

## Prognostic and Predictive Value of Tumor-infiltrating Leukocytes and of Immune Checkpoint Molecules PD1 and PDL1 in Clear Cell Renal Cell Carcinoma



Philipp J. Stenzel<sup>\*</sup>, Mario Schindeldecker<sup>\*,†</sup>, Katrin E. Tagscherer<sup>\*</sup>, Sebastian Foersch<sup>\*</sup>, Esther Herpel<sup>‡,§</sup>, Markus Hohenfellner<sup>¶</sup>, Gencay Hatiboglu<sup>¶</sup>, Juergen Alt<sup>#</sup>, Christian Thomas<sup>\*\*,+†</sup>, Axel Haferkamp<sup>\*\*</sup>, Wilfried Roth<sup>\*</sup> and Stephan Macher-Goepfing<sup>\*,†</sup>

<sup>\*</sup>Institute of Pathology, University Medical Center Mainz, Mainz, Germany; <sup>†</sup>Tissue Biobank, University Medical Center Mainz, Mainz, Germany; <sup>‡</sup>Institute of Pathology, University Hospital Heidelberg, Heidelberg, Germany; <sup>§</sup>Tissue Bank of the National Center for Tumor Diseases, Heidelberg, Germany; <sup>¶</sup>Department of Urology, University Hospital Heidelberg, Heidelberg, Germany; <sup>#</sup>Department of Hematology, Medical Oncology & Pneumology, University Medical Center Mainz, Mainz, Germany; <sup>\*\*</sup>Department of Urology, University Medical Center Mainz, Mainz, Germany; <sup>+†</sup>Department of Urology, University Hospital Carl Gustav Carus, Dresden, Germany

### Abstract

**INTRODUCTION:** Immune checkpoint inhibitors (ICI) have been approved for patients with clear cell renal cell carcinoma (ccRCC), but not all patients benefit from ICI. One reason is the tumor microenvironment (TME) that has substantial influence on patient's prognosis and therapy response. Thus, we comprehensively analyzed the TME of ccRCC regarding prognostic and predictive properties. **METHODS:** Tumor-infiltrating CD3-positive T-cells, CD8-positive cytotoxic T-lymphocytes (CTLs), regulatory T-cells, B-cells, plasma cells, macrophages, granulocytes, programmed cell death receptor 1 (PD-1), and its ligand PD-L1 were examined in a large hospital-based series of ccRCC with long-term follow-up information (n = 756) and in another patient collective with information on response to nivolumab therapy (n = 8). Tissue microarray technique and digital image analysis were used. Relationship between immune cell infiltration and tumor characteristics, cancer-specific survival (CSS), or response to ICI was examined. **RESULTS:** Univariate survival analysis revealed that increased tumor-infiltrating B-cells, T-cells, and PD-1-positive cells were significantly associated with favorable CSS and high levels of intratumoral granulocytes, macrophages, cytotoxic T-cells, and PD-L1 significantly with poor CSS. High CTL or B-cell infiltration and high PD-L1 expression of ccRCC tumor cells qualified as independent prognostic biomarkers for patients' CSS. Significantly higher densities of intratumoral T-cells, CTLs, and PD-1-positive immune cells were observed in ccRCC with response to ICI compared with patients with mixed or no response (CD3: p = 0.003; CD8: p = 0.006; PD-1: p = 0.01). **DISCUSSION:** This study shows that subsets of tumor-infiltrating leukocytes in the TME and also PD-1/PD-L1 provide prognostic and predictive information for patients with ccRCC.

*Translational Oncology (2020) 13, 336–345*

Address all correspondence to: Philipp J. Stenzel, Institute of Pathology, University Medical Center Mainz, Langenbeckstrasse 1, Mainz, Germany. E-mail: [philipp.stenzel@unimedizin-mainz.de](mailto:philipp.stenzel@unimedizin-mainz.de)

Received 5 August 2019; Accepted 6 November 2019

© 2019 The Authors. Published by Elsevier Inc. on behalf of Neoplasia Press, Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1936-5233/19

<https://doi.org/10.1016/j.tranon.2019.11.002>

## Introduction

Reports back to the 1960s reveal that cytoreductive nephrectomy can induce spontaneous remissions of metastatic clear cell renal cell carcinoma (ccRCC) [1–3]. This effect has been attributed to immune stimulation triggered by release of tumor antigens. Additional research gained insights into the immunobiology of tumor–immune cell interactions, and, at present, ccRCC is considered as an immunogenic malignancy [4–6]. As ccRCCs are highly resistant against conventional radio- and chemotherapy, historically immunotherapy consisting of either high-dose interleukin 2 (IL-2) or interferon alpha (IFN $\alpha$ ) became the treatment of choice in highly selected patients with systemic disease [7]. High-dose IL-2 is still the only therapy that can cure a minority of patients with ccRCC with complete and durable response in ~10% of patients [7–9]; however, substantial treatment-related toxicities occur, including treatment-related deaths in about 1–4% of treated patients [9,10]. Given that treatment with high-dose IL-2 is not applicable for many RCC patients, targeted therapy against the vascular endothelial growth factor and mammalian target of rapamycin pathways, which are available to nearly all patients with ccRCC and show less substantial adverse events, has been introduced and is currently the first-line treatment of ccRCC patients [11–13]. Although targeted therapies produce objective responses and prolong progression-free survival (PFS) and overall survival (OS) in ccRCC patients, these therapy options are not curative.

Currently, treatment landscape in ccRCC is shifting back towards immuno-oncology agents such as specific immune checkpoint inhibitors [14,15], which have been shown to improve OS in ccRCC patients. At present, nivolumab, a programmed cell death receptor 1 (PD-1) inhibitor, is approved for the treatment of advanced RCC after treatment with antiangiogenic therapy [16]. Furthermore, recently a randomized phase 3 study with nivolumab combined with ipilimumab, targeting immune checkpoint protein cytotoxic T-lymphocyte (CTL)–associated protein 4, showed significantly higher OS and response rates compared with sunitinib among intermediate- and poor-risk patients with previously untreated advanced RCC [17]. Although new immune modulatory agents improved treatment of ccRCC patients and will change the management of RCC for the years to come, better stratification of patients is essential. Indeed, less than half of the patients have objective response to ICI [15]; treatment-related adverse events grade 3 or 4 occur in ~ 50% of the patients [17]. To optimize patient benefit and minimize risk of toxicities, predictive biomarkers for ICI are needed. PD-L1 expression level of tumor cells is so far the most common used biomarker, but PD-L1 testing alone seems to be insufficient for patient selection in most malignancies. In the future, measuring immune activation including characterization of tumor-infiltrating mononuclear immune cells (TIMCs) and PD-L1 expression on tumor and immune cells as well as possibly assessment of gene signatures indicating preexisting adaptive antitumor immunity or myeloid inflammation seems to become more relevant [18,19].

Tumor mutational burden (TMB) is another biomarker for immuno-oncology agents that measures the number of mutations present in a tumor. For example, tumors with mismatch repair (MMR) deficiency have high response rates and the FDA-approved use of the anti-PD-1 agent pembrolizumab in patients with refractory tumor microsatellite instability or mismatch repair deficiency

(MMRd) solid tumors [20,21]. The mechanistic hypothesis is that tumor antigenicity generated by mutations promotes T-cell expansion, which enhances the anti-PD-1 response [22,23]. However, association between TMB and response to atezolizumab treatment in RCC patients was not observed, which is in contrast to results for other malignancies, such as lung cancer and metastatic urothelial carcinoma [19,24,25]. Hence, the underlying biological basis of immunogenicity in RCC remains to be identified.

In this study, we aimed to comprehensively characterize the tumor microenvironment (TME) in a large cohort of ccRCC tumor samples regarding its prognostic value. Furthermore, we addressed the question whether the TME holds predictive information regarding immune checkpoint inhibition in ccRCC patients.

