

Cancer Therapy: Clinical**Increased Intratumoral FOXP3-positive Regulatory Immune Cells during Interleukin-2 Treatment in Metastatic Renal Cell Carcinoma**Hanne Krogh Jensen,¹ Frede Donskov,¹ Marianne Nordmark,¹ Niels Marcussen,² and Hans von der Maase³

Abstract Purpose: The administration of interleukin-2 (IL-2) may increase the frequency of peripherally circulating FOXP3-positive regulatory immune cells, thus potentially compromising this treatment option for patients with metastatic renal cell carcinoma. The impact of IL-2-based therapy on the accumulation of FOXP3-positive immune cells in the tumor microenvironment in metastatic renal cell carcinoma is unknown.

Experimental Design: Baseline ($n = 58$) and on-treatment ($n = 42$) tumor core biopsies were prospectively obtained from patients with clear cell metastatic renal cell carcinoma before and during IL-2-based immunotherapy. Immunohistochemical expression of FOXP3 was estimated by stereological counting technique and correlated with other immune cell subsets and overall survival.

Results: A significant increase in absolute intratumoral FOXP3-positive immune cells was observed comparing baseline (median 23 cells/mm²; range, 0-183) and on-treatment biopsies (median, 89 cells/mm²; range, 11-388; $P < 0.001$). The relative increase in individual patients was median 4.7-fold, range 0.3 to 230. FOXP3-positive cells were positively correlated with CD3-positive, CD4-positive, and CD8-positive tumor-infiltrating immune cells at baseline and during treatment ($P < 0.05$ in all comparisons). All patients achieving high numbers (>180 cells/mm²) of on-treatment FOXP3-positive intratumoral immune cells were dead within 22 months ($n = 11$), whereas patients with low numbers (<180 cells/mm²) of on-treatment FOXP3-positive cells ($n = 31$) had a 5-year survival rate of 19% (hazard ratio, 2.2; confidence interval, 1.03-4.5; $P = 0.043$). All long-term survivors were characterized by low-baseline FOXP3-positive cells and a modest absolute rise in FOXP3-positive cells.

Conclusion: Intratumoral FOXP3-positive regulatory immune cells significantly increased during IL-2-based immunotherapy, and high numbers of on-treatment FOXP3-positive cells were correlated with poor prognosis in patients with metastatic renal cell carcinoma.

Regulatory T cells are highly specialized subsets of immune cells capable of preventing autoimmunity and limiting immune reaction in infectious diseases (1). Because most tumor-associated antigens are predominantly self-antigens, regulatory T cells may also play a role in preventing an effective antitumoral immune response (2–5). Increased frequencies of regulatory T cells have been reported in solid cancers of lung(6), ovary (4, 6), breast (7, 8), pancreas (8), melanoma

(9, 10), gastrointestinal (11), gastric (12, 13), esophageal (12, 13), head and neck (14), hepatocellular carcinoma (15), and renal cell carcinoma (16, 17). For these cancers, there seems to be a correlation between elevated levels of regulatory T cells and impaired overall survival (18). Regulatory T cells have traditionally been characterized by the CD4-positive CD25-positive phenotype, but a novel transcription factor, FOXP3, is considered a better marker for quantification of regulatory T cells (19, 20).

Interleukin-2 (IL-2) is the only curative treatment option for selected patients with metastatic renal cell carcinoma (21). During the past 20 years, reproducible durable tumor regressions have been noted after systemic administration of IL-2 in patients with metastatic renal cell carcinoma, but only in a minority of patients (22, 23). IL-2 activates several immune cells including T cells (24), natural killer (NK) cells (25), B cells (26), monocytes/macrophages (27), and neutrophils (28). However, recent studies have shown the administration of IL-2 to cancer patients to be accompanied by an increase in peripheral circulating FOXP3-positive immune cells, thereby potentially compromising the establishment of an effective antitumor immune response due to the induction of opposing immune cell functions (29–33).

