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Treatment-induced changes of lymphocyte subsets in patients with adenoid cystic carcinoma of the head and neck

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Abstract

Purpose Adenoid cystic carcinoma (ACC) of the head and neck is a rare and highly malignant tumor, characterized by perineural growth and early distant metastases. The composition of immune cells in the peripheral blood and the tumor microenvironment is critical to tumor growth and control. However, little is known about the frequency and function of the relevant immune cell subsets in this entity.

Methods In ACC patients ($n=11$) and matched healthy donors ($n=11$), the frequency of peripheral blood T and B cells was measured by flow cytometry at different treatment stages of disease (24 samples). Cells were further characterized by their expression of CCR7, PD-1, CD39 and CD73. Tumor-infiltrating lymphocytes (TIL) were analyzed by immunohistochemistry for ten patients and for three patients by flow cytometry.

Results $CD4^+$ T cells had significantly lower frequency after radiotherapy (RT). All other cell frequencies, including T_{reg}, were stable through course of the disease. In B cells, CD73 was reduced after RT. CCR7 expression on T and B cells in patients with relapse/metastases (R/M) differed significantly from patients with active disease. PD-1 remained stable. T_{reg} were more present in TIL compared to peripheral blood.

Conclusion Composition of lymphocyte subgroups behaves similar to squamous cell carcinoma in the head and neck, except for T_{reg}, which remained stable. Nevertheless, the $CD4^+/T_{reg}$ ratio was lower after RT, which could stand for an immunosuppressive effect in these patients. Therefore, it could be beneficial treating ACC with combined RT and immunomodulatory drugs.

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Abbreviations

ACC	Adenoid cystic carcinoma
TIL	Tumor-infiltrating lymphocytes
RT	Radiotherapy
R/M	Relapse/metastases
CAP	Cisplatin, doxorubicin, and cyclophosphamide
ADO	Adenosine
PBMC	Peripheral blood mononuclear cells
NC	Normal controls
AD	Active disease
mAbs	Monoclonal antibodies
FSC	Forward scatter
SSC	Side scatter
MFI	Mean fluorescence intensity
SRG	Surgery

Introduction

Adenoid cystic carcinoma (ACC) of the head and neck is a rare disease with an incidence of about 4.5 newly diagnosed cases per 100,000 persons [1]. The prognosis remains poor with reported 5 and 10-year survival rates of 77.3% and 59.6%. Involvement of lymph nodes significantly reduce median survival by threefold, the development of distant metastases by at least 20-fold, respectively [2]. ACC arise not only in salivary glands, but in a wide spectrum of locations within the head and neck area. Within the minor salivary glands, they represent up to 71% of all malignant tumors and arise mostly in the palate. The second most probable location is the submandibular gland with approximately 40% ACC of all malignant tumors, followed by the parotid gland and sinonasal tract [3, 4]. Therapy of these tumors is challenging, and it is performed according to other entities in the head and neck area. Currently, surgery is the gold standard in a locally confined disease followed by adjuvant radiation. However, radiation did not improve the overall survival in a large retrospective SEER analysis [2]. Non-resectable tumors and recurrent or metastatic diseases (R/M) are treated with conventional chemotherapy such as Cisplatin, 5-FU and CAP regimen (cisplatin, doxorubicin and cyclophosphamide)—all of them showing limited efficacy [1, 2].

For a long time, ACC remained an understudied entity because of its rareness. In the past years, more studies have emerged trying to characterize ACC molecularly. Whole-genome sequencing identified several somatic mutations and confirmed a MYB–NFIB fusion with high prevalence in salivary gland ACC, which could be an interesting therapeutic target [5]. The role of the immune system in ACC development and control; however, remains unclear as only very few studies have been published on this topic. Chang and colleagues showed a negative prognostic influence of low PD-L2 expression on relapse-free survival and, more interesting, a particularly low presence of $CD8^+$ tumor-infiltrating lymphocytes (TIL) in ACC compared with other salivary gland malignancies [6].

T cells are among the most interesting cell types in cancer development and control. For several cancer types $CD8^+$ T cells are linked to a good prognosis, while T_{reg} are usually linked to a worse prognosis in most cancer entities [7–9]. Tumor-infiltrating B cells on the other hand are associated with a good outcome in several cancer types additionally to HNSCC [10–13].

For $CD8^+$ T cells it has been shown, that a lower prevalence of $CD8^+CCR7^+$ T cells is linked to disease recurrence of HNSCC, for ACC the effect of CCR7 is unknown [14]. The PD-1/PD-L1 axis is known to be involved in immune escape of cancer cells [15]. It has been shown that PD-1 expression is increased both on circulating and intratumoral T cells of HNSCC patients with a significantly higher rate of $PD\text{-}1^+$ tumor-infiltrating T cells than $PD\text{-}1^+$ circulating T cells [16]. Therefore, the approval of the PD-1 inhibitor nivolumab for platinum-refractory HNSCC in 2017 has attracted particular attention and further indications are tested at the moment [17]. PD-1 may also be an interesting target for therapies in ACC patients. Concerning immunosuppression, adenosine (ADO) is a well known immunosuppressive nucleoside, published that it is highly accumulated in tumor tissue [18, 19]. ADO is metabolized via the ectonucleotidases CD39 and CD73 [20–25]. In addition, a CD73-antibody is currently tested in a clinical phase I study for solid tumors in combination with a PD-1 antibody (NCT02503774).

