Immune signature of tumor infiltrating immune cells in renal cancer

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Abbreviations: mAb, monoclonal antibody; MDSC, myeloid-derived suppressor cells; MFI, mean fluorescence intensity; NK, natural killer cells; PE, phycoerythrine; RCC, renal cell carcinoma; TIL, tumor-infiltrating lymphocytes.

Tumor-associated immune cells have been discussed as an essential factor for the prediction of the outcome of tumor patients. Lymphocyte-specific genes are associated with a favorable prognosis in colorectal cancer but with poor survival in renal cell carcinoma (RCC). Flow cytometric analyses combined with immunohistochemistry were performed to study the phenotypic profiles of tumor infiltrating lymphocytes (TIL) and the frequency of T cells and macrophages in RCC lesions. Data were correlated with clinicopathological parameters and survival of patients. Comparing oncocytoma and clear cell (cc)RCC, T cell numbers as well as activation-associated T cell markers were higher in ccRCC, whereas the frequency of NK cells was higher in oncocytoma. An intratumoral increase of T cell numbers was found with higher tumor grades (G1:G2:G3/4 1:3:4). Tumor-associated macrophages slightly increased with dedifferentiation, although the macrophage-to-T cell ratio was highest in G1 tumor lesions. A high expression of CD57 was found in T cells of early tumor grades, whereas T cells in dedifferentiated RCC lesions expressed higher levels of CD69 and CTLA4. TIL composition did not differ between older (>70 y) and younger (<58 y) patients. Enhanced patients’ survival was associated with a higher percentage of tumor infiltrating NK cells and Th1 markers, e.g. HLA-DR+ and CXCR3+ T cells, whereas a high number of T cells, especially with high CD69 expression correlated with a worse prognosis of patients. Our results suggest that immunomonitoring of RCC patients might represent a useful tool for the prediction of the outcome of RCC patients.

Introduction

RCC accounts for approximately 5% of all human malignancies in the Western world and its incidence is steadily increasing. It is a heterogeneous disease consisting of the most frequent clear cell carcinoma (ccRCC) (75%) and the renal papillary carcinoma with an incidence of 10–15%, both arising from epithelial cells of the proximal tubuli. In contrast, chromophobe RCC (5%) and the benign oncocytoma (3%–7%) arise from the intercalating cells of the collecting ducts of the kidney.1

The clinical outcome of RCC patients is highly variable. Due to the lack of symptoms approximately one-third RCC patients have already an advanced disease at the time of diagnosis.2 Furthermore, 25–30% of RCC patients treated for local disease develop metastasis. Radical or partial nephrectomy remain the only effective method of cure for localized disease.3 The outcome of RCC patients with metastatic disease is poor with a 5 y survival rate of less than 5%. Treatment options have been limited due to the resistance to chemotherapy and radiation. Recently, the treatment of metastatic RCC has been revolutionized by the development of targeted therapies, in particular by tyrosine kinase inhibitors (sunitinib, sorafenib, pazopanib, axitinib), anti-angiogenic agents such as bevacizumab, and mTOR inhibitors, which have been shown to increase the progression-free survival of RCC patients.4

Despite considerable progress in our understanding of the pathogenesis, genetics, and pathology of RCC, the prediction of the clinical outcome for individual cases remains difficult. RCC represents an immunosensitive tumor due to the relatively high spontaneous regression rate and the high levels of tumor infiltrating immune cells, including lymphocytes, macrophages and dendritic cells. Tumor-infiltrating lymphocytes (TIL) are influenced by the local cellular and soluble components of the tumor microenvironment.5 Despite antigen-specific HLA-restricted TIL clones can be found after culture of RCC tumor tissues,6,7,8 the function of these T cells in RCC is often impaired – due to their...
Tumor cells have developed different strategies to escape immune surveillance, such as loss or downregulation of MHC class I expression, expression of co-inhibitory molecules as well as secretion of cytokines and factors, which create an immune suppressive microenvironment. These processes lead to a failure of the local antitumor response and thus contribute to tumor progression. Recent studies demonstrated that the density of CD8+ T cells correlated with poor prognosis in RCC patients. Furthermore, high numbers of immune suppressive cells, such as regulatory T cells or myeloid derived suppressor cells (MDSC) in the peripheral blood and/or tumor lesions of RCC patients, were associated with a poor clinical outcome. In order to increase the knowledge about the control and dynamics of immune responses directed against RCC, the immune cell phenotype of renal tumor lesions was characterized in this study. Flow cytometric analysis combined with immunohistochemistry was performed and correlated with tumor histology, Fuhrmann grading, tumor size, age of patients as well as patients’ survival.