## Material and Methods

### Patients

Tissue samples from 756 patients with primary ccRCC, treated at the Department of Urology at the University of Heidelberg between 1987 and 2005, were collected (patient collective 1, for details see Table 1). The human tissue samples were provided by the Tissue Bank of the National Center for Tumor Diseases Heidelberg after approval by the ethics committee of the University of Heidelberg. No adjuvant treatment for localized disease was administered. Patients with metastasized disease and with a Karnofsky performance index of  $\geq 80$  and no medical contraindications received palliative IFN- $\alpha$ - and interleukin-2-based immunotherapy. Clinical follow-up was available for all cases. Survival was calculated from the date of surgery until last visit or death. Further detail has been described previously [26]. The tumors were graded in accordance with the three-tiered nuclear grading system [27].

**Table 1.** Clinicopathological Data of Patient Collective 1.

Item	
Collective size	<i>n</i> = 756
Grade	
G1	212 (28.0%)
G2	408 (54.0%)
G3	132 (17.5%)
Missing	4 (0.5%)
Tumor extent	
pT1	412 (54.5%)
pT2	59 (7.8%)
pT3	260 (34.4%)
pT4	25 (3.3%)
Local lymph node metastasis	
No	711 (94.0%)
Yes	45 (6.0%)
pNx	0
Distant metastasis	
No	635 (84.0%)
Yes	121 (16.0%)
Mx	0
Sex	
Female	292 (38.6%)
Male	464 (61.4%)
Age at surgery	
$\leq 65$	45 (6.0%)
$> 65$	711 (94.0%)
ECOG	
0	458 (60.6%)
$> 0$	298 (39.4%)

Tumor tissue of eight patients with clear cell renal carcinoma adjuvantly treated with nivolumab at the Department of Urology or at the University Center for Tumor Diseases (UCT) Mainz, University Medical Center of the Johannes Gutenberg University Mainz, Germany, was collected (patient collective 2, for details see [Supplemental Table 1](#)) (ethics approval: 837.031.15 (9799)). Information on response to therapy and duration was available for all cases.

### Immunohistochemistry

A tissue microarray (TMA) containing 756 primary ccRCC and corresponding normal tissue samples was used as described before [28]. For immunohistochemical staining of tissue, microarray slides or formalin fixed and paraffin-embedded tumor tissue antibodies directed against CD3 (IR503, Dako, Glostrup, Denmark), CD8 (C8/144B, Dako), CD20 (IR604, Dako), CD56 (123C3, Dako), CD68 (PG-M1, Dako), CD138 (MI15, Dako), FoxP3 (236A/E7, Abcam, Cambridge, United Kingdom), myeloperoxidase (MPO) (IR511, Dako), PD-1 (NAT105, Abcam), and PD-L1 (EPR19759, Abcam) were used. All slides were stained with automatized immunostainers (autostainer plus, Dako).

### Digital Image Analysis

All slides were digitalized using a digital whole slide scanner (Nanozoomer, Hamamatsu Photonics, Hamamatsu, Japan) and analyzed using the HALO® platform (Indica Labs, Corrales, NM, USA). Digital image analysis was performed as described before [29]: Briefly, for quantification of stain-positive cells, total amount of cells and analyzed area the CytoNuclear module (v1.4–1.6) including a tissue classifier to discriminate between tumor tissue and nontumor tissue, especially erythrocytes, was used. TMA slides were annotated with the TMA module and missing or erroneous TMA cores were excluded. Small areas with high artificial overlay and detritus were manually excluded from analysis by annotations layers. PD-L1- and CD138-stained TMA slides were manually prescored before digital image analysis because of high background noise: Cores with no specific PD-L1 or CD138 staining were manually set to 0% positive cells. In case of specific CD138-staining, positive cells were manually counted by an experienced pathologist, the amount of cells in each core quantified by digital image analysis and the percentage of positive cells calculated. Tumors were grouped into tumor cell–positive PD-L1 or nontumor cell–positive PD-L1 staining depending on the staining pattern by an experienced pathologist. For all stains, a representative set of cores was used to define analysis settings and thresholds ([Supplemental Table 2](#)). Results obtained from automated tissue analysis were manually controlled on a set of randomly selected cores. Invasive margin and tumor center were identified on whole slides of tumor tissue by an experienced pathologist and sketched on the scan. Invasive margin along the border of tumor to adjacent normal tissue was defined as the area of 500  $\mu\text{m}$  in direction of tumor and tumor-adjacent normal tissue, respectively. Tumor center was defined as a representative area in the core region of the tumor. Analysis of TIMC was performed in the selected regions only. Density of positive cells was calculated as the quotient of the number of positive cells and analyzed area.

### Statistical Analysis

For dichotomization of biomarker expression, the Charité Cutoff finder [30] was used for distinction of high and low biomarker levels

based on patient survival data. Association between biomarker and patient survival was calculated using the log rank test and depicted by Kaplan–Meier plots. Univariate and multivariate Cox regression model was used for statistical analysis of the influence of the marker on patients' outcome by calculation of hazards ratios and 95% confidence intervals. TIMC and PD-1/PD-L1 are given as percentage of total cells in the TMA core or as biomarker-positive cells/ $\text{mm}^2$  in the analysis of whole slides. Differences between three or more groups were tested using one way analysis of variance or Fisher's exact test for count data. Differences with error probabilities of  $<0.05$  were considered significant.

## Results

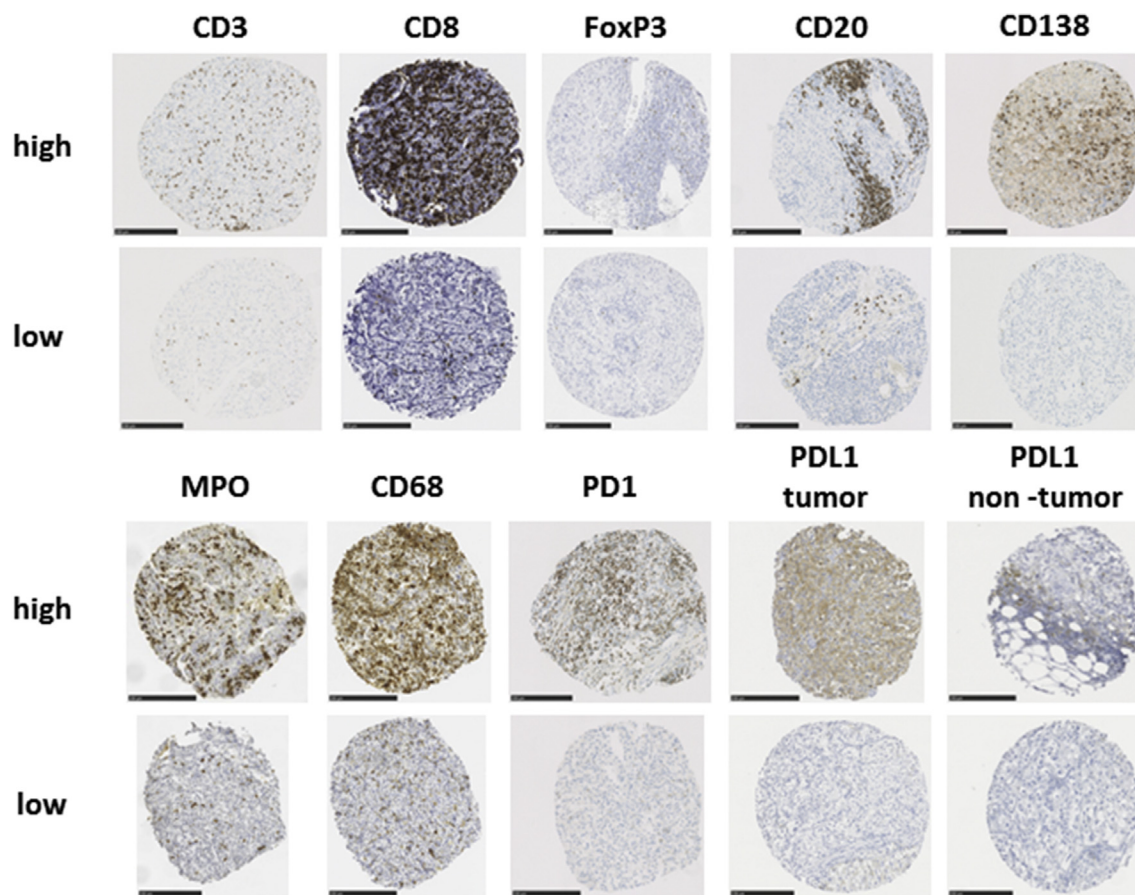
### Clinical Characteristics of the Patients and Immunohistochemistry

A TMA, containing ccRCC tissue of 756 patients, was immunohistochemically stained to examine TIMC and PD-1/PD-L1 in the TME. In total, 756 patients could be analyzed for at least one of the examined biomarkers. Clinical follow-up data and pathological tumor parameters are summarized in [Table 1](#). The median time of follow-up was 7.4 years (min 0 years, max 23.7 years, mean 7.8 years). By the end of follow-up, 248 patients (32.8%) had died from ccRCC. Among this subgroup, the median time of follow-up was 2.1 years (min 0.02 years, max 21.2 years, mean 3.5 years).