The present study is the first study to assess intratumoral FOXP3-positive regulatory cells in patients with metastatic renal

Authors' Affiliations: ¹Department of Oncology and Experimental Clinical Oncology, Aarhus University Hospital, Noerrebrogade 44, Aarhus C, Denmark; ²Department of Clinical Pathology, Odense University Hospital, Winsloewparken 15, Odense C, Denmark; and ³Department of Oncology, Rigshospitalet, Copenhagen University Hospital, Blegdamsvej 9, København Ø, Denmark
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Requests for reprints: Hanne Krogh Jensen, Department of Oncology Aarhus University Hospital Noerrebrogade 44 8000 Aarhus C, Denmark. Phone: 45-8949-2639; Fax: 45-86197109; E-mail: hajensen@rm.dk.

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Translational Relevance

Studies based on peripheral blood have shown FOXP3-positive regulatory immune cells to increase during interleukin-2 treatment, thereby potentially compromising a sufficient immune response.

This translational study is based on prospectively collected serial biopsies from patients with metastatic clear cell renal cell carcinoma. It is the first study systematically assessing the baseline and on-treatment expression of FOXP3-positive regulatory immune cells in patients before and during IL-2-based immunotherapy. We showed a significant absolute increase in intratumoral FOXP3-positive regulatory cells in metastatic renal cell carcinoma following IL-2 treatment, and also a relatively higher increase of FOXP3-positive compared with the increases of CD3-positive and CD8-positive immune cells. High numbers of on-treatment FOXP3-positive cells were correlated with poor prognosis in patients with metastatic renal cell carcinoma. All long-term survivors were characterized by low-baseline FOXP3-positive cells and a modest absolute increase in FOXP3-positive cells.

Aiming at understanding the relatively low response rate following IL-2-based therapy and the need for an improved outcome, this study shows a clear application to the future management of patients with metastatic renal cell carcinoma, and supports continued clinical research in combined FOXP3-positive depletion and immunomodulation.

cell carcinoma before and during IL-2-based immunotherapy. We report that intratumoral FOXP3-positive immune cells increased in the majority of patients during IL-2 therapy, and that high numbers of FOXP3-positive cells were associated with poor overall survival.

Material and Methods

Patient population. Paraffin-embedded tumor biopsies were collected among a cohort of 120 consecutive patients receiving IL-2-based immunotherapy in prospective phase II trials using low- or intermediate-dose s.c. IL-2 (34). Low-dose regimens consisted of one priming week of daily IFN- α , followed by cycles of 4 wk with IFN- α (3 mIU \times 1 s.c) 7 d/wk and IL-2 (2.4 mIU/m² \times 2) 5 d/wk, weeks 1 and 2 every cycle for up to 9 mo. Intermediate-dose regimens consisted of 5-wk treatment cycles with IL-2 (18 mIU \times 1) 5 d/wk for 3 wk, followed by 2 wk off-treatment, for up to four cycles. Histamine dihydrochloride 1 mg \times 2 for 5 d/wk was added in conjunction with IL-2 in some patients (Table 1). The reasons for not participating in the present study were non-clear cell histology ($n = 12$), lack of informed consent ($n = 19$), not evaluable for response ($n = 4$), fine needle biopsies only ($n = 2$), or insufficient tumor tissue or necrosis ($n = 25$). Among patients with clear cell histology, a total of 58 patients had a baseline biopsy. Out of these patients, 42 patients also had an on-treatment biopsy. The reasons for not having an on-treatment biopsy were withdrawal of informed consent or insufficient

tumor tissue. Core needle biopsies (18G cutting needle) were collected by standard ultrasound-guided procedures. The biopsies were obtained before treatment (baseline) and within the first 2 mo of treatment at predefined time points according to the treatment schedule (on treatment). The first and second biopsies were always taken from the same tumor site, either the primary tumor or a metastatic lesion. Objective response was defined according to standard WHO criteria (35). All studies were conducted in accordance with the Helsinki declaration II and approved by the local ethics committee and the Danish Medical Agency.

Immunohistochemical labeling of FOXP3-positive cells. The paraffin-embedded core needle biopsies were sectioned at 2 μ m and mounted on glass slides. Primary antibody was against FOXP3-positive (clone 236 A/E7, dilution 1:50; Biocare Medical). Immunohistochemistry was done using a BenchMark XT automated stainer (Ventana Medical Systems, Inc.). Deparaffinization, epitope retrieval, and immunostainings were done according to the manufacturer's instruction using Cell Conditioning solutions (CC1) and the ultra-VIEW Universal DAB detection system. Positive signals were amplified using ultra-VIEW Copper and sections were counterstained with hematoxylin and bluing reagent. In each run formalin-fixed paraffin-embedded tonsillar, pancreatic, appendix, and liver sections were included as positive controls.

