Orchestration and Prognostic Significance of Immune Checkpoints in the Microenvironment of Primary and Metastatic Renal Cell Cancer

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Translational Relevance:

Clear cell renal cell carcinoma (ccRCC) is an enigma of the tumor microenvironment studies since, in contrast to most malignancies, high densities of CD8+ T cells correlate with poor clinical outcome. We characterize the microenvironment of ccRCC primary tumors and lung metastases associated with an extensive CD8+ T cell infiltrate. In one group of patients, a T cell exhausted microenvironment characterized by high expression of immune checkpoints and the absence of fully functional mature dendritic cells (DC) was found and correlated with poor prognosis. In the second group, low expression of immune checkpoints and DC localized in peritumoral immune aggregates were found and conversely associated with good prognosis. Our data provide novel prognostic biomarkers highlighting the central role of PD-L2 and LAG-3 in the immunomodulation of ccRCC. The combination of these immune profiles could guide the selection of patients who are likely to respond to checkpoint blockade therapies.
Abstract:

Purpose: Clear cell Renal Cell Carcinoma (ccRCC) has shown durable responses to checkpoint blockade therapies. However, important gaps persist in the understanding of its immune microenvironment. This study aims to investigate the expression and prognostic significance of immune checkpoints in primary and metastatic ccRCC, in relation with mature dendritic cells (DC) and T cell densities.

Experimental Design: We investigated the infiltration and the localization of CD8+ T cells and mature DC, and the expression of immune checkpoints (PD-1, LAG-3, PD-L1, PD-L2) in relation with prognosis, in 135 primary ccRCC tumors and 51 ccRCC lung metastases. RNA expression data for 496 primary ccRCC samples were used as confirmatory cohort.

Results: We identify two groups of tumors with extensive CD8+ T cell infiltrates. One group, characterized by high expression of immune checkpoints in the absence of fully functional mature DC, is associated with increased risk of disease progression. The second group, characterized by low expression of immune checkpoints and localization of mature DC in peritumoral immune aggregates (tertiary lymphoid structures), is associated with good prognosis.

Conclusion: The expression of the immune checkpoints and the localization of DC in the tumor microenvironment modulate the clinical impact of CD8+T cells in ccRCC.
Introduction

A solid tumor is an intricate and dynamic ecosystem containing tumor and immune cells, fibroblasts, blood and lymphatic vessels (1). The density and composition of the immune microenvironment is heterogeneous among patients and tumor types, and it is becoming a robust tool to predict post-surgical recurrence and death (1, 2). In the vast majority of cancers, tumor infiltration by CD8+ memory cytotoxic T cells and Th1 cells is associated with good clinical outcome (1). In addition, it has been reported that an organized local immune reaction, characterized by the presence of mature dendritic cells (DC) localized in tumor-associated Tertiary Lymphoid Structures (TLS) is necessary to orchestrate this cytotoxic and Th1 immune contexture and is associated with good clinical outcome (3-8) and response to therapeutic vaccines (9, 10).

However, several recent findings challenge this concept of the unequivocal relation of effector memory CD8+ T cell infiltration with a good clinical outcome in cancer. Firstly, in diffuse large B cell lymphoma (DLBCL) (11), Hodgkin lymphoma (12) and clear cell Renal Cell Carcinoma (ccRCC) (13) high densities of tumor-infiltrating CD8+ T cells have been associated with poor prognosis in some studies. Secondly, a recent publication from our group showed that the infiltration of lung metastases from ccRCC by CD8+ T cells was correlated with poor overall survival (OS) while it was a factor of good prognosis in lung metastases from colorectal carcinoma (CRC) (14). Finally, in Non-Small-Cell Lung Cancer (NSCLC) where CD8+ T cells densities correlate with good prognosis, a low density of TLS-DC associated with high CD8+ T cell infiltration identifies a group of patients with high risk of death, suggesting a functional impairment of intra-tumoral CD8+ T cells in this situation (4).
Immune checkpoints on infiltrating T cells are key regulators of immune escape in cancer. In primary ccRCC, several studies have shown that the expression of the inhibitory receptor PD-1 on the immune cells (15, 16) or its ligand PD-L1 on tumor cells (17-19) is associated with a poor clinical outcome. Interestingly, antibodies that block the PD-1 axis have yielded a 20-30% response rate in metastatic ccRCC (20, 21) that seems to be related to the expression of PD-L1 by the tumor cells (20, 22). Another inhibitory molecule that has gained recent attention is the Lymphocyte Activation Gene-3 (LAG-3), which is co-expressed with PD-1 on CD8+ tumor-infiltrating lymphocytes in melanoma (23), and together with PD-1 synergistically regulates T cell function (24). We expected immune check points high ccRCC patients to have a worse prognosis than the immune check point low.

