Frequency of regulatory T cells in peripheral blood and in tumour-infiltrating lymphocytes correlates with poor prognosis in renal cell carcinoma

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OBJECTIVES
To compare the frequency of T regulatory cells (Tregs) in peripheral blood of patients (pPB) affected by renal cell carcinoma (RCC) both with the frequency of Tregs found in PB of healthy donors (hPB) and that of Tregs present in tumour infiltrating lymphocytes (TILs). To verify in vitro the inhibitory activity of tumour isolated Tregs on the effector T cells and, finally, to assess the prognostic role of Treg frequency determination.

PATIENTS AND METHODS
Treg frequency in hPB, pPB and TILs was evaluated in 30 patients and 20 healthy controls by measuring both membrane-CD25 and intracytoplasmic-Foxp3 expression by flow cytometry. Treg inhibitory activity was evaluated by an in vitro proliferation assay performed on total, CD25-depleted mononuclear cells (MNC) and CD25-depleted MNC cultured in the presence of purified CD25+ Tregs. Finally, Treg frequency in pPB and TIL were correlated with conventional prognostic factors and scores of University of California Los Angeles and Kattan predictive models.

RESULTS
Treg frequency was higher in TILs than in pPB (P = 0.002), whereas there were no important differences between hPB and pPB. CD25+ cells isolated either from PB and tumours showed the ability to significantly suppress in vitro both proliferation and interferon-γ production by CD25-depleted MNC, thus demonstrating that they are active Tregs. Treg frequency was found to significantly correlate both with pathological stage (pPB, P = 0.03; TIL, P = 0.04) and nuclear grade (TIL, P = 0.005), both for UCLA and Kattan models (all: P < 0.05 for both pPB and TIL).

CONCLUSION
Treg frequency is significantly higher in TIL than in pPB of patients with RCC. Tregs showed in vitro an inhibitory activity on effector T cells isolated from kidney tumours. The increase in both peripheral and intratumoral Tregs in subjects affected with RCC were associated with worse prognosis.

KEYWORDS
CD25, Foxp3, Treg, RCC, tolerance, tumour immunity

INTRODUCTION
RCC is considered an immunogenic cancer, with pathologic specimens frequently containing large numbers of tumour-infiltrating lymphocytes (TILs) [1]. Until recently, patients with advanced RCC had very few therapeutic options because RCC is refractory to conventional cytotoxic treatments such as chemotherapy or radiotherapy. Only recently has the treatment of RCC undergone a major change, with the development of novel promising therapeutic options such as tyrosine kinase inhibitors and anti-angiogenic agents, as used in first- and second-line therapies [2].

Immune therapy, consisting mainly of interleukin (IL)-2, was considered the mainstay of therapy in the past 30 years, even if the benefit of high-dose IL-2 is observed in a small percentage (15–20%) of highly-selected, good performance status patients with RCC [3]. The response of patients with RCC to IL-2 appears to be related to the stimulation of natural killer and T cells targeted against tumour cells [4]. Nevertheless, in a large proportion of patients, the neoplasm acquires the ability to escape from the immune response, which has been assumed to depend on a direct interaction between the cancer and immune effectors, with local recruitment/induction of immune-regulatory T cells (Treg) [5].
In 1995, a new population of CD4+ T lymphocytes, displaying in vivo and in vitro immunoregulatory activity, was described [6,7]. These cells show constitutive high level surface-expression of CD25 (α-chain of IL-2 receptor) and, for this reason, they were named CD25SUP Tregs. CD25 can be expressed also in the cell membrane of activated effector T cells; therefore, the identification of Tregs only by CD25 high expression can lead to overestimation. The gene foxp3 (fork-head box P3), known to be mutated in patients affected by the multiple autoimmune syndrome, IPEX (immune deregulation, polyendocrinopathy, enteropathy, X-linked) [8], has been identified as the master gene for Tregs development, and the protein product of this gene became the best marker for the identification of Treg [9].

