Polyfunctionality of CD4+ T lymphocytes is increased after chemoradiotherapy of head and neck squamous cell carcinoma

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Polyfunctionality of CD4+ T lymphocytes is increased after chemoradiotherapy of head and neck squamous cell carcinoma

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Abstract

Background For head and neck squamous cell cancer (HNSCC), standard therapy consists of surgery, radiation, and/or chemotherapy. Antineoplastic immunotherapy could be an option in an adjuvant setting and is already in palliation. A functional immune system is a prerequisite for successful immunotherapy. However, effects of the standard-of-care therapy on the patients' immune system are not fully understood.

Methods Peripheral blood mononuclear cells (PBMC) were collected from patients with HNSCC ($n=37$) and healthy controls ($n=10$). PBMC were stimulated with staphylococcal enterotoxin B (SEB). Simultaneous expression of various cytokines was measured in CD4+ and CD8+ T cells by multicolor flow cytometry, and polyfunctional cytokine expression profiles were determined on a single-cell basis.

Results Expression levels of all measured cytokines in CD4+ T cells were higher in patients after chemoradiotherapy (CRT) as compared to untreated HNSCC patients or normal controls. After CRT, the frequency of polyfunctional CD4+ T cells, which simultaneously expressed multiple cytokines, was significantly increased as compared to untreated patients ($p<0.01$).

Conclusion CRT increases polyfunctionality of CD4+ T cells in HNSCC patients, suggesting that standard-of-care therapy can promote immune activity in immune cells. These polyfunctional CD4+ T cells in the blood of treated HNSCC patients are expected to be responsive to subsequent immunotherapeutic approaches.

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Steigerung der Polyfunktionalität von CD4+ T-Lymphozyten nach Radiochemotherapie von Plattenepithelkarzinomen im Kopf-Hals-Bereich

Zusammenfassung

Hintergrund Die Standardtherapie für Plattenepithelkarzinome im Kopf-Hals-Bereich (HNSCC) beinhaltet chirurgische Verfahren, Strahlentherapie und/oder Chemotherapie. Seit Kurzem ist eine Immuntherapie als zusätzliche palliative Therapieoption verfügbar und könnte auch in der adjuvanten Therapie nach primärer Chirurgie angewandt werden. Ein funktionierendes Immunsystem ist hierbei eine Grundvoraussetzung. Allerdings sind die Auswirkungen der herkömmlichen Therapien auf das Immunsystem noch nicht vollständig klar und bedürfen weiterer Untersuchungen.

Methoden Es wurden periphere Monozyten („peripheral blood mononuclear cells“, PBMC) aus dem Blut von HNSCC-Patienten ($n=37$) und gesunden Probanden ($n=10$) zu unterschiedlichen Zeitpunkten isoliert. Zunächst wurden PBMCs mit Staphylokokken-Enterotoxin B (SEB) stimuliert. Anschließend wurde die Expression der Zytokine in $CD4^+$ und $CD8^+$ T-Zellen mittels Mehrfarben-Durchflusszytometrie gemessen. Die Expressionsprofile und Polyfunktionalität wurden auf Einzelzellbasis ermittelt.

Ergebnisse Die Expression aller gemessenen Zytokine in $CD4^+$ T-Zellen war für Patienten nach Radiochemotherapie höher, als für unbehandelte Patienten und gesunde Probanden. Die Polyfunktionalität der $CD4^+$ T-Zellen, also die Anzahl gleichzeitig exprimierter Zytokine, war nach Radiochemotherapie im Vergleich zu unbehandelten Patienten signifikant erhöht ($p<0{,}01$).

Schlussfolgerung Eine Steigerung der Polyfunktionalität von $CD4^+$ T-Lymphozyten nach Radiochemotherapie legt die Vermutung nahe, dass die Standardtherapie die Aktivität von Immunzellen fördert. Es ist daher gut möglich, dass diese polyfunktionalen $CD4^+$ T-Zellen auf eine nachfolgende Immuntherapie ansprechen könnten.

Introduction

In the past few years, new immunotherapeutic strategies have been successfully introduced into treatment of head and neck squamous cell cancer (HNSCC). The most prevalent agents are epidermal growth factor receptor (EGFR) antibodies, e. g., cetuximab, and immune checkpoint inhibitors against the programmed death receptor-1 (PD-1), e. g., pembrolizumab and nivolumab. However, radiotherapy in combination with cetuximab was not found to be superior compared to standard chemotherapy [1]. For pembrolizumab, an overall response rate of 18% was reported in the Keynote-012 cohort in a palliative setting [2]. Recently, several clinical trials have been started in order to evaluate the combination of various immunotherapeutic approaches or the combination of radio- and immune therapy, as this may induce abscopal effects and enhance the impact of checkpoint inhibitors [3, 4]. For breast cancer it has been shown that several chemotherapies inducing programmed-death-ligand 1 (PD-L1) surface expression on cancer cells promote immunoresistance [5]. Nevertheless, the overall influence of standard of care (SOC) therapy on the immune system is still not fully understood and there are no established tools to measure response to SOC on a molecular basis, although for locally advanced HNSCC there are several prognosticators discussed, e. g., hemoglobin and creatinine [6].

Measuring T cell responses to immunogens is highly complex and, depending on the specific antigen, there may be different patterns of simultaneously expressed cytokines in responding T cells [7]. Analyzing polyfunctionality of T cells is a recognized option in measuring T cell activation [8].

In conclusion, the newly available inhibition of the PD-1/PD-L1 pathway can release T cells, which are inhibited from exercising their anti-tumor functions by tumor-derived factors. Consequently, this inhibition of checkpoint inhibitors is beneficial for anti-tumor immune responses [9]. The availability of functional T cells is likely to be a prerequisite for the response to immunotherapeutic strategies and it has to be investigated which patients benefit from such an additional treatment after SOC. The first step is therefore to further elucidate influences on immune cells. The present study examines in detail lymphocyte subsets and T cell function in HNSCC patients at various disease stages.

