VIA MEDICA

ORIGINAL ARTICLE

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# The effect of different concentrations of anti-PD-1 and anti-PD-L1 antibodies on the activity of immune system cells in patients with non-small cell lung cancer

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Oncology in Clinical Practice
DOI: 10.5603/ocp.96792

ISSN 2450–1654
e-ISSN 2450–6478

## ABSTRACT

**Introduction.** The last century abounded in numerous scientific discoveries that allowed us to understand the operation and functioning of one of the most complex human systems, i.e. the immune system. One of the most important discoveries was the work of Prof. James Alison and Prof. Tasuko Honjo on the development of anti-cancer therapy inhibiting negative immune regulation (PD-1 and CTLA-4 molecules). Knowledge of these molecules' action and their huge role in inhibiting immune system activity, e.g. during cancer growth, created the basis for the development of specific monoclonal antibodies, without which clinicians from many specialties cannot imagine modern cancer therapies. However, side effects of these therapies are still quite troublesome.To minimize them, it would be necessary to reduce the dose while still maintaining the effective level of anticancer activity of immune system cells.

**Material and methods.** In this study, 24-hour culture of PBMCs isolated from blood and bronchoaspirate with various concentrations of nivolumab or atezolizumab was performed. Expression of the individual activation markers on cultured cells was compared to the expression of these markers on cells not subjected to cell culture.

**Results and conclusions.** The outcomes of our research may indicate that individualized dosages of anti-PD-1 and anti-PD-L1 antibodies may contribute to the effective activation of immune system cells while minimizing the side effects of the therapy.

**Keywords**: non-small cell lung cancer, immunotherapy, culture cells

Oncol Clin Pract 2024; 20, 2: 124–135

## Introduction

Currently, clinicians dealing with lung cancer patients cannot imagine modern therapies for this disease without the use of immunotherapy. The therapeutic possibilities of lung cancer treatment have been enhanced by the use of anti-PD-1 and anti-PD-L1 monoclonal antibodies that block the inhibition pathway of the immune system. The effectiveness of both groups of antibodies has been demonstrated in numerous clinical trials, which translated into widespreadregistration of immunotherapeutic drugs, not only for indication of lung cancer. However, a large percentage of patients do not respond to anti-PD-L1/ /anti-PD-1 treatment despite the presence of the predictive marker in the form of the PD-L1 molecule on the surface of cancer cells. Due to many mechanisms, cancer cells escape from immune surveillance, and cancer is very efficient in avoiding recognition by the immune system [1–3]. This study sought to elucidate immunological mechanisms of regulation of T lymphocyte activity in patients with non-small cell lung cancer as a result of stimulation with anti-PD-1 or anti-PD-L1 antibodies.

Received: 03.08.2023 Accepted: 29.08.2023 Early publication date: 06.11.2023

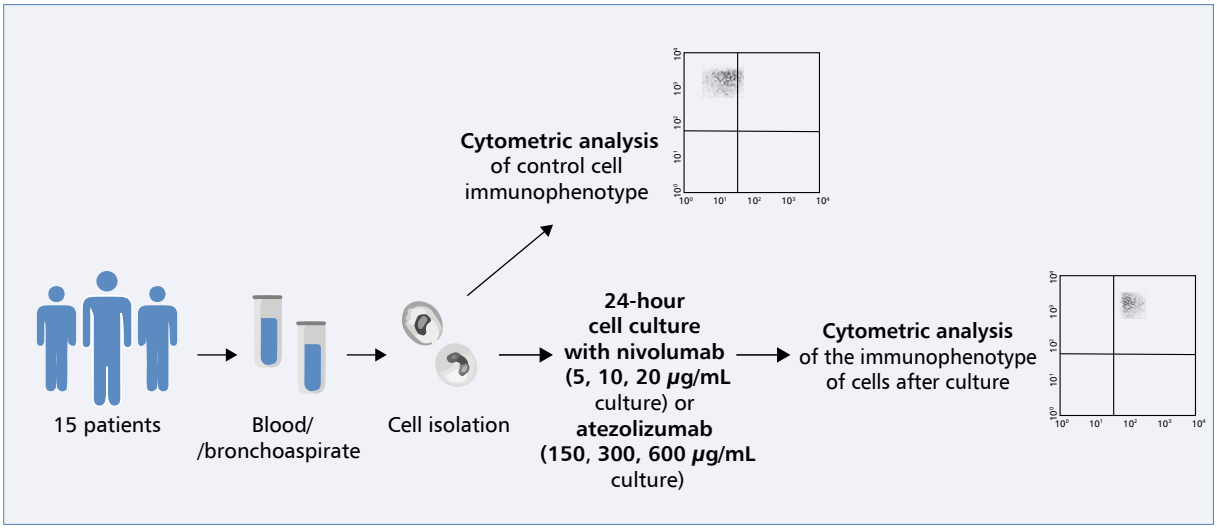


**Figure 1.** Culture methodology

## Material and methods

The study was conducted on a group of 15 patients with locally advanced or advanced lung adenocarcinoma. Six women and 9 men were qualified for the study, with an average age of 66 years. The patients had not previously received any systemic anticancer treatment, antibiotic therapy, drugs stimulating hematopoiesis, or drugs affecting the activity of the immune system; those patients had no diagnosed autoimmune diseases. All investigations were carried out according to relevant guidelines and regulations. Informed consent was obtained from all subjects. The project received a positive opinion from the Bioethics Committee at the Medical University of Lublin (nr KE-0254/318/2018).

### Isolation of peripheral blood mononuclear cells

Peripheral blood (9 mL) was collected into Monovette tubes with sodium heparin on the day of the planned bronchoscopy procedure. Bronchoaspirate (15–20 mL) was collected from the patients during bronchoscopy. Only patients diagnosed with lung adenocarcinoma without clinically significant molecular changes were qualified for the first-line anticancer treatment and were included in the final statistical analysis.

Peripheral blood mononuclear cells (PBMC) were generated by layering blood diluted with phosphate-buffered saline (PBS) without calcium and magnesium ions (1:1 ratio) on Lymphoprep. The isolated PBMCs were rinsed twice with a buffered saline solution, counted in a Neubauer chamber, and their viability was assessed using trypan blue. The bronchospirate was filtered through sterile gauze to remove conglomerates of exfoliated epithelial cells and mucus strands. Then, it was centrifuged for 5 min at 2000 rpm.

### Conducting short-term cell cultures

The PBMC and bronchoaspirate fraction was cultured for 24 hours in three 6-well plates with RPMI 1640 medium (PAA Laboratories, US) supplemented with antibiotics (1% penicillin-streptomycin-neomycin, Sigma Aldrich, US) at 37°C and 5% $CO_2$ in different concentrations of nivolumab (5 μg/mL, 10 μg/mL, 20 μg/mL culture) (Bristol-Myers Squibb, US) or atezolizumab (150 μg/mL, 300 μg/mL, 600 μg/mL culture) (Roche, France). Theculture methodology is shown in Figure 1. On the day of completion of the culture, the cells were recovered from the culture well and subjected to immunophenotyping.