### Results

#### Comparison of the frequency of infiltrating immune cells in oncocytoma and ccRCC

The frequency of intratumoral T cells (CD3+) was determined in paraffin-embedded sections of kidney tumor lesions demonstrating an approximately six-fold difference, with 16 ± 2 T cells per 2.5 mm² in oncocytoma and 105 ± 17 in ccRCC (p = 0.028). The frequency of intratumoral CD68+ macrophages was 10-fold higher in ccRCC (239 ± 17 per 2.5 mm²) when compared to oncocytoma (23 ± 2) (p = 0.004). The ratio of T cells to macrophages was 1–1.4 in oncocytoma, but 1–2.3 in ccRCC. In contrast to oncocytoma, the peritumoral number of T cells and macrophages in ccRCC was lower than the respective intratumoral number.

Next to immunohistochemical analysis, TIL obtained from different tumor histologies were stained with a large panel of mAbs followed by four-color flow cytometry. The viability of TIL was 97.6 ± 0.35. As summarized in Table 1, T cells represent 47–76% of TIL, the dominant lymphocytic population in most cases, while B cells were rarely detected (4–6% of TIL). Several characteristic features were found by comparing the frequency of lymphocytes in oncocytoma to that of ccRCC, while only marginal differences exist between papillary RCC and ccRCC. Interestingly, a high percentage of NK cells could be detected in oncocytoma (Fig. 1). While T cells and NK cells exert an approximately equal proportion in oncocytoma, NK cells represent only 20% of TIL in ccRCC, a value nearly comparable to peripheral blood. In oncocytoma CD8+ T cells dominated, while a balanced ratio between CD4+ and CD8+ T cells existed in ccRCC and papillary RCC. Additionally, T cells in oncocytoma showed a significant lower frequency of activation-associated surface molecules, such as CD26, CD69, and HLA-DR and a higher frequency of the immune senescence marker CD57 despite the latter difference was not significant. TIL of

