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Impaired ζ chain expression and IFN-γ production in peripheral blood T and NK cells of patients with advanced lung cancer

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Abstract. Recent studies show that low expression of ζ chain in T and NK cell leads to impaired anti-tumour immunity in patients with cancer, poor prognosis, and shorter overall survival. Therefore, monitoring ζ chain expression may be useful in assessing immune competence in lung cancer patients and in following changes during anticancer therapies. Such studies concerning small-cell and non-small cell lung cancer (SCLC and NSCLC, respectively) have not been published so far. The expression of ζ chain and IFN-γ in peripheral blood (PB) T and NK cells from SCLC and NSCLC patients at advanced (III, IV) stages were analysed before and after chemotherapy with etoposide and cisplatin using flow cytometry. Serum concentrations of TGF-ß1 and IL-10 were also estimated at each time point tested. Before therapy, impaired ζ chain expression was observed in all the patients corresponding with increased levels of immuno-suppressive cytokines in sera compared with controls. Decreased IFN-γ production in T cells from all patients was also demonstrated. In NK cells, IFN-γ was secreted at lower levels in NSCLC patients, while in the SCLC group it was normal. After chemotherapy, restoration of ζ expression in NK cells and its insignificant increase in T cells in SCLC patents corresponding with normalization of TGF-ß secretion were noted. In contrast, NSCLC patients retained impaired ζ expression in T and NK cells. SCLC and NSCLC patients showed a profound defect in IFN-γ secretion in T and NK cells upon treatment. There were no differences in studied parameters between NSCLC and SCLC groups before and after chemotherapy. This is the first report of impaired ζ expression in PB T and NK cells in patients with SCLC and NSCLC in advanced stages, which may result from higher levels of immunosuppressive cytokines in sera. After cytostatic treatment, all the studied patients, including those with initial good response to chemotherapy, remained with profound abnormalities in T and NK cells, which could have dramatic consequences regarding severely impaired anti-tumour immunity.

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Introduction

Several dysfunctions of tumour-infiltrating as well as peripheral blood T lymphocytes and NK cells have been described in patients with cancer. These include abnormalities in signalling via the T-cell receptor (TCR) in T cells and low-affinity Fc receptor for IgG (FcγRIII, also known as CD16) in NK cells (1,2), decreased tyrosine kinase activity following triggering with anti-CD3 or anti-CD16 monoclonal antibodies (1,2), reduced or absent Ca^{2+} flux (2), poor proliferative responses (1,3-6), defects in lytic capacity (5), decreased ability to cytokine production (1,7-9), and increased propensity for spontaneous apoptosis (6,10,11). These significant functional defects in many cases have been correlated with decreased expression of the ζ chain (1-4,6-9).

The ζ chain is a 16-kDa molecule expressed in T lymphocytes as well as NK cells. In T lymphocytes it is a component of the T-cell receptor (TCR), whereas in NK cells it is a part of the activating receptors NK-cell protein 46 (NKp46), NKp30, and the FcγRIII (12). The ζ chain plays a key role in the transduction of activation signals in T and NK cells. Reduced levels of the ζ chain result in a relative lack of tyrosine residues for phosphorylation and impaired recruitment and phosphorylation of downstream signal-transducing molecules, leading to failure of T- and NK-cell activation, proliferation, and cytokine production (12,13).

Studies in our and other laboratories showed decreased expression of the ζ chain in the tumour-infiltrating and peripheral T lymphocytes and NK cells isolated from

patients with various types of solid tumours (1,3-10,14-28) and haematological malignancies (29-31). Moreover, it has been observed that low expression of the ζ chain is associated with more advanced stage of tumour and poor clinical outcome (9,22). Based on these findings it has been suggested that low expression of the ζ chain in T and NK cell leads to impaired anti-tumour immunity in patients with cancer, poorer prognosis, and shorter overall survival and that ζ chain expression in these cells may be a putative biomarker of prognosis (9,22,33).

Lung cancer remains a major cause of malignancy-related death in many countries because of its frequency, its detection at advanced stages of disease (III and IV), and its relative resistance to currently available chemotherapy and radiation therapy (34). Lung cancers are divided histologically into two major groups: small-cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) (34,35). SCLC represents 15-20% of lung cancer cases and is a very aggressive disease characterised by rapid tumour growth, early development of widespread metastases, and overall poor prognosis despite its sensitivity to chemotherapy and radiation (34,35). Long-term survival of patients with SCLC is rare, since only 20-40% of patients with limited disease and <5% of patients with extensive disease are alive 2 years after diagnosis (36). NSCLC affects ~80% of lung cancer patients (34,35). In contrast to SCLC patients, response of NSCLC patients to chemotherapy is low (34). The median survival for advanced NSCLC patients without cytotoxic treatment is <6 months (37), which extends to ~8-10 months for treated patients (34). Therefore, a better understanding of the molecular biology of SCLC and NSCLC may be useful in identifying new targets for drug development that should help improve therapeutic options and patient outcomes.

Little information is available on the ζ chain expression in tumour-infiltrating and peripheral blood (PB) lymphocytes isolated from patients with lung cancer. Woo *et al* (32) examined the expression of this molecule in tumour-infiltrating and PB T cells from 9 patients with NSCLC at early stages of disease. Detailed information on the ζ chain expression in patients with SCLC and with NSCLC at advanced stages of disease is lacking so far.

Since the ζ chain is considered a putative biomarker of immune function and survival, and only limited data are available concerning its expression in lung cancer patients, we undertook the present study to evaluate the expression of the ζ chain in freshly isolated PB T ($CD3^+$) and NK ($CD3^-/CD56^+$) lymphocytes from patients with SCLC and NSCLC at advanced stages (III and IV) of disease in comparison with healthy individuals. We also sought to determine whether ζ chain expression differs in SCLC and NSCLC patients. We gave special attention to evaluating the effects of chemotherapeutic treatment of lung cancer patients on the ζ chain expression. Additionally, we examined the capability of T and NK PB cells from the two groups of lung cancer patients to produce IFN-γ and the obtained results were compared with those of corresponding cells from healthy controls. We also aimed to determine the possible prognostic significance of ζ chain expression and IFN-γ production on the survival of the patients with SCLC and NSCLC.

Table I. Characteristics of patients.