In these newly described scenarios where CD8+ T cell infiltration correlates with poor prognosis, it is important to define the combination of immune-based biomarkers that will predict patients’ prognosis and further guide immunotherapeutic approaches. This study aims to investigate the expression and prognostic significance of immune checkpoint receptors and paired ligands on primary and metastatic ccRCC in relation with TLS-DC and T cell densities.
Materials and Methods

Patients

A cohort of 135 primary ccRCC human tumors and another of 51 ccRCC lung metastases were collected. The primary cases derived from specimens of radical nephrectomy operated between 1999 and 2003 at the hospital Necker-Enfants Malades (Paris, France). The ccRCC lung metastases cohort resected at the Hotel Dieu hospital (Paris, France) or Hôpital Européen George Pompidou (HEGP, Paris, France) between 1992 and 2010 was already described in (14). This research was conducted according to the recommendations outlined in the Helsinki declaration and approved by the medical ethics boards of all participating institutions, and with the agreement of the Ile-de-France II ethics committee (no. 2012-0612). The demographic characteristics of the cohorts are depicted in Table S1 (14).

In addition, expression data for 496 primary ccRCC samples with complete follow-up was downloaded from The Cancer Genome Atlas’ KIRC study, using version 2 of the normalized RNA sequencing data. Corresponding clinical data (updated on 2013-04-06) were downloaded from (25).

Clinic and pathologic features

The original histologic diagnosis was confirmed on archival H&E stained slides, and histopathological features such as histologic subtype (26), tumor size, regional lymph node invasion, distant metastases at surgery, Fuhrman nuclear grade (27) and sarcomatoid features were collected. All tumors were pathologically staged according to the TNM (Tumor-Node-Metastasis) classification (28). The duration of follow up was calculated from the date of the
surgery (nephrectomy or metastasectomy) to the date of cancer progression, last follow up or death.

Immunohistochemical and immunofluorescence staining

Serial 5 µm formalin-fixed paraffin-embedded (FFPE) tissue sections from primary and metastatic ccRCC were stained using autostainerPlus Link 48 (Dako). Antigen retrieval and deparaffinization was carried out on a PT-Link (Dako) using the EnVision FLEX Target Retrieval Solutions (Dako). The antibodies used in this study for immunohistochemistry (IHC) and immunofluorescence (IF) are listed in Table S2. IF stained slides were scanned after secondary antibody incubation and mounting. For the IHC staining, peroxidase activity was detected using 3-amino-9-ethylcarbazole substrate (AEC) or Novared and alkaline phosphatase using alkaline phosphatase substrate III (Vector Laboratories).

Tests of the specificity and sensitivity of PD-1, PD-L1, PD-L2 and LAG-3 monoclonal antibodies for IHC experiments were performed utilizing generated FFPE cell pellets from transfected 300.19 cells [for PD-1, PD-L1 and PD-L2 (29)] and CHO cells (for hLAG-3), while parental un-transfected cells served as negative controls (Fig. S1) (Costim Pharmaceuticals, Cambridge, MA). Multiple-organs (n=32) human TMA (FDA999a, US Biomax) and malignant cancer tissues (n=10) from different oncology indications were also tested using the above mentioned protocols. Normal human FFPE tonsil sections for PD-1, LAG-3, PD-L2 and normal placenta for PD-L1 were used as positive controls (Fig. S1).

Immunohistochemical quantification
Stained slides were scanned with a Nanozoomer (Hamamatsu) and analyzed with Calopix software (Tribvn, France). For quantification purposes, tissues were divided into Invasive Margin (IM) and Tumor Center (TC) as previously described (30), and the density of positive cells was calculated in the whole tumor region (IM, TC). Due to the small size (or absence) of TC region in the majority of the metastases, the analysis on this cohort was done not discriminating between the two regions. The percentages of tumor cells stained positive for PD-L1 and PD-L2 were quantified by two independent reviewers (A.L. and N.G.) without prior knowledge of patient outcome, and the tumors above 5% tumor cell expression were considered as positive in accordance with studies in other types of cancer (20, 31).