The fraction of control cells from peripheral blood and bronchoaspirate, not intended for culture, was aliquoted into cytometric tubes and incubated with a panel of monoclonal antibodies for 30 min. at 4°C. Then, the cells were washed from the remains of unbound antibodies with PBS buffer without $Ca^{2+}$ and $Mg^{2+}$ ions (centrifugation parameters: 2000 rpm/5 min), and detailed analysis of the cell immunophenotype was performed in a flow cytometer.

In turn, cells subjected to short-term culture with individual anti-PD-1 or anti-PD-L1 antibodies, after 24-hour incubation, were incubated with the selected antibodies conjugated with appropriate fluorochromes (anti-CD4-FITC, anti-CD274-FITC, anti-CD14-FITC, anti-CD8-PE, anti-CD14-PE, anti-CD25-APC, anti-CD69-APC, anti-CD95-APC, anti-CD279-APC (Becton Dickinson, US).

Isolated cells from peripheral blood and bronchoaspirate were divided into 2 parts, the first of which was intended for control immunophenotype analysis on the day of material collection (so-called control cells), and the second part was intended for establishing short-term cell cultures.

## Results

### Evaluation of the percentage of helper and cytotoxic T lymphocytes and monocytes isolated from peripheral blood stimulated with anti-PD-1 antibody compared to unstimulated culture

**T helper or T cytotoxic lymphocytes expressed CD25+**

In the group of T helper (Th) lymphocytes, we observed a significant increase in the culture with the addition of 5 and 20 μg/mL nivolumab (p = 0.004 and p = 0.004, respectively). Similarly, in the $CD8^+$ T cell group, an increase was observed at all nivolumab concentrations, with the increase being statistically significant at a nivolumab concentration of 10 μg/mL culture (p = 0.032) (Fig. 2A).

**T helper or T cytotoxic lymphocytes expressed $CD69^+$**

The percentage of Th cells increased at all concentrations of nivolumab, with the increase being statistically significant at nivolumab concentrations of 10 μg/mL culture (p = 0.033) and 20 μg/mL culture (p = 0.016) and at a concentration of 20 μg/mL of culture compared to the lowest concentration of nivolumab used (p = 0.049). In the group of $CD8^+$ T cells, a significant increase was observed at each concentration of nivolumab (5, 10, 20 μg/mL culture) compared to the control culture (p = 0.017; p = 0.006, p = 0.004) (Fig. 2B).

**T helper or T cytotoxic lymphocytes expressed $CD95^+$**

The percentage of Th cells was higher at all concentrations of nivolumab, with a significant result obtained at the concentrations of 10 and 20 μg/mL (p = 0.01, p = 0.004, respectively). In the T cytotoxic (Tc) cell population, there was a significant increase after stimulation with 10 μg nivolumab (p = 0.016), with a significant decrease in the percentage stimulated with 20 μg nivolumab *vs.* the lowest concentration used (p = 0.033) (Fig. 2C).

**T helper or T cytotoxic lymphocytes expressed PD-1 and monocytes expressed PD-L1**

In the group of Th lymphocytes, a statistically significant increase in the percentage of cells was observed at each concentration of nivolumab (5, 10, and 20 μg/mL) compared to the unstimulated culture (p = 0.007; p = 0.004; p = 0.01). For cytotoxic T cells, the percentage of cells expressing the PD-1 molecule increased at all concentrations of nivolumab, with the increase being statistically significant at 10 μg/mL (p = 0.003) compared to the control culture and compared to the lowest concentration used (p = 0.008) and at 20 μg/mL (p = 0.006) (Fig. 2D).

### Evaluation of the percentage of helper and cytotoxic T lymphocytes and monocytes isolated from peripheral blood stimulated with anti-PD-L1 antibody compared to unstimulated culture

**T helper or T cytotoxic lymphocytes expressed $CD25^+$**

In the group of Th cells, the percentage of cells significantly increased after the use of atezolizumab at a concentration of 150 μg/mL (p = 0.008), 300 μg/mL (p = 0.013), and 600 μg/mL (p = 0.016) (Fig. 3A).

**T helper or T cytotoxic lymphocytes expressed $CD69^+$**

In the group of Th cells, a significant increase was observed in the concentration 150 μg/mL — p = 0.009, 300 μg/mL — p = 0.026, and 600 μg/mL — p = 0.008, and among the lymphocyte population Tc (respectively: 150 μg/mL — p = 0.003, 300 μg/mL — p = 0.041, 600 μg/mL — p = 0.003) (Fig. 3B).

**T helper or T cytotoxic lymphocytes expressed $CD95^+$**

For Th lymphocytes, a significant increase was observed at all concentrations of atezolizumab (150 μg — p = 0.041; 300 μg — p = 0.021; 600 μg — p = 0.026). The percentage of Tc lymphocytes increased significantly at all concentrations of atezolizumab: 150, 300, and 600 μg/mL (respectively: p = 0.013, p = 0.010, p = 0.003) (Fig. 3C).

**T helper or T cytotoxic lymphocytes expressed PD-1 and monocytes expressed PD-L1**

In the group of $CD4^+$ T lymphocytes, a significant increase was observed at each of the concentrations of atezolizumab compared to the control culture (150 μg — p = 0.021; 300 μg — p = 0.004; 600 μg — p = 0.026). For the Tc cells, a significant increase in the percentage of PD-1-positive cells was observed in each of the concentrations of atezolizumab used (respectively: 150 μg — p = 0.004, 300 μg — p = 0.006, and 600 μg — p = 0.006) compared to the control culture (Fig. 3D). In the group of monocytes, comparing the percentage of analyzed cells in the cultures stimulated with 150 μg and 300 μg atezolizumab, a significant (p = 0.041) decrease in the percentage of analyzed cells was observed at the concentration of 600 μg/mL (Fig. 3E).