The physiological role of Tregs is the protection against the autoimmune diseases through the direct suppression of T effector cells reacting against ‘self’, although they can be also involved in the control of immune response against exogenous antigens [10]. Because most antigens expressed by neoplastic cells are ‘self’-antigens [11], it is commonly considered that Tregs are also involved in the suppression of the immune response against tumours, favouring tumour escape from immune response [12].

The literature regarding the role of Tregs in RCC is poor and controversial: Griffiths et al. [13] and Li et al. [14] have shown that high frequency of these cells in the peritumoral area of RCC patients correlates with a negative prognosis of the disease, whereas Siddiqui et al. [15] showed no correlation between tumour-infiltrating Treg frequency and disease progression. Probably, the discrepancy among these two studies is secondary to the considerable difference in the methodology used to identify Tregs.

In the present study, Treg frequency was assessed in the peripheral blood of healthy controls (hPB), and patients with RCC (pPB), as well as in TILs. For this purpose, we evaluated CD25 and Foxp3 protein expression by flow-cytometry and Foxp3 mRNA expression by quantitative real-time quantitative PCR (RT-PCR), in TILs and in peripheral blood lymphocytes. We also assessed the in vitro inhibitory activity of Tregs obtained from PB and, for the first time in RCC, from TILs. Finally, we evaluated the prognostic role of Treg frequency by correlating their frequency (in both pPB and TILs) with conventional prognostic factors, to validate predictive models and clinical outcome during patient follow-up. The results of the present study provide clear evidence that functional Tregs are enriched inside the tumour and that their intratumoral and peripheral frequency correlates with worse prognosis in RCC.

PATIENTS AND METHODS

PATIENT SELECTION AND TISSUE SAMPLING

Patients who underwent nephrectomy for non-metastatic RCC between February 2005 and December 2006 were selected for the present study. Subjects with additional neoplasms, autoimmune diseases, lymphatic or lympho-proliferative disorders, or who were treated with chemotherapy and immunotherapy before surgery, were excluded. Preoperative clinical evaluations included chest X-ray and abdominal CT scan. Nephrectomy was performed by an open extraperitoneal approach, without preoperative embolization. Follow-up included blood count, blood chemistry, chest X-ray and abdomen ultrasound every 6 months after surgery; CT or MRI were performed according to a risk-stratified monogram. We collected two 7-mL EDTA vials of peripheral blood (PB) from each patient 1 h preoperatively, and from 20 healthy controls (hPB), and patients with RCC (pPB), and for the first time in RCC, from TILs. Finally, we evaluated the prognostic role of Treg frequency by

LABORATORY ASSESSMENT

Reagents

Medium used was RPMI 1640 (Seromed, Berlin, Germany), supplemented with 2 mM L-glutamine, 1% non-essential aminoacids, 1% pyruvate, 2 x 10⁻⁵ M 2-mercaptoethanol (Gibco Laboratories, Grand Island, NY, USA) and 5% autologous serum. Fluorochrome-conjugated anti-CD3, anti-CD4, anti-CD8, anti-CD14, anti-CD16, anti-CD19, anti-CD25 monoclonal antibodies (mAbs) were purchased from Becton Dickinson (San Jose, CA, USA); anti-Foxp3 (clone PCH101) was obtained by e-Bioscience (San Diego, CA, USA). Mouse anti-human CD25 mAb conjugated with magnetic beads was obtained from Miltenyi Biotec GmbH (Bisley, Germany). Purified, fluorochrome-conjugated isotype control and fluorochrome-conjugated anti-isotype mAbs were purchased from Southern Biotechnology Associates (Birmingham, AL, USA).

Preparation of PB mononuclear cells (MNCs) and isolation of TILs

PB MNCs were obtained from blood specimens by stratification through centrifugation on Ficoll-Hypaque gradient. To obtain TILs, tumour fragments were gently passed through a stainless-steel mesh (Medimachine; Becton Dickinson), which allowed single-cell suspensions to be obtained. Cells were then centrifuged on a Ficoll-Hypaque gradient, harvested and incubated on ice with microbead-conjugated mAbs against the ‘human epithelial antigen’ in accordance with the manufacturer’s instructions (Miltenyi Biotec GmbH) to remove tumour cells. After 20 min, the cells were washed and inserted in a LS+ column (Miltenyi Biotec GmbH) in a magnetic field (MidiMACS; Miltenyi Biotec GmbH); the negative fraction was harvested and evaluated by flow cytometry with anti-CD14, anti-CD19, anti-CD3 and anti-CD16 fluorochrome-conjugated mAbs to determine the phenotype and purity of TILs.

