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Prognostic value of the immunomonitoring of patients with renal cell carcinoma under therapy with IL-2/IFN-α-2 in combination with 5-FU

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Abstract After tumor nephrectomy, patients suffering from metastatic renal cell carcinoma (RCC) received interleukin-2 (IL-2), interferon (IFN)-α-2b and 5-fluorouracil (5-FU) in one to three treatment cycles over 8 weeks. Using flow cytometry, we investigated the immunophenotype of peripheral blood lymphocytes from 22 patients during therapy. In all patients, we found an increase in the absolute number of T lymphocytes, especially of the CD4 type, and in the number of HLA-DR$^+$, CD25$^+$ T cells and natural killer (NK) cells. The mean number of B cells did not increase during therapy. The numbers of CD4$^+$, CD8$^+$ and CD25$^+$ T cells correlated significantly with the clinical response. In addition, we found that the pretherapeutic number of T lymphocytes and B cells but not of NK cells was significantly higher in patients with a therapy-induced clinical response. In conclusion, we describe the predictive value of the number of lymphocytes from peripheral blood for the efficiency of IL-2/IFN-α-2b therapy in combination with 5-FU in patients with metastatic renal cell carcinoma.

Key words Renal cell carcinoma · Interleukin-2 therapeutic use · Interferon-α therapeutic use · T lymphocytes · CD4

Introduction

The prognosis of patients suffering from metastatic renal cell carcinoma (RCC) is extremely poor, although nowadays the disease can be diagnosed earlier. Sixty percent of patients with metastatic kidney tumor including relapses die within 1 year. The 5-year survival rate is less than 5% [6, 13]. The 5-year survival rate for tumors in stage $T_{3b}N_0M_0$, however, is 58% [13]. Conventional therapies are known to have a poor success rate. RCC is a highly immunogenic tumor with reported cases of spontaneous regression of metastatic lesions [7]. For this reason, immunotherapy is of increasing interest in the treatment of patients with RCC [30]. A large number of studies using interleukin-2 (IL-2) and IFN-α have been published over the last 5 years [3, 4, 15, 23]. Interferons (IFNs) are proteins produced by different nucleate cells in response to viral infections or other types of stimulus. They have direct antiproliferative as well as immunomodulating effects on some subpopulations of cells of the immune system. It is known that IFNs induce the differentiation of cells and exert an inhibitory action on the expression of some oncogenes responsible for malignant proliferation [9]. IFNs change the expression of antigens on the surface of tumor cells and make them recognizable for lymphocytes [5]. IL-2 is thought to act by augmenting the endogenous antitumor function of the host immune system rather than by direct tumoricidal action [9]. For therapeutic application to RCC, IFN-α-2 and IL-2 are used separately or in combination. Using a combined therapy regimen, it was possible to achieve rates of response in the order of 36% [10]. Combined chemo-/immunotherapy with IFN-α-2, IL-2 and 5-fluorouracil (5-FU) is a widely utilized protocol, providing an improvement of response rates [2, 16, 25].

For immunotherapy in cancer patients, it is essential to recognize the success of the therapy as early as possible. Different cellular and soluble factors of the immune system have been described as possible criteria for the response to such therapy. In the case of low-dose IL-2 therapy, for instance, the serum level of TNF-α is described as a prognostic factor [18]. Further soluble factors, the increase of which provides

