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## Impact of anti-inflammatory corticosteroids on changes in selected cytoimmunological parameters in selected interstitial lung diseases

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## Summary

**Introduction.** Glucocorticoids (GKS) hormones with strong immunosuppressive and anti-inflammatory activity are used, inter alia, in interstitial lung diseases (ILD). GKS inhibit excessive activity of inflammatory genes in the airways to induce apoptosis of immune cells, such as alveolar lymphocytes (AL).

**Objective.** Assessment of cytoimmunological changes including apoptosis occurring in alveolar lymphocytes in patients with selected ILD after treatment with systemic corticosteroids.

**Methods.** The material of the bronchoalveolar lavage (BAL) derived from patients with sarcoidosis (PS, n=66), idiopathic pulmonary fibrosis (IPF, n=27) and non-specific interstitial pneumonia (NSIP, n = 25; adequate number of patients treated with systemic GKS were 23, 8 and 6) were analyzed in cytoimmunological tests: a) percentage and the total values of BAL inflammatory cell populations; b) AL subset typing; c) CD4/CD8 index calculation; d) AL cell cycle analysis (DNA staining with propidium iodide, PI); in techniques mentioned in b) to d) items flow cytometry was used.

**Results.** In all patient groups, treatment with GKS resulted in a decrease in the total cell number, e.g. for PS (untreated:  $310 \pm 80 \times 10^3/\text{ml}$ ; treated:  $188 \pm 43 \times 10^3/\text{ml}$ , median  $\pm$  SEM,  $p < 0.05$ ) and lymphocytes (untreated:  $113 \pm 71 \times 10^3/\text{ml}$ ; treated:  $43 \pm 25 \times 10^3/\text{ml}$  median  $\pm$  SEM,  $p < 0.05$ ). There has also been significantly lower percentage of eosinophils in all groups in GKS-treated subgroups, e.g. for IPF (untreated:  $4,6 \pm 3,0\%$ , treated:  $0,3 \pm 0,5\%$ ), for NSIP (untreated:  $1,5 \pm 0,7\%$  and treated: with  $0,4 \pm 0,2\%$ ; median  $\pm$  SEM,  $p < 0.05$  for both). In contrast, AL apoptosis rate was significantly higher in treated patients, e.g. for PS (untreated:  $0,6 \pm 0,5\%$ ; treated:  $4,0 \pm 2,5\%$ ), NSIP (untreated:  $3,4 \pm 1,8\%$ ; treated:  $13,1 \pm 5,1\%$ ; median  $\pm$  SEM,  $p < 0.05$  for both). In all GKS-treated groups corticosteroid therapy caused lower CD4/CD8 index, but only on the level of statistical tendency (e.g. for PS untreated:  $4,7 \pm 0,5\%$ , treated: with  $2,9 \pm 0,9\%$ ) for IPF (untreated:  $1,2 \pm 0,5\%$ , treated: with  $1,1 \pm 0,5\%$ ; median  $\pm$  SEM for both).

**Conclusions.** Systemic inflammatory glucocorticoid therapy (GKS) in all included groups of patients results in a decrease in the total number of alveolar lymphocytes, which is most likely related to the significant increase apoptosis rate of these cells. GKS medication in a similar extent caused probably both the death of helper (Th) and cytotoxic lymphocytes (Tc), since the decline in the value of CD4/CD8 index in treated patients compared with untreated ones was insignificant. A characteristic BAL cytological change in all tested ILD after GKS administration was a remarkable decrease in eosinophil percentage.

**Key words:** Apoptosis, BAL, corticosteroids, interstitial lung disease

## Introduction

Interstitial lung diseases (ILD) is a heterogeneous group of diseases in which the main pathological phenomenon is chronic inflammation of the bronchioles and alveoli caused by exposure to a given factor. With the progression of the disease, this process involves the pulmonary vessels, the lower respiratory tract, which can lead to pulmonary fibrosis [1,2].

The examination, which has a significant importance in the diagnosis of interstitial diseases, is bronchoalveolar lavage (BAL) [3,4]. For the purpose of this study, using BAL material, attempts were made to assess alterations in the cytoimmunological image, specifically changes in immune system function and cells' phenotype after the treatment with glucocorticoid therapy (GKS) at patients with selected interstitial lung diseases: sarcoidosis (PS), idiopathic pulmonary fibrosis (IPF), nonspecific interstitial pneumonia (NSIP).

Sarcoidosis is a disease characterized by unknown etiology, the onset of the disease is chronic or subacute, fibrosis is rare, and inflammatory cells are lymphocytes and monocytes. In this disease, the population of CD4 + cells predominates. In IPF, the etiology is also not known, the beginning is of the type as in the case of sarcoidosis (the onset is chronic, when in PS - it is sometimes severe, as the so-called Löfgren syndrome). In addition, it differs from the previous disease entity, the presumed predominance of CD8 + lymphocytes, and the inflammatory cells are mainly eosinophils and neutrophils. Fibers are always present. However, fibrosis is common in NSIP, although in the form of so-called cell phones it appears late [5]. The treatment used in interstitial lung diseases is, among others corticotherapy.

