# Myeloid-derived suppressor cell accumulation in renal cell carcinoma is correlated with CCL2, IL-17 and IL-18 expression in blood and tumors

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#### **Abstract**

**Background.** Myeloid-derived suppressor cells (MDSC) play an important role in tumor-mediated immune evasion. Levels of MDSC in peripheral blood are increased in patients with cancer, correlating with cancer stage and outcome. Studies have confirmed the associations between MDSC and various cytokines in the peripheral blood of murine and human cancer hosts. However, little is known about the association between parenchymal MDSC subsets and cytokines, or the mechanism drawing MDSC into tumor parenchyma.

**Objectives.** The aim of this study was to analyze the correlation between MDSC subsets and tumor grade as well as stage in renal cell carcinoma (RCC) patients. The expression of chemokine (C-C motif) ligand 2 (CCL2), interleukin 17 (IL-17) and interleukin 18 (IL-18) in the peripheral blood and parenchyma of RCC patients was also detected to explore its correlation with MDSC accumulation.

**Material and methods.** Total MDSC, granulocytic MDSC (G-MDSC), monocytic MDSC (M-MDSC), and immature MDSC (I-MDSC) from the blood and parenchyma were isolated and analyzed by flow cytometry. Cytokines were detected by the enzyme-linked immunosorbent assay (ELISA), real-time polymerase chain reaction (PCR) and western blot in blood and tumors.

**Results.** Parenchymal levels of MDSC had a positive correlation with levels of CCL2, IL-17, and IL-18, suggesting these cytokines may attract MDSC into the parenchyma. Moreover, peripheral total MDSC, G-MDSC and I-MDSC were shown to correlate with tumor grade and stage. Gene and protein expression of CCL2, IL-17, and IL-18 was significantly increased in blood and tumors of RCC patients.

**Conclusions.** Our study has provided potential new targets for the risk stratification of patients with limited stages of renal carcinoma, in addition to elucidating a possible association between MDSC subsets and cytokine-induced migration into the tumor tissue.

**Key words:** chemokine (C-C motif) ligand 2, renal cell carcinoma, interleukin 17, interleukin 18, myeloid-derived suppressor cells

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#### Introduction

Renal cell carcinoma (RCC) is a kidney cancer originating in the lining of the proximal convoluted tubule and is the most common type of kidney cancer in adults, responsible for approx. 90–95% of cases. It accounts for approx. 3% of adult malignancies, with close to 64,000 new cases diagnosed every year and with a consistent increase in the incidence rate.1 It is well-known that tumor-mediated immunosuppression of the microenvironment and immune evasion contribute to decreased clinical efficacy of immune and targeted therapy.<sup>2,3</sup> Various cell types are involved in tumor-mediated immune suppression, such as regulatory T cells ( $T_{\text{reg}}\!$ ), tumor-associated macrophages (TAMs), and myeloid-derived suppressor cells (MDSC).<sup>4,5</sup> In particular, as a heterogeneous cell population, MDSC have become the focus of intense study in recent years because of their important role in tumor-associated immune suppression.<sup>6</sup>

Arising from myeloid progenitor cells, MDSC fail to differentiate into mature dendritic cells, granulocytes or macrophages, with the capacity to suppress T cell and natural killer (NK) cell function. These mechanisms to suppress antitumor immunity by MDSC include depletion of L-arginine to arrest T cells in mitosis, cystine sequestration to decrease proliferation, induction of FOXP3+  $T_{\rm reg}$  cells, down-regulation of CD4 and CD8 T cell homing to lymph nodes, etc. 8,9

Levels of MDSC in peripheral blood are significantly increased in patients with cancer, and correlate with metastatic burden and clinical cancer stage. <sup>10</sup> Granulocytic MDSC (G-MDSC) additionally express CD15 with CD14 negative, whereas monocytic MDSC (M-MDSC) express CD14 with CD15 negative, and immature MDSC (I-MDSC) express neither CD14 nor CD15. <sup>11</sup>

Granulocytic MDSC are the dominant population in peripheral blood in RCC, and have been associated with decreased overall survival (OS) time. Monocytic MDSC levels have also been shown to inversely correlate with OS, and have been described as an independent risk factor for recurrence in hepatocellular carcinoma. <sup>12–14</sup> However, all of these studies pertained only to peripheral MDSC, and to our knowledge, no correlation has been made between intratumoral MDSC levels.

