ORIGINAL ARTICLE

Increased levels of interleukin 27 in patients with early clinical stages of non–small cell lung cancer

Piotr Kopiński[1], Tomasz Wandtke[1], Andrzej Dyczek[2†], Ewelina Wędrowska[1], Adriana Rożyy[3], Tomasz Senderek[4], Grzegorz Przybylski[5], Joanna Chorostowska-Wynimko[3]

1 Department of Gene Therapy, Collegium Medicum, Nicolaus Copernicus University, Bydgoszcz, Poland
2 John Paul II Hospital in Krakow, Kraków, Poland
3 Department of Genetics and Clinical Immunology, National Institute of Tuberculosis and Lung Diseases, Warsaw, Poland
4 Department of Physiology and Patophysiology, Andrzej Frycz Modrzewski Krakow University, Kraków, Poland
5 Department and Clinics of Lung Diseases Tumors and Tuberculosis, Nicolaus Copernicus University, Bydgoszcz, Poland

KEY WORDS

alveolar lymphocytes, interleukin 27, interstitial lung diseases, non–small cell lung cancer

ABSTRACT

INTRODUCTION Interleukin 27 (IL-27) is a cytokine secreted mostly by antigen-presenting cells. It is important for the immune polarization of T helper-1 (Th1) cells, and its role in interstitial lung diseases (ILDs) and lung cancer has been investigated.

OBJECTIVES We assessed IL-27 expression in the lower airways of patients with selected ILDs and early-stage non–small cell lung cancer (NSCLC).

PATIENTS AND METHODS IL-27 concentrations were examined by an enzyme-linked immunosorbent assay in bronchoalveolar lavage (BAL) fluid supernatants collected from patients with pulmonary sarcoidosis (PS; n = 30), extrinsic allergic alveolitis (EAA; n = 14), idiopathic pulmonary fibrosis (IPF; n = 12), nonspecific interstitial pneumonia (NSIP; n = 14), and NSCLC stages I to IIa (n = 16) with peripheral localization, and in controls (n = 14). The major lymphocyte subsets in BAL fluid were phenotyped, and intracellular IL-27 expression was evaluated by flow cytometry.

RESULTS IL-27 concentrations in BAL fluid supernatants were significantly increased in Th1-mediated conditions such as EAA and PS, but not in IPF or NSIP. The highest IL-27 levels (median [SEM], 16.9 [17.5] pg/ml) were reported for NCSLC, and the lowest—for controls (median [SEM], 0.4 [0.2] pg/ml). IL-27 was undetectable in corticosteroid-treated patients with PS. Both $CD4^+$ and $CD8^+$ lymphocytes were positive for IL-27; they were a possible local source of IL-27 because the cytokine levels were positively significantly correlated with the total number of lymphocytes, including $CD4^+$ cells.

CONCLUSIONS Our results support the Th1-linked activity of IL-27 in ILDs. Early-stage NSCLC is characterized by high IL-27 expression in the lower airways. IL-27 is produced by a high percentage of $CD4^+$ and $CD8^+$ cells in BAL fluid, both in patients and controls.

Correspondence to:
Piotr Kopiński, MD, PhD, Zakład Genoterapii, Collegium Medicum, Uniwersytet Mikołaja Kopernika, ul. Marii Skłodowskiej-Curie 9, 85-094 Bydgoszcz, Poland, phone: +48 52 585 34 88, email: mpkopins@hotmail.pl
Received: September 8, 2017.
Revision accepted: November 28, 2017.
Published online: November 29, 2017.
Conflict of interest: none declared.
Pol Arch Intern Med. 2018; 128 (2): 105-114
doi:10.20452/pamw.4156

INTRODUCTION Interleukin (IL) 27 is a heterodimeric cytokine composed of 2 subunits: p28 (also named IL-30) and EBI3. It is classified as a member of the IL-12 family.[1] IL-27 exerts its biological activity and affects target cells exclusively by binding to its specific receptor, IL-27R. The receptor comprises 2 chains (subunits), IL-27Rα (WSX-1) and glycoprotein 130 (gp130), which are responsible for ligand binding and signal transduction, respectively, mainly in a signal transducer and activator of transcription (STAT)-1- and STAT-3-dependent manner.[2] Gp130 occurs in a wide variety of tissues, whereas the IL-27Rα expression seems to be restricted to the immune cells, including major lymphocyte subsets, monocytes, neutrophils, mast cells, macrophages, and Langerhans cells, as well as vascular endothelium and keratinocytes.[3,4]

It was formerly believed that IL-27 was produced only by myeloid cells, capable of

presenting antigens—dendritic cells and macrophages—due to stimulation of toll-like receptors (TLRs) by foreign molecular patterns, such as bacteria, viruses, fungi, and parasites, mainly by TLR types 3 and 4.[5] However, subsequent studies have shown that the spectrum of cells secreting IL-27 is much wider. Under physiological conditions, the IL-27 expression was observed in monocytes, microglial, endothelial, and trophoblast cells.[6]

IL-27 was initially described as a proinflammatory cytokine, because of its ability to activate STAT-1-dependent intracellular signaling pathways in naive $CD4^+$ T cells, resulting in their expansion and differentiation into T helper-1 (Th1) cells.[3] However, a number of studies have provided new data on the biology of IL-27 and shown that it affects also Th2, Th17, $Foxp3^-$ type 1 T regulatory, $Foxp3^+$ T regulatory, and T follicular helper cells.[7,8]

In this context, the major function of IL-27 in interstitial lung diseases (ILDs) is unclear. In pulmonary sarcoidosis (PS), IL-27 was shown to be overexpressed in the lungs and to participate in Th1-mediated immunity.[9] However, in patients with sarcoidosis, elevated IL-27 mRNA levels, but not the levels of IL-27 itself, were found only in mononuclear cells.[10] Ringowski et al[11] reported an increased IL-27 release after stimulation with lipopolysaccharide, but only in the blood and not in the lungs. Moreover, it was suggested that IL-27 is involved in immune suppression in sarcoidosis.[12] However, the literature data on the IL-27 expression in the lungs in other ILDs are scarce.

IL-27 exhibits antitumor properties by acting directly on cancer cells and indirectly via tumor microenvironment. It is consistent with the main postulated immune role, namely, APC--dependent Th1 polarization.[2] So far, IL-27 has been shown to stimulate a significant expression of human leukocyte antigen class I molecules on cancer cells[13] to reduce their proliferation, expansion, and survival, as well as to increase the expression of some death receptor ligands on melanoma cells.[2,14] Likewise, it directly affects tumor microenvironment by suppressing the activity of endothelial cells and by inducing the expression of antiangiogenic chemokines: CXCL9 and CXCL10.[15] However, there have been only a few studies analyzing the role of IL-27 in this context in a real-life setting, that is, in patients with non–small cell lung cancer (NSCLC), and the reported results are conflicting.[16,17] Therefore, the aim of this study was to investigate the role of IL-27 in the pathomechanism of lower airway diseases, ILDs and NSCLC.