Immunohistochemical staining of CD3 was used as a general marker for T-cells, CD8 for CTLs, and FoxP3 for regulatory T-cells ( $T_{\text{reg}}$ ). CD20 was used as a marker for B-cells, CD56 for natural killer cells (NK cells), CD68 for tumor-associated macrophages (TAMs), CD138 for plasma cells, and MPO for granulocytes. All examined biomarkers could be detected in ccRCC tissue. CD3, CD8, CD20, MPO, FoxP3, and PD-1 stained immune cells only, whereas PD-L1 stained tumor cells and nontumor cells, e.g., blood vessels and leukocytes. CD56, CD68, and CD138 stained immune cells, and, in some cases, the tumor tissue showed a membranous positivity. As we aimed to characterize the TME, CD56-, CD68-, or CD138-positive tumor cells were excluded from further analysis. The amount of TIMC and of PD1- or PD-L1-positive cells varied greatly among the various tumors ([Figure 1](#)).

### Prognostic Impact of TIMC in ccRCC Patients

Cutoff values regarding the examined TIMC could be calculated using the Charité Cutoff finder algorithm ([Supplemental Table 3](#)). The patient collective could accordingly be regrouped into tumors with low (immune cell content below calculated cutoff) and high (immune cell content above calculated cutoff) TIMC. Univariate survival analysis with respect to cancer-specific survival (CSS) was performed ([Figure 2A](#)): poor grading (HR 4.37; CI 3.36–5.68;  $p < 0.001$ ), advanced tumor size (HR 4.47; CI 3.44–5.82;  $p < 0.001$ ), positive lymph node (HR 5.37; CI 3.79–7.6;  $p < 0.001$ ), or distant metastases (HR 11.1; CI 8.49–14.5;  $p < 0.001$ ) as well as Eastern Cooperative Oncology Group (ECOG) performance status  $>0$  (HR 1.99; CI 1.55–2.56;  $p < 0.001$ ), a high infiltration of CTLs (HR 1.33; CI 1–1.77;  $p = 0.04$ ) ([Figure 3A](#)), granulocytes (HR 2.29; CI 1.61–3.26;  $p < 0.001$ ) ([Supplemental Figure S1A](#)), and TAMs (HR 1.77; CI 1.1–2.84;  $p = 0.02$ ) ([Supplemental Figure S2A](#)) were significantly associated with poor CSS. On the contrary, female sex (HR 0.672; CI



**Figure 1.** Immunohistochemical staining of TIMC and PD-1/PD-L1: examples are given for high- and low-infiltration/expression for every examined marker.

0.515–0.876;  $p = 0.003$ ) and a high infiltration of B-cells (HR 0.375; CI 0.266–0.528;  $p < 0.001$ ) (Figure 3B) and CD3-positive T-cells (HR 0.621; CI 0.421–0.916;  $p = 0.01$ ) (Supplemental Figure S3A) were significantly associated with favorable CSS.  $T_{reg}$  (HR 1.45; CI 0.963–2.18;  $p = 0.07$ ) (Supplemental Figure S4A) and plasma cells (HR 1.96; CI 0.97–3.98;  $p = 0.06$ ) (Supplemental Figure S5A) showed no significant association with CSS. In preliminary studies CD56-positive tumor-infiltrating immune cells could only be detected in 1 of 100 examined tumors.

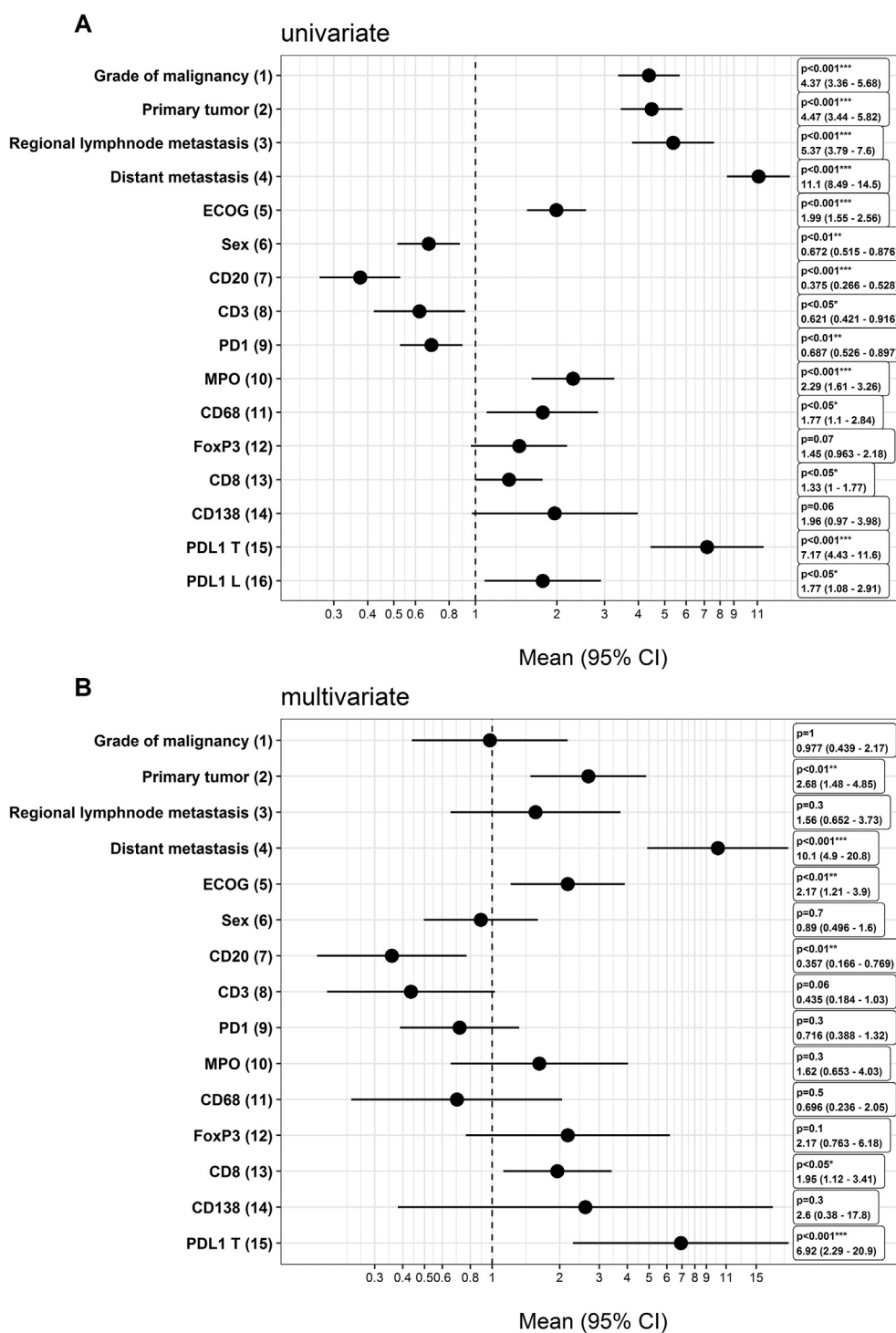
For further analysis, a multivariate analysis including grading, tumor size, lymph node and distant metastases, ECOG status, and sex, as well as all examined biomarkers was performed ( $n = 200$  patients) (Figure 2B): Tumor size (HR 2.68; CI 1.48–4.85;  $p < 0.01$ ), positive distant metastasis (HR 10.1; CI 4.9–20.8;  $p < 0.001$ ), ECOG status  $>0$  (HR 2.17; CI 1.21–3.9;  $p < 0.01$ ), and a high infiltration of CTLs (HR 1.95; CI 1.12–3.41;  $p < 0.05$ ) stayed significantly associated with poor CSS, whereas a high B-cell infiltration (HR 0.357; CI 0.166–0.769;  $p < 0.01$ ) was still significantly associated with favorable CSS. Thus, positive distant metastasis and a high infiltration of CTLs and B-cells in ccRCC were independent prognostic factors in this analysis. Grading (HR 0.977; CI 0.439–2.17;  $p = 1$ ), regional lymph node metastasis (HR 1.56; CI 0.652–3.73;  $p = 0.3$ ), sex (HR 0.89; CI 0.496–1.6;  $p = 0.7$ ), as well as CD3-positive T-cells (HR 0.435; CI 0.184–1.03;  $p = 0.06$ ), granulocytes (HR 1.62; CI 0.653–4.03;  $p = 0.3$ ), TAMs (HR 0.696; CI 0.236–2.05;  $p = 0.5$ ),  $T_{reg}$  (HR 2.17; CI 0.763–6.18;

