

ORIGINAL ARTICLE

Association of locally produced IL10 and TGFb1 with tumor size, histological type and presence of metastases in patients with lung carcinoma

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Summary

Purpose: Advanced lung carcinoma is characterized with fast disease progression. Interleukin (IL)10 and transforming growth factor (TGF)b1 are immunosuppressive mediators and their role in lung carcinoma pathogenesis and in the antitumor response has not yet been elucidated.

The purpose of this study was to correlate IL10 and TGFb1 levels in the serum and lung tumor microcirculation with clinical stage, disease extent, histological features and TNM stage.

Methods: The study included 41 lung cancer patients in clinical stage III and IV. Histological type was determined immunohistochemically, while tumor size, localization and dissemination were determined radiologically by multislice computerized tomography (MSCT). IL10 and TGFb1 levels were quantified with commercial flow cytometric test in serum and lung tumor microcirculation samples.

Results: Non small cell lung cancer (NSCLC) patients had

significantly elevated TGFb1 while small cell lung cancer (SCLC) patients had significantly increased IL10 in tumor microcirculation. IL10 was significantly elevated in patients with the largest tumors, as well as in patients with III clinical stage and without metastases, both in the serum and tumor microcirculation. TGFb1 was significantly increased in serum and tumor microcirculation in patients with larger tumors. We found significant correlation between these two immunosuppressive cytokines, IL10 and TGFb1, in tumor microcirculation but not in patient serum samples.

Conclusion: IL10 and TGFb1 in systemic and tumor microcirculation are significantly associated with particular histological type of lung cancer, tumor size and degree of disease extent.

Key words: IL10, lung cancer, tumor microcirculation, tumor size, TGF1

Introduction

Lung cancer represents one of deadliest malignant diseases [1]. Due to lack of symptoms at the beginning, frequently results in late admission of patients, often in stage when they have advanced or metastatic disease. Beyond surgical therapy of early disease, there is still no satisfactory therapy against

lung cancer, especially in late stages. Like other malignant lesions lung cancer begins as pre-neoplastic change of the respiratory mucosa. With time, this lesion acquires further genetic malformations together with numerous mechanisms that enables it to evade protective immune mediated destruction.

Several etiological factors have been identified to be associated with lung cancer, as well as most of them induce local pulmonary inflammation. The vast preeminence of evidence shows that tobacco smoke increases the risk of lung cancer and is almost certainly one the most common causes of it [2]. Chronic lung inflammation accompanied by lack of inflammatory response control is associated with active recruitment and functional changes of stromal and inflammatory cells, making the microenvironment pro-tumorigenic. Even without malignant lesion, tobacco smoke induced inflammation is represented with local overexpression of Th2 cytokines, especially IL10, resulting in mucus cell metaplasia, mucus hyperproduction, T cell mediated inflammation and subepithelial fibrosis of airways [3]. Using rat's trachea model, Wang et al. showed that tobacco smoke induces TGFb1 in epithelial cells [4]. A mouse model showed that chronic exposure to tobacco smoke induced impaired bacterial clearance by alveolar macrophages, mediated with increased local production of IL1, IL6, IL10 and tumor necrosis factor (TNF) [5].

These changes in local IL10 and TGFb1 production are probably important in early phases of lung cancer genesis, in initial tumor control during elimination/equilibrium phase, whereas malignant process is still relatively controlled by immune response. Once established, this inflammation generates further immunosuppressive mechanisms enabling selective intra tumor accumulation of myeloid derived suppressor cells (MDSC) and regulatory T lymphocytes (Treg) [6,7]. Local production of IL10 and TGFb1 by MDSC and Tregs, as well as by malignant cells has strong influence on tumor progression, enabling most aggressive and least immunogenic cell to escape completely immune response [8-14]. It has been still unclear whether IL10 and TGFb1 arbitrate in the immune response against already established tumor, besides their role in pro-tumorigenic settings.

Based on these data, the purpose of this study was to investigate if IL10 and TGFb1 levels in systemic circulation and local tumor microenvironment (more importantly) do correlate with clinical and pathological lung cancer stages.