PATIENTS AND METHODS

Study population

A total of 86 patients with distal airway abnormalities, including extrinsic allergic alveolitis (EAA, n = 14), PS (n = 30), idiopathic pulmonary fibrosis (IPF, n = 12), and nonspecific interstitial pneumonia (NSIP, n = 14), all nonsmokers, were included in the study. We also enrolled smoking patients diagnosed with early stages (Ia, Ib, or IIa) of NSCLC localized distally (n = 16).

Newly diagnosed PS was confirmed by a typical clinical presentation, patient-specific histology (noncaseating granulomas on biopsy), and typical findings on high-resolution computed tomography (HRCT).[18] Patients with PS were stratified according to conventional chest X-ray staging or, alternatively, according to the clinical phenotype into subgroups with Löfgren syndrome, chronic progressive sarcoidosis, and chronic stable sarcoidosis.[19]

EAA was diagnosed on the basis of clinical data, including typical symptoms following allergen exposure, specific presentation on HRCT, lung function test results, and the presence of precipitating antibodies in serum. In 5 patients (36%) with EAA, the diagnosis was confirmed by lung biopsy.[20] In patients with IPF (8 out of 12; 67%) and NSIP (100%), the diagnosis was based on a histological examination of the lung biopsy. In 4 patients with IPF, the biopsy was not performed and the diagnosis was established according to the joint criteria of the European Respiratory Society and the American Thoracic Society.[21]

Patients with PS (n = 5) who received ongoing oral corticosteroid therapy (prednisone, 15–60 mg for at least 3 months before bronchoalveolar lavage [BAL]) were analyzed separately. No other patients had a history of systemic steroid or immune suppressive therapy.

The NSCLC group consisted of 16 patients, stages I–IIa, all current smokers. BAL was performed as part of a routine diagnostic workup of a peripheral solitary pulmonary lesion, subsequently diagnosed as NSCLC by a histopathological examination of the biopsy material. Adenocarcinoma was diagnosed in 12 patients, while squamous carcinoma—in 4. In 5 of these 16 patients (31%), cancer cells were also present in BAL fluid samples.[22]

The control group consisted of 14 patients, including 7 smokers. None of the controls had any symptoms of any lung disorder. They showed no abnormalities on lung function tests, chest X-ray, or HRCT scans. They were not treated with corticosteroids or any other medications known to cause ILD. Five controls (3 nonsmokers and 2 smokers) were included in the control groups in a previous study.[23]

Bronchoalveolar lavage

The BAL procedure was part of the routine diagnostic workup. BAL was performed according to the European Respiratory Society guidelines, as described previously.[24] In brief, premedication with midazolam and local upper airway anesthesia with lidocaine (2%) was followed by bronchofiberoscopy with Olympus BF-H190 (Olympus, Tokyo, Japan). The middle lobe of the right lung (or alternatively the lingual of the left lung) was lavaged with 200 ml of sterile NaCl solution (0.9%), and instilled sequentially with four 50-ml aliquots. Then, BAL fluid fractions were retrieved by gentle suction,

pooled, filtered, and immediately transported to the laboratory. Fluid recovery was calculated as the percentage of instilled volume.

Informed consent was obtained from all subjects, and the study was approved by the Bioethics Committee of Nicolaus Copernicus University in Bydgoszcz, Poland (no. KB374/2011).

Assessment of interleukin 27 concentrations in bronchoalveolar lavage fluid IL-27 concentrations in BAL fluid supernatants were evaluated by an enzyme-linked immunosorbent assay (ELISA) specific kit, cat. no E08464h (Cusabio, Washington, Maryland, United States), according to the manufacturer's recommendations. The optical density was measured at 450 nm using a spectrophotometric reader ELx800 (Biotek Instruments, Inc., Winooski, Vermont, United States). The IL-27 levels in the supernatants were expressed in pg/ml rather than mmol/l to enable a comparison with the results published by other authors.[2]

Bronchoalveolar lavage cytology and immune cell typing The total cell count, cell viability (trypan blue exclusion test), and differential cell count of leukocytes in BAL fluid was calculated, as described previously.[25]

For lymphocyte typing, 3-color typing was applied, combining indirect (for IL-27) and direct (for CD4 and CD8) immunostaining. In brief, a sample containing 100 µl of BAL fluid cell suspension (2–10 × 10^6 cells/ml) was incubated with FACS Lysing Solution (cat. nº 349202, BD Biosciences, Franklin Lakes, New Jersey, United States), and then with FACS Permeabilizing Solution 2 (BD Biosciences, cat. nº 340973), and washed in phosphate buffered saline (PBS) with bovine serum albumin (BSA, 0.5%) (cat. nº 554657, BD Biosciences) and NaN_3 (0.1%). The cell pellet was then resuspended in PBS, incubated with a saturating amount (usually 10 µl) of murine anti–human IL-27 monoclonal antibody (cat. nº WH0246778M1, Sigma Aldrich, St. Louis, Missouri, United States) for 30 minutes in the dark, and washed with PBS. For secondary incubation, polyclonal rabbit, fluorescein isothiocyanate (FITC)-conjugated, anti–mouse serum (1 µl of serum diluted 1:10 in PBS; cat. nº F0313, DAKO Cytomation, Glostrup, Denmark) was applied for 10 minutes in the dark. The sample was washed twice in PBS/BSA/NaN_3, incubated with monoclonal antibodies directed against human superficial CD4 and CD8 antigens (10 µl both; cat. nº 345769 and 555368, respectively; BD Biosciences) for 30 minutes in the dark, washed in PBS/BSA/NaN_3, and then resuspended in 300 µl of PBS with formaldehyde (1%).

The internal control consisted of: 1) a sample proceeded identically, except for staining with primary antibody, which was replaced with the respective volume of PBS; 2) sample stained with negative isotype control (Tritest cat. nº 340385, BD Biosciences); and 3) sample stained with positive control, that is, murine monoclonal antibody directed against human superficial marker CD45 (cat. nº 345808, BD Biosciences). Flow cytometric data were acquired within 24 hours after staining, using a FACSCalibur cytometer (BD Biosciences). The flow cytometric acquisition was described previously.[26] The fluorochromes used in the study were as follows: FITC for the standard fluorescence channel FL1 (CD45, IL-27); phycoerythrin (PE) for FL2 (CD4); and phycoerythrin-cyanine 5 for FL-3 (CD8).

The panel of markers stained in the study was limited to CD4 and CD8, as a consequence of our previously published data[23,25,26] demonstrating that the majority of lymphocytes in BAL fluid comprise antigen-primed T cells, alternatively CD4 or CD8, and very few natural killer (NK) and B cells. This was observed for both patients with ILDs and healthy controls. Based on cytology and immunology results, the appearance of lymphocytes in BAL fluid was characterized by the total number of cells: all lymphocytes, CD4 cells, and CD8 cells per 1 milliliter of recovered fluid.

