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High number of CD14$^+$B7H4$^+$ monocytes is significantly associated with increased concentrations of IL-4, IL-13, IL-10, and TGF-β_1 in tumor microcirculation of lung carcinoma

Visoke vrednosti CD14$^+$B7H4$^+$ populacije monocita su značajno povezane sa povišenim koncentracijama IL-4, IL-13, IL-10 i TGF-β_1 u tumorskoj mikrocirkulaciji karcinoma pluća

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Abstract

Background/Aim. Lung cancer (LC) is one of the leading causes of mortality. Disease progression and advanced disease are characterized by the unprotective immune response due to M2 macrophage polarization, myeloid-derived suppressor cells (MDSC) activity, cytokine imbalance, and regulatory T lymphocyte activity. The aim of this study was to investigate the association between Th_1/Th_2 cytokines and CD14$^+$B7H4$^+$ monocyte (Mo) number in LC patients. **Methods.** We investigated principal Th_1/Th_2 cytokines and CD14$^+$B7H4$^+$ Mo number in blood and tumor microcirculation samples of 41 LC patients (III and IV clinical stage) and 30 healthy participants (control group). **Results.** The serum concentrations of investigated cytokines in all patients vs. heathy controls did not differ significantly. Stratification in groups according to tumor histology, disease extent, and tumor size revealed significant differences. LC patients with different histology types demonstrated significant differences, both in serum and tumor microcirculation samples. The presence of metastasis was associated with increased IFN-γ/IL-4 in blood and increased IL-13 in tumor microcirculation samples. Tumor microcirculation samples of the largest tumors were characterized by the Th_2 cytokine profile. Investigation of CD14$^+$B7H4$^+$ Mo in blood samples demonstrated a significant association of extreme value of this cell population with elevated IL-2/IL-13. Patients with the highest CD14$^+$B7H4$^+$ Mo number in tumor microcirculation samples demonstrated significant increments of IL-4, IL-13, IL-10, and TGF-β_1. **Conclusion.** LC patients demonstrated polarization of cytokine response associated with microenvironment origin, tumor histology type, tumor size, and disease extent. The highest number of CD14$^+$ B7H4$^+$ monocytes is significantly associated with the Th_2 cytokine profile.

Key words:
antigens, cd; blood; cytokines; histological techniques; lung neoplasms; monocytes.

Apstrakt

Uvod/Cilj. Karcinom pluća (KP) je jedan od vodećih uzroka smrtnosti. Napredovanje bolesti i uznapredovala bolest karakterišu se neprotektivnim imunološkim odgovorom zbog polarizacije M2 makrofaga, aktivnosti supresorskih ćelija mijeloidnog porekla (MDSC), neravnoteže citokina i regulatorne aktivnosti T limfocita. Cilj rada bio je da se utvrdi povezanost između Th_1/Th_2 citokina i CD14$^+$B7H4$^+$ broja monocita (Mo) kod bolesnika sa KP. **Metode.** Ispitali smo glavne Th_1/Th_2 citokine i broj CD14$^+$B7H4$^+$ Mo u uzorcima iz krvi i mikrocirkulacije tumora 41 bolesnika sa KP (III i IV klinički stadijum) i 30 zdravih ispitanika (kontrolna grupa). **Rezultati.** Koncentracija ispitivanih citokina u serumu bolesnika u odnosu na zdrave ispitanike nije se značajno razlikovala. Stratifikacija u histološkim grupama tumora, obimu bolesti i veličini tumora otkrila je značajne razlike. Kod bolesnika sa KP različitih histoloških tipova utvrđene su značajne razlike, kako u uzorcima seruma tako i u mikrocirkulaciji tumora. Prisustvo metastaza bilo je povezano sa povećanjem IFN-γ/IL-4 u krvi i povećanjem IL-13 u uzorcima mikrocirkulacije tumora. Uzorci

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mikrocirkulacije najvećih tumora okarakterisani su Th_2 citokinskim profilom. Ispitivanje $CD14^+B7H4^+$ Mo u uzorcima krvi pokazalo je značajnu povezanost ekstremne vrednosti te ćelijske populacije sa povišenim udelom IL-2/IL-13. Bolesnici sa najvećim brojem $CD14^+B7H4^+$ Mo u uzorcima mikrocirkulacije tumora pokazali su značajan porast IL-4, IL-13, IL-10 i TGF-β_1. **Zaključak.** Bolesnici sa KP pokazuju polarizaciju citokinskog odgovora povezanu sa vrstom mikrookoline, histološkim tipom tumora, veličinom tumora i proširenošću bolesti. Najveće vrednosti broja $CD14^+B7H4^+$ monocita značajno su povezane sa Th_2 citokinskim profilom.