Our aim was to investigate the role of the immune system with its effector as well as suppressive functions during the course of disease in patients suffering from ACC to gain a better understanding and identify potential immunotherapeutic approaches.

Materials and methods

Blood samples

Peripheral blood mononuclear cells (PBMC) were obtained from patients suffering from ACC ($n = 11$) and from normal controls (NC; $n = 11$), matched by age (± 5 years), who signed an informed consent form approved by the local ethical committee of the Ulm University (#255/14). At the time of blood withdrawal, eight patients suffered from an active disease (AD), five patients were within their first year after surgery (SRG), six patients had undergone radiotherapy (RT) within 12 months before blood withdrawal and five patients had a recurrent tumor or distant metastasis (R/M) within 1 month before blood withdrawal. Tumors of patients with surgery as primary treatment had negative margins, except for case #7, which had minimal residual disease before undergoing adjuvant RT. A total of 24 samples were collected of the patients during this study. Blood (50 ml) was collected in S-monovettes prefilled with trisodium citrate (Sarstedt) and centrifuged on Biocoll Separating Solution (Merck). PBMC were recovered, washed twice in PBS and stored for further experiments in a freezing medium containing FBS and DMSO in liquid nitrogen. The patients presented with different tumor sites and blood was drawn at different time points during treatment and course of disease. Detailed patient characteristics are listed in Table 1.

Table 1 Patient characteristics at initial diagnosis

Case #	Age	Location	T	N	M	DFS (m)	OS (m)	Subgroup
1	58	Base of tongue	2	0	0	19	51	AD, SRG, RT, R/M
2	61	Base of tongue	2	0	0	37	37	AD, SRG, RT
3	53	Lacrymal gland	4c	0	0	39	68	RT, R/M
4	55	Parotid gland	2	0	0	42	42	AD, RT
5	74	Soft palate	3	2b	0	16	50	AD, R/M
6	35	Nasopharynx	4b	0	0	22	61	SRG, RT, R/M
7	37	Nasal cavity	4a	0	0	40	40	AD, RT
8	53	Soft palate	2	0	0	33	33	AD, SRG
9	60	Hard palate	4a	0	0	3	3	AD, SRG
10	60	External auditory meatus	1	0	0	103	103	AD
11	63	Submandibulary gland	1	1	0	64	79	R/M

Staging is the initial pathological TNM (v8 AJCC) for most cases, except #3 and #6 (clinical staging before definite radiotherapy)

DFS disease-free survival, *OS* overall survival, subgroups: *AD* active disease, *SRG* surgery, *RT* radiotherapy, *R/M* recurrent/metastatic disease

Tumor-infiltrating lymphocytes

Tumor tissue samples of three patients were collected in sodium chloride directly after surgery. The tumor pieces were minced and collected in RPMI medium (gibco) containing 200 IU/ml Collagenase I (Worthington) for at least 3 h at 37 °C in a shaking water bath. The tissue pieces were mashed with a 100 µm EASY strainer (gibco) after digestion. The lymphocyte fraction was isolated via Biocoll centrifugation as mentioned above and stained for flow cytometry, after lysis of erythrocytes with Red Blood Cell Lysis Solution (Miltenyi Biotec).

Moreover, we performed immunohistochemistry for detecting TIL on ten patients with available archived specimen. Nine samples came from primary diagnosis and one sample from a distant metastasis. Immunohistochemistry was performed according to established protocols, details about antibodies are provided in Table S1.

Antibodies and reagents

The following anti-human monoclonal antibodies (mAbs) were used for flow cytometry: CD4 Alexa Fluor® 700, CD8 APC, CD39 PE-Cy7, CD73 FITC, and CD73 eFluor450, CCR7 (CD197) PE-Cy7, PD-1 (CD279) PE (eBioscience); CD19 PE-Cy5, CCR7 PE-CF594, CD45 AmCyan and CD45 FITC (Becton Dickinson), CD3 APC-H7, CD25 FITC, CD25 PE (MACS Miltenyi). All mAbs were titrated using PBMC of healthy donors to establish optimal dilution.

Surface staining

PBMC were thawed and immediately centrifuged with PBS to dispose DMSO. TIL were stained directly. The cells were incubated separately for the different stainings and labeled with mAbs at room temperature for 30 min in the dark. After the incubation time, PBMC were washed and collected in 250 µl PBS-containing 0.5% BSA for flow cytometry analysis. All flow cytometry measurements were performed using a Gallios 10-color-flow-cytometer equipped with Kaluza flow cytometry software (both Beckman Coulter). The acquisition and analysis gates were restricted to the lymphocyte gate based on characteristic properties of the cells in forward and side scatter (FSC and SSC). At least 10^5 cells were acquired for analysis.