$p = 0.1$ ), or plasma cells (HR 2.6; CI 0.38–17.8;  $p = 0.3$ ) showed no significant association in the multivariate analysis.

Moreover, comparison with pathological parameters showed that tumors with a high infiltration of CTLs had a significantly increased proportionate fraction in the groups of tumors with poor grading and advanced tumor size or positive lymph node or distant metastases compared with tumors with low infiltration (Figure 3C). The same, but consequently vice versa, was true for B-cells (Figure 3D). These analyses for granulocytes (Supplemental Figure S1B), TAMs (Supplemental Figure S2B), CD3-positive T-cells (Supplemental Figure S3B),  $T_{reg}$  (Supplemental Figure S4B), and plasma cells (Supplemental Figure S5B) are depicted in the supplements.

#### **Prognostic Impact of the Immune Checkpoint Molecules PD-1 and PD-L1 in ccRCC Patients**

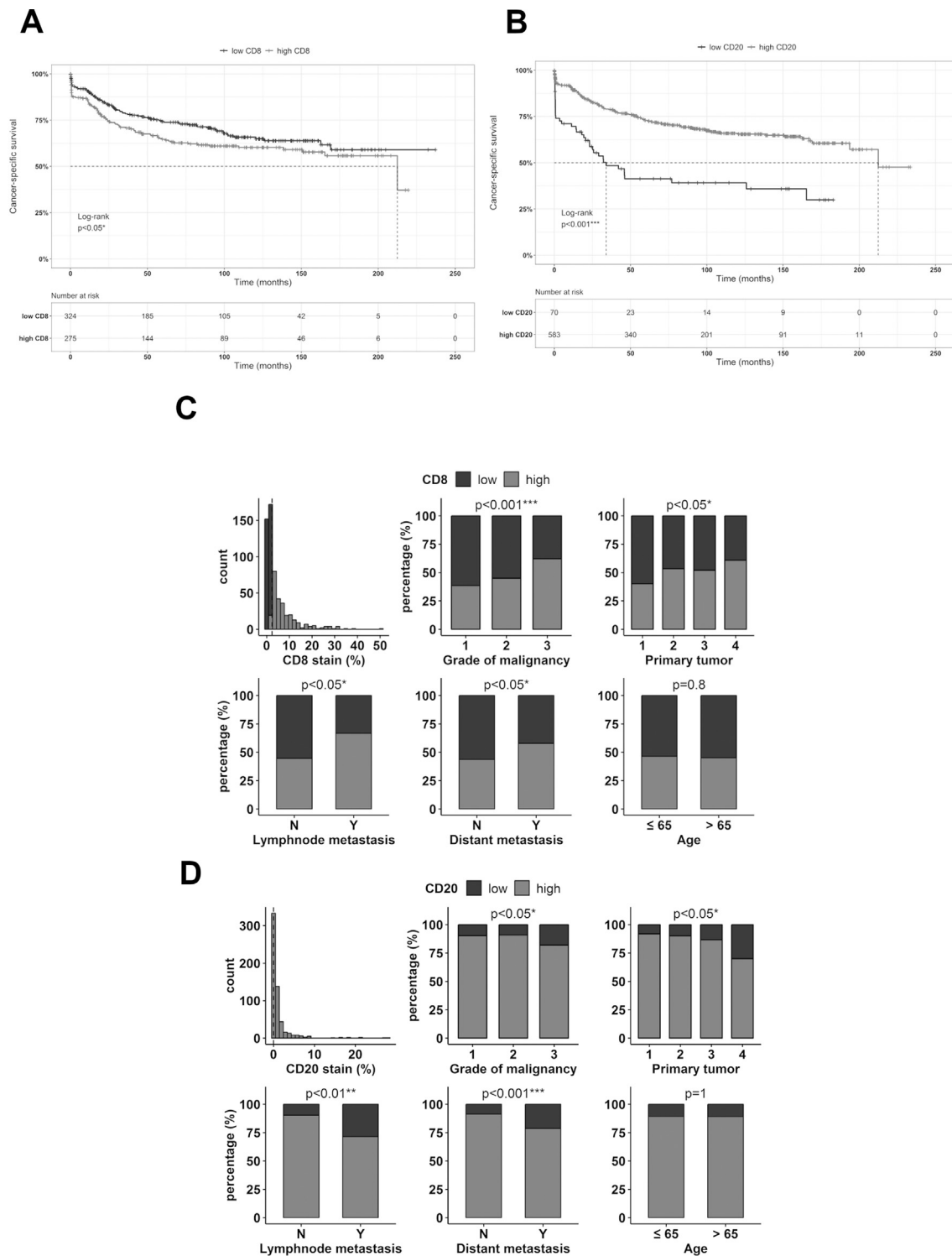
Cutoff values regarding PD-1 and PD-L1 could be calculated using the Charité Cutoff finder algorithm (Supplemental Table 3). The patient collective could accordingly be regrouped into tumors with low and high amount of PD-1 positive TIMC and PD-L1-positive tumor cells or nontumor cells, respectively. 441 (95.2%) tumors were either negative for PD-L1 or had a PD-L1 positive tumor cell portion below the cutoff. 22 (4.8%) tumors had a PD-L1 positivity above the cutoff. A high expression of PD-L1 in tumor cells (HR 7.17; CI 4.43–11.6;  $p < 0.001$ ) and nontumor cells (HR 1.77; CI 1.08–2.91;  $p = 0.023$ ) was significantly associated with poor CSS (Figure 2A). Kaplan–Meier analysis for



**Figure 2.** Uni- (A) and multivariate (B) analysis of prognostic factor influencing cancer-specific survival in ccRCC [1]; G3 vs. G1/G2 [2]; pT3/pT4 vs. pT1/pT2 [3]; pN1/pN2 vs. N0/pN0 [4]; M1 (synchronous distant metastasis) vs. M0 (no synchronous distant metastasis) [5];  $\geq 1$  vs. 0 [6]; female vs. male [7];  $>0.02\%$  vs.  $<0.02\%$  [8];  $>3.13\%$  vs.  $<3.13\%$  [9];  $>0.14\%$  vs.  $<0.14\%$  [10];  $>7.22\%$  vs.  $<7.22\%$  [11];  $>35.86\%$  vs.  $<35.86\%$  [12];  $>1.57\%$  vs.  $<1.57\%$  [13];  $>2.42\%$  vs.  $<2.42\%$  [14];  $>2.25\%$  vs.  $<2.25\%$  [15];  $>3.92\%$  vs.  $<3.92\%$  [16];  $>0.29\%$  vs.  $<0.29\%$ .