Double-stainings for FOXP3-positive/CD4-positive and FOXP3-positive/CD25-positive were done as described above

Table 1. Patient characteristics

Characteristics	Baseline ($n = 58$)	
	No	(%)
Age, y		
Median		60
Range		19-74
Sex		
Male	42	(72)
Karnofsky performance status		
100	21	(36)
90	19	(33)
80	8	(14)
70	10	(17)
MSKCC prognostic criteria		
Favorable	5	(9)
Intermediate	34	(59)
Poor	19	(33)
Prior nephrectomy		
Yes	21	(36)
No	37	(64)
Treatment		
IL2, IFN, and histamine	11	(19)
IL2 and IFN	9	(15)
IL2 and histamine	16	(28)
IL2	22	(38)
Treatment outcome		
PR	4	(7)
SD/PD	54	(93)
Biopsy specimen		
Primary tumor	30	(52)
Metastatic lesion	28	(48)

Abbreviations: MSKCC, Memorial Sloan Kettering Cancer Center; PR, partial response; SD, stable disease; PD, progressive disease.

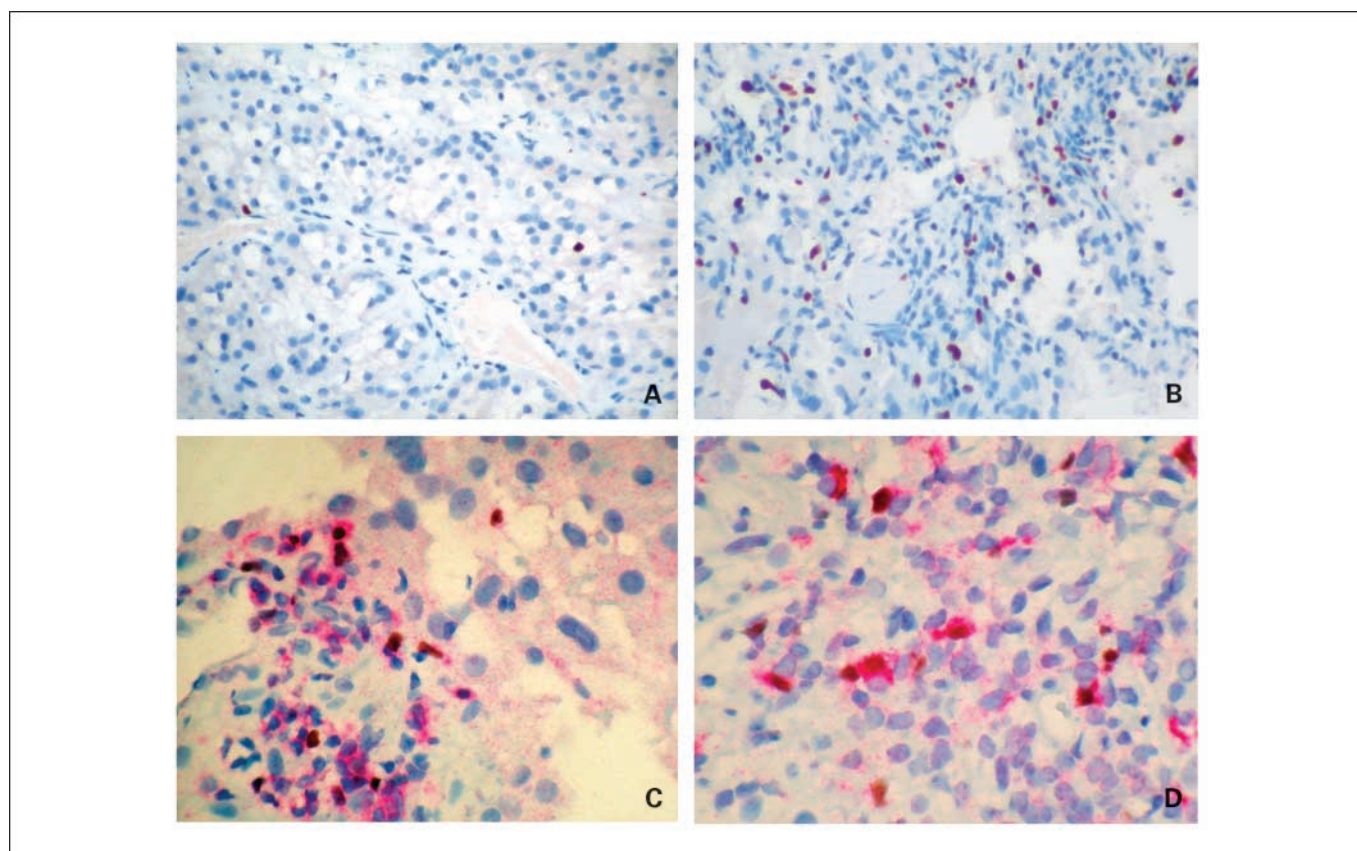


Fig. 1. Representative examples of FOXP3-positive immunostainings ($\times 200$). The images *A* and *B* are from the same patient at baseline (*A*) and on-treatment (*B*). They illustrate the infiltration of immune cells in general and the increase of FOXP3-positive in particular. *C*, double-labeled FOXP3-positive CD4-positive cells. *D*, double-labeled FOXP3-positive CD25-positive cells. The vast majority of the FOXP3-positive cells were CD4-positive and CD25-positive ($\times 400$).

using horseradish peroxidase and 3,3-diaminobenzidine tetrahydrochloride for FOXP3-positive, and alkaline phosphatase and Fast Red for CD4-positive and CD25-positive.

The staining and quantification of other immune cell subsets (CD3-positive, CD4-positive, CD8-positive, CD56-positive, CD57-positive, CD66b-positive, and macrophages) have been reported previously (34, 36, 37).