Statistical analysis Demographic and lung function data were presented as mean (SD). IL--27 concentrations in BAL fluid supernatants and BAL cytoimmunology data were presented as median (SEM) due to the nonparametric distribution of the values.[27] The Mann–Whitney test was used to compare the study groups (untreated patients with ILD/NSCLC versus controls; corticosteroid-treated versus untreated PS patients). The correlations between 2 random variables were tested using the Spearman's rank correlation coefficient. A P value of less than 0.05 was considered significant.

RESULTS The demographic and clinical characteristics of the study groups are presented in TABLE 1. The BAL cytological and immunological data are presented in TABLE 2. There were significant differences between patients with ILD and control nonsmokers, as well as those with NSCLC and control smokers.

IL-27 concentrations in the supernatants from patients with ILDs are shown in FIGURE 1 (as mentioned above, all patients with ILDs and healthy controls were nonsmokers). The IL-27 concentration was increased in patients with EAA (median [SEM], 5.4 [1.7] pg/ml; range, 2.0–18.4 pg/ml; P = 0.007) and in patients with PS (median [SEM], 3.2 [1.6] pg/ml; range, 0–35.6 pg/ml; P = 0.01), as compared with controls (median [SEM], 0.2 [0.5] pg/ml; range, 0–1.9 pg/ml). IL--27 levels were elevated in patients with IPF (median [SEM], 3.0 [1.4] pg/ml; range, 0–8.1 pg/ml) and those with NSIP (median [SEM], 4.0 [2.6]; range, 0–24.7 pg/ml), but the differences were not significant (P = 0.22 and P = 0.21, respectively), most probably due to a relatively low number of enrolled patients. No difference was observed between patients with early sarcoidosis (radiological stage I) and those with advanced sarcoidosis

TABLE 1 Demographic and clinical data of patients and controls included in the study

Parameter	PS		EAA	IPF	NSIP	NSCLC	Controls	
	Nonsmokers (n = 25)	Treated (n = 5)	Nonsmokers (n = 14)	Nonsmokers (n = 12)	Nonsmokers (n = 14)	Smokers (n = 16)	Nonsmokers (n = 7)	Smokers (n = 7)
Sex, male/female, n	10/15	1/4	6/8	8/4	4/10	3/13	4/3	5/2
Age, y	42.9 (10.7)	48.9 (18.2)	45 (9.7)	52.7 (10.5)	48.0 (13.9)	59.1 (11.1)	40.1 (14.4)	40.0 (13.0)
VC pred. val., %	103 (12.4)	94 (15.4)	87 (17.7)	89 (17.5)	88 (15.3)	106 (14.7)	98 (11.1)	111 (10.7)
DL_{CO} pred. val., %	86 (21.0)	75 (18.4)	53 (14.1)	50 (11.0)	67 (14.9)	86 (10.9)	99 (12.1)	89 (12.2)
Disease duration, mo	9 (3.1)	11.5 (5.8)	18.1 (13.5)	38.2 (17.4)	20.5 (8.1)	3.3 (1.9)	NA	NA
Pack-years of smoking, y	NA	NA	NA	NA	NA	35.4.5 (16.8)	NA	22.5 (7.2)

Data are presented as mean (SD) unless otherwise stated.

Abbreviations: DL_{CO}, diffusing capacity of the lungs for carbon monoxide; EAA, exogenous allergic alveolitis; IPF, idiopathic pulmonary fibrosis; NA, not applicable; NSCLC, non–small cell lung cancer; NSIP, nonspecific interstitial pneumonia; PS, pulmonary sarcoidosis; pred. val., predicted value; VC, vital capacity

TABLE 2 Bronchoalveolar lavage cytoimmunological data of patients and controls included in the study

Group	PS		EAA	IPF	NSIP	NSCLC	Controls	
	Nonsmokers (n = 25)	Treated (n = 5)	Nonsmokers (n = 14)	Nonsmokers (n = 12)	Nonsmokers (n = 14)	Smokers (n = 16)	Nonsmokers (n = 7)	Smokers (n = 7)
BAL fluid recovery, %	57 (3.7)	44 (11.6)	54 (5.1)	47 (7.0)	49 (9.2)	48 (3.6)	55 (4.0)	51 (9.3)
BAL fluid cell count, 10^3/ml	243 (43.1)[b]	134 (4.6)[a]	358 (30.3)[b]	136 (3.7)	184 (74.1)[a]	187 (97.4)	107 (15.1)	237 (14.9)
Macrophages, %	60 (3.1)[b]	50 (11.9)	46 (4.9)[b]	75 (3.5)[a]	74 (5.6)	81 (2.8)[a]	86 (3.5)	95 (4.1)
Neutrophils, %	1.1 (0.2)	0.7 (0.5)	3.0 (2.3)[a]	8.9 (1.3)[c]	2.8 (1.6)[b]	2.3 (1.9)[a]	1.0 (0.4)	1.3 (0.6)
Eosinophils, %	0.4 (0.2)[b]	0.2 (0.1)[a]	0.3 (0.2)	2.3 (2.5)[b]	2.0 (4.4)[a]	0.4 (0.3)	0.2 (0.1)	0.2 (0.1)
Lymphocytes, %	38 (3.0)[c]	49 (11.5)	47 (7.7)[c]	7.1 (5.4)[a]	17.9 (5.7)	15 (3.6)[b]	14 (3.3)	3.5 (0.4)
Total lymphocyte count in BAL fluid, 10^3/ml	60 (7.2)[c]	32.7 (3.9)[a]	91 (5.7)[b]	18.2 (4.4)	17.9 (5.7)	15 (3.6)[a]	14 (3.3)	3.5 (0.4)
Total $CD4^+$ cell count in BAL fluid, 10^3/ml	45 (36.1)[c]	26 (9.1)[a]	37 (20.7)[b]	10.9 (5.5)	12 (4.5)	18 (11.6)[a]	9.3 (2.5)	6.9 (3.6)
Total $CD8^+$ cell count in BAL fluid, 10^3/ml	9.0 (5.5)[b]	4.7 (2.6)	44 (7.3)[b]	3.6 (2.7)	3.7 (2.5)	11 (0.9)	3.7 (0.7)	7.4 (2.9)
CD4 / CD8, 1	7.2 (1.1)[c]	3.72 (0.9)	1.02 (0.9)[c]	3.48 (0.6)	1.98 (1.4)	1.77 (0.35)[b]	2.76 (0.4)	1.31 (0.8)

Data are presented as median (SEM). Nonsmokers and smokers were compared with respective controls, and treated patients with nonsmoking steroid-naive counterparts. Statistical differences were assessed with the Mann–Whitney test.

a $P < 0.05$, **b** $P < 0.01$, **c** $P < 0.001$

Abbreviations: BAL, bronchoalveolar lavage; others, see **TABLE 1**

(radiological stage II or III) (**FIGURE 2**). Likewise, the IL-27 concentration did not differ in the PS subgroups stratified according to the clinical presentation of the disease, that is, into Löfgren syndrome, chronic progressive, or chronic stable sarcoidosis (data not shown).