Materials and methods

Patients

Peripheral blood mononuclear cells (PBMC) of two independent cohorts were analyzed. Cohort 1 consisted of HNSCC patients ($n=24$) with a median age of 61 ± 7 years

Table 1 Cohort characteristics

	NC	NT	SRG	CRT
Cohort 1 (n)	–	11	10	3
Age ± SD; sex	–	61 ± 8; 0♀ 11♂	61 ± 7; 3♀ 7♂	58 ± 4; 1♀ 2♂
T1–2 (*n*)	–	7	7	1
T3–4 (*n*)	–	4	3	2
N + (*n*)	–	4	0	3
Radiation dose (median Gy)	–	–	–	66
Chemotherapeutic substance	–	–	–	Cisplatin 3/3
Time to blood drawing (median years)	–	0.11	0.77	1.15
Cohort 2 (n)	10	–	7	6
Age ± SD; sex	n/a	–	50 ± 13; 0♀ 7♂	62 ± 10; 1♀ 5♂
T1–2 (*n*)	–	–	2	3
T3–4 (*n*)	–	–	5	3
N +	–	–	0	3
Radiation dose (median Gy)	–	–	–	66
Chemotherapeutic substance	–	–	–	Cisplatin 4/6 Cetuximab 2/6
Time to blood drawing (median years)	–	–	0.61	0.26

NC normal control, *NT* no treatment, *SRG* surgery, *CRT* chemoradiotherapy, *n/a* not applicable, *SD* standard deviation

(4 female, 20 male). Blood was collected from untreated patients, meaning patients with active disease before any kind of treatment (NT; $n = 11$) and from patients with no evidence of disease after receiving surgery only (SRG; $n = 10$) or primary chemoradiotherapy (CRT) ($n = 3$) in curative intent. Patients with primary CRT received a median radiation dose of 66 Gy through intensity-modulated radiation therapy (IMRT) and concomitant cisplatin. Patients with a lower T stage were more favorably treated with surgery whereas patients with a higher T stage underwent primary CRT (table S1). For cohort 2, blood was collected from healthy individuals as normal controls (NC; $n = 10$) and from HNSCC patients with no evidence of disease after surgical treatment (SRG; $n = 7$) or primary CRT ($n = 6$). Four patients in the CRT group had also undergone surgical resection beforehand. The median radiation dose was 66 Gy through IMRT. Four patients received concomitant cisplatin and two cetuximab. Radiation dose was lower (64–66 Gy) for patients with concomitant cisplatin compared to cetuximab (70 Gy). Median patient age was 58 ± 12 years (1 female, 12 male). Distribution of T stage was balanced for patients treated with CRT. More advanced tumors were operated in this cohort (table S1). Cohort 2 was collected and analyzed as a control cohort after samples of cohort 1 had been tested. The study was approved by the local ethics committee (IRB #991206) and written consent was obtained from all patients. Patient details are presented in Table 1.

Cell preparation and stimulation

Peripheral blood was drawn from healthy volunteers as well as HNSCC patients at different stages of disease as described above. PBMC were isolated by centrifugation on Ficoll-Hypaque® (Merck, Darmstadt, Germany), washed with PBS, and stored at –80 °C. For experiments, PBMC were thawed quickly, washed with PBS, and incubated in RPMI medium (Thermo Scientific, Waltham, MA, USA) supplemented with IL-2 (150 IU/mL, CellGenix, Freiburg, Germany) at 37 °C and 5% CO_2 overnight. 2×10^6 cells were transferred to FACS tubes and stimulated with staphylococcal enterotoxin B (1 mg/mL, SEB, Sigma-Aldrich, St. Louis, MO, USA) in 200 µL RPMI and incubated for 6 h at 37 °C in an atmosphere of 5% CO_2.

FACS analysis

Stimulation of lymphocytes was done in the exact same manner and flow cytometry measurements were performed simultaneously for all samples of the respective cohorts in order to minimize intra-experimental bias. Cohort 1 and cohort 2 were measured at different time points.

Unstimulated PBMC were stained for CD4, CD14, CD39, and CD25 for measurement of T cell subpopulations (tube 1). Cells were stained at room temperature (RT) for 30 min. The gating strategy is shown in Fig. 1a. Antibodies used were CD39-FITC (eBioscience, Santa Clara, CA, USA), CD25-PE (Miltenyi, Bergisch Gladbach, Germany), CD4-PerCP-Cy5.5, and CD14-PE/Texas-Red (Beckman Coulter, Brea, CA, USA). Regulatory T cells