Glucocorticoids are hormones that are important regulators of the body's homeostasis [6,7,8,9]. Due to their multi-faceted effect, they have found application in the treatment of inflammatory diseases, among others in interstitial lung diseases, acting in the genomic and non-genomic mechanism. Steroids penetrating the cell connect with their receptors (GR, glucocorticoid receptor), which causes the detachment of chaperone proteins, then the GKS-GR complex travels to the cell nucleus where dimerization of the receptor takes place. This results in the exposure of domains rich in zinc fingers, called DNA-binding domains. In this manner, the GKS-GR complex is combined with the GRE (glucocorticoid response element) within the promoters of the target genes. We differentiate GRE positive, which enhances gene activation and GRE negative, which by combining with the target gene inhibit its expression [10].

The most important effect of steroid therapy is the silencing of numerous pro-inflammatory genes activated in the course of long-lasting inflammation [11]. According to the literature, one of the most significant mechanisms of triggering T lymphocyte apoptosis by GKS is the induction of Bim protein expression, along with the parallel inhibition of gene expression for the Bcl-xL anti-apoptotic protein [12]. Another hypothesis suggests the effect of GKS on the release of cathepsins by disintegrating lysosomes

### **Purpose of work**

The aim of the study was to determine cytoimmunological changes, with particular emphasis on the process of apoptosis in follicular lymphocytes from the lower respiratory tract in patients treated systemically with glucocorticosteroids in sarcoidosis, idiopathic pulmonary fibrosis and nonspecific interstitial pneumonias.

### **Materials and methods**

#### *Examined groups*

The study included 66 patients with sarcoidosis (pulmonary sarcoidosis, PS, including untreated, n = 43 and treated, n = 23), 27 with idiopathic pulmonary fibrosis (IPF, including untreated, n = 19 and treated, n = 8) and 25 with non-specific interstitial pneumonia (NSIP, including untreated n = 19 and treated n = 6). BAL material came from ill and untreated patients whose the therapy lasted for 6 to 22 months. The research included the consent of the Bioethical Commission of Collegium Medicum UMK (628/2014) with subsequent additions.

#### *Bronchoalveolar lavage*

BAL (BALF, bronchoalveolar lavage fluid) was performed in accordance with the recommendations of the European Respiratory Society [13]. Briefly, the Olympus Bf 20 bronchofiberscope was used, patients were premedicated (Midazolam 2.5-5mg intravenous, Atropina 0.5mg sub cutis); the epiglottis and trachea were anaesthetized with a local 2% xylocaine solution. Bronchofiberscopy was wedged in the bronchi of the medial lobe (right lung). Buffered saline (37°C) was used in four equal portions of 50 ml, after each fraction the

material was aspirated, filtered through sterile gauze, combined, mixed, collected in sterile vessels and transported to the laboratory on ice [14].

#### *Cytological and immunological tests of BAL material*

The principles of cytological and immunological analysis of BAL material have been previously reported [14,15]. The viability of BAL cells was determined with trypan blue, their total counts were calculated in a Bürker chamber; cytotoxic formulations (80g, 5min) were stained with the May-Grünwald-Giemsa method (MGG) and hematoxylin-eosin (HE); in the light microscope, the percentage formula of inflammation cells (inflow) BAL cells was calculated. The follicular lymphocyte (AL) subpopulations were typed by direct immunofluorescence technique. Briefly, the BAL material was centrifuged and rinsed in a PBS solution (300g, 8min), an amount of  $1-5 \times 10^5$  BAL cells was incubated with saturating amounts of mouse monoclonal antibodies (BD Pharmingen™). A set of anti-CD45 FITC / CD14 PE antibodies ("leucogate", used to define the field of lymphocytes in flow cytometry) was used as a positive control. To determine the expression of the appropriate cytokine receptors on Th (CD4) and Tc (CD8) cells, a set of antibodies against human HLA-DR, TRAIL, CD27, CD25, CD16 + 56, CD19, CD3, CD4 and CD8 antigens were used, conjugated with fluorescent dyes FITC, PE and PE-Cy5. Cells expressing the test antigen emitted light in the standard FL1 fluorescence channels (green light), FL2 (bright red) and FL3 (dark red), respectively. As a negative control, isotype-compatible mouse antibodies with working antibodies used in the work were used. Details, including the type of antibody-conjugated fluorochromes, are given in Table 1. Incubation of BAL cells with antibodies (30 min. No light) was discontinued with PBS solution with 0.1% sodium azide, cells were rinsed and suspended in 0.3ml PBS with 1 % formaldehyde additive [16]

The BD FACSCalibur flow cytometer (488nm argon laser) collected data for at least 10,000 cells of each individual sample, including their sizes (forward scatter, FSC), side scatter, SSC and fluorescence intensity FL1, FL2 and FL3 . The results were calculated as a percentage of positive BAL lymphocytes in a given stain. Defining the AL field. in the CD45 / SSC parameter system, detailed criteria for the qualification of BAL material for testing and the conditions of cytometric analysis were previously described [16].

Table 1. Monoclonal antibodies used in flow cytometry analysis.

