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Laboratory-Kidney cancer

NK and T cells with a cytotoxic/migratory phenotype accumulate in peritumoral tissue of patients with clear cell renal carcinoma

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Abstract

Objectives: Renal cell carcinoma (RCC) is the most lethal urologic malignancy with increasing incidence worldwide. The conventional treatment strategies for advanced or recurrent RCC are not efficient and show considerable toxicities. Adoptive cell transfer (ACT) has become a promising treatment option for multiple cancers, particularly in combination with other therapeutic approaches. ACT often utilizes extensively in vitro expanded tumor-infiltrating lymphocytes (TILs). However, TILs are a very heterogeneous mix of cell populations and only those populations that have a cytotoxic and migratory potential are thought to deliver a therapeutic impact in ACT. The identification and localization of these therapeutically potent populations are therefore needed.

Methods and materials: A total number of 57 tissue samples from 19 RCC patients who underwent radical nephrectomy was analyzed. The tissue samples were obtained from the tumor, peritumoral tissue, and the adjacent healthy renal tissue. The tissues were sliced, enzymatically dissociated into single cell suspensions and the obtained cells further analyzed by flow cytometry for the expression of markers of lymphocyte cytotoxicity – TRAIL and FasL, and a surrogate marker of lymphocyte migratory activity – PECAM-1. The analyzed data were next correlated with the clinical and histopathological data.

Results: Non-clear cell RCC (non-ccRCC) tumors showed a significantly decreased tumor infiltration with $TRAIL^+FasL^+$ NK cells but elevated infiltration with $FasL^+PECAM\text{-}1^+$ T cells as compared with clear cell RCC (ccRCC) tumors. Further analyses revealed that the peritumoral tissue of ccRCC patients is a reservoir of $TRAIL^+FasL^+$, $TRAIL^+PECAM\text{-}1^+$, or $FasL^+PECAM\text{-}1^+$ NK and T cells.

Conclusions: The cytotoxic/migratory lymphocytes were identified in tumors of ccRCC patients. These lymphocytes became excluded from the tumor and accumulated in the patient's peritumoral tissue.

Keywords: Renal cell carcinoma; Tumor-infiltrating lymphocytes; TRAIL; FasL; PECAM-1; peritumoral

Abbreviations: RCC, renal cell carcinoma; ccRCC, clear cell RCC; non-ccRCC, non-clear cell RCC; mRCC, metastatic RCC; ACT, adoptive cell transfer; TILs, tumor-infiltrating lymphocytes; NK, natural killer

1. Introduction

Renal cell carcinoma (RCC) is the most lethal of urologic malignancies and its incidence is rising globally with by far the highest rates in North America and Europe [1,2]. In 2012, RCC accounted for 35,000 deaths in the European Union [3]. RCC consists of diverse histological subtypes [4] and is often stratified into 2 major histological subtypes – clear cell RCC (ccRCC) and non-clear cell RCC (non-ccRCC) [5]. The ccRCC is the predominant histologic subtype of RCC accounting for approximately 75% of RCC [6] and belongs to the most lethal histological subtype, primarily due to increased incidence of metastasis [7]. The most prevalent non-ccRCC is papillary (~16%) and chromophobe (~7%) RCC [6] and both have a much better survival rate than ccRCC.

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The current treatment options of RCC are limited. In localized and regional disease, radical or partial nephrectomy can lead to complete recovery and accounts for a 5-year RCC survival rate of 60% [1]. However, approximately one third of newly diagnosed RCC patients have advanced stage of the disease with local invasions and distant metastases [8]. The current chemotherapy, radiotherapy and targeted therapy of metastatic RCC (mRCC) have low efficacy due to resistance and side effects [9–11]. The 5-year survival rate of mRCC is only around 10% [12,13].