Characteristics	SCLC	NSCLC
Number	15	32
Age (years)		
Mean	59	62
Range	43-81	47-78
Gender		
Male	9	17
Female	6	15
Performance status (ECOG)		
0	1	1
1	9	14
2	3	15
3	2	2
Stage		
LD	8	III A-B: 10
ED	7	IV: 22
Smoking		
Non-smoker	0	4
Smoker	15	28
Weight loss (>5%)		
No	13	17
Yes	2	15
Chronic obstructive pulmonary disease (COPD)		
No	11	24
Yes	4	8
Chemotherapy response		
Complete or partial response	7	9
Stable or progressive disease	8	23

Patients and methods

Patient characteristics. Peripheral blood samples were obtained after informed consent from 15 patients with histologically confirmed SCLC, 32 patients diagnosed with NSCLC, and 17 age- and gender-matched healthy volunteers. The patient characteristics are listed in Table I. Each patient underwent the following staging procedures: physical examination, chest radiograph, computed tomography (CT) of the chest and upper abdomen, brain CT, and hematologic and biochemical procedures. SCLC and NSCLC patients received etoposide (100 mg/m^2) and cisplatin (25 mg/m^2) on days 1-3. Chemotherapy was repeated every 3 weeks. Six and four cycles of therapy were administered to the patients with SCLC and NSCLC, respectively. Blood samples were taken twice from patients: at the time of diagnosis and again after chemotherapeutic treatment.

Mean percentages (and standard error) of peripheral blood $CD3^+$ and $CD3^-/CD56^+$ lymphocytes expressing the ζ chain and IFN-γ in all studied groups.

	$CD3^+/\zeta^+$	$CD3^-/CD56^+/\zeta^+$	$CD3^+/IFN\text{-}\gamma^+$	$CD3^-/CD56^+/IFN\text{-}\gamma^+$
SCLC (n=15)				
Before chemotherapy	26.99 (4.65)	37.85 (4.58)	8.2 (1.53)	13.92 (3.07)
	NS	P<0.0001	P=0.01	P=0.03
After chemotherapy	40.93 (4.23)	56.43 (6.85)	4.25 (0.86)	8.79 (1.37)
NSCLC (n=32)				
Before chemotherapy	30.05 (5.53)	45.09 (5.53)	6.19 (1.00)	9.57 (1.65)
	NS	NS	P=0.01	P=0.03
After chemotherapy	31.51 (5.48)	41.05 (5.75)	7.63 (1.97)	5.63 (0.85)
Control group (n=15)	75.12 (2.73)	65.54 (2.93)	9.90 (0.92)	19.21 (2.45)
SCLC vs. NSCLC				
Before chemotherapy	NS	NS	NS	NS
After chemotherapy	NS	NS	NS	NS
SCLC vs. Control group				
Before chemotherapy	P<0.0001	P<0.0001	P=0.05	NS
After chemotherapy	P<0.0001	NS	P<0.0001	P=0.0008
NSCLC vs. Control group				
Before chemotherapy	P<0.0001	P<0.0001	P=0.01	P=0.008
After chemotherapy	P<0.0001	P=0.002	P=0.0001	P<0.0001

Cell isolation. Peripheral blood mononuclear cells (PBMCs) were separated by buoyant density-gradient centrifugation on Lymphoflot (Biotest, Germany) from freshly drawn peripheral venous blood and washed 3 times in 0.9% saline.

The ζ chain staining and flow cytometric analysis. The experiments on fresh cells were carried out by double and triple labelling with anti-CD3/RPE, anti-CD3/PerCP, and anti-CD56/RPE monoclonal antibodies (MoAbs) (Becton Dickinson, San Jose, CA, USA) and anti-CD247/FITC MoAb (Serotec) directed against the cytoplasmic domain of the ζ chain.

Briefly, after isolation the cells were washed twice in phosphate-buffered saline (PBS) (without Ca^{2+} and Mg^{2+}), divided into tubes at a concentration of $5x10^5$ cells per tube, stained with anti-CD3/RPE MoAb (for detection of the ζ chain in T cells) or anti-CD3/PerCP and anti-CD56/RPE MoAbs (for detection of the ζ chain in NK cells) and incubated for 30 min at 4˚C in the dark. Excess unbound antibodies were removed by two washes with PBS. Following these washes, the cells were fixed and permeabilized with 2% paraformaldehyde and the BD Permeabilizing Solution 2 (Perm 2) (Becton Dickinson) according to the manufacturer's instructions. For the detection of the cytoplasmic domain of the ζ chain, cells were incubated for 30 min at 4˚C in the dark with AB serum and anti-CD247(the ζ chain)/FITC MoAb, then washed twice in PBS, resuspended in PBS, and analysed by flow cytometry using a FACScalibur flow cytometer (Becton Dickinson). Negative controls were always done by omitting the MoAb and by incubating the cells with mouse Ig of the same isotype as the MoAbs conjugated with FITC, PerCPor RPE. The results were expressed as the proportion of double-positive cells. At least 10,000 events per sample were analysed.

Culture conditions and intracellular IFN-γ staining. Cells ($2x10^6$/ml) were cultured in RPMI-1640 medium (Gibco, Paisley, UK) supplemented with 10% foetal calf serum (Flow Laboratories, UK), 2 mmol/l L-glutamine, and 50 μg/ml gentamycin (Gibco). In order to evaluate intracellular IFN-γ expression, cells were incubated with 25 ng/ml of PMA and 1 μg/ml of ionomycin (Ion) for 4 h at 37˚C in a 5% CO_2 atmosphere. Cytokine secretion was blocked with 10 μg/ml of brefeldin A (BFA), a protein transport inhibitor. Furthermore, this procedure was performed on non-activated lymphocytes using only BFA to assess the level of residual IFN-γ synthesis from *in vivo* activation. Cultured cells were washed twice in PBS, divided into tubes at a concentration of $5x10^5$ cells per tube, stained with anti-CD3/FITC or anti-CD3/PerCP and anti-CD56/FITC MoAbs (Becton Dickinson), and incubated for 20 min at room temperature. Following membrane staining, the cells were then fixed and permeabilized with the BD Permeabilizing Solution 2 (Perm 2) (Becton Dickinson) according to the manufacturer's instructions. Then the cells were incubated with anti-IFN-γ/RPE MoAb (PharMingen, San Diego, CA, USA) or IgG1 isotypic control. Finally, the cells were washed and analysed by flow cytometry directly after preparation.

