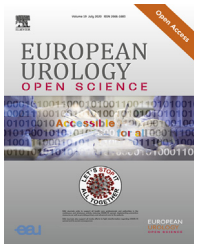
available at www.sciencedirect.com
journal homepage: www.eu-openscience.europeanurology.com



European Association of Urology

Kidney Cancer

Infiltration of M2 Macrophages and Regulatory T Cells Plays a Role in Recurrence of Renal Cell Carcinoma

Sabina Davidsson [a,*], *Michelangelo Fiorentino* [b], *Francesca Giunchi* [b], *Margareta Eriksson* [c], *Ann Erlandsson* [d], *Pernilla Sundqvist* [a,†], *Jessica Carlsson* [a,†]

[a] *Department of Urology, Faculty of Medicine and Health, Örebro University, Örebro, Sweden;* [b] *Department of Pathology, Maggiore Hospital and S. Orsola-Malpighi Hospital, University of Bologna, Bologna, Italy;* [c] *Department of Clinical Pathology and Cytology, Central Hospital Karlstad, Karlstad, Sweden;* [d] *Department of Environmental and Life Sciences/biology, Karlstad University, Karlstad, Sweden*

Article info

Article history:
Accepted June 11, 2020

Associate Editor: Axel Bex

Keywords:
Renal cell carcinoma
Immunohistochemistry
$CD163^+$ M2 macrophages
$FOXP3^+$ regulatory T cells

Abstract

Background: It has been hypothesized that M2 macrophages and regulatory T cells (Tregs) may contribute to tumor progression by suppression of antitumor immunity.

Objective: To investigate the association between infiltration of $CD163^+$ M2 macrophages and $CD4^+FOXP3^+$ Tregs with clinical outcomes in renal cell carcinoma patients.

Design, setting, and participants: A cohort of 346 patients diagnosed with renal cell carcinoma at Örebro University Hospital between 1986 and 2011 was evaluated for $CD163^+$ M2 macrophage and $CD4^+FOXP3^+$ Treg infiltration by immunohistochemistry.

Outcome measurements and statistical analysis: Associations between clinicopathological features and infiltration of $CD163^+$ M2 macrophages and/or $CD4^+FOXP3^+$ Tregs were estimated with chi-square or Fisher's exact tests. For survival analyses, Kaplan-Meier curves with log-rank tests and multivariate Cox proportional hazards regression models were used.

Results and limitations: We found that infiltration of $CD163^+$ M2 macrophages and $CD4^+FOXP3^+$ Tregs were associated with adverse clinical outcomes. Our data further demonstrate that $CD163^+$ M2 macrophages and $CD4^+FOXP3^+$ Tregs colocalize in tumor and normal tissue, and that this colocalization may have synergistic effects on tumor aggressiveness. The use of tissue microarrays rather than whole sections may be viewed as a limitation.

Conclusions: Infiltration of $CD163^+$ M2 macrophages and $CD4^+FOXP3^+$ Tregs is associated with recurrence of renal cell carcinoma, and colocalization of these cell types may have an association with clinical outcome.

Patient summary: The aim of this study was to investigate the association between infiltration of M2 macrophages and regulatory T cells with clinical outcomes in renal cell carcinoma. We demonstrated that renal cell carcinoma patients with high infiltration of both these cell types are at an increased risk of poor clinical outcomes.

† These authors contributed equally to this work.
* Corresponding author. Department of Urology, Örebro University Hospital, SE-701 85 Örebro, Sweden. Tel.: +46 196026604; Fax: +46 196026650.
E-mail address: sabina.davidsson@regionorebrolan.se (S. Davidsson).

http://dx.doi.org/10.1016/j.euros.2020.06.003

1. Introduction

One of the hallmarks of cancer is the capability of tumor cells to evade immune destruction, and one mechanism utilized by tumors is to recruit immune suppressive cells, such as M2 macrophages and regulatory T cells (Tregs), into the microenvironment [1].

Macrophages are the most abundant innate immune cells within the microenvironment of most tumors. Depending on the micro milieu, macrophages can acquire distinct subtypes, either pro- or anti-inflammatory [2]. M1 macrophages are immunostimulatory and antitumoral, while M2 macrophages are immunosuppressive and contributes to a protumoral environment [3]. High infiltration of $CD163^+$ M2 macrophages has been associated with worse clinical outcome in a number of malignancies including renal cell carcinoma (RCC) [4–8].

It has been suggested that Tregs are a key player in antitumor immune suppression and thereby promote tumor progression [9,10]. The most specific marker to identify Tregs is the transcription factor FOXP3 [11]. The prognostic value of intratumoral $FOXP3^+$ Tregs has intensively been evaluated previously, and positive associations were found for a number of different malignancies [12–14]. An association between infiltration of $FOXP3^+$ Tregs and worse clinical outcome has also been reported in the majority of studies investigating RCC patients [15–17]. However, conflicting data exist [18].

In the majority of studies evaluating the clinical impact of M2 macrophages and Tregs in cancer progression, the prognostic value has been assessed based on separate infiltration of either $CD163^+$ M2 macrophages or $FOXP3^+$ Tregs. The correlation between the synergistic effect and the clinical outcome has been reported only for a limited number of malignancies. Recently, Sun et al [19] found that laryngeal squamous cell carcinoma patients with a high number of $CD163^+$ M2 macrophages and $FOXP3^+$ Tregs have shorter overall survival than patients with low infiltration. To our knowledge, no previous study has evaluated the prognostic value and synergistic effect on clinical outcome of simultaneous infiltration of $CD163^+$ M2 macrophages and $FOXP3^+$ Tregs into tumor and tumor-adjacent normal tissue in RCC.

The aim of this study was to evaluate the prognostic value of separate infiltration and colocalized infiltration of $CD163^+$ M2 macrophages and $CD4^+FOXP3^+$ Tregs for disease recurrence and cancer-specific death.

2. Patients and methods

2.1. Case and tissue collection

The present study includes 346 patients diagnosed with RCC and treated with radical nephrectomy ($n = 303$) or nephron-sparing surgery ($n = 43$) at the Örebro University Hospital, Örebro, Sweden, between January 1986 and December 2011. The study cohort was followed for cancer-specific and all-cause mortality until December 2015. The cohort has previously been described by Grabowska et al [20].