Adoptive cell transfer (ACT) is a rapidly evolving treatment option for multiple cancers [14,15]. Although ACT has not previously shown significant efficacy in the treatment of mRCC [16], novel approaches in this field are however promising. These approaches are either based on a combination of ACT with other immunotherapies [17] or on enhancing its efficacy per se [18]. The traditional source material for ACT is tumor-infiltrating lymphocytes (TILs) isolated from excised tumors and non-specifically expanded in vitro [15]. Because TILs are a heterogeneous mix of cell populations that vary among different types of tumors, the presence of cell populations that can deliver a therapeutic impact is not ensured in the isolated TILs. Therefore, it is necessary to first identify and evaluate those populations of TILs which have the highest potential to deliver a therapeutic impact in ACT. Among these populations are lymphocytes that express cytotoxic markers TRAIL (CD253) [19,20] and FasL (CD178) [21], and also possess a transendothelial migratory capacity as determined through a surrogate marker PECAM-1(CD31) [22,23].

In this study, we identified and evaluated the therapeutically potent cytotoxic/migratory natural killer (NK) and T cells in tumors of ccRCC patients. We found that these cells became excluded from the tumor and accumulated in the peritumoral tissue. The peritumoral tissue was then found to be the main reservoir of these cytotoxic/migratory lymphocytes.

2. Methods

2.1. Patients

A total number of 19 patients with RCC who underwent primary radical nephrectomy without previous chemo- or radiotherapy between March 2017 and February 2018 at the Department of Urology, Charles University, 2nd Faculty of Medicine and Motol University Hospital in Prague were included in the study. The mean age of the patients was 68.9 years. All patients were ≥18 years old and consisted of 7 females and 12 males. The patient's demographic and clinical information were obtained from the medical record data of the UNIS central database of the Motol University Hospital in Prague. All the patient's information were acquired with the approval from the Ethics Committee of the Motol University Hospital in Prague (EK-377/13) and all patients provided written informed consent to participate in the research study.

2.2. Lymphocyte isolation

The tumor, peritumoral and the adjacent healthy renal tissue samples from each patient were resected from the extracted kidney after the radical nephrectomy. The mean size of the resected tissue was approximately 0.5 to 1.0 cm in diameter. The peritumoral tissue samples were resected 1 cm from the tumor edge and the adjacent healthy renal tissue sample from as the most distant location from the tumor edge as possible. Each tissue sample was sliced into small pieces of approximately 1 mm^3 and disintegrated by 30-min incubation at 37 °C with mild agitation in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 1 μg/ml of collagenase A and 1 μg/ml of DNase I (Roche Diagnostics GmbH, Mannheim, Germany). The disintegrated tissue was passed through a sterile 100 μm nylon cell strainer (Corning, Corning, NY) and the strainer rinsed with PBS. The obtained cell suspension was then supplemented with Ammonium-Chloride-Potassium Lysing Buffer (Thermo Fisher Scientific) for 10 to 15 minutes at room temperature to remove red blood cells. The cell suspension was then centrifuged at 500 × g for 10 minutes at room temperature and the pelleted cells resuspended in cold (4 °C) PBS containing 2 mM EDTA (PBS/EDTA). The cells were next analyzed by flow cytometry or cryopreserved for later analysis.

2.3. Flow cytometry

The isolated lymphocytes were stained in cold PBS/EDTA for 30 minutes in the dark with the following fluorophore-conjugated protein-specific antibodies: CD45 A700 (prod.# A7-222-T100, Exbio, Prague, Czech Republic), CD56 FITC (prod.# 1F-231-T100, Exbio), CD3 PerCP-Cy5.5 (prod.# 45-0036-42,Thermo Fisher Scientific), CD253 APC, CD178 PE and CD31 PC7 (prod.# 308210, 306407, and 303118, Biolegend, San Diego, CA). The isolated cells were then washed and analyzed by FACSAria II flow cytometer (Becton Dickinson, Franklin Lakes, NJ). The obtained data were analyzed by FlowJo software (Tree Star, Ashland, OR). The $CD45^+CD56^-CD3^-$ population was used to define negative staining for FasL and TRAIL. The fluorescence-minus-one (FMO) control was used to define negative staining for PECAM-1.

