Check for updates

Influence of cryoablation versus operation on circulating lymphocyte subsets in patients with early-stage renal cell carcinoma

Johanna Waidhauser[1*], Anna-Katharina Gantner[1], Paola Schifano[2], Katharina Rippel[3], Stefan Schiele[4], Tim Tobias Arndt[4,5], Gernot Müller[4], Julie Steinestel[2], Andreas Rank[1†] and Thomas Kröncke[3†]

Abstract

Immune response is known to play an important role in local tumor control especially in renal cell carcinoma (RCC), which is considered highly immunogenic. For localized tumors, operative resection or local ablative procedures such as cryoablation are common therapeutical options. For thermal ablative procedures such as cryoablation, additional immunological anti-tumor effects have been described.

The purpose of this prospective study was to determine changes in peripheral blood circulating lymphocytes and various of their subsets in RCC patients treated with cryoablation or surgery in a longitudinal approach using extensive flow cytometry. Additionally, lymphocytes of RCC patients were compared to a healthy control group.

We included 25 patients with RCC. Eight underwent cryoablation and 17 underwent surgery. Univariate and multivariable analysis revealed significantly lower values of B cells, CD4 and CD8 T cells, and various of their subsets in the treatment groups versus the healthy control group. Comparing the two different therapeutical approaches, a significant decline of various lymphocyte subsets with a consecutive normalization after three months was seen for the surgery group, whereas cryoablation led to increased values of CD69 + CD4 + and CD69 + CD8 + cell counts as well as memory CD8 + cells after three months.

Treatment-naïve RCC patients showed lower peripheral blood lymphocyte counts compared to healthy controls. The post-treatment course revealed different developments of lymphocytes in the surgery versus cryoablation group, and only cryoablation seems to induce a sustained immunological response after three months.

Keywords Renal cell carcinoma, Cryoablation, Surgery, Flow cytometry, Lymphocytes

---

†Andreas Rank and Thomas Kröncke shared last authorship.

*Correspondence:
Johanna Waidhauser
johanna.waidhauser@uk-augsburg.de
[1] Department of Hematology and Oncology, University Medical Center Augsburg, Stenglinstr.2, 86156 Augsburg, Germany
[2] Department of Urology, University Medical Center Augsburg, Augsburg, Germany
[3] Department of Diagnostic and Interventional Radiology, University Medical Center Augsburg, Augsburg, Germany
[4] Institute of Mathematics, Faculty of Mathematics and Natural Sciences, University of Augsburg, Augsburg, Germany
[5] General Pathology and Molecular Diagnostics, Medical Faculty, University of Augsburg, Augsburg, Germany

Introduction

Immune response is known to play an important role in local tumor control of renal cell carcinoma (RCC), and RCC is considered as one of the most immunogenic tumors, showing the highest rates of T cell infiltration compared to other kinds of cancer [1]. Although higher rates of infiltrating T cells are associated with poorer prognosis in general [2], differentiation between T-cell subgroups is needed. Whereas regulatory T cells and Th2 cells are associated with a poorer prognosis, higher rates of cytotoxic T cells are associated with better

Waidhauser *et al. BMC Cancer* (2024) 24:825

Page 2 of 11

prognosis [1, 3, 4]. This high immunogenicity is exploited by immune-mediated therapeutic approaches in the treatment of advanced RCC. Starting with Interleukin 2 and Interferon α as non-specific immunotherapy, checkpoint inhibitors are now the main players in the first line treatment of metastasized clear cell renal cancer [5]. Nephrectomy or, if feasible regarding tumor size and localization, partial nephrectomy is considered the first line therapy for localized tumors [6]. For patients not eligible for surgery due to age or comorbidities and tumors sized < 3 cm, local ablative procedures are gaining importance and are a promising approach [6–8]. For thermal ablative procedures such as radiofrequency ablation (RFA) or cryoablation, immunological anti-tumor effects have been described in animal models and in humans [9, 10]; however, clinical significance remains controversial.

Whether the described high immunogenicity of renal cell carcinomas is also reflected by alterations in circulating lymphocyte subsets and whether a potential influence of surgery or cryoablation can be seen in peripheral blood lymphocytes has not sufficiently been studied so far. In our prospective study, we investigate the potential differences in peripheral blood B cells, T cells, and NK cells as well as various of their subsets between patients with localized RCC and a healthy control group and address the question of whether the therapeutical method (cryoablation or surgery) has an impact on these cells in a longitudinal, hypothesis-generating approach.

Methods

Study design and patient selection

We included patients with newly diagnosed localized and histologically confirmed RCC who were either scheduled for surgery or for cryoablation according to the recommendation of the local tumor board between June 2020 and March 2021. Patients with chronic infections (especially hepatitis or HIV), a known history of autoimmune disorder, or inherent or acquired immunodeficiency were excluded. Additionally, a control group of healthy volunteers, mostly blood donors, were recruited. Written informed consent was obtained from patients and healthy controls. The study was approved by the medical ethical committee of Ludwig Maximilians University Munich (reference number N20-001).

Cryoablation and operation

Cryoablation was performed after diagnostic imaging by multi-detector spiral computed tomography (Siemens Somatom Definition AS, Forchheim, Germany) for treatment planning. Percutaneous CT-guided placement of up to five 17G cryoprobes (IceForce®, IceRod®, or IceSphere®; Boston Scientific, St Paul, MN, USA) into the tumor with an interprobe distance of less than 1 cm

ensuring complete coverage of the tumor including a > 5 mm safety margin was performed. Carbon dioxide or hydrodissection were utilized when appropriate to protect nearby structures. Cryoablation was performed with an argon-based cryoablation system (Visual Ice; Boston Scientific, St Paul, MN, USA) applying two freeze–thaw cycles as follows: 10 min of freezing with 100% freeze intensity (uninterrupted argon flow) with consecutive passive (8 min) and active (2 min) thawing while CT scans to assess expansion of the ice-ball were obtained at regular 4-min intervals. An effective core temperature of minus 40 degrees Celsius, variable in size depending on the type of cryoprobe, can be expected according to the manufacturer. After the procedure, patients were taken to a recovery area for monitoring and were subsequently sent to the ward for overnight surveillance and discharged the next day according to the local discharge policy.

Operative tumor removal was performed using complete or, if feasible, partial nephrectomy according to the urologist's assessment.

Analysis of lymphocytes and subsets

EDTA blood samples were taken before (V0), one day after (V1), one week after (V2), and three months after (V3) operative tumor resection or cryoablation. Flow cytometry was performed within 24 h in our local laboratory at University Medical Center Augsburg using FC500 (Beckman Coulter, Brea, California, USA) according to previously published studies by our research group [11]. Cell staining was done using commercial antibodies purchased from Beckman Coulter (Brea, California, USA) and Biolegend (San Diego, California, USA). Initial results for lymphocyte subsets were given as percentages. Absolute values were calculated using absolute leucocyte counts measured with Stem-Count (Stem-Kit, Beckman Coulter).