PD-L1 expression of tumor cells is depicted in Figure 4A. Further analyses on the influence of PD-L1 status on CSS after dichotomization of the patient collective regarding synchronous distant metastasis (negative vs. positive), tumor size (pT1/2 vs. pT3/4), or grading (G1/2 vs. G3) were performed: Strikingly, patients with synchronous distant metastases and high PD-L1 expression of tumor cells showed a significantly worse CSS in comparison to

synchronously metastasized patients with low PD-L1 expression (Figure 4B). PD-L1 expression was also able to significantly discriminate CSS with regard to tumor size (Figure 4C) and grading (Figure 4D). In multivariate analysis after adjustment for all other examined parameters and immune cells, high PD-L1 expression of tumor cells (HR 6.92; CI 2.29–20.9;  $p < 0.001$ ) stayed significantly associated with poor prognosis, thus being an



**Figure 3.** Analysis of cancer-specific survival in ccRCC: Association between survival times and tumor-infiltrating cytotoxic T-cells (A) or B-cells (B), represented by Kaplan–Meier plots. Relative distribution of tumor-infiltrating cytotoxic T-cells (C) or B-cells (D) in ccRCC and comparison with clinical and pathological features.

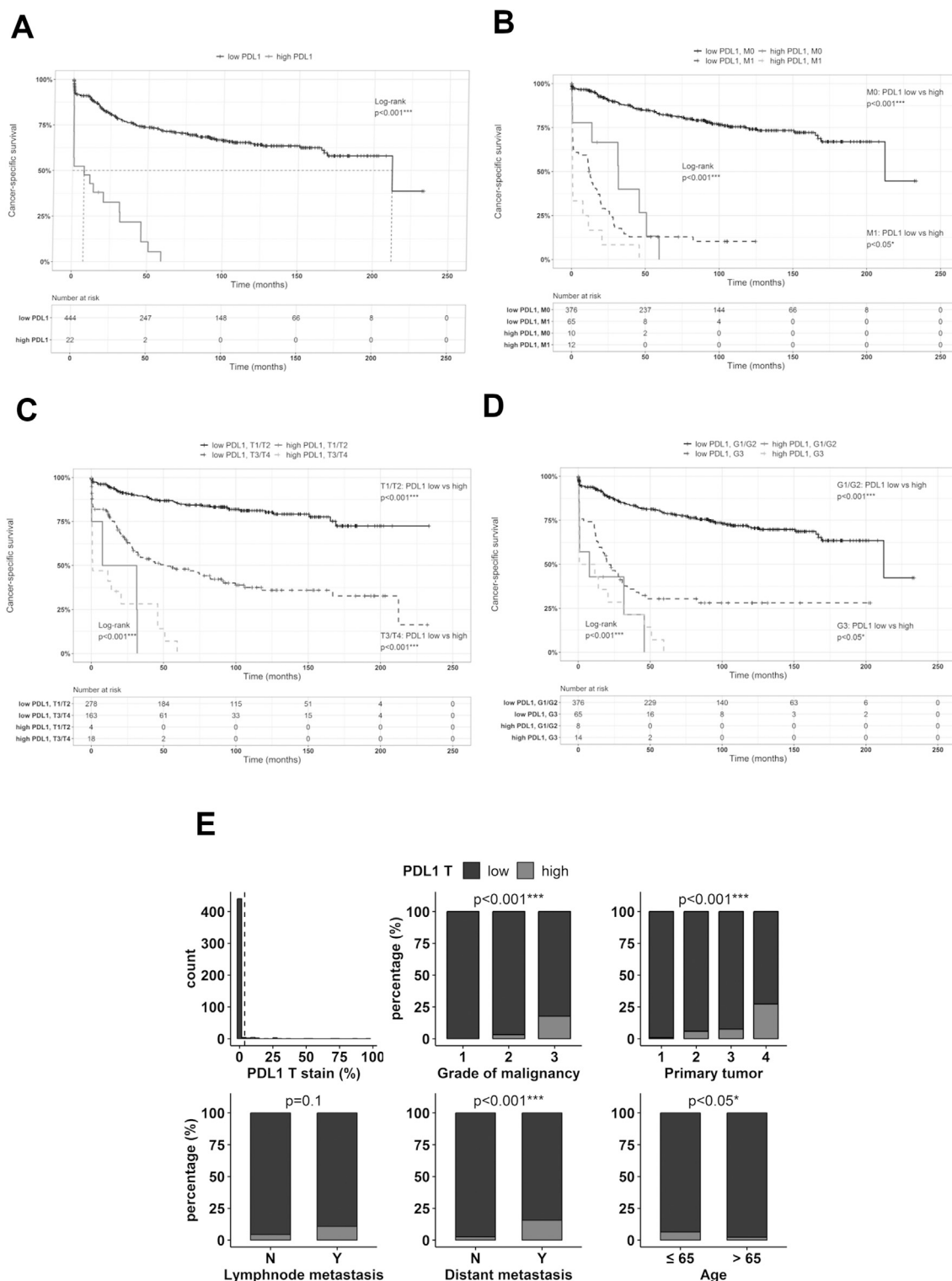
independent prognostic factor in ccRCC (Figure 2B). Furthermore, PD-L1 status was significantly connected with tumor grading and size, distant metastasis, and patient age (Figure 4E).

Tumors with high amount of PD-1-positive immune cells showed a significant association with favorable CSS in univariate analysis (HR 0.687; CI 0.526–0.897;  $p < 0.01$ ) (Supplemental Figure S6A) but not in multivariate analysis (HR 0.716; CI 0.388–1.32;  $p = 0.3$ )

(Figure 2B). Correlation with pathological parameters was not significant (Supplemental Figure S6B).

**Predictive Value of TIMC and PD-1/PD-L1 Regarding Nivolumab Therapy**

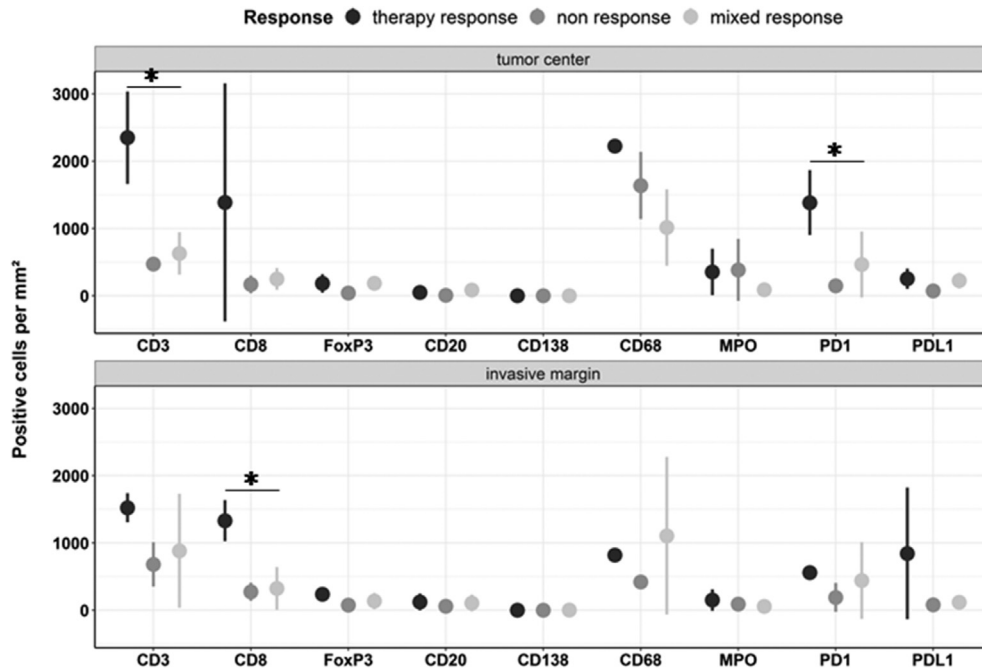
To investigate whether TIMC may predict treatment with nivolumab, RCC tissues of eight patients adjuvantly treated with