Methods

Patients

The study included 41 patients diagnosed with lung cancer and 30 healthy controls (Table 1). Patients were

diagnosed and treated at the Clinic for Lung Diseases, Military Medical Academy, Belgrade, Serbia, from March till December 2014. All necessary diagnostic procedures (histological, laboratory and radiological) were performed at Military Medical Academy, Belgrade, Serbia. All patients and healthy controls were consented and this study was approved by the local Research Ethics Committee, Military Medical Academy (11-03/2014).

Samples

Blood samples were taken from cubital vein. Tumor microcirculation samples were taken from accessible pathological blood vessels with needle aspiration during diagnostic bronchoscopy. Serum was separated from the samples after centrifugation (3000g, 10 min, at room temperature) and frozen at -70 °C until testing. Before testing, total TGFb1 in serum was converted from latent TGFb1 to active TGFb1 by acidification (20 min incubation at RT with 0.5 vol of 1 N HCl), followed by neutralization (mixed with the same volume of 1.2 N NaOH in 0.5M HEPES). Serum samples were diluted 1:100 for TGFb1 testing. IL10 and TGFb1 were quantified with commercial flow cytometric tests (eBiosci-

Table 1. Patient and disease characteristics

Characteristics		N
Healthy controls		
Male		22
Female		8
Age, years (mean±SD)		57±14
Patients		
Male		31
Female		10
Age, years (mean±SD)		62±8
Histological types		
NSCLC	adenocarcinoma	13
NSCLC	squamous cell carcinoma	9
NSCLC	large cell carcinoma	10
SCLC		9
Clinical TNM stage		
III		24
IV		17
Metastases		
M0		27
M1		14
TNM stage		
T1		7
T2		15
T3		12
T4		7

Table 2. IL10 and TGFb1 levels in serum and tumor microcirculation samples of lung cancer patients

Parameter	Type/ stage	IL10				TGFb1			
		Serum*	Tumor*	Change	p value	Serum*	Tumor*	Change	p value
Histological type	SCLC	16 ± 13	71 ± 36	▲	*	624 ± 347	174 ± 66	▼	**
	NSCLC Ad	32 ± 33	35 ± 27	▲	ns	754 ± 680	275 ± 266	▼	**
	NSCLC Sq	14 ± 10	18 ± 11	▲	ns	696 ± 676	760 ± 667	▲	ns
	NSCLC LC	37 ± 28	22 ± 12	▼	ns	843 ± 541	61 ± 29	▼	**
Clinical TNM stage	III	32 ± 25	50 ± 36	▲	*	907 ± 730	450 ± 626	▼	*
	IV	20 ± 23	21 ± 15	=/▲	ns	749 ± 600	202 ± 186	▼	**
Metastases	M0	39 ± 27	50 ± 33	▲	ns	833 ± 666	436 ± 560	▼	**
	M1	17 ± 16	22 ± 16	▲	ns	570 ± 264	210 ± 185	▼	**
T stage	T1	4 ± 3	20 ± 14	▲	*	484 ± 275	83 ± 74	▼	*
	T2	13 ± 11	32 ± 41	▲	ns	807 ± 694	278 ± 320	▼	**
	T3	38 ± 24	50 ± 23	▲	ns	868 ± 808	662 ± 720	▼	ns
	T4	73 ± 14	45 ± 13	▼	*	1360 ± 355	130 ± 48	▼	*

*All concentrations are presented as $x \pm SD$. *p < 0.05, **p < 0.01

ence kits, San Diego, USA) on Beckman Coulter flow cytometer FC500 (Nyon, Switzerland).

Statistics

All statistical tests were performed with Graph-Pad Prism 5 software, using ANOVA test (with Bonferroni post-testing) for multiple group comparisons, Mann-Whitney U test for comparisons of differences between two independent groups, Wilcoxon test for comparison of paired (serum/tumor) samples and Pearson correlation test. A p value < 0.05 was considered statistically significant.

Results

IL10 and TGFb1 in control serum samples

The mean IL10 and TGFb1 concentrations in control samples were 10 ± 7 pg/ml and 3429 ± 644 pg/ml, respectively. Naturally, we didn't take lung microcirculation samples from healthy controls due to ethical reasons.