Quantification of FOXP3-positive cells. The number of intratumoral FOXP3-positive immune cells per mm^2 was estimated using stereological counting technique (CAST software; ref. 38). In brief, the tumor section was encircled at the image projected onto the computer screen. A motorized stage controlled by the computer sampled the first counting field at random and then moved systematically throughout the biopsy section. The staining was very specific and only nuclear staining of leukocytes was observed. A minimum of 40 counting frames ($4,989 \mu\text{m}^2$ each) were chosen when the biopsy size allowed, analyzing the entire core needle biopsy. Counting frames were considered of use when tumor cells were present in the area. Areas including necrosis and artifacts were omitted. At a $\times 1024$ magnification a median of 81 counting frames (range, 23-112 frames), and a median area of the whole biopsy of 28% (range, 1.5-100%) was evaluated. Staining was analyzed by one blinded observer (HKJ).

The fraction of FOXP3/CD4-positive and FOXP3/CD25-positive cells of the total FOXP3 cells were estimated semiquantitatively at $\times 400$ by a senior pathologist (NM) and HKJ.

Statistical analysis. For comparisons of pretreatment and on-treatment biopsies and correlations between the different immune cell subtypes, nonparametric tests were used (Wilcoxon and Spearman's rank correlation). Comparisons between

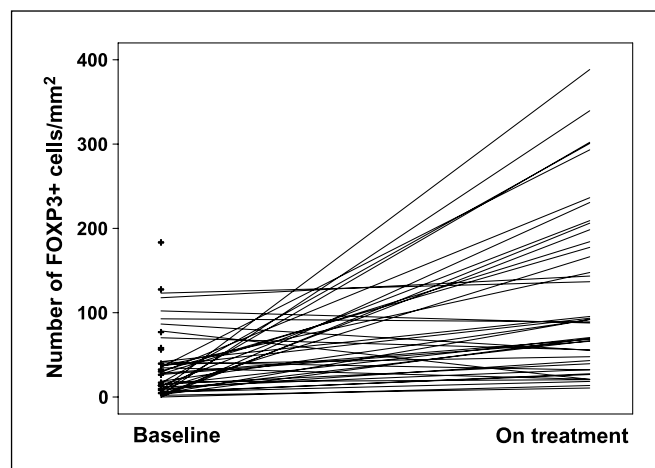


Fig. 2. The number of FOXP3-positive regulatory immune cells/ mm^2 at baseline and on-treatment in metastatic renal cell carcinoma. The crosses at baseline indicate patients with only baseline biopsies available (16 patients). For the 42 patients with both baseline and on-treatment biopsies, each line connects the two corresponding values for a single patient. The difference between baseline and on-treatment values is statistically significant ($P < 0.001$).