Statistical analysis

All data are presented as medians with interquartile ranges (except the TIL data, which were shown as median only, with all measured data points) of at least three separate experiments. Data were analyzed for Gaussian distribution with Kolmogorov–Smirnov test, as for other tests the sample numbers were too few. Unpaired *t* test or Wilcoxon–Mann–Whitney *U* test were used for comparison. p values ≤ 0.05 were considered to be significant. Statistical analyses were performed using GraphPad Prism Version 6.07 and SPSS (IBM) Version 23.

Results

To investigate the alterations of the immune system in ACC patients, we compared blood samples of normal controls (NC) with those of tumor patients at different time points in their course of disease, in particular before treatment, after radiotherapy and in case of recurrence or distant metastasis.

Figure 1 shows the gating strategy we used to identify the different subpopulations. We divided $CD3^+$ T cells and $CD8^+$ T cells with CD3- and CD8-mAbs (Fig. 1a) and B cells and $CD4^+$ T cells by CD19 and CD4 (Fig. 1b). T_{reg} were characterized by expression of CD4, CD25 and CD39 as published before (Fig. 1c) [21]. For further characterization we used CD39 and CD73 on the different subpopulations. Here we showed that B cells of healthy subjects are divided in two subpopulations, which are either $CD39^+$ or $CD39^+CD73^+$. $CD4^+$ T cells are either $CD39^+$ or $CD73^+$ or negative for both, while $CD8^+$ T cells are $CD73^+$ or negative for both markers (Fig. 1d). Figure 1e showed that all subpopulation express different frequencies of CCR7 but less PD-1.

Differences in lymphocytic cell populations at different treatment stages

After RT, the frequency of $CD3^+$ ($p > 0.01$) and $CD4^+$ T cells ($p = 0.03$) was decreased as compared to AD. The frequency of $CD3^+$ T cells was further decreased in patients with R/M disease ($p = 0.05$) (Fig. 2a–d). The frequencies of all T cell populations ($CD4^+$, $CD8^+$ and T_{reg}) showed no differences between NC and AD patients. For B cells, there were no differences measurable in the present cohort (Fig. 2e).

Decreased CD73 on B cells after radiotherapy

On B cells, the mean fluorescence intensity (MFI) of CD73 for AD was significantly higher compared with the MFI of RT patients ($p > 0.01$; Figure S1A). The MFI of CD73