IL-27 was undetectable in the supernatants obtained from corticosteroid-treated patients with PS (data not shown); therefore, its concentration in corticosteroid-naive patients with PS was significantly higher ($P = 0.02$). Similarly, the median (SEM) IL-27 concentration was higher in patients with NSCLC (stages I–IIa) (**FIGURE 3**), as compared with the respective control group, that is, healthy smokers: 17.5 (12.2) vs 0.4 (0.2) pg/ml ($P = 0.02$).

In patients with ILD and NCSLC, IL-27 levels in BAL fluid did not correlate with lavage fluid recovery, relative leukocyte, CD4, or CD8 cell count. However, when the lymphocyte count was expressed as the total cell number in 1 ml of recovered fluid, a significant correlation with the total lymphocyte count and total $CD4^+$ cell count was demonstrated (**FIGURE 4**). Interestingly, IL-27 intracellular staining was positive for BAL lymphocytes in all the study groups: patients with ILD and NSCLC, as well as in all healthy controls (**TABLE 3**). Of note, the IL-27 expression

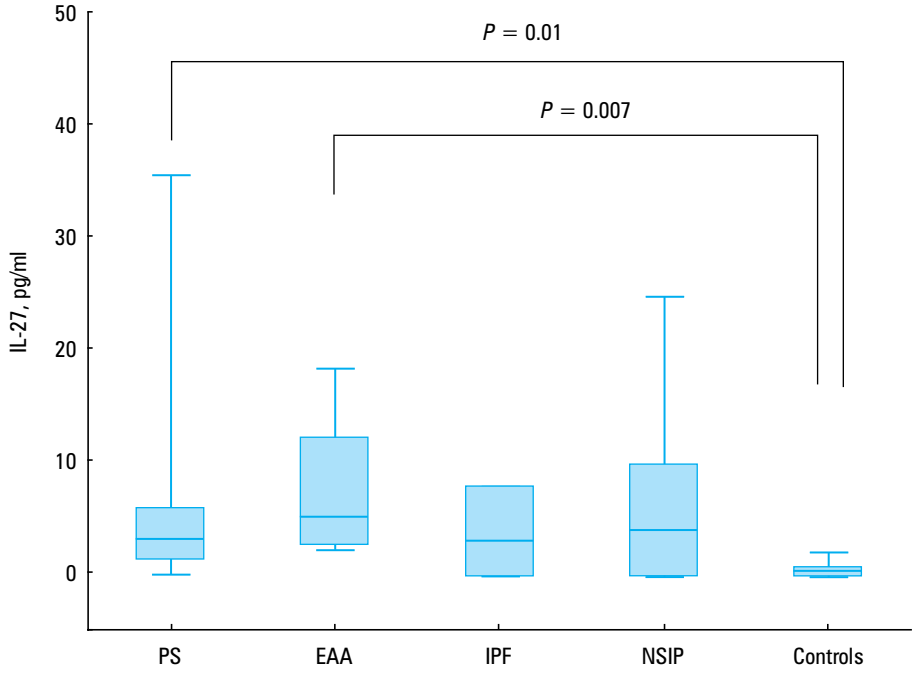

FIGURE 1 Interleukin 27 (IL-27) concentrations in bronchoalveolar lavage fluid supernatants from corticosteroid-naive patients with interstitial lung disease and healthy controls. Significant *P* values according to the Mann–Whitney test. The central horizontal line represents the median value; the square, interquartile range; and the vertical line, the minimum–maximum range.
Abbreviations: see **TABLE 1**

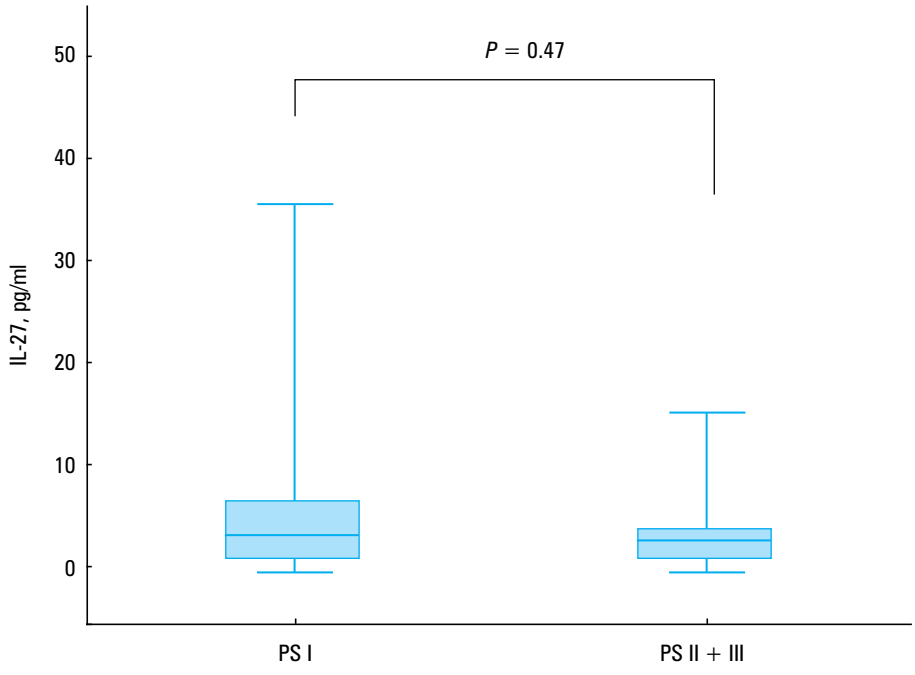

FIGURE 2 Interleukin 27 (IL-27) concentrations in bronchoalveolar lavage fluid supernatants from patients with pulmonary sarcoidosis (PS) stratified according to the radiological stage of the disease (stage I, II, or III). A *P* value according to the Mann–Whitney test. The central horizontal line represents the median value; the square, interquartile range; and the vertical line, the minimum–maximum range.