Ključne reči:
antigeni, cd; krv; citokini; histološke tehnike; pluća, neoplazme; monociti.

Introduction

Lung cancer (LC) is one of the leading causes of mortality. Advanced disease is associated with a minimal survival rate and lack of therapeutic possibilities [1]. Two histologically distinct entities, small cell LC (SC-LC) and group of non-small cell LC (NSC-LC), differ significantly in biological behavior, clinical manifestations, therapeutic response, and outcome [2].

Although immune cells establish a dynamic relationship with LC cells from the initial lesion, various mechanisms induce an inadequate anti-tumor response, resulting in the disease progression. M2 macrophage polarization [3], myeloid-derived suppressor cells (MDSC) activity [4], cytokine imbalance [5, 6], and regulatory T lymphocyte activity [7] are recognized as important factors that contribute to immunosuppression essential for LC progression. More than 20 years ago, Asselin-Paturel et al. [8] demonstrated that immune response in NSC-LC patients (both intratumor and systemic) corresponds to the so-called Th2 cytokine profile. They found that both tumor cells and tumor-infiltrating leukocytes produced cytokines and that advanced disease is associated with high interleukin (IL)-6, IL-10, transforming growth factor (TGF)-β_1, and absent/low IL-2, IL-4, interferon (IFN)-γ, granulocyte macrophage-colony stimulating factor (GM-CSF) (mRNA presence). From that point, further studies demonstrated that there is a polarization of cytokine response towards Th_2 type, at least in the population of patients with NSC-LC tumor type. Investigation of serum samples of LC patients demonstrated that IL-4/IL-10 are significantly increased, while IL-2/IFN-γ are significantly decreased in NSC-LC patients [9–12], surgical tumor reduction resulted in Th_2 decrease/Th increase [11] and that peripheral blood mononuclear cells of LC progressors contained a high volume of IL-4 mRNA [12]. Several studies confirmed these findings in the tumor tissue of LC patients, demonstrating a very high concentration of IL-4/IL-10 in pleural effusion samples [13], a significant predominance of IL-4/IL-10 over IL-2/IL-12/IFN-γ mRNA in tumor samples of LC patients [14, 15], and absent/low mRNA content for IFN-γ mRNA in tumor-infiltrating lymphocytes (TIL) extracted for LC tumor tissue [16]. Similar to previous studies, patients with SC-LC type also demonstrate Th_2 type predomination, represented with the finding of significantly increased IL-6 is in their sera [17]. This finding is particularly emphasized in the group of SC-LC patients with fast disease progression and in those that with unsuccessful response to therapy. Both experimental and clinical data implicate that various populations of myeloid cells found in LC are significantly associated with the disease progression and outcome [18] and connect their immunosuppressive capacity with the production of their specific mediators [19–21]. We have demonstrated that the number of $CD14^+B7H4^+$ monocytes (Mo) is significantly increased in blood and tumor microcirculation of LC patients and was correlated to tumor size and number of involved lymph nodes [22].

The aim of this study was to investigate the association between the Th_1/Th_2 cytokine profile and the number of $CD14^+B7H4^+$ Mo in blood and tumor microcirculation samples of LC patients.

Methods

Patients

We investigated 41 patients diagnosed for LC (31 males, 10 females, aged 62 ± 8 years) and 30 healthy controls (22 males, 8 females, aged 57 ± 14 years) (Table 1). Patients were diagnosed and treated at the Clinic for Lung Diseases, Military Medical Academy, Belgrade, Serbia for 18 months. All necessary diagnostic procedures (clinical, bronchoscopy, laboratory, histological, and radiological) were performed at the Military Medical Academy, Belgrade,

Table 1
Demographic and clinical characteristics of the study participants

Characteristics	Patients	Healthy persons
Sex, n		
male	31	22
female	10	8
Age (years), mean ± SD	62 ± 8	57 ± 14
Histological type of tumor, n		
NSC-LC (adenocarcinoma)	13	
NSC-LC (squamous cell carcinoma)	9	
NSC-LC (squamous cell carcinoma)	10	
SC-LC	9	
Clinical TNM stage, n		
III	24	
IV	17	
Metastases, n		
M0	27	
M1	14	
Tumor size, n		
T1	7	
T2	15	
T3	12	
T4	7	

SD – standard deviation; NSC-LC – non-small cell lung cancer; SC-LC – small lung cancer; TNM – tumor, node, metastasis.