Detailed information regarding flow cytometric identification of lymphocyte subsets, antibodies, and gating strategy is provided in Supplement Figure S1 [12], Table S1, and Table S2.

Charlson comorbidity Index

For all patients, we calculated the Charlson Comorbidity Index according to prior publications to quantify relevant comorbidities [13]. Healthy controls were rated with zero points, as pre-existing medical conditions would have excluded them from blood donation according to the national guidelines of blood donation.

Statistical analysis

The results of the descriptive analysis are given as median values and interquartile ranges. To detect differences

Waidhauser *et al. BMC Cancer*   (2024) 24:825

Page 3 of 11

between patients before treatment and the control group, as well as female and male study populations, we performed univariate analysis using the Mann–Whitney U test. We analyzed age-dependent alterations of lymphocyte subsets after logarithmic transformation and additionally performed a multivariable linear regression analysis on log-transformed cell counts including age, gender, and the presence of RCC. The results of the multivariable analysis were given as multiplicative factors (coefficient B). To detect changes in cell counts from V0 to the other timepoints (V1–V3), paired t-tests on the log-transformed cell counts were performed. P values < 0.05 were considered statistically significant. Data were analyzed with SPSS for Windows (IBM SPSS Statistics 24, Armonk, New York, USA) and R 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Population characteristics

We recruited a total of 25 consecutive RCC patients according to the inclusion criteria, of which 8 underwent cryoablation and 17 underwent conventional surgery, and additionally analyzed the blood of 50 healthy individuals comprising the control group. The median age of the RCC patients was 69 (range 40–93) years versus 43 (range 18–81) years in the control group ($p < 0.001$). There was also a significant age difference ($p = 0.019$) between the cryoablation group (median 77, range 66–87

years) and the surgery group (median 63, range 40–93 years). Further information on the demographic and disease characteristics is displayed in Table 1. T stage was determined based on prior imaging for the cryoablation and on final pathology for the surgery group and varied between T1a and T3a.

Univariate analysis of lymphocyte subsets before therapy

Univariate analysis revealed significantly lower lymphocyte counts for treatment-naïve RCC patients compared to healthy controls for total lymphocytes (median: 1459/μl vs. 1878/μl; $p = 0.010$); total B cells (110/μl vs. 206/μl; $p = 0.001$) and all of their subsets; total T cells (974/μl vs. 1175/μl; $p = 0.018$); CD4 cells (589/μl vs. 765/μl; $p = 0.042$) and their subsets of naïve CD4, effector memory CD4, EMRA CD4, CD69 + CD4, Th1, and regulatory (CD25 +) CD4 cells; CD8 cells (158/μl vs. 292/μl; $p = 0.001$) and their subsets of memory CD8, naïve CD8, effector memory CD8, and early, intermediate, late, and exhausted CD8 cells; as well as CD69 + CD8 + cells (see Table 2). Sex had a significant influence on naïve CD4 (339/μl vs. 223/μl; $p = 0.049$) and CD69 + CD4 cells (13/μl vs. 8/μl; $p = 0.009$), Th2 cells (71/μl vs. 45/μl; $p = 0.020$), and the CD4/CD8 ratio (3.6 vs. 2.1; $p < 0.001$), with higher values for women. Age had a significant influence with lower values of total lymphocytes; B cells and their subsets of transitory, naïve, and memory B cells; CD3 + cells; and CD4 + cells and CD8 + cells and various

Table 1 Demographic and disease characteristics of patients and healthy control group

Variables	Patients (*n* = 25)	Control group (*n* = 50)	*p*-value (Patients vs. Control)	Cryoablation (*n* = 8)	Operation (*n* = 17)	*p*-value (Cryoablation vs. Operation)
Age; *median (range)*	69 (40–93)	43 (18–81)	.000	77 (66–87)	63 (40–93)	.019
Sex						
male; *n (%)*	18 (72)	33 (66)	n.s	5 (63)	13 (76)	n.s
female; *n (%)*	7 (28)	17 (34)		3 (37)	4 (24)	
Histology						
Clear cell	9			3	6	
Chromophobe	9			3	6	
Papillary	7			2	5	
T Stage						
T1a	14			7	8	
T1b	3			1	2	
T2a	3				3	
T3a	5			0	4	
Charlson Comorbidity Index; *mean (range)*	3,6 (2–9)			4.5 (2–9)	3.2 (2–6)	n.s
Operative Procedure						
Nephrectomy					6	
Partial Nephrectomy					11	

Waidhauser *et al. BMC Cancer*   (2024) 24:825

Page 4 of 11

of their subsets. The strongest effect was seen for naïve CD4 + and naïve CD8 + cells (Fig. 1).

Due to these significant differences, the factors RCC, gender, and age were included in a multivariable analysis. As the CCI did not show significant differences between the control group and patients, it was not included in the multivariable analysis.

Multivariable analysis of lymphocytes before therapy

Multivariable analysis before the therapeutic procedure revealed an influence of age, with lower values of absolute lymphocyte count; B cells, especially naïve and transitory B cells; naïve and central memory CD4 + cells; total CD8 + cells and their subgroups of memory, naïve, central memory and early CD8 + cells; and NK cells and their subgroups of CD56 + CD16 + and CD56brightCD-16dim cells. Women had higher quantities of CD4 + cells and their subgroups of naïve, central memory, CD69 +, and Th2 cells as well as CD56brightCD16dim NK cells. Regarding the influence of the RCC on lymphocyte count, significantly lower values could be seen for total and class-switch B cells, and effector memory and

EMRA CD4 + cells, as well as Th1 cells and for EMRA, exhausted, CD69 +, and regulatory CD8 + cells. Higher values in RCC patients were only seen for naïve CD8 + cells and central memory CD4 + and CD8 + cells (Fig. 2, *Table S3*).