| % of lymphocytes | Oncocytoma n = 6 | ccRCC n = 92 | Papillary RCC n = 6 | oncocytoma-ccRCC |
|------------------|------------------|------------------|---------------------|------------------|
| T Cells          | 47.4 ± 7.9       | 76.4 ± 1.4       | 70.5 ± 4.1          | 0.014            |
| B Cells          | 6.1 ± 2.3        | 3.6 ± 0.4        | 5.0 ± 2.3           | 0.004            |
| NK Cells         | 46.6 ± 5.6       | 20 ± 1.3         | 24.4 ± 3.4          |                  |
| % of T cells     |                  |                  |                     |                  |
| CD4+             | 40.3 ± 3.6       | 49.7 ± 1.4       | 55.5 ± 5.1          | 0.046            |
| CD8+             | 55.4 ± 3.3       | 55.3 ± 1.7       | 51.3 ± 4.2          |                  |
| Ratio CD4/CD8    | 0.7 ± 0.09       | 1.06 ± 0.07      | 1.13 ± 0.2          |                  |
| CD26+            | 25.9 ± 4.6       | 48.3 ± 1.8       | 49.3 ± 5.8          | 0.0001           |
| CD13+            | 1.6 ± 0.5        | 32.4 ± 2.3       | 25.7 ± 5.6          | 0.003            |
| CD28+            | 41 ± 5.4         | 63.3 ± 1.9       | 70.1 ± 7.6          | 0.007            |
| CD57+            | 46.7 ± 7.8       | 32.5 ± 1.5       | 23.4 ± 3.0          | 0.0001           |
| CD69+            | 18 ± 2.6         | 58.2 ± 2.5       | 78 ± 4.1            | 0.0002           |
| HLA-DR+          | 40.2 ± 6.8       | 63.8 ± 1.8       | 60.7 ± 8.1          | 0.017            |
| CD107+           | 2.0 ± 0.6        | 6.1 ± 1.1        | 1.8 ± 0.4           | 0.002            |
| CCR5+            | 52.2 ± 9.1       | 55.7 ± 2.9       | 54.7 ± 13.8         |                  |
| CTLA4+           | 1.4 ± 0.3        | 1.0 ± 0.1        | 2.7 ± 0.9           |                  |
| CCX3+            | 52 ± 7.6         | 68.7 ± 2.1       | 62.7 ± 13.0         |                  |
| Vn24+ -NK+       | 3.6 ± 1.5        | 1.4 ± 0.2        | 4.0 ± 2.2           |                  |
| % of CD4+ T cells|                  |                  |                     |                  |
| CD4+ -CD7−       | 31.4 ± 8.2       | 29.3 ± 1.6       | 21 ± 5.7            |                  |
| % of CD8+ T cells|                  |                  |                     |                  |
| CD8+ -CD7−       | 13.7 ± 5.7       | 13.7 ± 1.2       | 5.6 ± 1.5           | 0.01             |
| CD28+ -CD8+      | 25.7 ± 5.0       | 44.8 ± 2.2       | 48.1 ± 12.4         |                  |
| % of NK cells    |                  |                  |                     |                  |
| NKp44+           | 8.7 ± 4.7        | 9.3 ± 1.2        | 36 ± 14.5           |                  |

Table 1. Composition of tumor infiltrating lymphocytes of renal tumors of different histology as result of flow cytometric analyses. Data were given as mean ± SE. Significant differences between oncocytoma and clear cell (cc) renal cell carcinoma (RCC) are shown (t test)
oncocytoma were nearly CD13 negative, whereas TIL of ccRCC expressed variable amounts of this surface peptidase as already described earlier. Moreover, CD107+ T cells were highest in ccRCC, whereas papillary carcinoma and oncocytoma had equally low values. In papillary carcinoma the highest values of CTLA4+ and CD69+ T cells and of NKp44+ NK cells were detected. (Table 1)

**Correlation of the ccRCC TIL phenotype with grading and tumor size**

Tumor stage and Fuhrmann grade are the most important outcome predictors for patients with localized RCC. By comparing the immune cell repertoire of ccRCC, differences in the frequency and functional markers of immune cell subpopulations were obvious for tumor grades than for tumor stages. Most of RCC lesions were classified as Fuhrmann grade 2 (n = 62).

Immunohistochemical analysis revealed an in particular intratumoral increase of the absolute T cell number in higher grades, ranging from a three-fold increase in G2 to a four-fold increase in G3/4 when compared to G1 tumors, respectively (Fig. 2A). The difference between higher intratumoral and lower peritumoral T cells was especially pronounced in G2 and G3/4 ccRCC. The number of macrophages slightly increased with dedifferentiation (Fig. 2B), also with higher intratumoral than peritumoral values. Intracellular CD68+ cells raised from 214 ± 70 per 2.5 mm² in G1 tumor lesions to 282± 61 in G3/4. The ratio of macrophage-to-T cell was (with 3.9) highest in G1 tumor lesions (Fig. 2C) suggesting that during tumor cell dedifferentiation T cell numbers raised more than macrophage numbers.