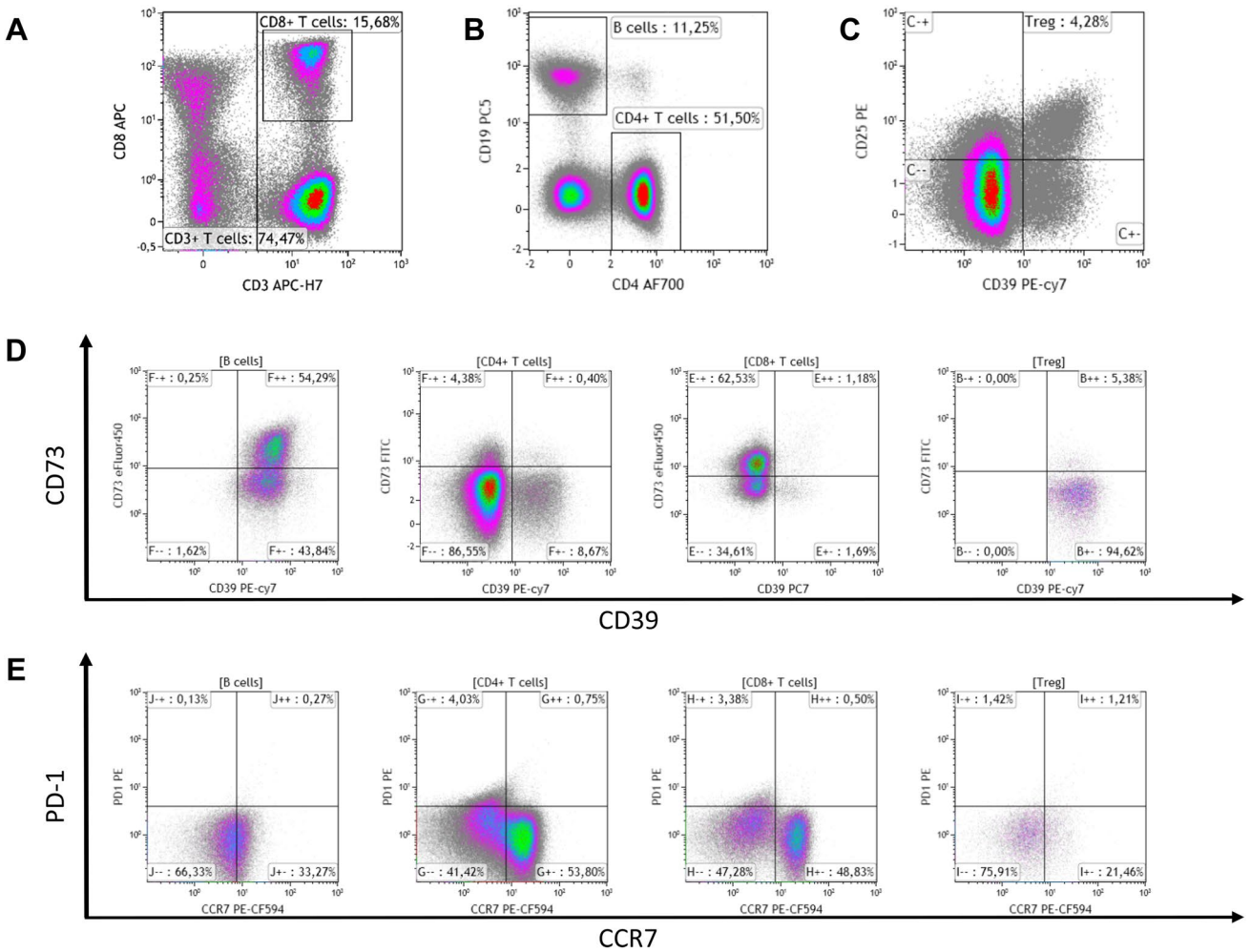

Fig. 1 Gaiting strategy. We analyzed the different lymphocytic fractions of PBMC by flow cytometry. The exemplary staining protocol of a normal control (NC) is shown. PBMC were stained for $CD3^+$, $CD4^+$, and $CD8^+$ T cells as well as for B cells and the $CD4^+$ T cell subpopulation $CD4^+CD25^+CD39^+$ T_{reg} **(a–c)**. For further analyses cells were labeled for CD39 and CD73 **(d)** and in a second step for PD-1 and CCR7 **(e)**

