Renal Cell Carcinoma-induced Immunosuppression: An Immunophenotypic Study of Lymphocyte Subpopulations and Circulating Dendritic Cells

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Abstract. Background: Renal cell carcinoma (RCC)-induced immune dysfunction in patients at first diagnosis was investigated. Patients and Methods: The main circulating lymphocyte subsets, the total number of circulating and intratumor dendritic cells and the titers of circulating VEGF were quantified in 47 RCC patients, using flow cytometric, immunohistochemical and ELISA assays. Results: Despite a significant activation of CD3/HLA-DR+ lymphocytes and of the CD56+ NK subset, RCC patients presented a marked immunosuppression of CD4/CD45RA naïve T-cells, CD4/CD45RO memory T-cells, CD16+ NK-cells, and total circulating dendritic cells, as well as a significant increase of lymphocytes co-expressing the CD4 and CD8 antigens. Finally, CD16+/CD56+ NK and DCs were poorly represented in tumor specimens. Conclusion: The complex immunological dysfunctions demonstrated involve different levels of immunocompetence and indicate a pattern of major disturbance of the immune system.

Experimental observations suggest that the immune system can play a key role in containing renal cell carcinoma (RCC) in situ (1); unfortunately, however, in the long term, the immune response against RCC fails to control the disease, which inevitably progresses towards invasive forms. This is true for most patients, even those diagnosed at a very early stage.

As for advanced disease, RCC may exhibit long periods of stabilization (either spontaneous or following immunotherapy) or even uncommon episodes of spontaneous regression (at metastatic sites, both after removal of the primary and in 6-7% of patients receiving placebo within randomized clinical trials). RCC natural history suggests that the immune system can indeed play a role in controlling tumor growth and progression, even in frankly metastatic disease (2).

Further evidence of the immune system involvement in RCC control comes from the frequent finding of major T-cell infiltration in the tumor; the presence of clones of antigen-specific T-cells in both the primary lesion and draining lymph nodes has been unquestionably demonstrated, and the clones appeared to be able to lyse renal carcinoma cells in vitro (3-5).

Thus, many different immunotherapy approaches have been proposed to treat RCC, especially since several RCC-related tumor antigens have been identified that can be sought, processed and presented by immune system cells (6). From the administration of different cytokines, to the development of vaccines, and even to allogeneic transplantation of circulating hematopoietic precursors after non-myeloablative conditioning, immunotherapy has been increasingly used for RCC treatment.

However, our everyday experience with immunotherapy is still far from satisfying, and a repeatable, clinically effective response, which can impact on survival in advanced RCC, is difficult to achieve.

Several experimental results confirm the hypothesis that no or poor recognition of RCC cells by immune system cells, activation of defense mechanisms by tumor cells, and finally different complex immunologic dysfunctions, are the
main obstacles to the development of an adequate anticancer response in vivo (7, 8), and not even immuno-stimulating therapies can overcome these drawbacks (9).

The hypothesis that RCC patients may present more or less complex immune function defects, even at diagnosis, was investigated.

**Patients and Methods**

**Patients.** Forty-seven consecutive RCC patients, 28 men (59.57%) and 19 women (40.42%), mean age 63.9 years (median: 63 years, range: 34 to 86 years), were enrolled in this study at first diagnosis, after giving their informed consent according to the institutional requirements. The patients’ characteristics are reported in Table I.

The main tumor histotype was clear cell renal carcinoma (34 patients, 72.34%), while other subjects had papillary, chromophoblc and sarcomatoid lesions.

Most patients (22, 46.80%) had a Furhman’s grade G2 lesion, i.e., a moderately differentiated neoplasm.

Most patients had an early stage lesion (pT1 according to Robson’s criteria in 61.7% of cases). Locoregional lymphadenectomy was hardly ever performed: given the low risk of recurrence in small lesions and the lack of any adjuvant treatment to decrease this risk, in Italy lymphadenectomy is often not performed in the absence of macroscopically suspicious lymph nodes. Only 7 patients (14.89%) had an advanced lesion, with distant metastases at the time of diagnosis.

None of the patients had received anticancer treatments or even immunosuppressants (including steroids) at the time of analysis.