Serbia. All patients and healthy controls gave their consent, and this study was approved by the local Ethics Committee, Military Medical Academy (11-03/2014, 12-02/2015).

All investigated cytokines were analyzed in groups of patients according to tumor histology type (SC-LC, adenocarcinoma (Ad) NSC-LC, squamous (Sq) NSC-LC, NSC-LC), tumor size, presence of metastasis and $CD14^+B7H4^+$ Mo number in blood, and tumor microcirculation samples. According to the $CD14^+B7H4^+$ Mo value, blood samples were qualified as basal level (less than 1% $CD14^+B7H4^+$), low (1–5%), intermediate (5–10%), and extreme value of $CD14^+B7H4^+$ (more than 10%). Tumor microcirculation samples were qualified as basal (less than 2% $CD14^+B7H4^+$), low (2–20%), intermediate (20–40%), and extreme value of $CD14^+B7H4^+$ (more than 40%).

Samples

Blood samples were taken from the cubital vein, while tumor microcirculation samples were taken from accessible pathological blood vessels with needle aspiration during diagnostic bronchoscopy. Both types of blood samples were taken with BD Vacutainer® Plus Plastic Serum Tubes. Serum was separated from the samples after centrifugation (3,000 g, 10 min, RT) and frozen at -70 ºC until testing. IL-2, IL-12, IFN-γ, IL-4, IL-5, IL-6, IL-10, and IL-13 were quantified with commercial flow cytometric tests (eBioscience kits) on Beckman Coulter flow cytometer FC500.

Both blood and tumor microcirculation samples were taken in the same way as described, but with the BD Vacutainer® spray-coated K2EDTA Tubes. The procedure for cell staining and immunophenotype analysis strategy was basically the same as previously referenced [22]. After removing erythrocytes with lysing buffer (NH_4Cl, EDTA, $KHCO_3$, 10 min with constant mixing) and further washing of nucleated cells (two times centrifuged with RPMI 1,640 culture medium complemented with 5% of normal human serum), cells were resuspended, enumerated (Beckman Coulter ACT differ blood counter) and concentration corrected to final suspension of 1×10^6 cells/100 μL per test tube. Final cell suspensions were stained with a cocktail of monoclonal antibodies. Multicolor analysis was performed with different combinations of CD15-FITC or PECy7, CD33-PE or PECy7, CD45-ECD or PECy5, HLA-DR PE/Cy5, CD14-FITC, CD16-PE, CD11b-PE, CD10-PECy7, CD3-FITC, CD19-FITC, CD56-FITC, B7H4-PECy7 (Biolegend, USA). The flow cytometry was performed using a Beckman Coulter FC 500 flow cytometer with CXP analysis software. Wanted subpopulations were identified from the initial $CD45^+$ /Side Scatter cell population, which was negative for T, B, and NK antigens. This triple-negative population of every sample was further gated on a CD11b versus HLA-DR dot plot histogram, and Mo was analyzed as lineage triple-negative ($CD3^-$, $CD19^-$, $CD56^-$), $CD45^+$, HLA-$DR^{-/low}$, $CD11b^+$ population. After further classification of this population according to the expression of CD15 or CD14, the $CD14^+$ Mo population was further investigated for B7H4 expression. Value of $CD14^+B7H4^+$ Mo cell population was expressed as % of all $CD45^+$ analyzed cells.

Statistical analysis

Data analysis was performed using the GraphPad Prism 5 software. Patients were stratified into groups according to lung tumor histology type, disease extent, and tumor node metastasis (TNM) classification. The difference in cytokine values was analyzed in two directions, either between groups (Mann-Whitney test) or as a ratio of tumor/serum sample of a particular patient (Wilcoxon matched pairs test). Comparison among multiple groups was done with nonparametric Kruskal-Wallis test, while identification of differences was performed with Dunn's multiple comparison test.

