

Quantitative changes within the peripheral blood CD8^{high}CD57⁺ T-cell subpopulation of patients with advanced renal cell carcinoma

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Background. Antitumor immunotherapy strategies, such as cytokine-based or cell-based immunotherapies, are designed to activate immune response against cancer cells, but considering that malignancy may be associated with the expansion of immunosuppressive components of antitumor immunity, it is likely that in such cases activation of the immune system would further enhance activity of these components, leading to more severe suppression of antitumor immune response thus making more favourable conditions for tumor progression.

Materials and methods. We studied the expression of biomarkers, representing immunosuppressive (FOXP3) and cytotoxic (perforin, IFN- γ) CD8^{high}CD57⁺ T-cell subpopulation functions in the peripheral blood of 34 patients with advanced clear cell renal cell carcinoma (RCC) and 26 controls by multicolour flow cytometry.

Results. CD8^{high}CD57⁺ T cell subpopulation of all CD8⁺ T cells in RCC patients was significantly higher compared to age-matched healthy controls ($p = 0.003$). It was found that the mean percentage of suppressive CD8^{high}CD57⁺FOXP3⁺ T-cell subset and cytotoxic CD8^{high}CD57⁺Perforin⁺ T-cell subset in the CD8^{high}CD57⁺ T-cell subpopulation was significantly increased in RCC patients compared to controls ($p = 0.0004$ and $p = 0.008$, respectively). There was no strong and biologically relevant negative correlation between the expression of FOXP3 and Perforin in the peripheral blood CD8^{high}CD57⁺ T-cell subpopulation of RCC patients.

Conclusions. Subsets of immunosuppressive FOXP3⁺ T cell and tumor-attacking (cytotoxic) Perforin⁺ T cells in the CD8^{high}CD57⁺ T-cell subpopulation are significantly increased in RCC patients compared to controls. These quantitative rearrangements are independent and individual for each RCC patient.

Key words: CD8^{high}CD57⁺ T cells, cytotoxic subsets, immunosuppressive subsets, renal cell carcinoma, individualized antitumor immunotherapy

INTRODUCTION

One of the functions of the immune system is to recognize and destroy tumor cells (1), thus various approaches enabling to promote and enhance antitumor immune response have been introduced into clinical practice (2). Antitumor immunotherapy strategies, such as cytokine-based or cell-based immunotherapies, are designed to activate immune response against cancer cells (3), but considering that malignancy may be associated with the expansion of immunosuppressive components of antitumor immunity (4), it is likely that in such cases activation of the immune system would further enhance activity of these components, leading to more severe suppression of antitumor immune response thus making more favourable conditions for tumor progression. Hence, it is likely that antitumor immunotherapy may be effective only when its prescription is based on immune system parameters, reflecting the nature of antitumor immune response (5). Hardly ever it will be possible to assess all parameters of closely interacting mechanisms of antitumor immunity, so it is important to identify such immune system components, which would reflect the nature of antitumor immune response as accurately as possible.

One of the components might be CD8^{high}CD57⁺ T-cell subpopulation, which expansion is associated with chronic antigenic stimulation (6-8), including tumor pathology (7, 9-11). Our previous results showed that treatment with adjuvant IFN- α_{2b} significantly increased the survival of renal cell carcinoma (RCC) patients with higher levels (>30%) of CD8^{high}CD57⁺ T cells in CD8⁺ T-cell population, whereas no increase in the survival was observed in RCC patients with lower levels (<30%) of CD8^{high}CD57⁺ T cells, moreover, a tendency towards decreased survival was observed in the latter patient group (9). We also found that during treatment with IFN- α_{2b} lower pre-treatment values of CD8^{high}CD57⁺ T cells tended to increase (IFN- α promotes their expansion and survival (12)), while higher pre-treatment values tended to decrease (9) (IFN- α may eliminate overactivated T cells (13)). Collectively our data suggested that immunosuppressive subsets of CD8^{high}CD57⁺ T-cell subpopulation tended to predominate in RCC patients.

Various authors provide contradictory data regarding effector functions of the CD8^{high}CD57⁺ T-cell subpopulation (14-17). There is evidence that CD8^{high}CD57⁺ T cells may express perforin, granzymes, granzysin and show high cytotoxic activity (14, 15), as well as they may secrete large amounts of proinflammatory cytokines tumor necrosis factor α (TNF- α) (18, 19), IL-5 (19) and interferon- γ (IFN- γ) (20, 21). The latter has dual effect on tumor cells – direct cytotoxic (1) and indirect through the promotion of innate and adaptive components of antitumor immunity (22). On the other hand, it is also known that CD8^{high}CD57⁺ T cells may exert immunosuppressive activity through the secretion of soluble inhibitory glycoprotein (16, 17). Considering the heterogeneity of CD8^{high}CD57⁺ T cells it appears that quantitative evaluation of general CD8^{high}CD57⁺ T-cell subpopulation in the peripheral blood of cancer patients would not disclose the nature of CD8^{high}CD57⁺ T-cell-mediated antitumor immune response. Thus it is necessary to assess the percentage of functionally competing T-cell subsets in the CD8^{high}CD57⁺ T-cell subpopulation.