2.4. Statistical analysis

Means and SEM were calculated from the indicated sample size (n) using GraphPad Prism 6 (GraphPad software, La Jolla, CA). Statistical significance ($P < 0.05$) was determined by the indicated test.

3. Results

3.1. Tumor infiltration with TRAIL$^+$FasL$^+$ NK cells is decreased in non-ccRCC tumors as compared with ccRCC tumors

In this study, 19 RCC patients who underwent radical nephrectomy were included (Table 1). The collected samples were tumor tissue, peritumoral tissue, and adjacent healthy renal tissue. The fresh tissue-isolated lymphocytes were stained with indicated antibodies and analyzed by flow cytometry (Fig. 1A). The content of T cells (CD3$^+$ cells) in the isolated leukocytes (CD45$^+$ cells) was 43.9% ($n = 19$, confidence interval [CI] 33.7–54.1) in the tumor, 36.8% ($n = 18$, CI 29.9–43.8) in the peritumoral tissue and 34.7% ($n = 19$, CI 29.0–40.3) in the adjacent healthy tissue. The content of NK cells (CD56$^+$CD3$^-$ cells) in the isolated leukocytes (CD45$^+$ cells) was 16.0% ($n = 19$, CI 11.7–20.4) in the tumor, 9.7% ($n = 18$, CI 6.6–12.7) in the peritumoral tissue and 15.2% ($n = 19$, CI 9.6–20.8) in the adjacent healthy tissue. The patients were stratified into 2 groups based on the histological subtype – 15 patients with ccRCC and 4 patients with non-ccRCC. The non-ccRCC group consisted of 3 patients with papillary and 1 patient with chromophobe RCC (Table 2). The ccRCC is more lethal than non-ccRCC [7]. We first compared the tumor infiltration with the cytotoxic/migratory NK cells in the group of ccRCC and non-ccRCC patients. As shown in Fig. 1B (left panel), the infiltration of the tumor with TRAIL$^+$FasL$^+$ NK cells was significantly higher in the group of ccRCC patients. No significant differences were observed with FasL$^+$PECAM-1$^+$ and TRAIL$^+$PECAM-1$^+$ NK cells (Fig. 1B, two right panels). These data showed that the less lethal histological subtypes of RCC were associated with decreased intratumoral infiltration with TRAIL$^+$FasL$^+$ NK cells.

Table 1
Demographic data of patients and their tumors.

Parameter	Value
Patients	19
Age (years)	
Median	68.9
Range	48–81
Males/Females	12/7
Histology	
Papillary	3
Chromophobe	1
Clear cell	15
Furhman tumor grade	
1	3
2	9
3	5
4	1
Not classified	1
TNM tumor stage	
T1a	3
T1b	8
T2	1
T2a	1
T2b	0
T3a	5
T3b	1
T3c	0
T4	0

3.2. Tumor infiltration with FasL$^+$PECAM-1$^+$ T cells is elevated in non-ccRCC tumors as compared with ccRCC tumors

We next compared the tumor infiltration with the cytotoxic/migratory T cells in the group of ccRCC and non-ccRCC patients. As shown in Fig. 1C (two left panels), no differences were observed with TRAIL$^+$FasL$^+$ and TRAIL$^+$PECAM-1$^+$ T cells. However, the infiltration with FasL$^+$PECAM-1$^+$ T cells was significantly higher in tumors of non-ccRCC patients (Fig. 1C, right panel). These data showed that the less lethal histological subtypes of RCC were associated with increased intratumoral infiltration with FasL$^+$PECAM-1$^+$ T cells.