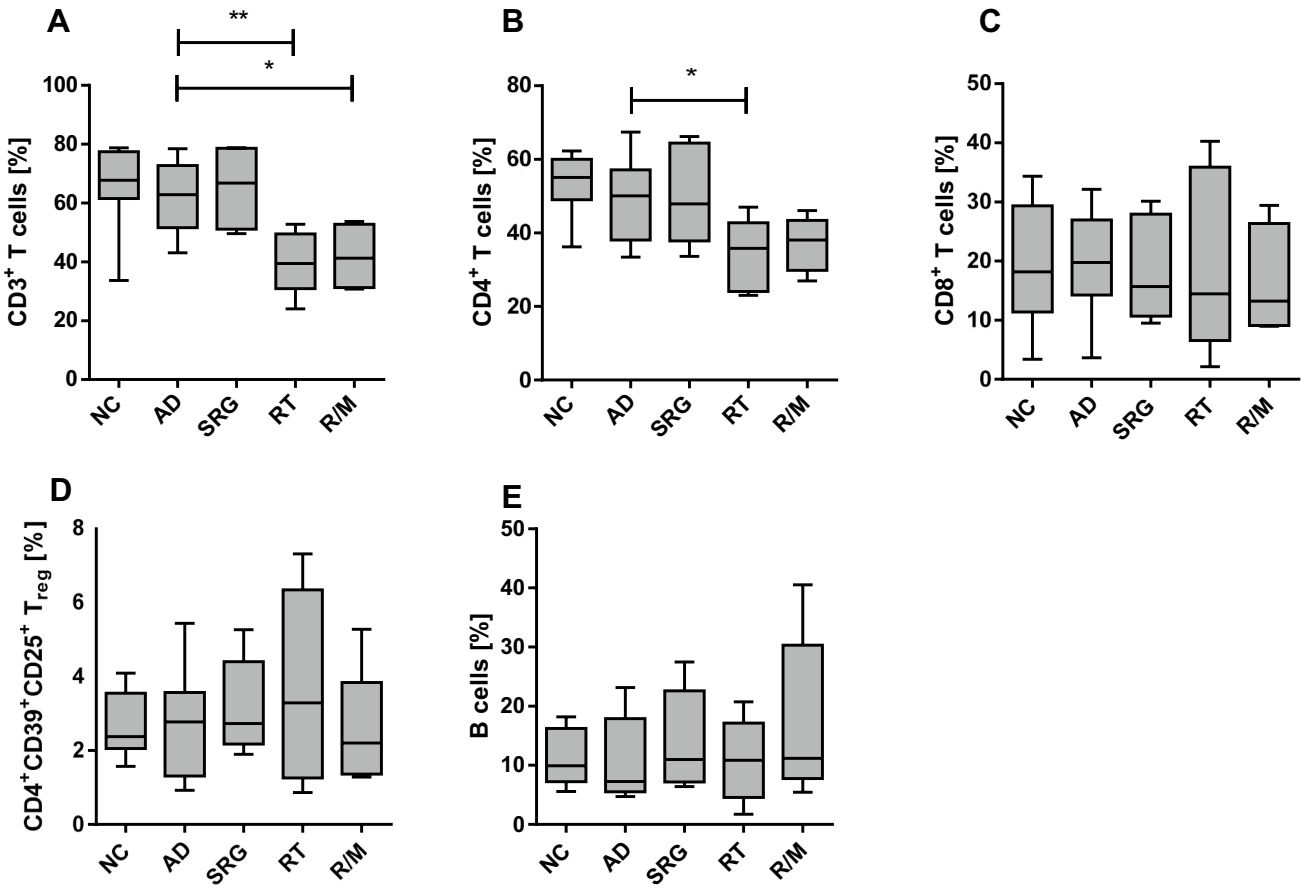

Fig. 2 Lymphocyte populations of different ACC patients for specific stages of cancer treatment compared to healthy donors. The frequencies of the lymphocytic cell populations $CD3^+$ T cells **(a)**, their subsets $CD4^+$, $CD8^+$ T cells **(b/c)** and T_{reg} **(d)**, and $CD19^+$ B cells **(e)** were measured by flow cytometry in PBMC of healthy donors and matched ACC patients at different stages of their treatment (*NC* normal control, *AD* active disease, *SRG* after surgery, *RT* after radiotherapy, *R/M* after recurrence/metastasis). $p \leq 0.05$ (*), $p \leq 0.01$ (**)

showed no significant changes for the T cell subpopulations (Figure S1B–D). The MFI of CD39 for all measured cell populations showed no significant differences (Figure S1E–H). Only $CD39^+CD73^+$ B cells showed a significantly lower frequency when comparing patients with AD and after RT ($p = 0.02$) (Figure S1I). Neither $CD4^+CD39^+$ nor $CD4^+CD73^+$ nor $CD8^+$ $CD73^+$ T cells showed any differences (Figure S1J-L).

Expression of $CCR7^+$ decreases in R/M disease, while PD-1 remained stable

We analyzed the expression frequencies of CCR7 on the lymphocytic populations of PBMC in ACC patients during their course of disease. The MFI of CCR7 on B and T cells was significantly lower in patients with R/M disease ($p = 0.04$) (Fig. 3a–c). The MFI of CCR7 on T_{reg} remained stable through the course of the disease (Fig. 3d). The same analysis was done for the expression of PD-1 (Fig. 3e–h) but no significant differences were measurable.

Differences between the PBMC and TIL of ACC patients

We were able to isolate TIL from three ACC patients and compared them with PBMC of the same day of surgery. Two of the samples, both T2 status and both of the base of the tongue (patient #1 and #2) were collected in the first surgery after diagnosis, while the third sample (#3) was obtained from a patient with advanced disease of the lacrimal gland with a T4c status and after the second relapse, having already developed a metastasis. As only three samples were available for comparison, statistical significance could not be reached. Nevertheless, we report about the observations to give a thought-provoking impulse. The tumor tissue had decreased frequency of B cells in two cases, but not in case #1, and decreased T_H cells in all cases (Fig. 4a, b). The frequency of T_{reg} as compared with the patients' peripheral blood was increased (Fig. 4c). In the TIL populations, we observed a higher value for both $CD4^+$ T cells and B cells in patient #1. This patient is, despite metastasis, still alive. The