Formalin-fixed, paraffin-embedded (FFPE) blocks and corresponding hematoxylin and eosin (H&E) slides from all cases were retrieved and re-reviewed by two experienced genitourinary pathologists (M.F. and F. G.). The pathologists re-evaluated tumor specimens based on the tumor, node, metastasis (TNM) classification according to American Joint Committee on Cancer (AJCC) 2010 eighth edition. The histological subtype of RCC was classified according to the 2008 World Health Organization (WHO) tumor classification. Moreover, each tumor was graded according to the WHO nucleolar grading system. In addition, the pathologists circled tumor and tumor-adjacent normal areas on H&E slides corresponding to the FFPE blocks.

This study was approved by the regional ethics review board (ethical approval number 2010/135 and 2015/353).

2.2. Immunohistochemistry

Tissue microarrays (TMAs) were constructed previously for this cohort, including three tissue cores with a diameter of 0.6 mm from each tumor and tumor-adjacent normal area. Four-micrometer sections were used for immunohistochemistry. The anti-CD163 antibody (Leika Biosystems, USA) were used to identify M2 macrophages and anti-CD4 and anti-FOXP3 antibodies (Agilent Dako, USA, and Thermo Fisher Scientific, USA) to identify Tregs. Immunohistochemical staining of CD163 and CD4/FOXP3 was performed on Autostainer Link 48 (Agilent Dako). The optimal conditions for the primary mouse monoclonal antibody anti-CD163 (clone 10D6) were to perform antigen retrieval by steam heat at 97 °C with HIER FLEX TRS low buffer, Envision Flex Target Retrieval Solution low pH, for 20 min (K805; Agilent Dako). The slides were then incubated with the antibody at 1:200 dilution for 30 min followed by visualization with an amplification system including EnVision FLEX/rabbit linker, EnVision FLEX/HRP, and FLEX DAB Sub-Chromogen (Agilent Dako).

The optimal conditions for the primary antibodies used for double staining, mouse monoclonal antibody anti-CD4 (clone 4B12, RTU) and mouse monoclonal antibody anti-FOXP3 (clone 236A/E7), were to perform antigen retrieval by steam heat at 97 °C with Envision Flex Target retrieval solution for 20 min at pH 9 (Dako K8004). The slides were then incubated with the anti-CD4 antibody followed by the anti-FOXP3 antibody diluted 1:25. Revelation was performed with an amplification system including EnVision FLEX/rabbit linker, EnVision FLEX/HRP, and FLEX DAB Sub-Chromogen (Agilent Dako) for CD4 and Vina Green Chromogen (Biocare, USA) for FOXP3. All slides were counterstained with Mayer's hematoxylin and mounted using Tissue-Tek coverslipping film (Sakura Finetek, USA). Tissue from tonsils was used as a positive control. The Panoramic 250 Flash II system (3DHISTECH, Hungary) was used to convert all CD163-, CD4-, and FOXP3-stained slides into high-resolution digital slides using the software Case viewer version 2.1 (3DHISTEC).

2.3. Evaluation of $CD163$ and $CD4^+Foxp3^+$ expression

We quantified M2 macrophages by CD163 expression and Tregs by simultaneous CD4 and FOXP3 expression. All Tregs cells were counted. For M2 macrophages, up to 200 cells were counted; a greater number of positive cells were recorded in a single category as >200. The observers (S.D. and A.E.) were blinded to the clinical data. The median number of $CD163^+$ M2 macrophages for the three cores was calculated. Patients with CD^+FOXP3^+ Tregs were defined as Treg-positive cases and patients without $CD4^+FOXP3^+$ Tregs as Treg-negative cases.

2.4. Statistics

The associations of $CD163^+$ M2 macrophages and $CD4^+FOXP3^+$ Tregs with clinicopathological characteristics were evaluated by chi-square test,

Fisher's exact test, or Mann-Whitney *U* test as appropriate. The correlation between CD163$^+$ M2 macrophages and CD4$^+$FOXP3$^+$ Tregs was evaluated by Spearman's rank coefficient test. Recurrence and cancer-specific death were used as endpoints, and a univariate analysis of the association of outcome was performed using the Kaplan-Meier methods. The significance between the curves was assessed by the log-rank test. For the multivariate analysis, a Cox proportional hazards regression model was used, adjusted for nucleolar grade, AJCC stage, and primary tumor size (40 mm). Two-side $p < 0.05$ was considered statistically significant. The statistical analysis was performed using SPSS version 22 (IBM, USA).

3. Results

In this study, we evaluated tissue obtained from 346 patients diagnosed with RCC (195 males and 151 females). The clinical pathological characteristics of the patients are summarized in Table 1. The median follow-up time was 81 mo (range, 0–325) and the median time to recurrence was 104 mo (range, 2–346). At the last follow-up, 174 of the 346 study participants were deceased, of whom 79 died from RCC within the follow-up time.

Table 1 – Characteristics of renal cell carcinoma patients

	Total cohort (*N* = 346), *n* (%)
Smoking	
Yes	96 (36.1)
No	170 (63.9)
Missing	80
Primary diameter (mm)	
Median (min–max)	60 (10–180)
WHO nuclear grade	
1	22 (6.9)
2	151 (47.3)
3	108 (33.9)
4	38 (11.9)
Missing	27
AJCC stage	
1	183 (52.9)
2	62 (17.9)
3	62 (17.9)
4	39 (11.3)
pT stage	
T1	193 (55.8)
T2	78 (22.5)
T3	64 (18.5)
T4	11 (3.2)
N stage	
N0	333 (96.2)
N1	12 (3.5)
Nx	1 (0.3)
M stage	
M0	316 (91.3)
M1	30 (8.7)
Recurrence	
Yes	92 (27.1)
No	248 (72.9)
Missing	6

AJCC = American Joint Committee on Cancer; WHO = World Health Organization.