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indicators as to the efficiency of IL-2 therapy, are soluble CD8-antigen and soluble IL-2 receptor [1, 17]. The number of eosinophiles and of $CD25^+$ (α-chain of the IL-2-receptor) T lymphocytes has been reported to correlate well with the efficiency of combined IL-2 and IFN-α-2 therapy [4, 21]. Furthermore, the pretherapeutic ratio CD4/CD8 is considered to provide information on the prognosis of patients [15]. Patients under IL-2/IFN-α therapy showing a clinical response had a higher CD4/CD8 ratio at the beginning of therapy than those who did not respond [20]. Moreover, Atzpodien et al. [3] found a significant difference in the number of NK cells of responders and nonresponders for low-dose therapies. Patients with complete or partial regression and those with stable disease had significantly higher numbers of $CD56^+$ lymphocytes in their peripheral blood than patients with progressive disease [23]. Furthermore, the intensity of antigen CD56 on NK cells before therapy has been described to correlate with the clinical response [8]. Using double-labeling and flow cytometry, we investigated the number of lymphocytes, CD4/CD8 ratio and number of HLA-DR^+ and $CD25^+$ T cells in the peripheral blood of RCC patients before and during therapy with IL-2/IFN-α-2b and 5-FU. We compared our results with respect to the lymphocytic phenotype in peripheral blood with the treatment response of patients.

Materials and methods

Patients

Twenty-two patients (17 male and 5 female) suffering from metastatic RCC, mostly of the clear cell type, were treated with IL-2 and IFN-α-2b in combination with 5-FU. Written informed consent was obtained from all patients. At the beginning of the immune therapy, patients had already undergone tumor surgery and showed progressive disease. Time from surgery to first metastasis ranged between 0 and 32 months, and time from metastasis to study entry ranged between 1 and 3 months. The 22 patients received a total of 39 cycles of treatment. During each treatment cycle phenotypic analysis of peripheral blood lymphocytes (PBLs) was carried out. The clinical state of patients was established by X-ray, computer tomography and bone scintigraphy after each cycle of therapy. The clinical state of patients allowed three groups to be distinguished at the end of each cycle of therapy. We categorized patients into "regression" (complete and partial remission), "stable disease", and "progression" groups. Partial response was defined as a 50% decrease in the sum of the products of the perpendicular diameters of measurable lesions sustained for at least 8 weeks with no development of any new lesion. Complete response required the disappearance of all lesions sustained for at least 8 weeks. Progressive disease was defined as a 25% increase in the sum of products of the perpendicular dimensions of all lesions, or the development of new lesions. All other patients were considered to have stable disease. Fifteen cycles of therapy were scored as "regression," the "stable disease" group was found for 14 cycles and "progression" was identified 10 times. We only evaluated complete cycles of therapy showing a clear result with regular phenotypic analysis. The side effects of the therapy were fever up to 39.5 °C and a flu-like syndrome. Side effects were well controlled by the appropriate medication. No systemic antibiotic prophylaxis was given.

Table 1 Pattern of a treatment cycle of immunotherapy with IL-2 and IFN-α-2b in combination with 5-FU

Week	IL-2	IFN-α-2b	5-FU
	Dose (/m^2 body surface), frequency (/week)		
1	2×10^7 IU 3×	5×10^6 U 1×	
2	5×10^6 IU 3×	5×10^6 U 3×	
3	5×10^6 IU 3×	5×10^6 U 3×	
4	2×10^7 IU 3×	5×10^6U 1×	
5		8×10^6 U 3×	750 mg 1×
6		8×10^6 U 3×	750 mg 1×
7		8×10^6 U 3×	750 mg 1×
8		8×10^6 U 3×	750 mg 1×

Pattern of therapy

The therapy with IL-2/IFN-α-2b/5-FU was carried out in cycles of therapy within 8 weeks according to the following pattern (Table 1): recombinant IL-2 (Proleukin, Chiron, Frankfurt) was injected in doses of 2×10^7 IU/m^2 body surface. This was done 3 times a week in the 1 and 4 weeks. IL-2 was given subcutaneously in amounts of 5×10^6 IU/m^2 body surface 3 times weekly in the 2nd and 3rd weeks. Recombinant IFN-α-2b (Roferon, Hoffmann La Roche, Grenzach) was applied subcutaneously in amounts of 5×10^6 U/m^2 body surface once per week in the 1st and 4th weeks. In addition, the scheme included identical applications 3 times weekly in the 2nd and 3rd weeks. A dose of 8×10^6 U IFN-α/m^2 body surface was used 3 times weekly in weeks 5–8. 5-FU was given i.v. as a bolus within 30 min once per week in weeks 5–8 of the therapy cycle. The dose was 750 mg/m^2 body surface.