Longitudinal comparison between surgery and cryoablation

Comparing the lymphocyte values before the therapeutical procedure with those one day after surgery revealed a significant decrease in total lymphocytes (1630/ μl vs. 1132/μl; $p = 0.018$), total T cells (1061/μl vs. 771/μl; $p = 0.011$), CD4 + cells (682/μl vs. 513/μl; $p = 0.020$) and their subgroups of memory (406/μl vs. 356/μl; $p = 0.031$), central memory (296/μl vs. 244/μl; $p = 0.022$) and naïve CD4 + cells (234/μl vs. 130/μl; $p = 0.004$), as well as Th1 (14/μl vs. 10/μl; $p = 0.016$) and Th2 cells (64/μl vs. 48/μl; $p = 0.034$), naïve CD8 + (48/μl vs. 40/μl; $p = 0.039$), EMRA CD8 + (40/μl vs. 23/μl; $p = 0.022$), late CD8 + (21/μl vs. 20/μl; $p = 0.044$), terminal effector CD8 + (22/μl vs. 11/μl; $p = 0.005$), NKT cells (18/μl vs. 11/μl; $p = 0.015$), NK cells (183/μl vs. 129/μl; $p = 0.001$) and all of their
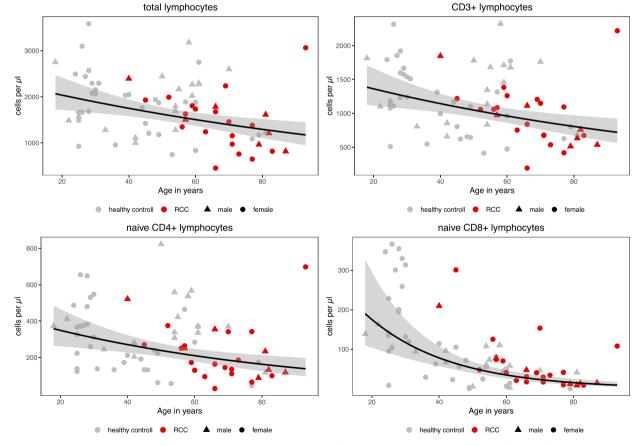

Fig. 1 Age-dependent distribution of cell counts and median decrease over lifetime. Black line = smoothed means of log transformed data, 95% confidence interval in gray

Table 2 Lymphocyte counts in healthy controls and patients at different time points (V0 = before procedure, V1 = after one day, V2 = after one week, V3 = after three months). Cell counts are given as median value/ µl (interquartile range). *P*-values are given for univariate analysis between total group of cancer patients before treatment and healthy controls

	NCC patients (*n* = 25) V0	NCC patients (*n* = 25) V1	NCC patients (*n* = 25) V2	NCC patients (*n* = 25) V3	Control group (*n* = 50)	*p*-value NCC patients V0 vs. Control
Total lymphocytes	1459 (970–1796)	1100 (677–1386)	1021 (820–1485)	1345 (1095–1803)	1878 (1282–2293)	.010
B lymphocytes	110 (53–197)	63 (46–205)	89 (38–162)	97 (46–150)	206 (146–276)	.001
transitory B	2 (1–8)	2 (1–11)	2 (1–6)	6 (1–13)	10 (5–18)	< .001
naïve B	58 (30–142)	50 (27–137)	51 (21–104)	64 (21–113)	126 (90–172)	.002
memory B	4 (2–10)	3 (1–7)	2 (2–4)	3 (2–8)	7 (5–13)	.009
class-switched B	12 (8–20)	9 (5–22)	9 (5–19)	12 (7–17)	24 (14–43)	.003
CD3 cells	974 (654–1178)	673 (443–985)	724 (515–996)	960 (667–1181)	1175 (806–1678)	.018
CD4_CD8	2 (1–7)	3 (1–6)	3 (1–5)	3 (2–7)	3 (2–7)	.719
CD4 cells	589 (438–777)	488 (296–640)	516 (375–653)	597 (475–812)	765 (525–1017)	.042
memory CD4	406 (311–460)	349 (153–416)	317 (226–428)	420 (348–504)	405 (309–590)	.323
naïve CD4	173 (114–306)	129 (71–237)	167 (97–262)	150 (112–301)	311 (173–417)	.013
CM CD4	292 (219–345)	203 (126–311)	233 (139–320)	313 (189–386)	269 (157–403)	.511
EM CD4	103 (77–135)	81 (39–113)	71 (50–124)	107 (83–173)	143 (93–238)	.022
EMRA CD4	1 (1–7)	1 (0–5)	1 (0–7)	2 (0–6)	4 (1–32)	.013
HLADR CD4	51 (31–76)	40 (23–53)	40 (23–61)	61 (38–80)	39 (27–60)	.134
CD69 CD4	8 (6–9)	7 (4–16)	10 (6–16)	8 (6–11)	12 (7–19)	.038
Th1	12 (6–31)	9 (4–19)	8 (5–19)	13 (7–25)	27 (14–58)	.001
Th2	54 (41–90)	43 (24–74)	47 (27–67)	58 (38–97)	45 (30–67)	.136
Th17	67 (36–86)	50 (25–75)	50 (32–66)	62 (46–102)	60 (35–82)	.727
IL2 (CD25 +) CD4	8 (6–13)	6 (4–11)	6 (4–10)	8 (5–11)	17 (8–21)	< .001
CD8 cells	158 (109–322)	138 (88–245)	162 (98–267)	197 (103–341)	292 (203–493)	.001
memory CD8	67 (40–113)	49 (31–112)	60 (29–90)	75 (53–112)	118 (63–165)	.023
naïve CD8	40 (16–75)	34 (15–61)	32 (13–57)	37 (16–72)	82 (35–140)	.045
CM CD8	23 (15–46)	18 (13–31)	20 (10–36)	25 (18–49)	29 (13–55)	.619
EM CD8	47 (32–84)	33 (22–81)	38 (21–75)	55 (40–86)	94 (55–144)	.004
EMRA CD8	40 (18–69)	25 (10–53)	36 (10–53)	49 (23–75)	51 (21–129)	.199
early CD8	105 (56–231)	102 (45–159)	102 (43–143)	98 (65–214)	180 (131–295)	.002
intermediate CD8	12 (5–22)	12 (3–22)	13 (3–24)	19 (6–26)	21 (12–34)	.012
late CD8	24 (14–79)	23 (7–49)	29 (8–73)	37 (13–68)	51 (26–124)	.045
exhausted CD8	42 (23–75)	46 (19–64)	49 (20–78)	68 (30–94)	86 (54–136)	.001
terminal effector CD8	22 (6–37)	11 (6–30)	14 (5–38)	22 (11–37)	24 (12–95)	.163
HLADR CD8	29 (10–66)	23 (9–51)	27 (9–56)	27 (14–78)	31 (17–61)	.694
CD69 CD8	12 (8–23)	15 (6–25)	23 (9–31)	18 (10–42)	32 (17–89)	< .001
IL2 (CD25 +) CD8	0 (0–1)	0 (0–1)	0 (0–1)	1 (0–1)	1 (0–1)	.093
NKT	19 (12–60)	17 (6–35)	16 (9–58)	33 (15–63)	45 (19–81)	.068
NK	177 (155–295)	141 (92–221)	131 (54–240)	187 (119–309)	226 (139–301)	.770
D56dimCD-16bright	17 (9–25)	10 (5–22)	10 (5–18)	18 (10–28)	15 (10–20)	.678
CD56brightCD-16bright	149 (120–254)	126 (59–187)	111 (42–195)	161 (98–279)	193 (111–274)	.621
CD56brightCD-16dim	11 (7–19)	7 (3–14)	5 (3–10)	14 (8–20)	15 (11–19)	.147
CD4/CD8 Ratio	4 (2–5)	3 (2–5)	3 (2–6)	3 (2–4)	2 (2–3)	.024