Positive selection of CD25+ T cells and obtainment of CD25-depleted MNCs

CD25+ T cells were enriched by immunomagnetic sorting, as described...
Previously [18]. Briefly, MNCs obtained from PB or TILs were incubated with an anti-CD25 microbead-conjugated mAb (Milteny Biotec GmbH) and, after washing, cells were separated on a LS+ column. The purity of recovered cells was consistently higher than 98%, and the purity of the eluted fraction (CD25-depleted MNCs) was higher than 95%.

**RT-PCR**

Total RNA was extracted and treated with DNase I (Qiagen, Hilden, Germany) to eliminate possible genomic DNA contamination. Taq-Man RT-PCR was performed as described previously [19]. Foxp3 quantitative analysis was performed using Assay on Demand (Applied Biosystems, Warrington, UK). All primers and probes did not react with DNA.

**Flow cytometry analysis**

MNCs obtained from PB or from TILs, were washed with PBS plus 0.5% BSA, incubated with the appropriate fluorochrome-conjugated mAbs at 4 °C for 15 min, washed with PBS plus 0.5% BSA, and acquired with a BDLSR II flow-cytometer in accordance with the manufacturer’s instructions (BD Biosciences). At least 10 000 cells were acquired and analyzed by using FACS Diva software (BD Biosciences).

**Cytokine production**

The concentration of the effector cytokine interferon (IFN)-γ was measured by flow-cytometry on the culture supernatants collected on day 5 from three out of the six proliferation experiments. In particular, we assessed cytokine concentration by using the Flex Set kit (BD Biosciences), in accordance with the manufacturer’s instructions. Samples were acquired by a BDLSR II flow-cytometer and the data obtained were analysed by FCAP array software (BD Biosciences).

**STATISTICAL ANALYSIS**

An unpaired samples t-test was used to assess the difference of Treg frequency between PB, pPB and TILs (Fig. 1), between low and high stage and low and high grade (Fig. 2), and between low, intermediate and high risk for the UCLA predictive model (Fig. 3, left). Pearson’s correlation coefficient was used to calculate the correlation rate between Treg frequency and the Kattan estimated probability of 5 years of disease-free survival (Fig. 3, right). P < 0.05 (two-tailed) was considered statistically significant.

**RESULTS**

**PATIENT CHARACTERISTICS**

Thirty patients (13 males and 17 females) with mean (range) age of 65.5 (31–82) years, and 20 healthy controls (11 males and nine females) with mean (range) age of 40.1 (19–67) years, were included in the present study. Twenty-five (83.33%) patients presented a conventional clear cell carcinoma, three (10%) with a chromophobe subtype and two (6.67%) with a papillary. Five (16.6%) tumours were classified as T1a, 13 (43.4%) as T1b, two (6.6%) as T2 and 10 (33.4%) as T3. Two patients (6.6%) presented positive lymph nodes by microscopic evaluation. G2 Fuhrman nuclear grade was reported in 25 (83.3%) patients, G3 in four (13.4%) and G4 in one (3.4%). Mean (range) tumour diameter was 5.4 (2–12) cm, with a mean (range) tumour volume of 105 (10–320) cm³.