After each treatment cycle, patients were evaluated for response. Responding patients (regressive metastases or stable disease) were treated with additional cycles of therapy, up to 2 times per patient (with a maximum of three cycles per patient).

Phenotypic analysis

The PBLs of the patients under therapy were studied once per week by flow cytometry. Double-staining was performed with directly labeled antibodies [fluorescein isothiocyanate (FITC) or phycoerythrin (PE)]. The monoclonal antibodies applied were the following: CD45FITC/CD14PE (leukogate), negative control, CD3/CD19, CD4/CD8, CD3/HLA-DR, CD3/CD16 + 56 (Simulset kit, Becton Dickinson, Heidelberg), CD3/CD8 (Becton Dickinson, Heidelberg) and CD3/CD25 (Dianova, Hamburg). We used the method of lysed whole blood staining without separation of PBLs. Fluorescence analyses were performed on a Becton Dickinson FACScan. Nonlymphoid and dead cells were excluded by the setting of appropriate forward and 90° light scatter gates. The lymphocyte clusters were essentially free of monocyte contamination. Two thousand cells/lymphocyte gate were counted.

Statistical analysis

The statistical analyses were done with the Statgraf 7.0 program. Data are expressed as means ± standard deviation (SD). Differences

Table 2 Pretherapeutic absolute numbers of lymphocyte subpopulations and of the CD4/CD8 ratio. Data are given as means ± SD

Parameter	Regression (1) ($n = 15$) (mean ± SD)	Stable disease (2) ($n = 14$) (mean ± SD)	Progression (3) ($n = 10$) (mean ± SD)	Significant differences between groups	Control group[a] ($n = 15$)
Age of patients (years)	51 ± 7	59 ± 7	56 ± 5		52 ± 7
Lymphocytes (cells/mm^3)	2196 ± 644	1534 ± 609	1139 ± 657	1–2, 1–3	1728 ± 206
T cells (cells/mm^3)	1611 ± 533	1136 ± 484	784 ± 508	1–2, 1–3	1240 ± 249
CD4$^+$ T cells (cells/mm^3)	1061 ± 380	762 ± 348	483 ± 405	1–2, 1–3	880 ± 193
CD8$^+$ T cells (cells/mm^3)	588 ± 339	369 ± 187	303 ± 157	1–2, 1–3	320 ± 156
HLA-DR$^+$ T cells (cells/mm^3)	155 ± 141	186 ± 158	137 ± 102	–	136 ± 76
CD25$^+$ T cells (cells/mm^3)	291 ± 166	182 ± 105	171 ± 148	–	151 ± 76
NK cells (cells/mm^3)	306 ± 185	225 ± 121	197 ± 117	–	514 ± 374
B cells (cells/mm^3)	239 ± 147	134 ± 82	121 ± 79	1–2, 1–3	190 ± 71
CD4/CD8	2.39 ± 1.39	2.28 ± 0.86	1.53 ± 0.99	–	2.0 ± 1.0

[a] Healthy volunteers (eight females, seven males) from our staff were investigated

between groups were compared with the Mann-Whitney U-test. A P value < 0.05 was considered to be statistically significant.

Results

For all patients during therapy, a numerical increase of the number of T cells and NK cells was observed compared to the initial values. This increase was discontinuous, but evident. Of the T cells, we found an increase in the CD4$^+$ as well as the CD8$^+$ T cells. Usually, the increase in CD4$^+$ T cells was more pronounced than that of the CD8$^+$ T cells. Consequently, an increase of the ratio CD4/CD8 was recorded. The absolute values of T cells bearing CD25 or HLA-DR on their surface also increased under the therapy. NK cells partially showed coexpression of CD8 in different amounts. The CD8$^+$CD3$^-$ cells were excluded from the CD8$^+$ cells when the CD4/CD8 ratio was calculated.

