Simultaneous Infiltration of Polyfunctional Effector and Suppressor T Cells into Renal Cell Carcinomas

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Abstract
Renal cell carcinoma is frequently infiltrated by cells of the immune system. This makes it important to understand interactions between cancer cells and immune cells so they can be manipulated to bring clinical benefit. Here, we analyze subsets and functions of T lymphocytes infiltrating renal cell tumors directly ex vivo following mechanical disaggregation and without any culture step. Subpopulations of memory and effector CD4+ Th1, Th2, and Th17 and CD8+ Tc1 cells were identified based on surface phenotype, activation potential, and multicytokine production. Compared with the same patient’s peripheral blood, T lymphocytes present inside tumors were found to be enriched in functional CD4+ cells of the Th1 lineage and in effector memory CD8+ cells. Additionally, several populations of CD4+ and CD8+ regulatory T cells were identified that may synergize to locally dampen antitumor T-cell responses. [Cancer Res 2009;69(21):8412–9]

Introduction
Tumor-infiltrating immune cells, especially T lymphocytes (TIL), can be found in many types of human cancer, but their contribution to tumor elimination or growth is unclear. They express many markers of activation and effector differentiation, recognize numerous tumor-associated antigens in a MHC-restricted fashion, and can kill tumor cells in vitro. Nonetheless, tumors commonly progress, suggesting that they have escaped immunity in vivo. The mechanisms that cause tumor escape have been extensively discussed and include T-cell suppression or dysfunction. The local presence of suppressive immune subsets such as natural regulatory T cells (nTreg) or myeloid-derived suppressive cells is currently considered to be one of the main mechanisms involved in tumor-associated T-cell tolerance (1–3). There is also increasing evidence that TIL include a variety of effector and regulatory subsets that can play opposite roles to the benefit or detriment of the patient.

Renal cell carcinomas (RCC) account for ~3% of adult malignancies, with an increasing incidence worldwide. RCC commonly contains T cells and natural killer cells, and earlier studies have shown that these cells express markers of activation and memory (4–6). On the functional level, Th1-associated [IFN-γ and interleukin (IL)-2], Th2-associated (IL-4 and IL-5), and suppression-associated (IL-10) cytokines have been detected (4, 5, 7). Antigen-specific, HLA-restricted CD8+ T cells, as well as CD4+, TIL have been described (8, 9), and T-cell epitopes derived from RCC-associated antigens subsequently identified (10, 11). However, most of these studies are based on tumor enzymatic digestion and/or in vitro expansion with IL-2 and therefore may not be representative of the immune cell populations actually residing in the tumor in vivo.

Several mechanisms contributing to the dysfunction of RCC-TIL have been described (reviewed in ref. 12). At the level of the cancer cell itself, downregulation of certain components of the antigen presentation machinery can occur; nevertheless, most renal tumors do express HLA class I as well as class II molecules on the cell surface (10, 13). Kidney tumor cells may also synthesize membrane-bound or soluble immunomodulatory molecules, such as inhibitory natural killer receptor ligands or PD-L1, and recruit immune cells secreting suppressive cytokines like transforming growth factor-β or IL-10, which favor the development of an anergizing tumor milieu. More recently, expansions of nTreg or myeloid-derived suppressive cells have been reported in the blood and tumor of RCC patients (14–17). Finally, downregulation of the CD3ζ chain at the surface of effector T cells is a major cause of dysfunction that has been observed in many cancer patients (18) and in a subset of RCC patients (12, 16).

Recent technological developments allow the refinement of human T-cell subset analysis and cast light on their heterogeneity and plasticity (19–21). It has also become possible to analyze phenotypic and functional parameters of CD4+ and CD8+ lymphocytes simultaneously. The capacity of single cells to exert several effector functions, so-called polyfunctionality, has been shown to correlate with protective immunity against several infectious diseases including after vaccination (reviewed in ref. 22). Here, we describe a detailed study of T-cell subsets present in kidney tumors. TIL were analyzed ex vivo, directly after mechanical tumor dissociation and without being cultured. This approach is likely to be more informative regarding different phenotypic and functional attributes of TIL compared with peripheral T cells of patients or healthy controls and may result in more rational strategies to manipulate immune reactivity within the tumor itself.