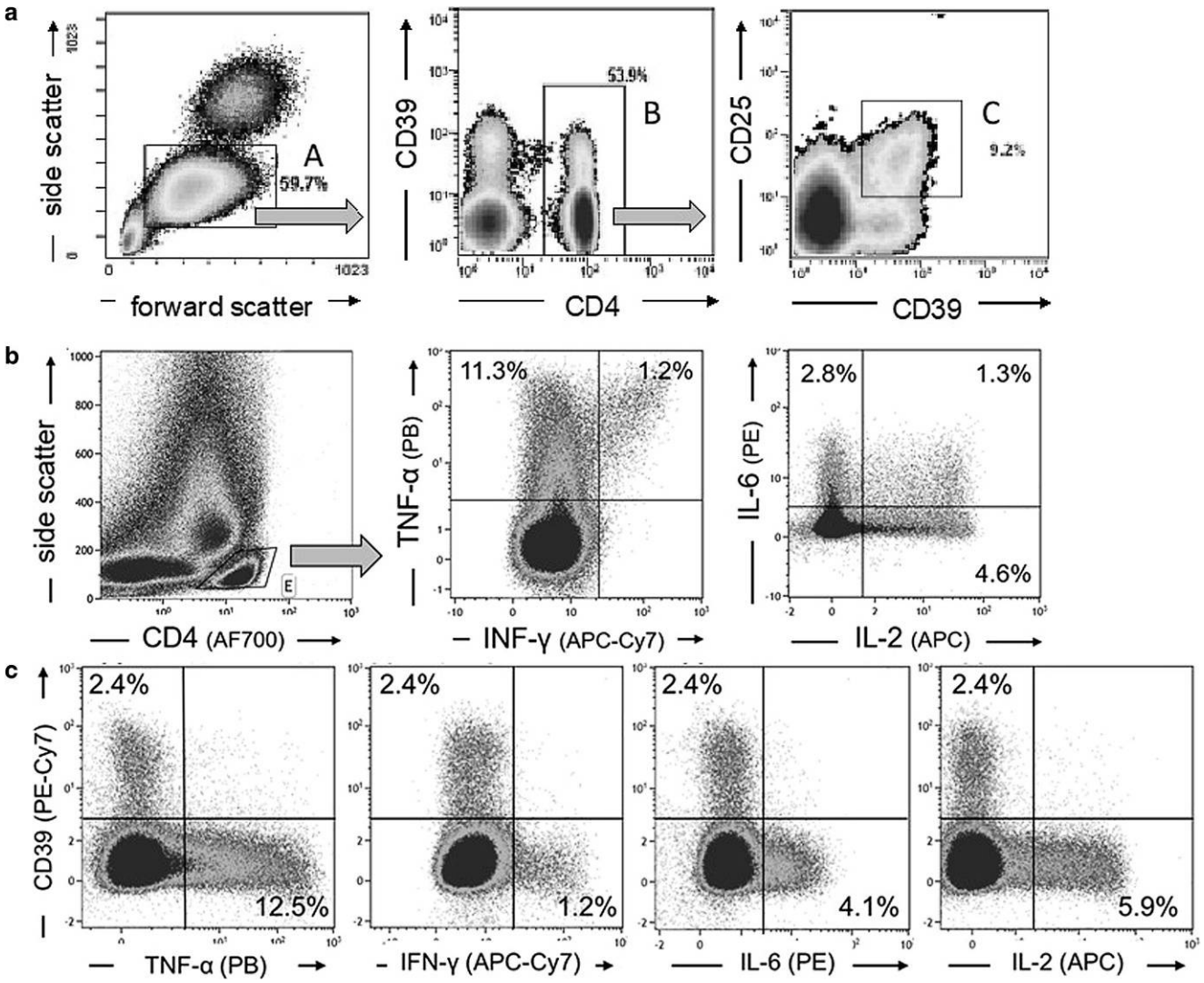

Fig. 1 Gating strategy flow cytometry analysis. **a** Gate A is set on lymphocytes based on forward and sideward scatter. Gate B is set on $CD4^+$ T cells. Gate C is set on regulatory T cells defined as $CD4^+CD39^+CD25^+$. **b** $CD4^+$ T cells express cytokines TNF-α, INF-γ, IL-2, and IL-6. INF-γ is mainly expressed together with TNF-α. IL-2 and IL-6 are almost not co-expressed. **c** Measured cytokines are only expressed by $CD4^+CD39^{neg}$ T-helper cells, but not by $CD4^+CD39^+$ regulatory T cells. *TNF-a* tumor necrosis factor alpha, *IFN-g* interferon gamma, *IL-2* interleukin 2, *IL-6* interleukin 6, *FOXP3* forkhead-box-protein P3, *TGF-ß* transforming growth factor beta

(Treg) were defined as $CD4^+CD39^+CD25^+$ lymphocytes. As previously published, this Treg population is $FOXP3^+$, TGF-β^+, and $CD127^{neg}$ [10, 11].

Next, stimulated PBMC (tube 2) were stained for CD4, CD8, and CD39 surface markers under the conditions described above. Cells were then stained for the intracellular markers FOXP-3, IL-2, IL-6, IFN-γ, and TNF-α using a FOXP3 staining kit (eBioscience). The antibodies used were FOXP-3-FITC, CD4-AF700, CD39-PE-Cy7, IL6-PE, TNF-α-Pacific-Blue (eBioscience); CD19-PE/Texas Red, CD8-PerCP-Cy5.5 (Beckman Coulter); IL2-APC, INF-γ-APC-Cy7 (Biolegend, San Diego, CA, USA). Directly after staining, cells were FACS analyzed on a 10-color flow cytometer (Gallios, Beckman Coulter, Brea, CA, USA). The applied gating strategies are shown in Fig. 1b, c.

Statistical analysis

Statistical testing showed a relevant variance between SRG and CRT subgroups of the two cohorts. Thus, pooling of the two cohorts was not performed in order to get uncompromised results. Cytokine expression in $CD4^+$ T cells was compared among the subgroups of the respective cohorts. Statistics were computed with SPSS v21 (IBM, Armonk, NY, USA). Due to the limited number of samples and the values not being normally distributed, the Mann–Whitney U test was used to compare cytokine expression and cell frequencies between subgroups; p-values <0.05 were considered statistically significant. Statistical graphics were created with SPSS v21 and Excel v15 (Microsoft, Redmond, WA, USA).