At a mean of 24 months follow-up, 25 patients were disease-free, whereas five patients presented a tumour recurrence, including one patient who was deceased, for RCC. Three of these patients underwent nephron-sparing surgery and two underwent radical nephrectomy. Tumour stage was pT1a.
specimens from patients (PCH101) was commercially available, because anti-human Foxp3 mAb (clone PCH101) was commercially available, specimens from patients (n = 19) and from healthy donors (n = 15) were evaluated for both CD25 and Foxp3 expression, and the mean frequency of CD4+CD25+Foxp3+ T cells in pPB was comparable to the levels observed in the hPB (P = 0.57), whereas the frequency of CD4+CD25+Foxp3+ Treg in TILs was significantly higher than in pPB (P = 0.002) (Fig. 1A). To provide additional evidence for this finding, mRNA for Foxp3 was also evaluated by real-time quantitative RT-PCR in PB of 10 healthy donors, as well as in PB and TILs from nine randomly selected patients. As shown in Fig. 1B, healthy donors and tumour-affected patients showed similar Foxp3 mRNA values in circulating lymphocytes (P = 0.49), whereas Foxp3 mRNA was expressed more in TILs than in PB (P = 0.08), although, differently from the Foxp3 flow cytometric evaluation, in this case, a significant difference was being obtained, probably as a result of the low number of patients studied. The mean ± SE difference between CD25+ T cells and part of the latter cells were added to CD25+ T cells (CD25+/CD25+) and part of the latter cells were added to CD25+ cells in a 1 : 1 ratio (CD25+/CD25+). The three populations were then stimulated in vitro by allogeneic irradiated PB MNCs (MLR) for 5 days; in the last 12 h, [3H]thymidine was added to cultures, and radioactivity incorporation was measured by using a β-counter. Data are the mean ± SE of three separate experiments. Right: the supernatants from three of the above reported experiments were harvested on day 5, before [3H]thymidine addition, and evaluated by flow-cytometry for the amount of cytokines. Data are the mean ± SE.

FIG. 2. Functional features of regulatory CD25+ T cells. MNCs were obtained from pPB and from TILs. Left: part of the MNC were depleted of CD25+ T cells (CD25−) and part of the latter cells were added to CD25+ cells in a 1 : 1 ratio (CD25+/CD25+). The three populations were then stimulated in vitro by allogeneic irradiated PB MNCs (MLR) for 5 days; in the last 12 h, [3H]thymidine was added to cultures, and radioactivity incorporation was measured by using a β-counter. Data are the mean ± SE of three separate experiments. Right: the supernatants from three of the above reported experiments were harvested on day 5, before [3H]thymidine addition, and evaluated by flow-cytometry for the amount of cytokines. Data are the mean ± SE.

EXPRESSION OF CD25+ AND FOXP3+ CELLS IS HIGHER IN TILS AND PB OF PATIENTS WITH RCC THAN IN PB OF HEALTHY CONTROLS

The frequency of CD4+CD25+ T cells was significantly higher in pPB than in hPB (P = 0.03), whereas no significant differences were observed between pPB and TILs (P = 0.07).

Because anti-human Foxp3 mAb (clone PCH101) was commercially available, specimens from patients (n = 19) and from healthy donors (n = 15) were evaluated for both CD25 and Foxp3 expression, and the mean frequency of CD4+CD25+Foxp3+ T cells in pPB was comparable to the levels observed in the hPB (P = 0.57), whereas the frequency of CD4+CD25+Foxp3+ Treg in TILs was significantly higher than in pPB (P = 0.002) (Fig. 1A). To provide additional evidence for this finding, mRNA for Foxp3 was also evaluated by real-time quantitative RT-PCR in PB of 10 healthy donors, as well as in PB and TILs from nine randomly selected patients. As shown in Fig. 1B, healthy donors and tumour-affected patients showed similar Foxp3 mRNA values in circulating lymphocytes (P = 0.49), whereas Foxp3 mRNA was expressed more in TILs than in PB (P = 0.08), although, differently from the Foxp3 flow cytometric evaluation, in this case, a significant difference was being obtained, probably as a result of the low number of patients studied. The mean ± SE difference between CD25+ and Foxp3 proportions was particularly evident in pPB (CD4+CD25+ = 4.61 ± 0.3%, CD4+CD25−Foxp3+ = 3.3 ± 0.5%, variation = 29%), whereas, in TILs, no significant differences were observed (CD4+CD25+ = 7.3 ± 1.3%, CD4+CD25−Foxp3+ = 6.6 ± 1%, variation = 10%).