Table 2 shows the absolute pretherapeutic values of the total PBL counts and of the lymphocyte subpopulations in the three patient groups, "regression," "stable disease," and "progression." Additionally, values of a control group of age-matched, healthy volunteers are shown which are in agreement with values cited in the literature [12]. We found that the pretherapeutic absolute values of total lymphocytes and of CD4$^+$ and CD8$^+$ T cells as well as of B lymphocytes correlated with the clinical response. Patients having values prior to systemic therapy below those of our control group often showed no response to the therapy. Figures 1 and 2 illustrate this result for two patients with different responses to the therapy. With respect to the absolute number of the whole T cells as well as of the CD4$^+$ and the CD8$^+$ T cells, we found significant differences both between the regression and progression groups and between the regression and stable disease groups. The absolute number of B cells was significantly lower in the progression group compared to the regression group and in the stable disease group compared to the regression group. No significant differences were found for the pretherapeutic values of NK cells, HLA-DR$^+$ T cells, CD25$^+$ T cells and the CD4/CD8 ratio between the three groups, though the number of CD25$^+$ T cells and the number of NK cells

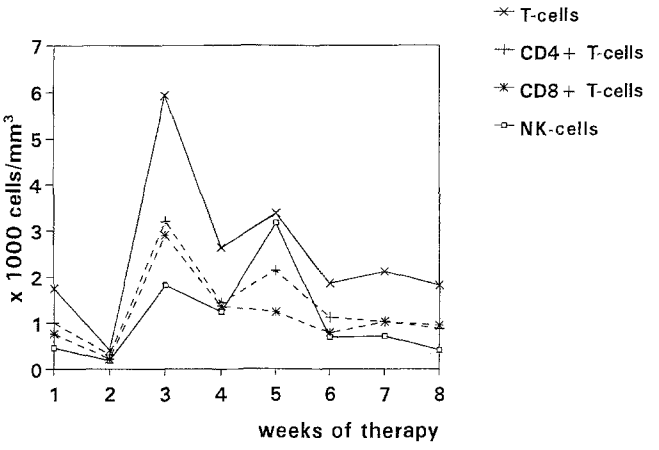

Fig. 1 Cell counts for a patient during 8 weeks of immunotherapy (response to the therapy)

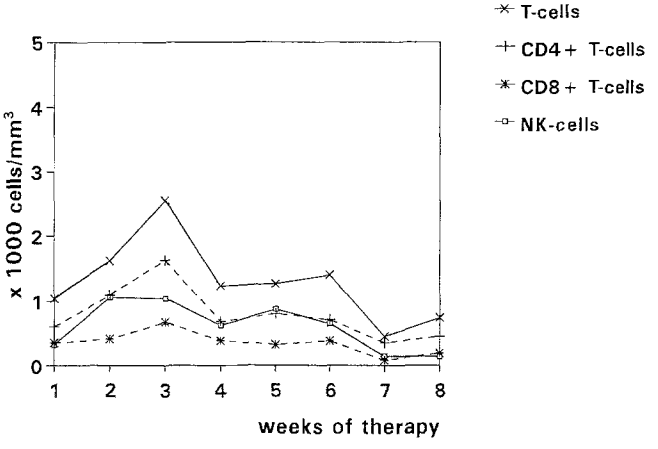

Fig. 2 Cell counts for a patient during 8 weeks of immunotherapy (no response to the therapy)

showed clearly higher figures in the regression group. In addition, the pretherapeutic CD4/CD8 ratio was lowest in the progression group. The mean ages of patients, 51, 59 and 56 years for regression, stable disease and progression groups; respectively, were similar. The male:female ratio was 14:1 in the group regression and 9:1 in the progression group; thus a gender bias in the observed differences is highly unlikely.