Results

The concentration of investigated cytokines did not differ significantly between LC patients and healthy controls (Table 2). Due to ethical reasons, we did not have adequate samples from healthy controls to compare them to LC tumor microcirculation samples. All investigated cytokines values demonstrated a higher tumor/ serum ratio.

Table 2

Comparison of serum cytokine concentrations of lung cancer (LC) patients and healthy persons (controls)

Cytokine	LC patients	Controls	*p*
IL-2			
serum	29 ± 29	19 ± 28	ns
tumor	32 ± 20	nd	nd
IL-12			
serum	9 ± 7	11 ± 29	ns
tumor	35 ± 28	nd	nd
IFN-γ			
serum	42 ± 41	57 ± 45	ns
tumor	58 ± 36	nd	nd
IL-4			
serum	24 ± 21	30 ± 21	ns
tumor	25 ± 14	nd	nd
IL-5			
serum	12 ± 15	9 ± 19	ns
tumor	39 ± 20	nd	nd
IL-6			
serum	45 ± 50	39 ± 38	ns
tumor	101 ± 91	nd	nd
IL-10			
serum	25 ± 21	19 ± 29	ns
tumor	37 ± 22	nd	nd
IL-13			
serum	53 ± 45	45 ± 38	ns
tumor	118 ± 53	nd	nd
IL-17	16 ± 13	9 ± 15	ns
serum	19 ± 11	nd	nd
tumor			
TGF-β1			
serum	729 ± 561	1010 ± 865	ns
tumor	320 ± 257	nd	nd

All values are expressed as mean ± standard deviation in pg/mL.
IL – interleukin; IFN – interferon; TGF– transforming growth factor.
ns – not significant; nd – not determined (Mann-Whitney test).

Serum cytokine concentrations between groups with particular lung tumor histology varied significantly. Patients with SC-LC type demonstrated highly increased levels of IL-2, IL-12, IL-6, and IL-13 (Tables 3 and 4). Patients from the Ad NSC-LC group had a profile of elevated IL-2, IFN-γ, IL-4, IL-10, IL-13, IL-17, and the lowest level of IL-6, compared to others. Patients with Sq NSC-LC demonstrated the highest IL-6 level of all but together with the significantly decreased value of all investigated cytokines. Contrary to others, patients with large cell (Lc) tumor type demonstrated the highest values of IL-2, IFN-γ, IL-10, and TGF-β_1 in their serum samples.

Analysis of tumor microcirculation samples demonstrated a completely different cytokine profile compared to that of serum samples. Patients with SC-LC tumor type had significantly increased IL-2, IL-12, IFN-γ, IL-6, IL-10, and IL-13. NSC-LC patients from the Ad group demonstrated increased IL-4, IL-6, and IL-17, Sq NSC-LC group had significantly increased IL-5 and TGF-β_1 value, while patients with Lc NSC-LC type demonstrated only increment of IL-5.

Patients with detectable metastasis (M1) had significantly increased IFN-γ and IL-4 compared to the M0 group. Patients with metastasis (M1) in tumor samples demonstrated a significant increase of IL-13.

Patients with the largest tumor size had significantly increased serum values of IL-2, IL-12, IFN-γ, IL-4, IL-5, IL-10, IL-13, and TGF-β_1. Interestingly, the T4 group demonstrated the lowest IL-6 and modest IL-17 level.

From all analyzed parameters, tumor size was clearly associated with a particular cytokine profile in tumor microcirculation. Smaller lung tumors were significantly associated with increased IL-2, IFN-γ, IL12 (T1), and IL-6 (T2), while larger sized tumors were associated with Th2 profile, namely, with increased IL-4, IL-10 (T3), IL-5, IL-13 (T4), and TGF-β_1 (T3, T4).

Stratification of patients with LC in groups according to the number of $CD14^+B7H4^+$ Mo in blood samples demonstrated specific cytokine profiles (Tables 5 and 6). Patients with the basal/low number of the $CD14^+B7H4^+$ Mo had significantly higher levels of IFN-γ and IL-12, together with IL-4, IL-5, IL-10, and TGF-β_1 compared to intermediate/extreme groups. On the other side, patients with intermediate/extreme high numbers of the $CD14^+B7H4^+$ Mo demonstrated a significant increase of IL-2 and IL-13, together with insignificantly elevated IL-6/IL-13 compared to basal/low groups.