Statistical analysis

Comparisons among the demographic and pathologic features, immune marker densities and PD-L1 and PD-L2 expression were evaluated by using $\chi^2$, Fisher’s exact and Wilcoxon rank-sum tests. Association of variables to prognosis was assessed using the Kaplan–Meier method, univariate and multivariate Cox regression analyses. To segregate patients in two groups based on numerical variables (cell density or gene expression), Log-rank P-values of each possible cutoff were computed. The cutoff that minimized the P-value of a Log-rank test for DFS was retained, and the corresponding P-value was corrected using the method published by Altman et al. (32). These cutoffs were later used to segregate patients into two groups, and their associated Kaplan-Meier curves are displayed throughout the Figures. To further confirm and validate the prognosis association of the cell densities, we determined the median and 3rd quartile cut-off and calculated the corresponding univariate Cox-regression P values (Table S3). Only those variables
univariately associated with prognosis were included in the multivariate Cox regression analysis. The duration of follow-up was calculated from the date of nephrectomy or metastasectomy to the date of death or last follow-up.
Results

Tumor infiltration by CD8+ T lymphocytes and expression of Th1 associated genes correlate with poor prognosis in ccRCC

The density of CD8+ T cells in the invasive margin (IM) of the primary tumors was heterogeneous in the cohort of 135 primary ccRCC. Based on the Optimal P value cut-off for DFS (OPv) (630 cells/mm²) we found that the CD8^High (n=41/135, 30%) group had a shorter Disease-Free Survival (DFS, P=0.0001, Fig. 1A) and Overall Survival (OS, P=0.001, Fig. 1A). The density of CD8+ T cells in the tumor center (TC) had no prognostic impact (Table S4). The density of CD8+ T cells in the IM correlated with the Fuhrman grade (Fig. S2) but not with any other pathologic variables including TNM (data not shown).

An independent cohort of lung metastases of ccRCC, in which the negative impact of high densities of CD8+T cells on OS had been previously described using semi-quantitative counting techniques (14), was reanalyzed using a quantitative approach on 51 cases. The OPv for CD8+ T cells density was 490 cells/mm² and the CD8^High (n=14/51, 27%) group displayed a shorter Overall Survival (OS, P=0.001, Fig. 1B). This result confirms our previous observations (14).

From The Cancer Genome Atlas (TCGA) public database of 496 primary ccRCC, we analyzed the expression of 844 immune-related genes, from which we extracted data concerning 7 genes expressed in a Th1 and CD8+ T cell oriented response according to Galon J et al. (30). We found that the expression of most of the genes associated with this cell signature correlated with poor prognosis: CD8A P=0.04, TBX21 P=0.03, IRF1 P=0.01, GZMB P=4.4x10^-5 and IFNG P=3.17x10^-7, that displayed the lowest P-value (Fig. 1C). Based on the OPv for IFNG, patients
that had high quantities of intratumoral mRNA for this gene displayed a shorter OS (P=0.006, Fig. 1D).

Simultaneous expression of immune checkpoints in primary and metastatic ccRCC identifies patients with poor clinical outcome

Primary tumors

To investigate the impact of immune check-point molecules on the negative prognostic impact of the CD8\textsuperscript{High} group, we analyzed the protein expression of PD-1, LAG-3, PD-L1 and PD-L2 molecules in this group of tumors (we could analyze 40 out of 41 tumors) and a randomly-matched group of the same size (n=40 out of 94) coming from the CD8\textsuperscript{Low}.

Based on the OPv cutoff, 15 tumors out of 80 were considered as PD-1\textsuperscript{High} in the IM and they displayed a shorter DFS (P=0.0005, Fig. 2A) and OS (P=0.03, Fig. 2A). The density of PD-1+ cells in the TC was not significantly associated with prognosis (Table S4). The Fuhrman grade was associated with the PD-1+ cell density in the IM (Fig. S2).

Out of the 80 patients, 9 were considered as LAG-3\textsuperscript{High} (subdivided by the OPv cutoff) in the IM and they displayed a shorter DFS (P=0.02, Fig. 2A), and did not reach significance for the OS (P=0.07, Fig. 2A). The density of LAG-3+ cells in the TC was not significantly associated with patients DFS or OS (Table S4). Representative pictures of the IHC staining of highly and poorly PD-1 and LAG-3 infiltrated lesions are shown in Fig. S3A and Fig. S3B, respectively.