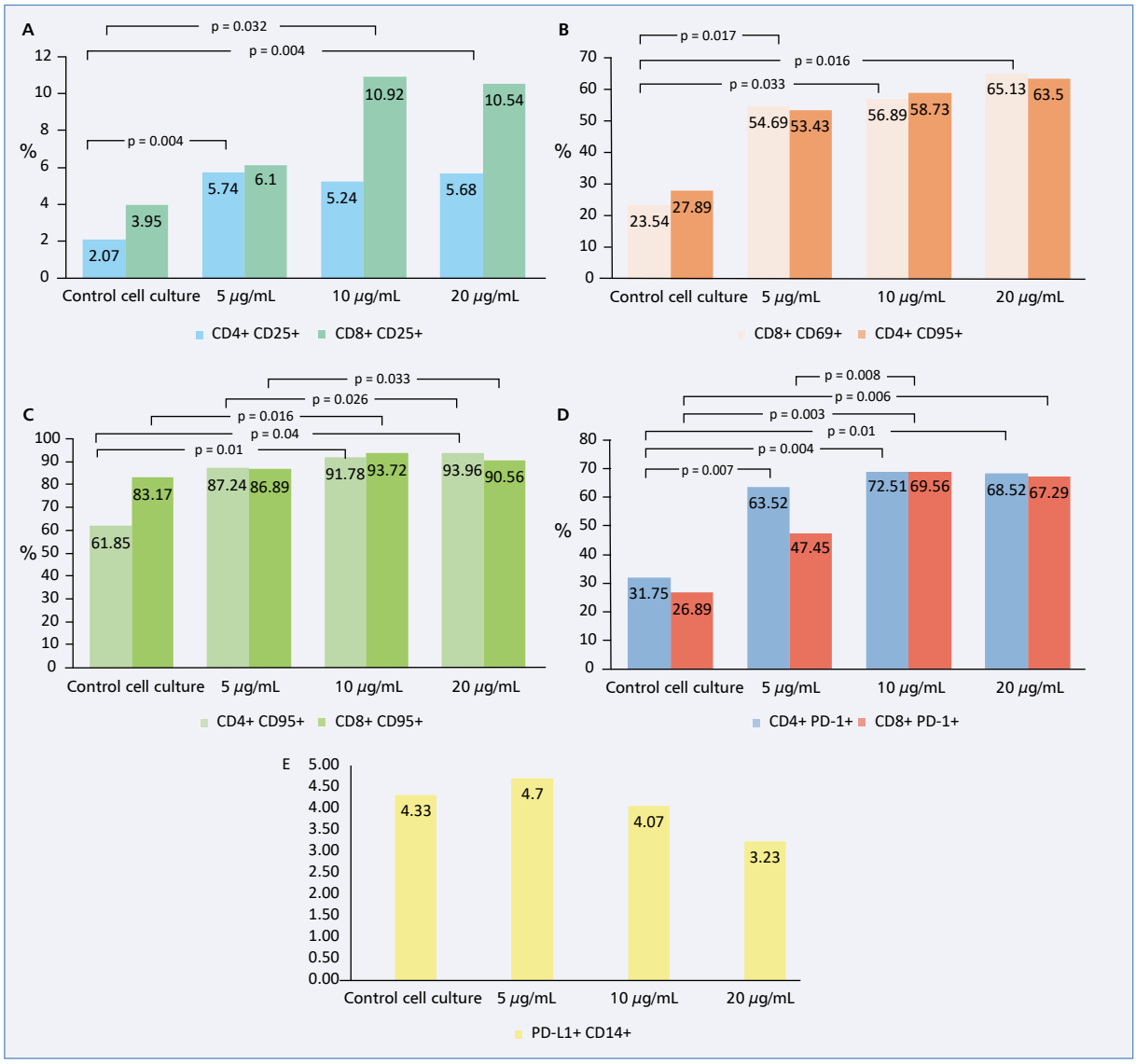


**Figure 2. A.** Percentage of CD4+ and CD8+ T lymphocytes expressing CD25; **B.** CD69; **C.** CD95; **D.** PD-1; **E.** Percentage of CD14+ monocytes expressing the PD-L1 molecule in the studied cell populations in the material isolated from blood stimulated with various concentrations of nivolumab

## Evaluation of expression of the PD-1 molecule on T helper or T cytotoxic lymphocytes isolated from blood and bronchoaspirate stimulated with anti-PD-1 and anti-PD-L1 antibodies compared to unstimulated culture

### T helper or T cytotoxic lymphocytes isolated from peripheral blood stimulated with nivolumab

Expression of the PD-1 molecule on the surface of the Th cells significantly decreased in 5 and 10 $\mu$g/mL concentrations of nivolumab (respectively $p = 0.01$ and $p = 0.013$), and expression of the PD-1 molecule on the surface of the Tc cells significantly decreased at all concentrations used (5 $\mu$g/mL, 10 $\mu$g/mL, 20 $\mu$g/mL) (respectively $p = 0.005$; $p = 0.003$; $p = 0.004$) (Fig. 4A).

### T helper or T cytotoxic lymphocytes isolated from bronchoaspirate stimulated with nivolumab

Expression of the PD-1 molecule on the surface of the Th cells after stimulations of 5 $\mu$g/mL and 10 $\mu$g/mL nivolumab non-significantly decreased (Fig. 4B).

### T helper or T cytotoxic lymphocytes isolated from peripheral blood stimulated with atezolizumab

We also observed that expression of the PD-1 molecule on Th cells decreased in each concentration of atezolizumab. The decrease was significant for 300 $\mu$g/mL ($p = 0.003$) and 600 $\mu$g/mL ($p = 0.004$) concentrations. PD-1 expression on $CD8^+$T cells decreased

