Regulatory T cells, dendritic cells and neutrophils in patients with renal cell carcinoma

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ABSTRACT

We evaluated dendritic cells (DC), regulatory T lymphocytes (Treg) and neutrophils in 37 patients with newly diagnosed renal cell carcinoma (RCC) in the tumor and peripheral blood (PB) and correlated these parameters with tumor staging (early-T1, 2, late-T3, 4 and metastatic disease). The number of myeloid and plasmacytoid DC in blood of RCC patients was higher than in healthy controls. The percentage of myeloid dendritic cells (mDC) from CD45+ cells in tumors was higher in comparison with peripheral blood irrespective of disease stage. Higher percentage of these cells expressed a maturation marker in the periphery in the early stage (CD83 expressing cells). The number of plasmacytoid dendritic cells (pDC) in PB was similar in both early and late stage groups, but the early group displayed a significantly higher percentage of pDC in tumor cell suspension. Neutrophil counts in the peripheral blood of RCC patients were higher than in healthy controls, but the counts in both tumor stage groups were similar. The proportion of neutrophils from CD45+ cells was higher in late stage tumors. Higher percentage of Treg from CD4+ cells was detected in renal carcinoma tissue in comparison to PB with no difference between stages of the disease. Our results reflect the complex interplay between various cells of the immune system and the tumor microenvironment. Activation of dendritic cell subpopulations at early stages of the disease course is counterbalanced by the early appearance of T regulatory cells both in the periphery and tumor tissue. Later stages are characterized by the accumulation of neutrophils in the tumor. Appropriate timing of antitumor strategies, especially immunotherapy, should take these dynamics of the immune response in RCC patients into account.

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1. Introduction

Renal cell carcinoma is a common urologic tumor and accounts for about 3% of all human malignancies. Reports of its incidence have increased steadily in recent decades. In 2008, 56,678 new cases were diagnosed in the USA and 73,171 in the European Union [1]. It is estimated that approximately 25–30% of all patients with RCC have metastases at the time of diagnosis and even following complete resection of the primary tumor by radical or partial nephrectomy, relapse occurs in 20–30% of RCC patients [2]. So far, patients with RCC profit only from immunotherapy (high dose IL-2, IFNα+ bevacizumab) or targeted therapy (tyrosine kinase inhibitors, mTOR inhibitors) as long as RCC is refractory to conventional treatment such as chemotherapy and radiotherapy.

The growth of tumor cells is greatly influenced by the immune system [3]. It is necessary to discover how the tumor cells resist and avoid immune system control. So the definition of tumor milieu is of utmost importance. Dendritic cells as the major antigen presenting cells induce reaction to foreign antigens as well as tolerance to self-antigens. Plasmacytoid dendritic cells initiate a response to viral CpG containing oligodeoxynucleotides by production of either IFNα or IL-6 [4]. Myeloid dendritic cells play significant roles in the development and maintenance of immune responses by production of Th1 or Th2 leading cytokines and by direct contact with effector cells. They are directly involved in the antitumor T-cell response [5] as well as preserving tolerance of tumor cells [6,7]. This dual role contributes to controversial results of many studies in different tumors: i.e., patients suffering from head-and-neck or liver cancer who have high levels of tumor-infiltrating DC had

Abbreviations: DC, dendritic cells; Treg, regulatory T lymphocytes; RCC, renal cell carcinoma; PB, peripheral blood; mDC, myeloid dendritic cells; pDC, plasmacytoid dendritic cells; CTL, cytotoxic T lymphocytes; NK, natural killer; TIN, tumor infiltrating neutrophils.

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significantly higher tumor-free survival rates compared to patients with low DC infiltration [8,9]. Increased infiltration of activated mDC in renal tumors was described by Verra et al. [10].