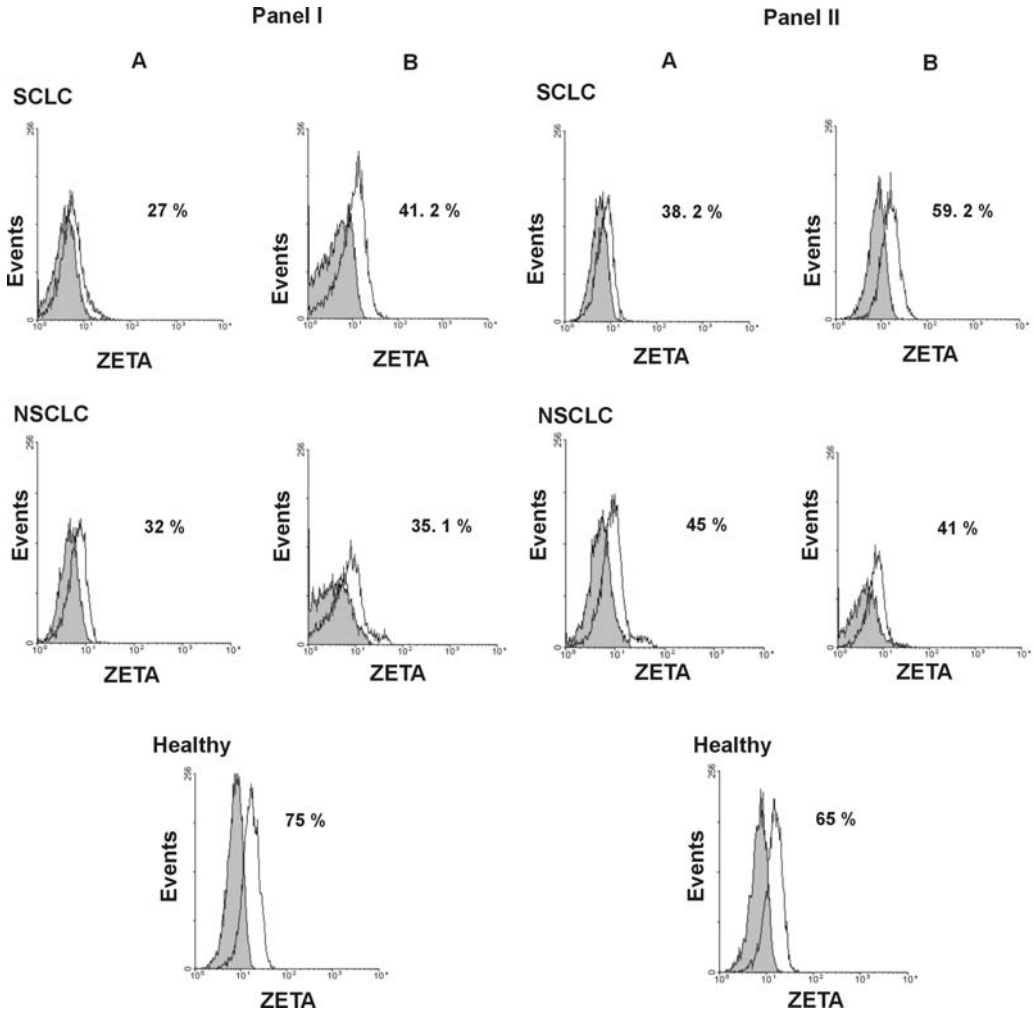

Figure 1. Representative data of the ζ chain expression (open histograms) in peripheral blood $CD3^+$ (Panel I) and $CD3^-/CD56^+$ (Panel II) cells from patients with SCLC and NSCLC before (A) and after chemotherapy (B), and from healthy individuals. The grey histograms represent the isotype controls. The numbers located on the histograms represent the percentages of $CD3^+$ (Panel I) and $CD3^-/CD56^+$ (Panel II) cells co-expressing the ζ chain.

Assessment of TGF-ß1 and IL-10 concentrations. Serum samples collected from all patients with SCLC and NSCLC were stored frozen (-70˚C) until analysed. Serum TGF-ß1 and IL-10 concentrations were determined by enzyme-linked immunonosorbent assay (ELISA) using a R&D Systems reagent kit (UK) according to the manufacturer's recommendations. Each sample was run in duplicate. The assay range is 7.8-500 pg/ml for IL-10 and 31.2-2000 pg/ml for TGF-ß1.

Statistical analysis. Repeated-measures one-factor analysis of variance (ANOVA) was used to compare all the studied groups with respect to ζ chain expression, IFN-γ production, and TGF-ß1 and IL-10 secretion. Survival was analysed using the Kaplan-Meier method. Survival was defined as the time between a moment of diagnosis and death. If dead had not occurred, survival time was considered censored as the last follow-up time. To study the potential influence of ζ chain expression and IFN-γ production on survival, the Cox's proportional-hazards model was used. Results were considered significant at $P \leq 0.05$.

Results

Expression of the ζ chain in freshly drawn T and NK lymphocytes from patients with SCLCL and NSCLC and healthy subjects. We performed intracellular staining for the ζ chain in freshly drawn T ($CD3^+$) and NK ($CD3^-/CD56^+$) PB lymphocytes from patients with SCLC or NSCLC and controls. The results, expressed as the mean proportions of $CD3^+/\zeta^+$ and $CD3^-/CD56^+/\zeta^+$ cells, are shown in Table II. We found that the mean percentages of T and NK cells co-expressing the ζ chain in the two groups of patients with lung cancer were significantly lower than the mean frequencies of the corresponding cells from healthy individuals (Fig. 1). Next we compared the studied groups of patients in the context of possible differences in the ζ chain expression. We observed that the mean percentages of $CD3^+/\zeta^+$ and $CD3^-/CD56^+/\zeta^+$ cells did not differ between the patients with SCLC and NSCLC.

Further we analysed the possible influence of chemotherapeutic treatment on ζ chain expression in the patients with lung cancer. We found that in the patients with SCLC,

Serum TGF-ß1 and IL-10 concentrations (pg/ml) in all studied groups (mean and standard error).