With respect to flow cytometric data, similar low percentages of B cells were found comparing G1 and G3/4 tumor lesions with a tendency to higher B cell percentages in G3/4 (Table 2). The frequency of NK cells was with 10% of TIL significantly lower in G3/4 tumors. The decrease of the NK cell proportion was accompanied by an increase in the percentage of T cells. No difference was found for the percentage of Vα24+ NKT cells between different tumor grades. Neither the proportion of CD4+ T cells significantly differed between different tumor grades nor did the ratio CD4+/CD8+ T cells. However, a tendency toward higher percentages of CD8+ T cells was observed in undifferentiated RCC lesions (Table 2, Fig. 3). With respect to function-associated molecules, the early activation marker CD69 exerted the highest frequency in G3/4 tumor grades (82% of T cells; Fig. 3). The activation marker HLA-DR was expressed at a high frequency on T cells (66–69%), without any significant difference between tumor grades. The percentages of CD7neg/CD4+ T cells as well as the percentage of CD57+ T cells were highest in G1 tumor lesions and decreased with dedifferentiation (Fig. 3). The percentage of CD107+ T cells was lowest in G1, whereas the frequency of CTLA4+ T cells was the highest in undifferentiated ccRCC, as was the proportion of NKp44+ NK cells (Table 2).

With respect to tumor size (Table S1) the percentage of T, B or NK cells did not significantly differ between early and late tumor stages. The percentage of NK cells showed a tendency to lower values in T2/3 stages compared to T1, whereas T cell percentages were higher in late stages. With respect to function-associated surface molecules, the percentage of CD69+ and CTLA4+ T cells increased with staging. The percentage of CD107+a+ T cells showed the highest values in T2 tumor lesions, decreasing in T3. CD7neg/CD4+ T cells were higher in T1 tumor stages, though the difference between G1 versus G3/4 (42 vs. 24% of T cells) was more compelling than the difference between T1a versus T3 (34 vs. 25% of T cells).

**Comparison of the TIL phenotype in ccRCC of younger versus older patients**

The average age of patients was 66.8 ± 9.7 y (range 41–91 y). We compared the TIL phenotype between patients <58 y (n = 12) with patients >70 y (n = 34), since young age (< 55 y) has been discussed as prognostic factor. As shown in Table S2 no significant difference could be found between the both groups of patients. We observed a tendency to higher percentages of CD8+ T cells as well as a higher CD69 expression of T cells in the group “patients >70 y.”

Correlation of the immune cell repertoire composition with the RCC patients’ survival. After a median follow up time of 25.8 mo (range 0–66 mo), 24% of RCC patients had died.
Addressing the prognostic value of the lymphocytic composition of TIL (Table S3, Fig. 4), a high percentage of NK cells was associated with an increased survival of RCC patients. Furthermore, a trend was observed toward shorter survival for high T cell percentages in univariate Kaplan–Meier analysis, but could not be confirmed in multivariate analysis (Cox regression). This was in line with the lack of a correlation between the frequency of CD4+ or CD8+ T cells, respectively, with the patients’ survival. Addressing the possible prognostic value of the expression of activation-associated molecules on T cells, a better survival was found in RCC patients with a high percentage of HLA-DR+ T cells and higher intensity of CXCR3 expression on T cells (Cox regression test). Furthermore, a lower frequency of CD69 expressing T cells was favorable for the survival of RCC patients, while a frequency of CD13+ T cells in a range of 20–45% correlated with worse prognosis (Kaplan–Meier). The absolute numbers of T cells and macrophages as well as the T cell-to-macrophage-ratio in stained paraffin-embedded RCC tissues did not correlate with survival in our analyses (data not shown).

**Discussion**

Immune cell infiltration is a common feature of RCC, but the contribution of lymphocytes to tumor elimination remains unclear. RCC are predominantly infiltrated by T- and NK cells expressing markers of activation and of memory suggesting a tissue-specific T cell activation in RCC. Despite convincing evidence of immune reactions to tumor cells exists, these immune responses remain ineffective, and the tumor infiltrated by immune cells continues to grow. The mechanisms that cause tumor escape include suppression of effector cells or immune cell dysfunction which could be mediated by immunosuppressive factors of the tumor microenvironment, including TGF-β, IL-10, gangliosides, adenosine, products of oxidative stress and thrombospondin, or by the tumor cells itself expressing e.g., the non-classical HLA-G molecules and co-inhibitory molecules of the B7-H family. Due to the interaction with KIR2DL4, ILT2 and/or ILT4, HLA-G negatively interferes with NK and T cell immune responses (for review see), while B7-H1 binds to PD1 thereby blocking T cell responses.