IL10 and TGFb1 values according to the lung cancer histological type

Large cell and adenocarcinoma patients had the highest mean serum IL10 concentrations, unlike squamous cell NSCLC patients, for which the mean value of IL10 was close to healthy controls. The mean serum IL10 concentration did not differ significantly between patients with different lung tumor histologies (Figure 1A). In contrast, the

highest mean IL10 concentration in the microcirculation of the tumor was detected in SCLC patients, which was significantly higher compared with all NSCLC types. Significant difference between serum and corresponding tumor IL10 values (within the individual lung carcinoma types) in favor of increased tumor IL10 value was noted only in SCLC patients (Table 2).

All serum and tumor samples contained significantly less TGFb1 compared to controls. Serum TGFb1 levels were insignificantly elevated in NSCLC patients compared with SCLC (Figure 2A). In tumor samples, patients with squamous NSCLC had the highest mean TGFb1 level, significantly increased compared with all other types. On the other hand, patients with large cell NSCLC morphology had significantly less TGFb1 than patients with adenocarcinoma and SCLC patients. Comparison of serum and tumor samples demonstrated insignificant TGFb1 increment only in NSCLC patients with squamous tumors and significant decrease in all other histological types (Table 2).

Increased IL10 and TGFb1 values in stage III patients

The mean IL10 concentration in patients with advanced lung tumor (stages III and IV) was significantly increased compared with the controls. Surprisingly, patients in stage III had increased mean IL10 levels, both in serum and tumor samples, compared with patients in stage IV (Figure 1B). This difference was significant in tumor samples. Also, comparison of serum with corresponding tumor samples demonstrated significant IL10 increment only in stage III patients (Table 2).