Similar controversial reports concern the role of regulatory T cells in cancer. Treg represent a subpopulation of T lymphocytes expressing receptor for IL-2 (CD25) and transcription factor Foxp3 (CD4+CD25highFoxP3+). They have the potential ability to suppress host immune responses, thus preventing autoimmune diseases by suppressing the activation of other T cells [11–14]. They also play a significant role in the suppression of specific and nonspecific antitumor immune responses by affecting the function of CD8+ CTL and NK cell [15,16]. Curiel et al. [17] were the first to prove that higher Treg cell frequencies in patients with ovarian cancer actually correlate with the tumor stage and significantly predict an inferior clinical outcome. Several reports have been published about elevated numbers of Treg cells in the peripheral blood of patients with different malignancies, including RCC [18–20]. On the one hand, high percentages of Treg in peripheral blood and/or cancer tissues were associated with poor prognosis in several studies concerning renal [21], ovarian [17,22], pancreatic [23] and liver cancer [24], but other studies showed no correlation between tumor-infiltrating Treg frequency and patient outcomes [25–28].

Most studies concerning tumor-infiltrating immune cells are focused on tumor-infiltrating lymphocytes, especially CD8+ effector T cells, Treg and myeloid suppressor cells. Surprisingly, little is known about neutrophils and different subtypes of DC within the tumor microenvironment as well as peripheral blood of cancer patients. To extend knowledge of the pattern of tumor-infiltrating immune cells in different stages of RCC, we evaluated the number and phenotype of mDC, pDC, neutrophils and Treg in the tumor tissue and peripheral blood in a cohort of newly diagnosed RCC patients. We correlated the findings with the tumor stage.

2. Patients and methods

2.1. Patients and sample collection

Blood samples and RCC tissue specimens were obtained intraoperatively from a total of 37 patients with newly diagnosed RCC. Complete blood count tests were also performed. The examined pathologic features included histological subtype, the 2002 tumor-node-metastasis (TNM) stage groupings and nuclear grade. Histological subtype was classified according to the Union Internationale Contre le Cancer, American Joint Committee on Cancer, and Heidelberg guidelines [29,30]. The collection of all tissue specimens was approved by the Institutional Review Board of the University Hospital Motol. For statistical analysis patients were divided into two groups according to the tumor stage. The stages T1 and T2 were classified as an early stage without metastases and the stages T3 and T4 as a late stage, including patients with metastases. The demographic and pathological parameters of patients after radical nephrectomy or nephron-sparing surgery are described in Table 1. Not all specimens were sufficient for all analyses which is reflected in different numbers of evaluated patients in different parameters. Eleven volunteers (average age 62) with no history of cancer were included in the study as healthy controls for the determination of peripheral blood T regulatory cells, neutrophils and DC subpopulation frequencies.

2.2. Cell preparation

Fresh tumor tissue samples were cut into small pieces and homogenized using gentleMACS™ dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany). Obtained cell suspension was then filtered through 70 μm nylon mesh (BD Biosciences, San Jose, CA, USA). Peripheral blood mononuclear cells (PBMC) were separated using Ficoll-Hypaque medium (Biocoll, Berlin, Germany) and gradient centrifugation.

2.3. Immune cell staining and flow cytometry

Whole peripheral blood was used for the assessment of DC. Two populations of dendritic cells (Fig. 1) were distinguished, namely mDC (CD45+, Lineage−, HLA-DR+, CD14−, CD11c−) and pDC (CD45+, Lineage−, HLA-DR+, CD14−, CD123+). Their relative number from the blood and dissociated tumor samples was determined as a percentage of all leukocyte gate CD45+, the absolute number of DC was calculated as a percentage of leukocytes from blood count tests. Mature mDC from isolated PBMC as well as from dissociated tumor samples were detected as cells expressing CD83 molecules within the population of mDC. Neutrophils defined as CD45+ CD14− CD65+ cells were identified in dissociated tumor samples. Circulating neutrophils were determined from the peripheral blood count. Treg were detected on density gradient isolated PBMC from the peripheral blood and determined in cell suspension from dissociated tumor tissue. For intracellular Foxp3 staining, cells were fixed and permeabilized using the Foxp3 staining buffer set (eBioscience, San Diego, CA, USA) according to the manufacturer’s instructions. Subsequently, the cells were stained with a specific FoxP3-Alexa Fluor® 488 antibody or isotype control (eBioscience, San Diego, CA, USA). Regulatory T cell subsets were determined from lymphocyte gate as CD4+ CD25+ FoxP3+ and reported as a percentage of CD4+ cells.