The phenotypic and functional profile of tumor-infiltrating immune cells varies depending on the tumor subtype and the tumor microenvironment and may influence prognosis and disease outcome. During the last years, the analysis of the microenvironment has kindled interest since the nature, the density and localization of cells of the adaptive immune system directly influence the risk of relapse and can be superior to the TNM classification in some cancers, in particular colorectal carcinoma. Using Cox regression analyses, the prognostic value of markers specifically expressed in B and T cells was correlated with overall survival. Whereas lymphocyte-specific genes were associated with a favorable prognosis in colorectal cancer, this was not the case for RCC. In this study, a combination of flow cytometry and immunohistochemistry revealed, that (i) a high NK cell percentage as well as Th1-associated markers, such as HLA-DR and CXCR3 were associated with better survival in ccRCC, (ii) the

**Table 2.** Composition of tumor infiltrating lymphocytes of renal carcinoma (clear cell type) of different nuclear grading, as estimated by 4-color antibody staining and flow cytometry. Data were given as mean ± SE. Significant differences between the 3 groups of nuclear grading are shown (n.s.: not significant).

|                  | Grading 1 n = 11 | Grading 2 n = 62 | Grading 3/4 n = 18 | ANOVA (Bonferroni) |
|------------------|------------------|------------------|-------------------|-------------------|
| % of lymphocytes |                  |                  |                   |                   |
| T cells          | 70.1 ± 5.4       | 78.5 ± 3.0       | 84.1 ± 4.2        | G1 versus G3/4, G2 vs. G3/4 |
| B cells          | 3.4 ± 1.6        | 2.3 ± 0.9        | 5.8 ± 1.2         | n.s.              |
| NK cells         | 25.3 ± 4.9       | 21.3 ± 2.8       | 10.1 ± 3.8        | G1 vs. G3/4, G2 vs. G3/4 |
| % of T cells     |                  |                  |                   |                   |
| CD4+            | 51.8 ± 5.5       | 52.9 ± 3.1       | 48.1 ± 4.3        | n.s.              |
| CD8+            | 47.3 ± 6.4       | 53.1 ± 3.5       | 58.7 ± 5.0        | n.s.              |
| Ratio CD4/CD8    | 1.1 ± 0.2        | 1.0 ± 0.1        | 0.8 ± 0.3         | n.s.              |
| CD26+           | 54.6 ± 7.5       | 53.7 ± 4.1       | 45.0 ± 5.8        | n.s.              |
| CD13+           | 36.7 ± 8.4       | 41.2 ± 4.7       | 34.5 ± 6.5        | n.s.              |
| CD28+           | 64.2 ± 7.3       | 68.5 ± 4.1       | 73.2 ± 5.7        | n.s.              |
| CD57+           | 35.8 ± 5.5       | 33.1 ± 3         | 26.4 ± 4.2        | G1 versus G3/4    |
| CD69+           | 56.2 ± 8.4       | 58.1 ± 4.7       | 78.9 ± 6.5        | G1 versus G3/4, G2 versus G3/4 |
| HLA-DR+         | 65.6 ± 6.9       | 68.7 ± 3.8       | 67 ± 5.3          | n.s.              |
| CD107a+         | 2.5 ± 2.1        | 7.9 ± 2.5        | 8.6 ± 3.4         | n.s.              |
| CCR5+           | 40.1 ± 10.9      | 60.9 ± 6.1       | 54.0 ± 8.5        | n.s.              |
| CTLA4+          | 0.7 ± 0.4        | 0.8 ± 0.2        | 1.8 ± 0.3         | G1 versus G3/4, G2 versus G3/4 |
| CXCR3+          | 70.5 ± 8.7       | 77.6 ± 4.8       | 71.3 ± 6.7        | n.s.              |
| Vα24+/NKT       | 0.7 ± 0.6        | 1.5 ± 0.4        | 0.8 ± 0.6         | n.s.              |
| % of CD4+ T cells |              |                  |                   |                   |
| CD7neg          | 41.7 ± 7         | 26.3 ± 3.9       | 23.9 ± 4.8        | G1 versus G2, G1 versus G3/4 |
| % of CD8+ T cells |              |                  |                   |                   |
| CD7neg          | 16.5 ± 5.3       | 17 ± 3           | 8.5 ± 3.8         | G2 versus G3/4    |
| CD28+           | 43.9 ± 8.9       | 49.6 ± 4.9       | 55.7 ± 6.9        | n.s.              |
| % of NK cells   |                  |                  |                   |                   |
| NKP44+ NK       | 3.8 ± 1.4        | 8.0 ± 2.3        | 16.9 ± 3.6        | G2 versus G3/4    |
percentage of macrophages to T cells was highest in G1 tumor grades as is the percentage of CD57+ T cells, (iii) the T cell percentage and the absolute number of T cells increased with dedifferentiation of ccRCC, (iv) a poor prognosis was associated with special T cell molecules, such as CD13 and CD69.