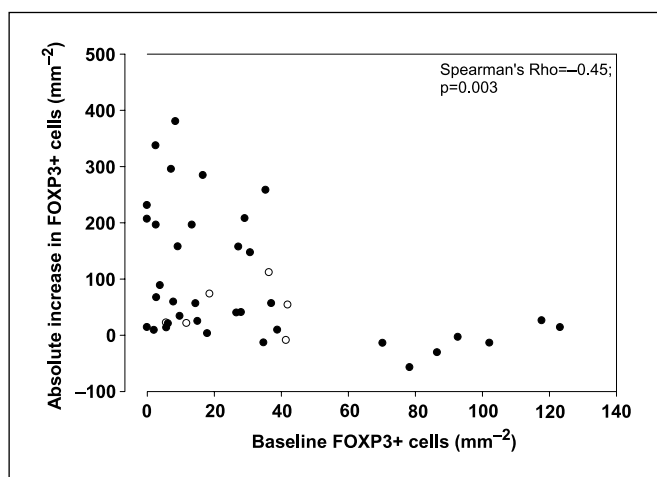


Fig. 3. The scatter plot shows the absolute increase in FOXP3-positive cells following IL-2–based treatment as a function of baseline values in the 42 patients. The increase in FOXP3-positive cells inversely correlated with the presence of FOXP3-positive cells at baseline. Open circles, patients alive (≥ 59 mo); closed circles, deceased patients.

the subgroups of patients were done using Fisher's exact test. Overall survival was estimated using the Kaplan-Meier method and log rank test. Survival was measured from the day of treatment initiation for the baseline values, and the day of the second biopsy for the on-treatment values, to the time of death or last follow-up. Survival status was updated on August 27, 2007. For the survival analysis, the absolute numbers of FOXP3-positive cells at baseline and on-treatment were divided into quartiles. The association of the number of on-treatment FOXP3-positive cells above the 75% percentile with overall survival was evaluated in a Cox proportional hazards regressions models analysis with the Memorial Sloan Kettering Cancer Center risk groups (poor risk group versus intermediate and good; ref. 39). Due to the low number of cases, no further variables were added. Statistical analyses were done using SPSS version 11.0 (SPSS Inc.) statistical software. All tests were two-sided, and $P < 0.05$ was considered statistically significant.

Results

Patient characteristics. A summary of the clinical characteristics of the baseline population is given in Table 1. The median survival for all 58 patients was 13 months (range, 0.5-102.7 months), and 52 of the 58 patients were dead at the time of analysis. The median age was 60 years (range, 19-74 years) and 72% were male. Sixty-four percent of the patients had the primary tumor *in situ* and 33% belonged to the Memorial Sloan Kettering Cancer Center poor prognostic group (39). Four had a partial response to treatment and 23 achieved stable disease. There were no significant differences between the total treatment population ($N = 120$) and the 58-patient study group as well as the 42-patient study group ($P = 0.11$ and $P = 0.17$, respectively) with respect to well-known prognostic factors (Memorial Sloan Kettering Cancer Center criteria; ref. 39).

FOXP3 baseline and during treatment. An example of the FOXP3 immunostaining is shown in Fig. 1. Fifty-five patients (95%) had intratumoral FOXP3-positive cells present at

baseline and all 42 patients had intratumoral FOXP3-positive cells present in the on-treatment biopsies. Comparing the baseline and on-treatment values in the individual patients, there was a significant difference in the number of FOXP3-positive cells following IL-2 treatment from 23 cells/mm² (range, 0-183 cells/mm²) in the baseline biopsies to 89 cells/mm² (range, 11-388 cells/mm²) in the on-treatment biopsies ($P < 0.001$; Fig. 2). The relative increase in FOXP3-positive immune cells in the individual patients was median 4.7-fold (range, 0.3- to 231-fold). Thus, there was a rise in 35 patients (83%) and a decrease in only 7 patients (17%). Interestingly, the absolute increase in FOXP3-positive cells during treatment was inversely correlated to the presence of FOXP3-positive cells before treatment (Spearman's $\rho = -0.45$; $P = 0.003$), and all six long-term survivors (alive ≥ 59 months) were characterized by low-baseline FOXP3-positive cells and a modest absolute rise in FOXP3-positive cells (Fig. 3).