Based on the 5% cut-off generally used in clinical trials with anti-checkpoint antibodies (20), 22 out of 80 (27%) and 27 out of 80 (34%) patients were PD-L1+ and PD-L2+, respectively (Fig.
S3C and Fig. S3D) ; among them, 9 tumors were double-positive. Patients with PD-L1+ tumors had a shorter OS than those with less than 5% positive tumor cells (P=0.02, Fig. 2A); a trend towards shorter DFS was also found (P=0.06, Fig. 2A). Univariately, patients with PD-L1+ tumors were 2.9 times more likely to die in the 5 years post-nephrectomy than patients with less than 5% PD-L1+ tumor cells (OS risk ratio 2.87; 95% confidence interval (CI) 1.2-7.1, P=0.02, Table S4). Patients with PD-L2+ tumors displayed a shorter OS compared to those with less than 5% positive tumor cells (P=0.005, Fig. 2A), but no impact on the DFS was found (P=0.13, Fig. 2A). Univariately, patients with PD-L2+ tumors were 3.4 times more likely to die than those with negative tumor cells (risk ratio 3.4; 95% CI 1.4-8.5, P=0.005, Table S4).

Patients with tumors exhibiting both high densities of PD-1+ lymphocytes in the IM and PD-L1+ and/or PD-L2+ tumor cells (n=11) had the worst prognosis, as assessed by DFS and OS (DFS, P=1.3x10^{-5}; OS, P=0.005; Fig. 2B). Patients that met these criteria had 6.1 times more risk to progress after resection than patients who did not (risk ratio 6.08; 95% CI 2.4-15.0; P<0.001, Table S4). Strikingly, 91% of the patients having a PD1High infiltrate and tumor cells expressing PD-L1 and/or PD-L2 progressed in the subsequent 5 years of surgery versus 36% in the negative group (complete 5-years follow-up n=70, Fisher’s exact test P=0.001, Fig. 2C). In the TCGA public datasets of 496 primary ccRCC, the gene expression of PD-1 (PDCD1), LAG-3 and PD-L2 (PDCD1LG) was associated with shorter OS (P=0.03, P=0.0001 and P=0.0003, respectively) while PD-L1 (CD274) was not (P=0.67) (Fig. 2D).

It has been reported that IFN-γ can induce PD-L1 expression on tumor cells (27). We found a significant positive correlation between the gene expression of IFNG with PD-L1 (R=0.13, P=0.004) and with PD-L2 (R=0.42, P=2.2x10^{-16}) in the TCGA cohort (Fig. 3A). In addition, a significant positive correlation was found between the densities of PD-1+ and CD8+ T cells...
(R=0.31, P=0.004), LAG-3+ and CD8+ T cells (R=0.42, P=0.001) and PD-1+ and LAG-3+ cells (R=0.55, P<0.0001) in the IM of the 80 primary ccRCC (Fig. 3B and 3C). An even stronger correlation was found at the gene expression level between PD-1 and LAG-3 (R=0.81, P=0.002), PD-1 and CD8A (R=0.95, P<0.001) and LAG-3 and CD8A (R=0.96, P<0.001) in the TCGA cohort of 496 primary tumors (Fig. 3B and 3C). In accordance with all these observations, we also detected the presence of triple positive CD8+/PD-1+/LAG-3+ cells in 6 out of 7 cases from the CD8High group of primary tumors by immunofluorescence on paraffin sections (representative pictures are displayed in Fig. 3D).

Metastases

Metastatic ccRCC has been one cancer where antibodies inhibiting the PD-1 axis have induced remarkable tumor regression in some patients (20), highlighting the necessity to define prognostic and predictive immune-based biomarkers in this disease. Out of the 51 patients with ccRCC lung metastasis, 13 were considered as PD-1High and the latter displayed a shorter OS (P=0.008, Fig. 4A). Seven out of 51 were considered as LAG-3High and again they displayed a shorter OS (P=0.048, Fig. 4A).

Based on a 5% cutoff, five out of 51 (10%) and 15 out of 51 (29%) metastases were PD-L1+ or PD-L2+, respectively; of them, 3 were double-positive. Whereas patients with PD-L1+ metastases did not have a significantly worse clinical outcome (P=0.12, Fig. 4A), the expression of PD-L2 on tumor cells was associated with a shorter OS (P=0.03, Fig. 4A). Univariately, patients with PD-L2+ metastasis were 2.2 times more likely to die compared with patients with PD-L2- ones (risk ratio 2.17, 95%CI 1.03-4.35, P=0.04, Table S4).
Patients with high densities of PD-1+ lymphocytes and PD-L1+ and/or PD-L2+ tumor cells in their ccRCC lung metastases (n=12) had the worst prognosis as assessed by OS (OS, P=0.003, Fig. 4B). The patients that met these criteria had 3.1 times more risk to die after metastasectomy than patients who did not (risk ratio 3.1; 95% CI 1.28-6.66; P=0.003, Table S4). Strikingly, 100% of the patients having a PD1High infiltrate and metastases simultaneously expressing one or both of its ligands (PD-L1 or PD-L2) died in the subsequent 5 years after surgery versus 57% in the negative group (Fisher tests, P value=0.004, Fig. 4C).

