Immunological stress in kidney cancer patients undergoing either open nephrectomy or nephron-sparing surgery: An immunophenotypic study of lymphocyte subpopulations and circulating dendritic cells

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Abstract. Immunosuppression is a characteristic hallmark of renal cell carcinoma (RCC), with several complex immune defects, almost solely at the level of cell-mediated immune function, well evident even in patients at first diagnosis. The main circulating lymphocyte subsets and the total number of circulating dendritic cells were quantified in 47 RCC patients at diagnosis (T0), 12 h (T1), 24 h (T2) and 8 days following either radical nephrectomy or nephron-sparing surgery using flow cytometry. RCC patients presented, at baseline, (T0) a profound state of immunosuppression involving naïve T-cells, memory T-cells, $CD16^+$ NK and total circulating dendritic cells, that worsened after 12 (T1) and 24 h (T2) from surgery, involving the majority of the analysed subsets; after 8 days (T3) from surgical removal of tumor, however, there was a return of all the analyzed parameters to the basal state. In conclusion, surgery causes transient but relevant immune suppression in RCC patients; even though, by day +8, this tends to return to baseline, immunostimulatory therapies could be considered in the peri-operative setting with the aim of reducing immunosuppression and, hopefully, also disease recurrence.

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Introduction

Immunosuppression is a characteristic hallmark of cancer, and of renal cell carcinoma (RCC) in particular. We have already demonstrated (1) the presence of several complex immune defects, almost solely at the level of cell-mediated immune function, in RCC patients at first diagnosis, even in patients without disseminated disease. Furthermore, several experimental studies clearly demonstrated that renal carcinoma cells may produce soluble immunosuppressive mediators (2) as well as suppress T-cell response through different mechanisms, such as induction of T cell apoptosis (2), 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 (3), and decreased activation of the nuclear factor NFκ-B in the lymphocytes effecting the immune response, resulting in increased susceptibility to apoptosis (4). Other immunosuppressive factors secreted by RCC cells include IL-10 (5), which is thought to minimize a T-helper 1 response and to skew the immune response toward a type 2 response, even though this cytokine could act differently in different experimental models (6).

Animal studies, as well as our everyday clinical practice, suggest that cancer immunotherapy efficacy is inversely proportional to tumor burden. Thus, at least theoretically, the surgical removal of primary tumor could revert such immunosuppressive status; this could, at least in part, explain why, differently from other cancer types, surgical removal of the primary kidney lesion has an impact on patients' overall survival even in the presence of a synchronous metastatic disease (7,8).

Only few studies have evaluated the immunological consequences of tumor resection in either mice (9,10) or humans (11,12). The authors found different levels of immunosuppression, which showed partial recovery after tumor removal. Danna *et al* (9) compared immune responses in

mice without tumor versus mice with intact primary tumors versus mice whose primary tumors had been removed, but who already had established, spontaneous metastatic disease; in their study, the authors demonstrated that although tumor-bearing animals had reduced B- and T-cell responses, the immunosuppression was reversed following primary tumor removal, even when metastatic disease was present.

In this study, we investigated the evolution of immune system in RCC patients after radical surgery (nefrectomy or nephron-sparing surgery).

Patients and methods

Patients. Thirty-nine consecutive RCC patients at first diagnosis were enrolled in this study, after giving their informed consent according to institutional requirements. They were 25 men (64.10%) and 14 women (45.90%), with an average age of 62.3 years (median, 63 years; range, 34-85 years). The predominant tumor histotype was clear cell renal carcinoma (29 patients, 74.36%), while other subjects had papillary, chromophobic and sarcomatoid lesions. Most patients (n=13, 33.33%) had a moderately differentiated, Furhman's grade 2, lesion. Most patients had an early stage lesion (pT1 according to Robson's criteria in 64.09% of cases).

Patients underwent either open radical nephrectomy (n=27, 62.23%) or nephron-sparing surgery (n=12, 30.77%); 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 (17.95%) had an advanced lesion, with distant metastases at the time of diagnosis. None of the patients had received anticancer treatments or even immunsuppressants (including steroids) at the time of analysis. The characteristics of the patients enrolled into this study are reported in Table I.

Table I. Patient characteristics.