	TGF-ß1		IL-10	
SCLC (n=15)				
Before chemotherapy	27995.4 (5458.0)	NS	8.5 (3.9)	NS
After chemotherapy	18750.9 (4656.9)		2.6 (0.9)	
NSCLC (n=32)				
Before chemotherapy	36839.9 (7719.6)	NS	5.4 (2.2)	NS
After chemotherapy	26894.5 (4911.2)		9.3 (3.1)	
Control group (n=15)	15318.6 (3976.8)		0.8 (0.6)	
SCLC vs. NSCLC				
Before chemotherapy	NS		NS	
After chemotherapy	NS		NS	
SCLC vs. Control group				
Before chemotherapy	P=0.04		P=0.02	
After chemotherapy	NS		P=0.03	
NSCLC vs. Control group				
Before chemotherapy	P=0.008		P=0.009	
After chemotherapy	P=0.04		P=0.001	

chemotherapy resulted in an increase in the mean frequencies of $CD3^+/\zeta^+$ as well as $CD3^-/CD56^+/\zeta^+$ cells, but this increment was statistically significant only in case of $CD3^-/CD56^+/\zeta^+$ lymphocytes, reaching the same level as that of controls. In patients with NSCLC, no significant effect of chemotherapeutic treatment on ζ chain expression was seen. In this group of patients the mean percentages of $CD3^+/\zeta^+$ and CD3-/CD56+/ζ^+ cells remained markedly lower compared with the mean frequencies of the corresponding cells from healthy volunteers. Moreover, after chemotherapy the mean proportions of $CD3^+$ and $CD3^-/CD56^+$ lymphocytes co-expressing the ζ chain did no differ between the patients with SCLC and NSCLC.

IFN-γ production in freshly drawn T and NK cells from patients with SCLC and NSCLC and healthy subject. To find out whether decreased ζ chain expression influences the functions of T and NK lymphocytes from patients with lung cancer, we performed functional studies by measuring IFN-γ production in these cells and the results were compared with the values found in healthy subjects. The mean percentages of $CD3^+/IFN\text{-}\gamma^+$ and $CD3^-/CD56^+/IFN\text{-}\gamma^+$ lymphocytes are shown in Table II. We observed that in patients with SCLC the mean proportion of $CD3^+/IFN\text{-}\gamma^+$ cells was markedly lower than in healthy individuals, whereas the mean frequency of $CD3^-/CD56^+/IFN\text{-}\gamma^+$ cells was comparable to the value found in the controls (Fig. 2). In patients with NSCLC the mean percentages of T and NK lymphocytes producing INF-γ were significantly lower than in the healthy subjects. No significant differences in the mean proportions of $CD3^+/IFN\text{-}\gamma^+$ and $CD3^-/CD56^+/\gamma^+$ cells were found between the patients with SCLC and NSCLC.

Furthermore, we tested the possible effect of chemotherapeutic treatment on IFN-γ synthesis. We found that in patients with SCLC the chemotherapy resulted in marked decreases in the mean frequencies of $CD3^+/IFN\text{-}\gamma^+$ as well as $CD3^-/CD56^+/IFN\text{-}\gamma^+$ cells. In patients with NSCLC the mean percentage of $CD3^+$ lymphocytes producing IFN-γ significantly increased after chemotherapeutic treatment, whereas the mean proportion of $CD3^-/CD56^+/IFN\text{-}\gamma^+$ cells markedly decreased. The mean frequencies of T and NK lymphocytes co-expressing IFN-γ in patients with SCLC and NSCLC after chemotherapy were comparable and remained significantly lower compared with those of controls.

The levels of TGF-ß1 and IL-10 in the sera of the patients with SCLC and NSCLC and healthy subjects. To explain whether the decrease in ζ chain expression and IFN-γ production in the group of lung cancer patients may be related to the presence of immunosuppressive cytokines such as TGF-ß1 and IL-10 in the microenvironment, we measured the levels of these cytokines in the sera of the lung cancer patients at the time of diagnosis and again after chemotherapeutic treatment and the results were compared with the values found in the healthy volunteers. The results are shown in Table III. We observed that the patients with SCLC and NSCLC had significantly higher median levels of TGF-ß1 and IL-10 in their sera before chemotherapy than the healthy individuals. In the patients with SCLC,

