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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].

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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 $CD25^{high}$ 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 intratumoural 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. After kidney removal, two samples (0.5–1.0 cm^3) were taken from the neoplasm to freshly obtain TILs. Patients and healthy controls provided their informed consent, and the procedures followed in the present study were carried out in accordance with the ethical standards of the Regional Committee on Human Experimentation.

PATHOLOGICAL ASSESSMENT AND PREDICTIVE MODELS

All tumour specimens were analyzed by a single uropathologist who had a much experience of RCC. The Heidelberg classification was used to assign the histologic type. All RCC were classified according to the 2002 TNM staging systems, as well as the Fuhrman nuclear grade. Two validated predictive models were used to assess the risk of tumour recurrence: the University of California Los Angeles (UCLA) model [16] to determine the low, intermediate or high class of recurrence risk, and that of Kattan *et al.* [17] to calculate the probability of 5 years of disease-free survival.

LABORATORY ASSESSMENT

Reagents

Medium used was RPMI 1640 (Seromed, Berlin, Germany), supplemented with 2 mM L-glutamine, 1% non-essential aminoacids, 1% pyruvate, 2×10^{-5} M 2-mercaptoethanol (Gibco Laboratories, Grand Island, NY, USA) and 5% autologous serum. Fluorocrome-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, fluorocrome-conjugated isotype control and fluorocrome-conjugated anti-isotype mAbs were purchased from Southern Biotechnology Associates (Birmingham, AL, USA).

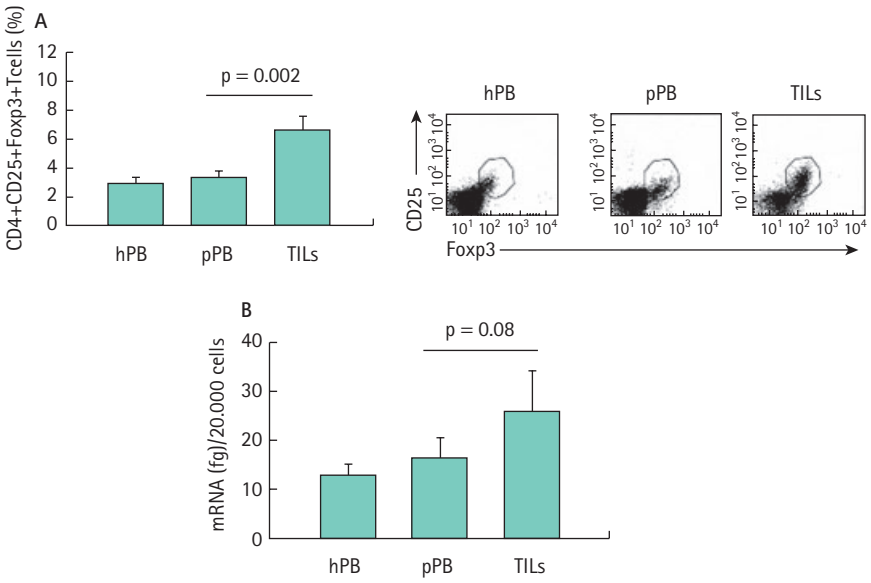
Preparation of PB mononuclear cells (MNCs) and isolation of TILs

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 than 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 determinate 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

*FIG. 1. $CD4^+CD25^+Foxp3^+$ Tregs are strongly expressed in TILs compared to PB. **A**, $CD4^+CD25^+Foxp3^+$ T cells were assessed as described above. Histograms represent the mean ± SE (healthy donors, n = 15; patients, n = 19). Three representative plots are shown on the right. **B**, mRNA levels for Foxp3 were measured by quantitative RT-PCR, and the values obtained were normalized on the absolute count of $CD4^+$ T cells evaluated in each specimen by flow cytometry. Data are the mean ± SE (healthy donors, n = 10; patients, n = 9).*



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

MNC obtained from PB or from TILs, were washed with PBS plus 0.5% BSA, incubated with the appropriate fluorocrome-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).