A significant and strong correlation was found between densities of PD-1+ and CD8+ T cells (R=0.51, P=0.0001), LAG-3+ and CD8+ T cells (R=0.4, P=0.004) (Fig. 4D) and PD-1+ and LAG-3+ cells (R=0.71, P<0.0001).

In conclusion, the combined analysis of the expression of PD-1, PD-L1 and PD-L2 identified a group of patients with a high risk of progression and death in primary and in an independent cohort of metastatic ccRCC.

**Opposite correlations of TLS-DC and NTLS-DC with prognosis and their association with immune checkpoints**

It has been shown that TLS-DC orchestrate intratumoral CD8+ cytotoxic T cells and Th1 responses in NSCLC (3, 4). In the primary ccRCC cohort (n=135), DC-Lamp+ cells were detected within TLS (TLS-DC) in the IM. They co-expressed CD83 and high amounts of MHC Class II, and were localized in the vicinity of PNAd+ High Endothelial venules (HEV) (Fig. 5A) as described in other tumor types (3). There was a trend where high densities of TLS-DC were associated with longer DFS (OPv=0.09) but not with OS (Table S4). TLS-DC densities in the TC
were not associated with prognosis. Interestingly, high densities of TLS-DC (based on the OPv cut-off) in the IM identified a group of patients with good prognosis among the CD8$^{\text{High}}$ group for both DFS (P=0.001, Fig. 5A) and OS (P=0.03, Fig. 5A).

However, in contrast to NSCLC where most of the DC-Lamp$^+$ cells are localized within TLS (4), in ccRCC a large percentage (79±20%) of DC-Lamp$^+$ cells with DC morphology was found isolated and outside TLS (NTLS-DC, Fig. 5B). They colocalized with CD34$^+$ blood vessels, but not with PNAd$^+$ HEV, expressed low amounts of MHC Class II and were CD83 negative (Fig. 5B). The majority of NTLS-DC was localized in the IM (71±14%) and their high densities (based on the OPv cut-off) were associated with poor clinical outcome in primary ccRCC patients (DFS P=0.006, OS P=5.1x10$^{-5}$, Fig. 5B).

The opposite influences of TLS-DC and NTLS-DC on prognosis prompted us to explore their relationships with the expression of PD-1 and its ligands in the CD8$^{\text{High}}$ group of patients. A negative correlation was found between the densities of TLS-DC and PD-1$^+$ cells ($r$=-0.23, P=0.04, Fig. 5C). Tumors containing PD-L1$^+$ and/or PD-L2$^+$ tumor cells exhibited less TLS-DC (P=0.014) (Fig. S4) but similar densities of CD8$^+$ T cells (P=0.96). In contrast, the density of NTLS-DC was associated with the tumor expression of PD-L1 (OR 6.54, 95%CI 1.23-45.45 P=0.012) and PD-L2 (OR 2.7, 95%CI 1.2-18.1 P=0.04). Most of the patients that were PD-L1$^{\text{High}}$&PD-L1$^+$and/or$L2^+$ (8 out of 11) were also CD8$^{\text{High}}$&TLS-DC$^{\text{Lo}}$ in primary ccRCC (OR 15.02, 95%CI 1.66-72.7, P=0.004) and the presence of one or both patterns correlated with shorter DFS (P<0.0001) and OS (P=0.001, Fig. 5D).

Identification of a group of patients with worst prognosis in primary and metastatic ccRCC
In order to define the independent prognostic significance of the previously mentioned immune-profiles (CD8^High&TLS-DC^Low or PD-1^High&PD-L1^+and/orPD-L2^+), we included them in a multivariate Cox regression analysis with the other significant prognostic clinical variables (TNM and Furhman grade). The strongest independent poor prognostic factors in primary ccRCC for DFS was to have a CD8^High&TLS-DC^Low (P=0.001, Table 1) or PD-1^High&PD-L1^+and/orPD-L2^+ (P=0.03, Table 1) immune profile. For the metastatic cohort, we found that the strongest independent worst prognostic variable for OS was being CD8^High (P=0.004, Table 1) or having a tumor with PD-1^High&PD-L1^+and/orPD-L2^+ immune profile (P=0.02, Table 1).
Discussion

