PHENOTYPE ANALYSIS OF TUMOUR-INFILTRATING LYMPHOCYTES AND LYMPHOCYTES IN PERIPHERAL BLOOD IN PATIENTS WITH RENAL CARCINOMA

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*Summary: **Introduction:*** When checking tumour growth, a number of observations indicate that the immune system plays a significant role in patients with renal cell carcinoma (RCC). Infiltration by lymphocytes (tumour infiltrating lymphocytes, TILs) is more prevalent in RCC than any other tumours. T lymphocytes are the dominant population of TIL cells. Views concerning the role of T lymphocytic subpopulations, B lymphocytes and NK cells in an anti-tumour response are not established. ***Aim:*** The aim is to determine the phenotype and activation of T and B lymphocytic subpopulations and NK cells and to compare their representation in tumour stroma and peripheral blood lymphocytes (PBL) in patients with RCC. ***Material and methods:*** Samples of peripheral blood taken from the cubital and renal veins and tumour stroma cells were obtained from 44 patients in the course of their surgeries carried out due to primary RCC. TILs were isolated from mechanically disintegrated tumour tissue. Immunophenotype multiparametric analysis of PBL and TILs was carried out. Their surface and activation characteristics were determined by means of flow cytometer. ***Results:*** CD3^{+} T lymphocytes (69.7 %) were the main population of TILs. The number of CD3^{+}/CD8^{+} T lymphocytes was significantly higher in TILs, 42.6 % (p< 0.01), while CD4^{+} T lymphocytes were the majority population in peripheral blood, 41.35 % (p < 0.001). The representation of CD3^{+}/69^{+} T lymphocytes was significantly higher in TILs, 32.9 %, compared to PBL (p<0.001). On the contrary, the numbers of CD3^{+}/CD25^{+}, CD8^{+}/57^{+} and CD4^{+}/RA^{+} (naïve CD4^{+} T lymphocytes) were higher in PBL (p<0.001). The differences in representation of (CD3^{-}/16^{+}56^{+}) NK cells and CD3^{+}/DR^{+} T cells in TILs and PBL were not significant. ***Conclusion:*** The above-mentioned results prove that the characteristics and intensity of anti-tumour responses are different in compared compartments (tumour/PBL). CD3^{+}/CD8^{+} T lymphocytes are the dominant lymphocytic population of TILs. The knowledge of the phenotype and functions of effector cells, which are responsible for anti-tumour response, are the basic precondition for understanding the anti-tumour immune response and the cause of its failure.

Introduction

Tumour diseases are one of the three most common causes of human death. Cytotoxic T lymphocytes and NK cells are responsible for the anti-tumour response. The cytotoxic activity of T lymphocytes may be realized through the exocytic pathway, during which perforins and granzymes are released, whose activity initiates the apoptosis process in target (tumour) cells (5). The second mechanism which leads to the initiation of tumour cell death is the pathway which includes pro-apoptotic activity of an Apo/Fas molecule and also the pathway mediated through TNFα (11). There is a great similarity between CD8^{+} cytotoxic T lymphocytes and NK cells in effector mechanisms of cyto-

toxicity. There are a number of studies concerning RCC which analyse tumour-infiltrating cells and their contribution to the anti-tumour response. However, no unified opinion exists concerning the representation and role of T lymphocytic subpopulations, B lymphocytes and NK cells (4, 8, 20, 23).

Aim

The aim is to determine, by means of multiparametric analysis using flow cytometer, the phenotype and activation of cytotoxic cells and compare their representation in TILs and in blood taken from the renal and cubital veins.

Group of patients

From April 2005 to February 2006, RCCs were removed by means of surgery in 44 patients. The group of patients consisted of 15 women and 29 men aged 31–82, with an average age of 65.9. In 42 patients, radical nephrectomy was carried out and in two patients partial resection of the kidney was performed. The bilateral findings of renal tumour was present in one patient, in whom a unilateral nephrectomy and tumour encapsulation were performed on the second affected kidney.

According to histological findings, 86 % of the cases (36 patients) had primary clear cell carcinoma, 9 % of the cases (4 patients) had papillary carcinoma, and in 5 % of the cases (2 patients) chromophobe carcinoma was discovered. In one patient sarcomatoid carcinoma was caused by the transformation of chromophobe carcinoma; in another patient, non-classified RCC was diagnosed probably based on Henle's loop origin.