Gender		
Male	25	(64.10%)
Female	14	(35.90%)
Age		
≤40	2	(5.13%)
>40	37	(94.87%)
Histology		
Clear cell carcinoma	29	(74.36%)
Papillary carcinoma	5	(12.82%)
Chromophobic carcinoma	4	(10.26%)
Sarcomatoid carcinoma[a]	1	(2.56%)
Staging (T)		
pT1a	14	(35.88%)
pT1b	11	(28.21%)
pT2	6	(15.38%)
pT3a	5	(12.82%)
pT3b	1	(2.57%)
pT3c	0	(0%)
pT4	2	(5.14%)
Staging (N)		
N-	3	(7.7%)
N^+	0	(0%)
Nx (nodal status not assessed)	36	(92.3%)
Staging (M)		
M^-	32	(82.05%)
M^+	7	(17.95%)
Surgery		
Nephron-sparing surgery	12	(30.77%)
Nephrectomy	27	(62.23%)

[a]Independently from the WHO histotype, >25% of the whole cell population should be sarcomatoid.

Lymphocyte immunophenotyping. A peripheral blood sample (3 ml) was taken 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 red blood cell lysis (immunoprep A); one, containing sodium carbonate (31.3 g/l) for white blood cells 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.; 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.); 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 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) thus obtained was washed 3 times in a PBS buffer (Sigma Chemicals) 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 cytofluorimetic analysis, starting from

Table II. The monoclonal antibodies used to identify each of the studied lymphocytic subset.

Subset	Fluorochrome	Clone	Function
CD4	FITC-PE	T4	T-helper lymphocyte
CD3	PE	T3	Mature T-cells, TCR complex
CD8	FITC	T8	T-cytotoxic lymphocyte
CD3/HLA-DR	PE FITC	T3 I2	Activated T-lymphocytes
CD2	PE	T11	Pan T
CD2/CD25	FITC PE	T11 B1.49.9	Activated T-lymphocytes
CD56	PE	NKH1	NK-cells
CD16	PE	3G8	NK-cells
CD16/CD56	PE PE	3G8 NKH1	NK-cells
CD20	FITC	B1	Pan B
CD19	PE	B4	Pan B
CD4/CD45RA	FITC/PE PE	T4 2H4	*Naïve* T-cells
CD4/CD45RO	FITC/PE FITC	T4 UCHLI	*Memory* T-cells
CD4/CD8	FITC/PE FITC	T4 T8	Immature, not yet committed, lymphocytes
CD20/CD38	FITC PE	B1 Leu17	*Early* B-cells

Table III. The monoclonal antibodies used for phenotyping of circulating dendritic cells and their DC1 and DC2 subsets.

Subset	Fluorochrome	Clone	Function
CD3	FITC	T3	Mature T-cells, TCR complex
CD19	FITC	B4	Pan-B
CD20	FITC	B1	Pan-B
CD16	FITC	3G8	NK-cells
CD56	FITC	T11	Pan T
CD11b	FITC	Bear1	Granulocytes, monocytes, NK-cells
CD14	FITC	Mo2	Monocytes
CD34	FITC	588	Hematopoietic precursors
HLA-DR	PC5	HLA-DR	HLA-DR locus
CD11c	PE	BU15	Granulocytes, monocytes, NK-cells T and B subsets
CDw123	PE	9F5	α chain of IL-3R

PBMC. 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 cocktail above marked T-, B- and NK cells, macrophages, granulocytes-monocytes and hematopoietic precursors

Table IV. Summary of the immune dysfunctions evidenced at the different time-points.[a]