The aim of the current prospective pilot study was to evaluate the expression of biomarkers, representing immunosuppressive (FOXP3) and cytotoxic (perforin, IFN- γ) T-cell functions in the peripheral blood CD8^{high}CD57⁺ T-cell subpopulation of RCC patients and age-matched healthy controls. To our knowledge, the expression of FOXP3 in CD8^{high}CD57⁺ T cells has not been investigated to date. Anyhow, currently FOXP3 is regarded as one of the main (but not absolute (23)) markers representing immunosuppressive properties of T cells (24, 25). Clear cell RCC is considered to be one of the most immunogenic malignancies in humans, however, non-individualized antitumor immunotherapy is effective only in about 10-30% of RCC patients (3, 26), thus the need for its individualization is obvious.

MATERIALS AND METHODS

Patients and controls

Thirty-four patients with advanced clear cell renal cell carcinoma (RCC) and 26 healthy individuals were enrolled in the study during the period 2008-2010. All patients were treated at the Section of

Oncology, Second Department of Oncological Surgery, Institute of Oncology, Vilnius University. RCC was proven histologically in all cases. Peripheral blood samples were collected once from each participant: for RCC patients blood samples were taken on the 7th day after nephrectomy. All patients have never been treated with chemotherapy, radiotherapy or immunotherapy.

Age-matched healthy control group subjects were enrolled in the study during their annual preventive medical examination. Control group included individuals without history of any oncological disease.

All participants (RCC patients and controls) were not affected with autoimmune diseases, acute or chronic infections (human cytomegalovirus, hepatitis B virus, hepatitis C virus, human immunodeficiency virus, *M. tuberculosis*), chronic alcoholism. Also they had no history of allogeneic bone marrow stem cell or solid organ transplantation.

The study was approved by the local Bioethical Committee and all individuals included in this study provided their informed written consent.

Flow cytometry

100 µl of ethylenediaminetetraacetic acid-collected blood samples were stained with the appropriate combination of three fluorochrome-labeled monoclonal antibodies (mAbs): anti-CD8-PerCP (peridinin-chlorophyll-protein) (BD Biosciences) and anti-CD57-FITC (fluorescein isothiocyanate) (BD Biosciences) combined with anti-FOXP3-PE (eBiosciences), anti-perforin-PE (BD Biosciences) or anti-IFN γ -PE (BD Biosciences).

For the detection of intracellular IFN- γ peripheral blood mononuclear cells (PBMCs) were separated from sodium citrate-collected peripheral blood by one-step density gradient centrifugation, using 8 ml (16 × 125 mm) BD Vacutainer[®] CPT[™] tubes (BD Biosciences) according to the instructions of the manufacturer. Staining with appropriate mAbs was performed after 6 hours of stimulation (1×10^6 cells per ml) with phorbol-12-myristate-13-acetate (5 ng/ml) (Fluka) and ionomycin (500 ng/ml) (Sigma Aldrich) in the presence of 10 µg/ml brefeldin A (Invitrogen) at 37 °C, 5% CO₂ atmosphere.

For surface antigen staining samples were incubated with 15 µl of the appropriate mAbs for

20 min at room temperature (RT) in the dark, then 1 ml of BD FACS[™] Lysing Solution (BD Biosciences) was added to each sample (except stimulated PBMCs separated by density gradient centrifugation) and the samples were incubated for 10 min at RT in the dark.

For intracellular antigen staining, the cells were fixed and permeabilized with 1 ml of Fixation / Permeabilization solution (eBioscience) for 30 min at RT in the dark, washed twice with 2 ml of Permeabilization buffer (eBioscience) and incubated with 20 µl of anti-FOXP3-PE (eBioscience), anti-Perforin-PE (BD Biosciences) or anti-IFN γ -PE (BD Biosciences) mAbs for 30 min at RT in the dark. Cells were washed with 2 ml of Permeabilization buffer (eBioscience) and 1 ml of CellWash buffer (BD Biosciences), resuspended in 500 µl of CellWash buffer (BD Biosciences) and applied to flow cytometric analysis.

Each experiment included samples incubated with isotype controls – IgG_{2a}-FITC (Dako), IgG₁-PE (phycoerythrin) (BD Biosciences) and IgG_{2b}-PE (BD Biosciences).

Assessment of intracellular IFN- γ expression after stimulation was also performed by comparing stimulated samples to non-stimulated controls.

Stimulation was not performed for the detection of intracellular perforin, since during initial experiments it was noticed that stimulation had no reliable impact on the expression level of perforin in either CD8^{high}CD57⁺ or CD8^{high}CD57⁻ T cells, however, increased non-specific staining with IgG_{2b} isotype control was observed after stimulation.

Flow cytometric analysis was performed on a BD FACS[™]Sort cytometer (BD Biosciences) with a single 488-nm argon ion laser and analyzed with the Cellquest software (BD Biosciences).

Statistical analysis

Distribution of the data was evaluated using the Shapiro-Wilk's W test. Differences between groups of parametrically-distributed data were evaluated using the Student's t test, while non-parametrically distributed data were evaluated using the Mann-Whitney's U test. Differences of the data within groups were evaluated using the Wilcoxon's criterion. Correlation between the expression of cytotoxic and immunosuppressive properties representing biomarkers was measured using the

Spearman's rank correlation analysis. The p values <0.05 were considered significant. Differences of the data are presented as mean values \pm standard deviation in the Results section. Statistical analyses were performed using the Statistica for Windows package version 8.0 (StatSoft Inc., Tulsa, OK, USA).

RESULTS

Quantitative changes within the peripheral blood CD8⁺ T-cell population in advanced RCC patients

We found that absolute counts of CD8⁺ T cells showed no significant differences between RCC patients and healthy controls, however, quantitative changes of various subpopulations were observed within the CD8⁺ T-cell population with the significant increase of CD8^{high}CD57⁺ T-cell subpopulation in RCC patients compared to age-matched healthy controls (Fig. 1).