IN VITRO EVALUATION OF TREG FUNCTION

T cell proliferation

To confirm their real regulatory nature, Tregs were purified, as previously described, from six patients, and tested for *in vitro* immunosuppressive activity. The suppressive activity of Tregs was assessed by evaluating the proliferation of total T cells or of CD25-depleted T cells in response to an allogeneic stimulus (mixed leucocyte reaction, MLR), as described previously [15,16]. Briefly total MNCs and CD25-depleted MNCs obtained from pPB and TILs, were cultured (0.5×10^6 per mL) in triplicate on 96-well U-bottomed plates (Nunc, Kamstrup, Denmark) for 5 days in the presence of MLR stimulation. After a 12-h pulse with 18 500 Bq [^{3}H]thymidine per well (Amersham International, Little Chalfont, UK), cultures were harvested and [^{3}H]TdR was measured by using a β-counter (Packard Instrument B. V. Chemical Operations, Brussels, Belgium).

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 Bioscences), 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 Bioscences).

STATISTICAL ANALYSIS

An unpaired samples *t*-test was used to assess the difference of Treg frequency between hPB, 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) cm^3.

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

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 radionuclide 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]thymidin addition, and evaluated by flow-cytometry for the amount of cytokines. Data are the mean ± SE.

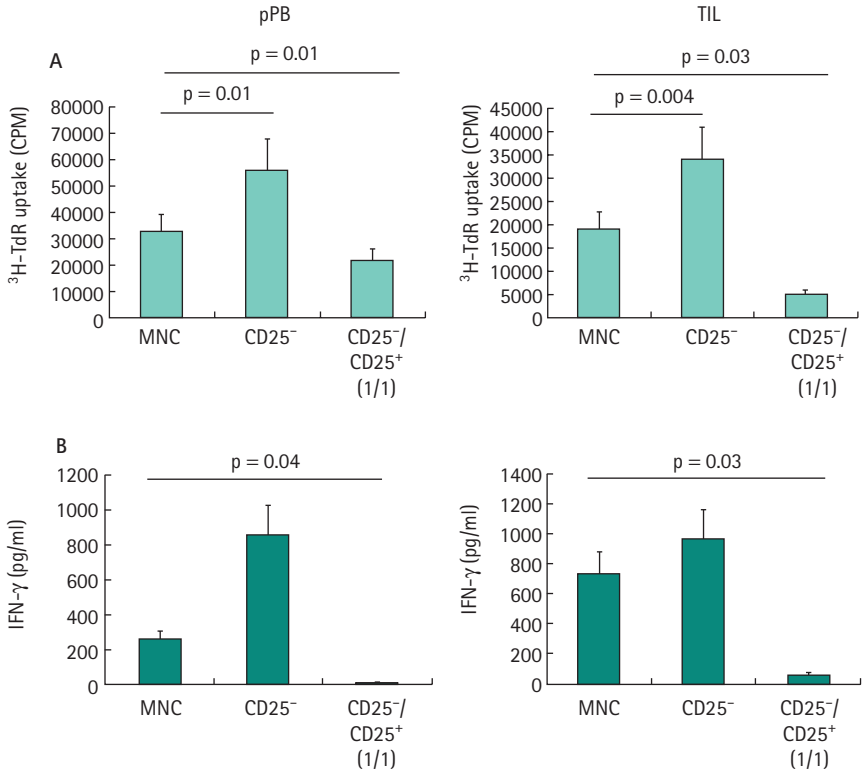


TABLE 1 Demographic and pathological characteristics of patients

Parameter	Value
Patients (*n*)	30
Age (years)	
Median	65.5
Range	31–82
Males/females	13/17
Histological subtype	
Conventional	25
Chromophobe	3
Papillary	2
Pathological stage	
T1a	5
T1b	13
T2	2
T3a	8
T3b	2
Fuhrman nuclear grading	
G2	25
G3	4
G4	1
Volume (cm^3)	
Mean ± SE	118 ± 33.1
Range	10.5–318.24

for two patients, pT1b for two of the other patients and pT3a for the final patient. All the tumours that recurred were graded G2, except for one G1. Mean (range) time to recurrence was 16.8 months (15–28) months.

All of the patient characteristics are summarized in Table 1.

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 $CD4^+CD25^{high}$ 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 pPB ($P = 0.08$), 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 $CD25^{high}$ and Foxp3 proportions was particularly evident in pPB ($CD4^+CD25^{high}$ = 4.61 ± 0.3%, $CD4^+CD25^+Foxp3^+$ = 3.3 ± 0.5%, variation = 29%), whereas, in TILs, no significant differences were observed ($CD4^+CD25^{high}$ = 7.3 ± 1.3%, $CD4^+CD25^+Foxp3^+$ = 6.6 ± 1%, variation = 10%).