Subpopulations		T0	T1	T2	T3	Changes over time overall p-value	Post-hoc comparisons p-value[b]
CD8	Median	403	219	271	369.5	<0.001	T0-T1=0.000
	25th	270	146	178	228.5		T0-T2=0.000
	75th	593	331	389	578		T0-T3=0.511
CD4	Median	647	383	483	653	<0.001	T0-T1=0.000
	25th	496	298	323	461.5		T0-T2=0.000
	75th	1039	485	642	804		T0-T3=0.035
CD3	Median	1046	548	728.5	968.5	<0.001	T0-T1=0.000
	25th	791	459	507	790.5		T0-T2=0.000
	75th	1352	730	877	1072		T0-T3=0.05
CD3/HLA-DR	Median	22	15	14.5	25	<0.001	T0-T1=0.002
	25th	14	8	8	13		T0-T2=0.000
	75th	35	22	25	43.5		T0-T3=0.156
CD2	Median	1204	676	827.5	1107.5	<0.001	T0-T1=0.000
	25th	926	556	697	890		T0-T2=0.000
	75th	1610	817	1001	1242.5		T0-T3=0.033
CD2/CD25	Median	5	3	4	4.5	0.042	T0-T1=0.019
	25th	2	1	2	3		T0-T2=0.147
	75th	10	6	7	10		T0-T3=0.909
CD56	Median	90	45	26	57	<0.001	T0-T1=0.006
	25th	22	8	11	31		T0-T2=0.002
	75th	153	107	80	220.5		T0-T3=0.389
CD16	Median	139	67	72	106	<0.001	T0-T1=0.000
	25th	76	34	34	45		T0-T2=0.000
	75th	215	165	118	197		T0-T3=0.233
CD16/CD56	Median	37	14	3	13.5	<0.001	T0-T1=0.082
	25th	3	1	1	3.5		T0-T2=0.007
	75th	68	44	36	87		T0-T3=0.000
CD20	Median	158	114	130	150.5	<0.001	T0-T1=0.002
	25th	96	67	105	95		T0-T2=0.711
	75th	239	161	198	198		T0-T3=0.870
CD19	Median	154	118	115	144	0.438	T0-T1=0.443
	25th	90	55	80	81		T0-T2=0.901
	75th	190	168	189	187.5		T0-T3=0.347
CD4/CD45RA	Median	187	99	109	156	<0.001	T0-T1=0.001
T naive	25th	90	58	61	100		T0-T2=0.002
	75th	286	145	173	231.5		T0-T3=0.683
CD4/CD45RO	Median	181	105	157.5	196.5	<0.001	T0-T1=0.000
T memory	25th	98	57	71	98.5		T0-T2=0.006
	75th	356	214	284	302.5		T0-T3=0.447
CD4/CD8	Median	10	5	7	7	0.355	T0-T1=0.111
	25th	5	3	3	3.5		T0-T2=0.493
	75th	20	10	12	14		T0-T3=0.161

Table IV. Continued.

Subpopulations		T0	T1	T2	T3	Changes over time overall p-value	Post-hoc comparisons p-value[b]
CD20/CD38	Median	10	10	9.5	8	0.680	T0-T1=0.854
	25th	6	4	5	3.5		T0-T2=0.703
	75th	21	14	20	14.5		T0-T3=0.253
DC	Median	0.58	0.45	0.39	0.75	0.126	T0-T1=0.404
	25th	0.38	0.26	0.24	0.2		T0-T2 = 0.041
	75th	0.79	0.80	0.63	0.91		T0-T3 = 0.736
DC 1	Median	39.5	42.6	39.65	18.25	0.012	T0-T1=0.863
	25th	18.8	23.3	22	9.63		T0-T2=0.473
	75th	46.9	68.3	62.2	25		T0-T3=0.002
DC 2	Median	25	13.9	19.4	18.45	0.586	T0-T1=0.288
	25th	16.6	9.57	12	11.95		T0-T2=0.919
	75th	36.4	26.6	33.3	28.35		T0-T3=0.892

[a]T0, basal state; T1, 12 h post-operatively; T2, 24 h post-operatively; T3, 8 days post-operatively. Results of the GEE analysis. [b]Statistical significance set at 0.017 for Bonferroni correction.