Surface marker	Fluorochrome	Catalog Number/Supplier
Simultest Isotype control (IgG1 FITC/IgG2a PE)	FITC/PE	342409/BD Pharmingen
Leucogate (CD45/CD14)	FITC/PE	340040/BD Pharmingen
CD45	PerCy5	555490/BD Pharmingen
HLA-DR	PE	347367/BD Pharmingen
CD4	FITC	345770/BD Pharmingen
CD8	PE	555367/BD Pharmingen
TRAIL	PE	550516/BD Pharmingen
CD27	FITC	340424/BD Pharmingen
CD25	PE	555432/BD Pharmingen
Simultest CD3/CD16+CD56	FITC/PE	340300/BD Pharmingen
CD19	FITC	345776/BD Pharmingen
CD3	PerCP	345766/BD Pharmingen

FITC - fluorescein isothiocyanate, PE - phycoerythrin, PerCy5 - phycoerythrin-cyanine 5, PerCP - peridinin chlorophyll protein

#### *Cell cycle analysis in flow cytometry with staining of DNA with propidium iodide*

The cell cycle was analyzed by flow cytometry with the staining of DNA with propidium iodide (PI). In this study, the ability of PI to stoichiometric DNA binding is used, making it possible to assess cells in the late stage of apoptosis. In the histogram presentation of the surface area of pulses in the FL2 band (and therefore the specific for the excited PI light), typical cell cycle phases are distinguished: G0 / G1 peak, S phase and G2 / M peak. In order to fully penetrate the interior of the cell by PI, the cell suspension is incubated with surface-active substances (e.g., Nonidet detergent). Briefly, the BAL material was centrifuged and rinsed first in a PBS solution (400g, 5min), an amount of  $1 \times 10^6$  BAL cells, and then incubated and under the same conditions 2 times centrifuged in NSS solution (Nonidet, propidium iodide, citrate buffer). 250µl of RNase solution was added to the cells and incubated for 15 min, then 0.5ml of PBS was added and tested in a flow cytometer. Caluza ®Flow Analysis Software was used to analyze and visualize the results.

#### *Statistical methods*

Analysis of the data in statistical terms was performed using Statistica 10.0. The numbered, non-parametricized groups studied were compared using the Mann-Whitney U test. The obtained results were presented as median  $\pm$  standard error of the mean (SEM), which were in accordance with the literature [17]. Statistically significant differences were found for  $p < 0.05$ ; where  $p$  is the level of significance. Differences for  $p < 0.1$  were also taken into account as a trend.

## Results

### *Cytological results of BAL material*

Figure 1 presents the percentage recovery of fluid BAL material from patients with selected disease entities: PS, IPF, NSIP before and after glucocorticoid therapy (GKS). There was observed a statistically significant, with a significance level of  $p < 0.05$ , percentage decrease in recovery after systemic treatment with GKS in the group of patients with IPF (untreated:  $50.5 \pm 1.8\%$ ; treated:  $45.0 \pm 2.9\%$ ; median  $\pm$  SEM). In the remaining groups, the percentage of BAL fluid recovery after steroid treatment was reduced, however, it was not statistically significant.

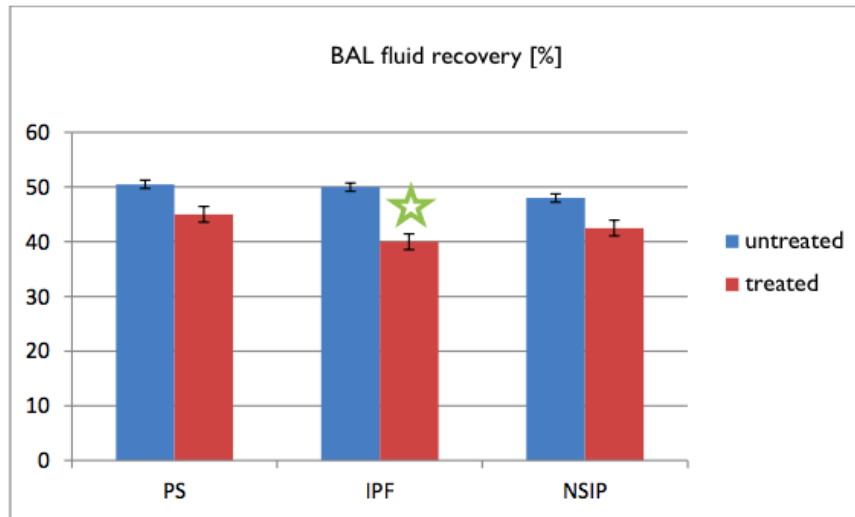


Fig. 1. BAL fluid recovery %, the results are presented as median  $\pm$  SEM values, ★  $p < 0.05$  was considered significant.