3.1. Infiltration of CD163$^+$ M2 macrophages and CD4$^+$FOXP3$^+$ Tregs is positively correlated with each other in RCC tissue and associated with adverse clinical outcomes

Here, we examined the infiltration of CD163$^+$ M2 macrophages and CD4$^+$FOXP3$^+$ Tregs into tumor and tumor-adjacent normal tissue obtained from 346 RCC patients (Fig. 1).

The median numbers of CD163$^+$ M2 macrophages were 65 (interquartile range [IQR] 58.5) and 141 (IQR 116.0) in tumor-adjacent normal area and tumor area, respectively. The mean numbers of CD4$^+$FOXP3$^+$ Tregs in corresponding areas were 1.2 (standard deviation [SD] 2.8) and 2.3 (SD 4.9), respectively (Figs. 2 and 3). When assessing the relationship between CD163$^+$ M2 macrophages and CD4$^+$FOXP3$^+$ Tregs, we observed that patients with CD163$^+$ M2 macrophage infiltration had a significantly higher number of CD4$^+$FOXP3$^+$ Tregs. The density between the two cell populations was positively correlated in both tumor-adjacent normal tissue ($R = 0.31$, $p < 0.001$) and tumor tissue ($R = 0.31$, $p < 0.001$).

To determine the clinical importance of CD163$^+$ M2 macrophages and CD4$^+$FOXP3$^+$ Tregs in tumor-adjacent normal tissue and tumor tissue, we analyzed their associations with clinicopathological characteristics. In tumor-adjacent tissue, the levels of infiltration of CD163$^+$ M2 macrophages were significantly higher in patients presenting with higher T ($p = 0.03$) and AJCC ($p = 0.02$) stages, and in patients with cancer recurrence ($p = 0.02$). Moreover, the levels of CD4$^+$FOXP3$^+$ Treg infiltration were significantly higher in patients with nuclear grades 3 and 4 than in those with nuclear grades 1 and 2 ($p = 0.03$). Table 2 summarizes the associations between clinicopathological characteristics and the results of the immunostaining in tumor-adjacent normal tissue. No associations between CD4$^+$FOXP3 positivity or CD163 positivity in tumor tissue and clinicopathological characteristics were found, even though a trend was observed for CD4$^+$FOXP3$^+$ Treg infiltration and cancer recurrence ($p = 0.06$). Table 3 summarizes the associations between clinicopathological characteristics and the results of the immunostaining in tumor tissue.

3.2. CD163$^+$ M2 macrophage and CD4$^+$FOXP3$^+$ Treg infiltration and clinical outcome

The median follow-up of the cohort used in the present study was 81 mo, and 79 patients died from RCC. According to the Kaplan-Meier analysis, patients with a high number of intratumoral CD163$^+$ M2 macrophages had a shorter median time to recurrence than those with a low number of CD163$^+$ M2 macrophages (82 vs 127 mo, $p = 0.038$; Fig. 4). A shorter median time to recurrence was also found for patients with a high number of CD163$^+$ M2 macrophages in tumor-adjacent normal tissue than in those with a low number of CD163$^+$ M2 macrophages (93 vs 127 mo, $p < 0.01$; Fig. 5). Further, there was a significant difference in median time to recurrence between patients with high intratumoral infiltration of CD163$^+$ M2 macrophages and

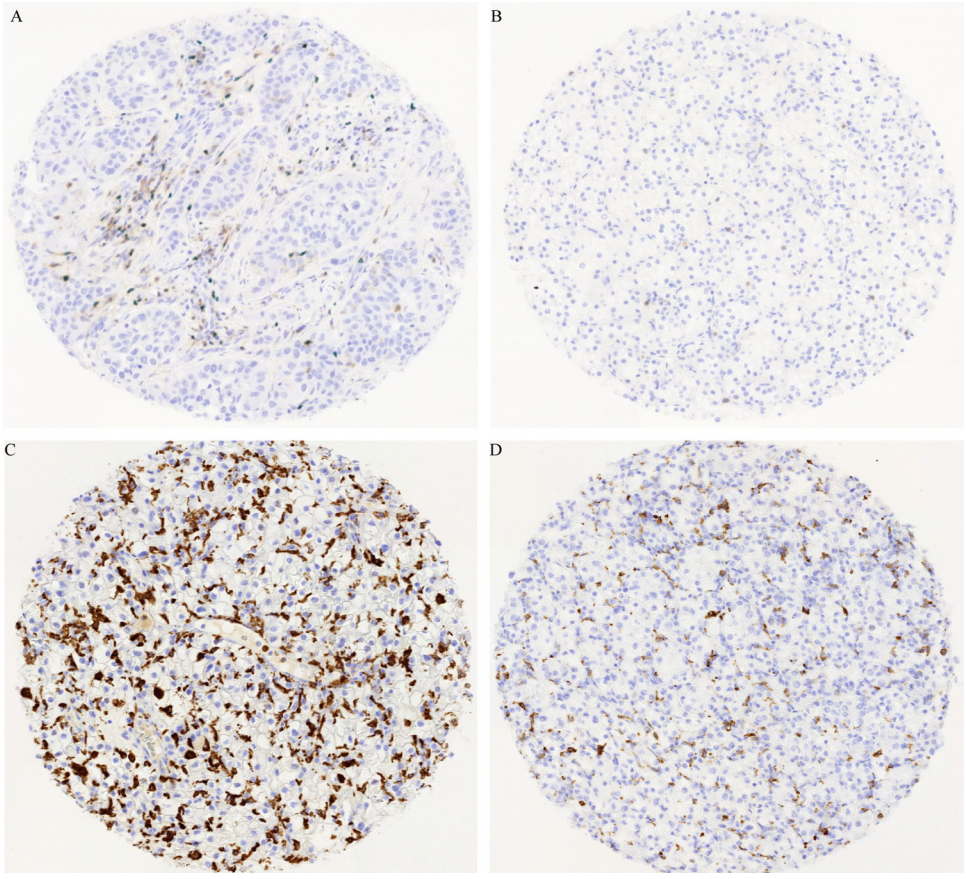


Fig. 1 – Representative immunohistochemical images of RCC cores stained to visualize the Treg markers CD4/FOXP3 (brown and green) and the M2 macrophages marker CD163 (brown): (A) a high number of Tregs, (B) a low number of Tregs, (C) a high number of M2 macrophages, and (D) a low number of M2 macrophages. Diameter of cores is 0.6 mm. RCC = renal cell carcinoma; Treg = regulatory T cell.