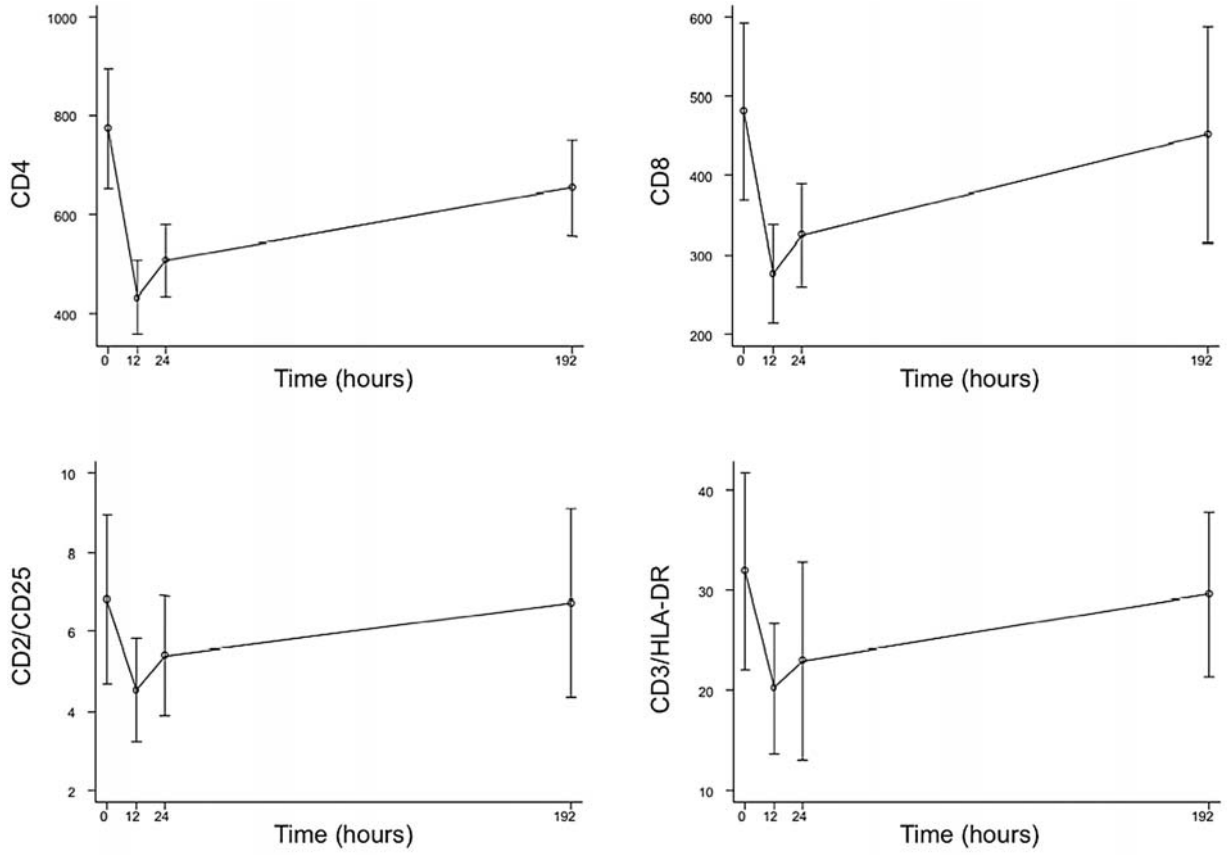

Figure 1. Time changes of selected lymphocyte subsets (CD4, CD8, CD2/CD25, CD3/HLA-DR). Mean and 95% confidence intervals are shown.

simultaneously, that is all the populations positive for HLA-DR (cocktail+/HLA-DR+); dendritic cells were thus identified as cocktail-/HLA-DR+.

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

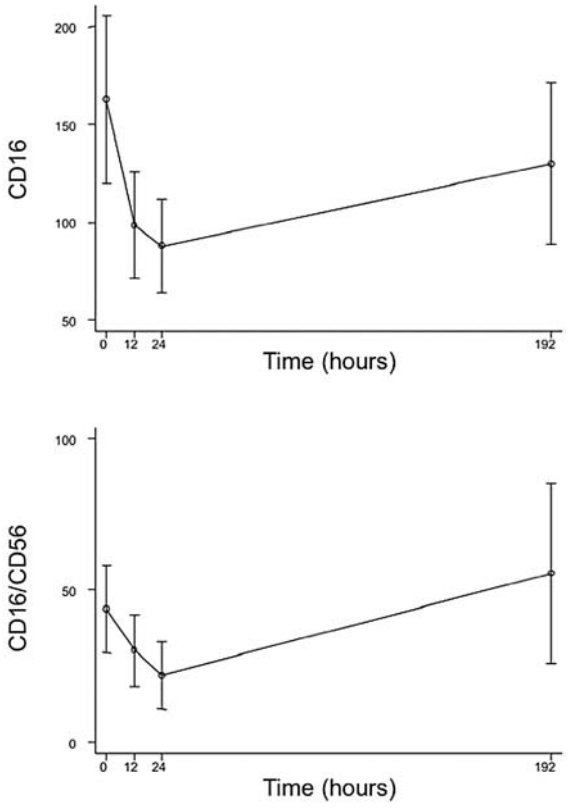

Figure 2. Time changes of two different NK cell subsets (CD16, CD16/CD56). Mean and 95% confidence intervals are shown.