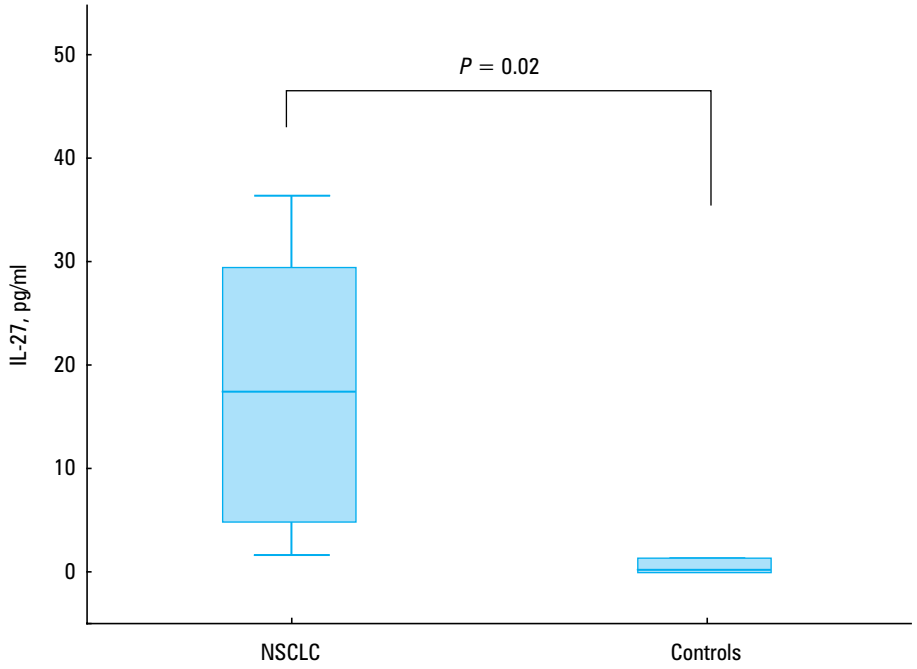

FIGURE 3 Interleukin 27 (IL-27) concentrations in bronchoalveolar lavage fluid supernatants from patients with non–small cell lung cancer as compared with controls. Significant *P* values according to the Mann–Whitney test. The central horizontal line represents the median value; the square, interquartile range; and the vertical line, the minimum–maximum range.
Abbreviations: see **TABLE 1**

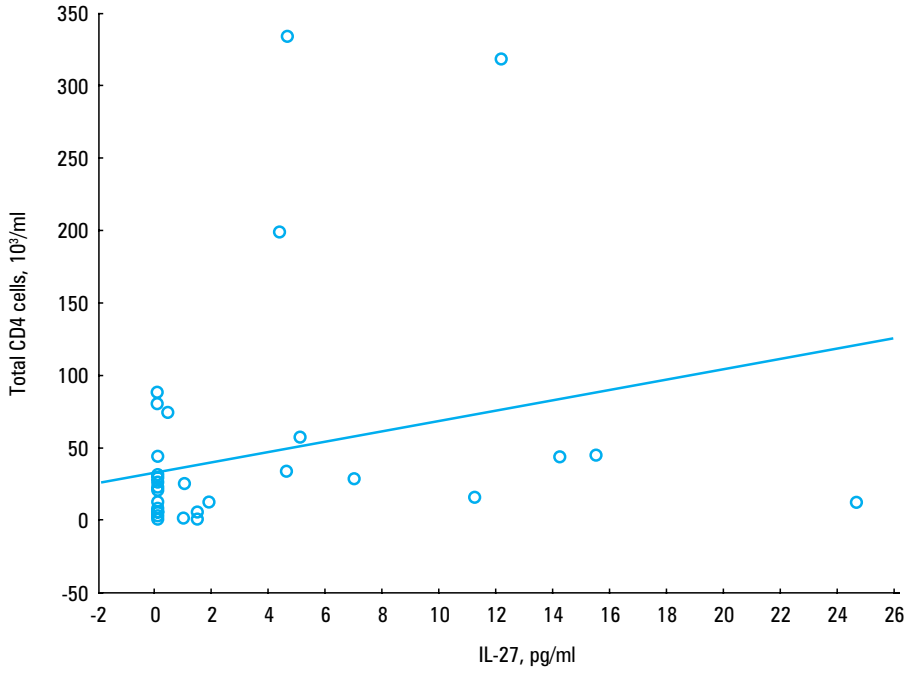

FIGURE 4 Correlation between interleukin 27 (IL-27) concentrations and total $CD4^+$ cell count in bronchoalveolar lavage fluid. Spearman's rank correlation coefficient: $r = 0.41$, $P = 0.01$.

was observed in $CD4^+$ and $CD8^+$ lymphocytes, and the proportion of positive cells was similar in both subsets. The representative flow cytometry sample is shown in **FIGURE 5**. IL-27 levels were not correlated with clinical parameters in patients with ILD, such as the predicted values of vital capacity or diffuse lung capacity for carbon monoxide.

DISCUSSION The most important findings of the study are as follows: 1) lymphocytes in BAL fluid produce IL-27, although they are not antigen-presenting cells; 2) this phenomenon was observed in patients with different types of ILDs, patients with early-stage NSCLC, and healthy controls; 3) PS is characterized by the highest percentage of $IL\text{-}27^+$ lymphocytes; 4)

TABLE 3 Interleukin 27 (IL-27) expression in bronchoalveolar lavage fluid lymphocytes

Group	PS		EAA	IPF	NSIP	NSCLC	Controls	
	Nonsmokers (n = 25)	Treated (n = 5)	Nonsmokers (n = 14)	Nonsmokers (n = 12)	Nonsmokers (n = 14)	Smokers (n = 16)	Nonsmokers (n = 7)	Smokers (n = 7)
BAL lymphocytes IL-27$^+$, %	89 (3.8)[b]	23 (10.7)	61 (10.0)	54 (17.6)	61 (12.1)	70 (5.6)	54 (7.4)	50 (9.5)
IL-27$^+$ cells in CD4$^+$ subset, %[a]	95 (3.3)[b]	33 (9.8)	68.5 (7.3)	55 (11.7)	68 (17.1)	75 (6.9)	48 (14.7)	49 (7.6)
IL-27$^+$ cells in CD8$^+$ subset, %[a]	90 (6.9)	22 (8.1)	61.5 (9.1)	52 (14.6)	60 (11.2)	81 (8.6)	53 (11.8)	57 (6.2)

Data are presented as median (SEM). Nonsmokers and smokers were compared with respective controls, and treated patients with nonsmoking steroid-naive counterparts. Statistical differences were assessed with the Mann–Whitney test.

a IL-27$^+$ lymphocytes are presented as percentage of respective subset (ie, CD4$^+$ or CD8$^+$), **b** $P = 0.04$