Compared to other neoplasia, the immune microenvironment in ccRCC has not yet been studied in detail. However, it is of paramount importance to understand its regulation in view of the paradoxical correlation of CD8+ T cell infiltration with poor prognosis (13, 14) and the recent advances of immunotherapy with anti-checkpoint antibodies (anti PD-1 and anti PD-L1) (20, 21).

ccRCC has been described as a pro-inflammatory neoplasia where tumor cells produce several cytokines (such as VEGF, IL-6 and TGF-β) (33-35) that may lead to the recruitment and activation of polyclonal CD8+ T cells (36-39). Our data suggest that these recruited CD8+ T cells could only be locally educated when high densities of TLS-DC are present, and in only in these cases their density correlates with favorable prognosis. ccRCC is the first tumor type where a large proportion of DC-Lamp+ cells outside TLS structures has been observed (NTLS-DC).

Interestingly, we found that these cells do not express activation and co-stimulatory markers and they are probably recruited directly from the blood into the tumor stroma –contrary to the usual path that DC-Lamp+ cells follow in other type of tumors (5, 40). Accordingly, several studies have shown that the ccRCC microenvironment can induce a dysfunctional DC maturation, a down-regulation of co-stimulatory molecules and tolerogenicity (34, 41, 42), while DC in the TLS are likely to be protected from these effects (43). Altogether, our results suggest that the particular proinflammatory milieu initiated by tumor cells induces the recruitment of CD8+ T cells that –due to the low number of fully functional mature DC present in specialized T cell priming-sites, or the presence of DC with suppressive phenotype– are not able to mount an effective anti-tumor immune response, but rather express exhaustion/inhibition molecules.
This work reinforces the concept that T cell exhaustion/inhibition (44) plays an important role in ccRCC pathogenesis. It has been described that the density of PD-1+ cells (15, 16) and the tumor expression of PD-L1 (17-19) in primary ccRCC are associated with a poor clinical outcome. In this study we confirm these findings, and extend them to ccRCC lung metastases. This information is highly relevant since metastatic ccRCC treated patients have shown one of the highest objective durable response rates to PD-1 blockade (approximately 30%) (20) and many efforts are being dedicated to define theranostic tools in this pathology. Furthermore, we describe for the first time the prognostic significance of PD-L2. This molecule seems to be expressed in a higher proportion of tumors than PD-L1, and up-to 30% of them might express it solely according to our results. This might be of clinical relevance since—although in two different non-randomized cohorts—the anti-PD-L1 treatment alone seems to have a lower response rate than the anti-PD-1 (12% and 27% respectively) (20, 21). Furthermore, there are PD-L1 negative tumors that respond to anti-PD-1 treatment (22), suggesting that indeed there are other molecules beside PD-L1 implicated in the PD-1-inhibition axis of ccRCC. Few publications have reported PD-L2 mRNA or protein expression in other tumors including primary mediastinal large B-cell lymphoma (29), NSCLC (45), ovarian (46) and esophageal (47) where it has shown a limited impact of patients’ prognosis.

To our knowledge, this is the first report on the poor prognostic impact associated with high densities of LAG-3+ cells in human tumors. Furthermore, we provide clear evidence that the expression of PD-1 and LAG-3 are highly correlated in ccRCC. Some studies on mouse models have shown a synergistic effect of the inhibition of both pathways in boosting anti-tumor immune response (24). Therefore, our data support the rationale of dual blockade of these molecules in ccRCC.
Although recent works have emphasized that PD-L1 is preferentially upregulated by IFN-γ (31, 48), while PD-L2 is regulated by IL-4 (49), the weak association of PD-L1 mRNA with IFNγ might suggest an important role for post-transcriptional regulation of PD-L1 expression in ccRCC as for other aggressive tumors (50). Furthermore, it is highly suggestive that IFN-γ can also induce PD-L2 upregulation in tumors, as in immune cells (51), and supports the rationale to use therapeutic antibodies targeting this ligand. Taken together, our results suggest that the expression of these molecules is related with a chronic inflammatory and highly suppressive process that is unselectively recruiting CD8+ and NTLS-DC cell from the circulation, and overall is associated with a poor prognosis.

Another characteristic of ccRCC is the lack of prognostic significance of the immune cells in the TC. While CD8+, PD-1+, LAG-3+, and NTLS-DC densities in the IM of the primary tumors were associated with poor prognosis, they had no prognostic significance when present in the TC. In CRC, both regions are important to define the best immune score, which correlates with patient’s longer survival (30). Whether this dichotomy between ccRCC and CRC reflects different tumor tissue organization or relates to other factors of the tumor microenvironment remains to be clarified.