While associations have been confirmed between MDSC and various cytokines in the peripheral blood of murine and human cancer hosts, little is known about the mechanism drawing MDSC directly into the tumor parenchyma. Studies have shown that inflammatory cytokines such as interleukin-17 (IL-17), interleukin-18 (IL-18) and chemokine (C-C motif) ligand 2 (CCL2) are related to stimulation of myeloid cells including tumor-promoting MDSC, inducing the accumulation of MDSC and enhancing their suppression of T cells. However, the association of cytokine production and accumulation of MDSC in RCC has not been fully investigated.

Here, we analyzed the relationship between circulating or intratumoral levels of MDSCs and tumor grade and stage in patients with RCC. In addition, we determined the correlation between MDSC subsets and levels of IL-17, IL-18, and CCL2 expression in the parenchyma and peripheral blood in patients with primary RCC tumors. This study elucidates some of the factors that might promote MDSC accumulation and lead to tumor immunosuppression.

#### **Material and methods**

#### Renal cell carcinoma tumor lysates

Primary RCC tumor samples were collected from 55 patients prior to nephrectomy according to the IRB (institutional review board)-approved protocol. A portion of the tumor sample was flash frozen to make lysates using FastPrep-24 (MP Biomedicals, Santa Ana, USA) according to the manufacturer's manual. Briefly, tumor tissue was placed in lysing matrix D tubes with RIPA buffer (Thermo Fisher Scientific, Waltham, USA), protease inhibitor (Sigma-Aldrich, St. Louis, USA) and Halt phosphatase inhibitors (Thermo Fisher), incubated and processed in the FastPrep-24. The lysates were then aliquoted and frozen at  $-80^{\circ}$ C.

### Phenotype analysis of myeloid-derived suppressor cells subsets from tumor

Phenotyping of MDSC was performed on fresh tumor samples. Granulocytic MDSC, M-MDSC and I-MDSC from the parenchyma of 55 RCC nephrectomy samples were phenotyped using flow cytometry. The tumor was cut into small fragments followed by digestion for 15 min with enzyme cocktail (collagenase 1 mg/mL, DNase 0.1 mg/mL, and hyaluronidase 2.5 U/mL; Sigma-Aldrich), and then filtered using 70 µm cell strainers (BD Falcon; BD, Franklin Lakes, USA). The single cell suspension was subjected to 30% percoll gradient over 70% percoll gradient to enrich for mononuclear cells, followed by staining cells with anti-CD33 APC, anti-HLA-DR FITC, anti-CD15 PE and anti-CD14 PerCP antibodies, along with appropriate isotype controls (all from BD) for flow cytometry analysis (BD FACSCalibur; BD) and computer analysis (CellQuest Pro; BD). Total MDSC were defined as CD33+ HLA-DRlow/-; G-MDSC were defined as CD33+ HLA-DRlow/- CD14-CD15+; M-MDSC were defined as CD33+ HLA-DRlow/- CD14+ CD15-, and I-MDSC were defined as CD33+ HLA-DRlow/- CD14- CD15-.

# Phenotype analysis of myeloid-derived suppressor cells subsets from peripheral blood

Peripheral blood was obtained prior to surgery from 55 patients with tumors, and 15 controls from healthy donors, in accordance with the IRB protocol. The blood was

subjected to Ficoll-Hypaque density centrifugation and the buffy layer containing peripheral blood mononuclear cells (PBMC), including MDSC, was stained with the same antibodies as the tumor for flow cytometry analysis. The flow data was presented as the percentage of MDSC subsets in the PBMC buffy layer.

### Cytokine levels from tumor and peripheral blood

The enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, USA) was used to quantitate levels of IL-17, IL-18 and CCL2 in tumor lysates and plasma according to the manufacturer's instructions. Protein was quantitated by the bicinhoninic acid assay (BCA) (Thermo Fisher) on both parenchymal and peripheral blood samples to ensure that equal amounts of protein were aliquoted in each well. Myeloid-derived suppressor cells subsets were screened with corresponding beads from the tumor lysates.