Abbreviations: see **TABLES 1** and **2**

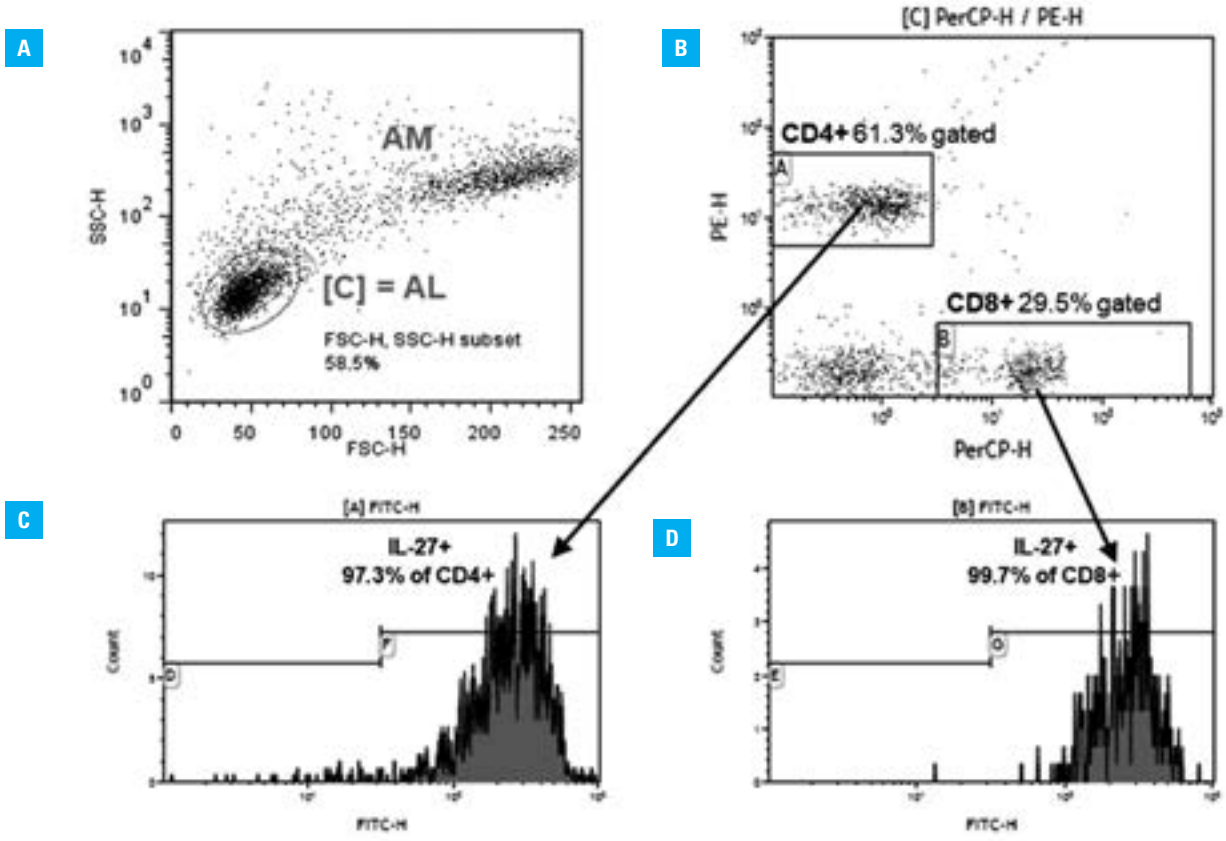

FIGURE 5 Flow cytometry results for CD4 and CD8 versus intracellular interleukin 27 (IL-27) coexpression in an untreated patient with sarcoidosis (radiological stage III). **A** – bronchoalveolar lavage (BAL) lymphocyte (AL) gating (gate C) according to FSC/SSC parameters. Technical difficulties (low AL percentage, contamination of red cells) are to be omitted with an additional sample with anti-CD45 staining ("leucogate", not shown); **B** – PerCP/PE analysis of gate C in the sample stained with monoclonal antibodies: anti–IL-27 (FITC; indirect immunotyping), anti-CD4 PEand anti-CD8 PerCP (direct immunotyping for both); gates A and B represent CD4$^+$ and CD8$^+$ BAL cells, respectively. Gate edges defined according to the control sample stained for CD4 and CD8 markers only; **C** and **D** – histograms presenting IL-27–FITC stained CD4$^+$ and CD8$^+$ cells, respectively. Positive cells labeled by markers (set according to control samples) F and G, respectively. CD4/CD8 index: 2.08. IL-27$^+$ cells: 97.3% and 99.7% of AL CD4$^+$ and CD8$^+$, respectively. Total AL IL-27$^+$: 95.0% (not shown).
Abbreviations: AM, alveolar macrophages; FITC, fluorescein isothiocyanate; FSC, forward scatter; H, height; PE, phycoerythrin; PerCP, peridinin--chlorophyll-protein complex; SSC, side scatter; others, see **TABLE 1**

a similar ratio of the key T lymphocyte subsets in BAL fluid, that is, CD4$^+$ and CD8$^+$, express IL-27; 5) the IL-27 concentration in BAL fluid supernatants is significantly increased in early-stage NSCLC, PS, and EAA, as compared with healthy controls; and, finally 6) in corticosteroid-treated PS patients, IL-27 concentrations in BAL fluid were undetectable.

As mentioned previously, antigen-presenting cells such as dendritic cells, macrophages, monocytes, and endothelial cells, are considered a major source of IL-27 in physiological

conditions. Meanwhile, in disease states, it is released by a number of other cell populations. Thus, intracellular IL-27 expression was observed, for example, in neutrophils in sepsis, in neoplastic cells in metastatic melanoma, in lung mesothelial cells in tuberculosis, and in blood $CD4^+FoxP3^-$ T-regulatory cells in acute malaria infection.[2,28,29]

We were the first to document the IL-27 expression in lower airway lymphocytes from patients with sarcoidosis.[27] Later the same year, Yang et al[28] observed IL-27^+ CD4 and CD8 cells in pleural effusion in patients with tuberculosis and identified other IL-27^+ cells in their pleura (ie, NK, NK T cells, B cells, monocytes and macrophages).[30] In the present study, we observed the same phenomenon in other Th1-driven disorders, like EAA and early-stage NSCLC.

It has been suggested that in tuberculosis IL--27 might protect against interferon-γ-induced apoptosis of peripheral blood mononuclear cells. Likewise, we have previously reported the low apoptosis rate of airway lymphocytes irrespective of the high interferon-γ concentration in BAL fluid from patients with PS and EAA.[25] We believe that this phenomenon might be explained by the protective effect of IL-27 activity, among other factors.