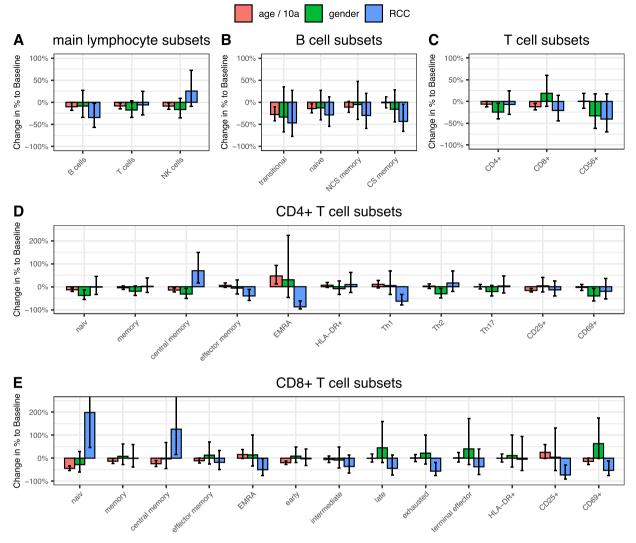Waidhauser *et al. BMC Cancer*   (2024) 24:825

Page 6 of 11

Fig. 2 Impact of age, gender, and renal cell carcinoma on lymphocyte subsets as calculated in multivariable analysis. **A** Main lymphocyte subsets, **B** B cell subsets, **C** T cell subsets, **D** CD4+T cell subsets, **E** CD8+T cell subsets

subsets. Regarding B cells, only memory B cells showed a significant decline at this time point after surgery (15/μl vs. 12/μl; $p=0.017$). After one week (V2), additional changes could be seen for total B cells (183/μl vs. 136/μl; $p=0.003$) and naïve (86/μl vs. 59/μl; $p<0.001$), transitory (4/μl vs. 3/μl; $p=0.004$), and class-switch B cells (15/μl vs. 13/μl; $p=0.013$), which all showed lower values at this time point compared to the preoperative values in the surgery group. For T cells, additional declines could be seen for effector memory CD4+ (115/μl vs. 61/μl; $p=0.011$), HLADR CD4+ (51/μl vs. 37/μl; $p=0.004$), Th17 (69/μl vs. 54/μl; $p=0.007$), regulatory (CD25+) CD4+cells (9/μl vs. 6/μl; $p=0.035$), CD8+cells (214/μl vs. 151/μl; $p=0.006$) and their subsets of memory (71/μl vs. 53/μl; $p=0.007$), central memory (32/μl vs. 20/μl;

$p=0.007$), effector memory (47/μl vs. 27/μl; $p=0.009$), early (136/μl vs. 104/μl; $p=0.010$), and intermediate CD8+cells (9/μl vs. 5/μl; $p=0.030$), as well as HLADR CD8+cells (25/μl vs. 11/μl; $p=0.005$) one week after surgery. Almost all subsets that showed significantly lower values the day after surgery remained reduced after one week with mostly even stronger declines. Three months after surgery, none of the reported declines persisted. The only significant change that could be seen in the surgery group at this time point was an increase of regulatory (CD25high) CD8+cells (0/μl vs. 1/μl; $p=0.030$) (Fig. 3, Table 3).

The type of surgery (partial vs. total nephrectomy) did not lead to significant differences between lymphocyte subsets at any timepoint in univariate analysis.

Waidhauser *et al. BMC Cancer*   (2024) 24:825

Page 7 of 11

In the ablation group, no significant changes could be observed one day after the procedure with the exception of reduced memory B cells (3/µl vs. 2/µl; $p=0.020$) and CD56brightCD16dim NK cells (13/µl vs. 10/µl; $p=0.020$). One week after the ablation, an increase in effector memory CD4+cells (80/µl vs. 105/µl; $p=0.027$), HLADR CD4+cells (49/µl vs. 52/µl; $p=0.045$), CD69+CD4+cells (5/µl vs. 10/µl; $p=0.017$), intermediate CD8+cells (13/µl vs. 17/µl; $p=0.015$), and CD69+CD8+cells (13/µl vs. 26/µl; $p=0.012$) could be seen. Three months after therapy, CD69+CD4+cells (5/µl vs. 9/µl; $p<0.001$) and CD69+CD8+cells (13/µl cs. 23/µl; $p=0.003$) showed a persisting increase. Additionally, an increase in memory CD8+cells (58/µl vs. 66/µl; $p=0.043$), central memory CD8+cells (18/µl vs. 23/µl; $p=0.013$), and NKT cells (26/µl vs. 41/µl; $p=0.026$) was found (Fig. 3, Table 3).

Discussion

Peripheral blood total lymphocyte counts, B cells, CD4+cells, CD8+cells and various of their subsets showed lower values in RCC patients compared to healthy controls in univariate analysis. Due to the known effects of age and gender [14, 15] on lymphocyte counts, these factors were included in a multivariable analysis in addition to the presence of cancer. Naïve CD8+cells and central memory CD4+and CD8+cells showed higher values in RCC patients. Central memory T cells play an important role in tumor antigen recognition and tumor control, which could be an explanation for their higher counts in RCC patients [16, 17]. In the majority of lymphocyte subsets, especially in activated and memory CD4+cells as well as different types of activated and memory CD8+cells, significantly lower values were observed in RCC patients independently of age or gender. A similar effect has been reported in patients with head and neck cancer [18], and in colorectal carcinoma patients compared to healthy controls [11], while a study by Wang et al. revealed lower values for some lymphocyte subsets and higher values for others [19]. The underlying mechanism of these lower lymphocyte counts in RCC patients could be some kind of immunosuppression caused by the cancer or attraction of lymphocytes to the tumor, with resulting lower peripheral blood lymphocytes. To answer this pathophysiological question is beyond the scope of this study, and further investigations comprising, for example, tumor-infiltrating lymphocytes are needed.
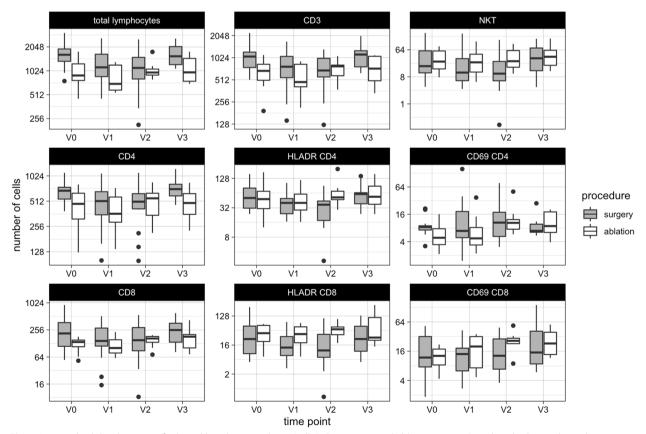