Table 3

Average value of investigated cytokines according to tumor histology type, presence of metastases and tumor, node, metastasis (TNM) classification

Cytokine	SC-LC	NSC-LC			Metastases		TNM stage			
		Ad	Sq	Lc	M0	M1	1	2	3	4
IL-2										
serum	26 ± 16	36 ± 43	6 ± 9	47 ± 46	37 ± 36	45 ± 55	31 ± 21	18 ± 22	58 ± 58	61 ± 16
tumor	46 ± 20*	32 ± 20	19 ± 19	31 ± 19	34 ± 22	34 ± 20	31 ± 26	33 ± 21	42 ± 5	43 ± 10*
IL-12										
serum	13 ± 10	9 ± 8	4 ± 2	11 ± 7	11 ± 10	6 ± 9	1 ± 1	11 ± 10	6 ± 5	28 ± 5
tumor	92 ± 77*	24 ± 23*	15 ± 7**	8 ± 6	30 ± 28*	31 ± 28*	56 ± 15*	19 ±22**	63 ± 43*	15 ± 5
IFN-γ										
serum	31 ± 20	55 ± 55	23 ± 45	59 ± 47	26 ± 23	192 ± 212	22 ± 20	23 ± 19	48 ± 33	47 ± 7
tumor	87 ± 64*	72 ± 58	43 ± 14	30 ± 9	123 ± 110*	55 ±28	77 ± 37*	74 ± 51***	65 ± 41	41 ± 44
IL-4										
serum	24 ± 19	46 ± 45	11 ± 8	15 ± 14	19 ± 14	90 ± 75	15 ± 12	40 ± 51	11 ± 11	55 ± 16
tumor	9 ± 11	40 ± 13	27 ± 20	23 ± 14	8 ± 17	38 ± 20	2 ± 15	33 ± 21	49 ± 12**	34 ±8*
IL-5										
serum	18 ± 20	14 ± 19	1 ± 1	14 ± 19	20 ± 11	35 ± 35	2 ± 1	15 ± 24	5 ± 12	49 ±5
tumor	20 ± 20	31 ± 23	51 ± 13*	55 ± 26**	29 ± 24*	34 ± 25	43 ± 8*	23 ± 23	48 ± 13**	40 ± 31
IL-6										
serum	78 ± 98	4 ± 4	88 ± 92	5 ± 4	26 ± 38	60 ± 90	2 ± 3	74 ± 112	41 ± 86	8 ± 3
tumor	146 ± 125	158 ± 155**	93 ± 80	7 ± 4	124 ±134**	87 ± 94	21 ± 13*	123 ± 110	118 ± 100**	70 ± 56
IL-10										
serum	16 ± 13	32 ± 33	14 ± 10	37 ± 28	39 ± 27	17 ± 16	4± 3	13 ± 11	38 ± 24	73 ± 14
tumor	71 ± 36*	35 ± 27	18 ± 11	22 ± 12	50 ± 33	22 ± 16	20 ± 14*	32 ± 41	50 ± 23	45 ± 13*
IL-13										
serum	72 ± 39	75 ± 67	40 ± 37	25 ± 35	61 ± 60	48 ± 35	2 ± 2	45 ± 50	49 ± 37	180 ± 83
tumor	173 ± 44**	118 ± 47	90 ± 70	91 ± 50**	97 ± 57*	159 ± 48***	127 ± 61*	111 ± 73**	163 ± 39***	125 ± 35
IL-17										
serum	13 ± 8	27 ± 26	11 ± 9	13 ± 8	20 ± 26	29 ± 29	3 ± 2	34 ± 49	44 ± 59	30 ± 11
tumor	22 ± 13	34 ± 21	8 ± 6	10 ± 5	24 ± 21	24 ± 17	24 ± 7*	22 ± 25	26 ± 11	19 ± 5
TGF-β_1										
serum	624 ± 347	754 ± 680	696 ± 676	843 ± 541	833 ± 666	570 ± 264	484 ± 275	807 ± 694	868 ± 808	1,360 ± 355
tumor	174 ± 66**	275 ± 266**	760 ±667	61 ± 29**	436 ± 560**	210 ± 185**	83 ± 74*	278 ± 320**	662 ± 720	130 ± 48*

All values are expressed as mean ± standard deviation in pg/mL.
***p* < 0.05, ***p* < 0.01, *****p* < 0.001 (Wilcoxon test).**
SC-LC – small cell lung cancer; NSC-LC – non-small cell lung cancer; TNM – tumor, node, metastasis, IL – interleukin; TGF– transforming growth factor; Ad – adenocarcinoma; Sq – squamous cell carcinoma; Lc – large cell carcinoma.