In summary, in this study we confirmed that lymphocytes in BAL fluid secrete IL-27. This was observed for ILD and NSCLC, as well as for healthy controls. In both lymphocyte subsets, $CD4^+$ and $CD8^+$, the relative number of the cells positive for IL-27 was similar, suggesting that this particular interleukin is generally produced by antigen-primed, activated T cells.[26] Moreover, while the IL-27 concentration in BAL positively correlated with the total number of lymphocytes in the lower airways of patients with ILD, no IL-27^+ lymphocytes were detected in the peripheral blood of healthy controls and patients with NSCLC (unpublished data). Based on these observations, the following assumptions can be made regarding the role of IL-27 in the regulation of immune response in the lower airways: 1) Th and Tc cells constitute an important source of IL-27 in the lungs; 2) IL-27 protects T cells from local apoptosis, as was suggested by other authors.[30]

Regarding the IL-27 expression in the lower airways in Th1-driven ILDs, we demonstrated its association with the pathogenesis of sarcoidosis and EAA. However, this is not entirely consistent with previous reports.[9,11,12] Importantly, the relative number of both CD4 and CD8 cells expressing IL-27 in BAL fluid was higher in patients with PS as compared with controls, which might suggest that lymphocytes are a relatively important source of IL-27 in the lower airways in PS. This phenomenon seems not to be linked to the clinical presentation of PS because no difference in the IL-27 expression in the lower airways was observed between the respective subgroups stratified according to the radiological stage of the disease. On the other hand, in patients treated with systemic corticosteroids, IL-27 levels in BAL fluid were below the detection range, suggesting the involvement of IL-27-secreting cells in the immune and inflammatory response in the lower airways.

Our observations on the role of IL-27 in PS and EAA cannot be directly extrapolated to other ILDs. In IPF and NSIP, conditions potentially resulting in lung fibrosis, the IL-27 concentration was slightly elevated, although not significantly. While it might reflect the nonspecific stimulation of inflammatory cells due to partial Th1 polarization or—as postulated for IPF—due to Th2 activation, it is necessary to collect more comprehensive data to interpret this finding. Importantly, research on the role of IL-27 in IPF might shed new light on the pathomechanism of this disease. It is commonly accepted that irreversible lung fibrosis is driven by fibroblast activation and proliferation, as well as myofibroblast development and epithelial-myofibroblast transition (EMT). Meanwhile, IL-27 was shown to exert an inhibitory effect on the EMT.[31] Similarly, IL-27 inhibits the expression of proangiogenic factors: vascular endothelial growth factor as well as chemokines CXCL5 and CXCL8.[32] The in vitro observations were confirmed by Dhong et al[33] who reported the suppressive effect of IL--27 in an experimental model of pulmonary fibrosis. Therefore, our results may actually reflect the unfavorable course of IPF and NSIP, when relatively too low expression of IL-27 does not preclude the progression of fibrosis.

The antitumor properties of IL-27 have been postulated by many authors. It has been suggested that the ability of IL-27 to induce a Th1--mediated response with subsequent T cytotoxic (Tc) cell activation is important for host immune response directed against cancer cells.[34]

Similarly to lung fibrosis, IL-27 was shown to suppress tumor-induced EMT transition, as well as angiogenesis, both phenomena being crucial for cancer progression and both eminently depending on WSX-1- and STAT-1-mediated signal transduction.[31] Importantly, there are also reports contradictory to the postulated antitumor activity of IL-27. Airoldi et al[35] suggested that in NSCLC IL-27 actually enhanced the expression of chemokine CXCR3 and VE-cadherin, which are known for their proangiogenic properties.[35]

Considering the ability of IL-27 to induce Th1 and Tc immune responses, we decided to examine its extra- and intracellular expression in BAL fluid cells collected directly from the lung lobe affected by the tumor. The IL-27 concentration in the BAL fluid from patients with NSCLC was not only significantly higher as compared with that in controls, but its median value was the highest of all the study groups.

The above findings are not corroborated by other studies. Naumnik et al[16] demonstrated no difference in IL-27 concentrations between patients with late-stage lung cancer and controls.

Duan et al[17] reported lower IL-27 concentrations as well as IL-27 mRNA expression in peripheral blood of patients with NSCLC than in controls. It is important to emphasize that our study group included only patients with stages I–IIa of NSCLC. We postulate that the local activation of the immune system at this stage of lung cancer is enhanced, hence we observed other indices of immune activation: a significantly increased relative number of BAL lymphocytes and a higher CD4/CD8 index in smokers with NSCLC. It is possible that some of our BAL fluid samples were obtained from patients with NSCLC before the final tumor escape from the immune system.[36] On the other hand, a meta-analysis of published lung cancer microarray datasets used to identify biomarkers related to survival of patients was conducted.[37] It revealed a significantly elevated IL-27 expression in patients with NSCLC with worse prognosis as compared with those with better prognosis (the median survival was applied as the cut-off point). The significance was especially high if smoking patients with early stage cancer were considered. However, the samples were obtained from the tumor tissue, and immune cells were not examined.[37] Anyway, the opinion on IL-27 as a double-edged sword is still valid.[2]

It should be emphasized that there was a considerable variation in IL-27 concentrations between the study groups, which means that individual participants may have different IL-27 levels.

CONTRIBUTION STATEMENT PK was responsible for the study design and team coordination, analysis of flow cytometry data, and drafting of the manuscript. TW and EW were responsible for BAL cytology and immunology procedure, including sample preparation for flow cytometry; TW drafted the manuscript, as a team specialist for IL-27 biology. AD compiled the control group and prepared clinical data from patients with IPF and NSIP. AR performed all ELISA assays. TS was responsible for the statistical analysis and figure preparation. GP performed the bronchoscopies and prepared clinical data from patients with IPF and NSIP. JC-W prepared clinical data from patients with EAA, analyzed clinical data, and prepared the final manuscript version. All authors, except AD, edited and approved the final version of the manuscript.

REFERENCES

1 Pflanz S, Timans JC, Cheung J, et al. IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4+ T cells. Immunity. 2002; 16: 779-790.

2 Jankowski M, Wandtke T. Interleukin-27: biological properties and clinical application. Switzerland: Springer International Publishing AG; 2016.

3 Yoshida H, Nakaya M, Miyazaki Y. Interleukin 27: a double-edged sword for offense and defense. J Leukoc Biol. 2009; 86: 1295-1303.

4 Swaminathan S, Dai L, Lane HC, Imamichi T. Evaluating the potential of IL-27 as a novel therapeutic agent in HIV-1 infection. Cytokine Growth Factor Rev. 2013; 24: 571-577.

5 Bosmann M, Haggadone MD, Hemmila MR, et al. Complement activation product C5a is a selective suppressor of TLR4-induced, but not TLR3--induced, production of IL-27(p28) from macrophages. J Immunol. 2012; 188: 5086-5093.

6 Pennock ND, Gapin L, Kedl RM. IL-27 is required for shaping the magnitude, affinity distribution, and memory of T cells responding to subunit immunization. Proc Natl Acad Sci U S A. 2014; 111: 16472-16477.

7 Yoshida H, Miyazaki Y. Regulation of immune responses by interleukin-27. Immunol Rev. 2008; 226: 234-247.

8 Fitzgerald DC, Fonseca-Kelly Z, Cullimore ML, et al. Independent and interdependent immunoregulatory effects of IL-27, IFN-β, and IL-10 in the suppression of human Th17 cells and murine experimental autoimmune encephalomyelitis. J Immunol. 2013; 190: 3225-3234.