Figure 2. Intratumoral T cells and macrophages increase during renal tumor dedifferentiation. T cells (CD3+) and macrophages (CD68+) were counted in 2.5 mm² (average of 10 representative fields) after immunohistochemical staining (peroxidase) of 2-μm paraffin-embedded sections of oncocytoma (OCT; n = 6) and renal cell carcinoma (clear cell histology) of different nuclear grading (n = 62). Absolute intratumoral and peritumoral cell numbers are shown for T cells (A) and macrophages (B). The highest ratio of “macrophages to T cells” was found in G1 tumor grades, as illustrated in (C). Results are given as mean value ± SE.

Using flow cytometry, we compared data of the benign oncocytoma with ccRCC. Oncocytoma are rare renal epithelial neoplastic lesions that exhibit antigenic characteristics of distal nephron intercalated cells.22 Data characterizing lymphocytic

Figure 3. The phenotype of tumor infiltrating lymphocytes differs with dedifferentiation of renal tumor lesions. Tissues of oncocytoma (OCT, n = 6) and renal cell carcinoma (clear cell type) of different nuclear grading (n = 7, 46, 9 for G1, G2, G3/4) were treated directly after mechanical tumor dissociation with antibody staining and analyzed by 4-color flow cytometry as described in materials and methods. Tumor dedifferentiation goes along with the enrichment of T cells (CD3+) and a parallel decrease of NK cell percentages (CD56+CD3neg). The percentage of CD8+ T cells does not correlate with tumor grading. Whereas CD69 expression of T cells clearly increases with tumor dedifferentiation, the percentage of CD57+ T cells and of CD4+CD7negative T cells rather decrease. Results are given as mean value ± SE. Asterisks mark significant differences (ANOVA, Bonferroni) with the respective reference parameter shown as an open circle.
infiltration of oncocytoma are lacking. In 1992 Stőrkel and co-authors described a low lymphocytic infiltration density in oncocytoma compared to ccRCC, which was confirmed by us and extended to NK cells and markers of T cell activation. The frequency of NK cells was nearly 50% in oncocytoma, since T cells are rare. TIL in oncocytoma compared to renal cancer differed with respect to the expression of function-associated markers, with a lower T cell expression of CD13, CD26, CD28, CD69 and HLA-DR and a high percentage of CD57+ T cells. A transition from markers expressed in oncocytoma, in good-differentiated and in poor-differentiated ccRCC tumor lesions was found in some markers, in particular in the percentage of NK cells, in T cell count or in the percentage of CD69+ T cells. The latter increased with grading and staging and correlated with poor patient’s survival. Tumor-induced CD69+ CD4+CD25neg T cells have been claimed to be a new subset of regulatory T cells, since they are not Foxp3+, but express membrane-bound TGF-1 and can suppress T cell proliferation in a cell–cell contact manner. Therefore, CD69+ T cells could play a major role in the transition from a renal tumor controlled by immune cells to a tumor which evades immune control. A high expression of CD69+CD25neg T cells was found in chronic pancreatitis as well as in pancreatic ductal adenocarcinoma (associated with nodal invasion and higher grading) and L1CAM/CD171 expression of tumor cells reduced proliferation, diminished CD25 expression and elevated CD69 expression in effector T cells.