The expression of immunosuppressive properties – representing FOXP3 in peripheral blood CD8^{high}CD57⁺ and CD8^{high}CD57⁻ T-cell subpopulations

It was found that the mean percentage of FOXP3⁺ T-cell subset in the CD8^{high}CD57⁺ T-cell subpopulation was significantly increased in RCC patients compared to controls, however, there were no statistically reliable changes in the expression of FOXP3 in the CD8^{high}CD57⁻ T-cell subpopulation between RCC patients and controls (Fig. 2a).

Both cancer patients and healthy controls could be divided into different groups according to the level of immunosuppressive CD8^{high}CD57⁺FOXP3⁺ T-cell subset (Table 1).

All subjects were grouped according to the percentage of FOXP3⁺ T lymphocytes in CD8^{high}CD57⁺ T-cell subpopulation of healthy controls, assuming that the percentage of CD8^{high}CD57⁺FOXP3⁺ T-cell subset in healthy individuals represents the normal value range.

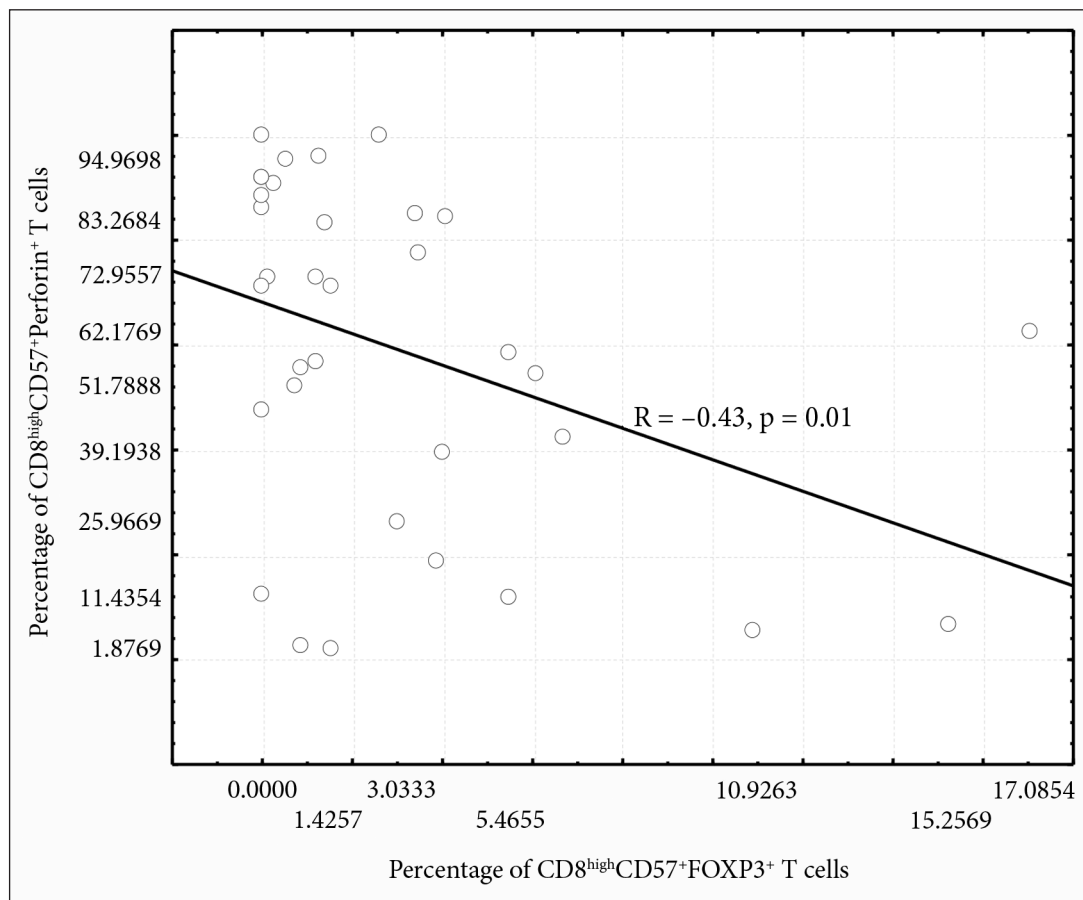


Fig. 1. Differences in the percentage of CD8⁺ T-cell subpopulations (CD8^{high}CD57⁻ and CD8^{high}CD57⁺) between healthy controls (n = 26) and RCC patients (n = 34), t test