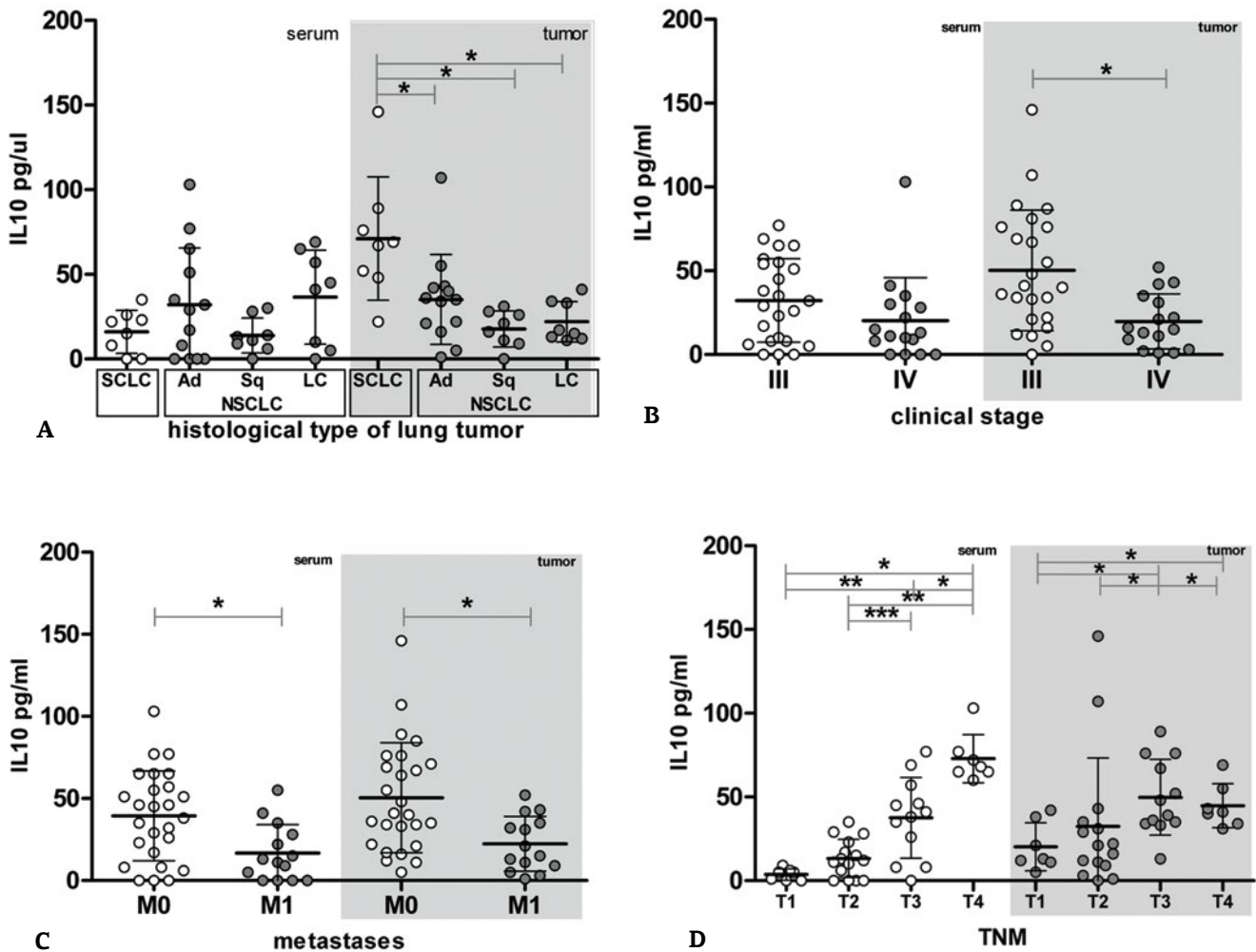


Figure 1. **A:** IL10 levels in serum and tumor samples of lung cancer patients with different histologies. **B:** IL10 concentrations in III and IV clinical stage patients. **C:** Patients without (M0) or with metastatic disease (M1). **D:** Patients with lung tumor according to TNM staging. Values are shown as $\bar{x} \pm SD$ pg/ml. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by Mann-Whitney U test.

Similarly as IL10, stage III patients had increased mean TGFb1 compared with stage IV patients, both in serum and tumor samples (Figure 2B). Comparing their serum values, the corresponding tumor TGFb1 concentration demonstrated significant decrease (Table 2).

Increased IL10 and TGFb1 are associated with absence of metastasis

Surprisingly, patients with proven metastasis (M1) had mean IL10 slightly above the mean values of healthy controls, while M0 patients had significantly increased IL10 (compared with M1 patients) both in serum and tumor samples (Figure 1C). Insignificant IL10 increase was demonstrated in tumor samples when compared with the corresponding serum samples, both in M0 and M1 patients (Table 2).

Patients without metastatic disease had in-

creased TGFb1 compared with M1 patients, both in serum and tumor samples (Figure 2C). Significant decrease of TGFb1 in tumor vs corresponding serum samples was demonstrated for both M0 and M1 patients (Table 2).

Patients with larger lung tumor have increased IL10 and TGFb1 concentrations

The mean serum IL10 values in patients with smaller tumors (T1,T2) corresponded to control values while those with larger lung tumors (T3,T4) showed significantly higher IL10 values (Figure 1D). Patients with T4 tumors had significantly increased IL10 compared with other T-staged patients (T1,T2,T3) in serum samples and T3 patients had significantly increased IL10 compared with T1 and T2 patients in tumor microcirculation. Analysis of serum and tumor microcirculation samples demonstrated increase