Recent unsupervised gene clustering of stage 4 primary ccRCC showed that tumors with high inflammatory immune infiltrate (approximately 15%) also have a high expression of PDCD1 (PD-1) and its ligands, and correlate with the worst prognosis (Beuselinck et al., in press). Indeed, this inflammatory/pro-angiogenic profile (probably originated from the ccRCC tumor cells) found in ccRCC primary tumors and in ccRCC lung metastases (14), is not found in CRC lung metastases (14), highly supporting that it may contribute to local immunosuppression process, the absence of fully mature DC, and high expression of immune checkpoints (52).
In summary, we identify a subset of primary and metastatic ccRCC patients characterized by (1) an extensive CD8+ T cell infiltrate, (2) expression of immune checkpoints, and (3) the absence of fully functional DC, which is associated with poor clinical outcome. Our results highlight novel independent prognostic factors in ccRCC based on the concomitant quantification of densities of DC, CD8+, PD-1+ and LAG-3+ lymphocytes in addition to PD-L1/PD-L2 expression by tumor cells. These immune profiles should guide the selection of suitable patients to receive immunotherapies and need to be further validated in larger and independent cohorts. Since this choice depends on both the extent of CD8+ T cell infiltration and the maturation and localization of DC, it invites revision of the idea that intratumoral CD8+ T cells are always associated with favorable prognosis in human tumors.

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Authors Contribution:

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Contributed reagents/materials/analysis tools: G.S., F.T., G.J.F.

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Table 1. Multivariate Cox regression analysis for DFS and OS of the pathologic and immune variables in primary and metastatic ccRCC
Figure Legends

Fig. 1. CD8+ T cells and CD8/Th1 gene signature are associated with poor prognosis in ccRCC. Overall and Disease-Free Survival (DFS) according to the presence of a High or Low density of CD8+ T cells in the IM of primary ccRCC (A) and ccRCC lung metastases (B).
values by univariate Cox regression analysis for OS on 7 Th1-related genes are displayed, P=0.05 dotted black line, HR>1.0 grey columns (C). Overall Survival (OS) according to IFNG gene expression in primary ccRCC (D).

**Fig. 2. Expression of immune checkpoints correlates with unfavorable clinical outcome for patients with primary ccRCC.** Overall Survival (OS) and Disease-Free Survival (DFS) according to the presence of a high or low density of PD-1+ and LAG-3+ cells, and the expression of PD-L1 or PD-L2 by >5% of the tumor cells in primary ccRCC (A). OS & DFS (B) and pie chart (C) representing the percentage of patients that had progressed after 5 years of surgery according to the expression of PD-L1 and/or PD-L2 on tumor cells related with high densities of PD-1+ lymphocytes. The P values by univariate Cox regression analysis for OS on 4 genes are displayed, P=0.05 dotted grey line, HR>1.0 grey columns (D).

**Fig. 3. Expression of immune checkpoints correlates with CD8+ T cells infiltration in primary ccRCC.** Dotplot of the gene expression of PD-L1 (red dots) and PD-L2 (blue dots) (y axis) against IFNG (x axis) (A). Dotplot of the Log_{10} cell density and gene expression of PD-1 (red dots) and LAG-3 (blue dots) (y axis) against CD8 (x axis) (B). Dot plot of the Log_{10} cell density and gene expression of PD-1 (y axis) against LAG-3 (x axis) (C). Pearson's r value and the number of samples for each correlation is displayed. Immunofluorescence staining on 1 paraffin-embedded ccRCC showing the colocalization of CD8 (green), PD-1 (red) and LAG-3 (white) proteins in lymphocytes (D); nuclei are stained with DAPI. TCGA, The Cancer Genome Atlas.

**Fig. 4. Expression of immune checkpoints correlates with unfavorable clinical outcome for patients with ccRCC lung metastases.** Overall Survival (OS) according to the presence of a
high or low density of PD-1+ and LAG-3+ cells, and the expression of PD-L1 or PD-L2 by >5% of the tumor cells in ccRCC lung metastases (A). OS (B) and pie chart (C) representing the percentage of patients that had died 5 years after the metastasectomy according to the expression of PD-L1 and/or PD-L2 on tumor cells and high densities of PD-1+ lymphocytes. Dot plot of the Log_{10} density of PD-1+ (black dots) and LAG-3+ cells (grey dots) (y axis) against CD8 (x axis) (D); Pearson's r value for each correlation is displayed.