In order to determine the degree of disease progression, we used TNM classification. Twenty-two patients (50 %) with renal carcinoma were classified as primary tumour T1, twelve patients (27 %) T2, nine patients (20.5 %) T3 and one patient (2.5 %) T4. The disease classification of stages complies with numeric indexes of the primary tumour (T – tumour). Only two patients who were classified T1 and one patient with T3 classification were ranked into stage IV due to the presence of metastases. N (noduli) in the TNM classification in two patients had the value of N1 due to metastases of regional lymphnodes. The other patients had negative findings in the nodes. M (metastases) in TNM classification informs us about the presence of metastases. Three patients (7 %) were classified M1 due to distant metastases. In 93 % of the patients no distant metastases were diagnosed and they were classified M0. On the day of operation, samples of tumour tissue and blood from cubital and renal veins were taken from each patient.

Methods

Heparinized test tubes were used in the collection of blood from cubital and renal veins. Tumour tissue was sampled in a physiological saline with heparin solution (25 j/ml) and mechanically disintegrated and homogenized into individual cellular elements by means of scissors, pincers and homogenizer. These cellular elements were filtrated, twice washed with physiological saline (Infusion Natrii Chlorati Isotonica, the IMUNA Company), and diluted to an approximate concentration of $5x10^5$ cells. The samples were processed up to 12 hours after their sampling.

Each time 25 μl of cellular suspension and heparinized blood were incubated for 20 minutes with 10 μl of diluted conjungated monoclonal antibodies (the Immunotech Company and the Beckman Coulter Company) in the following combinations: CD45-FITC/CD14-PE, CD3-FITC/CD16⁺56-PE/CD19-PC5, RO-FITC/RA-PE/CD4-PC, CD8-FITC/DR-PE/CD3-PC5, CD69-FITC/CD25-PE/CD3-PC5, CD57-FITC/TcRαβ-PE/CD8-PC5, CD34-FITC/CD105-PE. Monoclonal cells in CD44-FITC/CD105-PE combination were used for the evidence of cellular endotheliums.

After lysing erythrocytes with lysing solution (0.5 ml OptiLyse C, the Beckman Coulter Company), we added physiological saline with 5 % natrium azide (1.5 ml Natriumazid-MERCK ⁺ Infusio Natrii Chlorati Isotonica, the IMUNA Company). The measurement was carried out by means of Coulter® Epics® XL flow cytometer (the Coulter Company, Fullerton, USA) equipped with an air-cooled argon laser emitting light with a wave length of 488 nm. This cytometer is equipped with four filters which absorb light beams of various wave lengths. This is why we were able to use three different fluorescent stains in one sample. The evaluation of measured samples was carried out by means of CPX software analysing programme.

The Sigmastat version 2.0 programme was used for statistical evaluation. The statistics were processed by standard methods for statistical comparison of several groups. The normality value test was, in the evaluated cases, unsuccessful, therefore the "One Way Anova on Ranks" test was carried out to determine the level of statistical significance. The median was the indicator of value position.

Results

The mean purity of the lymphocyte suspensions isolated from the tumour was 86.5 % and did not cause any problems during measuring and comparing the findings in the samples of venous blood and tumoral cellular suspension. The results of measurements are shown in was Tab. 1. The number of cells carrying CD3⁺/69⁺, CD8⁺ and CD3⁺/8⁺ phenotype were significantly higher in TILs compared to PBL (with lymphocytes in blood from renal and cubital veins). On the contrary, the number of cells with CD4⁺, CD 19⁺, CD3⁻/8⁺, CD3⁺/16⁺/56⁺, CD3⁺/25⁺, CD57⁺, CD8⁺/57⁺, CD4⁺/RA⁺ phenotype was significantly higher in PBL compared to TILs. CD8⁺ T lymphocytes were the dominant lymphocytic population in tumour tissue. On the contrary, CD4⁺ T lymphocytes were the majority population in peripheral and renal venous blood. The representation of (CD3⁻/16⁺/56⁺) NK cells and CD3⁺/DR⁺, CD8⁺/DR⁺, CD25⁺ and CD4⁺/RO⁺ T lymphocytes showed no statistically significant differences (Fig. 1).