3.3. Cytotoxic/migratory NK and T cells accumulate in the peritumoral tissue of ccRCC patients

The finding that the histological subtype of RCC, which impacts survival of RCC, is associated with the cytotoxic/migratory signature of intratumoral NK and T cells prompted us to investigate this signature beyond the tumor itself and analyze the infiltration with these cells also in the peritumoral tissue and the adjacent healthy renal tissue. As shown in the left panels of Fig. 2, NK cells with all the 3 tested cytotoxic/migratory phenotypes significantly accumulated in the peritumoral tissue as compared with the tumor itself (Fig. 2, left panels). In addition, TRAIL$^+$PECAM-1$^+$ NK cells were also significantly increased in the adjacent healthy renal tissue as compared with the tumor itself (Fig. 2, middle left panel).

T cells with all the 3 tested cytotoxic/migratory phenotypes were also significantly increased in the peritumoral tissue as compared with the tumor itself (Fig. 2, right panels). Unlike NK cells, not only TRAIL$^+$PECAM-1$^+$ but also TRAIL$^+$FasL$^+$ and FasL$^+$PECAM-1$^+$ T cells were significantly increased in the adjacent healthy renal tissue as compared with the tumor itself (Fig. 2, right panels).

4. Discussion

In this work, we found that the peritumoral tissue of ccRCC patients constitute a reservoir of cytotoxic/migratory NK and T cells. As these cells were previously found to deliver a superior impact against cancer cells both in vivo and in vitro [19–21], the peritumoral tissue of ccRCC patients might then represent an invaluable source material for isolation and use of these cells for ACT of ccRCC.