9 Larousserie F, Pflanz S, Coulomb-L'Hermine A, et al. Expression of IL--27 in human Th1-associated granulomatous diseases. J Pathol. 2004; 202: 164-171.

10 Loke W, Freeman A, Garthwaite L, et al. T-bet and interleukin-27: possible TH1 immunomodulators of sarcoidosis. Inflammopharmacol. 2015; 23: 283-290.

11 Ringowski S, Loke J, Huang S, et al. Enhanced LPS activation of Il+27 signaling in sarcoidosis. Respir Med. 2016; 117: 243-253.

12 Hibbert L, Lieberman L, Wilson E, et al. The IL-27R (WSX-1) is required to suppress T cell hyperactivity during infection. Immunity. 2003; 19: 645-655.

13 Fabbi M, Carbotti G, Ferrini S. Dual roles of IL-27 in cancer biology and immunotherapy. Mediators Inflamm. 2017; 2017: 1-14.

14 Chiba Y, Mizoguchi I, Mitobe K, et al. IL-27 enhances the expression of TRAIL and TLR3 in human melanomas and inhibits their tumor growth in cooperation with a TLR3 agonist poly(I:C) partly in a TRAIL-dependent manner. PLoS One. 2013; 8: e76159.

15 Liu L, Wang S, Shan B, et al. IL-27-mediated activation of natural killer cells and inflammation produced antitumour effects for human oesophageal carcinoma cells. Scand J Immunol. 2008; 68: 22-29.

16 Naumnik W, Naumnik B, Niewiarowska K, et al. Novel cytokines: IL--27, IL-29, IL-31 and IL-33. Can they be useful in clinical practice at the time diagnosis of lung cancer? Exp Oncol. 2012; 34: 348-353.

17 Duan M, Ning Z, Fu Z, et al. Decreased IL-27 negatively correlated with Th17 cells in non-small-cell lung cancer patients. Mediators Inflamm. 2015; 2015: 802939.

18 Piotrowski WJ, Młynarski W, Fendler W, et al. Chemokine receptor CXCR3 ligands in bronchoalveolar lavage fluid: associations with radiological pattern, clinical course, and prognosis in sarcoidosis. Pol Arch Med Wewn. 2014; 124: 395-402.

19 Ziegenhagen MW, Rothe ME, Schlaak M, Müller-Quernheim J. Bronchoalveolar and serological parameters reflecting the severity of sarcoidosis. Eur Respir J. 2003; 21: 407-413.

20 Meyer KC, Raghu G, Baughman RP, et al. An official American Thoracic Society clinical practice guideline: the clinical utility of bronchoalveolar lavage cellular analysis in interstitial lung disease. Am J Respir Crit Care Med. 2012; 185: 1004-1014.

21 American Thoracic Society, European Respiratory Society. American Thoracic Society/European Respiratory Society International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias. Am J Respir Crit Care Med. 2002; 165: 277-304.

22 Alberts WM. Updated ACCP Guidelines for the diagnosis and management of lung cancer: what are the important changes in recommendations? Pol Arch Med Wewn. 2008; 118: 4-5.

23 Przybylski G, Chorostowska-Wynimko J, Dyczek A, et al. Studies of hepatocyte growth factor in bronchoalveolar lavage fluid in chronic interstitial lung diseases. Pol Arch Med Wewn. 2015; 125: 260-271.

24 Szlubowski A, Soja J, Grzanka P, et al. TGF-β1 in bronchoalveolar lavage fluid in diffuse parenchymal lung diseases and high-resolution computed tomography score. Pol Arch Med Wewn. 2010; 120: 270-275.

25 Kopiński P, Składek K, Szczeklik J, et al. Expression of insulin-like growth factor-I (IGF-I) in alveolar macrophages and lymphocytes obtained by bronchoalveolar lavage (BAL) in interstitial lung diseases (ILD). Assessment of IGF-I as a potential local mitogen and antiapoptotic cytokine. Folia Histochem Cytobiol. 2006; 44: 249-258.

26 Kopiński P, Balicka-Ślusarczyk B, Dyczek A, et al. Enhanced expression of Fas Ligand (FasL) in the lower airways of patients with fibrotic interstitial lung diseases (ILDs). Folia Histochem Cytobiol. 2011; 49: 636-645.

27 Kopiński P, Wandtke T, Chorostowska-Wynimko J, et al. Expression of interleukin-27 (IL27) in human lower airways. Pathophysiological implications in pulmonary sarcoidosis (PS). Eur Resp J. 2012; 40: 656-657.

28 Yang WB, Liang QL, Ye ZJ, et al. Cell origins and diagnostic accuracy of interleukin 27 in pleural effusions. PLoS One. 2012; 7: e40450.

29 Kimura D. Miyakoda M, Kimura K, et al. Interleukin-27-producing CD4+ T cells regulate protective immunity during malaria parasite infection. Immunity. 2016; 44: 672-682.

30 Ye ZJ, Xu LL, Zhou Q, et al. Recruitment of IL-27-producing CD4(+) T cells and effect of IL-27 on pleural mesothelial cells in tuberculous pleurisy. Lung. 2015; 193: 539-548.

31 Lee MH, Kachroo P, Pagano PC, et al. Combination treatment with apricoxib and IL-27 enhances inhibition of epithelial-mesenchymal transition in human lung cancer cells through a STAT1 dominant pathway. J Cancer Sci Ther. 2014; 6: 468-477.

32 Kachroo P, Lee MH, Zhang L, et al. IL-27 inhibits epithelial--mesenchymal transition and angiogenic factor production in a STAT1--dominant pathway in human non-small cell lung cancer. J Exp Clin Cancer Res. 2013; 32: 97.

33 Dhong Z, Lu X, Yayng Y, et al. IL-27alleviates the beleomycin-induced pulmonary fibrosis by regulating the Th17 differentiation. BMC Pulm Med. 2015; 15: 13.

34 Salledo R, Hixon JA, Stauffer JK, et al. Immunologic and therapeutic synergy of IL-27 and IL-2: enhancement of T cell sensitization, tumor--specific CTL reactivity and complete regression of disseminated neuroblastoma metastases in the liver and bone marrow. J Immunol. 2009; 182: 4328-4338.

35 Airoldi I, Tupone MG, Esposito S, et al. Interleukin-27 re-educates intratumoral myeloid cells and down-regulates stemness genes in non-small cell lung cancer. Oncotarget. 2015; 6: 3694-3708.

36 Dunn GP, Bruce AT, Ikeda H, et al. Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol. 2002; 3: 991-998.

37 Győrffy B, Surowiak P, Budczies J, Lánczky A. Online survival analysis software to assess the prognostic value of biomarkers using transcriptomic data in non-small-cell lung cancer. PLoS One. 2013; 8: e82241.