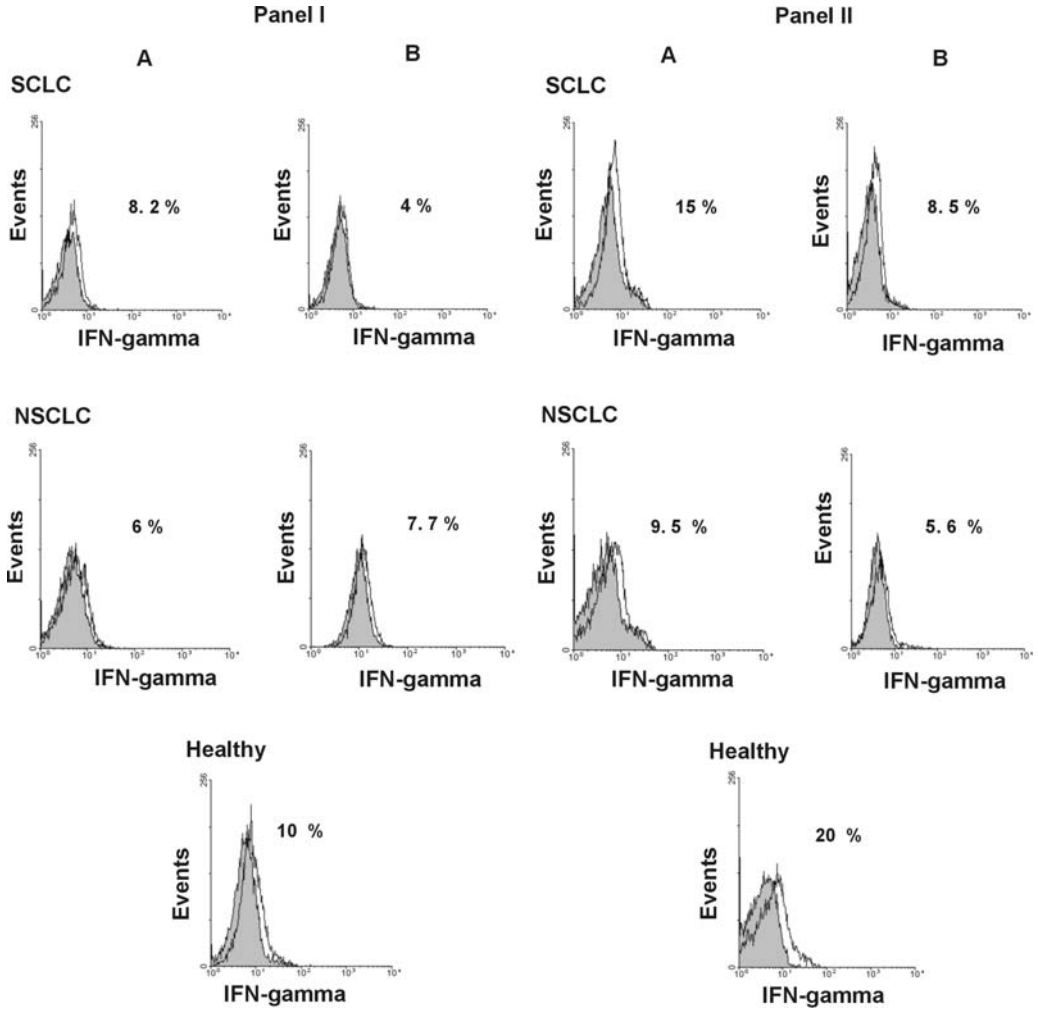

Figure 2. Cytometric presentation of IFN-γ expression (open histograms) by $CD3^+$ (Panel I) and $CD3^-/CD56^+$ (Panel II) lymphocytes from patients SCLC and NSCLC before (A) and after chemotherapeutic treatment (B), and from healthy individuals. The grey histograms represent the isotype controls. The numbers located on the histograms represent the percentages of $CD3^+$ (Panel I) and $CD3^-/CD56^+$ (Panel II) cells co-expressing the IFN-γ.

chemotherapeutic treatment resulted in a decrease in median levels of TGF-ß1 and IL-10, but only TGF-ß1 reached the levels found in the healthy volunteers.

The therapy in NSCLC patients decresed the levels of TGF-ß1. In contrast, IL-10 was produced at even higher amounts. However, these differences were not statistically significant compared with pre-treatment levels. In consequence, the mean concentrations of the studied cytokines in the patients with NSCLC after chemotherapy remained markedly higher than in the controls. No significant differences in the levels of TGF-ß1 and IL-10 in the sera were found among the patients with SCLC and NSCLC before and after chemotherapy.

ζ chain expression and IFN-γ production in T and NK cells with regard to response to chemotherapy in the groups of patients with SCLC and NSCLC. We also aimed to determine whether there is an association between ζ chain expression and IFN-γ production and effectiveness of chemotherapy in patients with SCLC and NSCLC. Based on the clinical data of the effect of chemotherapy, we divided the two groups of SCLC and NSCLC patients into responders, who reached partial or complete remissions of tumour upon chemotherapy, and non-responders, with stable or even increased tumour load after chemotherapeutic treatment. Next we analysed the changes in ζ chain expression and IFN-γ production after chemotherapy.

Among the patients with SCLC, in the responders the mean percentages of $CD3^+/\zeta^+$ cells increased in 6 of the 7 patients (to 335.7, 909.9, 130.6, 122.8, 185.5, and 116.6%) and decreased in 1 individual (to 79.5%) compared with the values before therapy (Fig. 3A). The mean proportions of $CD3^+$ cells co-expressing the ζ chain increased in 2 of the 4 non-responders (to 111.8 and 199.7%) and remained essentially unchanged in other 2 (to 96.2, and 106.2%) (Fig. 3B). The mean frequencies of $CD3^-/CD56^+/\zeta^+$ lymphocytes increased in 5 of the 7 responders (to 169.2, 181.1, 207.2, 147.1, 162.0%), remained essentially unchanged in 1 (to 90.7%) and decreased in 1 (to 66.0%) (Fig. 3A), whereas the mean percentages of these lymphocytes increased in all the non-responders (to 218.6, 117.4, 132.0, 317.0%) (Fig. 3B).

The mean proportions of $CD3^+$ cells co-expressing IFN-γ increased in 1 of the 7 responders (to 133.8%), remained unchanged in 1 (102.7%), and decreased in 5 (to 15.2, 41.9, 4.1, 45.8, and 89.0%) (Fig. 3A). In non-responders, the