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⁺ cells 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

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.

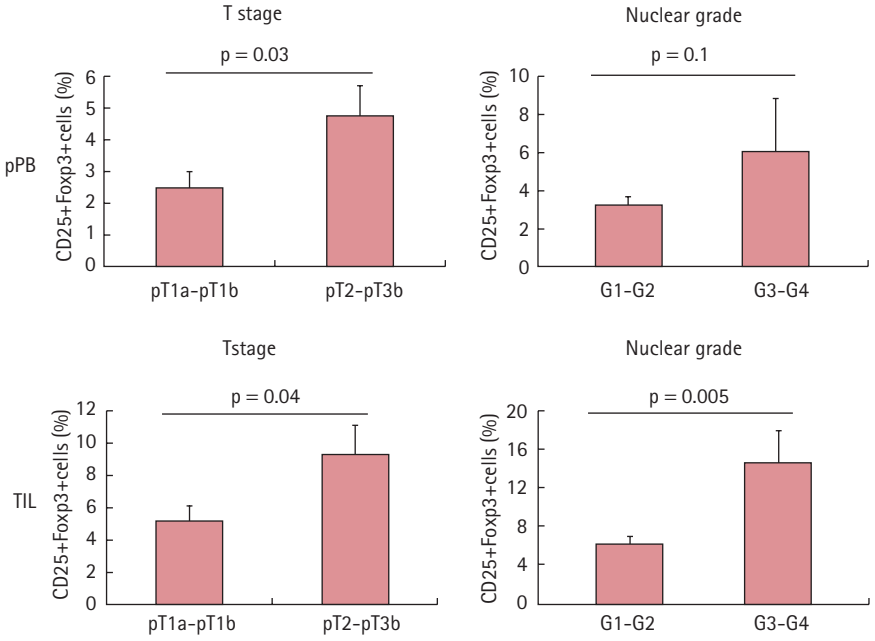
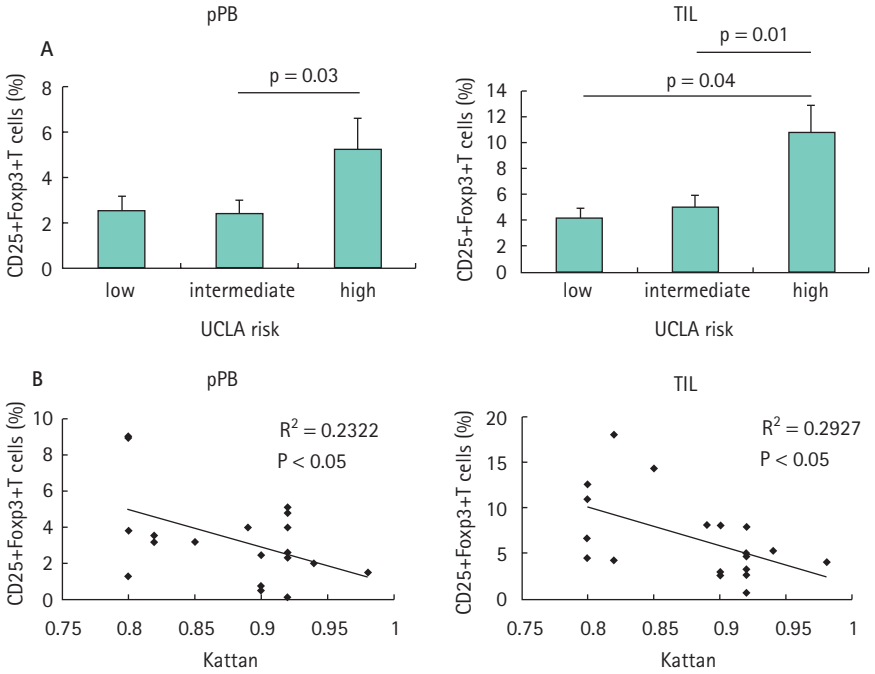


*FIG. 4. CD4⁺CD25⁺Foxp3⁺ Treg cells are represented more in both PB and TILs of patients with a higher UCLA score, and their frequency inversely correlates with the Kattan score. **A**, Patients were stratified on the basis of the UCLA risk score (high, intermediate and low) and the frequency of circulating (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.*



significantly inhibited the proliferation of the latter (Fig. 2A).