### Quantitative real-time reverse transcription polymerase chain reaction

Samples from the patients and healthy donors were collected and placed in 100  $\mu$ L of TRIzol reagent (Thermo Fisher) to extract the total RNA. Total RNA from samples was pooled together. Complementary DNA was generated by adding 1  $\mu$ g of the total RNA to SuperScript master mix (Thermo Fisher) and performing reverse transcription. Quantitative polymerase chain reaction (PCR) and melt-curve analyses were performed using iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, USA). The comparative  $C_t$  value method was used to quantify the expression of genes of interest in different samples. The mRNA levels were normalized to that of a housekeeping gene, *Rpl19* mRNA.

#### Western blot

Total cellular protein from the samples of the patients and healthy donors was pooled together and quantified with the BCA protein assay kit. After boiling, equal amounts of protein (50 µg) from each sample were subjected to electrophoresis on a 10% (v/v) sodium dodecyl sulfate (SDS)-polyacrylamide gel. The protein was then electroblotted from gel to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with phosphate buffered saline-Tween 20 (PBS-T), containing 5% non-fat milk at room temperature for 1 h, and incubated with the indicated primary antibodies (anti-CCL2 rabbit pAb ab9669, 1:2000; anti-IL-17 rabbit pAb ab79056, 1:1000; anti-IL-18 rabbit pAb ab68435, 1:1000; anti-glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH) rabbit pAb SC-25778, 1:2000) at 4°C overnight, followed by incubating with the goat-anti-rabbit horseradish peroxidase-conjugated secondary antibody for 1 h. After incubation, the membrane was washed 3 times, and the antigen–antibody complexes were visualized using the enhanced chemiluminescence system.

#### Statistical analysis

Categorical variables were summarized as frequency counts and percentages. The Wilcoxon rank-sum and Jonckheere-Terpstra tests were used for the comparison of MDSC between patient groups. Spearman rank correlations were used to assess the association between MDSC and inflammatory factors in tumor samples and blood. A p-value <0.05 was considered as significant difference. SAS v. 9.1 software (SAS Institute, Cary, USA) was used for all analyses.

#### **Ethics**

The present study was approved by the institutional Human Investigations and Ethics Committee in Shandong Provincial Hospital Affiliated to Shandong University and was conducted in accordance with the Helsinki Declaration. Written informed consent was obtained from patients and healthy donors.

#### Results

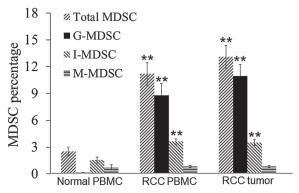
#### **Patient characteristics**

The present cohort included 55 patients with RCC prior to nephrectomy. Median age was 62 (range: 43–68), and 42 patients (76%) were male. 52 patients (94%) had clear cell carcinoma, and 3 patients had papillary carcinoma. 13 patients (24%) had grade 2 histology, 25 (45%) had grade 3, and 17 (31%) had grade 4 histology. 28 patients (51%) had stage

Table 1. Patient characteristics

Factor	N (%) or median (range)		
Age (range) at diagnosis [years]	62 (43–68)		
Gender			
Male	42 (76%)		
Female	13 (24%)		
Histology			
Clear cell	52 (95%)		
Papillary carcinoma	3 (5%)		
Grade			
2	13 (24%)		
3	25 (45%)		
4	17 (31%)		
Stage			
1	25 (45%)		
2	3 (6%)		
3	18 (33%)		
4	9 (16%)		

I–II, 18 (33%) had stage III, and 9 patients (16%) had metastatic (stage IV) disease at the time of nephrectomy (Table 1). Of the 9 patients with metastases, 5 had lung, lymph node or adrenal involvement, 3 had liver metastasis, and 1 had bone metastasis. No patient had received any systemic anti-cancer treatment prior to nephrectomy. The peripheral blood analysis of MDSC subsets in RCC patients was compared to the same populations in the PBMC fraction from healthy donors.