Fig. 3 Longitudinal development of selected lymphocyte subsets (cells/ µl) in surgery and ablation groups. Box plots display median values and interquartile ranges

Table 3 Lymphocyte counts in different study groups (surgery/ablation) at different time points (V0 = before procedure, V1 = after one day, V2 = after one week, V3 = after three months). Cell counts are given as median value/µl (interquartile range). *P*-values are given for longitudinal comparisons

	Ablation (*n* = 8) V0	Ablation (*n* = 8) V1	Ablation (*n* = 8) V2	Ablation (*n* = 8) V3	*p*-value V0 vs. V1	V2	V3
Total lymphocytes	897 (692 - 1340)	701 (569 - 1212)	975 (837 - 1142)	1003 (723 - 1529)	0.3780	0.2821	0.3076
B lymphocytes	54 (29 - 87)	47 (24 - 71)	41 (21 - 93)	63 (25 - 93)	0.1904	0.1823	0.5425
transitory B	1 (0 - 3)	1 (0 - 2)	1 (0 - 4)	2 (0 - 6)	0.1492	0.0029	0.6878
naiv B	34 (12 - 58)	24 (11 - 56)	23 (8 - 55)	40 (7 - 66)	0.1995	0.1735	0.3628
memory B	3 (1 - 4)	2 (1 - 4)	2 (1 - 3)	2 (1 - 3)	0.0204	0.3223	0.4772
class switched B	7 (6 - 10)	6 (3 - 9)	5 (4 - 8)	7 (5 - 10)	0.2578	0.3547	0.6768
CD3 cells	677 (448 - 1011)	477 (382 - 847)	781 (516 - 863)	733 (439 - 1076)	0.3752	0.1921	0.3196
CD4_CD8	2 (1 - 5)	3 (1 - 5)	4 (1 - 6)	3 (0 - 8)	0.3737	0.5634	0.5665
CD4 cells	479 (293 - 751)	363 (250 - 588)	556 (319 - 653)	489 (310 - 754)	0.5008	0.1401	0.3195
memory CD4	367 (203 - 453)	300 (148 - 385)	365 (233 - 507)	358 (245 - 462)	0.5025	0.0832	0.2563
naiv CD4	114 (73 - 290)	88 (60 - 214)	121 (71 - 246)	108 (65 - 289)	0.4973	0.5684	0.6654
CM CD4	242 (133 - 350)	198 (117 - 262)	247 (143 - 333)	224 (177 - 355)	0.5321	0.2742	0.2243
EM CD4	80 (49 - 139)	94 (43 - 116)	105 (66 - 140)	87 (64 - 157)	0.5310	0.0266	0.3583
EMRA CD4	1 (0 - 7)	1 (0 - 5)	4 (1 - 7)	1 (0 - 4)	0.9074	0.1125	0.4306
HLADR CD4	49 (30 - 70)	40 (21 - 82)	52 (45 - 77)	55 (36 - 102)	0.5497	0.0446	0.1449
CD69 CD4	5 (3 - 11)	5 (3 - 12)	10 (7 - 15)	9 (5 - 18)	0.5215	0.0174	0.0007
Th1	7 (7 - 29)	7 (4 - 39)	9 (6 - 37)	8 (6 - 25)	0.7297	0.6617	0.5357
Th2	38 (24 - 58)	36 (17 - 46)	43 (25 - 67)	40 (29 - 54)	0.5166	0.9841	0.2318
Th17	45 (27 - 66)	52 (21 - 64)	43 (32 - 75)	50 (34 - 66)	0.6029	0.1339	0.0747
IL2 CD4	6 (3 - 8)	4 (3 - 5)	6 (4 - 8)	6 (5 - 7)	0.0604	0.6813	0.8596
CD8 cells	138 (81 - 156)	101 (77 - 196)	165 (114 - 189)	182 (101 - 210)	0.7672	0.1453	0.0797
memory CD8	58 (31 - 77)	47 (41 - 60)	86 (44 - 93)	66 (53 - 93)	0.8104	0.1632	0.0428
naiv CD8	16 (12 - 38)	16 (11 - 33)	23 (10 - 32)	16 (14 - 40)	0.9114	0.8758	0.5349
CM CD8	18 (10 - 23)	16 (14 - 22)	21 (13 - 28)	23 (15 - 28)	0.7437	0.4504	0.0131
EM CD8	46 (22 - 58)	35 (26 - 49)	62 (33 - 78)	55 (42 - 75)	0.9144	0.0853	0.0621
EMRA CD8	41 (13 - 50)	28 (18 - 50)	43 (23 - 58)	47 (17 - 78)	0.8226	0.0663	0.1154
early CD8	55 (35 - 98)	48 (37 - 90)	76 (43 - 128)	70 (46 - 103)	0.6575	0.2302	0.2312
intermediate CD8	13 (10 - 18)	15 (10 - 22)	17 (14 - 24)	22 (13 - 26)	0.6512	0.0145	0.1667
late CD8	37 (11 - 68)	30 (14 - 56)	34 (13 - 82)	45 (9 - 114)	0.9589	0.3994	0.4449
exhausted CD8	48 (17 - 80)	50 (30 - 65)	64 (51 - 85)	55 (23 - 93)	0.3072	0.0529	0.2618
terminal effector CD8	19 (6 - 31)	13 (8 - 18)	20 (10 - 38)	22 (11 - 58)	0.7161	0.8076	0.1807
HLADR CD8	37 (15 - 67)	36 (14 - 62)	50 (28 - 62)	27 (19 - 127)	0.6550	0.1980	0.1166
CD69 CD8	13 (8 - 19)	20 (6 - 32)	26 (23 - 32)	23 (12 - 42)	0.1474	0.0121	0.0034
IL2 CD8	1 (0 - 1)	1 (0 - 1)	1 (0 - 1)	0 (0 - 1)	0.2516	0.4227	0.6034
NKT	26 (13 - 60)	25 (9 - 71)	28 (15 - 65)	41 (19 - 63)	0.5705	0.3442	0.0255
NK	156 (109 - 303)	152 (109 - 237)	181 (131 - 264)	288 (95 - 323)	0.2856	0.4503	0.3885
D56dimCD16bright	19 (9 - 25)	13 (7 - 34)	16 (7 - 21)	19 (13 - 28)	0.8912	0.3983	0.6392
CD56brightCD16bright	135 (80 - 269)	129 (73 - 181)	154 (101 - 244)	247 (72 - 303)	0.2311	0.2938	0.3636
CD56brightCD16dim	13 (7 - 23)	8 (6 - 21)	10 (8 - 23)	11 (6 - 24)	0.0203	0.8794	0.2213
CD4/CD8 Ratio	4 (2 - 5)	3 (2 - 4)	4 (2 - 4)	4 (2 - 4)	0.2675	0.2455	0.0941
	Operation (*n* = 17) V0	Operation (*n* = 17) V1	Operation (*n* = 16) V2	Operation (*n* = 12) V3	*p*-value V0 vs. V1	V2	V3
Total lymphocytes	1630 (1295 - 1960)	1132 (795 - 1688)	1113 (697 - 1528)	1560 (1181 - 2203)	0.0182	0.0029	0.9179
B lymphocytes	183 (82 - 251)	191 (55 - 272)	136 (62 - 203)	134 (97 - 254)	0.3541	0.0029	0.6055
transitory B	4 (1 - 12)	3 (1 - 15)	3 (1 - 7)	8 (3 - 15)	0.4175	0.0044	0.8161
naiv B	86 (50 - 163)	76 (34 - 205)	59 (36 - 117)	82 (40 - 176)	0.6622	0.0003	0.3679
memory B	6 (2 - 12)	6 (1 - 10)	3 (2 - 5)	7 (2 - 9)	0.0713	0.0021	0.4201
class switched B	15 (11 - 38)	12 (6 - 29)	13 (8 - 23)	16 (12 - 33)	0,0167	0.0133	0.6390
CD3 cells	1061 (714 - 1240)	771 (503 - 1089)	686 (515 - 1019)	1131 (761 - 1404)	0.0105	0.0038	0.5236
CD4_CD8	3 (1 - 8)	3 (1 - 6)	3 (1 - 5)	4 (2 - 7)	0.8023	0.6059	0.7711