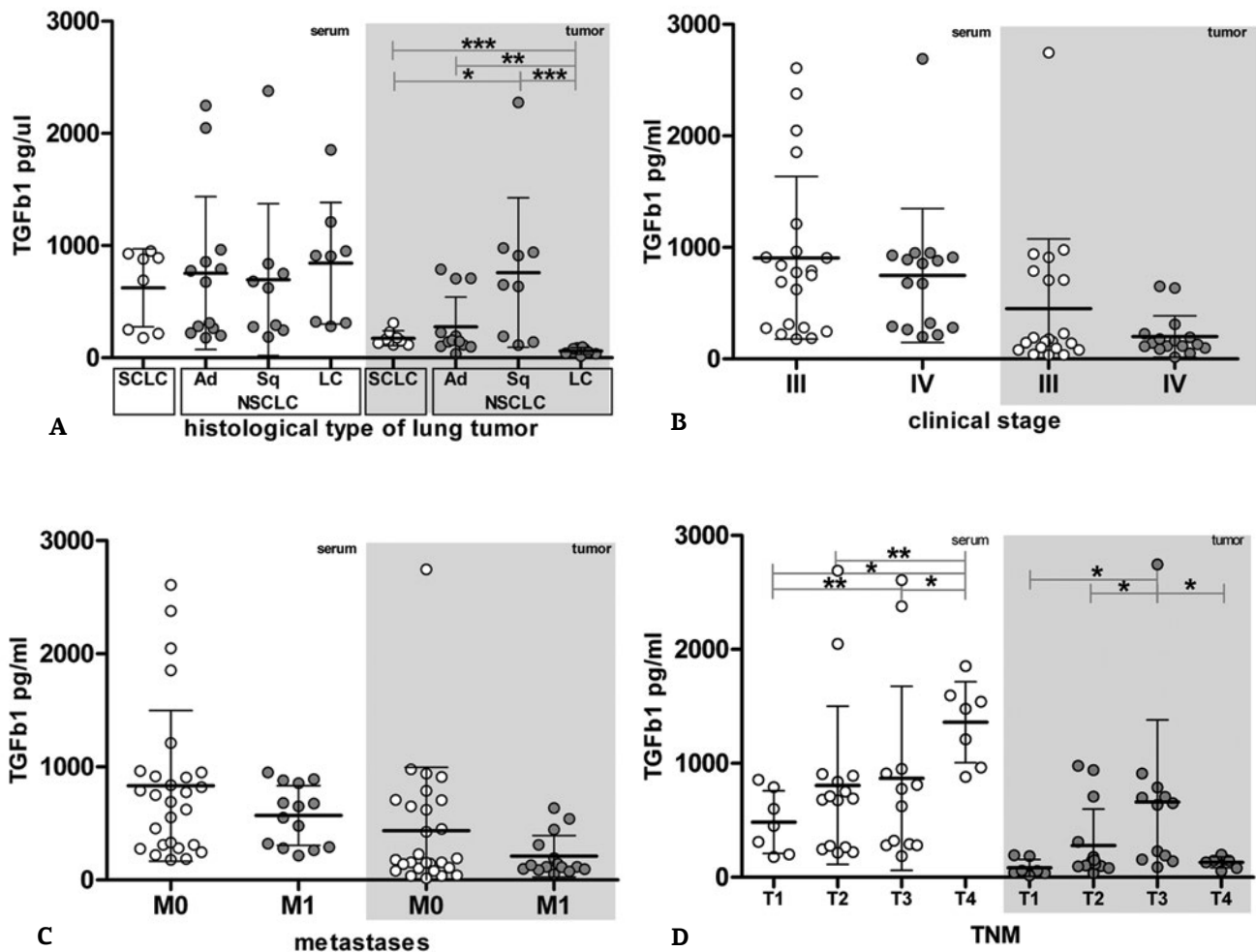


Figure 2. **A:** TGFb1 levels in serum and tumor samples of lung cancer patients with different histologies. **B:** TGFb1 levels in III and IV clinical stage patients. **C:** Patients without (M0) or with metastatic disease (M1). **D:** Patients with lung tumor according to TNM staging. Values are shown as $\bar{x} \pm SD$ pg/ml. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. by Mann-Whitney U test.

in T1, T2, T3 and decrease in T4 tumor samples. These changes were significant only in T1 and T4 patients (Table 2).

Like IL10, patients with larger tumors had increased mean TGFb1 values in serum compared with those with smaller tumors. TGFb1 was significantly increased in patients stage 4 (T4) compared with all others (T1, T2, T3) as well as in T3 patients compared with T1 (Figure 2D). In tumor samples, patients with stage 3 tumors had significantly increased TGFb1 compared with all others (T1, T2, T4). Comparison of serum/tumor samples demonstrated significant decrease of TGFb1 in stage T1, T2 and T4 patients (Table 2).