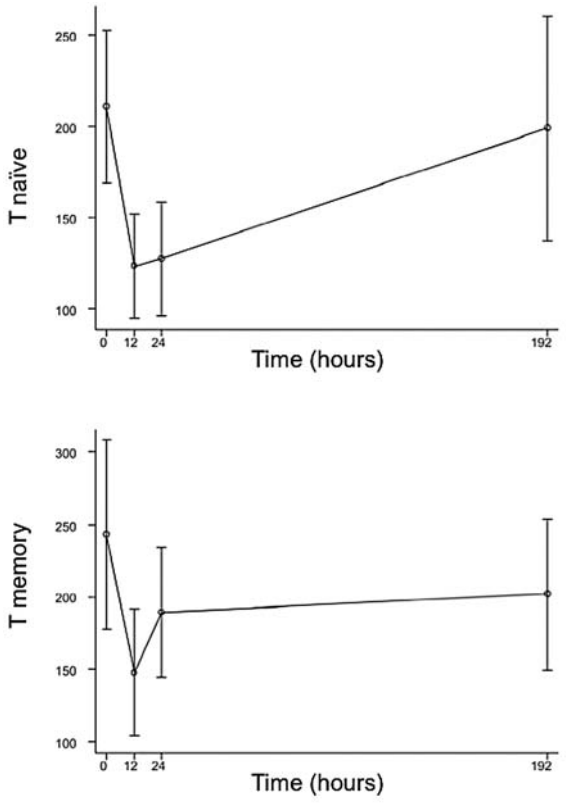

Figure 3. Time changes of selected lymphocyte subsets (T naïve, T memory). Mean and 95% confidence intervals are shown.

with the mixture of FITC, HLA-DR, PC5 and CD11c PE (DC1) or CDw123 PE (DC2). 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$^+$/Leu11c$^+$ and HLA-DR$^+$/CDw123$^+$, respectively).

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

Statistical analysis. Data were described as median and 25th-75th percentiles or as counts and percent. Mean and 95% confidence intervals (95%CI) at each time point were also computed. For the purpose of the analysis, due to sample size, stage and grade were collapsed into 2 categories (0 for stage and grade 1-2; 1 for stage and grade 3-4). Baseline comparisons of subpopulations between categories were performed by means of the Mann-Whitney U test. The association of age and each subpopulation was measured by means of the Spearman R. General estimating equations (GEE) were used (to account for intra-patient correlation) in order to evaluate changes over time in the subpopulation levels. Moreover, the interaction of time and type of surgery, stage, grade, presence of metastasis and gender in turn was included into the models to compare subpopulation profiles over time.

Stata 9.2 (StataCorp, College Station, TX, USA) was used for computation. A 2-sided p-value <0.05 was considered statistically significant. Bonferroni correction was used for multiple comparisons.

Results

Lymphocyte subsets and circulating dendritic cells. RCC patients presented a basal (T0) statistically significant activation of CD3/HLA-DR$^+$ aspecific lymphocytes, of lymphocytes co-expressing the CD4 and CD8 antigens and of the CD56$^+$ NK subset, but the prevalent finding was that of immunosuppression involving naïve T-cells, memory T-cells, CD16$^+$ NK and total circulating dendritic cells. The ratio of DC1 to DC2 cells appeared to be preserved in our RCC patients; nevertheless, the absolute number of circulating dendritic cells was significantly lower in cancer patients relative to controls (data not shown) (1).

Changes over time. For most of the analyzed lymphocytic subsets, we observed a significant change in their levels over time, mainly characterized by a marked initial (at T1) decrease, followed by a progressive return to baseline (at T2), with the exception of CD19$^+$ (Pan B), dendritic cells and their DC-2