Cells for the FACS analysis were diluted in PBS and stained with directly conjugated monoclonal antibodies by incubating 30 min in the dark according to manufacturer’s recommendation. The following monoclonal antibodies were used: HLA DR PE-Alexa Fluor 750 (BD Biosciences, San Jose, CA, USA), CD123-PE (BD Biosciences, San Jose, CA, USA), CD11c APC (Exbio, Prague, Czech republic), CD14-PE-Dyomics 590, CD45-PB or CD45-PE (Exbio, Prague, Czech republic), CD65-FITC, CD83-PE-Cy5 (BD Biosciences, San Jose, CA, USA), Lineage cocktail 1-FITC (mixture of CD3, 14, 16, 19, 20, 56) (BD Biosciences, San Jose, CA, USA), CD4-PE-Cy7 (Exbio, Prague, Czech republic), CD8-PE-Dy590 (Exbio, Prague, Czech republic), CD25-PE (Exbio, Prague, Czech republic).

Samples were analyzed with FACS Aria (BD Biosciences, San Jose, CA, USA). Data were analyzed using FlowJo software (Tree Star, Ashland, USA).

2.4. Statistical analysis

The StatView program was used for statistical assessment. An unpaired Student’s t-test, Mann–Whitney test and paired t-test or Wilcoxon matched pairs test were applied to determine the differences between groups. The data were verified for the normality with D’Agostino and Pearson omnibus normality test. Data were
expressed as a percentage of positive cells. Statistical significance was defined as \( p < 0.05 \).

3. Results

3.1. Dendritic cells in the peripheral blood and tumor tissue

The number of mDC and pDC in the peripheral blood of RCC patients \( (n=26) \) is significantly higher than in the healthy population \( (n=11) \) (median \( 15.13 \times 10^3/\text{ml} \) and \( 8.64 \times 10^3/\text{ml} \), respectively for mDC, \( p = 0.005 \); median \( 9795 \times 10^3/\text{ml} \) and \( 3.648 \times 10^3/\text{ml} \), respectively for pDC, \( p < 0.0001 \)). The percentage of mDC in the dissociated tumor cell suspension was significantly higher than in the peripheral blood in both early (0.81% and 0.22% of CD45+, respectively, \( p = 0.0005 \)) and late stage groups (0.62% and 0.19% of CD45+, respectively, \( p = 0.04 \)) (Fig. 2A and B), but the percentage in the blood and in tumor tissue did not correlate with the disease stage \( (p = 0.7 \) for blood, \( p = 0.35 \) for tumor).

The percentage of mature mDC (CD83+) in the tumor was significantly higher (mean 24.5%, \( n = 29 \)) in comparison with peripheral blood (mean 12.4%) in the entire unsorted RCC group \( (p = 0.01 \) Fig. 3), as well as in the early stages of RCC (mean 9.93% in blood and 24.24% in tumor, \( n = 18 \), \( p = 0.01 \) Fig. 4A), but not in the late stage (mean 15.5% and 20.1%, respectively, \( p = 0.58 \), Fig. 4B). We also compared portions of mature mDC in the tumor tissue in the early and late stage of the disease. The difference was not statistically significant (mean 24.2% and 20.1%, respectively, \( p = 0.59 \)). We did not prove the difference between mature mDC in the blood in the late and early stages (mean 15.5% and 9.93%, respectively, \( p = 0.29 \)) either.