Interestingly, in sarcoidosis and nonspecific interstitial pneumonia a statistically significant decrease in the total number of cells was observed after treatment with GKS, PS (untreated  $310 \pm 80 \times 10^3$  cells/ml, treated:  $188 \pm 43 \times 10^3$  cells/ml, median  $\pm$  SEM), for NSIP (untreated:  $168 \pm 62 \times 10^3$  cells/ml, treated:  $160 \pm 32 \times 10^3$  cells/ml, median  $\pm$  SEM). However, in the case of IPF we observe a slight increase (untreated:  $140 \pm 38 \times 10^3$  cells/ml, treated:  $147 \pm 62 \times 10^3$  cells/ml, median  $\pm$  SEM).

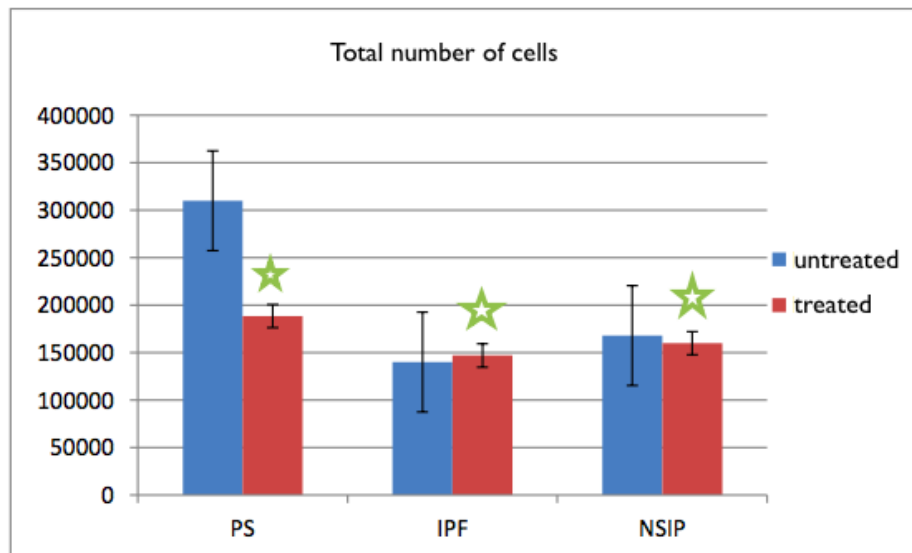


Fig. 2. Total number of BAL cells, the results are presented as median  $\pm$  SEM values, ★  $p < 0,05$  was considered significant.

Table 2 provides us with the cytological results of the BAL material considering the selected cell populations in all the studied groups. In the BAL fluid of patients undergoing corticotherapy in all three diseases, a statistically significant decrease in eosinophils is noticeable. In PS (untreated:  $1.1 \pm 0.3\%$ , treated:  $0.2 \pm 0.3\%$ , median  $\pm$  SEM), in IPF (untreated:  $4.6 \pm 3.0\%$ ; treated:  $0.3 \pm 0.5\%$ , median  $\pm$  SEM), in NSIP (untreated:  $1.5 \pm 0.7\%$ , treated:  $0.4 \pm 0.2\%$ , median  $\pm$  SEM).

Table. 2. Cytoimmunological results of BAL material [%]

		Macrophage s %	Lymphocyte s %	Neutrophils %	Eosinophils %
PS	untreated	56,0 $\pm$ 2,8 (12,7-87,4)	41,7 $\pm$ 2,9 (11,0-85,5)	1,1 $\pm$ 0,5 (0,0-11,6)	1,1 $\pm$ 0,3 (0,0-7,0)
	treated	63,2 $\pm$ 4,1 (25,6-94,7)	30,5 $\pm$ 4,0 (2,8-74,8)	2,1 $\pm$ 1,4 (0,0-29,0)	<b>0,2<math>\pm</math>0,3*</b> (0,0-6,9)
IPF	untreated	60,1 $\pm$ 4,7 (7,0-89,8)	14,0 $\pm$ 3,6 (2,5-59,0)	15,0 $\pm$ 2,9 (0,0-40,1)	4,6 $\pm$ 3,0 (0,0-50,0)
	treated	62,0 $\pm$ 5,7 (44,0-90,5)	17,3 $\pm$ 6,2 (3,0-58,0)	6,7 $\pm$ 5,5 (2,1-53,0)	<b>0,3<math>\pm</math>0,5*</b> (0,0-3,9)
NSIP	untreated	68,9 $\pm$ 3,9 (33,1-85,2)	18,2 $\pm$ 2,9 (4,6-45,0)	5,9 $\pm$ 3,8 (0,9-57,3)	1,5 $\pm$ 0,7 (0,0-12,2)
	treated	78,1 $\pm$ 4,8 (64,9-95,4)	13,9 $\pm$ 4,2 (3,5-28,6)	1,8 $\pm$ 2,8 (0,7-18,4)	<b>0,4<math>\pm</math>0,2*</b> (0,0-1,5)

All results were presented as median  $\pm$  SEM. The variable value range (minimum-maximum) is given in brackets, \*  $p < 0,05$  was considered significant.