Table 3 (continued)

CD4 cells	682 (525 - 819)	513 (317 - 723)	505 (410 - 690)	715 (591 - 828)	0.0200	0.0100	0.5520
memory CD4	406 (350 - 505)	356 (153 - 430)	308 (166 - 401)	441 (398 - 533)	0.0307	0.0058	0.5760
naiv CD4	234 (140 - 306)	130 (103 - 256)	181 (105 - 311)	195 (140 - 301)	0.0040	0.0611	0.9731
CM CD4	296 (250 - 345)	244 (126 - 318)	224 (121 - 300)	348 (280 - 390)	0.0221	0.0074	0.8763
EM CD4	115 (85 - 135)	78 (35 - 138)	61 (41 - 123)	131 (98 - 178)	0,.0621	0.0110	0.2353
EMRA CD4	2 (1 - 7)	1 (0 - 5)	1 (0 - 14)	2 (1 - 14)	0.3245	0.2630	0.5521
HLADR CD4	51 (31 - 85)	40 (23 - 51)	37 (18 - 52)	61 (38 - 68)	0.0522	0.0042	0.3859
CD69 CD4	8 (7 - 9)	7 (4 - 19)	11 (5 - 22)	7 (6 - 10)	0.8818	0.4579	0.9878
Th1	14 (6 - 31)	10 (4 - 19)	6 (4 - 19)	14 (10 - 25)	0.0159	0.0324	0.2352
Th2	64 (43 - 95)	48 (24 - 75)	47 (27 - 70)	76 (54 - 112)	0.0337	0.0034	0.4007
Th17	69 (43 - 88)	49 (27 - 77)	54 (23 - 66)	76 (55 - 107)	0.0792	0.0065	0.1451
IL2 CD4	9 (7 - 14)	9 (5 - 13)	6 (4 - 13)	10 (7 - 13)	0.3715	0.0353	0.4347
CD8 cells	214 (109 - 420)	145 (107 - 309)	151 (74 - 290)	259 (117 - 386)	0.0620	0.0059	0.2683
memory CD8	71 (40 - 161)	58 (28 - 131)	53 (24 - 81)	83 (54 - 163)	0.0620	0.0068	0.4400
naiv CD8	48 (26 - 116)	40 (19 - 86)	45 (15 - 90)	61 (29 - 102)	0.0387	0.0221	0.8138
CM CD8	32 (15 - 53)	24 (11 - 54)	20 (10 - 39)	28 (19 - 52)	0.1248	0.0069	0.7912
EM CD8	47 (37 - 119)	31 (15 - 105)	27 (15 - 64)	64 (36 - 125)	0.0620	0.0091	0.4670
EMRA CD8	40 (18 - 74)	23 (8 - 55)	21 (9 - 52)	49 (26 - 62)	0.0223	0.0187	0.1146
early CD8	136 (73 - 256)	120 (49 - 178)	104 (38 - 170)	149 (85 - 260)	0.0788	0.0096	0.4078
intermediate CD8	9 (4 - 28)	7 (2 - 23)	5 (2 - 23)	15 (4 - 33)	0.0538	0.0302	0.5301
late CD8	21 (14 - 91)	20 (6 - 49)	20 (7 - 73)	34 (17 - 58)	0.0435	0.0339	0.1080
exhausted CD8	42 (28 - 75)	31 (18 - 77)	38 (15 - 53)	70 (30 - 111)	0.1610	0.0507	0.1058
terminal effector CD8	22 (6 - 51)	11 (3 - 37)	9 (5 - 40)	22 (11 - 37)	0.0050	0.0122	0.6109
HLADR CD8	25 (8 - 69)	13 (7 - 35)	11 (6 - 37)	25 (10 - 70)	0.0527	0.0046	0.3817
CD69 CD8	12 (8 - 33)	14 (6 - 21)	13 (6 - 30)	15 (8 - 57)	0.3744	0.8077	0.1879
IL2 CD8	0 (0 - 1)	0 (0 - 1)	0 (0 - 1)	1 (0 - 1)	0.9939	0.7901	0.0297
NKT	18 (8 - 70)	11 (6 - 33)	10 (6 - 48)	33 (12 - 103)	0.0152	0.0174	0.2054
NK	183 (162 - 295)	129 (86 - 221)	83 (51 - 149)	183 (132 - 246)	0.0010	0.0015	0.2134
D56dimCD16bright	15 (9 - 26)	7 (4 - 20)	9 (5 - 18)	18 (8 - 30)	0.0081	0.0782	0.7013
CD56brightCD16bright	151 (129 - 254)	116 (52 - 201)	68 (34 - 135)	151 (112 - 191)	0.0009	0.0010	0.1808
CD56brightCD16dim	11 (8 - 17)	6 (2 - 14)	4 (3 - 7)	14 (8 - 20)	0.0068	0.0007	0.8576
CD4/CD8 Ratio	3 (2 - 6)	3 (2 - 5)	3 (2 - 6)	3 (2 - 5)	0.4180	0.1050	0.3195

Regarding the longitudinal development of peripheral blood lymphocytes, a decline of total lymphocytes, total T cells, and several subsets of CD4+and CD8+cells could be seen already one day after surgery with a prolonged and in parts even stronger effect after one week. This decline resolved after three months. It is known that surgical procedures can induce alterations of peripheral blood lymphocytes, which has been shown for cytotoxic T cells in patients after resection of hepatocellular carcinoma [20], and for total T cells and CD4+cells in patients with gastrointestinal cancer [21].