Materials and Methods

Patients and healthy donors. Peripheral blood as well as fresh tumor and autologous renal parenchyma tissues were obtained from 33 patients...
with the approval of the local ethics committee including written informed consent. All patients (n = 14 men and n = 19 women; mean age, 64 years) underwent surgery for RCC stages pT1 (n = 20), pT2 (n = 1), and pT3 (n = 12). Four patients had distant metastases at diagnosis. The majority were clear cell type (n = 26), with 1 sarcomatoid, 3 papillary, and 3 chromophobe tumors. Peripheral blood samples were additionally obtained from 20 consenting healthy donors or buffy coats provided by the local blood bank (n = 15 men and n = 5 women; mean age, 48 years).

Cell isolation. Peripheral blood mononuclear cells (PBMC) from healthy donors or RCC patients were isolated using a density gradient separation solution (PAA Laboratories). Fresh tumor samples were collected in Iscove's modified Dulbecco's medium containing 100 units/mL penicillin/ streptomycin (Lonza), 10% heat-inactivated FCS (PAA Laboratories), and 50 μg/mL gentamicin and then mechanically disaggregated using a Medimachine (DAKO) and 50 μm Medicon grids (Becton Dickinson). The cell suspension was then filtered through a 70 μm nylon mesh and density gradient separation was done to isolate the TIL. Collagenase/DNase treatment was avoided because it affects detection of surface molecules by some monoclonal antibodies (23, 24). PBMC and TIL samples were cryopreserved in FCS 10% DMSO.

Phenotypic analysis. PBMC and TIL were thawed in T-cell medium consisting of Iscove's modified Dulbecco's medium containing 100 units/mL penicillin/streptomycin, 50 μmol/L β-mercaptoethanol, and 10% heat-inactivated human serum (c.c.Pro). Between 0.5 × 10⁶ and 2 × 10⁶ cells were analyzed by polychromatic flow cytometry using five fixed antibody combinations. Fluorochrome-labeled antibodies were all pretested: CD4-APC-Cy7, CD8-PerCP, CD25-APC, CD56-PE-Cy7, CD57-FITC, CD103-FITC, CXCR3-APC, anti-CCR7-PE-Cy7, anti-NKG2D-PE, and anti-TCRV γδ-PECy7, anti-CCR5-PE (eBioscience); anti-TCRV α-PE-Cy7, CD28-APC, CD56-PE-Cy7, CD57-FITC, CD103-FITC, CXCR3-APC, anti-CCR7-PE-Cy7, anti-NKG2D-PE, and anti-TCRV γδ (all Becton Dickinson); CD127-FITC and anti-CD154-APC (both Becton Dickinson); CD127-FITC and anti-CD154-APC (eBioscience); anti-TCRβ-FITC (Beckman Coulter); anti-CCR4-FTTC (R&D Systems); CD3-Pacific Blue (Biolegend); and CD45RA-Pacific Blue (Caltag). Antibody cocktails were added in CD127-FITC and anti-CCR5-PE (eBioscience); anti-TCRβ-FITC (Beckman Coulter); anti-CCR4-FTTC (R&D Systems); CD3-Pacific Blue (Biolegend); and CD45RA-Pacific Blue (Caltag). Antibody cocktails were added in CD127-FITC and anti-CCR5-PE (eBioscience); anti-TCRβ-FITC (Beckman Coulter); anti-CCR4-FTTC (R&D Systems); CD3-Pacific Blue (Biolegend); and CD45RA-Pacific Blue (Caltag). Antibody cocktails were added in CD127-FITC and anti-CCR5-PE (eBioscience); anti-TCRβ-FITC (Beckman Coulter); anti-CCR4-FTTC (R&D Systems); CD3-Pacific Blue (Biolegend); and CD45RA-Pacific Blue (Caltag). Antibody cocktails were added in CD127-FITC and anti-CCR5-PE (eBioscience); anti-TCRβ-FITC (Beckman Coulter); anti-CCR4-FTTC (R&D Systems); CD3-Pacific Blue (Biolegend); and CD45RA-Pacific Blue (Caltag).
with each antibody combination. For the functional analysis, 7 patients were analyzed and compared with 5 healthy donors (except 3 healthy donors for IL-10 and IL-17 detection). Statistical significance was tested only between TIL and autologous PBMC. Significant differences (P < 0.05) are marked with an asterisk.