Discussion

Clinical monitoring of both spontaneous regressions of RCC primary and secondary foci and therapeutic responses in 15–20 % of patients after the administration of IL-2 shows an effective anti-tumour response. Tumour-infiltrating lymphocytes are responsible for the anti-tumour response. This is a heterogeneous population of cytotoxic cells that carry predominantly CD3⁺/CD8⁺ and CD3⁻/
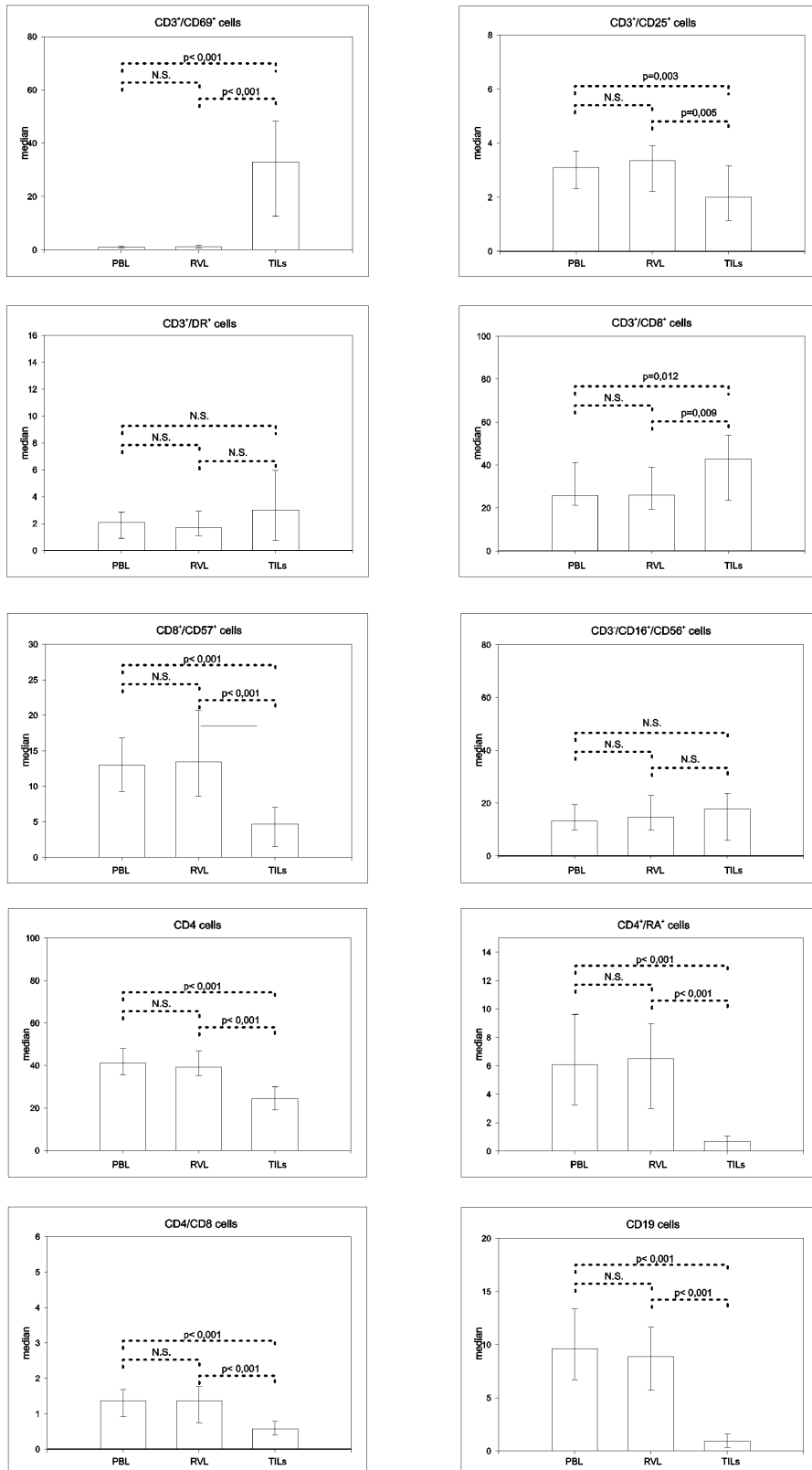
Fig. 1: Differencies in numbers of lymphocyte subpopulations in various compartments (peripheral blood lymphocytes – PBL, renal vein lymphocytes -RVL, tumor-infiltrating lymphocytes -TILs).