We found that the percentage of NK cells decreased in ccRCC with higher grading, while the number of T cells increased. Since the absolute numbers of T cells are four-fold higher in G3/4 tumors, the decrease in NK cells might be only in the percentage of lymphocytes and not in absolute cell numbers. However, a higher percentage of NK cells predicts a better survival of RCC patients in our analysis, which is in line with other reports. As recently shown, NK cells from TIL with a high percentage of NK cells (>20%) are CD16bright and non-cytolytic ex vivo, but developed cytotoxicity after a short-term activation with IL-2. Only a few CD16bright NK cells and lower perforin expression were found in tumors with low NK cell frequencies. These data suggest a functional difference according to NK cell density as well as an influence of T cells on NK cell function, which has to be clarified in future work.

This study confirms the high expression level of markers of T cell exhaustion in TIL of RCC, such as CD57. However, both the accumulation of CD57+ T cells as well as of CD7neg/CD4+ T cells was more pronounced in low grade and not in high grade RCC lesions and even T cells of oncocytoma showed a high expression of CD57. Though CD57 has been claimed to be a general marker of proliferative instability (for a review see

![Figure 4. Relationship between the phenotype of tumor infiltrating lymphocytes of renal cell carcinoma (clear cell type) and patient’s survival. Patients with higher T cell and lower NK cell percentage in tumor tissue were closely correlated with poorer overall survival. A better survival was correlated with a high percentage of TH1-associated markers, such as HLA-DR expression or CXCR3 intensity, as well as a lower CD69 expression of T cells. A tendency to a better overall survival was found for a low CD13 expression of T cells. Kaplan–Meier analysis is shown, patients with censored survival times are denoted by tick marks.](image-url)
ref.27), CD57 expression correlates strongly with simultaneous expression of granzymes A, B, and perforin.28 Therefore, our data might implement a high cytotoxic potential of CD57+ T cells in oncocytoma and early grade ccRCC, but not in poor-differentiated tumors. However, in melanoma CD8+CD57+ T cells have been described as incompletely-differentiated cytotoxic T cells with hybrid phenotypic and functional properties of both an early effector-memory type and a terminally-differentiated effector cell.29 These T cells co-expressed CD27, CD28, CD57, and granzyme B, but little or no perforin. They could be induced to proliferate, produce a high level of IFNγ, and to differentiate into perforin (high) mature cytotoxic T cells in vitro, but addition of TGF-β1 prevented further differentiation. Our data give no hint to an association of CD57 expression and the patients’ survival.

Using T-cell receptor clonotype mapping of expanded TIL, low numbers of distinct expanded T-cell clonotypes were found in RCC as compared with melanoma lesions.8 Comparing TIL in melanoma and RCC a more pronounced T-cell differentiation was reported in RCC (e.g., a higher percentage of effector-memory T cells).30 The authors discuss that CD70 expression as a peculiarity of RCC cells can result in T-cell costimulation and subsequently in proliferative exhaustion of T cells. However, no association was found between CD70 expression and staging or grading of RCC by other authors.30 A comparison of TIL from RCC and lung cancer demonstrated a higher frequency of polarized tumor-associated macrophages and showed that especially CD206/mannose receptor-positive macrophages (M2 type) are associated with reduced survival in RCC patients.38 Recently, markers for human MDSC have been developed (e.g., CD14+HLA-DRlow) therefore the inclusion of myeloid cells in flow cytometric analyses would be a worthwhile approach in future studies. A higher amount of HLA-DRlow (CD13high) monocytes in the blood of RCC patients has been described already especially in late tumor stages and might be the cause for poor results obtained with cancer vaccines of monocyte-derived dendritic cells.40

In conclusion, this study demonstrated peculiarities of tumor-infiltrating immune cells in renal tissues. Further understanding of the dynamics of intra-tumoral NK cells, T cells and macrophages would provide key insights into the process of immune suppression in renal cancer and help to develop successful immunotherapies, urgently needed especially for metastatic RCC.