In BAL fluid, the number of lymphocytes in all examined groups descends at a statistically significant level after the use of GKS preparations. For sarcoidosis (untreated:  $112 \pm 71 \times 10^3$  cells/ml, median  $\pm$  SEM; treated:  $43 \pm 25 \times 10^3$  cells/ml), for idiopathic pulmonary fibrosis (untreated:  $32 \pm 7 \times 10^3$  cells/ml; treated:  $18 \pm 11 \times 10^3$  cells / ml; median  $\pm$  SEM), for non-specific interstitial pneumonia (untreated:  $38 \pm 30 \times 10^3$  cells / ml, treated:  $20 \pm 8 \times 10^3$  cells/ml, median  $\pm$  SEM).

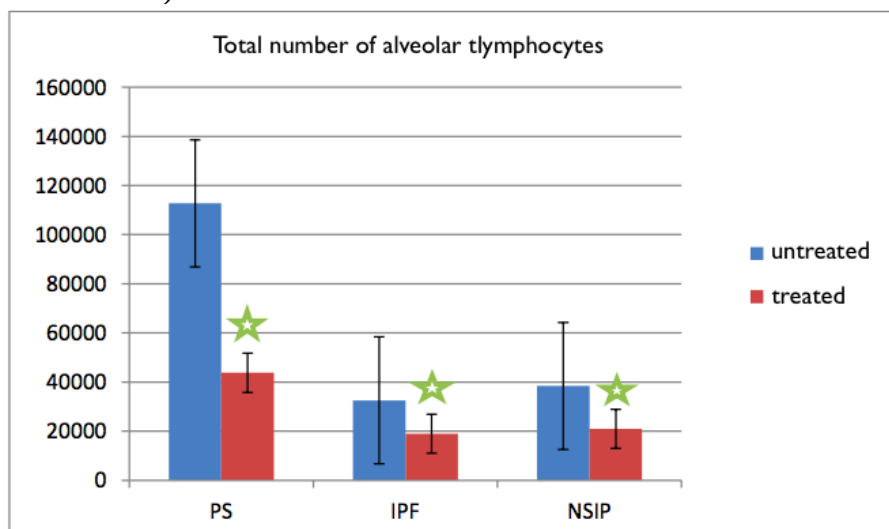


Fig. 3. Total number of alveolar lymphocytes, the results are presented as median  $\pm$  SEM values, ★  $p < 0,05$  was considered significant.

Figure 3 displays the so-called the CD4/CD8 index, namely the ratio of CD4 + cells to CD8 +. There was visible, at the level of statistical tendency ( $p < 0.1$ ), decrease in all groups examined after treatment with glucocorticosteroids. For sarcoidosis (not treated:  $4.7 \pm 0.5\%$ , treated:  $2.9 \pm 0.9\%$ , median  $\pm$  SEM), for idiopathic pulmonary fibrosis (untreated:  $1.2 \pm 0.5\%$ ; treated:  $1, 02 \pm 0.5\%$ , median  $\pm$  SEM), for non-specific interstitial pneumonia (untreated:  $1.7 \pm 0.7\%$ , treated:  $1.2 \pm 0.6\%$ , median  $\pm$  SEM).

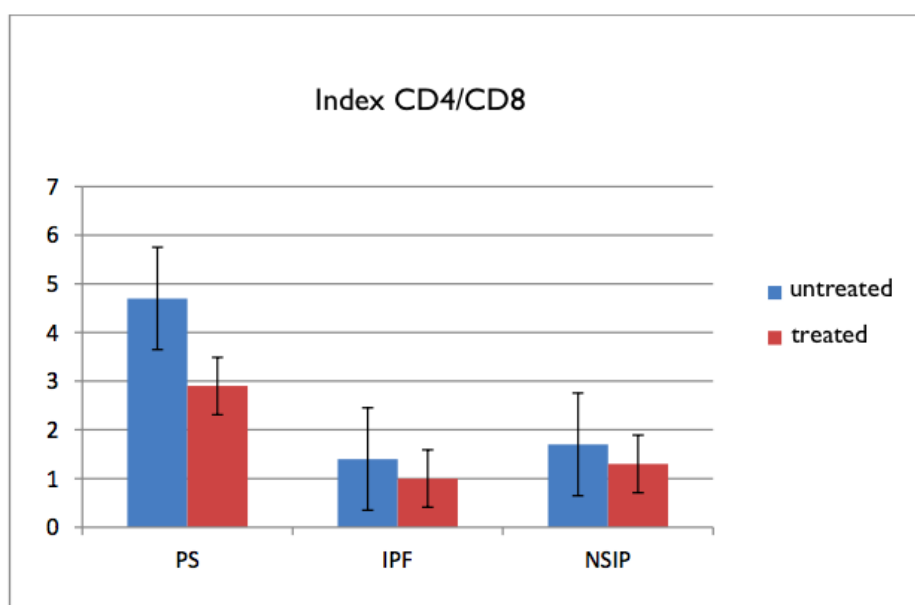


Fig. 4. The CD4+/CD8+ ratio, the results are presented as median  $\pm$  SEM values.