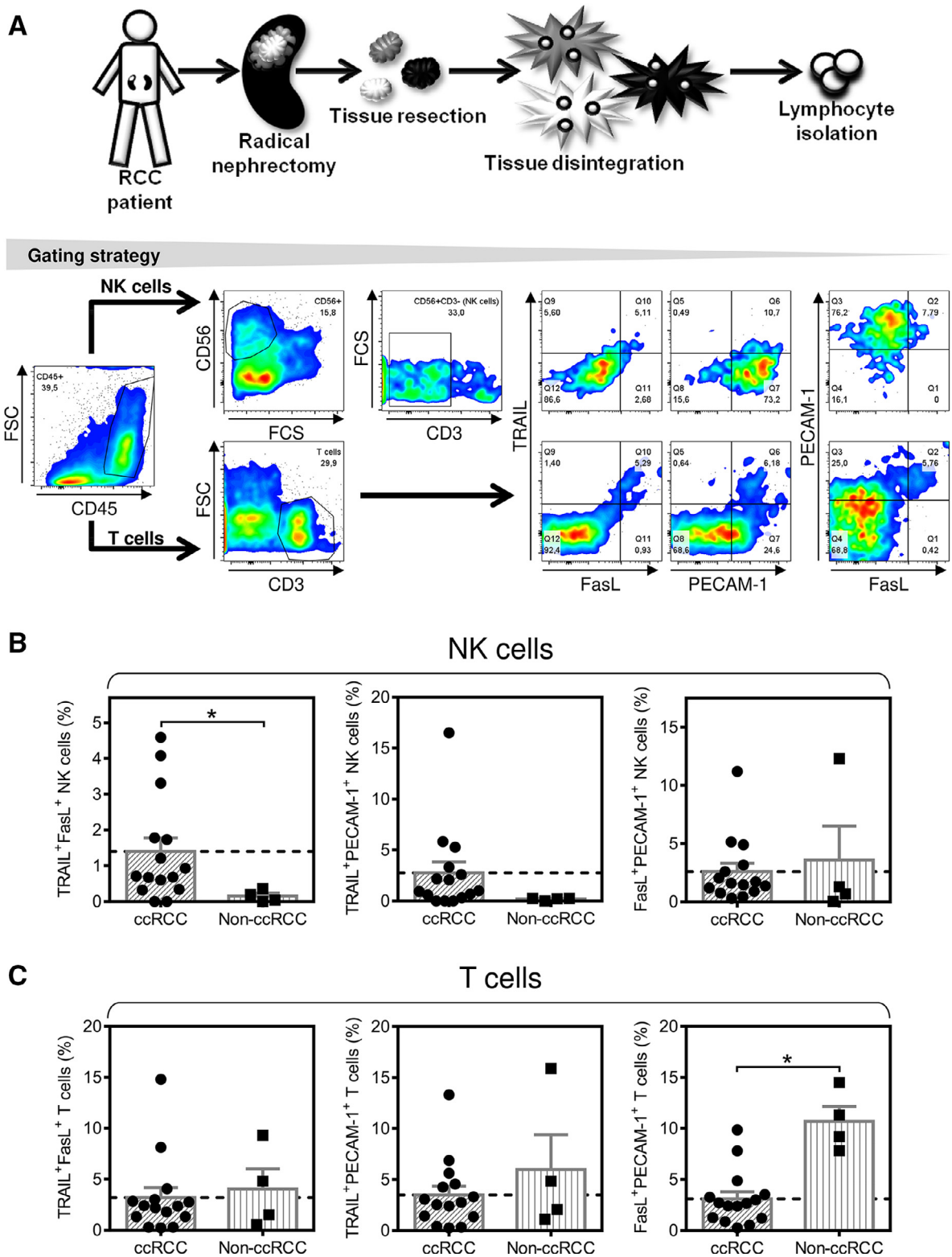

Fig. 1. The tumor and the surrounding milieu infiltration with cytotoxic/migratory NK and T cells in RCC patients. (A) Schema of the lymphocyte isolation from the tumor (white), peritumoral (grey) and the adjacent healthy renal (black) tissue after radical nephrectomy. The gating strategy for data analysis from flow cytometry. The isolated tissue lymphocytes were stained with CD45, CD3, CD56, TRAIL, FasL, and PECAM-1 specific antibodies. NK cells were gated as $CD45^+CD56^+CD3^-$ cells and T cells as $CD45^+CD3^+$ cells (left panels). The population of NK and T cells with the combined expression of cytotoxic markers (TRAIL, FasL) and/or the migratory marker (PECAM-1) was determined (right panels). (B–C) The population of $TRAIL^+FasL^+$ (left panels), $TRAIL^+PECAM\text{-}1^+$ (middle panels) and $FasL^+PECAM\text{-}1^+$ (right panels) tumor-infiltrating NK (B) or T (C) cells was determined in 15 ccRCC and 4 non-ccRCC (1 chromophobe and 3 papillary RCC) patients. The difference between the 2 groups was statistically evaluated by Mann-Whitney test (ccRCC, $n = 15$; non-ccRCC, $n = 4$). $^*P < 0.05$ was considered significant. The data are shown as mean+SEM.

Table 2
Demographic data of patients and their tumors stratified into 2 groups based on the histological subtype – 15 patients with ccRCC and 4 patients with non-ccRCC.

Characteristic	ccRCC patients (n)	ccRCC patients (%)	Non-ccRCC patients (n)	Non-ccRCC patients (%)
Age				
> 75	5	33.3	0	0
75 – 65	7	46.7	2	50
< 65	3	20	2	50
Sex				
Male	9	60	3	75
Female	6	40	1	25
Surgical nephrectomy				
Laparoscopic	11	73.3	3	75
Open	4	26.7	1	25
Fuhrman tumor grade				
1	2	13.3	0	0
2	8	53.3	1	25
3	4	26.7	1	25
4	1	6.7	0	0
Not classified	0	0	2	50
TNM tumor staging				
T1a	3	20	0	0
T1b	7	46.7	1	25
T2	1	6.7	0	0
T2a	0	0	1	25
T2b	0	0	0	0
T3a	4	26.7	1	25
T3b	0	0	1	25
T3c	0	0	0	0
T4	0	0	0	0
Smoking history				
Yes	3	20	2	50
No	12	80	2	50
Chronic diseases				
COPD	1	6.7	1	25
Diabetes mellitus	4	26.7	1	25
Ischemic heart disease	4	26.7	1	25
Hypertension	10	66.7	3	75
Stauffer syndrome				
Yes	5	33.3	1	25
No	10	66.7	3	75
Erythrocytosis				
Yes	0	0	0	0
No	15	100	1	100
Thrombocytosis				
Yes	1	6.7	0	0
No	14	93.3	0	0