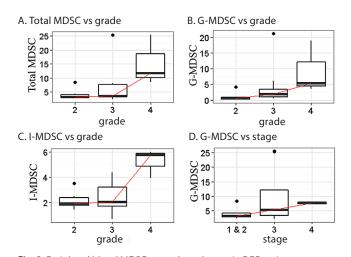
**Fig. 1.** Percentage of MDSC subsets from normal individuals, RCC patients' PBMC and tumors. Compared to healthy donors, the percentage of MDSC, particularly the G-MDSC and I-MDSC subsets, is significantly increased in the peripheral blood of RCC patients

The data was expressed as mean  $\pm$ SEM; \*\* p < 0.01 vs normal PBMC; PBMC – peripheral blood mononuclear cells; RCC – renal cell carcinoma; MDSC – myeloid-derived suppressor cells; G-MDSC – granulocytic myeloid-derived suppressor cells; M-MDSC – monocytic myeloid-derived suppressor cells; I-MDSC – immature myeloid-derived suppressor cells.

# Myeloid-derived suppressor cells subset levels were increased in renal cell carcinoma patients

Gates were set up for analysis of MDSC subsets based on staining for CD33, HLA-DR, CD15 and CD14. Compared to healthy donor PBMC (n = 15), the number of total MDSC, G-MDSC and I-MDSC was significantly increased in RCC patients (Fig. 1).

Peripheral blood was available from 55 patients with tumors, and 15 normal controls (Table 2). Comparing MDSC subset levels in each cohort, we found that total MDSC were 5.6% (median) in patients vs 2.3% in controls (a 2.43-fold increase, p < 0.001). In addition, G-MDSC were 23.75-fold elevated in RCC patients compared to controls (1.9% vs 0.08%, respectively; p < 0.001). Immature MDSC



**Fig. 2.** Peripheral blood MDSC vs grade and stage in RCC patients RCC – renal cell carcinoma; MDSC – myeloid-derived suppressor cells;

G-MDSC – granulocytic myeloid-derived suppressor cells; M-MDSC – monocytic myeloid-derived suppressor cells; I-MDSC – immature myeloid-derived suppressor cells.

were also elevated in patients compared to controls (2.7% vs 1.14%, a 2.37-fold increase; p < 0.001). There was no significant difference in the levels of M-MDSC between the 2 cohorts.

### Myeloid-derived suppressor cells levels correlate with tumor grade

Increasing peripheral levels of MDSC correlated with higher tumor grade. Specifically, total MDSC, G-MDSC and I-MDSC in the periphery of RCC patients correlated with increasing tumor grade (p < 0.05, p < 0.01 and p < 0.05, respectively) (Fig. 2 A–C). There was no correlation between peripheral M-MDSC level and tumor grade. Moreover, the peripheral level of G-MDSC correlated with higher tumor stage (p < 0.05) (Fig. 2 D).

### Cytokines were increased in renal cell carcinoma patients

Gene and protein expression of CCL2, IL-17 and IL-18 in PBMC and tumors was detected by real-time PCR and western blot. The results showed that both gene and protein expression was significantly increased in the RCC patients comparing to the healthy donors (Fig. 3–4).

Table 2. Comparison of MDSC subsets in peripheral blood between patients with cancer and healthy donors

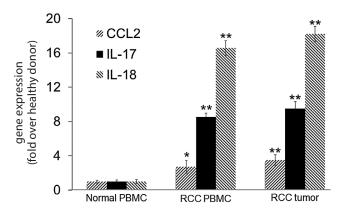
Factor	Controls (n = 15)	Patients (n = 55)	p-value*	
Total MDSC	2.3 (0.53, 6.8)	5.6 (2.6, 36.8)	<0.001	
G-MDSC	0.08 (0.02, 0.35)	1.9 (0.06, 25.7)	<0.001	
M-MDSC	0.41 (0.05, 3.4)	0.48 (0.12, 2.8)	0.55	
I-MDSC	1.14 (0.36, 4.9)	2.7 (0.73, 7.8)	<0.001	

<sup>\*</sup>Wilcoxon rank-sum test; MDSC – myeloid-derived suppressor cells; G-MDSC – granulocytic myeloid-derived suppressor cells; M-MDSC – monocytic myeloid-derived suppressor cells; I-MDSC – immature myeloid-derived suppressor cells; results expressed as medians.