$CD4^+FOXP3^+$ Tregs present, compared with those with low infiltration of $CD163^+$ M2 macrophages and no $CD4^+FOXP3^+$ Tregs present (79 versus 197 mo, $p < 0.01$). To evaluate the impact of both $CD163^+$ M2 macrophages and $CD4^+FOXP3^+$ Tregs in progression of RCC, we compared patients with concurrent infiltration of $CD163^+$ M2 macrophages and $CD4^+FOXP3^+$ Tregs with those with only high infiltration of $CD163^+$ M2 macrophages. A shorter median time to recurrence was seen in patients with concurrent infiltration (79 vs 91 mo), although it did not reach statistical significance ($p = 0.08$; Fig. 6).

3.3. *Prognostic value of $CD163^+$ M2 macrophages and $CD4^+FOXP3^+$ Tregs in RCC*

We then evaluated the significance of $CD163^+$ M2 macrophage and $CD4^+FOXP3^+$ Treg infiltration, in addition to a number of clinicopathological variables, as predictors of

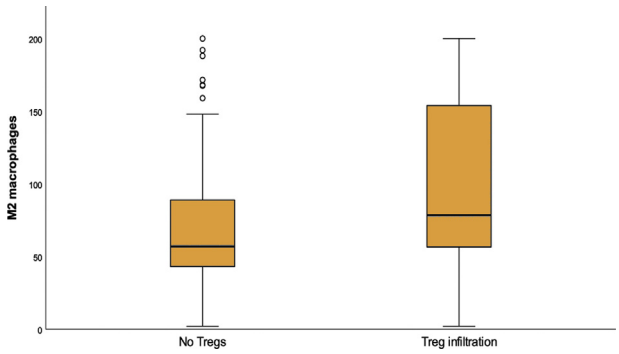


Fig. 2 – Comparison of $CD163^+$ M2 macrophage infiltration in tumor-adjacent normal tissue in RCC patients with and without $CD4^+FOXP3^+$ Treg infiltration. RCC = renal cell carcinoma; Treg = regulatory T cell.

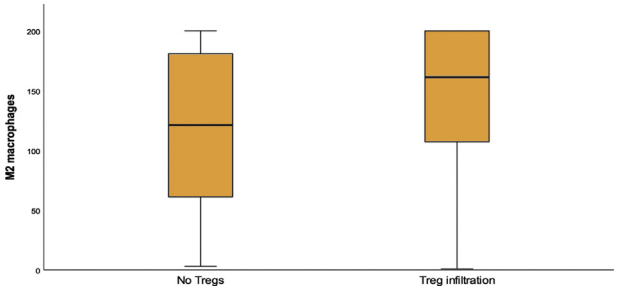


Fig. 3 – Comparison of $CD163^+$ M2 macrophage infiltration into tumor tissue in RCC patients with and without $CD4^+FOXP3^+$ Treg infiltration. RCC = renal cell carcinoma; Treg = regulatory T cell.

Table 2 – Associations between CD4/FOXP3 and CD163 immunoreactivity and clinical characteristics in tumor-adjacent normal tissue in patients with renal cell carcinoma

	$CD4^+FOXP3^+$ Tregs			$CD163^+$ M2 macrophages		
	Negative	Positive	*p* value	Low infiltration	High infiltration	*p* value
Gender			**0.05**[a]			**0.03**[a]
Male	94 (61)	61 (39)		95 (56)	75 (44)	
Female	56 (48)	60 (52)		54 (43)	71 (57)	
WHO nuclear grade			**0.03**[b]			0.27[a]
1	10 (53)	9 (47)		12 (63)	7 (37)	
2	53 (47)	61 (53)		65 (50)	66 (50)	
3	56 (68)	30 (32)		47 (51)	45 (49)	
4	20 (69)	9 (31)		11 (36)	20 (64)	
AJCC stage			0.34[a]			**0.02**[a]
1	73 (52)	67 (48)		89 (57)	66 (43)	
2	30 (64)	17 (36)		29 (54)	25 (46)	
3	27 (51)	26 (49)		19 (35)	35 (65)	
4	20 (65)	11 (35)		12 (38)	20 (62)	
pT stage			0.95[b]			**0.03**[b]
pT1	80 (55)	65 (45)		92 (56)	72 (44)	
pT2	35 (57)	26 (43)		35 (52)	33 (48)	
pT3	30 (56)	24 (44)		20 (36)	35 (64)	
pT4	5 (63)	3 (37)		2 (25)	6 (75)	
N stage			0.76[b]			0.99[a]
N0	141 (55)	116 (45)		141 (50)	140 (50)	
N1	7 (64)	4 (36)		6 (55)	5 (45)	
Nx						
M stage			0.29[a]			0.54[a]
M0	134 (54)	113 (46)		138 (51)	132 (49)	
M1	16 (67)	8 (33)		11 (44)	14 (56)	
Recurrence			0.89[a]			**0.02**[a]
Yes	41 (56)	32 (44)		29 (38)	47 (62)	
No	105 (54)	88 (46)		116 (55)	97 (45)	
Cause of death			0.48[a]			0.13[a]
Renal cell cancer	41 (63)	24 (37)		21 (33)	42 (67)	
Other reason	39 (56)	31 (44)		38 (47)	43 (53)	

AJCC = American Joint Committee on Cancer; Tregs = regulatory T cells; WHO = World Health Organization.
[a] Fisher's exact test was used.
[b] Chi-square test was used.

recurrence and cancer-specific death (Tables 4 and 5). A univariate Cox regression analysis revealed a significant positive association of time to recurrence with high $CD163^+$ M2 macrophage infiltration, into both tumor and tumor-adjacent normal tissue (hazard ratio [HR] 1.62; 95% confidence interval [CI] 1.02–2.56 and HR 1.86; 95% CI 1.16–2.98, respectively). A significant positive association of time to recurrence was also found with simultaneous infiltration of $CD163^+$ M2 macrophages and $CD4^+FOXP3^+$ Tregs into tumor tissue (HR 3.12; 95% CI 1.43–6.81). The association for infiltration of $CD163^+$ M2 macrophages into tumor tissue (HR 1.77; 95% CI 1.02–3.07) and for simultaneous infiltration of $CD163^+$ M2 macrophages and $CD4^+FOXP3^+$ Tregs into tumor tissue (HR 3.32; 95% CI 1.45–7.63) remained significant after adjusting for WHO nucleolar grade, AJCC stage, and primary tumor size.