Materials and Methods

Patients and tumor samples

The study was performed with the approval from the Ethics Committee of the University and comprised 104 RCC patients.

Table 3. Summary of patient’s characteristics and histological subtypes of renal tumors.

| Tumor grade | ccRCC | papillary RCC | oncocytoma |
|-------------|-------|---------------|------------|
| pT1a        | 27 (29.3%) | 2 (33.3%) | 0 |
| pT1b        | 26 (28.3%) | 2 (33.3%) | 1 (16.7%) |
| pT2         | 8 (8.7%) | 1 (16.7%) | 0 |
| pT3         | 3 (3.3%) | 1 (16.7%) | 0 |
| pT3a        | 10 (10.9%) | 0 | 1 (16.7%) |
| pT3b        | 18 (19.6%) | 0 | 0 |
| Number      | 92 | 6 | 6 |
| Gender      |       |       |   |
| Female      | 39 (42.4%) | 1 (16.7%) | 4 (66.7%) |
| Male        | 53 (57.6%) | 5 (83.3%) | 2 (33.3%) |
| pT stage    |       |       |   |
| G1          | 11 (12.0%) | 1 (16.7%) | 0 |
| G2          | 62 (67.4%) | 3 (50.0%) | 1 (16.7%) |
| G3/4        | 18 (19.6%) | 0 | 1 (16.7%) |
who underwent surgery of the primary tumor at the Department of Urology of the Martin Luther University Halle-Wittenberg. After informed consent, tumor samples were obtained immediately after surgical resection. Tumors were harvested into sterile antibiotic-containing RPMI1640 culture medium and processed within 6 h. Histological types of renal tumor samples were classified according to WHO criteria: 92 clear cell carcinoma, 6 papillary carcinoma, 6 samples oncocytoma. Patients’ characteristics are given in Table 3. The average age of patients was 66.8 ± 9.7 y (range 41–91 y). 84 patients were male and 20 were female. Patients received no anticancer therapy before surgery. For analysis of overall survival, patients were censored at the time of their last clinical follow-up appointment or at their date of death.

Antibodies and TIL sample preparation
TIL were analyzed directly after mechanical tumor dissociation and without being cultured. Tumors were dissected into 5 mm³ pieces and filtered through a coarse wire grid. Cells were used directly after passing through a 70 µm cell strainer for phenotypical analysis with 4-color staining of cells. The monoclonal antibodies (mAbs) purchased from BD Biosciences were CD3 PerCP, CD8 APC, CD13 PE, CD45 PerCP, CD56 FITC, CD69 PE, CD107 FITC, CCR5 PE, CTLA4 (cytotoxic T lymphocyte-associated antigen 4)/CD152 APC, HLA-DR PE; those purchased from Miltenyi Biotec encompassed CD3 FITC, CD4 PE, CD7 FITC, CD28 PE, CD45 FITC, CD57 FITC. Antibodies from Beckman Coulter GmbH included NKp44 PE and TCVRo24 FITC, mAbs from DAKO were CD19 PE and CD26 FITC. The CXCR3 FITC was from R&D Systems. After incubation, red cell lysis and washing, cells were fixed and examined using a FACS Calibur and the Cellquest software (BD Biosciences, Heidelberg, Germany). Cell viability was investigated inched using Student’s t-test, ANOVA, Bonferroni correction, Wilcoxon–Mann–Whitney test, and chi-squared test, respectively. All p-values are exploratory. The duration of follow up was calculated from the date of nephrectomy to the date of death or last follow-up. Survival analysis firstly comprised a descriptive presentation of the cumulative survival functions according to Kaplan–Meier. After univariate analyses, factors were examined in a multivariate Cox regression model adjusted to prognostic relevant parameters such as Fuhrmann grading (analyzed using two indicator variables for grades 3 and 4, with grades 1 and 2 as the reference), tumor size (analyzed using three indicator variables for stages II, III, IV, with stage I as the reference), and age of patients.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Supplemental Material
Supplemental data for this article can be accessed on the publisher’s website.
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