There was no impact of biopsy site (primary tumor or metastases; $P = 0.61$) or whether the treatment comprised intermediate- or low-dose IL-2 ($P = 0.56$), IFN- α containing regimens ($P = 0.56$), or histamine ($P = 0.89$) on the absolute numbers of FOXP3-positive cells following IL-2–based treatment.

Phenotypic characteristics of the FOXP3-positive cells. In order to show the type of cells expressing FOXP3, we did double-staining of FOXP3/CD4 and FOXP3/CD25 (Fig. 1C and D). For these analyses, sufficient tumor samples were available in 19 biopsies from 12 patients. Double-labeling of FOXP3-positive and CD4-positive cells revealed a median of 90% (range, 60-100%) of the FOXP3-positive cells to be CD4-positive. A median of 70% (range, 50-90%) of the FOXP3-positive cells were positive for CD25. No difference in the cell fraction of double-positive cells following IL-2 treatment compared with baseline was observed.

Correlation to other intratumoral immune cells. We have previously reported on tumor infiltration of CD3-positive T cells, CD4-positive T cells, CD8-positive T cells, CD56-positive NK cells, CD57-positive NK cells, CD66b-positive neutrophils, and macrophages in these patients, both baseline and during therapy (36, 37). Analyses of the correlations between FOXP3-positive and immune cell subsets revealed a significant positive correlation at baseline with CD3-positive, CD4-positive, CD8-positive, and CD57-positive cells ($P < 0.05$). On-treatment, there were significant positive correlations between FOXP3-positive and CD3-positive, CD4-positive, CD8-positive, and CD66b-positive cells (Table 2).

The increase in numbers of FOXP3-positive cells were compared with the changes in CD3-positive and CD8-positive immune cells. At baseline, the median FOXP3/CD3 ratio was 0.12 (range, 0-11.1), whereas the on-treatment median value was 0.26 (range, 0.02-9.6). Similarly, the baseline median FOXP3/CD8 ratio was 0.21 (range, 0-5.8), and on-treatment 0.62 (range, 0.03-9.6). Both differences were statistically significant with increasing percentages being most prominent ($P = 0.004$ and $P = 0.001$, respectively).

Correlation between FOXP3-positive cells and response. There was no impact of baseline FOXP3-positive densities when comparing responders and nonresponders ($P = 0.8$), or comparing progressive disease and non–progressive disease patients ($P = 0.3$). In addition, no impact of the on-treatment

densities on response or progression of disease was seen ($P = 0.9$ and $P = 0.2$, respectively).

FOXP3-positive cells and survival. The impact of the baseline and on-treatment FOXP3-positive cells on survival following IL-2-based therapy was explored by Kaplan-Meier curves and log rank test dividing the patients into quartiles. There was no difference in overall survival probability when comparing the upper quartile with the lower three quartiles in the baseline group of patients ($n = 58$; $P = 0.5$). For the on-treatment biopsies ($n = 42$), however, a significant difference was observed comparing patients above the 75% percentiles with the remaining lowest quartiles ($P = 0.04$; Fig. 4). Thus, all patients achieving high numbers (>180 cells/mm²) of on-treatment FOXP3-positive intratumoral regulatory immune cells were dead within 22 months ($n = 11$), whereas patients with low numbers (<180 cells/mm²) of FOXP3-positive cells ($n = 31$) had a 5-year survival rate of 19%. In the multivariate analysis, the difference remained statistically significant (hazard ratio, 2.2; confidence interval, 1.03-4.5; $P = 0.043$).

Discussion

The present study is to our knowledge the first study to evaluate the clinical relevance of IL-2-induced intratumoral FOXP3-positive regulatory immune cells in patients with metastatic renal cell carcinoma. The frequency and absolute number of intratumoral FOXP3-positive cells was significantly increased following IL-2-based therapy, and high numbers of on-treatment FOXP3-positive cells correlated with poor prognosis in patients with metastatic renal cell carcinoma. These results are consistent with and extend previous data based on circulating FOXP3-positive cells in blood samples from patients with metastatic renal cell carcinoma treated with IL-2.