Tumor lysate	Cytokines					
	IL-17		IL-18		CCL2	
	COF	p-value	COF	p-value	COF	p-value
Total MDSC	0.71	<0.01	0.62	<0.01	0.49	<0.01
G-MDSC	0.68	<0.01	0.66	<0.01	0.56	<0.01
M-MDSC	-0.21	0.22	-0.28	0.15	-0.17	0.46
I-MDSC	0.39	<0.05	0.12	0.32	0.65	<0.01

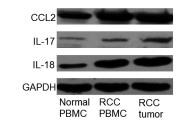
Table 3. Correlation between MDSC and IL-17, IL-18 and CCL2 expression in tumor samples from patients with RCC

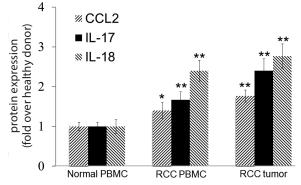
COF – coefficient; MDSC – myeloid-derived suppressor cells; G-MDSC – granulocytic myeloid-derived suppressor cells; M-MDSC – monocytic myeloid-derived suppressor cells; IL-17 – interleukin-17; IL-18 – interleukin-18; CCL2 – chemokine (C-C motif) ligand 2.



**Fig. 3.** Cytokine gene expression was increased in the PBMC and tumor lysate of RCC patients

\* p < 0.05; \*\* p < 0.01 vs normal PBMC; PBMC – peripheral blood mononuclear cells; RCC – renal cell carcinoma; IL-17 – interleukin-17; IL-18 – interleukin-18; CCL2 – chemokine (C-C motif) ligand 2.





**Fig. 4.** Cytokine protein expression was increased in the PBMC and tumor lysate of RCC patients

\* p < 0.05; \*\* p < 0.01 vs normal PBMC; PBMC – peripheral blood mononuclear cells; RCC – renal cell carcinoma; IL-17 – interleukin-17; IL-18 – interleukin-18; CCL2 – chemokine (C-C motif) ligand 2; GAPDH – glyceraldehyde 3-phosphate dehydrogenase.

### Myeloid-derived suppressor cells subsets correlate with cytokines

Renal cell carcinoma tumor tissue was examined for expression of cytokines known to promote the trafficking of MDSC and neutrophils. The results showed that parenchymal levels of total MDSC and G-MDSC had a positive correlation with IL-17 (p < 0.01), IL-18 (p < 0.01) and CCL2 (p < 0.01) (Table 3). In addition, levels of I-MDSC correlated positively with IL-17 (p < 0.05) and CCL2 (p < 0.01), but not IL-18 (Table 3).

#### **Discussion**

Myeloid-derived suppressor cells have emerged as key effectors in the tumor microenvironment in many solid tumors, and the factors that influence MDSC recruitment and function continue to expand. In this study, MDSC subset levels in the peripheral blood of RCC patients vs healthy donors were compared and the results showed that total MDSC, as well as G-MDSC and I-MDSC, are elevated in patients, which is consistent with several other studies.<sup>22,23</sup> The levels of M-MDSC did not change significantly, probably due to its lowest subset proportion or its potential differentiation into G-MDSC or TAMs. 24,25 Total MDSC, G-MDSC and I-MDSC in the peripheral blood of RCC patients correlated with increasing tumor grade. Moreover, peripheral levels of G-MDSC correlated with higher tumor stage (p < 0.05). Although several other studies have shown a correlation between peripheral MDSC levels and patient outcomes, to the best of our knowledge, this is the first time a correlation has been shown between MDSC subset levels and tumor grade in RCC patients.