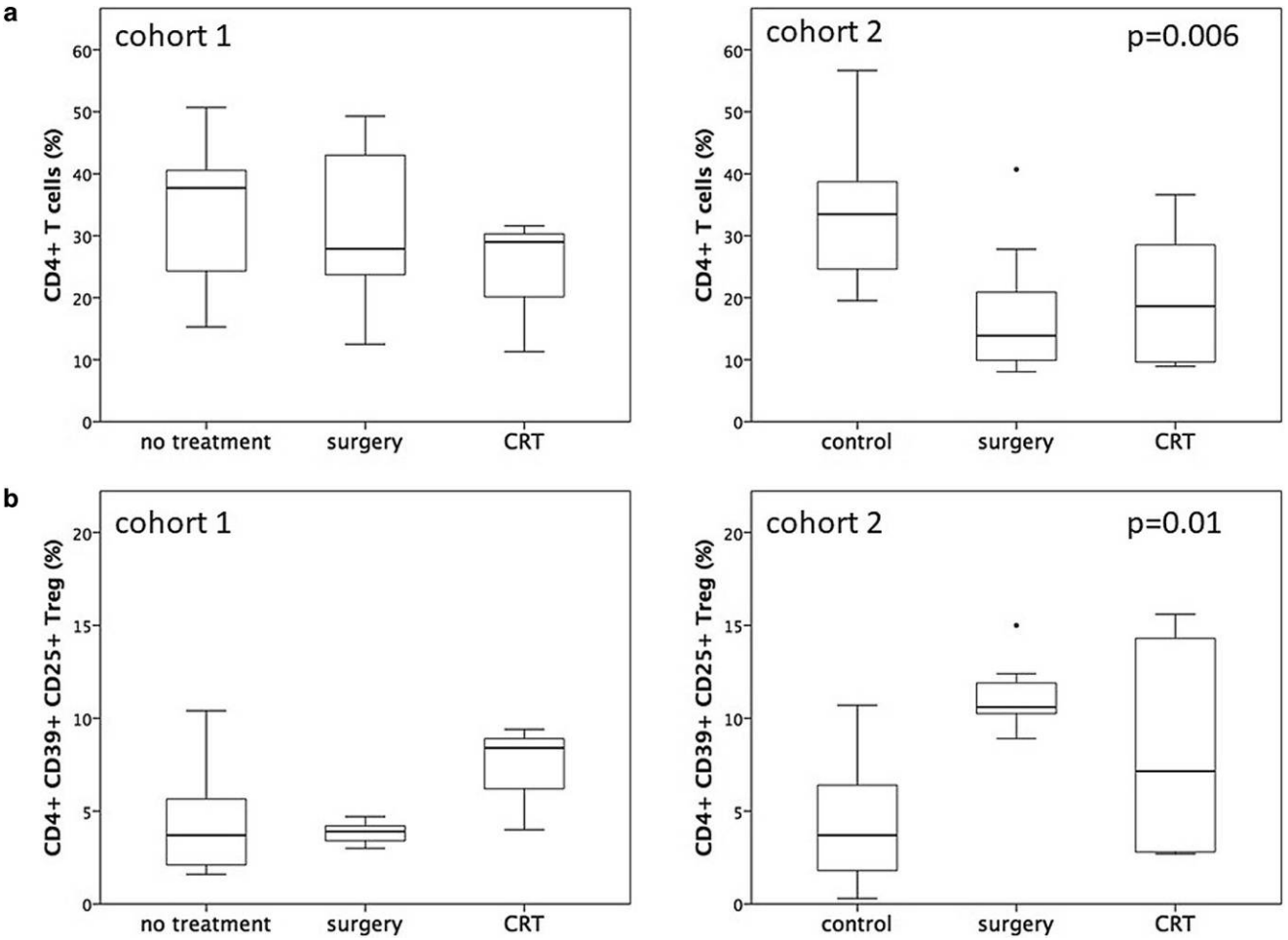

Fig. 2 Changes in lymphocyte frequencies according to treatment. **a** In both cohorts, the frequency of $CD4^+$ T cells is lower after surgery or chemoradiotherapy (*CRT*), which is significant in cohort 2 ($p=0.006$). **b** In both cohorts, the frequency of $CD4^+CD39^+CD25^+$ regulatory T cells (*Treg*) is elevated after CRT, which is significant in cohort 2 (0.01). *IL* interleukin, *TNF* tumor necrosis factor

Results

Lymphocyte frequency

The frequency of lymphocyte populations in each sample was measured in unstimulated cells and is presented in Fig. 2. In cohort 1, the frequency of $CD4^+$ T cells was lower after CRT (29.0 ± 11%) and even after SRG (27.9 ± 12.4%) in comparison to NT (37.7 ± 11.4%), without reaching significance. In addition, a higher frequency of $CD4^+CD39^+CD25^+$ Treg was observed after CRT (8.4 ± 2.9%) as compared to NT (3.7 ± 2.8%) and SRG (3.9 ± 0.6%) without reaching significance ($p=0.14$ and $p=0.09$, respectively). In cohort 2, the frequency of $CD4^+$ T cells within the analyzed lymphoid cell population was found to be higher ($p=0.006$) in NC (33.5 ± 11.9%) as compared to SRG (13.9 ± 12.1%) and CRT (18.9 ± 0.8%). The frequency of Treg was significantly ($p=0.01$) higher after CRT (7.2 ± 5.6%) and SRG (10.6 ± 2%) as compared to NC (3.7 ± 3.5%). No significant differences were measured in the frequency of $CD8^+$ T cells in the subgroups of both cohorts (data not shown).

Cytokine expression patterns

Cytokine expression patterns are shown in Table 2 and figure S2.