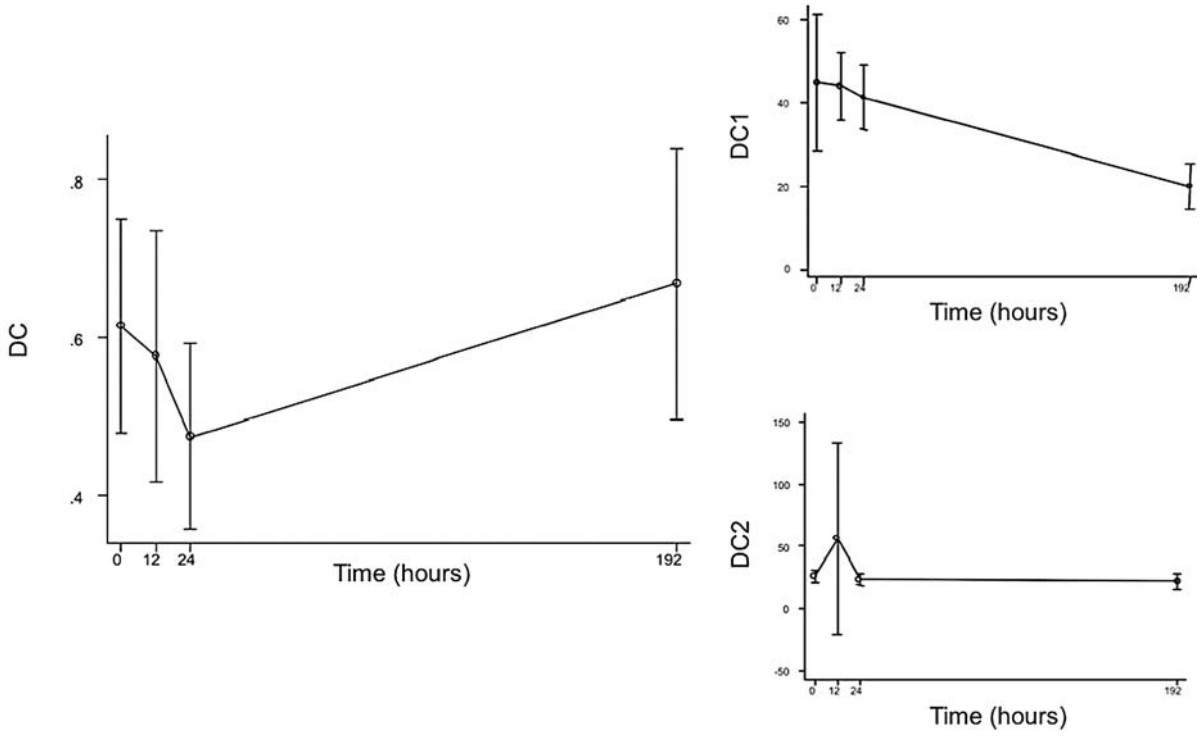

Figure 4. Time changes of DCs and their subsets (DC1, DC2). Mean and 95% confidence intervals are shown.