**Figure 4.** Analysis of cancer-specific survival in ccRCC: (A) Association between survival times and PD-L1 expression of tumor cells, represented by Kaplan–Meier plots. Subset analysis: (B) localized (M0: no synchronous distant metastasis) vs. distant disease (M1: synchronous distant metastasis), (C) advanced vs. restricted tumor extent, (D) high-grade vs. low-grade ccRCC. (E) Relative distribution of PD-L1 expression of ccRCC, broken line represents cutoff value (3.92%), and comparison with clinical and pathological features.

nivolumab (mean therapy duration 8.2 months, median therapy duration 4 months) were selected. Two patients (25%) showed a stable disease under nivolumab treatment (responders), two patients (25%), a mixed response, and four patients (50%), a progressive

disease (nonresponders) (Supplemental Table 1). In the responders group, density of CD3-positive T-cells in the tumor center ( $2349.5 \text{ cells/mm}^2 \pm 685.9$ ) was significantly higher compared with that of nonresponders ( $471.6 \text{ cells/mm}^2 \pm 104.8$ ) and mixed



**Figure 5.** Comparison of density of tumor-infiltrating mononuclear cells (TIMCs) and PD-1/PD-L1 between tumor with response, mixed response, or no response to nivolumab: TIMC and PD-1/PD-L1 were examined in the tumor center and in the invasive margin. Data are given as mean  $\pm$  standard deviation. \*:  $p < 0.05$ .

responders ( $628.4 \text{ cells/mm}^2 \pm 314.7$ ) ( $p = 0.003$ ) (Figure 5). The same was true for PD-1-positive TIMC in the tumor center (responders:  $1383.6 \text{ cells/mm}^2 \pm 482.7$ ; nonresponders:  $146.9 \text{ cells/mm}^2 \pm 100.2$ ; mixed responders:  $463.9 \text{ cells/mm}^2 \pm 490.7$ ;  $p = 0.01$ ) (Figure 5) and CTLs in the invasive margin (responders:  $1330.5 \text{ cells/mm}^2 \pm 305.3$ ; nonresponders:  $274.3 \text{ cells/mm}^2 \pm 134.8$ ; mixed responders:  $322.9 \text{ cells/mm}^2 \pm 317.4$ ;  $p < 0.01$ ) (Figure 4B). Density of PD-L1-positive cells in the invasive margin of tumors with response to nivolumab therapy ( $843.9 \text{ cells/mm}^2 \pm 977.7$ ) was by tendency higher compared with nonresponders ( $80.4 \text{ cells/mm}^2 \pm 76.6$ ) or mixed responders ( $118.4 \text{ cells/mm}^2 \pm 95.6$ ) ( $p = 0.2$ ) (Figure 5).  $T_{reg}$ , B-cells, plasma cells, TAMs, and granulocytes showed no significant differences.

**Discussion**

This study aimed to comprehensively investigate the immune cells in the TME of ccRCC in a large patient cohort regarding patients' prognosis and prediction of response to ICI using digital image analysis. Tumor-infiltrating B-cells and CTLs as well as PD-L1 expression of tumor cells were identified as independent prognostic markers for CSS. Despite the small size of the study cohort, patients with response to treatment with nivolumab had a significantly higher amount of tumor-infiltrating CD3-positive T-cells, CTLs, and PD-1 positive TIMCs than patients with mixed or no response.

PD-L1 has previously been studied intensively in both ccRCC and non-ccRCC. While PD-L1 expression of tumor cells seems not to be of prognostic value in non-ccRCC, a high PD-L1 expression in ccRCC provides a strong negative prognostic value regarding patients' survival [31,32]. However, when investigating PD-L1 expression of tumor cells, composition and quantity of tumor-infiltrating immune cells are relevant, because tumor cells upregulate PD-L1 in response to T-cell-produced interferon  $\gamma$  (IFN $\gamma$ ) [33]. Thus, we wanted to analyze if PD-L1 expression of tumor cells still holds predictive value

under simultaneous consideration of TIMC and well-established prognostic factors such as tumor grading and size, lymph node, and synchronous distant metastasis. A high expression of PD-L1 of tumor cells was associated with a more aggressive tumor phenotype and proved to be an independent prognostic factor for poor CSS. These findings emphasize the prognostic value of PD-L1 in ccRCC and support its role in the immune escape. Thus, PD-L1 expression of tumor cells provides valuable prognostic information and should be determined in diagnostic routine in addition to the other prognostic parameters. Our data on a limited number of patients with nivolumab therapy show a tendency of higher PD-L1 expression in the responders' group. PD-L1 assessment is so far not required for initiation of therapy with ICI for patients with ccRCC. This might also change in the future because of ongoing clinical investigations [34]. PD-L1 alone, although of strong prognostic value, would not be sufficient to collect all the prognostic and predictive information the immune cells in the TME provide. The relationship between TIMC and patient's prognosis has been investigated for many tumor entities: The immunoscore for colorectal cancer (CRC) is only one outstanding example [35]. Furthermore, TIMC have been proposed as one component of CRC classification with potential impact on therapy [36]. Application of the immunoscore for CRC in pancreatic cancer showed that a high immunoscore was associated with improved survival [37]. There are similar attempts for other cancer entities, e.g., lung or gastric cancer, to implement the TME in the tumor staging system [38,39].

Our data show that tumor-infiltrating CTLs and B-cells are independent prognostic markers for patients with ccRCC. CTLs are involved in elimination of tumor cells and thus a high CTL infiltration is considered to be associated with good prognosis. In RCC, however, a high infiltration of CTLs is connected with unfavorable outcome [40,41]. The same is also true for lung metastases of RCC [42]. Strikingly, CTL density was significantly



higher in tumors with response to nivolumab, thus quantification of tumor-infiltrating CTLs could help identify the patients with ccRCC and a higher risk for adverse events and at the same a better chance of response to treatment with ICI. Additional prognostic and predictive information can be obtained, if not only quantity of CD8-positive CTL is examined but also presence of the immune checkpoint receptors PD-1, lymphocyte activation gene 3 (LAG3), and T-cell immunoglobulin 3 on the cell surface [43,44]. A high amount of tumor-infiltrating B-cells was an independent prognostic marker for a good prognosis in this study. Tumor-infiltrating B-cells can act antitumorogenic through production of antibodies and cytokines as well as antigen presentation. Certain subtypes however, especially regulatory B-cells ( $B_{reg}$ ), can suppress T-cell function and impair elimination of tumor cells [45]. Investigation on tumor-infiltrating B-cells in 361 cases of RCC, including all histologic subtypes, found an association of high B-cell infiltration with poor overall survival [46]. A different composition of B-cell subtypes in the study cohorts could explain this discrepancy. Thus, future research should focus on the B-cell activation status or subtypes, because this could yield new insights in how B-cells affect antitumor immunity. Our analysis of  $T_{reg}$  showed a nonsignificant association of high  $T_{reg}$  infiltration with poorer survival. They seem not to possess great predictive value, because there were no differences in  $T_{reg}$  density between nivolumab therapy responders and nonresponders. Nonetheless, one should keep in mind that  $T_{reg}$  can also express PD-1 and therefore the tumor-supporting effects are enhanced by nivolumab. A high infiltration of PD-1 positive tumor-infiltrating immune cells was a dependent prognostic marker for favorable patients' survival in ccRCC. The data in the literature however are heterogenous, showing either a correlation of high infiltration of PD-1 positive TIL with reduced CSS or no correlation with survival in RCC [47]. PD-1 is considered to be a marker of exhausted T-cells, often leading to the assumption that a high expression on TIL is adverse regarding prognosis. But PD-1 is also upregulated during physiologic activation of T-cells preventing an unbalanced immune response [48]. Still, PD-1 positive cell density was higher in patients with response to treatment with ICI. Moreover, PD-1 is also expressed on other immune cells than T cells [48]. Thus, PD-1 is a marker for a heterogenous group of immune cells with a wide range of activation. In accordance with our data, PD-1 seems to select tumors with a high amount of TIL which comes along with a high antitumor immune response and consequently favorable CSS. TAM contributes essentially to the pathogenesis of ccRCC [49]. Our analyses show that a high amount of TAM in ccRCC is associated with poor prognosis compared with patients with a low amount. Examining CD68 only, a common marker for macrophages, is a relatively rough approach: TAM exhibits different profiles, thereby either inhibiting (M1 polarization) or promoting (M2 polarization) tumor growth. The association of TAM with poor prognosis is suggestive for a predominance of M2-polarized TAM in the TME. There was a trend for a higher density of TAM in the invasive margin of tumors with no response to treatment with ICI, whereas the density was higher in the center of tumors with response to treatment with ICI: Presumably, there is a regional distribution of M1-polarized TAM in the tumor center and M2-polarized TAM in the invasive margin. Tumor-associated neutrophils (TANs) can promote tumor growth and the development of metastasis [50]. A higher amount of TAN was found in patients with advanced or metastasized RCC compared with patients with localized disease [51]. Also, any presence of TAN in