Granulocytic MDSC derived from the peripheral blood have been shown to suppress T cell production of interferon gamma (IFN- $\gamma$ ) and T cell proliferation, whose mechanism of suppression is partly dependent on the arginase pathway.<sup>8,12,26</sup> These findings support the idea that G-MDSC have the potential to block the development of an effective antitumor immunity, thereby promoting tumor progression. Another study showed that G-MDSC promoted immune suppression and reduced the

effectiveness of anti-PD-1 antibody therapy in tumor bearing mice. The reduction of G-MDSC with anti-CXCR2 antibody treatment decreased tumor growth and significantly improved in vivo activity of anti-PD-1 treatment.<sup>27</sup> Tumor-associated inflammation contributes to cancer growth and spread, and intratumoral immune cells influence patient prognosis and survival. Various immunomodulatory factors have been linked to increased cancer metastatic potential.<sup>28</sup>

By recruiting monocytes, memory T cells and dendritic cells to the sites of inflammation, CCL2 has been shown to be a chemoattractant for MDSC and functions as a neoplastic factor that regulates MDSC accumulation and function and fosters a tumor permissive microenvironment that influences early-stage carcinogenesis. Chemokine (C-C motif) ligand 2 contributes intratumoral MDSC accumulation and influences MDSC-mediated suppression of CD4+ and CD8+ T cells via distinct pathways. Constitutive deletion or antibody-mediated neutralization of CCL2 halts neoplastic progression in an inflammation-associated tumor model. <sup>20,29</sup>

Interleukin-17 is a pro-inflammatory cytokine that is thought to promote neutrophil chemotaxis and degranulation, and increased expression of IL-17 has been shown in cancer cells and TAMs, suggesting it may play an important role in the regulation of the tumor microenvironment. Previous studies have suggested a causal relationship between IL-17 production and the level of circulating MDSC. Indeed, in addition to IL-6 and tumor necrosis factor alpha (TNF- $\alpha$ ), IL-17 has been shown to be significantly increased in RCC tissue, and its expression is dependent on the degree of malignancy.  $^{16,30-32}\,\mathrm{Here}$  , we showed that parenchymal levels of IL-17 correlate with increased total MDSC (p < 0.01), G-MDSC (p < 0.01) and I-MDSC (p < 0.05). While this data is associative, it again suggests that IL-17 plays an important role in immune modulation of the tumor microenvironment.

Interleukin-18 has been reported to stimulate expression of hematopoietic cytokines and growth factors such as IL-3, IL-6, G-CSF and GM-CSF, which regulate the development of neutrophils and differentiation of hematopoietic cells. Many studies have demonstrated the pivotal role of IL-18 in immune cell activation and tumor progression. Interleukin-18 supplementation significantly enhances the immunosuppressive activity of MDSC.<sup>6,33</sup> As MDSC have a wide range of effects in cancer progression and metastasis, we hypothesized that IL-18 may have certain effects on MDSC biology in RCC.

Although MDSC accumulation correlated with cytokine expression in this study, our sample size was small, which may have led to type II error, such as the inability to discern the difference between chemokine levels in the patients and healthy donors. Moreover, one PBMC sample from a healthy donor and 2 PBMC samples from patients with RCC used for chemokine analysis had been re-thawed one time, which may have led to inter-sample variability.

However, based on our experience and results, the difference of chemokine analysis between fresh samples and re-thawed samples is negligible and it does not undermine the reliability of our results. Despite these limitations, our results showed that total MDSC, G-MDSC and I-MDSC were significantly increased in the blood and tumors of RCC patients, and total MDSC, G-MDSC and I-MDSC in peripheral blood correlated with tumor grade. Myeloid-derived suppressor cells were found to correlated with intratumoral levels of IL-17, IL-18 and CCL2, suggesting that these cytokines promote accumulation of MDSC in the parenchyma of the RCC host and MDSC were drawn into the RCC tumor parenchyma by these factors in the tumor milieu. Blocking these cytokines may inhibit MDSC recruitment and delay tumor progression.

#### **Conclusions**

Our study has provided potential new targets for the risk stratification of patients with limited stage renal carcinoma, in addition to elucidating a possible association between MDSC subsets and cytokine-induced migration into the tumor tissue. Targeting these cytokines may reduce MDSC number and function, which could enhance the efficacy of immunotherapy and targeted therapy approaches, including immune checkpoint blockade in RCC patients.

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