**Fig. 5. Characteristics of TLS-DC and NTLS-DC and their relationships with prognosis and immune checkpoints.** IHC photomicrographs of DC-Lamp(Red)/CD3(Blue) illustrating the presence of mDC in TLS (A, white arrows) or outside TLS (B, black arrows); DC-Lamp(Red)/PNAd(Blue) and DC-Lamp(Red)/CD34(Blue) illustrating TLS-DC near HEV (A) and NTLS-DC near endothelial cells (B) in primary ccRCC. IF staining showing the co-localization of CD3 (green), DC-Lamp (red) with HLA-DR or CD83 (white) expression in TLS-DC (A) but not in NTLS-DC (B); nuclei are stained with DAPI. Overall Survival (OS) and Disease-Free Survival (DFS) according to the presence of a High or Low Density TLS-DC in the CD8^{High} group (A, bottom) or NTLS-DC in the entire cohort (B, bottom). Dot plot of the PD-1+ against TLS-DC+ cell densities (blue dots) (C); Pearson's r value is displayed. OS and DFS according to the presence of a CD8^{High} & TLS-DC^{Low} and/or PD-1^{High} & PD-L1^{and/or}L2+ immune profiles (D).
Figure 1

A

CD8+ cell density

Disease-Free Survival (%)

Time (months)

CD8Low (n=94) vs CD8High (n=41)
P=0.001

B

CD8+ cell density

Overall Survival (%)

Time (months)

CD8Low (n=37) vs CD8High (n=14)
P=0.001

C

IFNG gene expression

P healthcare

Gene expression

n=436

D

Overall Survival (%)

Time (months)

IFNGLow (n=287) vs IFNGHigh (n=209)
P=0.006
Figure 2

A

PD1+ cell density

LAG3+ cell density

PD-L1+ Tumor Expression

PD-L2+ Tumor Expression

B

Tumor: PD-L1 and/or L2+
TILs: PD1High

Disease-Free Survival (%)

Overall Survival (%)

Time (months)

C

Primary ccRCC
TILs: PD1Low
Tumor: PD1+ and L2-

Primary ccRCC
TILs: PD1High
Tumor: PD1+ and/or L2+

D

q=0.05

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Figure 3

A) Primary ccRCC TCGA

B) Primary ccRCC

C) Primary ccRCC

D)

In each panel, the scatter plots show correlations between different gene expressions and immune cell densities. The Pearson correlation coefficient (r) is indicated for each relationship. The numbers of samples (n) are also provided for each dataset.
Figure 4

A

- **PD1+ cell density**
  - PD1Low (n=38)
  - PD1High (n=13)
  - Overall Survival (%)
  - Time (months)
  - P=0.008

- **LAG3+ cell density**
  - LAG3Low (n=44)
  - LAG3High (n=7)
  - Overall Survival (%)
  - Time (months)
  - P=0.05

- **PD-L1+ Tumor Expression**
  - <5% Tumor cells (n=68)
  - >5% Tumor cells (n=6)
  - Overall Survival (%)
  - Time (months)
  - P=0.12

- **PD-L2+ Tumor Expression**
  - <5% Tumor cells (n=36)
  - >5% Tumor cells (n=15)
  - Overall Survival (%)
  - Time (months)
  - P=0.03

B

- **Tumor: PD-L1 and/or L2+**
- **TILs: PD1+**
  - Negative (n=39)
  - Positive (n=12)

- Overall Survival (%)
- Time (months)
- P=0.002

C

- **ccRCC Lung Metastases**
  - TILs: PD1 Low
  - OR
  - Tumor: PD1- and L2-

- **ccRCC Lung Metastases**
  - TILs: PD1 High
  - AND
  - Tumor: PD1+ and/or L2+

- 43% 57% 100%
- 5y not dead 5y dead
- n=39 n=12

- Fisher test P=0.004

D

- **Log10CD8+ cell density**
- PD1 r=0.51
- LAG3 r=0.41
- n=51
Figure 5

A) TLS-DC

B) NTLS-DC

C) CD8 High Group TLS-DC-Lamp+ cell density

D) CD8 High Group NTLS-DC-Lamp+ cell density

C) PD-1+ Cells/mM IM

D) CD8\textsuperscript{High}/TLS-DC\textsuperscript{Low} and/or PD\textsuperscript{High}/PDL\textsubscript{1} and/or L2+
Orchestration and Prognostic Significance of Immune Checkpoints in the Microenvironment of Primary and Metastatic Renal Cell Cancer

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