Finally, culture supernatants from three out of the six experiments described above were harvested on day 5 and evaluated by flow cytometry for IFN-γ content. As shown in Fig. 2B, a significant decrease of IFN-γ production was observed when $CD25^+$ cells were added to $CD25^-$ cells in a 1 : 1 cell ratio, confirming the immunoregulatory nature of Tregs present in PB and in TILs of patients affected with RCC.

FREQUENCY OF TREGS CORRELATES WITH A HIGHER GRADE AND STAGE IN PATIENTS WITH RCC, AND WITH A HIGHER RISK OF TUMOUR RECURRENCE FOR THE UCLA AND KATTAN PREDICTIVE MODELS

No correlation was found between $CD4^+CD25^+Foxp3^-$ T cells from PB or TILs and hystotype, tumour size and lymph node metastasis. By contrast, significant correlations were observed between Tregs frequency and the tumour stage and grade. In particular, patients with a pT1 tumour show a lower Treg frequency than those with a ≥pT2 RCC both in PB ($P = 0.03$) and TILs ($P = 0.04$) (Fig. 3, left). We did not observe significant differences between PB Tregs of patients with low (G1–G2) compared to high (G3–G4) Fuhrman nuclear grade tumours ($P = 0.10$), whereas a significant difference was observed in TILs ($P = 0.005$) (Fig. 3, right).

In addition, we observed a strong correlation between Tregs frequency and the risk of tumour recurrence both for UCLA and Kattan predictive models. In particular, the mean frequency of Treg both in PB and TILs was significantly higher in patients with a higher UCLA risk of relapse than in the intermediate or the combined low plus intermediate risk group ($P = 0.03$ and $P = 0.01$, respectively) (Fig. 4A). Moreover, we reported a significant inverse correlation between the frequency of Treg in PB and TILs and the probability of 5 years of disease-free survival in the Kattan model ($P < 0.05$) (Fig. 4B).

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 $CD4^+CD25^{high}$ 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 $CD4^+CD25^{high}$ T cells counts that were higher than those in patients who did not experience any relapse: mean Treg frequency: 17.12 ± 4.7% vs 5.17 ± 1.4% respectively, $P = 0.004$; a similar but tighter trend was observed at the PB level: 5.47 ± 0.69% in patients with relapse vs 3.9 ± 0.26% in disease-free patients ($P = 0.02$).

DISCUSSION

Tregs are involved in the peripheral control of autoimmune diseases through the direct suppression of T effector cells reacting against 'self'. Because most antigens expressed by neoplastic cells are 'self'-antigens [11], Tregs are considered to be involved in the suppression of the immune response against tumours [12]. Accordingly, mice *in vivo* models and studies in humans have confirmed this concept. With regard to the studies in humans, Wolf *et al.* [20] were the first to report an increase of Tregs in PB of patients with cancer. In addition, Curiel *et al.* [21] showed that tumour-infiltrating Tregs were specific for tumour antigens, emphasizing the effective role of these cells in suppressing the anti-tumour immune response. Furthermore, higher percentages of Tregs in PB or TILs were associated with poor survival outcomes in several cancers, such as ovarian [21], pancreas [22] and liver [23] carcinomas.

The role of Tregs in RCC is still controversial: Griffiths *et al.* [13] and, more recently, 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.

The discrepancies among these studies depend on the significant differences in the methodologies and controls used to address the research objective: Siddiqui *et al.* [15] evaluated Tregs exclusively at the tumoural level by confocal microscopy and Li *et al.* [14] analysed Treg infiltration at the peri-tumoural level by immunohystochemestry. Both techniques have low statistical power and accuracy for the determination of rare cell populations (i.e. cells represented at low frequencies). Moreover, they did not investigate the frequency of circulating Tregs, nor their functional properties. Griffiths *et al.* [13] evaluated Tregs at both intratumoural and peripheral levels by flow cytometry, although functional tests on intratumoural Tregs were not performed; moreover, Treg frequency was correlated with patients' outcome (median follow-up: 19.4–24.5 months), but conventional prognostic parameters were not evaluated, nor were predictive models validated. For this reason, 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 to validate predictive models and clinical outcome.

A robust, positive correlation between $CD25^{high}$ 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 pPB, 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 neoplasm. 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.