The number of pDC in peripheral blood was similar in both early and late stage groups (median \( 10.14 \times 10^3/\text{ml} \) and \( 9.01 \times 10^3/\text{ml} \), respectively, \( p = 0.89 \)). However, we found a significant difference between these 2 groups in the tumor environment: a higher proportion of pDC was found in early stage (mean 0.248% and 0.06% of CD45+ cells, respectively; \( p = 0.001 \), Fig. 5). When we compared pDC in the early stage in the blood and tumor tissue, the difference nearly reached significance \( (p = 0.06 \) Fig. 6A). The same comparison in the late stage of the renal carcinoma proved the significant difference \( (p = 0.003 \) Fig. 6B) in terms of lower percentage in the tumor than in the blood.

3.2. Neutrophils in the peripheral blood and tumor tissue

We further focused on neutrophil counts in the peripheral blood and their infiltration in each tumor. The peripheral blood

![Fig. 1. Samples were analyzed on FACS Aria. Dendritic cells were identified as CD45+, CD14−, Lineage 1− and HLA-DR+ (A) and subdivided into myeloid (CD11c+) and plasmacytoid (CD123+) dendritic cells (B).](image1)

![Fig. 2. Comparison of mDC in early (A) and late (B) stage groups in the peripheral blood (PB) and tumor tissue (T). Myeloid dendritic cells were determined in the blood or dissociated tumor samples by staining with monoclonal antibodies (CD45+, Lineage 1−, HLA-DR−, CD14−, CD11c+). Horizontal bars represent the median. P-value was calculated by Wilcoxon signed rank test.](image2)
was analyzed with regular blood count tests. Patients with tumor had a higher number of neutrophils in the peripheral blood compared to healthy controls (mean $4.99 \times 10^3/\mu l$ and $3.19 \times 10^3/\mu l$, respectively, $p = 0.01$). There was no difference in blood neutrophils between early and late stages (mean $5.56 \times 10^3/\mu l$ and $4.56 \times 10^3/\mu l$, respectively, $p = 0.27$). Neutrophils from the tumor were gated as CD45+CD14–CD65+ cells. There was a significant difference between early and late tumor stages: in the early stages we found lower percentages of neutrophils (mean 8.7% of CD45+ cells, range 0–19%, $n = 10$) in comparison with late stages (mean 46.6% of CD45+ cells, range 9.0–92.0%, $n = 10$, $p < 0.001$, Fig. 7).

### 3.3. Treg cells in the peripheral blood and tumor tissue

In an effort to enumerate CD4+ Treg cells in renal carcinomas, we used FoxP3 transcription factor assessed by flow cytometry.

**Fig. 3.** Comparison of mature mDC in blood and tumor tissue. Myeloid dendritic cells from 29 patients with RCC were determined in the blood or dissociated tumor samples by staining with monoclonal antibodies (CD45+, Lineage 1–, HLA-DR+, CD14–, CD11c+). Mature DC were recognized by expression of CD83 molecule. Horizontal bars represent the mean. $P$-value was calculated by paired t-test.

**Fig. 4.** Comparison of mature mDC in (A) early stages ($n = 18$) and (B) late stages of RCC ($n = 11$) in tumor (T) and peripheral blood (PB). mDC were determined from blood or dissociated tumor samples by staining with monoclonal antibodies (CD45+, Lineage 1–, HLA-DR+, CD14–, CD11c+). Mature mDC were recognized by expression of CD83 molecule. Horizontal bars represent the mean. $P$-value was calculated by paired t-test.

**Fig. 5.** Comparison of pDC present in RCC in the early ($n = 14$) and late ($n = 14$) stages of the disease. pDC were determined from dissociated tumor samples by staining with monoclonal antibodies (CD45+, Lineage 1–, HLA-DR+, CD14–, CD123+). Horizontal bars represent the mean. $P$-value was calculated by unpaired t-test. T: tumor.