Results

CD4+ and CD8+ differentiated effectors predominate in TIL. Multicolor flow cytometry was used to compare RCC-TIL with blood T cells from the same patients or from healthy controls. Results are shown in Fig. 1 for the CD4+ and CD8+ subsets.

Because the number of nTreg is elevated in different tumors (1, 15), we first asked whether they accumulate in kidney cancers. An increased proportion of CD4+CD25highFoxp3 cells were present in patients′ TIL compared with PBMC (mean 2.6% versus 0.4%, respectively). In 4 of 15 patients, CD4+CD25highFoxp3 cells represented at least 4% of the CD4+ TIL but were not elevated in the PBMC (Figs. 1 and 2A). Weak expression of the IL-7 receptor (CD127) on these cells was consistent with the likelihood that they are nTregs (data not shown).

We next analyzed selected markers associated with the lineage and differentiation status of T cells. The expression pattern of chemokine receptors has been shown to vary among T-cell subsets, CXCR3 being highly expressed on Th0, Th1, and memory CD8+ cells, whereas CCR4 is generally associated with Th2 activity; furthermore, CCR5 is preferentially expressed by effector memory and effector T cells (19, 21). We found that CD3+CD4+ TIL were enriched in CCR4+CXCR3+ cells compared with RCC-PBMC (mean 47.0% versus 13.8%) and, in approximately half of the patients, in cells expressing CCR5 (mean 47.5% versus 13.3%). A similar pattern of CXCR3 (mean 73.0% versus 32.4%) and CCR5 (mean 44.7% versus 25.6%) expression was observed in CD8+ TIL. Paired analysis revealed that increased expression of CCR5 was concomitant in the CD4+ and CD8+ subsets and was reflected, albeit to a lesser extent, in the peripheral T lymphocytes (data not shown).

TIL were further investigated assessing surface markers of effector status. CD28 expression was not significantly different in TIL versus autologous PBMC but clearly reduced compared with healthy donors-PBMC in both CD4+ and CD8+ subsets. In contrast, an increase in CD57 expression was detected. Figure 2B illustrates that CD28+CD57+ cells were enriched in some patients, especially in TIL, a phenotype that is a hallmark of late-differentiated effector T cells (26, 27). Finally, we tested the expression of the natural killer cell activating receptor NKG2D, which is known to play an important role in CD8+ T-cell activation (28), and observed a decrease in the CD8′NKG2D′ subset in TIL compared with autologous PBMC (Fig. 1B).

Overall, the pattern of chemokine receptors and other markers suggested that tumor-infiltrating CD4+ T cells were preferentially Th1 effectors but also contained an increased proportion of nTregs. CD8+ cells were enriched in differentiated, presumably highly cytotoxic, effector cells. In addition, the expression of CD28, CD35, NKG2D, and CXCR3 was different in patients’ peripheral CD8+ compared with healthy donors, revealing an increase in effector CTL. Finally, we observed that ‘TCR-gamma’ cells and natural killer T cells (TCRαβ24+) were not enriched in the TIL, suggesting that most are TCR-alpha lymphocytes (data not shown).