Cytokine expression was measured in stimulated cells. In each cohort, the single cytokine expression level was compared in the respective subgroups (NT/NC vs. SRG vs. CRT). In both cohorts, expression levels of the cytokines IL-2 and IL-6 were significantly higher in $CD4^+$ T cells after CRT. In particular, median expression of IL-2 was 7.2% in NT and 13.8% after CRT in cohort 1 ($p=0.02$), and 6.9% in NC as compared to 12.8% after CRT in cohort 2 ($p=0.05$). IL-6 expression differed from 5.3% (NT) to 17.3% (CRT) in cohort 1 ($p=0.01$), and from 4.9% (NC) to 10.1 (CRT) in cohort 2 ($p=0.04$). A significant difference for IFN-γ expression was observed in cohort 1 (1.1% in NT and 7.8%

Table 2 Expression profiles for individual cytokines expressed in T lymphocytes

	Group	IL-2	*p*-value	IL-6	*p*-value	IFN-γ	*p*-value	TNF-α	*p*-value
CD4+ T cells	C1 NT ($n=11$)	7.2 ± 4.6	0.02	5.3 ± 2.6	0.01	1.1 ± 2.7	0.05	28.9 ± 12.2	0.19
	C1 CRT ($n=3$)	13.8 ± 9.2		17.3 ± 6.1		7.8 ± 1.7		41.3 ± 8.2	
	C2 NC ($n=10$)	6.9 ± 3.9	0.05	4.9 ± 4.8	0.04	1.7 ± 2.4	0.28	31.2 ± 11.5	0.02
	C2 CRT ($n=6$)	12.8 ± 8.1		10.1 ± 6.0		3.7 ± 4.8		48.8 ± 10.0	
CD8+ T cells	C1 NT ($n=11$)	10.6 ± 3.9	0.07	0.1 ± 0.07	0.03	6.6 ± 10.7	0.31	13.0 ± 10.5	0.03
	C1 CRT ($n=3$)	14.4 ± 7.3		0.2 ± 0.06		27.0 ± 17.8		29.3 ± 7.2	
	C2 NC ($n=10$)	37.9 ± 3.7	0.66	0.2 ± 0.3	0.54	9.2 ± 5.6	0.82	16.0 ± 9.9	0.19
	C2 CRT ($n=6$)	40.0 ± 11.2		0.2 ± 0.1		8.4 ± 22.7		22.4 ± 20.0	

All values are given as median % ± standard deviation
NC normal control, *NT* no treatment, *CRT* chemoradiotherapy

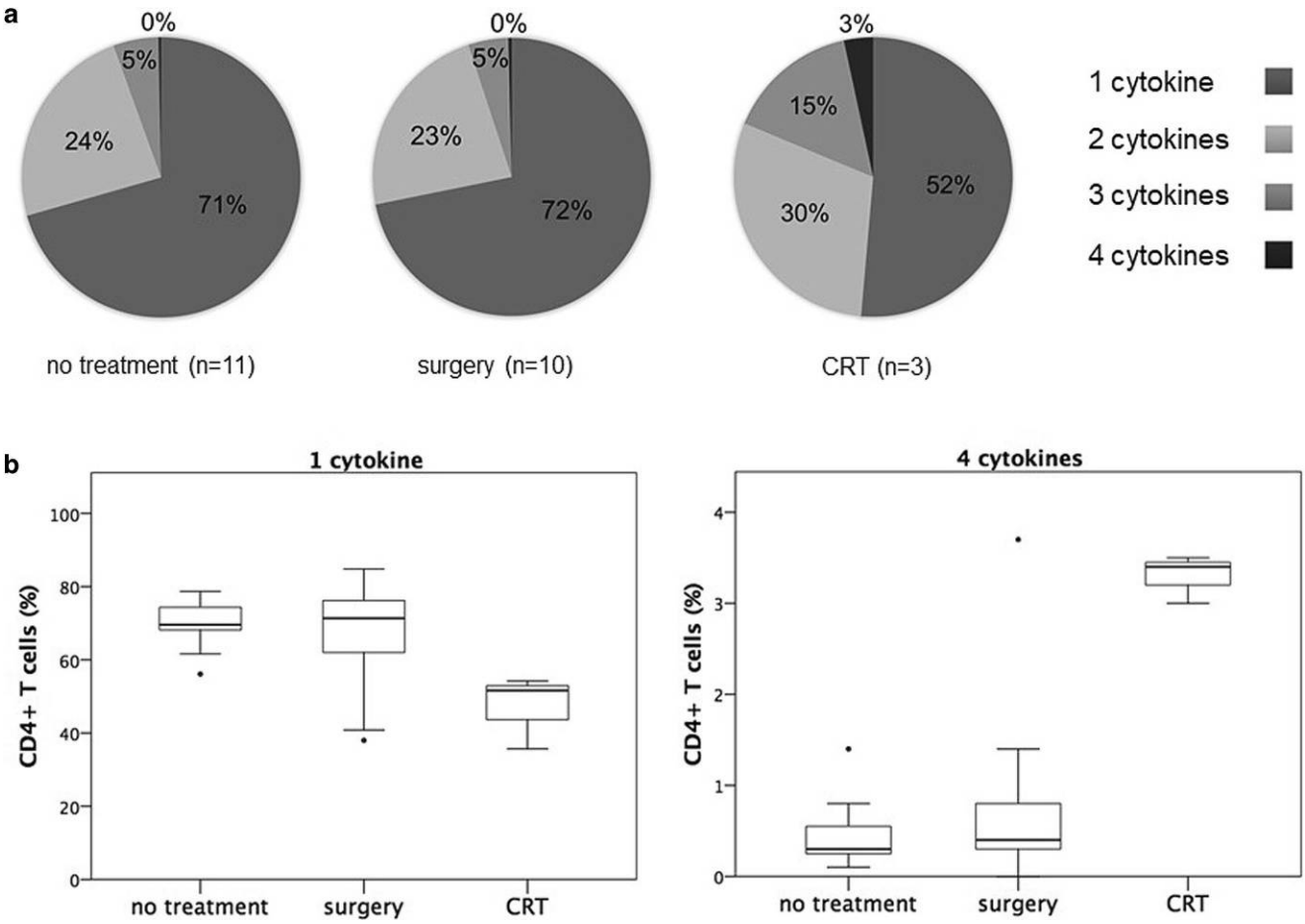

Fig. 3 Polyfunctional cytokine expression in cohort 1. **a** *Pie charts* show distribution of cytokine expression according to treatment. Surgery has almost no influence on the proportion of simultaneously expressed cytokines, whereas CRT leads to a higher number of T cells expressing more than one cytokine at a time. **b** *Boxplots* display changes for $CD4^+$ T cells expressing one cytokine or four cytokines simultaneously. It can be shown that the proportion of $CD4^+$ T cells expressing only one cytokine after CRT is lower as the proportion of cells expressing four cytokines is elevated at the same time ($p=0.01$ and 0.009, respectively)