The impact of IL-2 on peripheral circulating FOXP3-positive cells has previously been shown in different tumor types. In pediatric sarcoma patients receiving either IL-2 or no IL-2, Zhang et al. explored regulatory T cells and observed IL-2 recipients to have significantly higher FOXP3-positive expression (33). Similarly, in patients with malignant melanoma and renal cell carcinoma, Ahmadzadeh et al. showed high-dose bolus IL-2 to significantly increase the proportion of CD4-positive CD25-positive T cells expressing FOXP3-positive in the peripheral blood (29). Two other studies testing high-dose IL-2 schedules showed similar results (30, 31). Despite the s.c. administration and the low and intermediate levels of IL-2 used

in the present study, our intratumoral data agree with existing peripheral blood data obtained in these high dose studies. The impact of high-dose IL-2 on intratumoral FOXP3 cells needs to be clarified.

In ovarian cancer, evidence of IL-2 as a promoter of regulatory T-cell tumor infiltration was reported by Wei et al., who observed that regulatory T cells efficiently migrated with ovarian tumor ascites following IL-2 treatment, suggesting that specific cytokines in the microenvironment attract regulatory T cells in response to IL-2 treatment (32). In addition, the IL-2-induced regulatory T cell proliferation was negatively influenced by the existing regulatory T cells (32). This is consistent with our results, as we observed the increase in FOXP3-positive cells inversely correlating with the number of FOXP3-positive cells at baseline. In other words, FOXP3-positive cells may have a self-inhibitory effect.

To our knowledge, no literature regarding the effect of IFN on FOXP3-positive cells has been published. Consistent with this we observed identical on-treatment FOXP3-positive cell densities in patients treated with or without IFN-containing regimens.

IL-2 functions entirely through activation of the patient's endogenous immune system, and orchestration of an effective antitumor immune response is prerequisite for durable tumor eradication. However, the exact mechanism of action is unclear. Thus, although IL-2-based immunotherapy may induce durable tumor regression in some patients with metastatic renal cell carcinoma, it also seems that treatment with IL-2 may compromise the establishment of an effective antitumor immune response in some patients by the development of reciprocal alterations of immunoregulatory cells (31).

In the present study, the increase in FOXP3-positive density following IL-2 treatment was associated with poor overall survival. It was, however, most prominent in patients having levels of FOXP3-positive cells above the 75% percentile, suggesting that the threshold for immune suppression is relatively high. We observed that the number of FOXP3-positive cells increased more than the number of effector T cells following IL-2 administration as the FOXP3/CD8-positive ratio increased from 0.21 to 0.62. This indicates an existence of a balance between FOXP3-positive cells and CD3-positive or CD8-positive cells causing either tumor immune response or tolerance. The association between IL-2-induced rise in FOXP3-positive cells and overall survival needs further investigation.

Table 2. Correlations between intratumoral FOXP3-positive cells and intratumoral T-cells, NK cells, neutrophils, and macrophages at baseline and on-treatment

Cell type	Baseline FOXP3 positive			On-treatment FOXP3 positive		
	Spearman's ρ	<i>P</i>	Biopsies evaluated	Spearman's ρ	<i>P</i>	Biopsies evaluated
CD3-positive T cells	0.50	<0.001	54	0.37	0.02	42
CD4-positive T cells	0.32	0.02	57	0.43	0.005	42
CD8-positive T cells	0.58	<0.001	57	0.57	<0.001	41
CD57-positive NK cells	0.46	<0.001	56	0.20	0.2	42
CD56-positive NK cells	0.22	0.1	57	0.07	0.66	41
CD66b-positive Neutrophils	0.16	0.23	57	0.34	0.03	42
Macrophages	0.08	0.57	57	0.13	0.43	41

NOTE: Missing values are due to nonevaluable sections.