**Controls.** As controls, 40 healthy donors, matched by gender and age were used; tumor and/or immune conditions were excluded for all of them.

**Lymphocyte immunophenotyping.** A peripheral blood sample (3 ml) was taken from all patients by venipuncture, in EDTA-containing vials. Samples were then lysed using a commercial kit (Immunoprep Reagent System, Coulter Co., Miami, FL, USA) with 3 different reagents: one, containing formic acid (1.2 ml/l) for RBC lysis (Immunoprep A); one, containing sodium carbonate (31.3 g/l) for WBC stabilization (Immunoprep B); and one, containing paraformaldehyde (10 g/l) and buffers, for cell membrane fixation (Immunoprep C).

IgG1, IgG2a, FITC and PE histotype controls were provided by Coulter Co. (Miami, FL, USA); the characteristics of the monoclonal antibodies used to identify the studied subsets are listed in Table II.

Samples were analyzed by means of an EPICS-XL cytometer (Coulter Co, Miami, FL, USA); 4 parameters were acquired and saved in List Mode: 2 on a linear scale (forward scatter, FS, and side angle scatter, SS) and 2 on a logarithmic scale, fluorescence 1 (FL1-FITC) and fluorescence 2 (FL2-PE).

The acquisition gate was set by determining a bitmap on the FS vs. SS cytogram delimiting the lymphocyte area; 5000 events were calculated on that population. Histotype control reading allowed calipers to be positioned to discriminate positive from negative events and to correctly compensate for fluorescence.

Finally, the percentage values of the studied subsets obtained by cytometric reading were converted into absolute values according to white blood cells and whole blood count values.
Evaluation of circulating dendritic cells (DCs) in peripheral blood. Peripheral blood samples, collected in heparinized vials, were diluted 1:1 with RPMI-1640 supplemented with L-glutamine (Sigma Chemicals, St. Louis, MO, USA) and then layered on a Ficoll-Paque concentration gradient (density 1.077 g/l, Amersham Biosciences, Uppsala, Sweden) at a 1:1 ratio, and centrifuged at 2000 rpm for 20 min; the ring of mononuclear cells (PBMC) obtained was washed three times in a PBS buffer (Sigma Chemicals, St. Louis, MO, USA), and then centrifuged again at 1400 rpm for 10 min. Cells were finally resuspended in 1 ml PBS and counted with a Bürker chamber.

Dendritic cells were sought by direct immunofluorescence with 2- and 3-color cytofluorimetric analysis, starting from PBMC (Figure 1a). One million cells were incubated for 20 min at 4°C with a cocktail of lineage-specific antibodies conjugated with FITC, that is CD3, CD19, CD20, CD11b, CD56, CD16, CD34, CD14 and HLA-DR PC5. At the end of incubation, the cells, washed twice in a PBS buffer and centrifuged at 1400 rpm for 10 min, were ready for cytometric reading. The above cocktail marked T-, B- and NK-cells, macrophages, granulocytes-monocytes and hematopoietic precursors simultaneously – that is, all the populations positive for HLA-DR (cocktail+/HLA-DR+).

![Figure 1](image_url)

**Figure 1.** Identification of circulating dendritic cells (DCs) and of DC subsets by flow cytometry. Panels show representative contour plot data illustrating the analytical methods used to identify DCs in peripheral blood mononuclear cells after immunostaining as described in Materials and Methods. a) Mononuclear cells analysis regions applied to FS/SS data acquired for exclusion of granulocytes and debris. b) The same identified cell population after labelling with a cocktail of FITC-conjugated monoclonal antibodies recognizing the lineage-associated antigens listed in Materials and Methods and with a PC5-labeled anti-HLA-DR monoclonal antibody. c) Representative contour plot of DC1 subset, identified after labelling lin-/HLA-DR+ DCs with CD11c PE. d) Representative contour plot of DC2 subset, identified after labelling lin-/HLA-DR+ DCs with CD123 PE.
Table III. List of the monoclonal antibodies used for phenotyping of circulating dendritic cells and their DC1 and DC2 subsets.