In the study of BAL cell apoptosis in the sub-G1 phase of the cell cycle, attention is paid to the higher percentage of apoptotic T lymphocytes in all examined groups after treatment with glucocorticosteroids ( $p < 0.05$ ) for PS (untreated:  $0.6 \pm 0.5\%$ ;  $4.0 \pm 2.5\%$ ), IPF (untreated:  $5.0 \pm 8.5\%$ , median  $\pm$  SEM, treated:  $5.9 \pm 1.3\%$ ), NSIP (untreated:  $3.4 \pm 1.8\%$ ; treated:  $13.1 \pm 5.1\%$ , median  $\pm$  SEM,  $p < 0.05$ ).

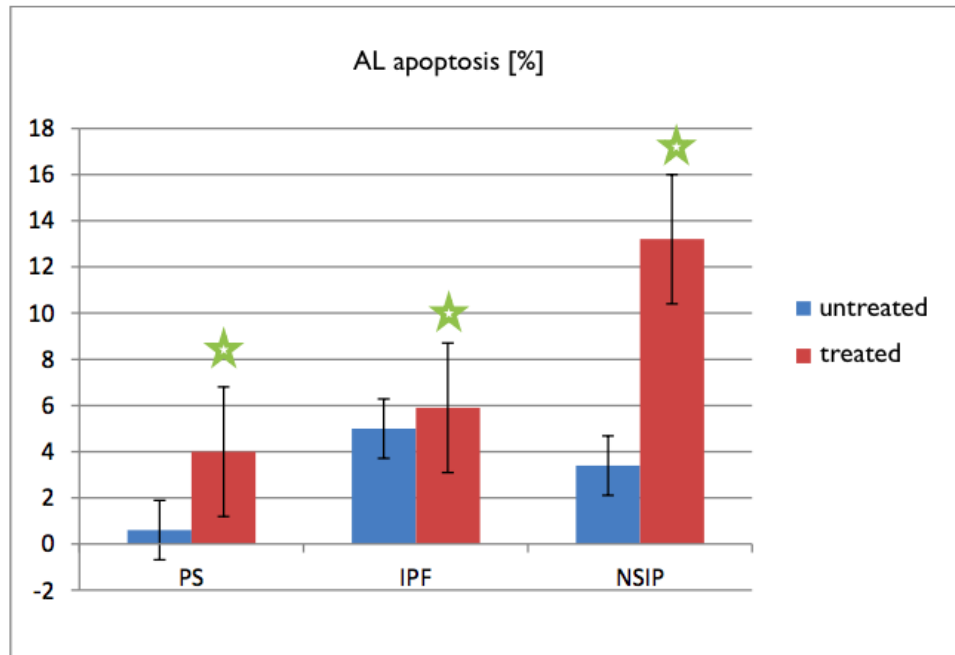


Fig. 5. Alveolar lymphocytes (AL) apoptosis [%]; the results are presented as median  $\pm$  SEM values,  $p < 0.05$  was considered significant.

## Discussion

In studies carried out on experimental models, it was demonstrated that the Fas ligand transcription factor, specifically the NF- $\kappa$ B complex, is inhibited by GKS preparations. The beneficial effect of corticosteroids in sarcoidosis and in NSIP probably results from the inhibition of the NF- $\kappa$ B nuclear factor. In T lymphocytes, it becomes active due to ligation of the TCR receptor with antigen. It works anti-apoptotic because it enhances the expression of the Bcl-2 and Bcl-xL genes. Due to its activity, it influences the intrinsic pathway of apoptosis. The inhibition of its action by, for example, GKS preparations results in activation of the above-mentioned Bim protein, expression of the gene that suppresses the p73 cell cycle dependent on the p53 protein [18]. NF- $\kappa$ B is naturally blocked by an I $\kappa$ B inhibitor, whereas phosphorylation of this inhibitor by IKK kinase leads to the activation of the NF $\kappa$ B factor, which leads to the inhibition of a programmed cell death. In addition, the p22-FLIP factor degradation product activates IKK kinase owing to which it can perform its function [19].

Literature data confirm that glucocorticoids activate the pro-apoptotic Bim protein [20]. This polypeptide is important from the point of view of the NID mechanism. The combination of the TCR receptor with the antigen inhibits the Bim protein in the JNK / PKC kinase signaling pathway. However, the Bim protein inhibits the antiapoptotic proteins of the Bcl-2 family, which allows the opening of mitochondrial channels through other proapoptotic factors Bak and Bax. Following, free radicals penetrate the mitochondria, damage the DNA, and activate the Puma-dependent protein, which inevitably tends to the death of the

lymphocyte [21]. Considering proteins from the Bcl-2 family, it is worth mentioning interleukin 7, which acts as a factor in the survival of follicular lymphocytes. Through JAK kinases and STAT-5 family transcription factors, it activates the expression of Bcl-2 factor [22]. Unfortunately, this cytokine promising to inhibit the inflammatory reaction seems to be insensitive to the use of preparations from the GKS group [17].