When evaluating the significance of $CD163^+$ M2 macrophages and $CD4^+FOXP3^+$ Tregs as predictors of median time to cancer-specific death, $CD163^+$ M2 macrophages in tumor-adjacent normal tissue were found to be an independent prognostic marker for median time to cancer-specific death in the multivariate analysis ($p = 0.02$).

4. Discussion

In the present study, we demonstrate, for the first time in kidney cancer, that $CD163^+$ M2 macrophages and $CD4^+FOXP3^+$ Tregs colocalize in tumor and tumor-adjacent normal tissue, and that this colocalization may have an association with tumor recurrence.

M1 macrophages are suggested to be immunostimulatory and antitumoral, while M2 macrophages are immunosuppressive and contribute to a protumoral environment [2]. We found that high infiltration of $CD163^+$ M2 macrophages, into both tumor and the normal adjacent tissue, was associated with worse clinical prognosis. The present study also confirmed the prognostic role of $CD163^+$ M2 macrophages since a positive association of time to recurrence was found. In addition, our results showed that high infiltration of $CD163^+$ M2 macrophages into tumor-adjacent normal tissue was associated with shorter time to RCC-specific lethality. The prognostic role of $CD163^+$ M2 macrophage infiltrate in the adjacent normal tissue, rather than in the tumor tissue, is somewhat unexpected but not surprising. In a previous

Table 3 – Associations between CD4⁺FOXP3 and CD163 immunoreactivity and clinical characteristics in tumor tissue in patients with renal cell carcinoma

	CD4⁺FOXP3⁺ Tregs			CD163⁺ M2 macrophages		
	Negative	Positive	*p* value	Low infiltration	High infiltration	*p* value
Gender			0.40[a]			0.91[a]
Male	66 (41)	97 (59)		77 (50)	77 (50)	
Female	57 (46)	67 (54)		63 (51)	61 (49)	
WHO nuclear grade			0.11[a]			0.75[b]
1	11 (65)	6 (35)		10 (56)	8 (44)	
2	54 (42)	76 (58)		60 (47)	68 (53)	
3	36 (42)	50 (58)		39 (46)	46 (54)	
4	9 (28)	23 (72)		16 (55)	13 (45)	
AJCC stage			0.82[a]			0.91[a]
1	61(41)	88 (59)		75 (51)	71 (49)	
2	22 (42)	31(58)		23 (47)	26 (53)	
3	26 (48)	28 (52)		28 (53)	25 (47)	
4	14 (45)	17 (55)		14 (47)	16 (53)	
pT stage			0.39[b]			0.82[b]
pT1	65 (42)	91 (58)		80 (52)	74 (48)	
pT2	28 (42)	39 (58)		28 (45)	34 (55)	
pT3	28 (51)	27 (49)		28 (52)	26 (48)	
pT4	2 (22)	7 (78)		4 (50)	4 (50)	
N stage			0.99[b]			0.99[b]
N0	118 (43)	155 (57)		134 (51)	131 (49)	
N1	5 (46)	6 (54)		5 (50)	5 (50)	
Nx						
M stage			0.28[a]			0.99[a]
M0	110 (42)	153 (58)		128 (50)	126 (50)	
M1	13 (54)	11 (46)		12 (50)	12 (50)	
Recurrence			**0.06**[a]			0.08[a]
Yes	26 (33)	52 (67)		32 (43)	45 (57)	
No	94 (46)	110 (54)		105 (54)	91 (46)	
Cause of death			0.99[a]			0.99[a]
Renal cell cancer	27 (43)	36 (57)		33 (52)	30 (48)	
Other reason	33 (42)	45 (58)		44 (51)	42 (49)	

AJCC = American Joint Committee on Cancer; Tregs = regulatory T cells; WHO = World Health Organization.
[a] Fisher's exact test was used.
[b] Chi-square test was used.

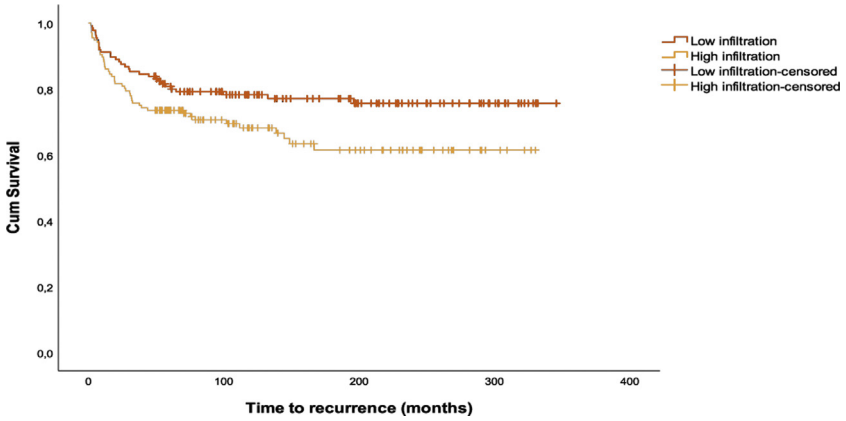


Fig. 4 – Kaplan-Meier analysis of median time to recurrence in terms of intratumoral CD163⁺ M2 macrophages showing shorter median time to recurrence in patients with a high number of CD163⁺ M2 macrophages. Low infiltration specified as under 141 CD163+M2 macrophages. Cum = cumulative.