For the statistical analysis of all data during one treatment cycle, we calculated the mean values of all parameters to avoid the scatter of single data. As shown in Table 3, the absolute numbers of T cells, $CD4^+$ T cells and $CD25^+$ T cells was significantly higher in treatment cycles with the regression and stable disease results than in cycles scored progression. $CD4^+$ T cells were significantly higher in the regression than in the progression group and significantly higher in the stable disease group than in the progression group. No significant differences could be found for the absolute numbers of NK cells and $HLA\text{-}DR^+$ T cells between the three groups. The mean absolute number of B cells did not increase during the treatment cycles and remained higher in the regression than in the stable disease and progression groups. Though the progression group had a distinctly lower CD4/CD8 ratio, this difference was not statistically significant. Figure 3 illustrates the differences between the three response groups to the therapy with respect to the absolute numbers of T cells, $CD4^+$ and $CD25^+$ T cells, and CD4/CD8 ratio (mean values during therapy). The high standard deviations of the data, especially in the regression group (1), have to be taken into consideration. It is obvious from Fig. 3 that patients with complete or partial remission have a mean CD4/CD8 ratio ranging from 0.5 to 5.5. In contrast to the number of T-cells, the CD4/CD8 ratio is not a reliable marker for the estimation of treatment success. Table 4 shows the mean values and SD of the relative amounts of lymphocyte subpopulations over the therapy. Only for the $CD4^+$ and not for the $CD8^+$ T-cell subpopulation did we find significantly lower percentages in the progression group than in the regression or stable disease groups. This perhaps points to a special role of the $CD4^+$ T cells in the peripheral blood as an indicator of the clinical outcome for patients.