Table 4

Comparison of cytokine data between groups of patients according to lung cancer histological type, presence of metastasis (M), and tumor size (T)

Sample	Group	/Group	IL-2	IL-12	IFN-γ	IL-4	IL-5	IL-6	IL-10	IL-13	IL-17	TGF-β_1
Serum	SC	/ Ad						*				
	SC	/ Sq	**	*								
	SC	/ Lc								*		
	Ad	/ Sq			*			**			*	
	Ad	/ Lc										
	Sq	/ Lc	*				*	*				
Tumor microcirculation	SC	/ Ad		*	*				*	*		
	SC	/ Sq		*	*	*	*		*	*		
	SC	/ Lc		*	**	*	*	***		**		
	Ad	/ Sq							*			
	Ad	/ Lc					*					
	Sq	/ Lc						***				
	M0	/ M1			*	**						
	M0	/ M1								**		
Serum	T1	/ T2										
	T1	/ T3							*			
	T1	/ T4		*		*	*		*	*		*
	T2	/ T3										
	T2	/ T4	*	**					**	*		*
	T3	/ T4		**		**	**		*	*	*	
Tumor microcirculation	T1	/ T2		*								
	T1	/ T3		*								
	T1	/ T4										
	T2	/ T3		*			*			*		
	T2	/ T4										
	T3	/ T4										

***p* < 0.05; ****p* < 0.01; *****p* < 0.001 (Mann-Whitney test).**

SC – small cell lung cancer; Ad – adenocarcinoma non-small cell cancer; Sq – squamous cell non small cell lung cancer; Lc – large cell non-small cell lung cancer; IL – interleukin;TGF – transforming growth factor.

Table 5

Concentrations of investigated cytokines in serum and lung tumor microcirculation samples according to the number of $CD14^+B7H4^+$ monocyte (Mo) in peripheral blood

Cytokine	Number of $CD14^+B7H4^+$ Mo in peripheral blood				Number of $CD14^+B7H4^+$ Mo in tumor microcirculation			
	basal	low	intermediate	extreme	basal	low	intermediate	extreme
IFN-γ	50 ± 57	71 ± 57	30 ± 79	28 ± 24	40 ± 12	99 ± 71	56 ± 39	41 ± 15
IL-2	29 ± 29	22 ± 25	26 ± 25	52 ± 72	19 ± 18	40 ± 7	27 ± 19	26 ± 22
IL-12	19 ± 8	5 ± 10	10 ± 9	3 ± 3	10 ± 7	15 ± 15	27 ± 33	66 ± 58
IL-4	29 ± 23	22 ± 17	22 ± 21	7 ± 8	21 ± 16	105 ± 59	31 ± 20	142 ± 76
IL-5	34 ± 31	50 ± 60	13 ± 22	0 ± 0	7 ± 13	38 ± 28	16 ± 13	28 ± 26
IL-6	11 ± 9	8 ± 8	17 ± 30	21 ± 26	126 ± 121	36 ± 35	134 ± 210	71 ± 65
IL-10	35 ± 36	26 ± 19	26 ± 19	30 ± 36	17 ± 18	36 ± 19	35 ± 22	42 ± 37
IL-13	38 ± 45	70 ± 52	114 ± 57	47 ± 60	94 ± 63	105 ± 59	80 ± 63	142 ± 76
IL-17	13 ± 11	17 ± 10	13 ± 10	21 ± 23	21 ± 23	18 ± 9	13 ± 9	20 ± 16
TGF-β_1	902 ± 608	1,325 ± 1,411	782 ± 564	446 ± 320	151 ± 43	122 ± 46	315 ± 343	363 ± 303

All values are expressed as mean ± standard deviation in pg/mL.

IFN – interferon; IL – interleukin;TGF – transforming growth factor.

The number of $CD14^+B7H4^+$ Mo that we detected in tumor microcirculation was significantly higher in all samples compared to blood values (Tables 5 and 6). Patients with basal/low number of the $CD14^+B7H4^+$ Mo demonstrated significant increment of IFN-γ, IL-2, and IL-6. Patients with extreme numbers of $CD14^+B7H4^+$ Mo in tumor microcirculation samples had significantly elevated levels of IL-12, IL-4, IL-10, IL-13, TGF-β_1 and TNF-α (Table 7).

