson; CTLA-4, cytotoxic T lymphocyte associated antigen 4; FITC, fluorescein isothiocyanate; FL, fluorescence; IL-5R, interleukin-5 receptor: LCA, leukocyte common antigen; PD-1, programmed death receptor 1; PD-L1 (2), programmed death ligand 1 (2); PE Cy 5.5, phycoerythrin cyanine 5.5; PerCP, peridinin-chlorophyll-protein

Table S2 Authors' range of normal phenotype of alveolar (BAL) lymphocyte in nonsmokers. Comparison with HP and sarcoidosis nonsmoker results

Variable	HP (n=12)	Sarcoidosis (n=21)	Normal value range[a]	Notes for normal value range
	Median (Interquartile range)	Median (Interquartile range)	5-95 percentile	
T cells (CD3+), %	92 (88-96)	93 (89-96)	78-96	Generally higher percentage than in parallel PB typing; low MFI value
B cells (CD19+), %	1 (1-3)	2 (1-2)	0-6	Generally lower percentage than in parallel PB typing
NK cells CD3-(CD16+56)+, %	4 (2-6)	3 (2-5)	0-11	Generally lower percentage than in parallel PB typing; increasing NK percentage may reflect upper airway contamination
T helper cells (CD4+), %	53 (37-66)[b]	75.5 (64.5-82.5)	38-77	Low MFI value, necessary distinction from small monocytic macrophages (by CD14 staining);
T cytotoxic cells (CD8+), %	39 (29-58)[b]	17 (13-24)	19-46	
CD4 / CD8, 1	1.37 (0.65-2,20)[c]	4.50 (2.76-6.62)	0.9-4.0	High value variability

All results from nonsmokers only; [a] unpublished data; n=56; [b] $P < 0.01$ compared to sarcoidosis; [c] $P < 0.001$ compared to sarcoidosis, Mann-Whitney U test.

Abbreviations: BAL, bronchoalveolar lavage; HP, hypersensitivity pneumonitis; MFI, mean fluorescence intensity; NK, natural killer; PB, peripheral blood.