localized RCC was an independent prognostic marker for shorter recurrence-free survival, CSS, and overall survival [52]. Thus, our data support the tumor-promoting effects of TAN in ccRCC. Our preliminary data showed a low frequency of tumor-infiltrating CD56-positive NK cells, thus we resigned on further investigation. RCC tumors are however infiltrated by NK cells with lytic effects on tumor cells [53,54].

Taken together, we could show that the immune cells in the TME and PD-L1/PD-1 qualify as prognostic and predictive biomarkers for patients with ccRCC. In the future, comprehensive analysis of the immune cells in the TME could lead to an immunoscore for ccRCC with impact on therapeutical decisions.

### Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

### Acknowledgments

We thank Jutta Richter, Silke Mitschke, and Bonny Adami for excellent technical assistance. This project was supported by the Tissue Bank of the University Medical Center Mainz and the Tissue Bank of the National Center for Tumor Diseases Heidelberg.

### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.tranon.2019.11.002>.

### References

- Montie JE, Stewart BH, Straffon RA, Banowsky LH, Hewitt CB and Montague DK (1977). The role of adjunctive nephrectomy in patients with metastatic renal cell carcinoma. *J Urol* **117**(3), 272–275.
- Dekernion JB, Ramming KP and Smith RB (1978). The natural history of metastatic renal cell carcinoma: a computer analysis. *J Urol* **120**(2), 148–152.
- Marcus SG, Choyke PL, Reiter R, Jaffe GS, Alexander RB and Linehan WM, et al (1993). Regression of metastatic renal cell carcinoma after cytoreductive nephrectomy. *J Urol* **150**(2), 463–466.
- Raman R and Vaena D (2015). Immunotherapy in metastatic renal cell carcinoma: a comprehensive review. *BioMed Res Int* 2015. Article ID:1–8.
- Mazza C, Escudier B and Albiges L (2017). Nivolumab in renal cell carcinoma: latest evidence and clinical potential. *Ther Adv Med Oncol* **9**(3), 171–181.
- Itsumi M and Tatsugami K (2010). Immunotherapy for renal cell carcinoma. *Clin Dev Immunol* 2010, 284581.
- Biswas S and Eisen T (2009). Immunotherapeutic strategies in kidney cancer – when TKIs are not enough. *Nat Rev Clin Oncol* **6**, 478–487.
- Rosenberg SA, Yang JC, Topalian SL, Schwartzentruber DJ, Weber JS and Parkinson DR, et al (1994). Treatment of 283 consecutive patients with metastatic melanoma or renal cell cancer using high-dose bolus interleukin 2. *J Am Med Assoc* **271**(12), 907–913.
- Fyfe BG, Fisher RI, Rosenberg SA, Sznol M, Parkinson DR and Louie AC (1995). Results of treatment of 255 patients with metastatic renal cell carcinoma who received high-dose recombinant interleukin-2 therapy. *J Clin Oncol* **13**(3), 688–696.
- Amin A and White RL (2014). Interleukin-2 in renal cell carcinoma: a has-been or a still-viable option? *J Kidney Cancer VHL* **1**(7), 74–83.
- Lalani AKA, McGregor BA, Albiges L, Choueiri TK, Motzer R and Powles T, et al (2019). Systemic treatment of metastatic clear cell renal cell carcinoma in 2018: current paradigms, use of immunotherapy, and future directions. *Eur Urol* **75**(1), 100–110.
- Rini BI, McDermott DF, Hammers H, Bro W, Bukowski RM and Faba B, et al (2016). Society for Immunotherapy of Cancer consensus statement on immunotherapy for the treatment of renal cell carcinoma. *J Immunother Cancer* **4**(81), 1–15.
- Motzer RJ, Hutson TE, Tomczak P, Michaelson MD, Bukowski RM and Rixe O, et al (2007). Sunitinib versus interferon Alfa in metastatic renal-cell carcinoma. *N Engl J Med* **356**(2), 115–124.
- Bedke J, Stähler V, Stenzl A and Brehmer B (2017). Immunotherapy for kidney cancer. *Curr Opin Urol* **28**(1), 8–14.