Table 6

Significant differences in concentrations of investigated cytokines in serum and tumor microcirculation between groups with different $CD14^+B7H4^+$ monocyte (Mo) number

$CD14^+B7H4^+$ Mo number	Serum		Tumor microcirculation	
	cytokine	significance	cytokine	significance
Basal/low	IL-12	*	IFN-γ	*
Basal/low	IL-4	*	IL-2	*
Basal/low			IL-5	**
Basal/intermediate	IL-12	*	IL-10	*
Basal/intermediate	IL-4	*		
Basal/intermediate	IL-13	**		
Low/intermediate	IFN-γ	*		
Basal/extreme	IL-12	*	IL-12	*
Basal/extreme	IL-5	*	IL-10	*
Basal/extreme	TGF-β_1	*	TGF-β_1	*
Low / extreme	IFN-γ	*	IFN-γ	*
Low / extreme	IL-5	*	IL-5	*
Low / extreme	TGF-β_1	*		
Intermediate/ extreme	IL-12	*		
Intermediate/ extreme	IL-4	**	IL-4	*
Intermediate/ extreme	IL-13	*	IL-13	*

IFN – interferon; IL – interleukin;TGF – transforming growth factor.
***p* < 0.05; ****p* < 0.01 (Mann-Whitney test).**

Table 7

Cytokines with the highest average concentration according to the number of $CD14^+B7H4^+$ monocytes (Mo) in peripheral blood and tumor microcirculation samples

$CD14^+B7H4^+$ Mo number	Serum	Tumor microcirculation
Basal	IL-12, IL-4, IL-10, IL-1β, TNF-α	IL-9, IL-1β
Low	IFN- γ, IL-5, IL-17, IL-9	IFN- γ, IL-2, IL-5, IL-27
Intermediate	IL-6, IL-13,	IL-6, IL-22
Extreme	IL-2, IL-22, IL-27	IL-12, IL-4, IL-13, IL-10, TGF-β_1, TNF-α

IFN – interferon; IL – interleukin;TGF – transforming growth factor; TNF – tumor necrosis factor.

Discussion

Data from numerous experimental and clinical studies indicate that the cytokine network is an important factor that shapes the anti-tumor response. Although immune response – tumor is an extremely complex and dynamic relation that depends on individual tumor characteristics, disease stage, and immune response characteristics, the prevailing attitude is that Th_1 is associated with protective anti-tumor response while Th_2 characterize pro-tumor microenvironment. Numerous investigations stated that there is a domination of Th_2 over Th_1 profile in samples of LC patients. The presence of high IL-6, IL-10, GM-CSF, and IFN-γ with low IL-2 mRNA level [8], domination of IL-4/IL-10 over IL-2/ IL-12/IFN-γ in lung tumor biopsy and pleural effusion of NSC-LC patients [9, 13, 14], the prevalence of Th_2 in the peripheral blood [10] and depressed cytotoxicity paralleled with decreased IFN-γ production in NK/NKT cells [16] was demonstrated in tumor tissue from LC patients (NSC-LC). It was also shown that successful therapy reduced Th_2 and increased Th_1 profile [10, 11]. The first indication of the biological significance of Th_2 profile domination in lung cancer pathophysiology came from Zhang et al. [15]. They demonstrated that more than 75% of macrophages corresponded to M2 type, which was significantly associated with high IL4/IL10 and low IFN-γ/IL-12 found in the tumor tissue of NSC-LC patients but not benign lesion samples. Two studies from Ito et al. [23, 24] demonstrated the complexity and dynamic change of Th_1/Th_2 profile related to the different microenvironment and disease progression. Twenty years ago, they demonstrated that tumor-infiltrating lymphocytes of NSC-LC patients are dominantly Th_1, while T lymphocytes in peripheral blood of the same patients corresponded to Th_2 population [23]. They expanded their investigation at the cytotoxic population of T lymphocytes (Tc), and after 5 years of follow-up, they demonstrated a predomination of Th_2/Th_1 associated with Tc_2/Tc_1 [24]. Surprisingly, this high Th_2/Th_1 and Tc_2/Tc_1 ratio found in peripheral blood was significantly associated with better prognosis in NSC-LC patients with advanced disease (clinical stage II and III), but not in those with early disease (stage I). These studies showed a significant difference between lung tumor and peripheral blood in the profile of cytokine response and that there is an important change that follows or reflects disease progression.