**Fig. 6.** Comparison of pDC in the peripheral blood (PB) and tumor tissue (T) in the (A) early ($n = 15$) and (B) late ($n = 16$) stages of RCC. pDC were determined from dissociated tumor samples by staining with monoclonal antibodies (CD45+, Lineage 1–, HLA-DR+, CD14–, CD123+). Horizontal bars represent the mean. $P$-value was calculated by paired t-test.
as a marker for regulatory T cells. FoxP3 expression was readily detected in tumor infiltrating CD4+ T cell population but not in CD8+ T cells. The difference between percentage of circulating FoxP3+CD4+ Treg in peripheral blood of healthy donors (mean 1.7% of CD4+ cells, range 0.85–3.0%, n = 11) and renal carcinoma patients (mean 6.3% of CD4+ cells, range 2.5–10.0%, n = 28, Fig. 8) was highly significant (p < 0.0001). High percentages of CD4+ T cells in the tumors expressed FoxP3 (mean 13.4%, range 0–35.0%, n = 19; Fig. 9) compared to the percentage of FoxP3+CD4+ T cells found in the peripheral blood samples from renal carcinoma patients (mean 6.3%, range 2.5–10.0%, n = 28). The difference was statistically significant (p = 0.001). If stratified according to stage groups the mean Treg infiltration in tumor tissue in the early and late stages were 12.5% and 14.4% of CD4+ cells, respectively (n = 19, Fig. 10). The difference was not significant (p = 0.63). The difference between these two groups in the blood was also insignificant (mean 6.29% and 6.31% of CD4+ cells, respectively).

4. Discussion

Besides tumor cells, the tumor microenvironment is composed of a wide spectrum of immune cell types, which can significantly affect cancer progression and patient outcome. To enhance knowledge of the dynamics of anti-tumor immune response, we have studied proportions of DC, Treg and neutrophils in peripheral blood and fresh tumor tissues of patients with early and advanced RCC. As we were not able to isolate sufficient amount of tumor tissue, we have not tested the function of these cells in this study.

Dendritic cells as a major antigen presenting cells play an essential role in initiating adaptive immune response. As tumor cells are eliminated by effector cytotoxic T lymphocytes, the presence of DC in tumors could be the sign of active immune reaction. In the studies with colorectal and other cancers [31–35] the higher number of dendritic cells in tumors correlated rather with worse prognosis. It has been described that tumor cells produce various cytokines (e.g., EGF, G-CSF, IL-6, IL-8, IL-10, TGF-β, VEGF) and surface molecules (PD-L1, FasL, TRAIL, etc.) that may suppress local immune response and thus enduring dendritic cells ineffective [36,37]. On the contrary, in other studies high dendritic cell infiltration in tumors correlated with better prognosis [38]. If more stratified by maturation status, the presence of dendritic cells with high expression of CD83 in renal tumors was the sign of a better response to future immunotherapy [25,39], whereas in other studies mature dendritic cells correlated instead with worse prognosis [32]. Little is known about the frequency of plasmacytoid DC in tumors. In our study we divided patients with clear cell carcinoma into 2 groups according to assumed prognosis (early stage confined to kidney with better clinical prognosis, and late stage with worse clinical prognosis).
based on TNM classification). The concentration of mDC and pDC in the peripheral blood of RCC patients was higher than in healthy controls. We observed a higher percentage of mDC in tumor tissue than in the peripheral blood of patients, however, there was no statistically significant difference between stage groups. Similar results were observed when comparing maturation status of mDC in the tumor infiltrate, a higher proportion of mDC expressed maturation marker CD83 than in the peripheral blood, but without the correlation with disease stage. As to pDC, we found a significantly higher ratio in the tissue cell infiltration in early stage patients while there was no difference in the peripheral blood number of these cells. As described by Gigante et al. pDC are virtually absent in normal kidney tissue, on the other hand, they observed tumor infiltration by pDC [40]. Conrad et al. observed higher pDC numbers close to regulatory T cells in ovarian cancer. These women had worse clinical prognosis [41]. Our findings of a lower number of pDC in the later stages of renal cancer compared with the early stages implies possible roles in local immune responses in the early course of the disease.