- [15] Flippot R, Escudier B and Albiges L (2018). Immune checkpoint inhibitors: toward new paradigms in renal cell carcinoma. *Drugs* **78**, 1443–1457.
- [16] Topalian S, Hodi F, Brahmer J, Gettinger S, Smith D and McDermott D, et al (2012). Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med* **366**(26), 2443–2454.
- [17] Motzer RJ, Tannir NM, McDermott DF, Arén Frontera O, Melichar B and Choueiri TK, et al (2018). Nivolumab plus ipilimumab versus sunitinib in advanced renal-cell carcinoma. *N Engl J Med* **378**(14), 1277–1290.
- [18] Grigg C and Rizvi NA (2016). PD-L1 biomarker testing for non-small cell lung cancer: truth or fiction? *J Immunother Cancer* **4**(48), 1–10.
- [19] McDermott DF, Huseni MA, Atkins MB, Motzer RJ, Rini BI and Escudier B, et al (2018). Clinical activity and molecular correlates of response to atezolizumab alone or in combination with bevacizumab versus sunitinib in renal cell carcinoma. *Nat Med* **24**(6), 749–757.
- [20] Mouw KW, Goldberg MS, Konstantinopoulos PA and D'Andrea AD (2017). DNA damage and repair biomarkers of immunotherapy response. *Cancer Discov* **7**(7), 675–693.
- [21] Goodman AM, Kato S, Bazhenova L, Patel SP, Frampton M and Miller V, et al (2017). Tumor mutational burden as an independent predictor of response to immunotherapy in diverse cancers. *Mol Cancer Ther* **16**(11), 2598–2608.
- [22] Boyiadzis MM, Kirkwood JM, Marshall JL, Pritchard CC, Azad NS and Gully JL (2018). Significance and implications of FDA approval of pembrolizumab for biomarker-defined disease. *J Immunother Cancer* **6**(35), 1–7.
- [23] Cristescu R, Mogg R, Ayers M, Albright A, Murphy E and Yearley J, et al (2018). Pan-tumor genomic biomarkers for PD-1 checkpoint blockade-based immunotherapy. *Science* **362**(6411), eaar3593.
- [24] Fehrenbacher L, Spira A, Ballinger M, Kowanzet M, Vansteenkiste J and Mazieres J, et al (2016). Atezolizumab versus docetaxel for patients with previously treated non-small-cell lung cancer (POPLAR): a multicentre, open-label, phase 2 randomised controlled trial. *Lancet* **387**, 1837–1846.
- [25] Rosenberg JE, Hoffman-Censits J, Powles T, Van Der Heijden MS, Balar AV and Necchi A, et al (2016). Atezolizumab in patients with locally advanced and metastatic urothelial carcinoma who have progressed following treatment with platinum-based chemotherapy: a single-arm, multicentre, phase 2 trial. *Lancet* **387**, 1909–1920.
- [26] Macher-Goeppinger S, Aulmann S, Wagener N, Funke B, Tagscherer KE and Haferkamp A, et al (2008). Decoy receptor 3 is a prognostic factor in renal cell cancer. *Neoplasia* **10**(10), 1049–1056.
- [27] Sobin L, Gospodarowicz M and Wittekind C (2009). TNM classification of malignant tumours. 7th ed. 2009.
- [28] Macher-Goeppinger S, Aulmann S, Tagscherer KE, Wagener N, Haferkamp A and Penzel R, et al (2009). Prognostic value of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and TRAIL receptors in renal cell cancer. *Clin Cancer Res* **15**(2), 650–659.
- [29] Foersch S, Schindeldecker M, Keith M, Tagscherer KE, Fernandez A and Stenzel PJ, et al (2017). Prognostic relevance of androgen receptor expression in renal cell carcinomas. *Oncotarget* **8**(45), 78545–78555.
- [30] Budczies J, Klauschen F, Sinn BV, Gyorffy B, Schmitt WD and Darb-Esfahani S, et al (2012). Cutoff finder: a comprehensive and straightforward web application enabling rapid biomarker cutoff optimization. *PLoS One* **7**(12), e51862.
- [31] Thompson RH, Kuntz SM, Leibovich BC, Dong H, Lohse CM and Webster WS, et al (2006). Tumor B7-H1 is associated with poor prognosis in renal cell carcinoma patients with long-term follow-up. *Cancer Res* **66**(7), 3381–3385.
- [32] Leite KRM, Reis ST, Junior JP, Zerati M, Gomes D and de O, et al (2015). PD-L1 expression in renal cell carcinoma clear cell type is related to unfavorable prognosis. *Diagn Pathol* **10**(189), 1–6.
- [33] Teng MWL, Ngiew SF, Ribas A and Smyth MJ (2015). Classifying cancers based on T-cell infiltration and PD-L1. *Cancer Res* **75**(11), 2139–2145.
- [34] Donskov F, McDermott D, Lee J, Szczylik C, Malik J and Alekseev BY, et al. *Ann Oncol* **29**(suppl\_8), viii303–viii331. <https://doi.org/10.1093/annonc/mdy283>.
- [35] Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B and Lagorce-Pages C, et al (2006). Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* **313**, 1960–1963.
- [36] Colangelo T, Polcaro G, Muccillo L, D'Agostino G, Rosato V and Ziccardi P, et al (2017). Friend or foe? The tumour microenvironment dilemma in colorectal cancer. *Biochim Biophys Acta Rev Cancer* **1867**(1), 1–18.
- [37] Tahkola K, Mecklin J-P, Wirta E-V, Ahtriainen M, Helminen O and Böhm J, et al (2018). High immune cell score predicts improved survival in pancreatic cancer. *Virchows Arch* **472**(4), 653–665.
- [38] Kurebayashi Y, Emoto K, Hayashi Y, Kamiyama I, Ohtsuka T and Asamura H, et al (2016). Comprehensive immune profiling of lung adenocarcinomas reveals four immunosubtypes with plasma cell subtype a negative indicator. *Cancer Immunol Res* **4**(3), 234–247.
- [39] Zeng D, Li M, Zhou R, Zhang J, Sun H and Shi M, et al (2019). Tumor microenvironment characterization in gastric cancer identifies prognostic and immunotherapeutically relevant gene signatures. *Cancer Immunol Res* **7**(5), 737–750.
- [40] Nakano O, Sato M, Naito Y, Suzuki K, Orikasa S and Aizawa M, et al (2001). Proliferative activity of intratumoral CD8+ T-lymphocytes as a prognostic factor in human renal cell carcinoma: clinicopathologic demonstration of antitumor immunity. *Cancer Res* **61**, 5132–5136.
- [41] Giraldo NA, Becht E, Pagès F, Skliris G, Verkarre V and Vano Y, et al (2015). Orchestration and prognostic significance of immune checkpoints in the microenvironment of primary and metastatic renal cell cancer. *Clin Cancer Res* **21**(13), 3031–3040.
- [42] Remark R, Alifano M, Cremer I, Lupo A, Dieu-Nosjean MC and Riquet M, et al (2013). Characteristics and clinical impacts of the immune environments in colorectal and renal cell carcinoma lung metastases: influence of tumor origin. *Clin Cancer Res* **19**(15), 4079–4091.
- [43] Granier C, Dariane C, Combe P, Verkarre V, Urien S and Badoual C, et al (2017). Tim-3 expression on tumor-infiltrating PD-1+CD8+ T cells correlates with poor clinical outcome in renal cell carcinoma. *Cancer Res* **77**(5), 1075–1082.
- [44] Pignon JC, Jegede O, Shukla SA, Braun DA, Horak CE and Wind-Rotolo M, et al (2019). irRECIST for the evaluation of candidate biomarkers of response to nivolumab in metastatic clear cell renal cell carcinoma: analysis of a phase II prospective clinical trial. *Clin Cancer Res* **25**(7), 2174–2184.
- [45] Shen M, Sun Q, Wang J, Pan W and Ren X (2016). Positive and negative functions of B lymphocytes in tumors. *Oncotarget* **7**(34), 55828–55839.
- [46] Sjöberg E, Frödin M, Lövrot J, Mezheyeuski A, Johansson M and Harmenberg U, et al (2018). A minority-group of renal cell cancer patients with high infiltration of CD20+B-cells is associated with poor prognosis. *Br J Cancer* **119**, 840–846.
- [47] Erlmeier F, Weichert W, Schrader AJ, Autenrieth M, Hartmann A and Steffens S, et al (2017). Prognostic impact of PD-1 and its ligands in renal cell carcinoma. *Med Oncol* **34**(99), 1–8.
- [48] Sharpe AH and Pauken KE (2018). The diverse functions of the PD1 inhibitory pathway. *Nat Rev Immunol* **18**, 153–167.
- [49] Kovaleva OV, Samoilova DV, Shitova MS and Gratchev A (2016). Tumor associated macrophages in kidney cancer. *Anal Cell Pathol* 2016. Article ID: 1–6.
- [50] Hurt B, Schulick R, Edil B, El Kasmí KC and Barnett C (2017). Cancer-promoting mechanisms of tumor-associated neutrophils. *Am J Surg* **214**, 938–944.
- [51] Minárik I, Laš'ovička J, Budinský V, Kayserová J, Špíšek R and Jarolím L, et al (2013). Regulatory T cells, dendritic cells and neutrophils in patients with renal cell carcinoma. *Immunol Lett* **152**, 144–150.
- [52] Jensen HK, Donskov F, Marcussen N, Nordmark M, Lundbeck F and Von Der Maase H (2009). Presence of intratumoral neutrophils is an independent prognostic factor in localized renal cell carcinoma. *J Clin Oncol* **27**(28), 4709–4717.
- [53] Schleypen JS, Von Geldern M, Weiß EH, Kotzias N, Rohrmann K and Schendel DJ, et al (2003). Renal cell carcinoma-infiltrating natural killer cells express differential repertoires of activating and inhibitory receptors and are inhibited by specific HLA class I allotypes. *Int J Cancer* **106**(6), 905–912.
- [54] Schleypen JS, Baur N, Kammerer R, Nelson PJ, Rohrmann K and Gröne EF, et al (2006). Cytotoxic markers and frequency predict functional capacity of natural killer cells infiltrating renal cell carcinoma. *Clin Cancer Res* **12**(3), 718–725.