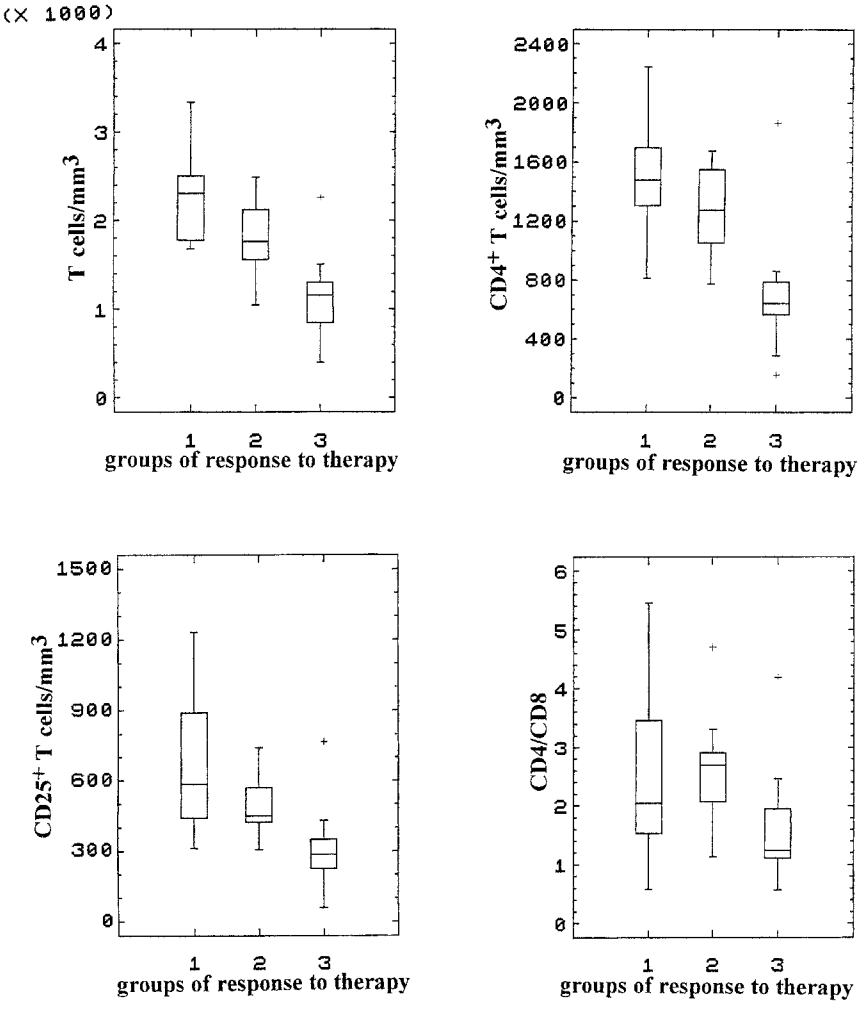

Fig. 3 Mean values of the absolute numbers of T cells, $CD4^+$ T cells, $CD25^+$ T cells and the ratio CD4/CD8 in the three groups of therapy response: *1* Regression, *2* Stable disease, *3* Progression during treatment cycles. Data are given as box plots with the median (*continuous line*). The box encompasses the 25th through 75th percentiles. The 5th and 95th percentiles are displayed as error bars

Table 3 Absolute amounts of the investigated lymphocyte subpopulations and of the CD4/CD8 ratio within the regression, stable disease and progression groups over the whole treatment cycles. Data given as means ± SD of all measurements taken during a cycle

Parameter	Regression (1) ($n = 15$) (mean ± SD)	Stable disease (2) ($n = 14$) (mean ± SD)	Progression (3) $n = 10$ (mean ± SD)	Significant differences between groups
T cells (cells/mm^3)	2295 ± 481	1829 ± 409	1183 ± 491	1–2, 1–3, 2–3
CD4$^+$ T cells (cells/mm^3)	1535 ± 380	1270 ± 301	705 ± 459	1–3, 2–3
CD8$^+$ T cells (cells/mm^3)	787 ± 413	541 ± 233	462 ± 135	1–2, 1–3
HLA-DR$^+$ T cells (cells/mm^3)	353 ± 117	369 ± 239	272 ± 117	–
CD25$^+$ T cells (cells/mm^3)	639 ± 266	499 ± 118	319 ± 185	1–3, 2–3
NK cells (cells/mm^3)	664 ± 326	580 ± 277	490 ± 205	–
B cells (cells/mm^3)	207 ± 114	139 ± 59	133 ± 170	1–2, 1–3
CD4/CD8	2.58 ± 1.49	2.59 ± 0.92	1.60 ± 1.07	–

Table 4 Relative amounts of the investigated lymphocyte subpopulations and the CD4/CD8 ratio within the regression, stable disease and progression groups over the whole treatment. Data are given as means ± SD of all measurements taken during a cycle

Parameter	Regression (1) ($n = 15$) (mean ± SD)	Stable disease (2) ($n = 14$) (mean ± SD)	Progression (3) ($n = 10$) (mean ± SD)	Significant differences between groups
T cells (% lymphocytes)	72 ± 9	73 ± 5	64 ± 10	1–3, 2–3
CD4$^+$ T cells (%)	48 ± 8	50 ± 7	36 ± 12	1–3, 2–3
CD8$^+$ T cells (%)	25 ± 12	22 ± 6	27 ± 7	–
HLA-DR$^+$ T cells (%)	15 ± 9	18 ± 8	25 ± 12	1–3
CD25$^+$ T cells (%)	27 ± 8	26 ± 6	25 ± 5	–
NK cells (% lymphocytes)	15 ± 8	20 ± 6	26 ± 8	1–3
B cells (% lymphocytes)	7 ± 4	6 ± 2	7 ± 3	–

Discussion

In this paper, we monitored the changes in PBLs of patients suffering from advanced RCC during therapy with IL-2, IFN-α and 5-FU. The data presented show a significant correlation of the clinical response to the immunotherapy applied with the absolute values of CD4$^+$ and CD8$^+$ T cells. Furthermore, the number of CD25$^+$ but not of HLA-DR$^+$ T cells during a therapy cycle correlated with the response of patients to the therapy. Remarkably, we found higher pretherapeutic numbers of total lymphocytes, of CD4$^+$ and CD8$^+$ T cells and of B cells in patients with partial or complete remission compared to the progression group of patients. No statistical difference was observed for the pretherapeutic numbers of NK cells, HLA-DR$^+$ or CD25$^+$ T cells and CD4/CD8 ratio.