<table>
<thead>
<tr>
<th>Subset</th>
<th>Fluorochrome</th>
<th>Clone</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>FITC</td>
<td>T3</td>
<td>Mature T-cells, TCR complex</td>
</tr>
<tr>
<td>CD19</td>
<td>FITC</td>
<td>B4</td>
<td>Pan-B</td>
</tr>
<tr>
<td>CD20</td>
<td>FITC</td>
<td>B1</td>
<td>Pan-B</td>
</tr>
<tr>
<td>CD16</td>
<td>FITC</td>
<td>3G8</td>
<td>NK-cells</td>
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<tr>
<td>CD56</td>
<td>FITC</td>
<td>T11</td>
<td>Pan-T</td>
</tr>
<tr>
<td>CD11b</td>
<td>FITC</td>
<td>Bear1</td>
<td>Granulocytes, monocytes, NK-cells</td>
</tr>
<tr>
<td>CD14</td>
<td>FITC</td>
<td>Mo2</td>
<td>Monocytes</td>
</tr>
<tr>
<td>CD34</td>
<td>FITC</td>
<td>588</td>
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</tr>
<tr>
<td>HLA-DR</td>
<td>FITC</td>
<td>PC5</td>
<td>HLA-DR locus</td>
</tr>
<tr>
<td>CD11c</td>
<td>PE</td>
<td>BU15</td>
<td>Granulocytes, monocytes, NK-cells, T and B subsets</td>
</tr>
<tr>
<td>CDw123</td>
<td>PE</td>
<td>9F5</td>
<td>A chain of IL-3R</td>
</tr>
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Dendritic cells were thus identified as cocktail-/+HLA-DR+ (Figure 1b).

Three-color analysis allowed identification and counting not only of the total number of circulating dendritic cells but also of DC1 and DC2 subsets. In this case, cells were marked with the mixture of FITC, HLA-DR, PC5 and CD11c PE (DC1, Figure 1c) or CDw123 PE (DC2, Figure 1d). The IgG1 FITC and PE, IgG2a FITC and IgM FITC histotype controls were provided by Coulter Co.

The characteristics of the monoclonal antibodies used for the phenotyping of circulating dendritic cells and their DC1 and DC2 subsets are reported in Table III.

The data from the samples were then acquired and saved, and 5 parameters were evaluated, 2 on a linear scale, FS and SS, and 3 on a logarithmic scale, FL1, FL2 and FL3.

Cytometric reading was performed using a bitmap on mononuclear cells designed on the cytogram with two physical parameters (FS vs. SS), counting 50,000 events. Applying a gate on dendritic cells (that is cocktail-/+HLA-DR+ cells), the DC1 and DC2 subsets were shown (HLA-DR+/CDw123+, respectively).

The histotype control permitted the differentiation of positive event from negative event areas and to correctly compensate for the three fluoroscences.

Immunohistochemical assessment of dendritic and NK-cells tumor infiltration. Sections of formalin-fixed, paraffin-embedded RCC samples from all patients were immunoreacted with antibodies against S100 protein (Dako Cytomation, Carpinteria, CA, USA), CD56 (123c3, Zymed San Francisco CA, USA) and CD16 (2H7 Novocastra, Newcastle, UK), according to standardized protocols (Ullman et al., 2004). Rabbit polyclonal antiserum against S100 protein did not require antigen unmasking pretreatment and was used at 750W at 1:40,000 dilution; mouse monoclonal antibodies for CD56 and CD16 were used after microwave antigen retrieval (2 cycles of 5’ at 750W) at 1:100 and 1:20 dilution. Positive and negative controls were included in each reaction set. The density of S100+ dendritic cells, and of CD16+ and CD56+ NK lymphocytes per cm2 of tumor tissue was evaluated with a video-assisted microscopy system (Nikon Coolscope, Nikon, Tokio, Japan) on three different samples from each tumor, by manually counting the number of immunoreactive cells and instrumentally measuring the surface of vital tumor area.

VEGF assay. The blood sample for VEGF assay was drawn early in the morning after overnight fasting in all patients; a blood aliquot was then collected into a whole blood (non-heparin) tube and allowed to separate at room temperature for 30 minutes before being centrifuged at 1,000 xg for 15 min. The serum thus obtained was then immediately frozen at –80°C for subsequent assay. Serum samples from a control population of healthy donors were also obtained and stored under the same conditions. VEGF was then measured using a commercial kit (Quantikine™, R&D Systems Inc., Minneapolis, MN, USA) based on a common ELISA method.