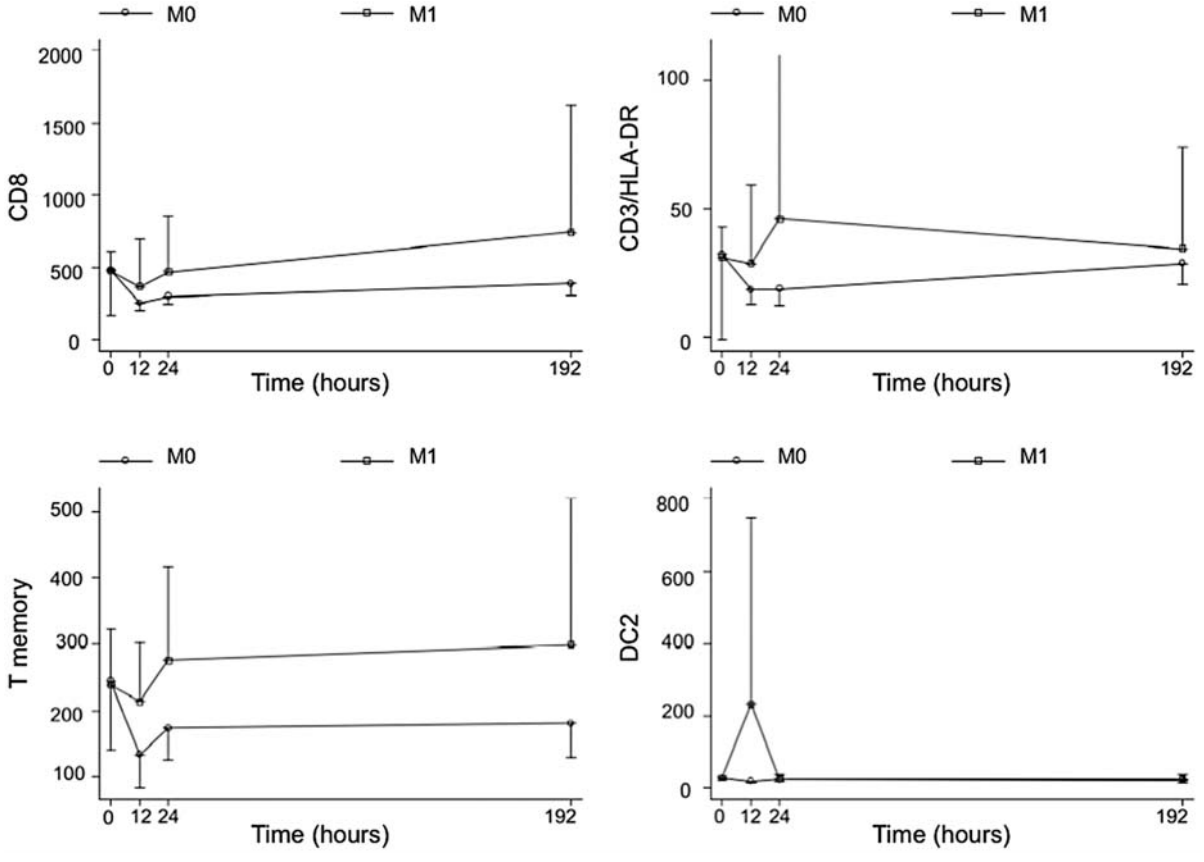

Figure 5. Time changes for CD8, CD3/HLA-DR, CD4/CD45RO T memory and DC 2 according to the presence/absence of metastasis.

subsets, as well as of CD20/CD 38 (early B) and CD4/CD8, DC and DC2. After 8 days (T3) from surgical removal of tumor, however, there was a return to the basal state, except for NK cells CD16/ CD56 and DC 1, which continued to present a statistically significant reduction. Overall results are summarized in Table IV and illustrated in Figs. 1-4.