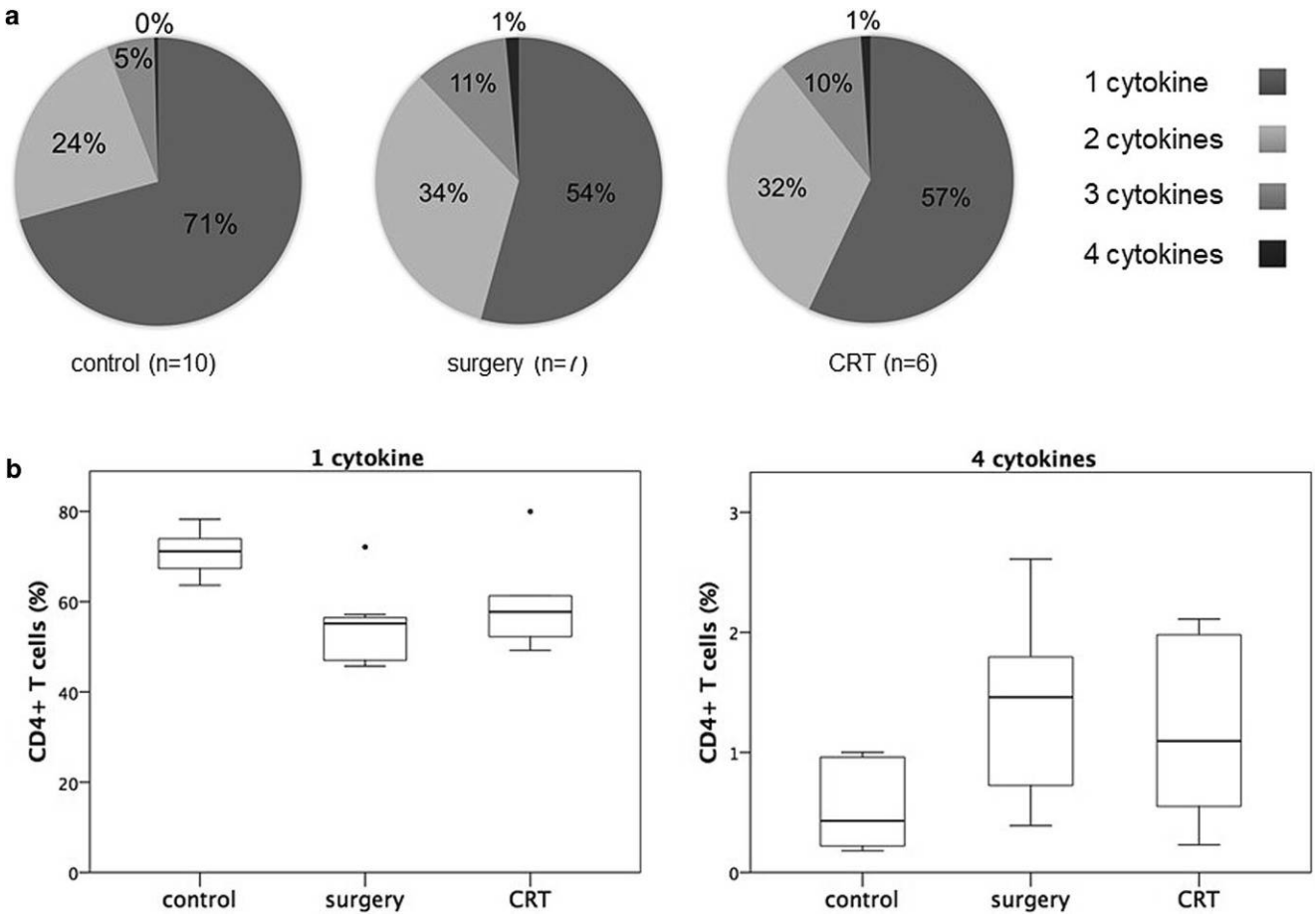

Fig. 4 Polyfunctional cytokine expression in cohort 2. **a** *Pie charts* show distribution of cytokine expression according to treatment. In comparison to healthy controls, the proportion of $CD4^+$ T cells expressing more than one cytokine is higher after surgery and chemoradiotherapy (CRT). **b** *Boxplots* display changes for $CD4^+$ T cells expressing one cytokine or four cytokines simultaneously. The proportion of $CD4^+$ T cells expressing one cytokine is significantly lower after surgery and CRT ($p = 0.03$) whereas more $CD4^+$ T cells show expression of four cytokines simultaneously after surgery and CRT ($p = 0.01$ and $p = 0.07$, respectively)

after CRT; $p = 0.05$) and for TNF-α expression in cohort 2 (31.2% in NC and 48.8% after CRT; $p = 0.02$).

In Fig. 1b and figure S1, examples of simultaneously expressed cytokines are shown. IFN-γ was only expressed in combination with TNF-α IL-2 and IL-6 were mainly co-expressed with TNF-α. Moreover, there was almost no co-expression of IL-2 and IL-6, and TNF-α was mostly expressed by itself. There were no significant differences in cytokine expression for NT/NC and CRT as compared to SRG. $CD4^+CD39^+CD25^+$ Treg did not express the measured cytokines at all (Fig. 1c).