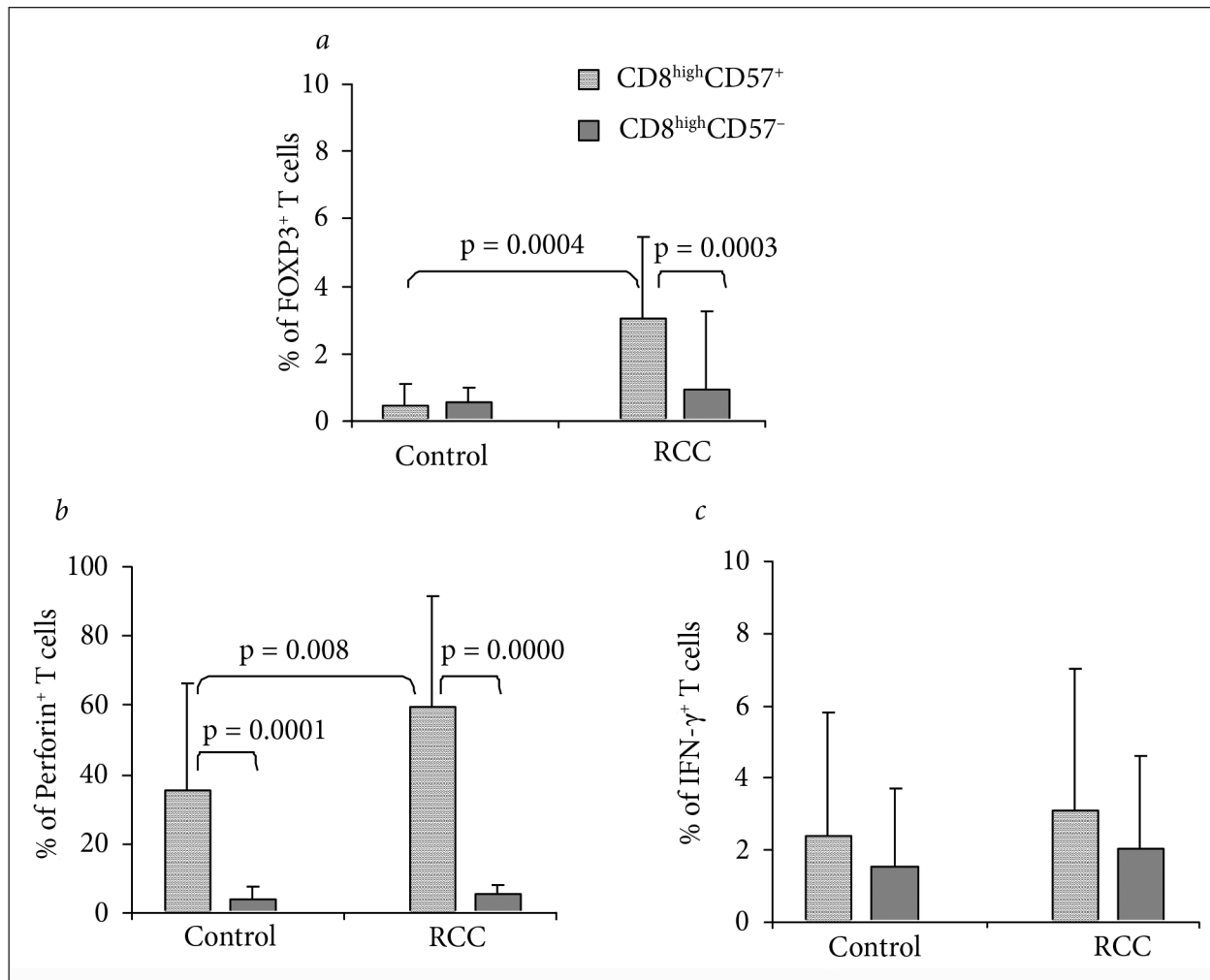


Fig. 2. Differences in the percentage of (a) FOXP3-positive, (b) perforin-positive and (c) IFN- γ -positive T cells in the peripheral blood CD8^{high}CD57⁺ and CD8^{high}CD57⁻ T-cell subpopulations of healthy controls (n = 26) and RCC patients (n = 34), U test

It was also found that in healthy controls the percentage of FOXP3⁺ T cells was slightly greater in CD8^{high}CD57⁻ T-cell subpopulation compared to CD8^{high}CD57⁺ T cells, while in cancer patients the increase of FOXP3 expression was obviously preferential to the CD8^{high}CD57⁺ T-cell subpopulation (Fig. 2a).

The expression of cytotoxic properties-representing perforin and IFN- γ in peripheral blood CD8^{high}CD57⁺ and CD8^{high}CD57⁻ T-cell subpopulations

Expression of perforin was significantly increased in peripheral blood CD8^{high}CD57⁺ T-cell subpopulation of advanced RCC patients compared

Table 1. Groups of RCC patients and healthy controls according to the percentage of immunosuppressive CD8^{high}CD57⁺FOXP3⁺ T-cell subset in the CD8^{high}CD57⁺ T-cell subpopulation

Percentage of CD8 ^{high} CD57 ⁺ FOXP3 ⁺ T-cell subset in CD8 ^{high} CD57 ⁺ T-cell subpopulation	RCC patients (n = 34)	Healthy controls (n = 26)
0	n = 8 (23.5%)	n = 17 (65.4%)
0.01–1 (low)	n = 6 (17.6%)	n = 4 (15.4%)
1.01–2 (medium)	n = 6 (17.6%)	n = 5 (19.2%)
>2 (2.01–17.09) (high)	n = 14 (41.2%)	n = 0 (0%)

Table 2. Groups of RCC patients and healthy controls according to the percentage of cytotoxic CD8^{high}CD57⁺Perforin⁺ T-cell subset in the CD8^{high}CD57⁺ T-cell subpopulation

Percentage of CD8 ^{high} CD57 ⁺ Perforin ⁺ T-cell subset in CD8 ^{high} CD57 ⁺ T-cell subpopulation	RCC patients (n = 34)	Healthy controls (n = 26)
<13.74 (low)	n = 6 (17.6%)	n = 12 (46.1%)
13.75–55.55 (medium)	n = 8 (23.5%)	n = 6 (23.1%)
55.6–84.74 (high)	n = 11 (32.4%)	n = 6 (23.1%)
>84.74 (very high)	n = 9 (26.5%)	n = 2 (7.7%)

to healthy controls (Fig. 2b), whereas there were no significant differences in the expression of intracellular IFN- γ in CD8^{high}CD57⁺ T cells (Fig. 2c).