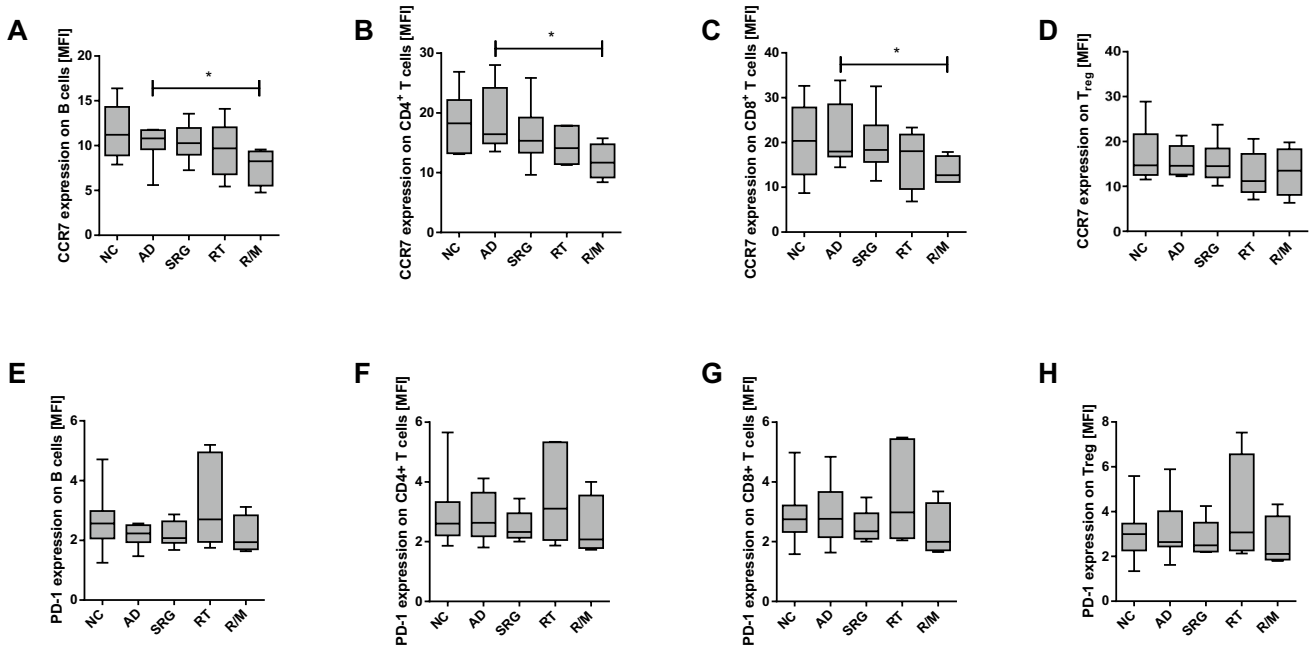

Fig. 3 The expression of CCR7 and PD-1 on lymphocytes of ACC patients. The MFI of CCR7 on B **(a)** and T cells **(b/c)** and T_{reg} **(d)** on ACC patients in different stages of their treatment compared to healthy subjects The MFI of PD-1 on B cells **(e)**, $CD4^+$ **(f)**, $CD8^+$ T cells **(g)** and T_{reg} **(h)** were plotted for PBMC of ACC patients and matched healthy donors; $p < 0.05$ (*)

metastasis developed more than 1 year after collecting the analyzed blood sample (Fig. 4a, b). The expression frequencies of CD39 and CD73 on B and $CD4^+$ T cells showed no significant differences in TIL (Fig. 4d, e).

Additionally, we evaluated TIL in formalin-fixed sections of ten patients (Fig. 5). We found seven of ten tumors to present with only very few TIL and with cytotoxic T cells localized mainly at the borders of the tumor (Fig. 5a), which is understood as an infiltrated–excluded distribution pattern [26]. One tumor was completely void of any TIL. Only two tumors had a relevant number of TIL within the tumor, forming an infiltrated–inflamed phenotype. In both tumors we could additionally identify tertiary lymphoid structures (Fig. 5b). Due to the limited number of patients no statistically significant survival differences could be calculated. Because the timepoints of the archived formalin-fixed material and blood withdrawal were differing, no correlation could be done.

Discussion

We investigated the role of the immune system in 11 patients suffering from ACC by flow cytometry. In a first step, we analyzed frequencies of lymphocyte subsets and looked for differences between matched normal controls and patients with active disease as well as differences through the course of disease, in particular after any form of radiotherapy and in patients with recurrent or metastatic ACC. In a next step, we analyzed the lymphocytes for different surface markers and compared them as described above.

Compared with normal controls, T_{reg} frequencies did not differ significantly in patients with active disease. This is contrary to results for HNSCC, for which higher T_{reg} frequencies in patients with active disease could be shown [21], while the frequencies of other lymphocyte populations do not differ. These observations result in the hypothesis that immunosuppression through ACC is lower compared to HNSCC.

After RT we observed significant lower frequencies of $CD3^+$ and $CD4^+$ T cells. Moreover, we observed significant lower frequencies of $CD3^+$ T cells in patients with R/M disease. Sridharan and colleagues found a slightly different pattern with stable $CD8^+$ T cells, but slightly increased T_{reg} 7 weeks after completion of CRT in a single-patient analysis. However, in this study no data about $CD4^+$ T cells were presented, the definition of T_{reg} was different and the patients did not receive RT alone, but a combination with concurrent cisplatin [27]. When comparing these data to an earlier work of our group on HNSCC, differences in T_{reg} frequency become obvious, as we reported about elevated frequencies over 3 years after CRT [28]. In the current study, stable T_{reg} frequencies with lower $CD4^+$ frequencies after RT result in a lower CD4/T_{reg} ratio, which leads to the suggestion of an immunosuppression of non-regulatory $CD4^+$ T cells by T_{reg} within the 1st year after RT of ACC.