Polyfunctionality of T cells

Using a 10-color flow cytometer allowed for measurements of simultaneous expression of four cytokines (IL-2, IL-6, TNF-α, and IFN-γ) on a single-cell basis. As a surrogate marker for polyfunctionality, the frequency of T cells expressing all four cytokines simultaneously was established. In cohort 1, a higher polyfunctionality was observed in $CD4^+$ T cells after CRT (3.4 ± 0.3%) as compared to NT (0.3 ± 0.3%; $p = 0.009$; Fig. 3). Accordingly, a significantly ($p = 0.01$) lower frequency of $CD4^+$ T cells expressing only one cytokine was observed after CRT (51.6 ± 10%) as compared to NT (69.9 ± 6.9%).

In cohort 2, polyfunctionality of $CD4^+$ T cells was increased after CRT (1.1 ± 0.8%) as compared to NC (0.4 ± 0.3%; $p = 0.07$; Fig. 4). Accordingly, a significantly ($p = 0.03$) lower frequency of $CD4^+$ T cells expressing only one cytokine was observed after CRT (57.8 ± 10.9%) as compared to NC (71.2 ± 4.8%). All data are listed in detail in Table 3. Interestingly, in cohort 2 the polyfunctionality in $CD4^+$ T cells was also increased after surgery (1.5 ± 0.8%) as compared to NC (0.4 ± 0.4%; $p = 0.01$). In $CD8^+$ T cells there was no simultaneous expression of four cytokines but only three at a time (Table 4). In cohort 1, there was a significant difference ($p = 0.04$) between patients with NT (2.1 ± 1.3) and patients after CRT (6.3 ± 2.3). There was

Table 3 Polyfunctionality of CD4+ T cells

	Group	1 cytokine	*p*-value[a]	2 cytokines	3 cytokines	4 cytokines	*p*-value[a]
CD4+ T cells	C1 NT (*n* = 11)	69.6 ± 6.9	–	23.5 ± 5.8	5.2 ± 1.7	0.3 ± 0.4	–
	C1 SRG (*n* = 10)	71.4 ± 15.5	NSD	23.0 ± 8.7	4.6 ± 6.6	0.4 ± 1.1	NSD
	C1 CRT (*n* = 3)	51.6 ± 10	0.01	30.0 ± 3.7	15.4 ± 6.3	3.4 ± 0.3	0.009
	C2 NC (*n* = 10)	71.2 ± 4.8	–	23.5 ± 3.3	5.4 ± 2.4	0.4 ± 0.3	–
	C2 SRG (*n* = 7)	55.2 ± 9.2	0.03	34.2 ± 6	10.8 ± 3.3	1.5 ± 0.8	0.01
	C2 CRT (*n* = 6)	57.8 ± 10.9	0.03	32.8 ± 7.7	9.6 ± 3.3	1.1 ± 0.8	0.07

All values are given as median % ± standard deviation
NC normal control, *NT* no treatment, *SRG* surgery, *CRT* chemoradiotherapy, *NSD* no significant difference
[a]*p*-values refer to subgroup C1 NT or C2 NC

Table 4 Polyfunctionality of CD8+ T cells

	Group	1 cytokine	*p*-value[a]	2 cytokines	3 cytokines	*p*-value[a]	4 cytokines
CD8+ T cells	C1 NT (*n* = 11)	58.2 ± 17.8	–	37.8 ± 17.1	2.1 ± 1.3	–	0
	C1 SRG (*n* = 10)	52.3 ± 17.4	NSD	45.1 ± 15.8	2.6 ± 3.3	NSD	0
	C1 CRT (*n* = 3)	39.7 ± 8.3	0.07	52.9 ± 6.8	6.3 ± 2.3	0.04	0
	C2 NC (*n* = 10)	74.6 ± 11.4	–	19.0 ± 10.0	5.2 ± 4.3	–	0
	S2 SRG (*n* = 7)	54.4 ± 11.7	0.01	31.8 ± 6.7	13.4 ± 6.2	0.03	0
	C2 CRT (*n* = 6)	60.7 ± 22.2	0.08	31.8 ± 15.6	7.1 ± 12.8	NSD	0

All values are given as median % ± standard deviation
NC normal control, *NT* no treatment, *SRG* surgery, *CRT* chemoradiotherapy, *NSD* no significant difference
[a]*p*-values refer to subgroup C1 NT or C2 NC

a trend towards a lower frequency of T cells expressing one cytokine ($p = 0.07$; figure S3). A slightly different pattern could be observed in cohort 2, where polyfunctionality differed from healthy controls and patients after surgery but not after CRT. The difference was also significant for reduction of cells expressing one cytokine (NC 74.6 ± 11.4 and SRG 54.4 ± 11.7; $p = 0.01$) and for the change in cells expressing three cytokines simultaneously (NC 5.2 ± 4.3 and SRG 13.4 ± 6.2; $p = 0.03$).

Discussion

In this study, we compared the lymphocyte frequency and the cytokine expression profiles of T cells before and after standard oncological treatment in HNSCC patients. While the frequency of CD4+ T cells was significantly lower after CRT, the mean frequency of Treg was strongly elevated and almost doubled. This is concordant to the results of our previous work, showing that Treg elevation is consistent for up to three years after CRT, while the absolute number of Treg is stable and the absolute number of CD4+ T cells is decreased. We therefore concluded that the increase in Treg frequency after CRT was due to their relative resistance to CRT as compared to CD4+ T cells [12]. Similar results for various cancer entities were found by others, who reported on an increased Treg frequency in patients with progressive disease after CRT [13]. However, these observations differ from entity to entity, and the exact influence of peripheral Treg is not clear yet [14]. In our present patient cohort, untreated patients were compared with samples after SRG or CRT. The frequency of Treg was only elevated after CRT, but not after SRG, suggesting that conventional CRT induces higher Treg levels in the peripheral blood, but not the cancer itself. However, when comparing all samples, a lower Treg frequency and a higher frequency of CD4+ T cells was observed for healthy individuals. Interestingly, the frequency of CD8+ T cells did not change significantly after treatment, which may be due to a relative resistance of CD8+ T cells to platinum-based CRT.