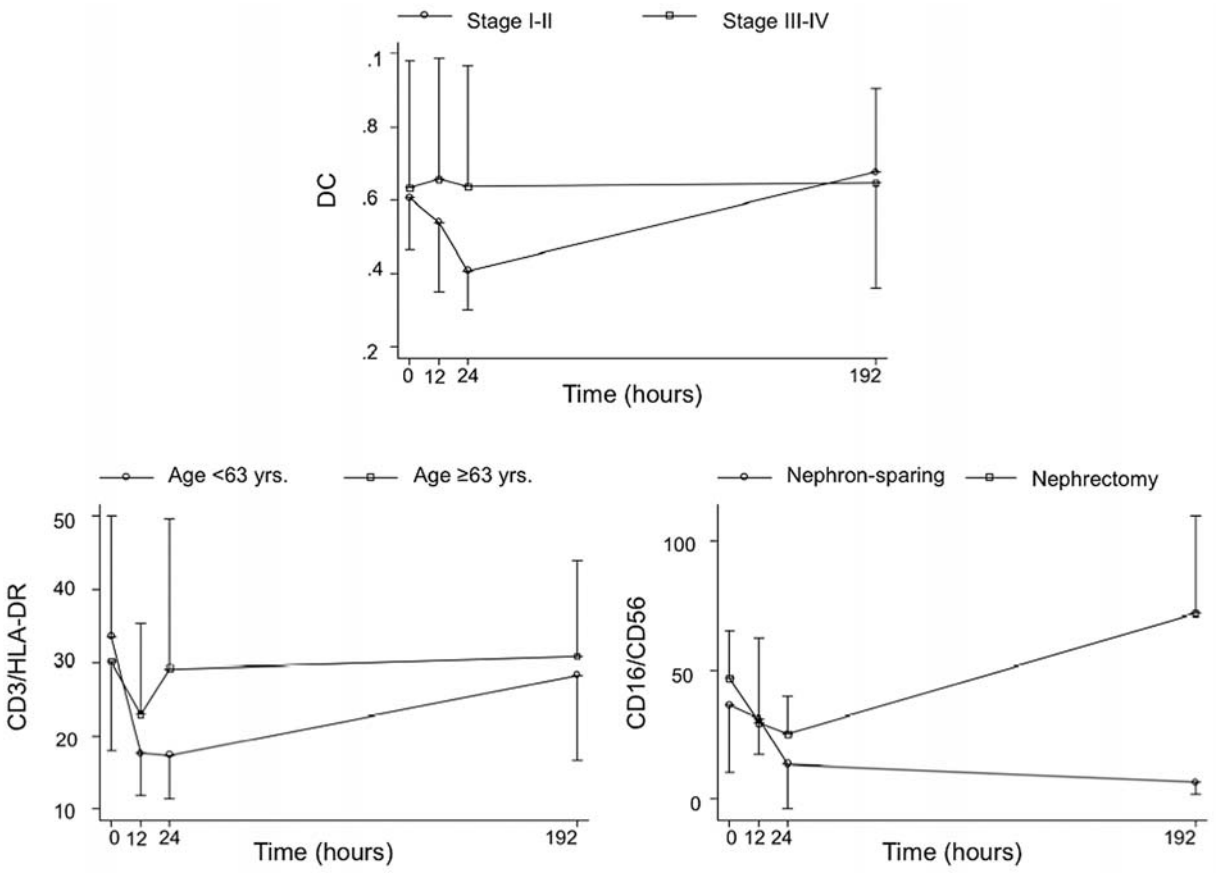

Figure 6. Time changes for CD16/CD56, according to the type of surgery, for DC1, according to stage, and for CD3/HLA-DR, according to age (dichotomized at the median of 63).

Association with patient characteristics. No statistically significant association was found between lymphocyte subset and circulating dendritic cells and any of the following parameters measured at T0: age, gender, disease stage (in terms of TNM), tumor grading or type of surgical approach. However, as shown in Figs. 5 and 6, different time profiles for CD8, CD3/HLA-DR, CD4/CD45RO T memory and DC2 were observed according to the presence/absence of metastasis; for CD16/CD56 according to the type of surgery; for DC1 according to stage; and finally for CD3/HLA-DR according to age (dichotomized at the median of 63).

Discussion

Renal carcinoma patients, especially (but not only) with metastatic disease, usually exhibit several immunological dysfunctions (13,14). The underlying mechanism for immune dysfunction in cancer patients are probably multifactorial (15); indeed, many factors (advanced age, nutritional status, etc.) contribute to the poorly functioning immune system often observed in neoplastic patients (16). However, the role of the tumor itself was clearly demonstrated years ago in a fibrosarcoma mouse model (17); upon inoculation with tumoral cells, these mice became immune suppressed, as demonstrated by impaired antitumor reactivity of T cells, associated with decreased expression of signalling molecules. This phenotype was completely reversed following surgical removal of the tumor, unmasking a population of primed T cells that were capable of rejecting a subsequent tumor challenge.

However, immune suppression, as well as metabolic alterations, are established consequences of surgical stress and trauma, which may expose patients to a higher risk of post-operative complications (18-20). Furthermore, a prospective comparison of the immunological and stress response following either laparoscopic or open surgery for localized RCC clearly showed that both approaches induce similar alterations (21), a result that appears to be in agreement with similar observations done in different tumors (22,23).

In this study, we assessed the evolution (before and after surgery) of immune dysfunctions in patients with RCC undergoing either open radical nephrectomy or nephron-sparing surgery.

We confirmed the presence of relevant basal immune defects in RCC patients, a finding that have already been described, both by our group as well as by other researchers (14,24,25); these alterations involved expecially cell-mediated immunity. Immediately after surgical removal of the tumor, we found a transient, but relevant worsening of the above immunosuppression, that touched the majority of the analyzed subpopulations, with the sole exception of pan B, early B, lymphocytes CD4/CD8 and dendritic cells.

It is well known that surgical trauma may induce immunosuppression through imbalance of pro-inflammatory and anti-inflammatory cytokines, as well as a hypoactivity of

monocytes, which is characterized by a reduced expression of HLA-DR receptor, a defeat of antigen-presenting ability and a reduction in their production of TNF-α upon stimulation with LPS *in vitro* (26).

All patients analyzed in this study had returned to immunological basal conditions one week after surgical tumor removal; however, we should keep in mind that the basal status of these patients was already characterized by a profound state of immune suppression (1).

The fact that immune suppression did not completely resolve with the removal of the tumor bulk in those patients that had no metastasis, could have different causes, including the persistence of soluble mediators of immunosuppression (e.g. TGF-ß, gangliosides) despite tumor removal, or alternatively, the persistence of a microscopical residue of neoplastic cells, that could represent a continous source of immunosuppressive molecules. This latter supposition could be supported by the findings reported by several authors, who, using RT-PCR, demonstrated the frequent presence of circulating tumoral cells in radically operated patients.

The lack of significant correlations between clinico-pathological features and immunological state, could be explained by the fact that immunosuppression is independent from tumor burden. These findings suggest the possibility that immunostimulatory therapies should be applied in the peri-operative setting with the aim of reducing immuno-suppression and, hopefully, also disease recurrence. Prospective studies could thus be warranted in this setting.

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