In contrast to patients who underwent surgery, cryoablation had no remarkable effect on circulating lymphocytes after one day. One week after the procedure, an increase in different types of CD4+and CD8+cells could be observed including mainly activated cells as CD69+CD4+cells, HLADR CD4+cells, and CD69+CD8+cells. Higher values of CD69+CD4+and CD69+CD8 cells persisted after three months. This

effect might be caused by the differing therapeutical approach. The intention of cryoablation is the destruction of tumor tissue with a resulting release to the bloodstream and antigen presentation to lymphocytes [22], while surgery tends to be performed using a no-touch technique to avoid the circulation of tumor cells. Immune stimulation after cryoablation is dependent on tumor cell necrosis, while apoptosis seems to induce immunosuppression [23]. The effects of cryoablation-induced immune activation on long-term prognosis are controversial [22], and longer-lasting effects have been described for combination with other immunotherapeutic approaches [24, 25]. The increase in activated T cells three months after cryoablation in our study population could be evidence for a persisting immune activation in this group of patients in contrast to the patients who underwent surgery. HLA-DR positive cells in particular seem to be crucial for immunological tumor control, as HLA-DR loss is linked to tumor escape from

Waidhauser *et al. BMC Cancer*   (2024) 24:825

Page 10 of 11

immunosurveillance [26], and higher numbers of activated HLA-DR + CD4 + cells were associated with a better prognosis in B-NHL patients [27]. For breast cancer patients, a positive correlation of tumor-infiltrating and circulating HLA-DR positive cytotoxic T cells has been shown with lower rates of lymph node metastases and better response to chemotherapy in HLA-DR^{hi} patients [28]. However, some studies report adverse prognostic effects of elevated HLA-DR T cells in patients with solid tumors or hematological malignancies [29, 30]. CD69 + T cells display another type of activated cells for which an important role in anti-tumor immunity has been reported [31]. Whether elevated activated T cells have an impact on long-term prognosis can, however, not be answered by our study population.

The question whether surgical technique (partial vs. total nephrectomy) makes a difference cannot be finally answered. Comparing these two subgroups revealed no significant differences in lymphocyte subsets at the different time points in univariate analysis. Because of this and the small sample size no multivariate analysis including this parameter was performed.

Missing long time follow-up is one limitation of our study, as prognostic differences between surgery and ablation, possibly caused by the different development of lymphocyte counts, cannot be detected. Further, we are unable to state whether the cryoablation-induced effect of cellular immune system activation seen at three months was specifically directed against RCC. Another limitation is the relatively small number of patients in the ablation group. However, longitudinal comparison revealed significant changes of lymphocyte counts regardless of the low patient numbers.

In summary, our data show that lymphocyte counts of RCC patients are lower than those of healthy controls and that the two therapeutical approaches of surgery and cryoablation lead to different developments of circulating lymphocytes. Cryoablation induces a sustained activation of CD4 + and CD8 + T cells three months after the procedure. This observation may indicate a longer-term immunostimulatory effect of cryoablation, which could support immunological tumor control.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12885-024-12596-w.

Additional file 1.

Authors' contributions

JW wrote the main manuscript and performed basic statistical analysis. A-KG, PS, JS and KR were involved in patient recruitmend and follow up, A-KG and PS performed data acquisition. SS, TTA and GM performed statistical analysis and prepared all figures. AR and TK conceptualized the study. All authors reviewed the manuscript. JS, AR and TK revised the manuscript.

References

1. Şenbabaoğlu Y, Gejman RS, Winer AG, Liu M, Van Allen EM, de Velasco G, et al. Tumor immune microenvironment characterization in clear cell renal cell carcinoma identifies prognostic and immunotherapeutically relevant messenger RNA signatures. Genome Biol. 2016;17(1):231. https://doi.org/10.1186/s13059-016-1092-z.
2. Vuong L, Kotecha RR, Voss MH, Hakimi AA. Tumor Microenvironment Dynamics in Clear-Cell Renal Cell Carcinoma. Cancer Discov. 2019;9(10):1349–57. https://doi.org/10.1158/2159-8290.Cd-19-0499.
3. Giraldo NA, Becht E, Vano Y, Petitprez F, Lacroix L, Validire P, et al. Tumor-Infiltrating and Peripheral Blood T-cell Immunophenotypes Predict Early Relapse in Localized Clear Cell Renal Cell Carcinoma. Clin Cancer Res. 2017;23(15):4416–28. https://doi.org/10.1158/1078-0432.Ccr-16-2848.
4. Zhang S, Zhang E, Long J, Hu Z, Peng J, Liu L, et al. Immune infiltration in renal cell carcinoma. Cancer Sci. 2019;110(5):1564–72. https://doi.org/10.1111/cas.13996.
5. Choueiri TK, Motzer RJ. Systemic Therapy for Metastatic Renal-Cell Carcinoma. N Engl J Med. 2017;376(4):354–66. https://doi.org/10.1056/NEJMra1601333.
6. Campbell SC, Clark PE, Chang SS, Karam JA, Souter L, Uzzo RG. Renal Mass and Localized Renal Cancer: Evaluation, Management, and Follow-Up: AUA Guideline: Part I. J Urol. 2021;206(2):199–208. https://doi.org/10.1097/ju.0000000000001911.
7. Breen DJ, King AJ, Patel N, Lockyer R, Hayes M. Image-guided Cryoablation for Sporadic Renal Cell Carcinoma: Three- and 5-year Outcomes in 220 Patients with Biopsy-proven Renal Cell Carcinoma. Radiology. 2018;289(2):554–61. https://doi.org/10.1148/radiol.2018180249.
8. Yanagisawa T, Mori K, Kawada T, Motlagh RS, Mostafaei H, Quhal F, et al. Differential efficacy of ablation therapy versus partial nephrectomy between clinical T1a and T1b renal tumors: A systematic review and meta-analysis. Urol Oncol. 2022;40(7):315–30. https://doi.org/10.1016/j.urolonc.2022.04.002.
9. Kato T, Iwasaki T, Uemura M, Nagahara A, Higashihara H, Osuga K, et al. Characterization of the cryoablation-induced immune response in kidney cancer patients. Oncoimmunology. 2017;6(7):e1326441.
10. Takaki H, Cornelis F, Kako Y, Kobayashi K, Kamikonya N, Yamakado K. Thermal ablation and immunomodulation: From preclinical experiments to clinical trials. Diagn Interv Imaging. 2017;98(9):651–9. https://doi.org/10.1016/j.diii.2017.04.008.
11. Waidhauser J, Nerlinger P, Arndt TT, Schiele S, Sommer F, Wolf S, et al. Alterations of circulating lymphocyte subsets in patients with colorectal carcinoma. Cancer Immunol Immunother. 2021. https://doi.org/10.1007/s00262-021-03127-8.