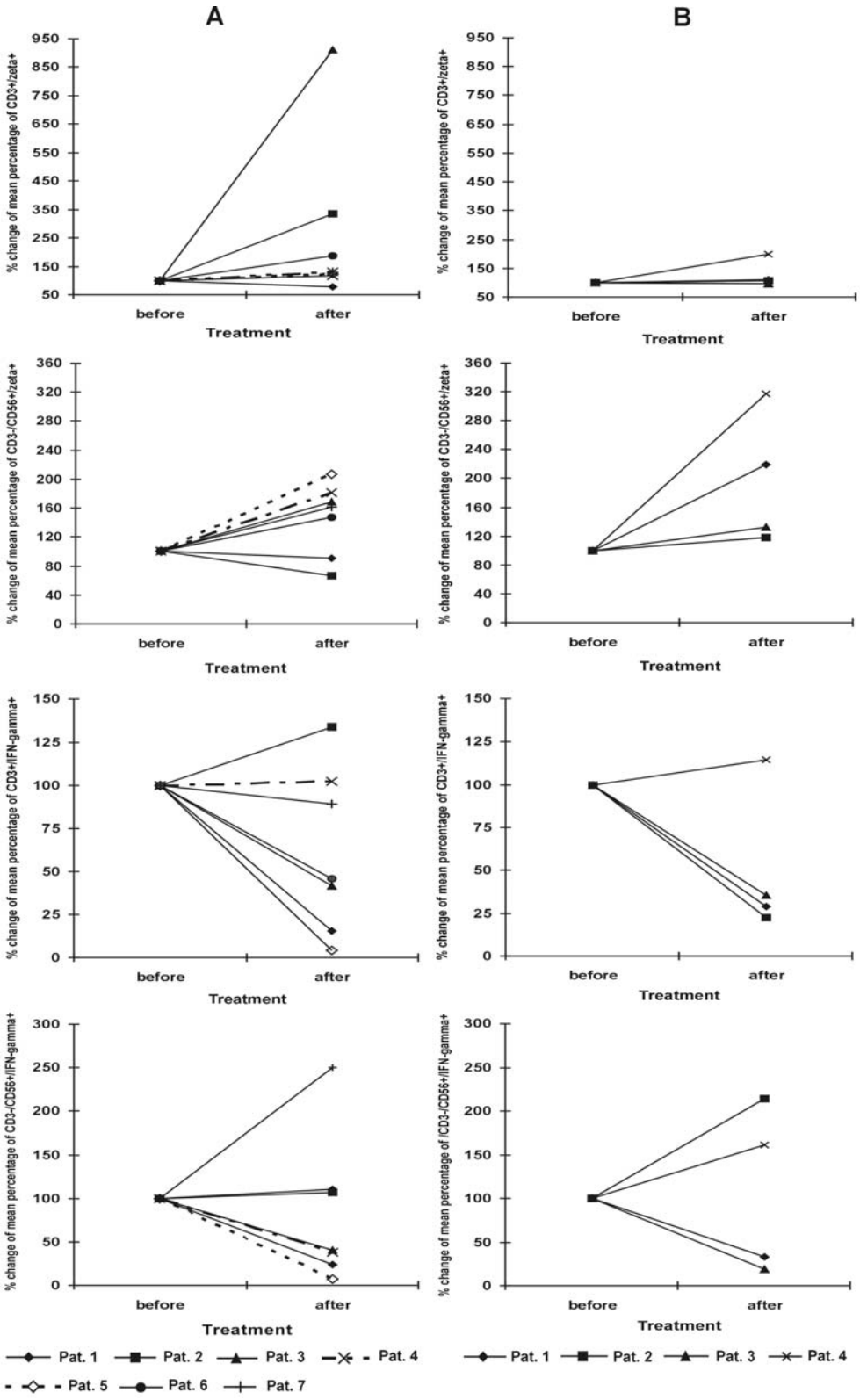

Figure 3. The changes in ζ chain and IFN-γ expression in peripheral blood T and NK cells from patients with SCLC after effective (A) or ineffective chemotherapy (B). The change >10% was considered as significant.

mean frequencies of $CD3^+/IFN\text{-}\gamma^+$ cells increased in 1 of the 4 (to 114.5%) and decreased in 3 (to 29.0, 22.5, and 35.6%) (Fig. 3B). The mean percentages of NK lymphocytes co-expressing IFN-γ increased in 2 of the 7 responders (to 110,1 and 250.5%), remained essentially unchanged in 1 (to 107.3%), and decreased in 4 (to 24.1, 39.7, 38.5, and 7.3%) (Fig. 3A), whereas the mean proportions of these lymphocytes increased in 2 of the 4 non-responders (to 213.9 and 160.6%) and decreased in the other 2 (to 32.5 and 18.4%) (Fig. 3B).

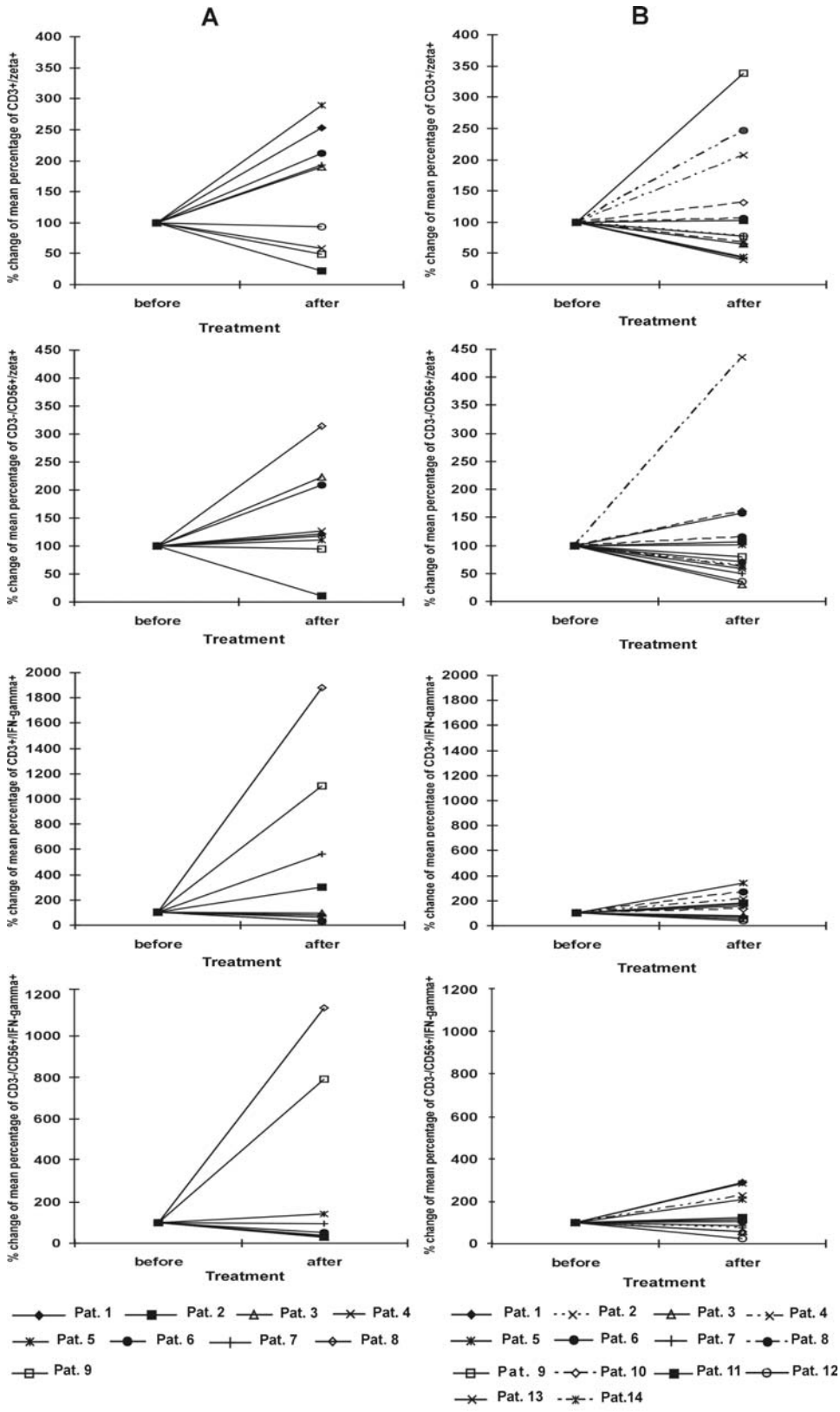

Figure 4. The changes in ζ chain and IFN-γ expression in peripheral blood T and NK cells from patients with NSCLC after effective (A) or ineffective chemotherapy (B). The change >10% was considered as significant.