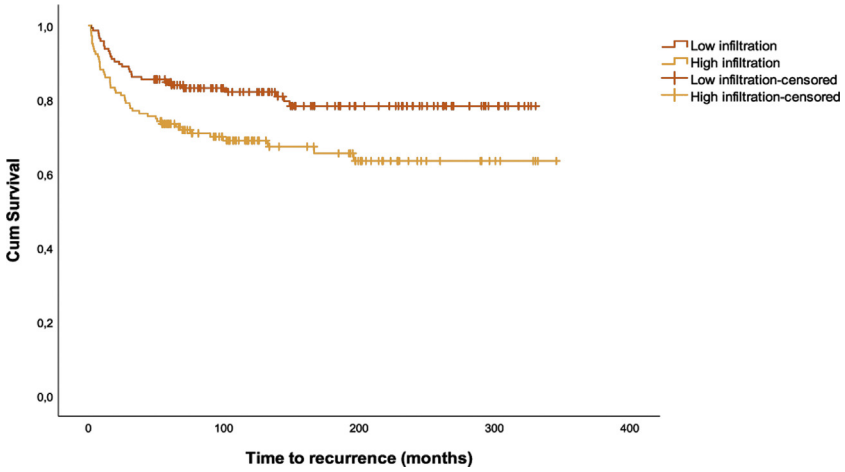


Fig. 5 – Kaplan-Meier analysis of median time to recurrence in terms of CD163⁺ M2 macrophages in tumor-adjacent normal tissue showing shorter median time to recurrence in patients with a high number of CD163⁺ M2 macrophages. Low infiltration specified as under 65 CD163+M2 macrophages. Cum = cumulative.

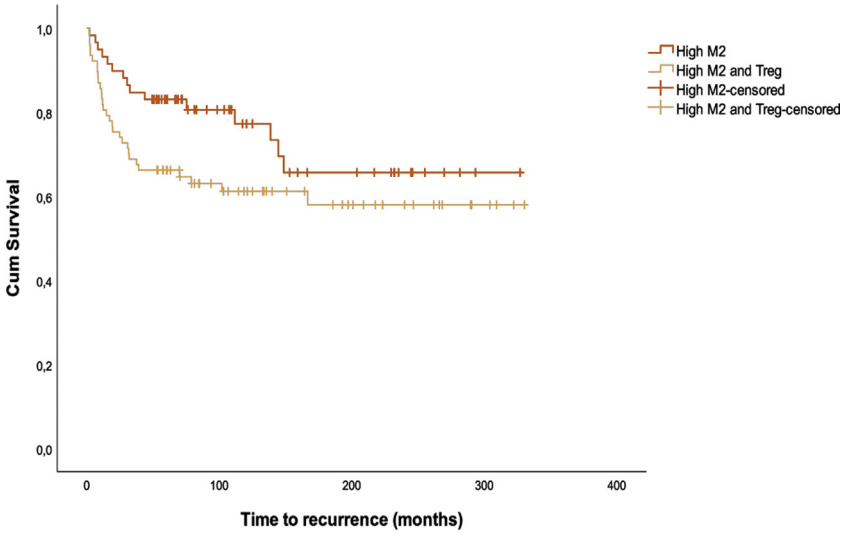


Fig. 6 – Kaplan-Meier analysis of median time to recurrence in terms of combination of intratumoral CD4⁺FOXP3⁺ Tregs and CD163⁺ M2 macrophages showing shorter median time to recurrence in patients with high numbers of CD163⁺ M2 macrophages and CD4⁺FOXP3⁺ Tregs. Cum = cumulative; Treg = regulatory T cell.

study on hepatocellular carcinoma, the prognostic gene profile was found in the surrounding liver tissue and not in the actual tumor [21].

CD163⁺ M2 macrophages contribute to immune suppression by several different mechanisms, including recruiting FOXP3⁺ Tregs [22–24]. In the present study, we observed colocalization of CD163⁺ M2 macrophages and CD4⁺FOXP3⁺ Tregs in tumor and tumor-adjacent normal tissue in patients diagnosed with RCC. This finding adds support to the postulated activation of FOXP3⁺ Tregs by M2 macrophage–released cytokines [25].

Supporting data for a contributing role of these cell types in accelerating tumor progression were recently shown for laryngeal squamous cell carcinoma patients [19]. Sun et al [19] found that infiltration of a high number of intratumoral CD163⁺ M2 macrophages in combination with the presence of CD4⁺FOXP3⁺ Tregs was an independent prognostic marker for overall survival.

No previous study has, to our knowledge, evaluated the synergistic effect of CD163⁺ M2 macrophages and CD4⁺FOXP3⁺ Tregs in RCC. The results of the present study, based on an RCC cohort, also suggest that colocalization of CD163⁺ M2 macrophages and CD4⁺FOXP3⁺ Tregs might have a synergistic effect on tumor aggressiveness. We showed that an increased number of CD163⁺ M2 macrophages, presence of CD4⁺FOXP3⁺ Tregs, or their colocalization were associated with a higher rate of RCC recurrence. We further confirmed the prognostic role of CD163⁺ M2

Table 4 – Univariate and multivariate Cox regression analyses of time to recurrence in RCC patients

	Univariate analysis HR (95% CI)	*p* value	Multivariate analysis 1 HR (95% CI)	*p* value	Multivariate analysis 2 HR (95% CI)	*p* value
$CD4^+FOXP3^+$ Tregs, TAN						
Treg negative	1 (reference)		1 (reference)			
Treg positive	0.99 (0.62–1.59)	0.98	1.1 (0.68–1.78)	0.70		
$CD4^+FOXP3^+$ Tregs, tumor						
Treg negative	1 (reference)		1 (reference)			
Treg positive	1.56 (0.97–2.52)	0.07	1.55 (0.95–2.52)	0.08		
$CD163^+$ M2 macrophages, TAN						
CD163 low	1 (reference)		1 (reference)		1 (reference)	
CD163 high	**1.86 (1.16–2.98)**	**0.01**	1.23 (0.76–1.99)	0.40	1.13 (0.67–1.89)	0.66
$CD163^+$ M2 macrophages, tumor						
CD163 low	1 (reference)		1 (reference)		1 (reference)	
CD163 high	**1.62 (1.02–2.56)**	**0.04**	**1.66 (1.03–2.66)**	**0.04**	**1.77 (1.02–3.07)**	**0.04**
Tregs/M2 macrophages, TAN						
Treg negative/CD163 low	1 (reference)		1 (reference)			
Treg positive/CD163 high	1.63 (0.85–3.12)	0.14	1.19 (0.60–2.34)	0.62		
Tregs/M2 macrophages, tumor						
Treg negative/CD163 low	1 (reference)		1 (reference)			
Treg positive/CD163 high	**3.12 (1.43–6.81)**	**0.01**	**3.32 (1.45–7.63)**	**0.01**		