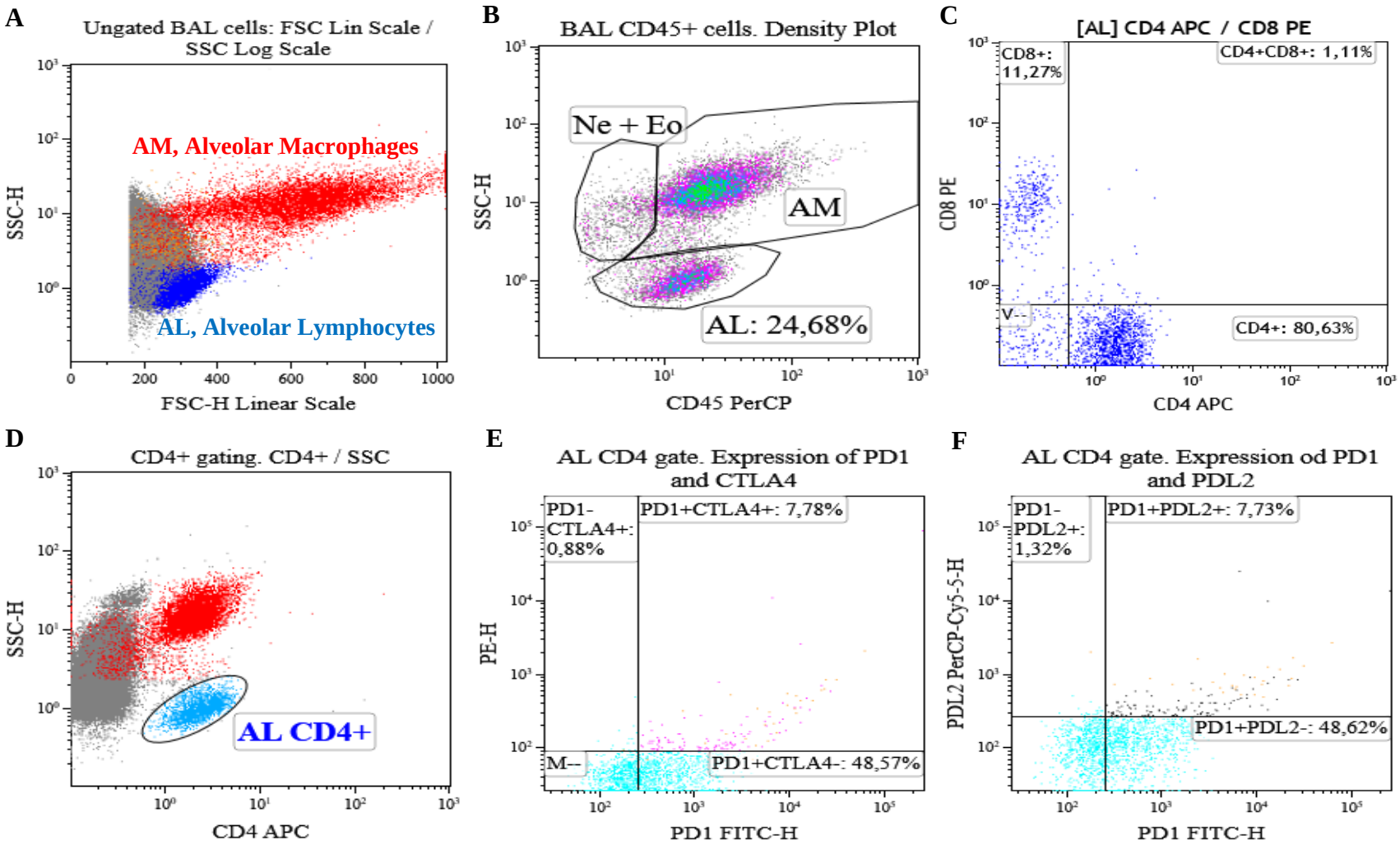

Figure S1 Sample of flow cytometry analysis in sarcoidosis patient. Expression of immune checkpoints on BAL (alveolar) CD4+ cells