A noteworthy protein is tumor necrosis factor  $\alpha$  (TNF $\alpha$ ). T-lymphocytes expressing the TNFR2 receptor use TNF $\alpha$  as a survival signal. The TNFR2 receptor at the death domain has a structural TRAF motif that attaches the TRAF1 and 2 adapter proteins. The MAPK kinase, JNK kinase pathway is activated and, most importantly, the activation of the IKK inhibitor that triggers NF $\kappa$ B factors, the role of which has been described above. This mechanism is worth noting due to the fact that in one of the studies, researchers showed that in people suffering from sarcoidosis after using corticotherapy TNF $\alpha$  level decreased according to the above-mentioned mechanism of anti-inflammatory action of GKS preparations - by inhibition of proinflammatory cytokines as well as TNF [23].

GKS preparations also limit the expression of other inflammatory cytokines reactions, such as IL-1, IL-2, INF $\gamma$ . It is these cytokines that are secreted by Th1 lymphocytes and, as is known, sarcoidosis is a disease in which the Th1 cell subpopulation predominates, while the steroids acting on the GILZ leucine zipper (glucocorticoid-induced leucine zipper) additionally inhibit gene expression for T-bet (a factor protein transcriptional genes promoting Th1 subpopulations [4]) The above hypotheses quoted from the literature may be supported by the results obtained in the present study on the increase in alveolar cell (AL) apoptosis, especially in sarcoidosis and NSIP, thus in diseases with a key role of Th1 cells. this was statistically significant.

According to literature data, the problem of apoptotic effector T cells is not completely clear. It is not certain whether the apoptosis of these cells is determined by the mechanism of cell death induced by activation of AICD (activation of the induced cell death) or death by NID (neglect induced death as a special example of cell death in the mitochondrial pathway). The literature suggests that the interaction between Bim / Bcl-2 proteins may have a special role in the process of apoptosis. Patients with significantly low apoptosis of follicular lymphocytes showed high expression of BCL-2 protein. Conversely, significantly more frequent apoptosis was observed in groups of patients with a decreased percentage of BCL-2 + follicular lymphocytes [22]. Therefore, further research should focus on analyzing proteins, transcription factors that bind to particular signaling pathways, in order to be able to assess which one (extrinsic, internal, and possibly pseudoreceptor) is more often activated in the context of the death of follicular lymphocytes.

The study shows that after the use of GKS preparations in all studied groups, the CD4 / CD8 index, that is the ratio of Th helper cells to Tc cytotoxic lymphocytes, decreased in treated patients compared to untreated patients. These differences were not statistically significant. It follows that CD4 + cells are more damaged by apoptosis induced by steroids. For one group of patients this requires a comment. In the subgroup of sarcoidosis untreated GKS, only cases of chronic sarcoidosis were considered, because in our opinion only such a group can be compared with treated patients. The division proposed by Ziegenhegen [24] was used, according to which three forms of sarcoidosis can be distinguished: a) Loeffgren's syndrome, b) chronic progressive disease not requiring treatment, c) chronic progressive disease requiring treatment [24]. The individual cases of Loeffgren's syndrome treated with GKS have been omitted because the treatment of GKS is criticized in the literature as well as due to the small number of cases. Therefore, the group of untreated patients included only patients with chronic sarcoidosis, in which I, II, III stage of radiological progression is

distinguished [25]. Perhaps that is why the results from previous work [26], that is statistically significant decrease in the CD4 / CD8 index in patients treated with sarcoidosis, were not confirmed. Well, in this study, this observation could not be unambiguously confirmed despite the fact that the group treated with GKS preparations had a marked decrease in CD4 / CD8 values, compared to untreated patients (more cases are necessary).

The conducted research also shows that the percentage and activity of follicular lymphocytes decreases in all three diseases in patients treated with glucocorticosteroids, which would indicate a beneficial effect of GKS preparations, and this is in accordance with the literature [27, 28]. An interesting fact from the point of view of this work are the results indicating an insignificant increase in the percentage of neutrophils, which is in conflict with literature reports that signal the reduction of neutrophil chemotaxis, eg by inhibiting the expression of IL-8 [29]. Nevertheless, this issue requires further research on a more representative group of patients.

For unknown reasons, lymphocytes at people suffering from IPF treated with GKS are unlikely to be resistant - the conclusion is due to the lack of statistically significant differences between the groups - despite the fact that the initial (in untreated patients) AL apoptosis is the highest of all three analyzed units disease. This corresponds to the increasingly common belief that GKS preparations do not benefit in the treatment of IPF [23] but they cause many side effects [30]. Despite this, doctors use GKS in default of other therapeutic options. In the light of the results of this work, this is not a rational position, there are no interest changes (in treated versus untreated) BAL neutrophils, currently considered important fibroblast inducing cells. The case is still unclear and may be the subject of further research, however, because another cell responsible for the deterioration of vital functions of IPF patients, probably the progression of pulmonary fibrosis, eosinophilia, seems to be very sensitive, according to these results, to the therapeutic effect of GKS. The results are confirmed in Meagher's work [31]. Finally, it should be noted that corticotherapy is not indicated if fibrosis has already occurred [32]. This would amplify the view present in the literature data about their inhibitory effect, first of all, differentiation into the Th1 subpopulation. The steroid preparations - as already mentioned - activate the GILZ protein (glucocorticoid-induced leucine zipper) which suppresses the expression of the T-beta transcription factor involved in the maturation of Th1 lymphocytes. It is believed that the predominance of Th2 (and perhaps also Th17) over Th1 is responsible for lung fibrosis and this is the case with IPF [33]. However, the current findings regarding modifications in the T cell subpopulations, and in particular the Th1 / Th2 / Th17 polarization disorder are not definitive. The next step that could be done is to study the markers of individual subpopulations of helper lymphocytes and to evaluate their expression.