RCC belongs to tumors where increased infiltration with immune cells is, in contrast to many other tumors, associated with rather worse prognosis and overall survival [24,25]. More detailed analysis however also found that a better prognosis was associated with the infiltration where increased labeling with the proliferation-associated antigen Ki-67 in $CD8^+$ T cells was observed [26]. Our data showed that tumor infiltration with cytotoxic/migratory ($FasL^+PECAM\text{-}1^+$) T cells was significantly elevated in non-ccRCC patients as compared with tumors of ccRCC patients. Surprisingly, the opposite was found for the cytotoxic ($TRAIL^+FasL^+$) NK cells. Since non-ccRCC patients have much better survival than ccRCC patients, these data implicate that tumor infiltration with the cytotoxic/migratory T cells might be also associated with better patients' survival, whereas the infiltration with the cytotoxic NK cells might indicate the opposite.

Recent studies have shown that the characterization of immune signatures beyond the tumor itself can broaden the view on the disease status and its prognosis [27]. Recently, Giraldo et al. showed that the presence of mature dendritic cells in the peritumoral immune aggregates in combination with tumor infiltration with $CD8^+$ T cells with low expression of checkpoint molecules in ccRCC patients is associated with good prognosis [28]. Our results also corroborate the necessity for a more detailed characterization of the