It should be noted that perforin, but not IFN- γ , was preferentially expressed in CD8^{high}CD57⁺ T-cell subpopulation compared to CD8^{high}CD57⁻ T-cell subpopulation both in RCC patients and healthy controls (Fig. 2b–c).

Both RCC patients and controls could be divided into different groups according to the level of cytotoxic CD8^{high}CD57⁺Perforin⁺ T-cell subset (Table 2).

All subjects were grouped according to the percentage of perforin-expressing T lymphocytes in the CD8^{high}CD57⁺ T-cell subpopulation of healthy controls, assuming that the percentage of CD8^{high}CD57⁺Perforin⁺ T-cell subset in healthy individuals represents the normal value.

Correlation between the expression of FOXP3 and perforin in the CD8^{high}CD57⁺ T-cell subpopulation of advanced RCC patients

There was no strong and biologically relevant negative correlation between the expression of FOXP3 and perforin in the peripheral blood CD8^{high}CD57⁺ T-cell subpopulation of RCC patients (Fig. 3). Thus quantitative rearrangements of immunosuppressive and tumor-attacking (cytotoxic) subsets in the CD8^{high}CD57⁺ T-cell subpopulation are independent and individual for each RCC patient.

Importantly, in the RCC patients' group there were 8 subjects (23.5% of all RCC patients) with the predominance of cytotoxic chain of the CD8^{high}CD57⁺ T-cell subpopulation, i. e. they had very high percentage (>84.74%) of potentially cytotoxic CD8^{high}CD57⁺Perforin⁺ T-cell subset, while the immunosuppressive CD8^{high}CD57⁺FOXP3⁺ T-cell subset was absent or low (Table 3).

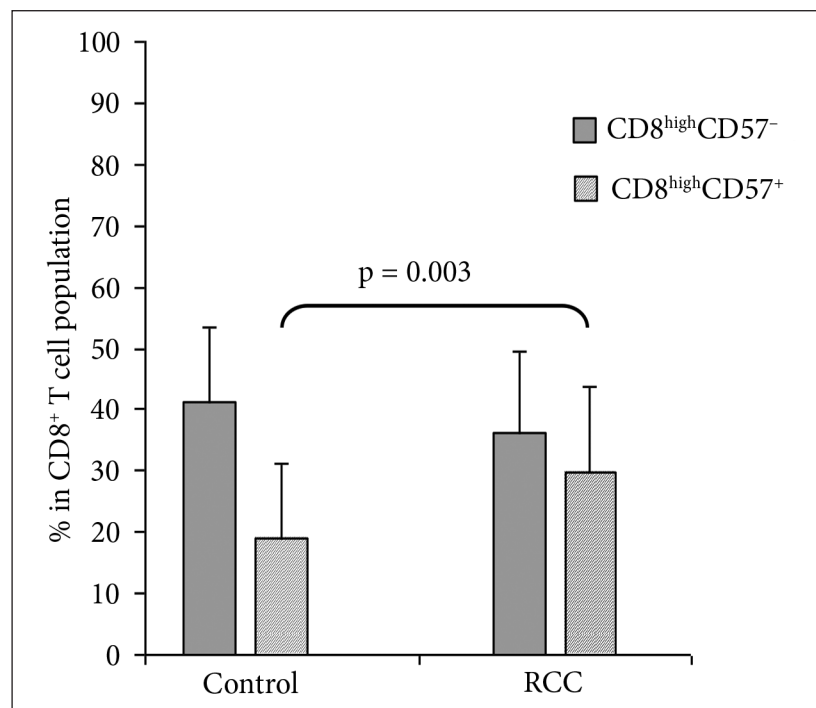


Fig. 3. Correlation between the expression of immunosuppressive properties representing FOXP3 and cytotoxic properties representing perforin in the peripheral blood CD8^{high}CD57⁺ T-cell subpopulation of advanced RCC patients (n = 34)

Table 3. Percentage of FOXP3 or perforin-expressing T cells in the peripheral blood CD8^{high}CD57⁺ T-cell subpopulation of advanced RCC patients

Patient No.	Percentage of FOXP3 ⁺ T cells in CD8 ^{high} CD57 ⁺ T-cell subpopulation		Percentage of Perforin ⁺ T cells in CD8 ^{high} CD57 ⁺ T-cell subpopulation	
1	0.85	low	55.4	intermediate
2	3.03	high	25.97	intermediate
3	0	absent	99.58	very high
4	1.53	intermediate	71.22	high
5	6.06	high	73.21	high
6	0.25	low	90.62	very high
7	0	absent	12.43	low
8	1.19	intermediate	72.98	high
9	0	absent	85.85	very high
10	4.04	high	39.19	intermediate
11	3.4	high	84.70	high
12	0	absent	91.82	very high
13	0	absent	47.27	intermediate
14	0	absent	91.70	very high
15	0.16	low	72.96	high
16	4.09	high	84.10	high
17	0	absent	88.28	very high
18	3.46	high	77.17	high
19	0.77	low	51.79	intermediate
20	15.26	high	6.32	low
21	0.54	low	94.97	very high
22	1.23	intermediate	56.62	(high)
23	10.93	high	5.63	low
24	0.85	low	2.60	low
25	1.55	intermediate	1.88	low
26	1.43	intermediate	83.27	high
27	17.09	high	62.18	high
28	5.5	high	11.44	low
29	3.89	high	18.45	intermediate
30	5.47	high	93.38	very high
31	6.72	high	42.06	intermediate
32	2.59	high	71.11	high
33	0	absent	99.7	very high
34	1.27	medium	95.76	very high