Tumor-infiltrating neutrophils are known to make up a significant part of immune cells within the tumor microenvironment in different types of human cancer [42–44]. Despite their origin in the peripheral blood, TIN have been shown to exhibit impaired bactericidal and enhanced angiogenic activities [45]. The presence of intratumoral neutrophils has been reported to be associated with poor prognosis in primary breast cancer [46] and RCC [43]. Consistently within these studies, neutrophil depletion experiments led to an inhibited tumor growth [47], limited metastasis numbers [48] and reduced endothelial recruitment to the tumors [49]. Our results confirm the rather harmful role of TIN as we found significantly higher ratio of these cells in dissociated tumor cell suspension from advanced RCC patients with T3–4 and metastatic disease.

Contrary to TIN, the role of tumor-infiltrating and peripheral blood Treg in cancer prognosis is still controversial. High percentages of Treg in peripheral blood and/or cancer tissues were associated with poor prognosis in several studies concerning renal [21], ovarian [17,22], pancreatic [23] and liver cancer [24]. However, some studies showed no correlation between tumor-infiltrating Treg frequency and patients' outcome [25–28]. Recently [50] Jeron et al. reported that Treg frequencies were increased in the peripheral blood of RCC patients. Griffiths et al. [51] systemically assessed Treg frequencies in predominantly metastatic RCC patients. They found significantly increased number of Treg cells in blood compared to normal donors. Moreover, there was a clear positive correlation in the relative levels of Treg cells in the peripheral blood and the tumor microenvironment of RCC patients. In addition, using univariate Kaplan–Meier survival analysis, they revealed an adverse outcome for those patients who presented with higher Treg counts in the periphery.

In our study, we have shown that RCC patients have higher proportions of Treg in peripheral blood than healthy controls. Higher percentage of Treg of CD4+ cells was found in the tumor tissue in the comparison with the peripheral blood of RCC patients irrespective of the disease stage. These findings may reflect the possibility of cancer-induced systemic as well as local immunosuppression which both seem to be the early event in the course of the disease.

Additionally, we observed higher percentage of both TIN and Treg within the RCC tissue during the disease progression. Indeed, patients in early stages of RCC had significantly lower percentage of TIN than patients in late disease stages. The results may be in conjunction with the findings that neutrophil chemokines are secreted by a wide spectrum of cell types within the tumor microenvironment [52]. Recently, it has been shown that human Treg are also capable to produce substantial amounts of IL-8 and thus induce neutrophil migration [53].

Our results reflect the complex interplay between various cells of immune system and the tumor microenvironment. Signs of the activation of dendritic cell subpopulations at early stages of the course of the disease are counterbalanced by the early appearance of T regulatory cells both in the periphery and tumor tissue. With the disease progression, the presence of mDC even in the mature state does not prevent the accumulation of tumor infiltrating neutrophils which are probably attracted by chemokines released by Treg. Tumor infiltrating neutrophils further suppress the efficient immune response namely by the release of reactive oxygen species and enabling the tumor to progress and metastasize. Anticancer strategies, especially immunotherapy, should take into account the dynamics of the immune response. Immunotherapy should not only boost specific antitumor immunity, but target regulatory T cells even in the early stages of the disease preventing their further hampering role in suppressing effector T cells as well as attracting neutrophils. Considering the high amounts of neutrophils within RCC tissue and their strong prognostic significance in the late stage of the disease, anti-cancer strategies in renal cancer should also target recruitment and/or activation of TIN.

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References