Correlation of IL10 and TGFb1 values

Significant correlation ($R = +0.375$, $p = 0.032$) was found between IL10 and TGFb1 values in tumor microcirculation, but not in serum samples

of lung cancer patients (Figure 3). Further analysis demonstrated significant correlation between IL10 and TGFb1 in tumor samples of stage III patients ($R = +0.468$, $p = 0.030$), T2 patients ($R = +0.700$, $p = 0.005$) and T4 patients ($R = +0.948$, $p = 0.017$; data not shown).

Discussion

Lung cancer is associated with surrounding inflammation, which promotes further tumor growth and disease progression by immune and non-immune mechanisms [15]. Events contributing to carcinogenesis refer to oxidatively damaged cell DNA (contributing directly to genetic instability) by abundant local reactive oxygen species (ROS) overproduction, vascular endothelial growth factor (VEGF) secretion enabling pathological microvasculature development and metalloproteinases (MMPs) secretion, which reorgan-

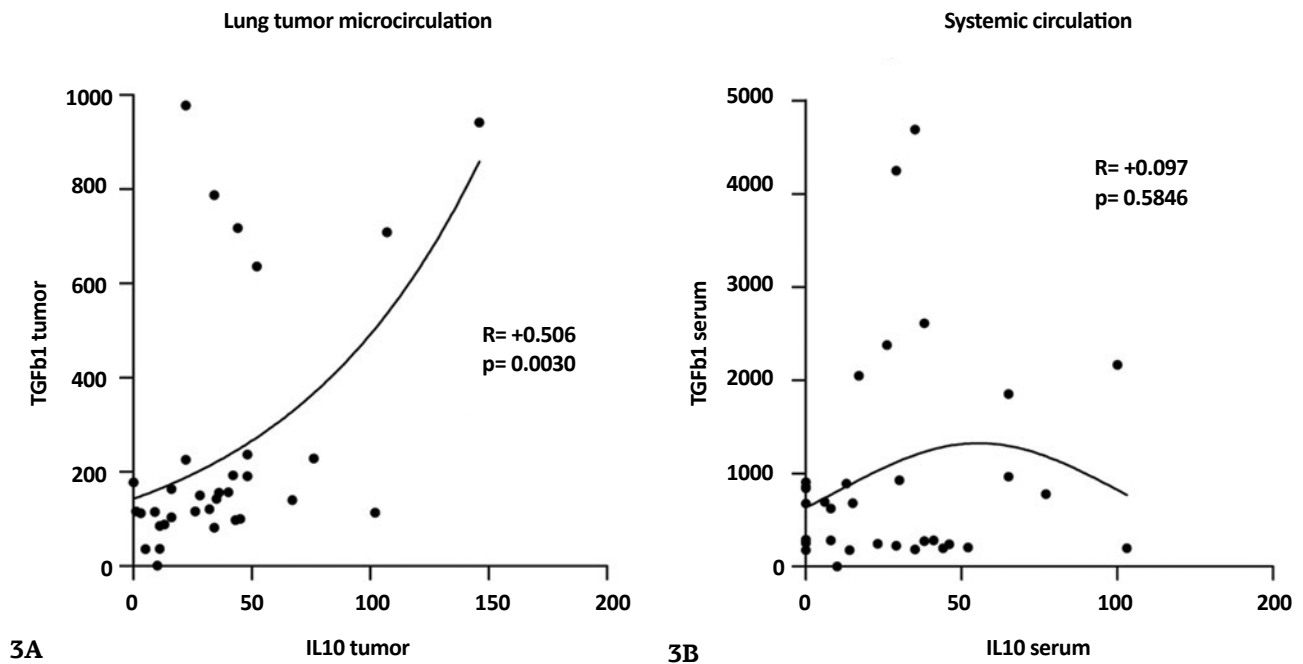


Figure 3. Correlation between IL10 and TGFb1 values in samples from tumor microcirculation (**3A**) and serum samples (**3B**) of patients with lung cancer. Values are shown as $x \pm SD$ pg/ml. $p < 0.05$ by Pearson's correlation test.