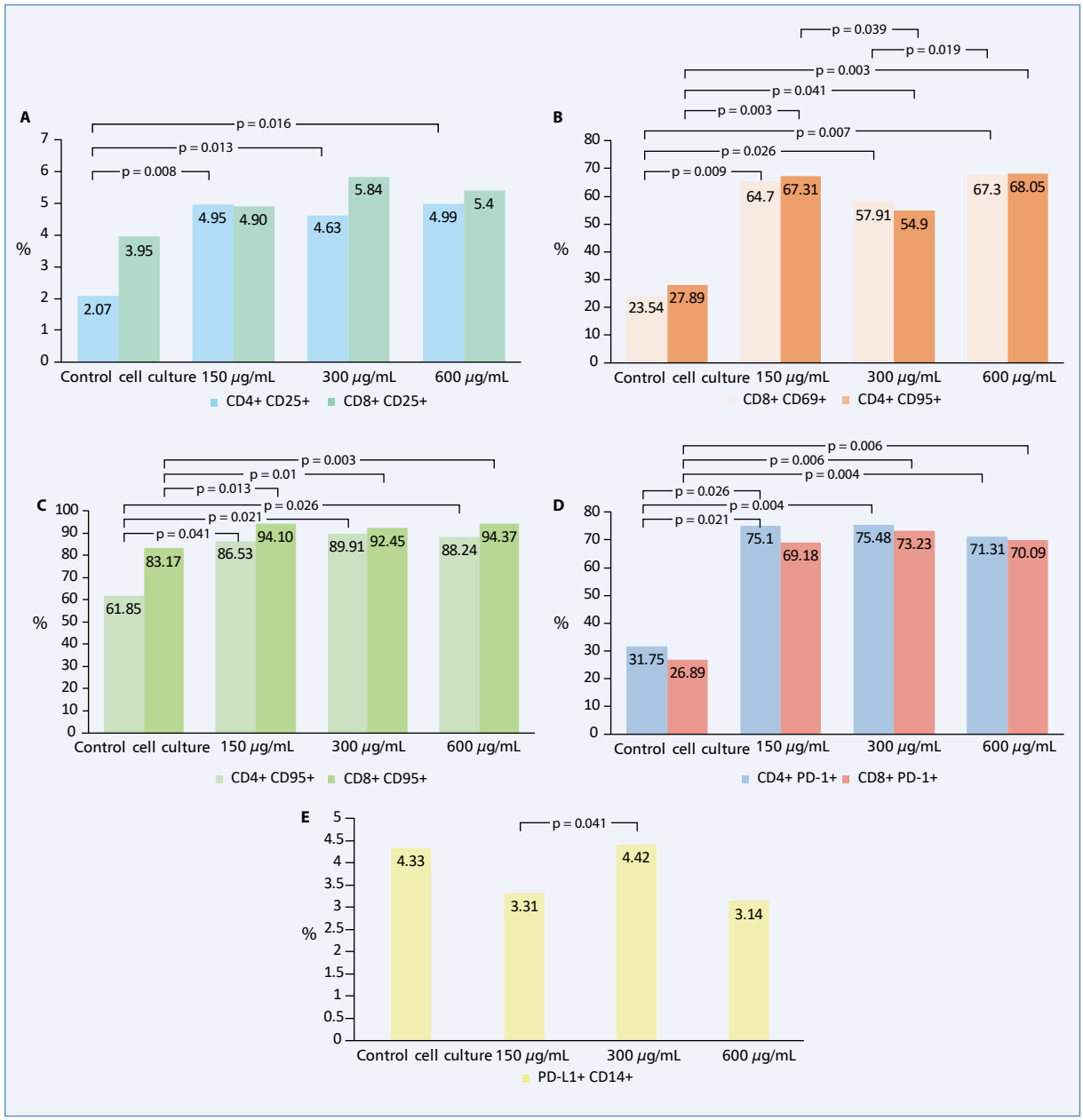


**Figure 3. A.** Percentage of CD4+ and CD8+ T lymphocytes expressing CD25; **B.** CD69; **C.** CD95; **D.** PD-1; **E.** Percentage of CD14+ monocytes expressing the PD-L1 molecule, in the studied cell populations in the material isolated from peripheral blood stimulated with various concentrations of atezolizumab

after atezolizumab stimulation in each concentration (respectively $p = 0.003$, $p = 0.013$, $p = 0.003$) (Fig. 4C).

### T helper or T cytotoxic lymphocytes isolated from bronchoaspirate stimulated with atezolizumab

Expression of the PD-1 molecule on Th lymphocytes was significantly higher in the cell culture stimulated 600 μg/mL of atezolizumab in comparison to the cell culture stimulated by atezolizumab in 300 μg/mL concentrations ($p = 0.041$) (Fig. 4D).

Tables listing all percentages of analyzed cells isolated from peripheral blood and bronchoaspirate (with standard deviations) stimulated with nivolumab or atezolizumab are in Supplementary Tables S1 and S2.

The results on the culture of cells isolated from bronchoaspirate are included in Supplementary material.

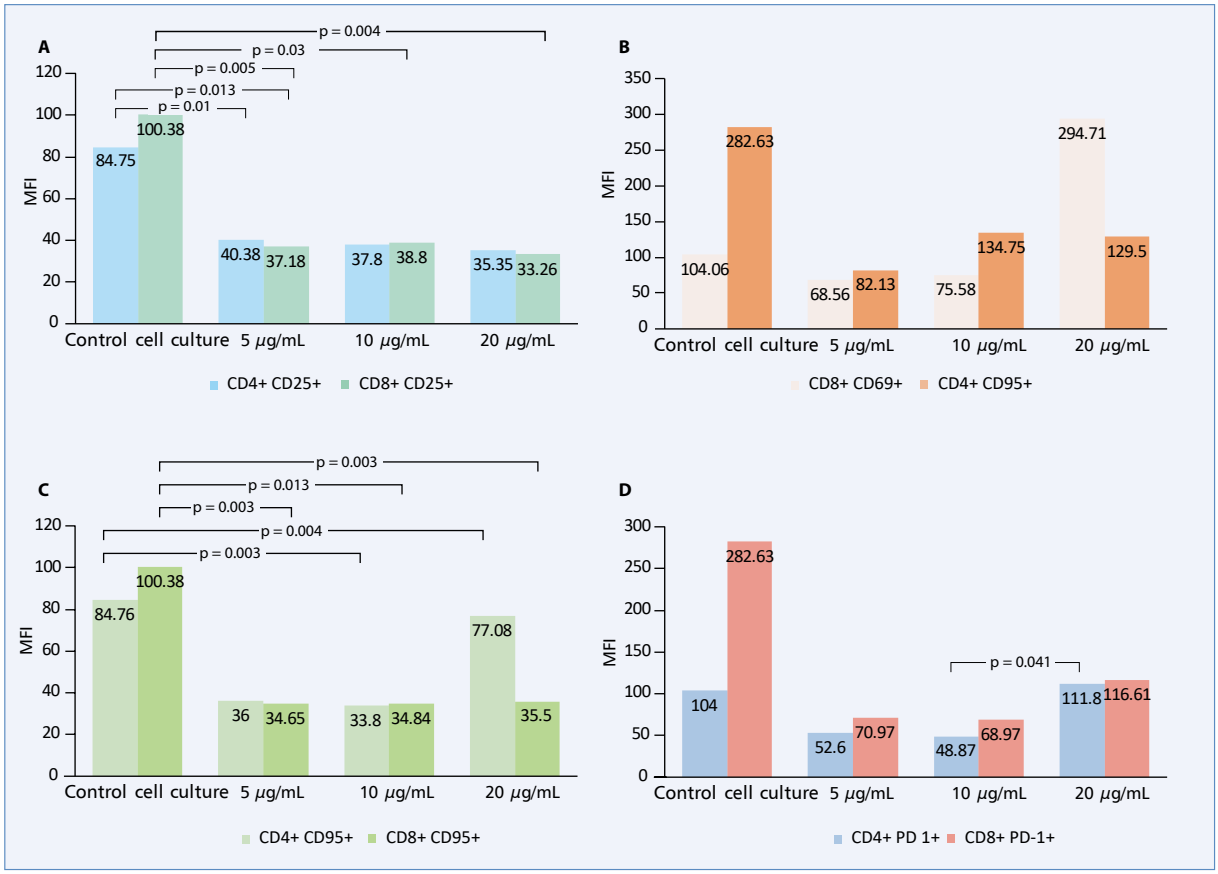


**Figure 4.** Mean PD-1 fluorescence intensity on the tested populations of CD4+ and CD8+ T cells isolated from blood and bronchoaspirate stimulated by nivolumab (**A, B**) and atezolizumab (**C, D**); MFI — mean fluorescence intensity

## Discussion

In this study, we attempted to analyze *in vitro* changes in the immunophenotype and T lymphocyte activity after the use of anti-PD-1 and anti-PD-L1 antibodies. The study showed that checkpoint inhibitors (nivolumab and atezolizumab) have an impact on the blockade of the PD-1/PD-L1 connection. They also influence many functions of immune cells as shown by variable expression of markers analyzed in the study on the surface of T lymphocytes or monocytes, which indicate the current state of the immune system.