Statistical analysis. The following statistical analyses were carried out: Fisher’s exact test for comparison of quantitative variables, Mann-Whitney U-test for nonparametric analysis of continuous variables, and Pearson’s coefficient to evaluate age correlation with the absolute values of the various lymphocyte subsets studied.

Results

Lymphocyte subsets and circulating dendritic cells. Overall results are summarized in Table IV. Though RCC patients presented a statistically significant activation of CD3+/HLA-DR+ aspecific lymphocytes (21 µl vs. 12.5 µl in controls, p<0.0001; Figure 2a) and of the CD56+ NK subset (90 µl...
vs. 46 µl, p=0.04; Figure 2b), the prevalent finding was that of immunosuppression of CD4/CD45RA naïve T-cells (184 µl in patients vs. 409 µl in controls, p<0.0001; Figure 3a), CD4/CD45RO memory T-cells (294 µl in patients vs. 366.5 µl in controls, p=0.003; Figure 3b), CD16+ NK (154 µl in patients vs. 240.5 µl, p=0.004; Figure 3c), and total circulating dendritic cells (0.6% in patients vs. 0.9% in controls, p=0.0004; Figure 3d).

Also, relative to controls, tumor patients exhibited a statistically significant increase (10 µl vs. 0 µl, p<0.0001) of lymphocytes co-expressing the CD4 and CD8 antigens, a subset not yet committed toward either helper or suppressor T-cells.

The ratio of DC1 to DC2 cells appeared to be preserved in our RCC patients, as the mean proportion of DC1 cells was 38.03% and that of DC2 was 26.6%, as expected under normal conditions; nevertheless, the absolute number of circulating dendritic cells was significantly lower in cancer patients relative to healthy controls.

Correlations with patient characteristics. No statistically significant correlation was found between lymphocyte subset and circulating dendritic cells and any of the following parameters: age, gender, disease stage (in terms of TNM) or tumor grading.

Furthermore, patients with metastatic disease at disease presentation demonstrated no significantly different immunological alterations with respect to patients with localized tumors.

NK and dendritic cell infiltration of RCC. CD16+ and CD56+ NK lymphocytes were virtually absent in all RCC samples studied; indeed, only occasional immunoreactive cells were found in some sections within lymphocytic aggregates. S100-immunoreactive dendritic cells were present in all RCC interposed between tumor cells. Their number varied from 2 to 34 cm² (Figure 4).

VEGF assay. VEGF was significantly higher in patients with RCC than in healthy controls (455.14 pg/ml in patients vs. 211.09 in controls; p=0.004), the higher VEGF titers being observed in patients with metastatic disease at diagnosis.

Discussion

Renal carcinoma patients, especially (but not only) with metastatic disease, usually exhibit several immunological dysfunctions (9, 10).

Aside from the active and passive immune-escape mechanisms that can be found in most tumors (11-13), other mechanisms seem to be specific to renal carcinoma; Schwaab et al. demonstrated defective antigen presentation by dendritic cells, partly related to the down-regulation of co-stimulatory molecules, such as B7.2, and a consequently decreased recruiting of CD8+ cytotoxic lymphocytes infiltrating the tumor (14). Also, experimental research demonstrates that renal carcinoma cell production of soluble immunosuppression mediators (15), can suppress T-cell response through different mechanisms, such as:

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induction of T-cell apoptosis (15), decrease in IL-2 production by CD4+ helper T-cells, inhibition of the activity of Jak-3 kinase involved in trasduction of the signal from IL-2 receptor activation (16), and decreased activation of the nuclear factor NFκB in the lymphocytes effecting the immune response, resulting in increased susceptibility to apoptosis (17). All these mechanisms ultimately contribute to providing tumor cells with an advantage in terms of survival, both absolute, and relative to immune system cells.

However, when immune system modulation represents one of the most commonly used treatment options available, as in RCC, such complex immune system dysfunctions also risk affecting our capability of actually modulating an adequate immune response against cancer.