ize local tissue architecture enabling malignant cells to invade and make metastatic foci. Lung cancer causes significant defects in myelopoiesis and hemopoiesis, ultimately leading to cell mediated antitumor response dysfunction. Besides other mechanisms, principal immunosuppressive components, regulatory T lymphocytes (Tregs), myeloid derived suppressor cells (MDSC) and tumor associated macrophages (TAM) induce negative immune regulation through IL10 and TGFb1 production [8-12]. Tumor cells themselves produce mediators with immunosuppressive and inflammatory effects, including IL10, TGFb1 and enzyme indoleamine 2,3-dioxygenase (IDO) [13].

According to the histological characteristics of malignant cells, lung tumors are defined as SCLC and NSCLC. It is still unclear whether these various histological tumor types could induce different types of antitumor immune response. There is some evidence that SCLC and NSCLC differ in their capacity to secrete and/or induce IL10 secretion. Investigations of cytokine genes in lung cancer patients showed conflicting data. A study on IL10 gene polymorphisms in 117 patients with lung cancer (77 NSCLC, 40 SCLC) and 243 healthy controls showed significant correlation between the IL10-1082 G allele and the presence of SCLC [16]. Shih et al. found significant association between gene polymorphism for IL10 (1082, 819 and 592 alleles) in 154 NSCLC patients compared with healthy controls [17]. Analyzing IL10 genetic

polymorphism in tumor tissue samples of NSCLC patients, Wang et al. reported IL10 mRNA expression (which prevails in non-ATA haplotype samples) in 63% of the patients (241/385) [18]. They noticed that these patients had shorter overall and disease free survival. These types of studies did not consider the disease stages or any immune response parameter, so, although comprehensive, they reported very general conclusions.

Increased serum IL10 concentration in lung cancer patients is associated with important clinical features. De Vita et al. investigated IL10 level in serum samples of 60 NSCLC patients (stages III and IV) who underwent conventional platinum-based chemotherapy [19]. Lung cancer patients had significantly increased serum IL10 compared with healthy controls (18 ± 4 vs 9 ± 2 pg/mL). Among them, metastatic patients had significantly higher IL10 than those with limited disease (21 ± 4 vs 14 ± 1 pg/mL), while responders had significantly decreased IL10 compared with non-responders (15 ± 2 vs 21 ± 4 pg/mL). The authors concluded that IL10 value before treatment could be independent prognostic factor in NSCLC patients.

High IL10 values associated with lung cancer could originate from both, tumor cells and tumor stimulated lymphocytes. In an article published almost 20 years ago, Huang and Sharma showed that NSCLC cell lines induce IL10 overproduction in healthy peripheral blood lymphocytes [20]. This effect was partially based on tumor prosta-

glandin E (PGE) production, because treatment with indomethacin, a PGE inhibitor, reduced this phenomenon. Increased plasma PGE2 and IL10 [21] together with low interferon (IFN) γ production from peripheral blood lymphocytes of NSCLC patients is a result of deteriorated regulation of transcription factors T-bet and GATA-3, probably caused by PGE2 produced from lung cancer cells [22]. Furthermore, some SCLC tumor cell lines constitutively secrete molecules that suppress the proliferation of activated CD4 T cells from healthy donors *in vitro*, and induce their differentiation into regulatory T lymphocytes (CD4⁺, CD25⁺, FOXP3⁺, CD127^{low}, Helios⁺, Treg), which infiltrate tumor tissue and negatively correlate with patient survival [23]. Another immunosuppressive population, tumor associated macrophages (TAM) express high levels of IL10 and cathepsin B mRNA in NSCLC patients [12]. IL10 TAM expression was significantly associated with stage, tumor size and degree of lymphovascular invasion [12]. Expression of IL10 mRNA in TAMs was significantly associated with histological types of NSCLC, with lowest value in squamous and highest in adenocarcinoma.