CD4+ and CD8+ TIL are of the effector memory phenotype. We next stained TIL for CD45RA and the chemokine receptor CCR7, markers employed to distinguish naive from memory (central memory TCM and effector memory TEM) and differentiated effectors (TEMA) T cells (19). Figure 3 shows the distribution of these four subsets among CD4+ (Fig. 3A) and CD8+ (Fig. 3B) cells in TIL and PBMC. The proportion of CD45RA′CCR7+ naive cells was markedly lower in the TIL compared with patients’ PBMC (CD4+ mean 6.6% versus 24.4%; CD8+ mean 4.9% versus 15.3%). Additionally, TCM in the CD4+ subset (CD45RA′CCR7−) and CD8+ differentiated effectors (CD45RA′CCR7+) were diminished. These changes were reciprocated by an enrichment of CD45RA′CCR7+ TEM (CD4+ mean 74.0% versus 43.2%; CD8+ mean 56.7% versus 29.8%). We also found significant differences in the distribution of naive, effector memory and effector CD4+ and CD8+ PBMC between patients and healthy donors. Together, these findings suggest a role of the tumor in modeling T-cell differentiation. Especially, TEMA are predominant inside the tumor in both CD4+ and CD8+ subsets.

E-cadherin and its receptors are expressed on kidney tumor cells and infiltrating T cells. Expression of the inhibitory receptor CCR7 is increased during chronic antigenic stimulation and may therefore contribute to the reduction of T-cell effector function inside tumors. Figure 4A shows that KLRG1 was more frequently expressed by peripheral CD8+ cells (but not CD4+ cells) from RCC patients than healthy donors. Two groups of patients could be distinguished: in 9, the percentage of CD8+ TIL coexpressing KLRG1 was similar to that observed in the blood (mean 68.2% versus 67.9%), whereas, in the remaining 12, there was a reduction in the proportion of KLRG1+ cells (mean 26.4%). Therefore, we asked whether the percentage of CD8′KLRG1′ TIL was related to the expression of its ligand, E-cadherin, on kidney tumor cells (29). Figure 4C presents the results of a representative immunostaining.

Figure 2. Dot plots of PBMC and autologous TIL from RCC patients and healthy donors′ PBMC. A, increased numbers of cells expressing Treg markers (CD25+, Foxp3+) in CD4+ TIL of one patient. CD8+ lymphocytes are gated. CD4+Foxp3+ cells are highlighted in black and percentage among CD4+ cells is shown. B, expression of CD28 and CD57 on CD4+ (top) or CD8+ (bottom) lymphocytes in two other donors. Percentages of positive cells are indicated in each quadrant.

Figure 4. Immunohistochemical analysis of RCC specimens. A, B, and C illustrate that E-cadherin and its receptors are expressed in kidney tumors and infiltrating T cells. E-cadherin (A) is expressed on RCC cells and infiltrating T cells. E-cadherin and its receptors are expressed on kidney tumor cells and infiltrating T cells. Expression of the inhibitory receptor CCR7 is increased during chronic antigenic stimulation and may therefore contribute to the reduction of T-cell effector function inside tumors. Figure 4A shows that KLRG1 was more frequently expressed by peripheral CD8+ cells (but not CD4+ cells) from RCC patients than healthy donors. Two groups of patients could be distinguished: in 9, the percentage of CD8+ TIL coexpressing KLRG1 was similar to that observed in the blood (mean 68.2% versus 67.9%), whereas, in the remaining 12, there was a reduction in the proportion of KLRG1+ cells (mean 26.4%). Therefore, we asked whether the percentage of CD8′KLRG1′ TIL was related to the expression of its ligand, E-cadherin, on kidney tumor cells (29). Figure 4C presents the results of a representative immunostaining.
showing that E-cadherin is found on normal distal tubules as well as in autologous tumor tissue, confirming previous findings (30).

The integrin αE(β7) is a second described ligand of E-cadherin and is expressed by TIL (31). In contrast to KLRG1, we found a strong increase of CD103 expression on CD8+ TIL and, to a lower extent, on CD4+ TIL compared with PBMC (Fig. 4A; CD4+CD103+ mean 3.3% in TIL versus 0.9% and 1.0% in RCC-PBMC and healthy donors-PBMC; CD8+CD103+ mean 17.0% versus 1.3% and 2.7%)

Double-antibody stainings revealed that KLRG1+ and CD103+ cells represent partially overlapping but distinct subpopulations of CD4+ and CD8+ cells as illustrated for one donor in Fig. 4B.