The CD25 molecule forms a receptor for interleukin 2 and appears on activated T cells (but does not occur on naive T cells) [4]. In this study, helper and cytotoxic T lymphocytes expressing the CD25 molecule were analyzed and an almost 10-fold higher percentage of $CD4^+/CD25^+$ and $CD8^+/CD25^+$ T cells was observed in the bronchoaspirate than in peripheral blood, which may indicate increased inflammation and increased cell activation in the bronchoaspirate around the tumor. In addition, immunohistochemical analysis confirmed that the presence of activated T lymphocytes has a beneficial effect on the prognosis of NSCLC patients [5].

The CD69 molecule is widely known as an early marker of leukocyte activation, and its main function is to stimulate the proliferation of T lymphocytes in various tissues. Despite this, it turned out that it is also involved in inhibiting the activation of T lymphocytes in the tumor microenvironment [6]. In the presented study, after using different concentrations of nivolumab or atezolizumab, the percentage of CD4-positive and CD8-positive cells expressing the CD69 molecule increased in the cultures of cells isolated from peripheral blood, while expression of this molecule significantly decreased with the increase in the concentration of anti-PD-1 or anti-PD-L1 antibodies compared to the control culture. Such relationships were not observed in cultures of cells isolated from bronchoaspirate. This may indicate a slight response of these cells to stimulation with anti-PD-1 or anti-PD-L1 antibodies and confirm the results of the cited studies on the inhibition of T lymphocyte activation in the tumor microenvironment.

The CD95 molecule (Fas receptor) on the surface of T lymphocytes is primarily a marker of apoptosis of

the cell that will undergo this process after binding to the FasL ligand [7]. The results of our study indicate that with the increase in the concentration of nivolumab, with which the cells isolated from bronchoaspirate were stimulated, the percentage of helper and cytotoxic T cells expressing the CD95 molecule decreased, and the expression intensity of this molecule decreased. In the case of stimulation of the studied cell population with atezolizumab, at the initial concentration (150 μl/mL), the percentage of analyzed T lymphocytes decreased, then increased with increasing concentration, to reach a value comparable to the initial value in the control culture at the highest concentration (600 μl/mL). This may indicate the inhibition of apoptosis of helper and cytotoxic T lymphocytes in the tumor microenvironment after the use of atezolizumab, which may significantly increase the infiltration of tumor tissue by active cells of the immune system. On the other hand, in the cultures of cells isolated from peripheral blood, the opposite situation was observed. When the percentage of tested lymphocytes increased after both nivolumab and atezolizumab, expression of the CD95 molecule decreased. This situation may indicate the influence of anti-PD-1 or anti-PD-L1 antibodies on the stimulation of early apoptosis of circulating helper and cytotoxic T lymphocytes.

Studies conducted over the last few years indicate that cytotoxic T lymphocytes are mainly responsible for direct destruction of cancer cells. Many cells present in the tumor microenvironment cooperate directly or indirectly with CD8-positive T lymphocytes as pro- or anti-cancer cells (including dendritic cells, natural killer cells, and tumor-associated macrophages). For CD8-positive T cells to begin their cytotoxic function, dendritic cells must present them with a tumor antigen in the context of major histocompatibility complex (MHC) class I molecules. During this time, helper T cells secrete cytokines that directly support the differentiation and activation of cytotoxic T cells. In addition, another indirect mechanism of CD8+ T lymphocyte support is the secretion by NK cells and T helper cells of chemokines affecting the maturation and chemotaxis of other innate response cells, including macrophages and dendritic cells [8, 9].

Overexpression of the PD-1 molecule on cytotoxic T lymphocytes and its stimulation by specific ligands contributes to T-cell receptor (TCR) dysfunction and, consequently, to blocking the activity of these cells. Immunotherapy with the use of anti-PD-1 antibodies inhibits the extinction of the activity of these cells, leading to the re-activation of their functions. However, recent studies have shown that reactivated T cells are more likely to be from a group of freshly tumor-infiltrating cells, as they are less regenerative than originally thought [10].

Jin et al. [11] correlated the presence of tumor-infiltrating T cells with expression of PD-L1 on the surface of tumor cells and observed that a high percentage of (tumor-infiltrating lymphocyte (TILs) also had high expression of PD-L1 on tumor cells. The authors conclude that the induction of high PD-L1 expression is certainly one of the mechanisms of defense of cancer cells against the activity of the immune system. At the same time, it is also a predictive marker of the response of such patients to immunotherapy [11].

Gros et al. [12] observed that CD8-positive T cells expressing TCR specific for melanoma antigens were present in the CD8+/PD-1+ lymphocyte fraction, but not in the fraction of cells without the PD-1 molecule. This may suggest that these cells are ready to recognize cancer antigens, and only require unlocking their cytotoxic activity [12, 13].

The tumor microenvironment has a huge and undeniable impact on the activity of the cells of the immune system. The division of tumor types according to the presence of the immune system cells is well described in the literature. The hot type is characterized by strong infiltration of cancer cells by the inactive immune system; the cold type does not have components of the immune system in the tumor tissue; and in the infiltrating type, the immune system marginally penetrates the tumor tissue, but the strong immunosuppressive microenvironment does not allow it to do so [14, 15]. Moreover, it should be borne in mind that effective anti-tumor defense requires the cooperation of both the specific response cells and active non-specific response cells. In our study, no significant effect of the applied antibodies on the activity of monocytes isolated from bronchoaspirate was observed. This may indicate that a single-point approach to immunotherapy — aimed only at stimulating T lymphocyte activity — may not be sufficient to achieve a clinical effect. A comprehensive approach to immunotherapy, in which the activity of T lymphocytes is reactivated and at the same time the activity of non-specific response cells is stimulated, seems to be an interesting approach in the modern treatment of cancer.

## Conclusions

In conclusion, our comprehensive analysis of changes in the percentages of T lymphocytes and monocytes examined allows us to draw four significant conclusions:

1. Both anti-PD-1 and anti-PD-L1 antibody stimulation had a more significant effect on the activation of the specific response of PBMCs compared to cells in bronchoaspirate, which may be due to their functional extinction in bronchoaspirate. This material may be a model of the influence of the neoplastic environment on the immune system in the lungs.
2. CD25-positive and CD69-positive helper and cytotoxic T cells are present in the bronchoaspirate of NSCLC patients, but these cells seem unable to form an immune synapse due to the low expression of the CD28 molecule.
3. A decrease in expression of the PD-1 molecule on the surface of the cells of the specific response was observed on mononuclear cells of peripheral blood and bronchoaspirate after stimulation with both anti-PD-1 and anti-PD-L1 antibodies, regardless of the concentration of antibodies used. This indicates the possibility of restoring T lymphocyte function with the use of a minimal dose of anti-PD-1 or anti-PD-L1 antibodies.

## Article Information and Declarations

### Data availability statement

Original contributions presented in the study are included in the article and further inquiries can be directed to the corresponding author.

### Ethics statement

The project received a positive opinion from the Bioethics Committee at the Medical University of Lublin (nr KE-0254/318/2018).

### Author contributions

A.B.: conception and design, execution and interpretation of the data being published, wrote the paper; K.W.-K.: conception and design, execution and interpretation of the data being published, supervision; M.N.: supervision; P.K.: execution and interpretation of the data being published.