DISCUSSION

CD8^{high}CD57⁺ T cells are absent at birth and gradually accumulate throughout lifetime as a result of persistent immune activation (21, 27, 28). Their expansion is associated with chronic intracellular infections (viral, some bacterial) (6, 7, 14, 29), cancer (7, 9, 10, 17, 30–32), bone marrow or solid organ allogeneic transplantation (7, 16, 33), some autoimmune diseases (6, 7, 33), chronic alcoholism (33). The expansion of CD8^{high}CD57⁺ T-cell subpopulation also increases with ageing,

however, this increase is not associated with age per se, but most likely is a result of persistent common viral infections, especially human cytomegalovirus infection (7).

Repeated stimulation with the same antigen gradually induces the loss of CD28 expression and gain of CD57 expression on memory CD8⁺ T cells (and to a substantially lesser extent on memory CD4⁺ T cells (7, 34, 35)), eventually leading to the generation and accumulation of highly differentiated CD28⁻/CD57⁺ T cells with eroded telomeres (6, 7, 34, 36). These oligoclonally-

expanded T cells are not the ballast of the immune system, since they retain strong effector activity and play an important role in the immune system's function (19, 20, 32, 34, 35, 37). The loss of CD28 and gain of CD57 expression in circulating T cells during persistent immune stimulation is a definite immunological feature of humans and non-human primates but not of mice (34, 35).

Initially it was suggested that CD28 and CD57 expressions are mutually exclusive on human T cells (6, 38), however, Brenchley et al. clearly demonstrated that both CD28⁺CD57⁺ and CD28⁻CD57⁻ T cells were definitely detectable in peripheral blood of HIV patients and comprised up to 20% of memory CD8⁺ T-cell pool (6). Nevertheless, CD8⁺ T cells defined either by the loss of CD28 or by the gain of CD57 expression are antigen specific, oligoclonally-expanded, functionally competent and generated in response to chronic antigenic stimulation (6, 7, 19, 32, 34, 36), thus they appear to be of very similar functional status, although some minor differences may exist, depending on the expression pattern of CD28 and CD57.

For some time CD8^{high}CD57⁺ (CD8^{high}CD28⁻) T cells were characterized by their inability to proliferate under stimulation (due to critically shortened telomeres and loss of telomerase activity) (18, 34, 35), however, recently it was clearly shown that these T cells can transiently up-regulate telomerase activity and proliferate, but they need a special milieu, consisting of particular costimulatory signals and / or cytokines (19, 39). Also contradictory data is provided concerning the apoptotic potential of CD8^{high}CD57⁺ (CD8^{high}CD28⁻) T cells (37) – some authors (6, 40) claim that these T cells are very sensitive to activation-induced apoptosis, while others (20, 41) state that they show high resistance to apoptosis. Summarizing conflicting data of various authors it appears that a proportion of CD8^{high}CD57⁺ (CD8^{high}CD28⁻) T cells may acquire resistance to apoptosis (presumably due to persistent antigenic stimulation-related reasons (42)) and become long-lived inhabitants of the immune system, while others die rapidly after activation by cognate antigen.

In our studies antigen experienced, late-stage differentiated CD8^{high} T cells are defined by the expression of CD57, i.e. CD8^{high}CD57⁺, since several years ago we started our research based on this phenotype (9, 10) as in our initial studies we found

that the expression of CD28 and CD57 were almost reciprocal, moreover, there was emerging data that expression CD57 more precisely characterized late-differentiated CD8⁺ T cells (6).

Various authors describe either cytotoxic (14, 21) or immunosuppressive (16, 17) properties of CD8^{high}CD57⁺ T cells and collectively these data suggest that CD8^{high}CD57⁺ T-cell subpopulation is heterogenous and composed of various functionally competing subsets, which may preferentially predominate in certain diseases associated with chronic antigenic stimulation. Therefore it appears that evaluation of general CD8^{high}CD57⁺ T-cell subpopulation alone cannot display the final outcome of CD8^{high}CD57⁺ T-cell-mediated antitumor immune response since the proportion of competing subsets may influence the overall effect of antitumor immunity and determine individual response to antitumor immunotherapy.

In our previous study we have found that treatment with adjuvant IFN- α_{2b} significantly increased the survival of RCC patients with higher levels (>30%) of CD8^{high}CD57⁺ T cells in the peripheral blood CD8⁺ T-cell population, whereas no increase in the survival was observed in RCC patients with lower levels (<30%) of CD8^{high}CD57⁺ T cells. Moreover, we observed a tendency towards decreased survival in the latter RCC patient group (9). We have also showed that during treatment with IFN- α_{2b} lower pre-treatment values of CD8^{high}CD57⁺ T cells tended to increase (IFN- α promotes their expansion and survival), while higher pre-treatment values tended to decrease in RCC or melanoma patients (9, 10) (IFN- α may eliminate overactivated T cells (13)). Collectively our data suggested that the expansion of immunosuppressive subsets in CD8^{high}CD57⁺ T-cell subpopulation might be prevailing in RCC patients. To check this hypothesis, in the current study we analyzed the CD8^{high}CD57⁺ T-cell subpopulation and proportion of its various subsets in patients with advanced RCC and age-matched healthy controls. It was found that although CD8⁺ T-cell concentration showed no significant differences in the peripheral blood of RCC patients compared to healthy controls, quantitative rearrangements within the CD8⁺ T-cell subpopulation were observed with the significant increase of CD8^{high}CD57⁺ T-cell subpopulation in RCC patients. These results are consistent with