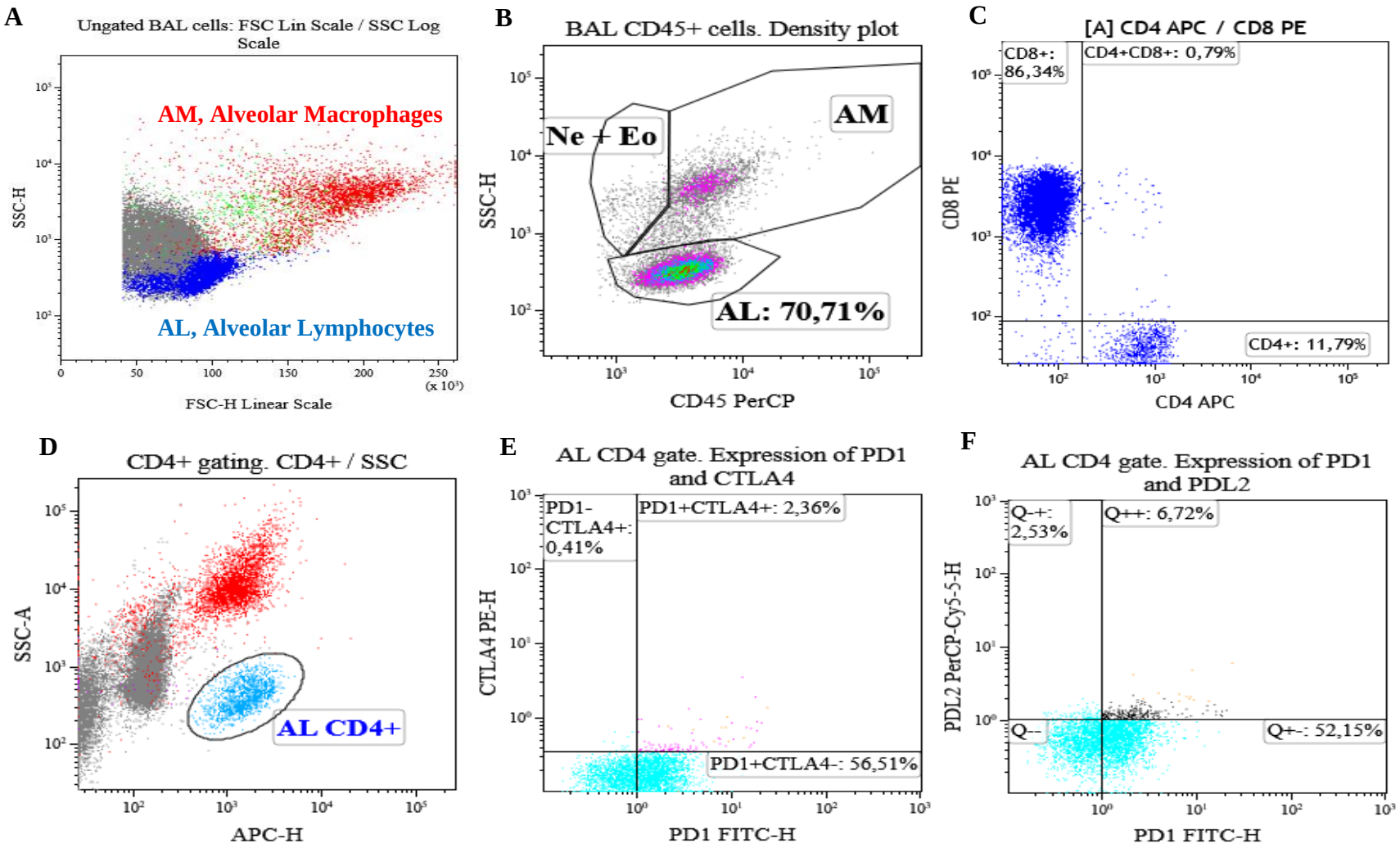

Figure S2 Sample of flow cytometry analysis in HP patient. Expression of immune checkpoints on BAL (alveolar) CD4+ cells

Figures description

Figure S1. 36-years old man with chronic sarcoidosis, radiological stage III. Non-smoker, untreated with corticosteroids. All cytometry variables presented on logarithmic scale and as dot plots unless otherwise noted. **A.** Standard FSC/SSC presentation. The alveolar macrophages (red) and alveolar lymphocytes (blue) are defined here according to anti-CD45 staining because they are difficult to distinguish from red blood cells, cell debris, and epithelial cells (gray) if only their size (forward scatter) and granularity (side scatter) is considered. Therefore, the first step when analyzing BAL material is to gate the leukocytes using an anti-CD45 antibody as shown in Figure S1.B. **B.** Only CD45+ events are presented. Populations of macrophages and lymphocytes as well as granulocytes (neutrophils along with eosinophils) are easily distinguished. The percentages obtained by cytometry are indicative because the standard for BAL differential count is a routine cytological staining. The boundary between macrophages and granulocytes is taken authoritatively, despite the routine use of anti-CD125 antibody (unpublished data). Between lymphocytes and macrophages young monocytic forms of macrophages are situated, so staining with an anti-CD14 antibody is necessary to phenotype BAL lymphocytes (table S1, sample 2). **C.** AL gate from figure B is analyzed for CD4/CD8 index (table S1, sample 3), it is 6.83 (high value is common in sarcoidosis). Markers inserted according to isotype control. **D.-F.** Results of cytometric analysis according to the samples 1 and 5 (table S1). **D.** CD4+ alveolar cells gated according to anti-CD4 staining and side scatter properties. **E.** Staining of CD4+ cells for expression of PD-1 and CTLA-4 immune checkpoints. Cells with co-expression of both markers are shown in purple. Markers inserted according to isotype control. **F.** Staining of CD4+ cells for expression of PD-1 and PD-L2. Cells with co-expression of both markers are shown in black. Markers inserted according to isotype control.

Figure S2. 45-years old woman with HP, non-smoker, treated with budesonide inhalations 2 x 400 μg daily. **A.** Standard FSC/SSC presentation. The alveolar macrophages (red) and alveolar lymphocytes (blue), according to anti-CD45 staining. Of note, in the FSC/SSC parameter set, lymphocytes form two populations. Smaller cells (with lower FSC value) are apoptotic due to corticosteroid intake by the patient (author's own experience, unpublished data). **B.** Apoptotic lymphocytes do not differentiate from the others in staining with anti-CD45. **C.** AL gated in figure B show low value of CD4/CD8 index (0.15, as it is characteristic for HP). Markers inserted according to isotype control. **D.-F.** Results of

cytometric analysis according to the samples 1 and 5 (table S1). Commentaries for figure S2, points D-F, are the same as in the description of Figure S1 (applied to D, E, and F, respectively).

Abbreviations: APC, allophycocyanin; BAL, bronchoalveolar lavage; CTLA-4, cytotoxic T lymphocyte associated antigen 4; HP, hypersensitivity pneumonitis; FITC, fluorescein isothiocyanate; FL, fluorescence; FSC, forward scatter; IL-5R, interleukin-5 receptor: PD-1, programmed death receptor 1; PD-L1 (2), programmed death ligand 1 (2); PE Cy 5.5, phycoerythrin cyanine 5.5; PerCP, peridinin-chlorophyll-protein; SSC, side scatter.