Supposing that only certain lung cancer types produce significant IL10, Hatanaka et al. investigated both IL10 and IL10R mRNA expression, cellular localization (immunohistochemically assessed) and serum concentrations in samples of 82 patients with NSCLC lung cancer [24]. RT-PCR confirmed IL10 in 83% and IL10R expression in 96% of investigated NSCLC tumors. Serum IL10 increment was not always associated with mRNA expression in tumor, but those NSCLC patients with increased serum IL10 concentration had significantly poorer prognosis compared with others. The authors concluded that IL10 expression in NSCLC patients is associated with significantly poorer prognosis of survival.

Most clinical data came from studies with advanced disease. Very few studies on stage I NSCLC patients with completely resectable tumors have shown that squamous type had the most frequent IL10 expression (immunohistochemically determined) compared with the other histological types. Soria et al. in their study reported that lack of IL10 expression in tumor tissue was associated with a significantly worse outcome [25].

To the best of our knowledge this is the first study in which tumor microcirculation samples of lung cancer are used for investigation. We do consider this kind of sample is more relevant

than serum because it reflects local microenvironment processes. Herein, we showed that IL10 values from tumor microcirculation and serum samples differ according to histological types and TNM stages. Surprisingly, stage III M0 patients had higher average IL10 level compared with patients with more advanced disease. Interestingly, IL10 from SCLC microcirculation showed the highest IL10 average value. Based on these results, we confirmed different capacities and ability of the investigated lung cancer histological types and the disease stages to induce other host immune components to intensify IL10 production.

Results from several studies are consistent in finding elevated serum of TGFb1 levels in lung cancer patients compared with healthy controls or other nonmalignant pulmonary diseases [26-29]. Different types of tumor cells, including SCLC as well as NSCLC cells, overexpress TGFb [30,31]. SCLC and NSCLC cell lines *in vitro* exposed to TGFb1 acquire different pro-tumorigenic features such as stemness maintenance, tumorigenicity, invasion and migration [32]. Increased TGFb1 expression and increased serum levels are associated with progression of lung cancer in patients with NSCLC [28,33,34].

All of our patients with lung cancer had decreased serum TGFb1 compared with the control samples. The highest TGFb1 in tumor microcirculation was detected in squamous cell NSCLC patients, which was significantly elevated compared with the others. Serum TGFb1 values correlated with tumor size, being highest in the T4 patient group, as documented by others [28,33]. Surprisingly, patients with more advanced disease, stage IV or with M1, had lower TGFb1 (compared with stage III and M0) both in serum and tumor microcirculation. It could be assumed that immunosuppressive cell interactions and mediator release are more important in early disease stages, creating essential conditions for disease spreading.

Analysis of both cytokines in the samples of serum and tumor microcirculation demonstrated significant correlation between IL10 and TGFb1 values in tumor microcirculation, but not in serum samples. Jarnicki et al. [35] reported on tumor infiltration with CD4⁺ T regulatory cells (which produce IL10 and TGFb1), exploiting various pathways during lung cancer growth (experimental study). They demonstrated that significant production of IL10 and TGFb1 by cancer cells induced infiltration of growing tumor by CD4⁺ and

CD8⁺ Treg cells. However, depletion of CD8⁺ T regulatory cells reduced tumor mass, stressing their ability to inhibit antitumor response.

Conclusion

The roles of IL10 and TGFb1 in lung cancer are still far from being fully elucidated. IL10 could be protective in early stages, and tumor promoting in later stages of lung cancer. Lung cancer cells of particular histological types are associated with different profiles of these cytokines in systemic circulation vs tumor microenvironment, which could be considered for antitumor response monitoring. Substantial contribution in understanding IL10 and TGFb1 roles maybe achieved by investigating mediators and phenotype of immune cells in tumor microcirculation in all lung cancer stages.

Authors' contributions

Karlicic Vukoica, Vukovic Jelena, Sotirovic Je-

lena, Peric Aleksandar and Vlado Cvijanovic performed all diagnostic and therapeutic procedures of lung tumor patients. Jovic Milena performed histological analysis of tumor samples. Djukic Mirjana and Saso Luciano performed mediator testing and wrote the paper. Stanojevic Ivan, Banovic Tatjana and Vojvodic Danilo designed the study, performed cytokine testing and wrote the paper.

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Conflict of interests

The authors declare no conflict of interests.

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