Tab. 1:

	Med. PBL 25–75%	Med. RVL 25–75%	Med. TILs 25–75%	PBL-RVL p	PBL – TILs p	RVL – TILs p
Suspensionpurity	99.2 (98.7–99.4)	98.9 (97.8–99.3)	86.5 (74.7–94.7)	0.166	<0.001	<0.001
CD3$^-$/16$^+$56$^+$	13.2 (9.9–19.4)	14.8 (9.9–22.9)	17.7 (5.9–23.8)	0.362	0.528	1.000
CD3$^+$/DR$^+$	2.1 (0.9–2.9)	1.7 (1.1–2.9)	3.0 (0.8–5.9)	0.872	0.211	0.204
CD3$^+$/69$^+$	1.0 (0.7–1.4)	1.1 (0.7–1.7)	32.9 (12.6–48.4)	0.288	<0.001	<0.001
CD4$^+$	41.4 (35.5–48.1)	39.2 (30.3–46.9)	24.6 (19.0–30.1)	0.484	<0.001	<0.001
CD8$^+$	32.0 (26.4–43.4)	32.8 (26.2–43.7)	47.2 (26.3–58.2)	0.816	0.010	0.017
CD3$^+$	71.9 (62.8–79.4)	72.8 (64.1–79.0)	69.7 (60.9–76.8)	0.812	0.314	0.390
CD4$^+$/CD8$^+$	1.4 (0.9–1.7)	1.4 (0.7–1.8)	0.6 (0.4–0.8)	0.857	<0.001	<0.001
CD19$^+$	9.7 (6.7–13.4)	9.1 (5.8–12.2)	0.9 (0.3–1.6)	0.316	<0.001	<0.001
CD3$^+$/8$^+$	25.8 (21.3–41.2)	26.1 (19.5–38.9)	42.6 (23.5–53.9)	0.975	0.009	0.012
CD3$^-$/8$^+$	4.0 (2.7–7.2)	3.7 (2.3–7.3)	3.3 (1.6–5.7)	0.958	0.195	0.167
CD3$^+$/16$^+$56$^+$	5.5 (2.2–9.7)	5.6 (3.0–9.4)	2.9 (1.6–5.4)	0.766	0.018	0.004
CD8$^+$/DR$^+$	1.1 (0.5–2.4)	1.2 (0.6–1.5)	1.6 (0.5–5.1)	0.619	0.282	0.114
CD25$^+$	3.3 (2.7–4.1)	3.6 (2.5–4.6)	2.6 (1.4–4.7)	0.495	0.102	0.071
CD3$^+$/25$^+$	3.1 (2.3–3.7)	3.4 (2.2–3.9)	2.0 (1.1–3.1)	0.669	0.003	0.005
CD57$^+$	21.9 (15.9–28.5)	21.7 (16.1–30.2)	7.3 (3.2–13.0)	0.746	<0.001	<0.001
CD8$^+$/57$^+$	13.0 (9.3–16.8)	13.5 (8.6–20.7)	4.7 (1.5–7.1)	0.613	<0.001	<0.001
CD4$^+$/RA$^+$	6.1 (3.3–9.6)	6.5 (3.0–8.9)	0.7 (0.0–1.1)	0.980	<0.001	<0.001
CD4$^+$/RO$^+$	25.5 (22.8–35.9)	23.9 (22.5–32.2)	22.2 (14.6–29.1)	0.376	0.123	0.212
CD8$^+$/$\alpha\beta^+$	24.7 (20.8–37.7)	26.7 (19.4–41.9)	37.4 (19.9–52.6)	0.880	0.065	0.107

Abbreviations: PBL – peripheral blood lymphocytes; RVL – renal vein lymphocytes; TILs – tumour-infiltrating leukocytes; Med – median

Analysis of lymphocyte populations infiltrating renal cell carcinoma primary tumor compared with peripheral blood lymphocytes and renal vein lymphocytes.