Contrary to all studies performed mainly on samples from NSC-LC patients, our study included patients with both histology tumor types. When we analyzed the patient group without stratification to any of the parameters, we did not find any significant differences in the concentration of investigated cytokines. Our data underlined significant

differences in Th_1/Th_2 cytokines according to microenvironment (tumor/blood). Furthermore, we demonstrated that patients with SC-LC histology type, without metastasis, and with the smallest tumors had the highest local IL-12/IL-2, IFN-γ concentration.

New data indicate that IL-4/IL-13 could have an important immunosuppressive role in LC, based on effects on TAM and MDSC. IL-4 as a key Th_2 cytokine indirectly induces neoplastic proliferation of LC by stimulating TAM to gain M2 phenotype, produce insulin-like growth factor 1 (IGF1) locally [25], and secrete cathepsin, which increases tumor cells capacity to invade surrounding tissues [26]. Gocheva et al. [26] demonstrated that IL-4 producing cells in LC tumor tissue were both infiltrating T lymphocytes and tumor cells. Importantly, infiltrating T lymphocytes represented a minority, only 2% of total IL-4 producing cells, while up to 90% of the dominant IL-4 producing population are tumor cells themselves. These data indicate that tumor cells manipulate the intratumor monocyte population, transforming them into TAM or/and M2 macrophages due to the secretion of Th_2 cytokines. A study from Feng et al. [27] demonstrated a significant role of $CD11b^+CD14^+S100A^+$ suppressive monocytes isolated from peripheral blood samples of NSC-LC patients. NSC-LC patients, especially those with advanced disease and unsuccessful therapy response, had significantly more these cells compared to healthy controls. This monocyte population was highly immunosuppressive *in vitro*, potently inhibiting $CD8^+$ T cell activation, inhibited IFN-γ production, and secreted high amounts of iNOS, arginase, IL-8, IL-10, tumor necrosis factor (TNF)-α, hepatocyte growth factor (HGF), and IL-13. The authors concluded that $CD14^+$ $S100A9^+$ inflammatory monocytes in patients with NSC-LC represent a distinct MDSCs subset capable of suppressing T lymphocytes by the production of arginase, iNOS, and the IL-13/IL-4Rα axis. TAM and MDSC are not the only sources of IL-13 in tumors of LC patients, since in almost historic study Huang et al. [28] demonstrated that tumor lung cells are potent producers of IL-13. The importance of IL-13 in regulating tumor invasion and spreading is already described in ovarian [29] and pancreatic cancer [30].

Our patients with SC-LC had the highest average IL-13 value, particularly in tumor microcirculation. IL-13 was the only cytokine of all investigated whose concentration significantly increased in tumor samples of all patients compared to sera values, regardless of histologic type of the tumor. Additionally, our patients with the intermediate/extreme number of the $CD14^+B7H4^+$ monocytes in tumor microcirculation samples demonstrated significantly elevated levels of IL-4, IL-5, IL-10, IL-13, and $TGF\text{-}\beta_1$, which is in concordance with data from the previous studies. In our previous paper [22], we have demonstrated that lung cancer patients had significantly more $CD14^+$ $B7H4^+$ Mo than healthy people. The highest $CD14^+$ $B7H4^+$ Mo number was associated with N3 nodal status, with the largest tumors, but also with clinical stage III and patients without metastasis. In this research, we investigated values of key Th_1/Th_2 cytokines estimated in those LC patients and represented them in the context of $CD14^+$ $B7H4^+$ monocyte number, indicating a significant association of Th_2 profile with the highest number of this Mo population in tumor samples.

Conclusion

Samples of LC patients demonstrated polarization of cytokine response, which is significantly associated with microenvironment origin, tumor histology type, tumor size, and disease extent. Stratification of LC patients according to $CD14^+$ $B7H4^+$ monocyte number offers a new possibility for the interpretation of cytokine profiles. The highest number of $CD14^+$ $B7H4^+$ monocytes is significantly associated with a particular cytokine profile in tumor samples, dominated with Th_2 cytokines.

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Conflict of interest

The authors state no conflict of interest.

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