**CD4+ and CD8+ infiltrating renal tumors are functional.**

We then asked whether the functionality of the T lymphocytes in RCTIL was compromised, whether this was reflected in patients’ PBMC, and whether this was different in healthy donors. We stimulated cells via the TCR with a combination of CD3 and CD28 antibodies or in a TCR-independent manner with PMA and ionomycin. Activation (CD154), degranulation (CD107a), and production of effector cytokines (TNF-α, IFN-γ, IL-10, IL-17, and IL-2) were measured by flow cytometry. Figure 5 shows the results obtained for the CD4+ (Fig. 5A) and CD8+ (Fig. 5B) subsets. The most striking observation was the increased production of the suppressive cytokine IL-10 in CD4+ and CD8+ TIL, but not autologous PBMC, in the majority of the patients tested (4 of 7). After PMA and ionomycin treatment, up to 5.9% of the CD4+ and 5.3% of the CD8+ lymphocytes produced IL-10 (see also Supplementary Fig. S1).

**Figure 3.** Distribution of differentiation subsets according to CD45RA and CCR7 expression among CD4+ (A) and CD8+ (B) cells in PBMC and TIL from RCC patients and healthy donors’ PBMC. Mean percentages in each group and significant differences are shown. *, P < 0.05.

**Figure 4.** Expression of E-cadherin and its receptors. A, expression of KLRG1 and CD103 among CD4+ (top) and CD8+ (bottom) in PBMC and TIL from RCC patients and healthy donors’ PBMC. *, P < 0.05. B, dot plots illustrating the coexpression pattern of KLRG1 and CD103 on CD4+ (top) and CD8+ (bottom) TIL from one patient compared with autologous PBMC. C, example of E-cadherin expression in one renal tumor and autologous normal tissue.
these four cases, high IL-10 production was simultaneously detected in the CD4+ and CD8+ subsets.

The proportion of cells producing inflammatory cytokines in response to activation was slightly increased in PBMC and TIL of patients compared with healthy donors as illustrated for TNF-α (CD4+ and CD8+ subsets) and IFN-γ (CD8+ cells). We also found that IL-17 production was upregulated in the CD4+ TIL from 2 of 7 patients (up to 6.7%), suggesting that Th17 cells may specifically infiltrate the tumor tissue.

CD107a surface expression was then tested as a surrogate for cytotoxic capacity. In the CD8+ compartment, a clear increase of degranulating cells was seen in the TIL, with a mean of 52.8% of CD8+CD107a+ after PMA and ionomycin stimulation compared with 20.8% and 17.0% in CD8+ PBMC from RCC and healthy donors, respectively. A subpopulation of CD4+ cells also expressed CD107a after activation, and this subset was increased in TIL compared with autologous PBMC (CD3 and CD28 antibodies mean 3.7% versus 1.0%; PMA and ionomycin mean 13.0% versus 2.0%). Finally, we also observed upregulation of the early activation marker CD154 on stimulation and, consistently, production of IL-2 in both CD4+ and CD8+ TIL subsets and of IFN-γ in CD4+ cells (Supplementary Fig. S1).

Thus, functional Th1 and CD8+ effectors are present inside the tumor together with potentially suppressive cells.

Polylfunctional CD4+ T cells are increased in TIL. Recent work has shown that the expansion of polyfunctional T cells is associated with both resistance to infection and protective vaccine-induced immunity (reviewed in ref. 22). We therefore examined the polyfunctionality of peripheral and tumor-infiltrating T cells, focusing on the upregulation of CD154 and CD107a, and the production of IL-2 in both CD4+ and CD8+ TIL subsets and of IFN-γ in CD4+ cells (Figure 6A).

Figure 5. Cytokine and CD107a expression in PBMC and TIL from RCC patients and healthy donors’ PBMC. Analysis was done after a 5 h stimulation period in medium (alone), in the presence of CD3 and CD28 antibodies (3+28), or after incubation with PMA and ionomycin (P+I) in CD4+ (A) and CD8+ (B) cells. *, P < 0.05.