AJCC = American Joint Committee on Cancer; CI = confidence interval; HR = hazard ratio; RCC = renal cell carcinoma; TAN = tumor-adjacent normal; Treg = regulatory T cell; WHO = World Health Organization.
Multivariate analysis 1: adjusted for WHO nucleolar grade, AJCC stage, and primary tumor size (40 mm).
Multivariate analysis 2: adjusted for WHO nucleolar grade, AJCC stage, primary tumor size (40 mm), and Tregs.

Table 5 – Univariate and multivariate Cox regression analyses of cancer-specific death in RCC patients

	Univariate analysis HR (95% CI)	*p* value	Multivariate analysis HR (95% CI)	*p* value
CD4+$FOXP3^+$ Tregs, TAN				
Treg negative	1 (reference)		1 (reference)	
Treg positive	1.07 (0.64–1.78)	0.80	1.35 (0.77–2.39)	0.30
$CD4^+FOXP3^+$ Tregs, tumor				
Treg negative	1 (reference)		1 (reference)	
Treg positive	0.97 (0.59–1.60)	0.97	0.95 (0.55–1.65)	0.86
$CD163^+$ M2 macrophages, TAN				
CD163 low	1 (reference)		1 (reference)	
CD163 high	1.90 (1.11–3.23)	0.18	**1.96 (1.10–3.47)**	**0.02**
$CD163^+$ M2 macrophages, tumor				
CD163 low	1 (reference)		1 (reference)	
CD163 high	1.05 (0.64–1.73)	0.84	0.88 (0.53–1.47)	0.62
Tregs/M2 macrophages, TAN				
Treg negative/CD163 low	1 (reference)		1 (reference)	
Treg positive/CD163 high	1.42 (0.72–2.82)	0.32	1.62 (0.72–3.65)	0.24
Tregs/M2 macrophages, tumor				
Treg negative/CD163 low	1 (reference)		1 (reference)	
Treg positive/CD163 high	1.23 (0.58–2.59)	0.59	1.03 (0.46–2.26)	0.95

AJCC = American Joint Committee on Cancer; CI = confidence interval; HR = hazard ratio; RCC = renal cell carcinoma; TAN = tumor-adjacent normal; Treg = regulatory T cell; WHO = World Health Organization.
Multivariate analysis: adjusted for WHO nucleolar grade, AJCC stage, and primary tumor size (40 mm).

macrophages or $CD4^+FOXP3^+$ Tregs in our multivariate analysis, where we demonstrated that the levels of $CD4^+FOXP3^+$ Tregs and mainly $CD163^+$ M2 macrophages are independent markers of time to recurrence in RCC patients. Our results are in line with the mRNA data presented by Dannenmann et al [26].

Approximately 70% of RCC patients are diagnosed with localized disease, and this group of patients has a good clinical course. However, about 20–30% of the patients will present with or develop metastatic disease, and for them the survival rate is dramatically lower [27]. Inhibition of different immune checkpoints, including CTLA-4, PD-1, and PD-L1, has shown remarkable impact in the treatment of cancer patients with solid tumors, including RCC [28]. Recently, the European Medicines Agency approved an immunotherapy for advanced or metastatic RCC using the combination of the anti-CTLA-4 drug ipilimumab and the anti-PD-1 drug nivolumab. This combined immunotherapy was reported to be superior to the standard of care with antiangiogenic targeted therapies in the phase III Check-

Mate 214 trial. In the CheckMate 214 trial, the objective response rate to ipilimumab plus nivolumab was approximately 40%, and the reason for the response failure in the remaining 60% of patients is still largely unclear [29]. One might speculate that our results may provide important information since $CD163^+$ M2 macrophages and $CD4^+FOXP3^+$ Tregs in the tumor microenvironment would represent a potential mechanism of resistance to immunotherapies aiming to reinforce the antitumor immune response.

The major strength of this study is the well-defined cohort used, including complete follow-up of a large number of study participants, with well-defined outcome measurements from the Swedish Cause of Death register. The use of TMAs rather than whole sections for the assessment of CD163 and FOXP3 immunohistochemistry could be viewed as a limitation.

5. Conclusions

We found that infiltration of $CD163^+$ M2 macrophages and $CD4^+FOXP3+$ Tregs is associated with an increased RCC recurrence, and that colocalization of these cell types might have a synergistic effect on clinical outcome.

***Author contributions*:** Sabina Davidsson had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Davidsson, Sundqvist, Carlsson.
Acquisition of data: Davidsson, Eriksson, Erlandsson, Fiorentino, Giunchi.
Analysis and interpretation of data: Davidsson, Erlandsson, Fiorentino.
Drafting of the manuscript: Davidsson, Fiorentino, Sundqvist.
Critical revision of the manuscript for important intellectual content: Fiorentino, Giunchi, Sundqvist, Carlsson.
Statistical analysis: Carlsson.
Obtaining funding: Davidsson.
Administrative, technical, or material support: Eriksson, Erlandsson, Sundqvist.
Supervision: Davidsson.
Other: None.

Financial disclosures: Sabina Davidsson certifies that all conflicts of interest, including specific financial interests and relationships and affiliations relevant to the subject matter or materials discussed in the manuscript (eg, employment/affiliation, grants or funding, consultancies, honoraria, stock ownership or options, expert testimony, royalties, or patents filed, received, or pending), are the following: None.