### Funding

Project hasn't additional funding received.

### Acknowledgments

None.

### Conflict of interest

Authors declare no conflict of interests.

### Supplementary material

Tables S1, S2 and results on the culture of cells isolated from bronchoaspirate.

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**Table S1. Cells isolated from blood stimulating by different concetrations of antibodies anti-PD-1 or anti-PD-L1**

| | | Nivolumab | | | Atezolizumab | | |
|---|---|---|---|---|---|---|---|
| Percentage of cells [%] | Control cell culture | 5 μg/mL | 10 μg/mL | 20 μg/mL | 150 μg/mL | 300 μg/mL | 600 μg/mL |
| $CD4^+$ $CD25^+$ | 2.07 ± 2.22 | 5.74* ± 5.57 | 5.24 ± 5.47* | 5.68** ± 5.18 | 4.95** ± 3.93 | 4.63* ± 4.44 | 4.99* ± 5.09 |
| $CD8^+$ $CD25^+$ | 3.95 ± 7.04 | 6.1 ± 6.07 | 10.92* ± 15.91 | 10.54 ± 17.05 | 4.9 ± 7.62 | 5.84 ± 10.02 | 5.4 ± 5.08 |
| $CD4^+$ $CD69^+$ | 23.54 ± 27.70 | 54.69 ± 26.52 | 56.89* ± 21.19 | 65.13* ± 23.52 | 64.7** ± 18.43 | 57.91* ± 21.59 | 67.3** ± 17.99 |
| $CD8^+$ $CD69^+$ | 27.89 ± 18.40 | 53.43* ± 24.60 | 58.73** ± 23.47 | 63.5** ± 18.32 | 67.31** ± 14.16 | 54.9* ± 23.62 | 68.05** ± 16.64 |
| $CD4^+$ $CD95^+$ | 61.85 ± 29.08 | 87.24 ± 13.76 | 91.78** ± 6.80 | 93.96* ± 5.24 | 86.53* ± 17.87 | 89.91* ± 15.83 | 88.24* ± 15.59 |
| $CD8^+$ $CD95^+$ | 83.17 ± 7.67 | 86.89 ± 17.33 | 93.72* ± 5.04 | 90.56 ± 15.01 | 94.1* ± 5.75 | 92.45** ± 6.96 | 94.37** ± 6.22 |
| $CD4^+$ CTLA-$4^+$ $FoxP3^+$ | 88.37 ± 8.20 | 92.23 ± 5.92 | 90.47 ± 17.74 | 86.16 ± 19.88 | 93.26 ± 7.33 | 87.87 ± 16.65 | 89.82 ± 3.42 |
| $CD14^+$ B7-$H4^+$ | 15.14 ± 10.71 | 9.08 ± 6.02 | 7.7* ± 4.24 | 7.8 ± 4.54 | 6.27* ± 4.66 | 5.45* ± 3.12 | 8.67 ± 7.65 |
| $CD14^+$ B7-$H4^+$ IL-$10^+$ | 96.73 ± 2.41 | 97.16 ± 2.04 | 94.07 ± 11.57 | 94.5 ± 11.21 | 94.84 ± 9.68 | 91.06 ± 18.56 | 98.02 ± 1.60 |
| $CD4^+$ PD-$1^+$ | 31.75 ± 25.04 | 63.52** ± 24.54 | 72.51** ± 20.80 | 68.52* ± 23.89 | 75.1* ± 17.5 | 75.48* ± 11.56 | 71.31* ± 19.86 |
| $CD8^+$ PD-$1^+$ | 26.89 ± 18.31 | 47.45 ± 26.46 | 69.56** ± 16.98 | 67.29* ± 21.21 | 69.18* ± 14.59 | 73.23* ± 14.02 | 70.09** ± 13.15 |
| PD-$L1^+$ $CD14^+$ | 4.33 ± 4.24 | 4.7 ± 2.48 | 4.07 ± 2.72 | 3.23 ± 2.33 | 3.31 ± 1.86 | 4.42 ± 2.95 | 3.14 ± 2.19 |

*p < 0.05; **p < 0.01

**Table S2. Cells isolated from bronchoaspirate stimulating by different concetrations of antibodies anti PD-1 or anti-PD-L1**

| | | Nivolumab | | | Atezolizumab | | |
|---|---|---|---|---|---|---|---|
| Percentage of cells [%] | Control cell culture | 5 μg/mL | 10 μg/mL | 20 μg/mL | 150 μg/mL | 300 μg/mL | 600 μg/mL |
| $CD4^+$ $CD25^+$ | 35.54 ± 27.33 | 25.64 ± 24.87 | 25.26 ± 26.12 | 27.71 ± 21.99 | 30.25 21.23 | 36.82 25.62 | 24.85 22.88 |
| $CD8^+$ $CD25^+$ | 46.13 ± 27.73 | 34.05 ± 24.02 | 38.54 ± 27.20 | 39 ± 23.32 | 36.01 ± 29.98 | 48.19 ± 23.34 | 42.52 ± 27.95 |
| $CD4^+$ $CD69^+$ | 48.06 ± 30.01 | 56.52 ± 29.05 | 43.21 ± 20.97 | 55.23 ± 21.57 | 52.36 ± 24.98 | 57.51 ± 27.54 | 58.58 ± 23.46 |
| $CD8^+$ $CD69^+$ | 54.52 ± 24.44 | 62.37 ± 21.57 | 52.45 ± 26.18 | 61.95 ± 24.85 | 57.13 ± 27.92 | 68.23 ± 22.74 | 71.72 ± 24.15 |
| $CD4^+$ $CD95^+$ | 72.14 ± 27.87 | 65.13 ± 29.42 | 57.54 ± 37.48 | 58.92 ± 33.28 | 63.44 ± 26.60 | 65.52 ± 29.03 | 73.14 ± 20.67 |
| $CD8^+$ $CD95^+$ | 81.71 ± 22.14 | 70.4 ± 19.69 | 67.4 ± 31.87 | 64.81 ± 34.71 | 70.5 ± 33.52 | 70.72 ± 30.36 | 81.03 ± 26.74 |
| $CD4^+$ CTLA-$4^+$ $FoxP3^+$ | 92.78 ± 1.61 | 92.28 ± 5.73 | 93.35 ± 5.47 | 92.48 ± 5.92 | 94.9 ± 4.61 | 93.99 ± 6.25 | 94.12 ± 6.99 |
| $CD14^+$ B7-$H4^+$ | 46.03 ± 41.76 | 50.88 ± 34.96 | 58.52 ± 27.91 | 58.62 ± 21.12 | 57.31 ± 32.01 | 47.25 ± 31.20 | 52.37 ± 29.99 |
| $CD14^+$ B7-$H4^+$ IL-$10^+$ | 99.17 ± 0.68 | 99.08 ± 1.36 | 98.43 ± 2.64 | 99.27 ± 0.93 | 99.33 ± 0.99 | 99.34 ± 1.13 | 99.33 ± 1.03 |
| $CD4^+$ $PD1^+$ | 70.87 ± 24.96 | 57.62 ± 34.82 | 60.69 ± 31.12 | 42.26* ± 30.31 | 62 ± 33.46 | 68.21 ± 27.91 | 68.69 ± 27.53 |
| $CD8^+$ PD-$1^+$ | 72.23 ± 23.65 | 62.85 ± 28.12 | 68.77 ± 27.79 | 55.62* ± 23.46 | 63.43 ± 35.66 | 70.28 ± 31.16 | 79.59 ± 16.43 |
| PD-$L1^+$ $CD14^+$ | 40.55 ± 37.01 | 35.13 ± 35.74 | 28.4 ± 31.51 | 27.13 ± 30.62 | 22.83 ± 25.46 | 36.55 ± 30.33 | 28.81 ± 30.66 |