Waidhauser *et al. BMC Cancer*   (2024) 24:825

Page 11 of 11

12. Löhr P, Schiele S, Arndt TT, Grützner S, Claus R, Römmele C, et al. Impact of age and gender on lymphocyte subset counts in patients with COVID-19. Cytometry A. 2021. https://doi.org/10.1002/cyto.a.24470.

13. Charlson ME, Pompei P, Ales KL, MacKenzie CR. A new method of classifying prognostic comorbidity in longitudinal studies: development and validation. J Chronic Dis. 1987;40(5):373–83. https://doi.org/10.1016/0021-9681(87)90171-8.

14. Qin L, Jing X, Qiu Z, Cao W, Jiao Y, Routy JP, et al. Aging of immune system: Immune signature from peripheral blood lymphocyte subsets in 1068 healthy adults. Aging (Albany NY). 2016;8(5):848–59. https://doi.org/10.18632/aging.100894.

15. Kverneland AH, Streitz M, Geissler E, Hutchinson J, Vogt K, Boës D, et al. Age and gender leucocytes variances and references values generated using the standardized ONE-Study protocol. Cytometry A. 2016;89(6):543–64. https://doi.org/10.1002/cyto.a.22855.

16. Liao Y, Geng P, Tian Y, Miao H, Liang H, Zeng R, et al. ked anti-tumor effects of CD8(+)CD62L(+) T cells from melanoma-bearing mice. Immunol Invest. 2015;44(2):147–63. https://doi.org/10.3109/08820139.2014.944980.

17. Beckhove P, Feuerer M, Dolenc M, Schuetz F, Choi C, Sommerfeldt N, et al. Specifically activated memory T cell subsets from cancer patients recognize and reject xenotransplanted autologous tumors. J Clin Invest. 2004;114(1):67–76. https://doi.org/10.1172/jci20278.

18. Kuss I, Hathaway B, Ferris RL, Gooding W, Whiteside TL. Imbalance in absolute counts of T lymphocyte subsets in patients with head and neck cancer and its relation to disease. Adv Otorhinolaryngol. 2005;62:161–72. https://doi.org/10.1159/000082506.

19. Wang YY, Zhou N, Liu HS, Gong XL, Zhu R, Li XY, et al. Circulating activated lymphocyte subsets as potential blood biomarkers of cancer progression. Cancer Med. 2020;9(14):5086–94. https://doi.org/10.1002/cam4.3150.

20. Liu HZ, Deng W, Li JL, Tang YM, Zhang LT, Cui Y, et al. Peripheral blood lymphocyte subset levels differ in patients with hepatocellular carcinoma. Oncotarget. 2016;7(47):77558–64. https://doi.org/10.18632/oncotarget.13041.

21. Lin CY, Bai DJ, Yuan HY, Wang K, Yang GL, Hu MB, et al. Perioperative cimetidine administration promotes peripheral blood lymphocytes and tumor infiltrating lymphocytes in patients with gastrointestinal cancer: Results of a randomized controlled clinical trial. World J Gastroenterol. 2004;10(1):136–42. https://doi.org/10.3748/wjg.v10.i1.136.

22. Slovak R, Ludwig JM, Gettinger SN, Herbst RS, Kim HS. Immuno-thermal ablations - boosting the anticancer immune response. J Immunother Cancer. 2017;5(1):78. https://doi.org/10.1186/s40425-017-0284-8.

23. Mehta A, Oklu R, Sheth RA. Thermal Ablative Therapies and Immune Checkpoint Modulation: Can Locoregional Approaches Effect a Systemic Response? Gastroenterol Res Pract. 2016;2016:9251375. https://doi.org/10.1155/2016/9251375.

24. Duffy AG, Ulahannan SV, Makorova-Rusher O, Rahma O, Wedemeyer H, Pratt D, et al. Tremelimumab in combination with ablation in patients with advanced hepatocellular carcinoma. J Hepatol. 2017;66(3):545–51. https://doi.org/10.1016/j.jhep.2016.10.029.

25. den Brok MH, Sutmuller RP, Nierkens S, Bennink EJ, Frielink C, Toonen LW, et al. Efficient loading of dendritic cells following cryo and radiofrequency ablation in combination with immune modulation induces anti-tumour immunity. Br J Cancer. 2006;95(7):896–905. https://doi.org/10.1038/sj.bjc.6603341.

26. Higashi M, Tokuhira M, Fujino S, Yamashita T, Abe K, Arai E, et al. Loss of HLA-DR expression is related to tumor microenvironment and predicts adverse outcome in diffuse large B-cell lymphoma. Leuk Lymphoma. 2016;57(1):161–6. https://doi.org/10.3109/10428194.2015.1038708.

27. Ansell SM, Stenson M, Habermann TM, Jelinek DF, Witzig TE. Cd4+ T-cell immune response to large B-cell non-Hodgkin's lymphoma predicts patient outcome. J Clin Oncol. 2001;19(3):720–6. https://doi.org/10.1200/jco.2001.19.3.720.

28. Saraiva DP, Jacinto A, Borralho P, Braga S, Cabral MG. HLA-DR in Cytotoxic T Lymphocytes Predicts Breast Cancer Patients' Response to Neoadjuvant Chemotherapy. Front Immunol. 2018;9:2605. https://doi.org/10.3389/fimmu.2018.02605.

29. Kågedal Å, Hjalmarsson E, Farrajota Neves da Silva P, Piersiala K, Georén SK, Margolin G, et al. Activation of T helper cells in sentinel node predicts poor prognosis in oral squamous cell carcinoma. Sci Rep. 2020;10(1):22352, https://doi.org/10.1038/s41598-020-79273-3.

30. Váróczy L, Gergely L, Miltényi Z, Aleksza M, Illés A. Can CD3+/HLA-DR+ activated T cells predict the prognosis of non-Hodgkin's lymphoma patients? Immunol Lett. 2005;97(1):155–7. https://doi.org/10.1016/j.imlet.2004.10.005.

31. Lan B, Zhang J, Lu D, Li W. Generation of cancer-specific CD8(+) CD69(+) cells inhibits colon cancer growth. Immunobiology. 2016;221(1):1–5. https://doi.org/10.1016/j.imbio.2015.08.010.

Publisher's Note