the data of others (17, 30–32) and our previous studies (9, 10) and imply that CD8^{high}CD57⁺ T-cell subpopulation is involved in antitumor immunity and may contribute to or regulate the final immune response against tumors (11). Moreover, here we show that essential changes in the expression of markers representing different effector functions of T cells (FOXP3, perforin) are observed particularly within the CD8^{high}CD57⁺ (but not CD8^{high}CD57⁻) T-cell subpopulation of RCC patients, further reinforcing the arguments that CD8^{high}CD57⁺ T cells are substantial participants in antitumor immune response.

CD8^{high}CD57⁺FOXP3⁺ T cells may represent an immunosuppressive component of CD8^{high}CD57⁺ T-cell subpopulation since the intranuclear transcription factor FOXP3 is claimed to be one of the main markers of many (but not all) regulatory / suppressive T cells (24, 25). However, it should be noted that unlike in mice, in humans FOXP3 is not an absolute marker of T cells with regulatory / suppressive activity since its expression (together with CD25) is transiently induced in virtually all activated and proliferating “conventional” CD4⁺ and CD8⁺ T cells (24). Yet considering that CD8^{high}CD57⁺ T cells are highly antigen specific, late-differentiated memory / effector T cells which exert their effector function under TCR activation (19, 32), but proliferate only under certain activation conditions (19, 39), it is unlikely that induction of FOXP3 expression would be associated with CD8^{high}CD57⁺ T-cell activation. It was shown by several groups that suppressive activity of CD8^{high}CD57⁺ T cells was mediated by the secretion of soluble inhibitory glycoprotein (16, 17), however, to our knowledge, expression of FOXP3 has never been investigated in CD8^{high}CD57⁺ T-cell subpopulation to date. Although the Suciú-Foca group thoroughly characterized CD8⁺CD28⁻FOXP3⁺ Ts cells with evident suppressive activity, these Ts seem not to express CD57 (43) and may represent that a minor subset of highly-differentiated CD8⁺ T cells that are negative for both CD28 and CD57. We investigated the expression of FOXP3 in CD8^{high}CD57⁺ T cells for the first time, and regarded these cells to represent a potentially immunosuppressive subset of CD8^{high}CD57⁺ T-cell subpopulation. Our results show that expression of FOXP3 in the CD8^{high}CD57⁺ T-cell subpopulation is very diverse

between RCC patients and healthy controls. We found that even 65% of healthy individuals had no immunosuppressive CD8^{high}CD57⁺FOXP3⁺ T-cell subset in the CD8^{high}CD57⁺ T-cell subpopulation, while only 23.5% of such subjects were observed in the advanced RCC patient group (Table 1). Worth noting is the fact that antitumor immunotherapy is clinically effective in about 10–30% of advanced RCC patients (3, 26) and the assumption that it is beneficial only for patients without the immunosuppressive CD8^{high}CD57⁺FOXP3⁺ T-cell subset cannot be refuted and should be investigated in future studies. Also of great importance is the fact that even 41% of advanced RCC patients had a very high percentage (>2%) of CD8^{high}CD57⁺FOXP3⁺ T-cell subset in the peripheral blood CD8^{high}CD57⁺ T-cell subpopulation, while in age-matched healthy controls we have not found any subject with so highly pronounced immunosuppressive component (Table 1). These data suggest that the increase of the immunosuppressive CD8^{high}CD57⁺FOXP3⁺ T-cell subset may be associated with malignancy and is consistent with the data of Montes et al. who found that late-differentiated (yet defined as senescent by the authors) T cells with suppressor function were tumor-induced (4). Looking from the clinical viewpoint, prescription of antitumor immunotherapy for cancer patients with highly pronounced immunosuppressive subset can be not only ineffective but even harmful because in such cases immune system stimulation inevitably causes activation of its immunosuppressive components which may finally lead to more severe suppression of antitumor immune response.

CD8^{high}CD57⁺Perforin⁺ and CD8^{high}CD57⁺IFN γ ⁺ T cells represent the cytotoxic subsets of CD8^{high}CD57⁺ T-cell subpopulation since perforin is one of the main cytolytic molecules involved in killing the target cells (1), while IFN- γ has a dual destructive effect on tumor cells – direct cytotoxic (1) and indirect through the activation of various components of innate and acquired immunity (22). In this study we found that cytotoxic CD8^{high}CD57⁺Perforin⁺ T-cell subset was significantly increased in CD8^{high}CD57⁺ T-cell subpopulation of advanced RCC patients compared to healthy controls, whereas CD8^{high}CD57⁺IFN γ ⁺ T-cell subset showed no statistically relevant quantitative changes (Fig. 2b–c). Most importantly there was no biologically relevant correlation between the

expression of perforin and FOXP3 in CD8^{high}CD57⁺ T cells and there were 17.6% of RCC patients who had very high percentage (>84.74%) of potentially cytotoxic CD8^{high}CD57⁺Perforin⁺ T-cell subset in the CD8^{high}CD57⁺ T-cell subpopulation, but had no immunosuppressive CD8^{high}CD57⁺FOXP3⁺ T-cell subset, i. e. these patients demonstrated absolute predominance of a cytotoxic component in the CD8^{high}CD57⁺ T-cell subpopulation. Again, worth noting is the fact that an objective clinical response to antitumor immunotherapy is achieved in about 10–30% of RCC patients (3, 26), thus it is reasonable to presume that activation of the immune system (prescription of antitumor immunotherapy) might be the most effective, particularly for the patients with highly pronounced cytotoxic component in the absence of immunosuppressive chain, but this hypothesis should be confirmed in further clinical studies, one of which is being performed in our institution.