*p < 0.05; **p < 0.01

## Results on the culture of cells isolated from bronchoaspirate

### Evaluation of the percentage of helper and cytotoxic T lymphocytes and monocytes isolated from bronchoaspirate stimulated with the anti-PD-1 antibody

**T helper or T cytotoxic lymphocytes expressed CD25+, CD69+, or CD95+**

In the population of Th lymphocytes, a non-significant decrease in the percentage of cells expressing the CD25 molecule was observed at each of the used anti-PD-1 concentrations compared to the unstimulated culture. In the Tc cell group, the percentage of cells expressing the CD25 molecule was non-significantly lower at all used nivolumab concentrations than in the control culture (Fig. S1A). In the Th lymphocyte population, a non-significant increase in the percentage of CD69-positive cells was observed at the lowest concentration of nivolumab (5 $\mu$g/mL) compared to the control culture. Then, at the concentration of 10 $\mu$g/mL, the percentage decreased insignificantly, and after the application of 20 $\mu$g nivolumab in culture, the percentage of $CD4^+/CD69^+$ cells increased insignificantly compared to the unstimulated culture. In the Tc cell group, the percentage of CD69-positive cells increased non-significantly at the lowest nivolumab concentration of

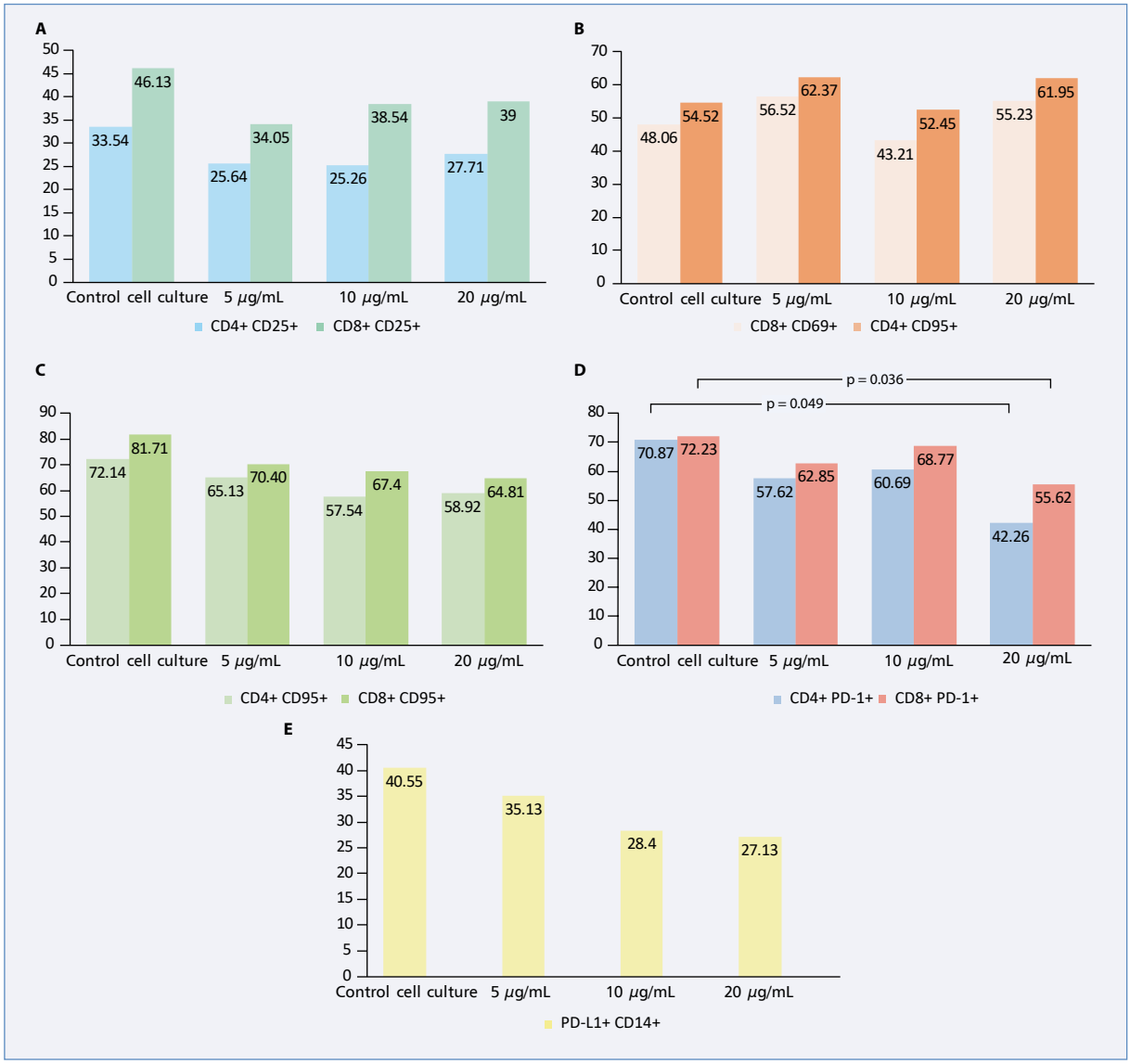


**Figure S1. A.** Percentage of CD4+ and CD8+ T lymphocytes expressing CD25; **B.** CD69; **C.** CD95; **D.** PD-1; **E.** Percentage of CD14+ monocytes expressing the PD-L1 molecule on the molecules on the studied cell populations in the material isolated from bronchoaspirate stimulated with various concentrations of nivolumab

5 μg/mL relative to the control culture, then decreased at the next concentration (10 μg/mL) compared to the control culture, and increased again non-significantly at the highest (20 μg/mL) concentration of nivolumab relative to the control culture (Fig. S1B). In the group of Th lymphocytes, a decrease in the percentage of cells expressing the CD95 molecule was observed at all concentrations of nivolumab compared to the control culture. Similarly, in the group of $CD8^+$ T cells, a non-significant decrease in the percentage of cells expressing the CD95 molecule was observed, with increasing nivolumab concentration compared to the unstimulated culture (Fig. S1C).