CD16$^+$/CD56$^+$ phenotype. CD4$^+$ T lymphocytes and B lymphocytes are found by a majority of the authors as a minority population in TILs (26). In all the above-mentioned cellular populations isolated from RCC, the signs of early and late activation are detected as dendritic (5). For an effective anti-tumour response, tumour cells must be recognized by tumour-infiltrating monocytes and T lymphocytes. In this regard, the role of cells (DCs) is considered key. Dendritic cells present tumour antigens to cytotoxic lymphocytes and may prefer CD4$^+$ subsets, and Th1 or Th2 response (2) with its cytokine spectrum (22).

In up to 70 % of renal tumours, CD8$^+$ T lymphocytes are the main cellular population of TIL cells (16). Our observations comply with this finding. The prevalence of CD3$^+$CD8$^+$ cell population was also described in the stroma of other tumours (7, 15, 23, 32, 33).

CD4$^+$ T lymphocytes, which are the main producer of IL-2, show anti-tumour activity. The differentiation and activation of cytotoxic T lymphocytes and NK cells are induced by the effect of IL-2. The Th1 subset of CD4$^+$ T lymphocytes produces IFN γ and increases the cytotoxic function of CD8$^+$ T lymphocytes. On the contrary, if the Th2 subset activity prevails, the antibody response is preferred through IL-4 and IL-5 and the function of Th1 subtype is inhibited. The presence of tumour-infiltrating effector cells and their activation are evidence of a local immune response (12).

Igarashi et al. compared the representation of lymphocytic subpopulations in TILs. Their study evaluated the correlation between the TIL phenotype and the response to interferon-α therapy. In patients with an advanced stage of disease (stages III and IV), in whom CD4$^+$ T lymphocytes represented more than 40 % of TIL cells, the partial remission or stabilization of the disease was achieved by the administration of interferon-α. On the contrary, in patients with CD8$^+$ TILs number higher than 25 %, no therapeutic response was achieved. The increase in CD8$^+$ and decrease in CD4$^+$ tumour-infiltrating lymphocytes were associated with a worse prognosis. Moreover, in vitro functional analyses of TILs confirmed that CD4$^+$ T lymphocytes showed signs of activation, while CD8$^+$ T lymphocytes, in some cases, did not express (exprime) the surface molecules, which was evidence of activation, and these cells were not able to induce effectively the cytolysis of target cells (14). On the contrary, in patients with localized disease, the phenotype of TILs does not have a prognostic significance (28, 30).

After presentation of the antigen, specific T lymphocytes are activated, which is associated with the change of expression of many molecules, for example HLA-DR molecule, the receptor for IL-2 (CD25) and the CD69 molecule (17, 24). The CD25 molecule reflects an early stage of cellular activation and is present in a higher number of tumour-infiltrating T lymphocytes compared to T lympho-

cytes in peripheral blood (27). An increased expression of the CD25 molecule (an α chain of the receptor complex for IL-2) on T lymphocytes in renal cell carcinoma is considered to be evidence of local anti-tumour activity. An increased expression of this receptor is described in CD3^{+} T lymphocytes after the specific and non-specific stimulation in vitro and in vivo conditions (1, 2, 18, 25). The expression of early activation molecule CD69 is detectable 2–3 hours after stimulation. Non-specific stimulation could have an effect on the expression of early marker activation during the separation of TILs from the tumour stroma, but the time of sample processing (the preparation of suspension up to the evaluation on the flow cytometer) did not exceed 60 minutes.

The expression of HLA-DR molecules is the manifestation of prolonged stimulation. Class II HLA-DR molecules may be seen in cells with a delay of 48–72 hours after antigen stimulation (6). The results of our study comply with other studies which describe the expression of HLA-DR molecules in CD3 and CD8 positive T lymphocytes (9, 29, 30). Although the representation of CD3^{+}/DR^{+} and CD8^{+}/DR^{+} cells was higher in TILs than PBL in our patients, these differences were not significant

NK cells in peripheral blood are a phenotype-heterogeneous population. CD 56 molecule is present in nearly all NK cells, while CD 57 molecule is present only in mature NK cells. The presence of NK cells is important for the effectiveness of treatment with IL-2. Donskov et al. described the absence of CD 56^{+} cells in tumours during the course of treatment of clear cell renal carcinoma (10). On the contrary, it was proven that the presence of CD57 positive cells is necessary for achieving the therapeutic response after the administration of IL-2. It was confirmed that the presence of CD57^{+} NK cells in the tumour has an influence on the survival of patients with renal, colorectal and stomach carcinomas. NK cells are considered to be the key population that checks tumour growth. The low number of NK cells among TILs is associated with a higher risk of tumour progression. The lack of CD3^{-}CD56^{+}CD16^{+} cells in peripheral blood and/or among TIL cells is associated with RCC patients at risk of metastasis development, which proves the protective role of these cells (13, 31). The representation of NK cells in peripheral blood and tumour stroma showed no difference in our patients.