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CONFLICT OF INTEREST

None declared.

REFERENCES

1 **Bromwich EJ, McArdie PA, Canna K.** The relationship between T-lymphocyte infiltration, stage, tumor grade and survival in patients undergoing curative surgery for renal cell cancer. *Br J Cancer* 2003; **89**: 1906–8
2 **Kruck S, Kuczyk MA, Gakis G, Kramer MW, Stenzl A, Merseburger AS.** Novel therapeutic options in metastatic renal cancer – review and post ASCO 2007 update. *Rev Recent Clin Trials* 2008; **3**: 212–6
3 **Dutcher J.** Current status of interleukin-2 therapy for metastatic renal cell carcinoma and metastatic melanoma. *Oncology* 2002; **16**: 4–10
4 **Donskov F.** Interleukin-2 based immunotherapy in patients with metastatic renal cell carcinoma. *Dan Med Bull* 2007; **54** (4): 249–65

5 **Nishikawa H, Kato T, Tawara I *et al.*** Accelerated chemically induced tumor development mediated by CD4+CD25+ regulatory T cells in wild-type hosts. *Proc Natl Acad Sci U S A* 2005; **102** (26): 9253–7

6 **Sakaguchi S, Sakaguchi N, Shimizu J *et al.*** Immunologic tolerance maintained by CD25+CD4+ T regulatory cells: their common role in controlling autoimmunity, tumorimmunity and transplantation tolerance. *Immunol Rev* 2001; **182**: 18–32

7 **Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M.** Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 1995; **155** (3): 1151–64

8 **Gambineri E, Torgerson TR, Ochs HD.** Immune dysregulation, polyendocrinopathy, enteropathy, and X-linked inheritance (IPEX), a syndrome of systemic autoimmunity caused by mutations of FOXP3, a critical regulator of T-cell homeostasis. *Curr Opin Rheumatol* 2003; **15** (4): 430–5

9 **Fontenot JD, Gavin MA, Rudensky AY.** Foxp3 programs the development and function of CD4+CD25+ regulatory Tcells. *Nat Immunol* 2003; **4**: 330–6

10 **Maggi L, Santarlasci V, Liotta F *et al.*** Demonstration of circulating allergen-specific CD4+CD25highFoxp3+ T-regulatory cells in both nonatopic and atopic individuals. *J Allergy Clin Immunol* 2007; **120** (2): 429–36

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 U S A* 2003; **100** (19): 10902–6

12 **Baecher-Allan C, Anderson DE.** Immune regulation in tumour-bearing hosts. *Curr Opin Immunol* 2006; **18** (2): 214–9

13 **Griffiths R, Elkord E, Gilham D *et al.*** Frequency of regulatory T cells in renal cell carcinom patinets and investigation of correlation with survival. *Cancer Immunol Immunother* 2007; **56**: 1743–53

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.*** Tumorinfiltrating Foxp3-CD4+CD25+ T cells predict poor survival in renal cell carcinoma. *Clin Cancer Res* 2007; **13**: 2075–81

16 **Zisman A, Pantuck AJ, Dorey F.** Improved prognostication of renal cell carcinoma using and integrated staging system. *J Clin Oncol* 2001; **19**: 1649–57

17 **Kattan M, Reuter V, Motzer R, Katz J, Russo P.** A postoperative monogram for renal cell carcinoma. *J Urol* 2001; **166**: 63–7

18 **Annunziato F, Cosmi L, Liotta F *et al.*** Phenotype, localization and mechanism of suppression of CD4+CD25+ human thymocytes. *J Exp Med* 2002; **196**: 379–87

19 **Cosmi L, Liotta F, Lazzeri E *et al.*** Human CD8+CD25+ thymocytes sharing phenotypic and functional features with CD4+CD25+ regulatory thymocytes. *Blood* 2003; **102**: 4107–14

20 **Wolf A, Wolf D, Steurer M.** Increase of regulatory T cells in the peripheral blood of cancer patients. *Clin Cancer Res* 2003; **9**: 606–12

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

22 **Hiraoka N, Onozato K, Kosuge T, Hirohashi S.** Prelavence of Foxp3+ regulatory cells increases during the progression of pancreatic ductal adenocarcinoma and its premalignant lesions. *Clin Cancer Res* 2006; **12**: 5423–34

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.