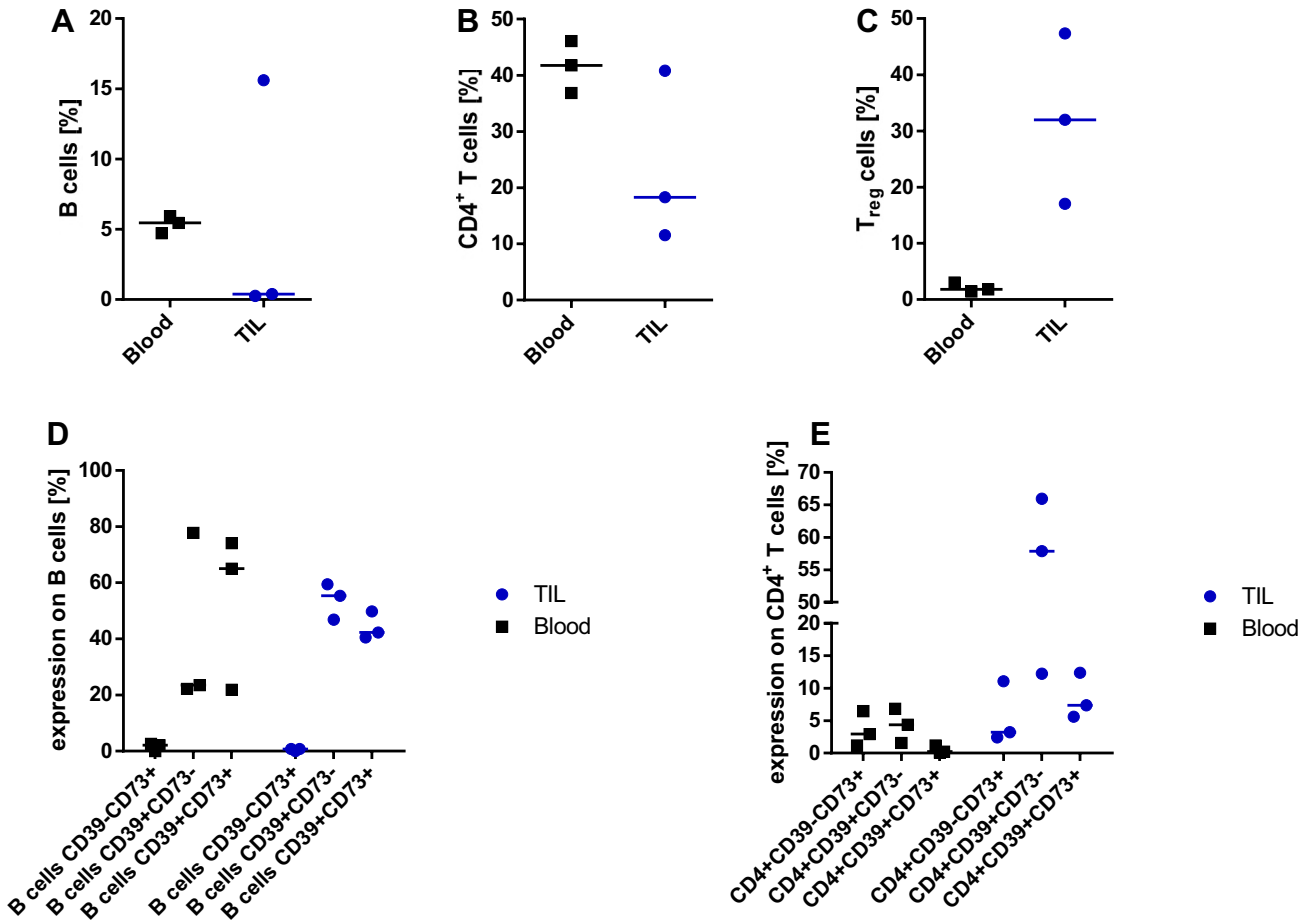

Fig. 4 Differences of peripheral blood lymphocytes and TIL. The lymphocytes isolated out of the tumor were stained and analyzed by flow cytometry for $CD4^+$ T cells **(a)**, B cells **(b)** and T_{reg} **(c)** and compared to the frequencies of the patients' blood. For further analyses the B **(d)** and $CD4^+$ T cells **(e)** were stained for CD39 and CD73

The MFI of CD73 on B cells in our cohort is significantly lower after RT, as the frequency of $CD39^+CD73^+$ B cells is, too (Fig. 3a, i). This is an interesting observation as $CD39^+CD73^+$ B cells could be responsible for an immunosuppressive microenvironment in the tumor. This effect was also visible in HNSCC, where the expression frequency of CD73 significantly decreased after CT [24]. ADO is an immunosuppressive nucleoside that is highly accumulated in tumor tissues and is produced by the ectonucleotidase CD73, which can metabolize 5′-AMP to ADO [19, 20]. B cells are the best ADO-producing cells in peripheral blood as these cells are the only ones that additionally express CD39 for the production of 5′-AMP from ATP (Fig. 1) [20]. Downregulation of CD73 may be indicative for a response to treatment and a downregulation of the immunosuppressive capacity of the B cells. However, we could not compare the response rate to a low CD73 expression because of a limited patient number.

T cells and dendritic cells normally express CCR7 for trafficking to lymph nodes [29]. Our results show a lower MFI of CCR7 on B and T cells in patients with recurrent disease. This fits to observations made for HNSCC where a low $CD8^+CCR7^+$ T cell presence was associated with a higher risk of disease recurrence [14]. Further analysis of this observation would probably be worthwhile to identify patients with a higher risk for recurrent ACC. Nevertheless, our cohort of 11 patients was very small and partly inhomogeneous with different tumor sites due to the rarity and heterogeneous locations of origin of ACC in general. To verify the above results, we encourage scientist to join together for multicentric analyses of this entity.

When looking at the differences of PBMC and TIL, we saw a predominance of T_{reg} in the tumor tissue as shown earlier for HNSCC [16]. As we had only three patient samples available for these analyses, the results have to be taken carefully. However, since T_{reg} in HNSCC and other carcinomas is elevated [9, 30], it might well be a characteristic of ACC. In immunohistochemical analysis, we found most tumors to be infiltrated–excluded by cytotoxic T cells, which is associated with a promoted immune escape compared to