Because we have observed an increased proportion of IL-10+ TIL, we also asked whether these cells produce other cytokines. As exemplified for one RCC patient in Fig. 6B, we observed that IL-10-producing cells were mainly contained in the TNF-α‘IFN-γ’ fraction of both CD4+ and CD8+ subsets.

Together, these data show that T lymphocytes infiltrating renal tumors have the potential for activation, cytotoxicity, and simultaneous production of inflammatory cytokines. However, RCC-TIL are also enriched in CD4+ and CD8+ cells capable of secreting the immunosuppressive factor IL-10 that may contribute to weakened effector function in the tumor microenvironment.

Discussion

This study was undertaken to gain insights into the different T-cell subsets infiltrating RCC, their differentiation stages, and their functional features. Our results show that highly functional CD4+ T cells are present within kidney tumors, with the phenotype (CCR4−CXCR3+) and cytokine secretion pattern (TNF-α, IFN-γ, and IL-2 but only little IL-5; data not shown) pointing toward the preferential recruitment of Th1 cells and extending previous data (4, 32). Interestingly, expression of CXCR3 in tumor tissue was previously associated with clinical responses in RCC and melanoma (33, 34). In contrast, low levels of IFN-γ secretion by PBMC from RCC patients were reported after in vitro stimulation with CD3 and CD28 antibodies (16, 17). Different readout assays (ELISA 48-72 h versus intracellular cytokine production after 5 h) may explain the difference between these results and ours; alternatively, because TIL...
have been shown to be especially susceptible to apoptosis (35, 36), short-term assays may be more informative. In other studies, antigen-specific Th2 responses were shown to predominate over Th1 in patients with advanced and active RCC, a skewing that is likely to correlate with disease progression (7, 37).

We observed that CD4+ TIL are predominantly antigen-experienced effector memory cells with a heterogeneous differentiation status (CD45RA−CCR7−, CD45RA−CCR7−, CD45RA−CCR7−, and CD45RA−CCR7−) and also detected CD107a after activation. Mobilization of CD107a and production of perforin and granzymes has been observed in antiviral CD4+CD57+ effectors (27), suggesting that similar cells within TIL should also be functional and potentially mediate antitumor activity. Finally, concurrent analysis of several functional parameters shows a high proportion of polyfunctional CD4+ TIL.

We also asked whether Th17 can be found in RCC patients; in two of seven patients, a subset of CD4+IL-17+ TIL was clearly detectable, but their expansion was modest (<7%) compared with that reported for instance in ovarian carcinoma (38, 39). Additional experiments including larger numbers of patients will be necessary to determine the role of Th17 cells in various stages of renal cell cancer.

Similar to CD4+ cells, CD45RA−CCR7− T<sub>EM</sub> CD8+ cells were enriched in TIL. In contrast to CD4+, however, a variable subset of CD45RA−CCR7− differentiated effectors was present inside the tumor. This may relate to the tumor stage, because we observed that all pT<sub>3</sub> tumors presented an increased proportion of T<sub>EM</sub>CD8+ T cells (data not shown). Expression of CD28, CD57, KLRG1, and chemokine receptors was heterogeneous, suggesting dynamic memory and effector differentiation (19, 20). Our findings are in line with earlier studies on melanoma showing that antigen-specific CD8+ cells are mostly of the T<sub>EM</sub> type (40, 41). Effector memory T cells have been associated with vaccine protection, and importantly, with the absence of metastatic invasion in colon carcinoma (22, 42). Up to now, however, the relationship between T-cell phenotype and immune efficacy remains elusive and our observation may well indicate that the tumor environment impairs full CD8+ differentiation into strong cytotoxic effectors. On the functional level, we detected elevated amounts of CTL-TIL (CD107a<sup>+</sup>), whereas IFN-γ, TNF-α, and IL-2 production was equivalent to that of autologous PBMC.