***Funding/Support and role of the sponsor*:** This research was supported by the Örebro County Council Research Committee and the Lions Cancer Foundation, Sweden.

References

[1] Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57–70.
[2] Wang LX, Zhang SX, Wu HJ, Rong XL, Guo J. M2b macrophage polarization and its roles in diseases. J Leukoc Biol 2019;106:345–58.
[3] Murray PJ, Wynn TA. Obstacles and opportunities for understanding macrophage polarization. J Leukoc Biol 2011;89:557–63.
[4] Erlandsson A, Carlsson J, Lundholm M, et al. M2 macrophages and regulatory T cells in lethal prostate cancer. Prostate 2019;79:363–9.
[5] Yagi T, Baba Y, Okadome K, et al. Tumour-associated macrophages are associated with poor prognosis and programmed death ligand 1 expression in oesophageal cancer. Eur J Cancer 2019;111:38–49.
[6] Behnes CL, Bremmer F, Hemmerlein B, Strauss A, Strobel P, Radzun HJ. Tumor-associated macrophages are involved in tumor progression in papillary renal cell carcinoma. Virchows Arch 2014;464:191–6.
[7] Komohara Y, Hasita H, Ohnishi K, et al. Macrophage infiltration and its prognostic relevance in clear cell renal cell carcinoma. Cancer Sci 2011;102:1424–31.
[8] Ma C, Horlad H, Ohnishi K, et al. CD163-positive cancer cells are potentially associated with high malignant potential in clear cell renal cell carcinoma. Med Mol Morphol 2018;51:13–20.
[9] Nishikawa H, Jager E, Ritter G, Old LJ, Gnjatic S. CD4+ CD25+ regulatory T cells control the induction of antigen-specific CD4+ helper T cell responses in cancer patients. Blood 2005;106:1008–11.
[10] Nishikawa H, Sakaguchi S. Regulatory T cells in cancer immunotherapy. Curr Opin Immunol 2014;27:1–7.
[11] Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003;299:1057–61.
[12] Davidsson S, Ohlson AL, Andersson SO, et al. CD4 helper T cells, CD8 cytotoxic T cells, and FOXP3(+) regulatory T cells with respect to lethal prostate cancer. Mod Pathol 2013;26:448–55.
[13] Peng GL, Li L, Guo YW, et al. CD8(+) cytotoxic and FoxP3(+) regulatory T lymphocytes serve as prognostic factors in breast cancer. Am J Transl Res 2019;11:5039–53.
[14] Shang B, Liu Y, Jiang SJ, Liu Y. Prognostic value of tumor-infiltrating FoxP3+ regulatory T cells in cancers: a systematic review and meta-analysis. Sci Rep 2015;5:15179.
[15] Zhu G, Pei L, Yin H, et al. Profiles of tumor-infiltrating immune cells in renal cell carcinoma and their clinical implications. Oncol Lett 2019;18:5235–42.
[16] Liotta F, Gacci M, Frosali F, et al. Frequency of regulatory T cells in peripheral blood and in tumour-infiltrating lymphocytes correlates with poor prognosis in renal cell carcinoma. BJU Int 2011;107:1500–6.
[17] Griffiths RW, Elkord E, Gilham DE, et al. Frequency of regulatory T cells in renal cell carcinoma patients and investigation of correlation with survival. Cancer Immunol Immunother 2007;56:1743–53.
[18] Siddiqui SA, Frigola X, Bonne-Annee S, et al. Tumor-infiltrating Foxp3-CD4+CD25+ T cells predict poor survival in renal cell carcinoma. Clin Cancer Res 2007;13:2075–81.
[19] Sun W, Wei FQ, Li WJ, et al. A positive-feedback loop between tumour infiltrating activated Treg cells and type 2-skewed macrophages is essential for progression of laryngeal squamous cell carcinoma. Br J Cancer 2017;117:1631–43.
[20] Grabowska B, Ulvskog E, Carlsson J, et al. Clinical outcome and time trends of surgically treated renal cell carcinoma between 1986 and 2010: results from a single centre in Sweden. Scand J Urol 2018;52:206–12.
[21] Hoshida Y, Villanueva A, Kobayashi M, et al. Gene expression in fixed tissues and outcome in hepatocellular carcinoma. N Engl J Med 2008;359:1995–2004.
[22] Han Q, Shi H, Liu F. CD163(+) M2-type tumor-associated macrophage support the suppression of tumor-infiltrating T cells in osteosarcoma. Int Immunopharmacol 2016;34:101–6.
[23] Liu G, Yang H. Modulation of macrophage activation and programming in immunity. J Cell Physiol 2013;228:502–12.
[24] Kubota K, Moriyama M, Furukawa S, et al. CD163(+)CD204(+) tumor-associated macrophages contribute to T cell regulation via interleukin-10 and PD-L1 production in oral squamous cell carcinoma. Sci Rep 2017;7:1755.

[25] Zhu Q, Wu X, Wu Y, Wang X. Interaction between Treg cells and tumor-associated macrophages in the tumor microenvironment of epithelial ovarian cancer. Oncol Rep 2016;36:3472–8.
[26] Dannenmann SR, Thielicke J, Stöcki M, et al. Tumor-associated macrophages subvert T-cell function and correlate with reduced survival in clear cell carcinoma. Oncoimmunology 2013;2:e23562.
[27] Smith-Bindman R, Kwan ML, Marlow EC, et al. Trends in use of medical imaging in US health care systems and in Ontario, Canada, 2000-2016. JAMA 2019;322:843–56.
[28] Rini BI, Plimack ER, Stus V, et al. Pembrolizumab plus axitinib versus sunitinib for advanced renal-cell carcinoma. N Engl J Med 2019;380:1116–27.
[29] Motzer RJ, Rini BI, McDermott DF, et al. Nivolumab plus ipilimumab versus sunitinib in first-line treatment for advanced renal cell carcinoma: extended follow-up of efficacy and safety results from a randomised, controlled, phase 3 trial. Lancet Oncol 2019;20:1370–85.