Next, we analyzed the influence of cancer activity, surgery, and CRT on expression levels of various cytokines in CD4+ T cells. Higher expression levels of IL-2 and IL-6 was found after CRT as compared to levels of cytokines in lymphocytes of patients before treatment (NT) or normal controls (NC). This observation might be explained as follows:

- (I) Although the frequency of Treg is increased, these have lost their suppressive potential after CRT;
- (II) T cells with the potential to express cytokines may be more resistant to CRT than silent T cells;
- (III) CRT induces cytokine production independent of specific antigens in a subset of T cells.

In our previous studies, we have shown that Treg are functional after CRT and produce more TGF-β than before CRT [12]. This is contradictory to hypothesis I, and may support hypotheses II and III. However, hypotheses II

and III should be tested by additional experiments with a larger cohort and paired samples, as it is still not clear whether the cytokine-expressing T cells already existed before CRT or not.

Agarwal et al. compared expression of mRNA specific for IFN-γ and IL-2 in $CD4^+$ T cells in the peripheral blood of HNSCC patients and healthy controls. A higher cytokine expression was found in early tumor stages as compared to advanced stages. Moreover, patients with local lymph node metastases displayed lower cytokine levels for IFN-γ and IL-2, but increased levels for Th2-defining cytokines IL-4 and IL-10 [15]. These results suggest a Th1 predominance in early stages, with a shift towards a Th2 response in advanced stages. Consequently, testing the polyfunctionality in a larger cohort with an emphasis on tumor stages could be of interest. Others have shown that antigen-specific T cells produce interferon-γ (IFN-γ) after activation [16]. The ability of IFN-γ to induce gene products which inhibit the cell cycle and even promote apoptosis make this cytokine an important player in the prevention of tumor development [17]. In HNSCC cell lines, it has been found that IFN-γ downregulates CXCR4 expression and consequently reduces proliferation and migration [18]. Another work on non-small cell lung cancer measured increased levels of IL-2 and IL-6 in PBMC of cancer patients as compared to healthy donors [19]. Although we were not able to directly compare NT with NC for expression levels of these cytokines in the present study, as they were measured in different cohorts, IL-6 was found at a similar level in both groups. HNSCC is associated with chronic inflammation, and serum levels of interleukin-6 (IL-6), an inflammatory cytokine, are elevated in those patients [20]. IL-6 is a well-characterized cytokine which is known to influence survival, invasion, and migration, as well as other tumor-promoting mechanisms in many human solid cancers [21]. On the other hand, IL-6 has an impact on the differentiation of T cells and is supposed to regulate the balance between regulatory T (Treg) cells and Th17 cells, which both play an important role in pro- and anti-tumoral effects [22]. Lastly, it has been shown that elevated serum levels of IL-6 are predictive for recurrence in HNSCC patients [23]. With low IL-6 expression in untreated patients, our present data are not able to support the established role of IL-6 in HNSCC progression [20].

Finally, we analyzed the polyfunctionality of T cells. Unexpectedly, the polyfunctionality of T cells increased after CRT as compared to untreated cancer patients or NC. Macchia et al. suggest that the polyfunctionality of T cells may serve as a monitoring marker for cancer treatment by immune vaccination. These investigators have established a standard procedure on how to measure changes in functionality of antigen-specific T cells in patients undergoing immunotherapy [24]. In addition, Yuan et al. reported on highly functional NY-ESO-1 antigen-specific T cells in metastatic melanoma patients responding to cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) treatment, suggesting that these T cells are potent effectors. However, it was not clear whether the potent T cells were already present at baseline [25]. Others have analyzed polyfunctional T cells even in non-malignant diseases, e. g., infection with the human immunodeficiency virus (HIV). In these patients, polyfunctional T cells were more powerful in reducing the viral load [26].

For future translational projects, we hypothesize that a minimum of functional T cells is a prerequisite for a successful immune therapy with, e. g., anti-PD1-inhibitors. The fact that after CRT a subset of polyfunctional T cells remains in the peripheral blood could serve as a rationale for subsequent or even concomitant immunotherapy that could benefit HNSCC patients. Therefore, it would be important to elucidate which patients would benefit from such an additional treatment. Due to the small number of samples after CRT available for our analyses, we could not determine whether responders differ from non-responders in terms of polyfunctionality. Also, it is believed that CRT equally activates specific and non-specific T cells. The small sample number and heterogeneous composition of the cohorts were two of the main limitations of the present study, although there were significant differences between treatment groups. Results have to be confirmed in larger cohorts. Moreover, T cell stimulation was performed with the unspecific antigen SEB. Therefore, the frequency of tumor antigen-specific T cells remains unclear. Possibly, an antigen-specific stimulation with, e. g., melanoma-associated antigen (MAGE), p53, or human papilloma virus (HPV) could give further insight into the behavior of cytokine-expressing T cells in cancer patients and this is the object of further experiments.

Conclusion

CRT induces an increased polyfunctional cytokine expression profile in peripheral T cells of HNSCC patients. These remaining T cells may be an important source for anti-tumor activities and their subsequent expansion by immunotherapy could represent a critical step towards long-term tumor control. The combination with a Treg-depleting approach could be beneficial, as the frequency of fully functional Treg is significantly increased after CRT. Our results may help in coordinating multiple regimens of cancer therapy in the future, including surgery, chemoradiotherapy, and immune therapy.

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Compliance with ethical guidelines

Conflict of interest J. Doescher, S. Jeske, S.E. Weissinger, C. Brunner, S. Laban, E. Bölke, T.K. Hoffmann, T.L. Whiteside, and P.J. Schuler declare that they have no competing interests.

Ethical standards All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

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