Several of our observations suggest that CTL antitumor responses may be dampened down in RCC. First, the expression of the activating receptor NKG2D was decreased on CD8+ TIL. MIC molecules released from tumor cells have been shown to induce NKG2D cell surface downregulation and functional impairment (28). Second, upregulation of the costimulatory molecule CD154 on activation was greatly reduced in the CD8+ TIL: although CD154 has been mostly associated with CD4+ T-cell activation, it is also found on CD8+ lymphocytes and this may be of relevance in the tumor context. Finally, we show that the proportion of polyfunctional CD8+ cells was not increased in RCC patients, neither in the blood nor in the TIL, compared with healthy individuals. Additionally to a protective role against infections, first reports in mouse and patients show that the polyfunctionality of CTL is important for efficient antitumor responses (43, 44). Thus, effector CD8+ cells are present in the renal tumors, but their activation and polyfunctional status may be suboptimal. Further investigations should address the dysfunction of tumor antigen-specific T cells.

Several subpopulations of CD4+ and CD8+ cells have been shown to have immunosuppressive activity in vitro (1, 14, 45), and these may be crucial for the clinical outcome. In RCC patients, it has been shown that CD4<sup>+</sup>CD25<sup>hi</sup>nTreg cells exert suppressive activity (14, 17). We found a modest enrichment of these cells in TIL (mean 2.6% of the CD4+ subset; maximum 6%) but not in the blood, whereas previous reports describe variable amounts of Treg in RCC-PBMC compared with healthy donors-PBMC (mean range 2-7% and 0.7-2%, respectively; refs. 14, 15, 17). An immunochemistry-based study reported the presence of CD4<sup>+</sup>CD25<sup>hi</sup>nTreg cells in ~25% of the RCC tissues analyzed, at a mean of 4% of all CD4+ cells (46), in line with our findings. The different frequencies of Treg found in all these studies may be due to the antibody combinations used for characterization and/or to the patient collectives. Apart from these natural CD4+ Treg, suppression by CD4+ and CD8+ subsets expressing the integrin α<sub>4</sub>(CD103)<sup>+</sup> has been described in mice and humans (45). We found that RCC-TIL contained elevated levels of both CD4+CD103<sup>+</sup> and CD8<sup>+</sup>CD103<sup>+</sup> cells, suggesting that at least two further distinct populations of regulatory T cells are recruited into renal tumors. In addition, CD4+ and CD8+ T cells producing IL-10 were detected in the majority of patients' tumors. Intratumoral CD4<sup>+</sup>Foxp3<sup>+</sup> but not CD4<sup>+</sup>Foxp3<sup>+</sup> cells have recently been reported to be associated with poor survival in RCC patients and to produce IL-10 upon stimulation (46). We found that most of
the CD4+IL-10+ TIL also produced TNF-α and IFN-γ, indicating that they are Th1 cells, not Th2 or Treg. Production of IL-10 by Th1 cells has been observed in mouse models of pathogen infection and in tuberculous patients and is proposed to serve as a self-control mechanism (47). Due to the limited amount of material, we could not enrich the identified Treg subsets to test their suppressive activity in vitro. Nevertheless, our results reveal that several T-cell–based mechanisms may synergize locally to induce immunosuppression. These findings call for further investigations, especially on the role of CD4–CD103–CD8– cells in RCC. At the T-cell level, signaling deficiencies may occur and CD3ζ expression defects have been described in some RCC patients (12, 16).

In colon carcinoma, the density of effector T cells (CD3ζ+CD45RO+, CD8+, and granzyme B+) and the expression of Th1-related genes correlate positively with absence of tumor relapse and increased survival (48). In RCC, expression of Th1-type genes was associated with favorable outcome (49). We could not observe any association between clinical outcome and presence of a particular subset of T cells (data not shown). However, our patient collective was small and clinical follow-up is still ongoing. Analysis of relevant TIL subsets, such as those identified in this study, on larger cohorts of patients should be done to definitively determine the role of TIL in the clinical outcome of renal cancer.

In summary, our study reveals the heterogeneity of intratumoral T-cell subsets, which include both highly functional effectors and likely immunosuppressive cells. Such information can contribute to a better understanding of the complex interactions between tumor and immune cells and guide approaches to manipulate the tumor microenvironment for the benefit of patients.

Disclosure of Potential conflicts of interest were disclosed.

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