**T helper or T cytotoxic lymphocytes expressed PD-1 and monocytes expressed PD-L1**

Among the helper T cells, a decrease in the percentage of cells expressing the PD-1 molecule was observed at all concentrations of nivolumab compared to the control culture, and it was statistically significant at the nivolumab concentration of 20 μg/mL ($p = 0.049$) compared to the control culture. In the group of Tc cells, the percentage of cells expressing the PD-1 molecule was lower compared to the control culture, but significantly lower at the nivolumab concentration of 20 μg/mL ($p = 0.036$) (Fig. S1D). At each of the used concentrations of nivolumab, the percentage of monocytes expressing the PD-L1 molecule was non-significantly lower compared to the control culture, with this value being the lowest at the highest concentration used (Fig. S1E).

## Evaluation of the percentage of helper and cytotoxic T lymphocytes and monocytes isolated from bronchoaspirate stimulated with anti-PD-L1 antibody

**T helper or T cytotoxic lymphocytes expressed CD25+, CD69+ or CD95+**

In the helper T cell group, a statistically significant decrease in the percentage of $CD4^+/CD25^+$ cells ($p = 0.016$) compared to 300 μg/mL was observed in the cultures stimulated with atezolizumab at a concentration of 600 μg/mL. At the other concentrations of atezolizumab (150 μl and 300 μg/mL), a non-significant decrease and an increase in the percentage of cells expressing the CD25 molecule were observed, respectively, compared to the control culture. In the group of $CD8^+$ T cells, only cultures stimulated with the anti-PD-L1 antibody at a concentration of 300 μg/mL showed a non-significant increase in the percentage of $CD8^+/CD25^+$ cells compared to the control culture. In the remaining concentrations of this antibody, the percentage of analyzed cells was insignificantly lower than in the control cultures (Fig. S2A). Both helper (CD4-positive) and cytotoxic (CD8-positive) T cell groups showed a non-significant increase in the percentage of cells expressing the CD69 molecule at all atezolizumab concentrations compared to the control culture (Fig. S2B). The percentage of CD95-positive Th cells was non-significantly higher compared to the control culture only in cultures stimulated with the anti-PD-L1 antibody at a concentration of 600 μg/mL. At the remaining concentrations of the anti-PD-L1 antibody, the percentage of these cells was non-significantly lower than in the control culture. The percentage of CD95-positive Tc cells was non-significantly lower at each used concentration of atezolizumab compared to the control culture (Fig. S2C).

**T helper or T cytotoxic lymphocytes lymphocytes expressed PD-1 and monocytes expressed PD-L1**

In the helper T cell population, the percentage of cells expressing the PD-1 surface molecule was non-significantly lower at all atezolizumab concentrations compared to the control culture. In the group of cytotoxic T cells, a non-significant decrease in the percentage of cells expressing the PD-1 molecule was observed (Fig. S2D). In the monocyte group, a non-significant decrease in the percentage of cells expressing the PD-L1 molecule was observed (Fig. S2E).

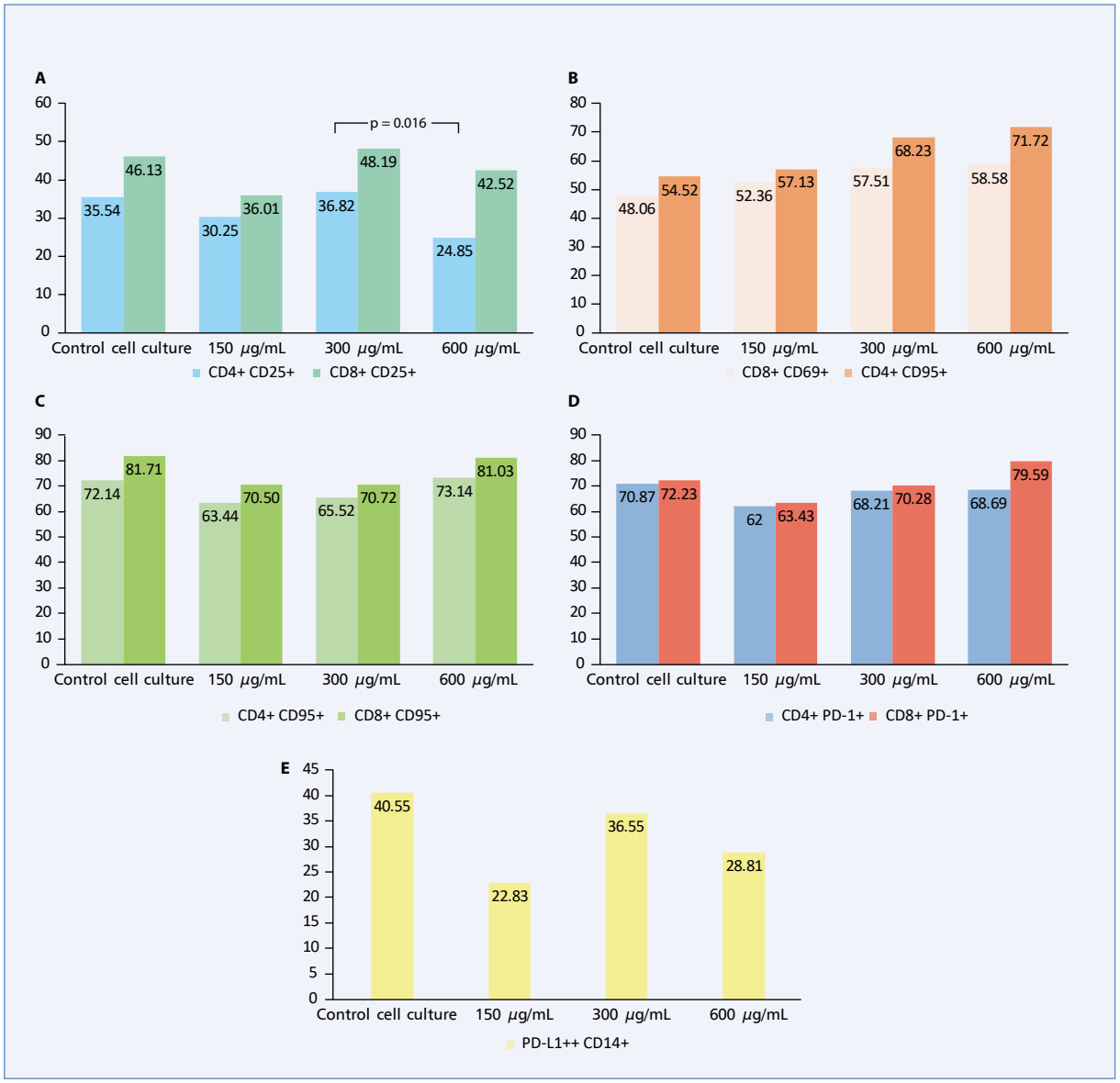


**Figure S2. A.** Percentage of CD4+ and CD8+ T lymphocytes expressing CD25; **B.** CD69; **C.** CD95; **D.** PD-1; **E.** Percentage of CD14+ monocytes expressing the PD-L1 molecule on the molecules on the studied cell populations in the material isolated from bronchoaspirate stimulated with various concentrations of atezolizumab