Among the NSCLC patients, the mean frequencies of $CD3^+/\zeta^+$ cells increased in 5 of the 9 responders (to 253.2, 189.8, 289.5, 212.6, and 193.3%), remained unchanged in 1 (93.5%), and decreased in 3 (to 21.9, 57.9, and 49.8%) (Fig. 4A). In contrast, the mean percentages of $CD3^+$ cells co-expressing the ζ chain increased in only 4 of the 14 in non-responders (to 207.8, 246.3, 337.8, and 130.8%), remained unchanged in 2 (106.2 and 103.5%), and decreased in 8 (to

, 65.1, 43.9, 42.5, 77.6, 39.7, and 67.9%) (Fig. 4B). In percentages of $CD3^-/CD56^+/\zeta^+$ lymphocytes increased in 7 of the 9 responders (to 122.1, 223.0, 126.2, 110.5, 209.2, 117.0, and 314.4%), remained essentially unchanged in 1 (95.4%), and decreased in 1 (to 10.6%), whereas in non-responders the mean proportions of these lymphocytes increased only in 4 of the 14 (to 435.7, 157.2, 115.3, 160.7%), remained unchanged in 2 (to 105.5% and 100.6%), and decreased in 8 (to 70.1, 63.2, 30.8, 49.6, 79.1, 36.3, 59.0, and 61.4%).

The mean proportions of $CD3^+$ cells co-expressing IFN-γ increased in 4 of the 9 responders (to 303.4, 561.8, 1880.0, and 1100.0%) and decreased in 5 (to 80.4, 91.3, 30.7, 62.9, and 32.3%) (Fig. 4A). In contrast, the mean frequencies of $CD3^+$/IFN-γ^+ cells increased in 7 of the 13 non-responders (to 160.9, 218.1, 342.6, 272.4, 130.2, 178.6, and 177.9%) and decreased in 6 (to 71.8, 77.6, 53.6, 55.0, 52.5, and 36.1%). The mean percentages of $CD3^-/CD56^+$/IFN-γ^+ lymphocytes increased in 3 of the 9 responders (to 142.6, 1140.0, and 791.6%), remained unchanged in 1 (96.6%), and decreased in 5 (to 29.6, 36.4, 32.4, 33.6, and 52.2%), whereas in non-responders the mean proportions of these lymphocytes increased in 6 of the 12 (to 288.8, 229.7, 210.5, 114.7, 125.6, and 285.2%), remained unchanged in 1 (104.3%), and decreased in 5 (to 73.8, 57.2, 84.4, 55.9, and 23.8%) (Fig. 4A and B).

Correlations between ζ chain expression, IFN-γ production, and TGF-ß 1 and IL-10 serum levels in lung cancer patients. We analysed the correlations of ζ chain expression, IFN-γ production, and TGF-ß 1 and IL-10 serum levels among themselves in all the studied groups of lung cancer patients. No correlations were found.

ζ chain expression, INF-γ production, and survival analysis. Next we aimed to determine the possible prognostic significance of ζ chain expression and IFN-γ production on survival of the patients with SCLC and with NSCLC. No significant impact of ζ chain expression on survival of the patients with SCLC or those with NSCLC was seen. Similarly, there was no association between survival probability and the median proportion of T or NK lymphocytes co-expressing IFN-γ in the two groups of lung cancer patients.

Discussion

Due to the key role of the ζ chain in the transduction of signals delivered via the receptors of T and NK cells, reduced expression of this molecule impairs T and NK cell signalling and contributes to immune cell dysfunction. Therefore, monitoring the ζ chain expression is useful in assessing immune competence in patients with cancer and in following changes in immune competence during anticancer therapies (33). To the best of our knowledge, such a study concerning lung cancer patients has not been published so far. We found that the mean proportions of T and NK cells co-expressing the ζ chain in patients with SCLC and NSCLC before chemotherapy were significantly lower than the mean frequencies of the corresponding cells from healthy volunteers. There is a discrepancy between our results and data shown by Woo *et al* (32). The authors did not observe decreased expression of ζ chain in tumour-infiltrating or PB T cells isolated from patients with NSCLC. This discrepancy may result from the fact that Woo *et al* (32) investigated patients with NSCLC at early stages of disease only, whereas we performed the studies in patients at advanced stages of disease. In the light of recent findings, decreased expression of ζ chain in PB lymphocytes is observed in patients with advanced, but not early stages of disease (9,18,22,32). Our results also showed that the level of ζ chain expression did not depend on the histological form of the lung cancer, since the mean frequencies of $CD3^+/\zeta^+$ and $CD3^-/CD56^+/\zeta^+$ cells in SCLC and NSCLC patients were comparable.

An interesting question was whether chemotherapeutic treatment with cisplatin and etoposide, the combination of drugs commonly used in the therapy of lung cancer patients, leads to restoration of ζ chain expression in these patients, since recent studies showed that normalization of the ζ chain expression in PB lymphocytes following therapy may be a good prognostic sign (33). We showed that in patients with SCLC, the chemotherapeutic treatment resulted in the normalization of ζ chain expression only in PB NK cells, while the increase in ζ chain expression in T cells, although substantial, was not statistically significant. Moreover, the tendency to up-regulate the ζ chain was more profound among the SCLC patients with good response to cytostatic treatment, while ζ chain expression usually remained unchanged in the non-responding patients. In contrast, no treatment-related modification of ζ chain expression was observed in the patients with NSCLC. These findings suggest that only the patients with SCLC have a potential to up-regulate ζ chain expression after cytostatic treatment. The frequency of tumour regression was higher in the group of patients with SCLC.