The tumour infiltration by lymphocytes, which show cytotoxic activity targeted against tumour cells, is considered a sign of effective anti-tumour response. In spite of these findings, spontaneous tumoral regression and therapeutic response to biological treatment are seen in only a small amount of patients with renal carcinoma. The presence of activated T lymphocytes does not have to reflect the anti-tumour response. The increased expression of CD 69 and HLA DR activation molecules and the low density of CD25 molecules on T lymphocytes are also seen in other diseases caused by or associated with immunopathological reactivity, for example in inflammatory diseases of con-

nective tissue. It is probable that, in part of the patients, the tumour infiltration by activated lymphocytes is caused by cytokine and chemokine tumoural microenvironments. Stimulation by tumour antigens may be, in most cases, insufficient or completely missing. The growing tumour tissue is exposed to ischemia and undergoes necrosis if there is an insufficient vascular supply. The necrotic tissue is invaded by phagocytating cells that produce chemoatractants and pro-inflammatory cytokines. Endothelial cells of newly-created vessels produce a number of factors that also stimulate proliferation of CD4^{+} T lymphocytes. Th2 lymphocytes produce IL-13, which has a pro-angiogenic effect. Non-specific stimulation may be a consequence which is responsible for failure of immunological supervision (16, 29).

B lymphocytes are the minority population of TILs. Although the precise role of B lymphocytes is unknown, the expression of CD69 molecules in tumour-infiltrating B lymphocytes shows their participation in the anti-tumour response (19). In case of tumours, an antibody response to tumour antigens may lead to the blunting of epitopes recognized by specific cytotoxic T lymphocytes and thus making possible tumour growth. Higher representation of B lymphocytes among TILs was observed in patients with a predominance in CD4^{+} infiltrating lymphocyte and Th2 population activity, respectively (22).

The significance of the role of antigen-presenting cells was not unambiguously proven in tumour diseases in case of B lymphocytes (3, 21, 26, 32, 33). In our patients, B lymphocytes were less than 1 % of TILs and their numbers were significantly lower compared to their numbers in PBL.

Shabtai had a different opinion in his study that comparing representation of T and B lymphocytes in TILs and peripheral venous blood in 8 patients with advanced renal carcinoma. The ratio of the number of tumour-infiltrating B lymphocytes to B lymphocytes in peripheral blood was 2.4, while the ratio of tumour-infiltrating T lymphocytes to T lymphocytes in peripheral blood was 0.48. Moreover, the T lymphocytic population was represented predominantly by CD3^{+}/CD4^{+} lymphocytes (27). In these patients Th2 response probably prevailed over Th1 response with production of antibodies blunting specific tumour antigens and cytokines, and consequently allowed the tumour growth (12).

Conclusion

We may sum up that immunocompetent cells are activated in renal cell carcinoma. However, transformation of these immunocompetent cells to effector cytotoxic T lymphocytes and NK cells is inhibited by so far unknown, but most probably multifactoral, mechanisms. It is not possible to exclude that in a majority of patients non-specific stimulation of TILs prevails. Th2 lymphocyte activation, characterized by the production of IL-4 and IL-5, or regulating Tr (TH3) subset activity, producing TGF β and IL-10, may be

one of the causes of local cellular specific anti-tumour response failure. In this way, the desired protective activity of the Th1 subset (with production of IFNγ), which is important for cytotoxic activity against the tumour, is restricted. Due to the activity of Th2 lymphocytes, the predominant immune response is antibody production and the release of cytokines, for example IL-13 with its angiogenic activity, which may stimulate tumour growth. IL-10 inhibits the expansion of cytotoxic $CD3^+/CD8^+$ T lymphocytes.

Cytokine tumoural environment, angiogenic factors and insufficient tumour antigenic stimuli are probably responsible for non-purposeful local inflammatory reactions and tumour progression. In most cases, intervention using interleukin-2 and interferon α does not make it possible to break through the conditions under which tumour structures are tolerated.

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