**TABLE 1 Demographic and pathological characteristics of patients**

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<td>Patients (n)</td>
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<td>G4: 1</td>
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<td>Volume (cm3)</td>
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All of the patient characteristics are summarized in Table 1.

**Figures**

**A** 3H-TdR uptake (CPM)

**B** IFN-γ (pg/ml)

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TREGS ISOLATED FROM TILS OF RCC PRESENT IN VITRO INHIBITORY ACTIVITY

To determine whether Tregs from pPB and from TILs exhibited *in vitro* immunosuppressive activity, we obtained PB and TIL MNCs from six patients. All samples were divided into: (i) total MNC, containing the physiological proportion of effector T cells and Tregs; (ii) MNC depleted of CD25+ cells (indicated as CD25−); and (iii) CD25+ cells reconstituted with CD25+ in a 1 : 1 ratio. With regard to TILs specimens this latter condition (iii) was performed only in three out of the six patients because of the low number of cells obtained from biopsies.

As shown in Fig. 2A, CD25+ cells from both PB and TILs showed significantly higher proliferation compared to total MNC. Moreover, the addition of the CD25+ to CD25− cell fraction in a cell–cell ratio of 1 : 1
CD4+CD25 Foxp3+ Tregs correlates with T stage, whereas TIL CD4+CD25 Foxp3+ Tregs correlates with both T stage and nuclear grading. Patients were stratified in groups on the basis of T stage and Fuhrman nuclear grading. These groups were then evaluated for Treg frequency at the peripheral level (pPB) and at the intratumoural level (TIL). Histograms represent the mean ± SE frequency of CD4+CD25 Foxp3+ Treg cells in the 19 patients.

**Fig. 3.** Frequency of circulating CD4+CD25 Foxp3+ Tregs correlates with T stage, whereas TIL CD4+CD25 Foxp3+ Treg correlates with both T stage and nuclear grading. Patients were stratified in groups on the basis of T stage and Fuhrman nuclear grading. These groups were then evaluated for Treg frequency at the peripheral level (pPB) and at the intratumoural level (TIL). Histograms represent the mean ± SE frequency of CD4+CD25 Foxp3+ Treg cells in the 19 patients.

**Fig. 4.** Frequency of circulating CD4+CD25 Foxp3+ Tregs is associated with UCLA risk score, and their frequency inversely correlates with the Kattan score. A, Patients were stratified on the basis of the UCLA risk score (high, intermediate and low) and the frequency of circulating (pPB) and of intratumoural (TIL) Tregs was evaluated by flow cytometry. Histograms represent the mean ± SE Treg frequency in each subgroup of patients. B, Correlations between circulating (left) or tumour-infiltrating (right graph) Treg (CD25 Foxp3+) cells and the Kattan value are shown.

To further determine the relationship between Tregs frequency and tumour recurrence, the 30 patients were followed-up for an evaluation of disease relapse (mean ± SE: 24.9 ± 1 months; median: 25 months). As previously noted, the mAb for Foxp3 was not available at the beginning of the present study; thus, those patients recruited in...
the first year were monitored for only CD4+CD25high expression in both PB and TILs; for this reason in this part of the present study, we identify Tregs as CD4+CD25high T cells, instead of CD4+CD25+Foxp3+ T cells. Five patients out of 30, all of having been recruited at the beginning of the study, reported a relapse of tumoural disease, and had TIL patients out of 30, all of having been recruited for this reason in this part of the present study, we identify Tregs as CD4+ T cells.

In the present study, we evaluated Tregs in both PB and TILs. These cells were defined on the basis of the expression of selective markers at protein and RNA levels. Moreover, the real regulatory nature of intratumoural and circulating Tregs was investigated by two different functional assays. Finally, to define the role of Tregs in the prognosis of RCC, the presence of these cells was correlated with prognostic parameters validated for predictive models and clinical outcome.