Several complex immune defects in RCC patients, almost solely at the level of cell-mediated immune function, were demonstrated; such dysfunctions are already present at diagnosis and, therefore, not necessarily consequent to disseminated disease.

Indeed, though aspecific lymphocyte activation has been demonstrated, with an increase in activated T-cells (co-expressing the antigens CD3 and HLA-DR) and in cells with NK activity characterized by positivity only to the CD56 antigen (NK-like T-cells and mature NK-cells), our dysfunctions seem to be more important from an immunologic viewpoint.

This is particularly true for the defect in naïve T-cells, which include immunocompetent cells that have not met any antigen yet. By definition, these cells possess an immunological repertoire directed against a broad spectrum of antigens and can originate an effector cell population once they meet a target antigen. Thus, a defect in this population is thought to favor tumor growth and progression (18).

![Figure 3. Number of (a) CD4/CD45RA+, (b) CD4/CD45RO+ and (c) CD16+ NK cells, as well as (d) percentage of circulating DCs in RCC patients and healthy controls.](image-url)
As for defects in memory T-cells, animal studies suggest that this subset plays an essential role in inducing and maintaining the so-called tumor dormancy, a delicate balance between tumor cells and the immune system (and thus between cell proliferation and apoptosis) that prevents a clinically undetected lesion from progressing to more invasive forms (19).

While a statistically significant increase was found in CD56+ NK-cells relative to controls, the defect in CD16+ NK-cells appears to be equally important. Indeed, if CD56+ NK-cells are involved in mechanisms of indirect cytotoxicity (mainly for CD56dim cells, which we did not study) (20), only NK-cells characterized by the CD16 (FcγIIIR) phenotype can mediate antibody-dependent toxicity (21). Interestingly, no defect in mature NK-cells (CD16/CD56+) could be found in our patients; it is however worth reminding that NK-cells are more involved in seeking and destroying non-self cells in the general circulation rather than in developed cancer sites.

We also demonstrated a statistically significant increase in T-cells co-expressing CD4 and CD8 antigens in RCC patients relative to healthy controls. In agreement with classic T-cell differentiation patterns, mature CD4+ and CD8+ T-cells are mutually exclusive subsets in peripheral blood, though very low rates (1-3%) of these cells can be found there, usually following aspecific lymphocyte activation (22). Thus, we would be speaking of an aspecifically activated cell population not yet committed toward a frank T-helper nor, much less, toward a T-suppressor phenotype (CD4 is usually more expressed than CD8 in this cells population), and therefore with minimal anticancer activity.

Finally, though the DC1 to DC2 subset ratio in circulating dendritic cells appears to be preserved, thus...
assuring a preferential Th1 response, we found a significant decrease in our RCC patients in circulating dendritic cells, which further complicates the above immunoincompetence state. The evidence of VEGF titers significantly higher than in healthy controls could be one of the main causes of such an impairment in DC number, since this pro-neoangiogenic cytokine was proven able to diminish host immunity by altering the function of major antigen-presenting cells, such as DCs (23).

The observation of reduced numbers of NK and dendritic cells in the systemic circulation made us wonder whether these cells could be recruited – and thus sequestered – by the tumor. However, the immunohistochemical finding of scarce NK or dendritic cells within the tumor confirms the state of deep immunosuppression seen in peripheral blood.

In conclusion, the complex immunological dysfunctions discussed above involve different levels of immunocompetence and indicate a pattern of major disturbance of the immune system, which is difficult to address therapeutically. Also, they appear to be unrelated to the main clinicopathological features of the patient population examined.

Moreover, although the use of dendritic cells (which can also be selected from peripheral blood) seems to be a promising treatment strategy for RCC patients, we cannot underestimate the marked decrease in this cell subset resulting from the tumor presence itself. If we consider that the dendritic cells found in RCC patients already exhibit poor function in terms of antigen presentation (11), the use of autologous dendritic cells within adoptive immunotherapy appears less promising than expected.

Another practical implication emerging from our study is the possible administration of an adjuvant immunostimulating treatment (e.g., using recombinant IL-2, with(out) GM-CSF) in patients that may now be considered at high risk of recurrence from both pathological and immunological viewpoints.

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