In conclusion, our data indicate that the immune system often attempts to combat cancer by mobilizing its cytotoxic effector components, but at the same time the competitive immunosuppressive chain which abolishes the cytotoxic antitumor immune response may increase as well. Exact mechanisms inducing expansion of the immunosuppressive component are not fully elucidated, although there is data that the increase of late-stage differentiated T cells with suppressor function may be tumor-induced (4). The crucial point is that the amount of cytotoxic and immunosuppressive components of antitumor immunity is different in an individual cancer patient (at least with RCC) and the overall antitumor immune response may influence individual efficiency of antitumor immunotherapy. Evaluation of these differences may serve as one of possible immune system parameters enabling to assess the overall status of antitumor immune response and select cancer patients most suitable for antitumor immunotherapy while dismissing those to whom it would be ineffective or potentially harmful.

CONCLUSIONS

CD8^{high}CD57⁺ T cell subpopulation of all CD8⁺ T cells in RCC patients was significantly higher compared to age-matched healthy controls. The mean percentage of immunosuppressive FOXP3⁺

T-cell subset in the CD8^{high}CD57⁺ T-cell subpopulation was significantly increased in RCC patients compared to controls. There was no strong and biologically relevant negative correlation between the expression of FOXP3 and perforin in the peripheral blood CD8^{high}CD57⁺ T-cell subpopulation of RCC patients. Thus, quantitative rearrangements of immunosuppressive (FOXP3⁺ T cell) and tumor-attacking (cytotoxic) (perforin⁺ T cell) subsets in the CD8^{high}CD57⁺ T-cell subpopulation are independent and individual for each RCC patient.

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CD8^{HIGH}CD57⁺ T LIMFOCITŲ SUBPOPULIACIJŲ KIEKYBINIAI POKYČIAI IŠPLITUSIA INKSTŲ LĄSTELIŲ KARCINOMA SERGANČIŲ PACIENTŲ PERIFERINIAME KRAUJYJE

Santrauka

Priešvėžinė imunoterapija, pavyzdžiui, citokinių arba ląstelių imunoterapija, yra skirta aktyvuoti imuninę atsaką, nukreiptą prieš vėžines ląsteles. Tačiau piktybinis navikas gali aktyvinti priešnavikinio imuniteto imunosupresinius komponentus, todėl tikėtina, kad tokiais atvejais imuninės sistemos aktyvinimas gali dar labiau sustiprinti šių komponentų aktyvumą ir dar labiau slopinti priešnavikinį imuninę atsaką, sudarydamas palankesnes sąlygas navikui progresuoti.

Tyrimo medžiaga ir metodai. Daugiaspalvės tėkmės citometrijos būdu tyrėme biologinių žymenų, atspindinčių imunosupresines (FOXP3) ir citotoksines (perforinas, IFN- γ) CD8^{high}CD57⁺ T limfocitų sub-

populiacijos funkcijas, raišką. Ištirti 34 išplitusia šviesiųjų ląstelių tipo inkstų karcinoma sergantys pacientai ir 26 sveiki kraujo donorai.

Rezultatai. Tyrimo metu nustatyta, kad CD8^{high}CD57⁺ T limfocitų išplitusia šviesiųjų ląstelių tipo inkstų karcinoma sergančių pacientų kraujyje yra reikšmingai daugiau nei kontrolinės grupės ($p = 0,003$). Tiriant CD8^{high}CD57⁺ T limfocitų subpopuliaciją nustatyta, kad tiek imunosupresinių CD8^{high}CD57⁺FOXP3⁺, tiek citotoksinių CD8^{high}CD57⁺Perforin⁺ T limfocitų statistiškai reikšmingai buvo daugiau inkstų karcinoma sergančių pacientų periferiniame kraujyje, palyginti su kontroline grupe (atitinkamai $p = 0,0004$ ir $p = 0,008$). Reikšmingos neigiamos koreliacijos tarp šių subpopuliacijų nenustatyta.

Išvados. Imunosupresinių CD8^{high}CD57⁺FOXP3⁺ ir citotoksinių CD8^{high}CD57⁺Perforin⁺ T limfocitų kiekis inkstų karcinoma sergančių pacientų periferiniame kraujyje buvo statistiškai reikšmingai didesnis, palyginti su kontroline grupe. Nustatyta, kad imunosupresinių ir naviką atakuojančių (citoloksinių) komponentų kiekybiniai pokyčiai CD8^{high}CD57⁺ T limfocitų subpopuliacijoje yra nepriklausomi ir individualūs kiekvieno išplitusia inkstų ląstelių karcinoma sergančio paciento.

Raktažodžiai: CD8^{high}CD57⁺ T limfocitai, citotoksiniai T limfocitai, imunosupresiniai T limfocitai, inkstų ląstelių karcinoma, individualizuota priešnavikinė imunoterapija