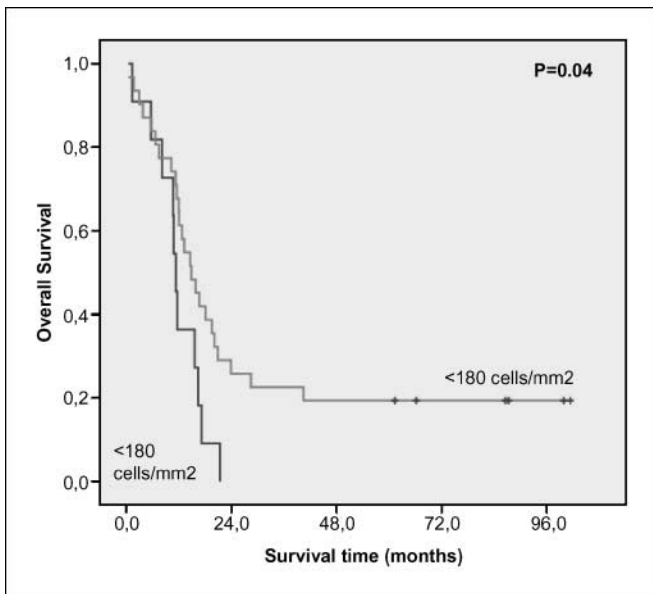


Fig. 4. Survival curves showing overall survival probability based on separation of the patients into two groups above and below the 75% percentile of the on-treatment FOXP3-positive value. *Solid line*, patients in the fourth quartile ($n = 11$; events = 11); *dotted line*, remaining three quartiles ($n = 31$; events = 25).

For this FOXP3 analysis, we only had tumor material available and were therefore not able to assess peripheral blood FOXP3-positive cells. Griffiths et al. compared intratumoral and peripheral blood FOXP3-positive cells in five renal cell carcinoma patients and observed a positive correlation between these two compartments (17). Indeed, Griffiths et al. observed the highest frequency in the tumor site, consistent with the high frequencies observed in the present study. This suggests similar frequencies in the two compartments, but needs to be confirmed in a considerable larger material.

Baseline FOXP3-positive tumor-infiltrating immune cells were not prognostic for survival in the current study. This observation concurs with a study by Siddiqui et al. of 170 clear cell renal cell carcinoma tumor samples, in which the presence of CD4-positive CD25-positive FOXP3-negative cells, rather than FOXP3-positive cells, was related to cancer-specific survival (16). The Siddiqui study population involved 15% stage IV patients, and only a minority of these patients (21%) had FOXP3-positive cells present, whereas our cohort exclusively contained patients with metastatic disease and 95% had FOXP3-positive cells present at baseline. This different prevalence of FOXP3-positive cells could reflect the use of two

different antibodies, frozen tissue technique, and differences in case mix.

We evaluated the phenotype of the FOXP3-positive cells using double-labeling with CD4-positive and CD25-positive, respectively. The vast majority of the FOXP3-positive cells were CD4 positive and CD25 positive, both at baseline and following IL-2. This is consistent with several other studies having revealed similar phenotypic and functional characteristics regardless of the induction stimulus, at least regarding peripheral, circulating FOXP3-positive cells (29, 30, 33). Also, the antibody used in the present study has previously been shown to specifically label FOXP3-positive cells of regulatory, suppressor subtype (7, 40).

The findings in the current study have improved our understanding of the complexity following IL-2 administration, and have also emphasized the vital therapeutic option of reversal tumor-mediated immunosuppression by eliminating regulatory T cells. It should be noted that all long-term survivors in our study were characterized by low-baseline FOXP3-positive cells and a modest absolute increase in FOXP3-positive cells. Promising preclinical findings inhibiting and depleting FOXP3-positive cells have been investigated using approaches such as vascular endothelial growth factor blockade (41), local CTL Antigen-4 blockade, systemic regulatory T cell depletion (by cyclophosphamide or anti-CD25 antibodies; refs. 42, 43), and vaccination against FOXP3-positive cells (44). Most of these approaches have led to tumor regression and improved survival of tumor-bearing mice. However, translating these results into the clinical setting has so far been without the desired improvement in response rate and survival, despite the ability to reduce the number of regulatory T cells in the tumor (45, 46).

In conclusion, the present study showed that intratumoral regulatory FOXP3-positive cells increased during IL-2 treatment and adversely correlated with overall survival. These results support further clinical studies depleting regulatory T cells in combination with IL-2 in metastatic renal cell carcinoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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