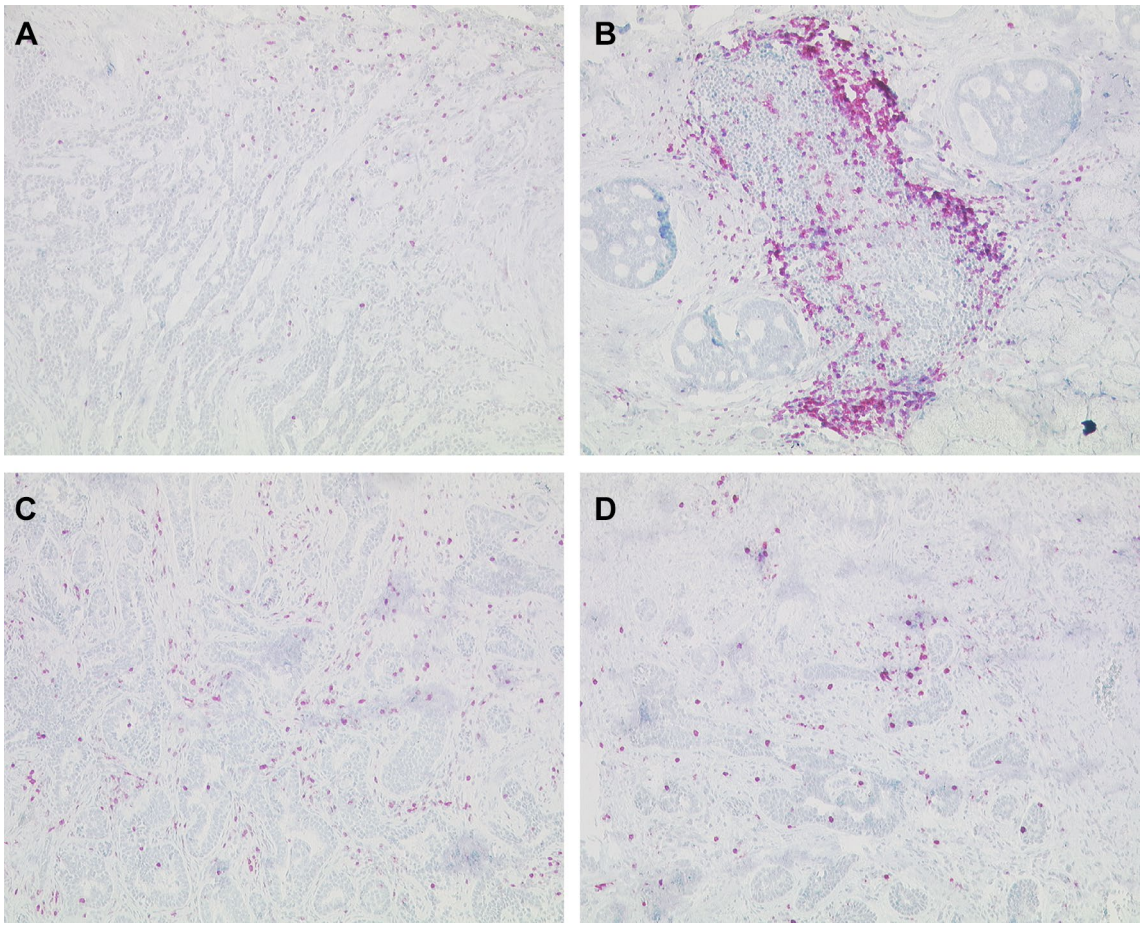

Fig. 5 Examples of TIL staining by CD3 and CD8 immunohistochemistry. Three different TIL distribution patterns could be analyzed. TIL (CD3) distribution mainly at the tumor borders is considered "infiltrated–excluded" (**a**). TIL (CD3) can form tertiary lymphoid structures (**b**). A prognostic more favorable distribution pattern is "infiltrated–inflamed" with a higher density of cytotoxic lymphocytes within the tumor; CD3 in **c** and CD8 in **d**

infiltrated–inflamed phenotypes [31]. This could explain the observation that even small primary ACC regularly recur as distant metastasis. The observation that the patient with higher B and T cell frequencies in the tumor microenvironment was alive despite recurrence could indicate a potentially active immune system. This patient could benefit of immunostimulatory drugs. In the future, analysis of TIL in ACC should be pursued to get more detailed insights in how the tumor microenvironment of this entity works and could be modified to eliminate the tumor.

Taken together, ACC seems to have al lower impact on immunosuppression compared to HNSCC in the peripheral blood. However, the $CD4^+/T_{reg}$ ratio is lower after RT of ACC, which could lead to an increased immunosuppressive milieu. Therefore, it could be beneficial to combine radiotherapy with immune checkpoint inhibition in the future.

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Author contributions SSJ, UH and SEW performed experiments; SSJ and SEW prepared figures; JV and JD provided patient samples; SSJ, PJS and JD designed research and wrote the paper; MNT, CB, and TKH edited the paper.

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Compliance with ethical standards

Conflict of interest There are no potential conflicts of interest to disclose. The research was supported by the German Research Foundation (DFG) Grant # SCHU 2536/3 (PJS) and by the International Graduate School in Molecular Medicine Ulm (SSJ).

Ethical approval This article does not contain any studies with animals performed by any of the authors. All procedures performed in studies involving human participants were in accordance with the ethical standards approved by the local ethical committee (#255/14).

Informed consent Informed consent was obtained from all individual participants included in the study.

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