Higher CD4/CD8 ratios have been reported for the peripheral blood T cells of patients suffering from RCC [20] than for persons suffering from other malignant diseases. During therapy, the authors found a mean value of 2.55 for the CD4/CD8 ratio in the case of RCC. For the patients considered in our study, a mean value of 2.07 was found. This difference could be explained by the use of other clones of monoclonal antibodies for CD4 and CD8. According to Raymond et al. [20], responders had higher pretherapeutic levels of the CD4/CD8 ratio in comparison to nonresponders. Also for small-cell lung cancer, increased survival rates were found [27] for patients having a higher CD4/CD8 ratio in their peripheral blood before chemotherapy and in follow-up. One could speculate that – with higher numbers of patients and lower standard deviations – in patients with renal cancer at least a significant correlation between a low CD4/CD8 ratio and decreased survival rates may exist. Other authors [3] reported that the number of NK cells is significant for the clinical response. In contrast to these results, we were unable to find significant differences in the number of NK cells before and during therapy within the different clinical response groups. Though the lowest numbers of NK cells could be found in patients with progression of disease, this difference was not statistically significant.

The fact that patients with partial or complete remission have significantly higher pre-therapeutic numbers of CD4$^+$ and CD8$^+$ T cells as well as of B cells is indicative of an ongoing immune response against the tumor cells in these patients. Obviously, the therapy with IL-2 and IFN-α supports this immune response, at least with respect to the T lymphocytes and NK cells. B-cell numbers in the peripheral blood did not increase during the treatment cycles, though they remained significantly higher in patients with partial or complete response. One could speculate that – also supporting B-cell proliferation with cytokines such as IL-4 or IL-6 – one might further improve the immunotherapy in

RCC patients. Indeed, initial attempts have been made in RCC using IL-6 or IL-4 as single agents in adoptive immunotherapy [26, 29], with a modest response in most patients. It would be interesting to study the immunophenotype during immunotherapy, e.g., with IL-2/IFN-α combined with IL-4 or IL-6.

$CD4^+$ T cells, which we found to be elevated in the peripheral blood of patients with a regressive disease, are considered to play a special role in the defense of RCC. Thiounn et al. [28] found an improvement in IL-2 therapy for patients with RCC by additional applications of $CD4^+$ tumor-infiltrating lymphocytes. Tumor-infiltrating lymphocytes of RCC are assumed preferentially to release cytokines of the Th2 type such as IL-4 [24]. Human RCC tumor lines express high-affinity IL-4 receptors [19] and IL-4 has been shown to specifically inhibit the growth of these cell lines in vitro [14, 19]. More recent studies demonstrate that RCC-specific cytotoxic T lymphocytes do exist [22] or compare the cytolytic activity of $CD4^+$ and $CD8^+$ tumor-infiltrating lymphocytes in RCC [11].

In conclusion, this study shows that investigation of the immunophenotype in the peripheral blood provides essential information on the clinical outcome of patients. The pretherapeutic monitoring of the number of lymphocytes and their subpopulations may help to select patients with metastatic RCC which will respond to immunotherapy. It is clear that without a true control group (patients without systemic immunotherapy), which, however, in our opinion is not feasible for ethical reasons, it is difficult to evidence directly the efficacy of immunotherapy. The data presented here clearly show, however, the response of patients to therapy in the increase of lymphocyte counts, and this response was significantly higher in the regression group than in the progression group. We are aware that therapy response is only one side of the coin. A higher rank has to be given to parameters such as survival time or prolongation of the time free of relapse as well as to the quality of life of patients. Further investigations by ourselves will deal with the question of whether the number of PBLs correlates with the time of survival of patients, or whether a different immunotherapy regimen may cause differences in the immunophenotype in the peripheral blood. Still more important to us seems the question of whether the correlation between lymphocyte numbers in peripheral blood and response to therapy will also be found in other forms of cancer.

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