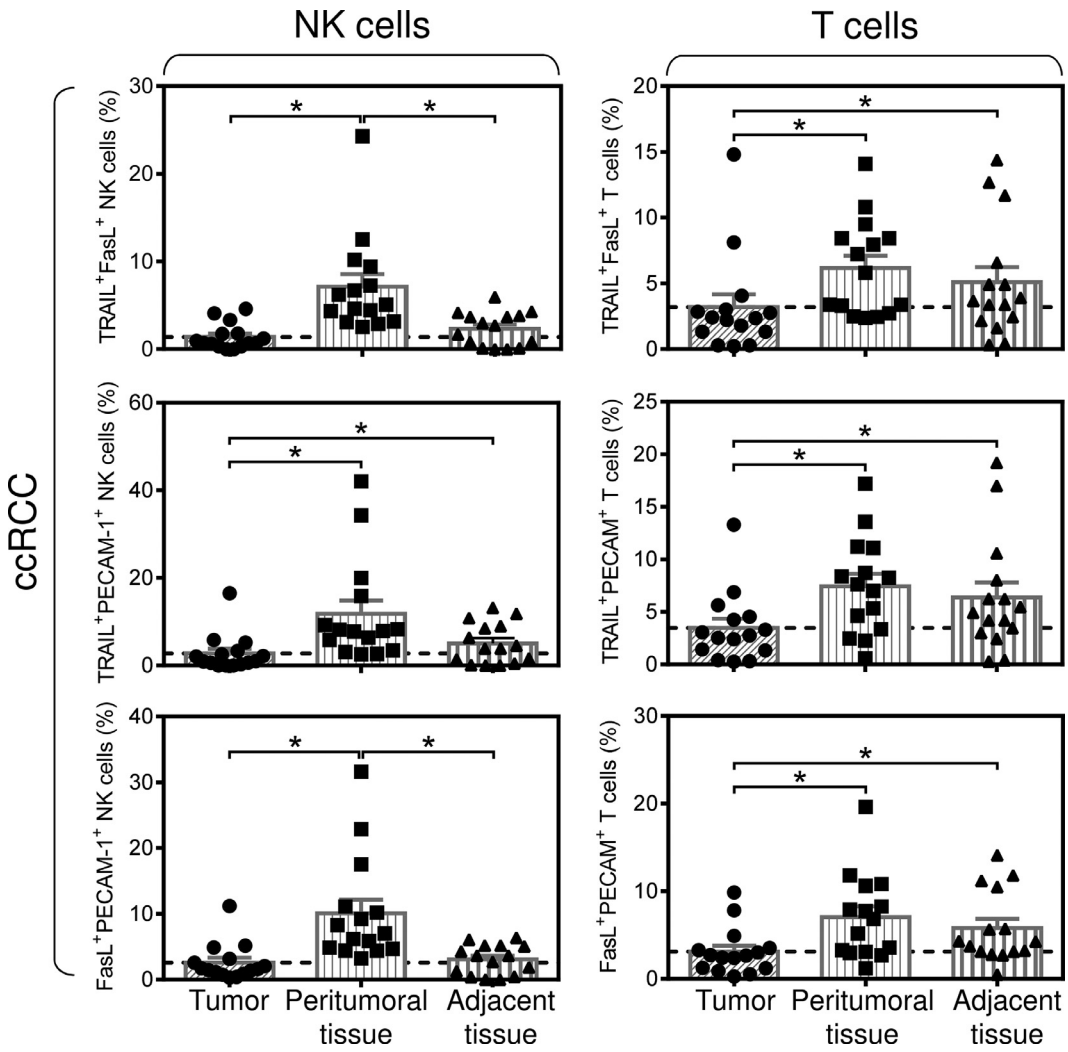

Fig. 2. Infiltration of the tumor, peritumoral and adjacent healthy renal tissue (Adjacent tissue) with cytotoxic/migratory NK and T cells in ccRCC patients. The lymphocytes of 15 ccRCC patients were isolated from the indicated tissues, stained and processed as in Fig. 1A. The population of TRAIL$^+$FasL$^+$ (top row), TRAIL$^+$PECAM-1$^+$ (middle row) and FasL$^+$PECAM-1$^+$ (bottom row) infiltrating NK (left panels) and T (right panels) cells was determined. The differences among the populations from the tumor (left bars), peritumoral (middle bars) and adjacent healthy renal tissue (Adjacent tissue; right bars) were statistically evaluated by Wilcoxon matched-pairs signed-ranks test ($n = 15$). *$P < 0.05$ was considered significant. The data are shown as mean+SEM.

immune cell signatures beyond the tumors themselves and also the necessity to correlate these signatures with factors that impact severity and/or prognosis of the disease. Through these extended analyses the immune cells with more promising phenotypes for ACT could be identified and exploited.

Many studies point to the important role of TILs not only in the prognosis and survival of multiple cancers but also as invaluable source material for ACT. However, very few studies considered the importance of peritumoral lymphocytes [28] and their plausible use in ACT. By going beyond the tumor itself in this study, we revealed that peritumoral tissue of ccRCC patients constituted a depo of NK and T cells with a cytotoxic/migratory phenotype. NK and T cells with this phenotype were previously found to have enhanced capability to eliminate cancer cells [19–21] and to contribute to their migration in vivo [23]. Moreover, one type of these cells, FasL$^+$PECAM-1$^+$ T cells, showed higher infiltration into those histological subtypes of RCC tumors that are associated with less metastatic potential and better overall survival [7]. Taken together, we show that peritumoral tissue of ccRCC patients is an invaluable source of immune cells with a cytotoxic/migratory phenotype. Which of these cells also have the potential to deliver a therapeutic impact once used as source material for ACT needs to be however determined.

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Conflict of interests

SP and JB are part-time employees and JB also a minority shareholder of SOTIO, a.s., a biotech company developing cell-based immunotherapy. ZS, PT, DmS, KH, SV, and DaS declare no conflict of interest.

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