Nonspecific interstitial pneumonitis is a disease of rather ambiguous pathogenesis. It is postulated that this is a disorder characterized by a higher percentage of lymphocytes and lower granulocytes compared to PS [34], and it is assumed, similarly to sarcoidosis (PS), that shifting the immune response towards the Th1 subpopulation, and therefore Perhaps for this reason, patients respond well to the use of corticotherapy [27, 28]. These arguments also support the results of the present study, because the apoptosis of follicular lymphocytes was the highest of all the groups examined after treatment with GKS and it was statistically significant increase. A similar result is seen in the assessment of lymphocyte activity (decrease in the percentage of lymphocytes with the CD3 + HLA-DR + phenotype). The issue of programmed death in BAL inflammatory cells has been very rarely studied [17]. Therefore, it would be worth considering expanding research towards apoptosis and concentrating on death receptors, their activity and role in pathogenesis.

According to the literature, little is known about the effect of neutrophils on lymphocyte apoptosis, and these cells are noteworthy because they are a source of oxygen free radicals and cathepsins, and recently discovered that such enzymes are involved in apoptosis of cells by inducing caspase-dependent death [35, 36], an interesting observation is the fact that in this study after steroid therapy only sarcoidosis had a non-significant increase in the percentage of neutrophils, and in the NSIP we noted a decrease, although the immune response in both cases shifted towards the polarization of the cell phenotype Th1.

Earlier, it was reported that steroid-induced apoptosis was primarily damaged by CD3 + lymphocytes [37]. However, in the light of this work, there is no unambiguous data confirming this hypothesis. Submission of two observations: 1) decrease in the number and percentage of lymphocytes in BAL material, 2) no statistically significant differences between subgroups of treated and untreated patients in the subpopulation of lymphocytes would suggest that apoptosis under the influence of GKS preparations in more or less the same range all three main BAL cell lymphocyte populations, namely T, B and NK cells [17].

In summary, in the light of the results we have received, we can come to three basic conclusions. In all diseases studied: sarcoidosis, idiopathic pulmonary fibrosis and non-specific interstitial pneumonia after the use of steroids, there is a significant decrease in the number of follicular lymphocytes (AL) and an increase in apoptosis of these cells. Therefore, the study of AL apoptosis can be used to assess the activity of the disease process, predict remission of the disease, namely the absence of symptoms, and determine the sensitivity of the inflammatory process to treatment with GKS. In addition, it is assumed that the main point of regulation of apoptosis are CD4 + accessory lymphocytes, because the value of the CD4 / CD8 index was reduced in all groups after taking steroid preparations (however unprecedentedly, which we emphasize once more, but was explained, too little of the examined cases).

IPF, because of the other two diseases, postulated by the literature response to glucocorticoids is a disorder that should be paid more attention. In the light of this research, the frequency of apoptosis increased after treatment with GKS. However, due to the conflict between the reports and the research carried out for the purpose of this study, further research into the determination of inflammatory mediators should be considered, among others interleukin 2, interferon  $\gamma$ , as well as BCL-2 protein, which overexpresses, as suggested by Herold and co-authors, has a protective function against apoptosis induced by GKS. Studies on transcription factors for individual lymphocyte subpopulations should not be omitted [17]. In the future, new therapeutic approaches, potentially protecting alveolar cells against apoptosis, may be considered, as the use of glucocorticosteroids is unfavorable in diseases of the occurring fibrosis, the more so as it has already been mentioned there probably is an unknown mechanism by which the Th2 subpopulation, which predominates in IPF escapes from the programmed death of a cell induced by steroids. Perhaps using gene therapy techniques and making genetic modifications of transcription factors inducing or inhibiting the programmed cell death process, we will be able to influence their expression in the course of interstitial diseases and presumably change the molecular mechanism of the action of steroid drugs.

## Conclusions

1. Systemic treatment with anti-inflammatory glucocorticosteroids in all the studied groups of patients results in a significantly lower number of inflammatory cells, including lymphocytes in the BAL material.

2. The use of glucocorticosteroids causes a decrease in the percentage of eosinophils and lymphocytes significantly for all groups of patients, and an increase in the percentage of neutrophils for sarcoidosis.
3. The probable mechanism associated with a decrease in the number and percentage of follicular lymphocytes after GKS administration is a significant increase in the frequency of apoptosis, which was found in all examined groups. The frequency of BAL lymphocyte apoptosis significantly correlates negatively with BAL lymphocytosis.
4. In all examined groups, GKS treatment reduced the CD4 / CD8 lymphocyte index value, but that was not of significant nature. Presumably, GKS drugs in a similar range caused the death of accessory lymphocytes as well as cytotoxic lymphocytes

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