It was recently suggested that one of the mechanisms responsible for low ζ chain expression in patients with various forms of cancer may be the secretion of immunosuppressive cytokines by tumour cells. Many malignant cells, including SCLC and NSCLC, and/or stromal cells produce TGF-ß1 and IL-10 (38-41) and, more importantly, the elevated serum levels of these cytokines have been correlated with decreased expression of the ζ chain (24). Recent studies show that high concentrations of TGF-ß1 and IL-10 in sera are usually associated with tumour progression and poor response to immunotherapy (38,42). Our study confirmed previous data (39-41) that before chemotherapy SCLC and NSCLC patients had higher serum levels of TGF-ß1 and IL-10 compared with healthy volunteers. Thus it seems possible that the decreased mean proportions of $CD3^+/\zeta^+$ and $CD3^-/CD56^+/\zeta^+$ PB cells from patients with SCLC and NSCLC may result from the immunosuppressive activity of TGF-ß1 and IL-10 in the microenvironment. It is of note that in the group of SCLC patients, chemotherapeutic treatment resulted in decreases in serum levels of TGF-ß1 to the levels found in healthy volunteers, which corresponded with the normalization of ζ chain expression in NK cells and its insignificantly up-regulation in T lymphocytes. It is possible, therefore, that

the increase in ζ chain expression observed in SCLC patients may result, at least in part, from normalization of the levels of immunosuppressive cytokines in the sera of these patients. However, the treatment did not normalize the levels of IL-10 in SCLC patients or the levels of the two immunosuppressive cytokines in NSCLC patients, suggesting that lung cancer patients maintained immunosuppression despite the cytostatic treatment.

Since the down-regulation of the ζ chain expression in T and NK cells leads to functional impairment of these lymphocytes, we were also interested in assessing the intracellular production of IFN-γ in T and NK cells from SCLC and NSCLC patients. Recent studies demonstrated significantly lower amounts of IFN-γ in whole blood cell cultures from the two groups of lung cancer patients compared with healthy individuals (43-46). Intracellular staining of IFN-γ in T lymphocytes showed that the proportion of $CD3^+/IFN\text{-}\gamma^+$ cells was also lower in lung cancer patients compared with controls, although the differences were not statistically significant, probably due to the high variability even in the healthy volunteers (46). Additionally, no significant differences between SCLC and NSCLC patients in the levels of IFN-γ secretion were found (45). Our findings are in agreement with these studies. The production of IFN-γ by $CD3^+$ cells from SCLC patients and $CD3^+$ and $CD3^-/CD56^+$ PB lymphocytes from NSCLC patients was significantly suppressed, remaining on comparable levels among the studied patients.

It has been found that TGF-ß1 and IL-10 inhibit the secretion of a variety of cytokines by immunocompetent cells, such as IFN-γ (38,42,43), which is one of the major anti-tumour cytokines (47,48). The mechanisms underlying this process are still not clear. Based on other (38,42,43) and our present findings, it seems that TGF-ß1 and IL-10 secreted at high levels in the sera of patients with SCLC and NSCLC decrease the mean proportions of T and NK cells co-expressing the ζ chain, leading to functional impairment of these cells, including a decrease in IFN-γ production. A recent study confirmed a high frequency of IL-10- and TGF-ß-producing T cells corresponding with a low frequency of IFN-γ-secreting T cells in growing tumour in lungs (49). These findings support the observation that T regulatory cells (Treg) secreting IL-10 and TGF-ß are recruited/activated in the tumour and, to a lesser degree, in peripheral blood, thus suppressing the development of IFN-γ-producing effector T cells (32,49).

Since chemotherapeutic treatment partially normalizes the expression of ζ chain in SCLC patients, we were also interested in assessing whether chemotherapy leads to reconstitution of suppressed IFN-γ production in T and NK PB lymphocytes from lung cancer patients. Unexpectedly, in patients with SCLC we observed further decreases in the ability to produce IFN-γ by $CD3^+$ and $CD3^-/CD56^+$ cells after chemotherapy despite the restoration of ζ chain expression and partial normalization of the immunosuppressive cytokine network in the microenvironment. In addition, we noted a tendency to down-regulate IFN-γ production in SCLC patients with remission as well as with stabilization or progression of the disease. Given the demonstrated severe and long-term impact of chemotherapy on the number and function of lymphocytes in cancers (46) and the fact that tumour cells from SCLC patients exert relative sensitivity to chemotherapy, we suggest that SCLC cells remained functionally more affected after cytostatic treatment than chemotherapy-resistant NSCLC cells.

In contrast, among the patients with NSCLC we found a high variability in IFN-γ production by T and NK lymphocytes. It is noteworthy, however, that a slightly higher frequency of up-regulated IFN-γ production after treatment was observed among those with no response to chemotherapy compared with patients in remission. Our results are in line with the findings showing that in NSCLC, the presence of tumour cells or tumour-derived factors in PB following tumour growth favours the differentiation of T cells towards tumour-specific Th1 ($CD4^+/IFN\text{-}\gamma^+$ cells) and Tc1 ($CD8^+/IFN\text{-}\gamma^+$) (50). A high Th1/Th2 ratio in PB may be a result of tumour-mediated Th1-dominant differentiation in association with tumour-derived substances in PB. Thus a high Th1/Th2 ratio in PB seems to be a negative prognostic factor, especially in advanced stages of NSCLC. The above observation may indicate that among patients with NSCLC, the individuals with increased IFN-γ expression after chemotherapy seem to have a significantly worse prognosis. However, the present study clearly showed that the mean percentage of $CD3^+/IFN\text{-}\gamma^+$ cells significantly increased after chemotherapy in patients with NSCLC, but this increment did not reach the normal levels, indicating a substantial defect in T-cell responses. It appears that the impaired IFN-γ production may be a characteristic of a compromised tumour status in lung cancer patients and also a result of cytostatic treatment, at least in chemotherapy-sensitive SCLC. The profound impairment in IFN-γ secretion in lung cancer patients could have dramatic consequence, since it is known to be involved in effective anti-tumour immunity, exerting antiproliferative, antiangiogenic, and proapototic effects on tumour cells (47,48).

In conclusion, we report here for the first time impaired ζ chain expression in PB T and NK cells in patients with SCLC and NSCLC in advanced stages, which may result from the increased levels of immunosuppressive cytokines in the sera. Our present data thus clearly confirmed cancer-associated immunosuppression in patients with advanced SCLC and NSCLC. We also found that SCLC and NSCLC patients, including individuals with initial good response to chemotherapy, maintained profound abnormalities in T and NK cells after cytostatic treatment. Further studies are therefore needed to develop novel strategies to improve the cellular immune response and achieve long-term survival in lung cancer patients.

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