A robust, positive correlation between CD25high and CD25+Foxp3+ was present in TILs and a smaller, but still significant, correlation was present in PB, suggesting that CD25 high circulating T cells in RCC patients include not only regulatory T cells, but also probably activated CD25+ effector lymphocytes. The inhibitory nature of CD25+Foxp3+ cells was confirmed by in vitro immunosuppressive assays, performed on PB and, for the first time, on TILs from patients suffering from RCC. The first remarkable result of the present study is that Tregs are particularly represented in TILs compared to PB, and that these cells express an in vitro inhibitory activity on effector cells isolated from the same RCC patients.

In addition, we show a significant positive correlation not only between Treg frequency at both PB and TIL levels and pathological stage, but also between Tregs frequency in TILs and Fuhrman nuclear grade. The prognostic significance of Tregs was confirmed by using two survival predictive models: the UCLA integrated staging system and the Kattan postoperative nomogram; both of them confirmed a negative prognostic value of a high Tregs frequency at peripheral and at intratumoural levels. Notably, our preliminary data on Treg frequency and disease-free survival (mean follow-up of 24.9 months) show a clear correlation between high Treg frequency at both peripheral and tumoural levels and tumour recurrence.

In conclusion, the present study shows that Tregs are enriched in TILs of patients with RCC. We also show, for the first time, the in vitro inhibitory activity of Tregs on effector cells isolated in the kidney neoplasms. Finally, the increase of both peripheral and intratumoural Tregs in subjects with renal tumours was associated with a worse prognosis. These data confirm that Tregs may play a significant role in the tumour microenvironment of patients with RCC.

Additional studies are necessary to investigate the possible implications for improving prognostic evaluation, planning tailored follow-up and developing targeted immunotherapies.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

None declared.

REFERENCES


5 Nishikawa H, Kato T, Kawara I et al. 

Immunologic tolerance maintained by CD25+CD4+ T regulatory cells: their common role in controlling autoimmunity, tumourimmunity and transplantation tolerance. Immunol Rev 2001; 182: 18–32

7 Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. 

8 Gambineri E, Torgerson TR, Ochs HD. 

9 Fontenot JD, Gavin MA, Rudensky AY. 

10 Maggi L, Santarlasci V, Liotta F et al. 

11 Nishikawa H, Kato T, Tanida K et al. 
CD4+CD25+ T cells responding to serologically defined autoantigens suppress antitumorimmune responses. Proc Natl Acad Sci USA 2003; 100 (19): 10902–6

12 Baecher-Allan C, Anderson DE. 

13 Griffiths R, Elkord E, Gilham D et al. 

14 Li JF, Chu YW, Wang GM et al. 
The prognostic value of peritumoral regulatory T cells and its correlation with intratumoral cyclooxygenase-2 expression in clear cell renal cell carcinoma. BJU Int 2009; 103 (3): 399–405

15 Siddiqui S, Frigola X, Bonne-Annee S et al. 

16 Zisman A, Pantuck AJ, Dorey F. 

17 Kattan M, Reuter V, Motzer R, Katz J, Russo P. 

18 Anunziato F, Cosmi L, Liotta F et al. 

19 Cosmi L, Liotta F, Lazzeri E et al. 

20 Wolf A, Wolf D, Steurer M. 

21 Curiel T, Coukos G, Zou L. 
Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predict reduced survival. Nat Med 2004; 10: 942–9

22 Hiraoka N, Onozato K, Kosuge T, Hirohashi S. 

23 Fu J, Liu Z, Shi M et al. 
Increased regulatory T cells correlate with CD8 T-cell impairment and poor survival in hepatocellular carcinoma patients. Gastroenterology 2007; 132: 2328–39

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Abbreviations: IFN, interferon; IL, interleukin; TIL, tumour-infiltrating lymphocyte; hPB, peripheral blood of healthy controls; mAb, monoclonal antibody; MLR, mixed leucocyte reaction; MNC, mononuclear cells; pPB, peripheral blood of patients with RCC; Treg, regulatory T cells; UCLA, University of California Los Angeles.