

# Comparison of the Composition of Lymphocyte Subpopulations in Non-relapse and Relapse Patients With Squamous Cell Carcinoma of the Head and Neck Before, During Radiochemotherapy and in the Follow-up Period: a Multicenter Prospective Study of the German Cancer Consortium Radiation Oncology Group (DKTK-ROG)

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# Abstract

**Background:** Radiochemotherapy (RCT) has been shown to induce changes in immune cell homeostasis which might affect antitumor immune responses. In the present study, we aimed to compare the composition and kinetics of major lymphocyte subsets in the periphery of patients with non-locoregional recurrent (n=23) and locoregional recurrent (n=9) squamous cell carcinoma of the head and neck (SCCHN) upon primary RCT.

**Methods:** EDTA-blood of non-locoregional recurrent SCCHN patients was collected before (t0), after application of 20 to 30 Gy (t1), in the follow-up period 3 (t2) and 6 months (t3) after RCT. In patients with locoregional recurrence blood samples were taken at t0, t1, t2 and at the time of recurrence (t5). EDTA-blood of age-related, healthy volunteers (n=22) served as a control (Ctrl). Major lymphocyte subpopulations were phenotyped by multiparameter flow cytometry.

**Results:** Patients with non-recurrent SCCHN had significantly lower proportions of CD19<sup>+</sup> B cells compared to healthy individuals before start of any therapy (t0) that dropped further until 3 months after RCT (t2), but reached initial levels 6 months after RCT (t3). The proportion of CD3<sup>+</sup> T and CD3<sup>+</sup>/CD4<sup>+</sup> T helper cells continuously decreased between t0 and t3, whereas that of CD8<sup>+</sup> cytotoxic T cells and CD3<sup>+</sup>/CD56<sup>+</sup> NK-like T cells (NKT) gradually increased in the same period of time in non-recurrent patients. The percentage of CD4<sup>+</sup>/CD25<sup>+</sup>/FoxP3<sup>+</sup> regulatory T cells (Tregs) decreased directly after RCT, but increased above initial levels in the follow-up period 3 (t2) and 6 (t3) months after RCT. Patients with locoregional recurrence showed similar trends with respect to B, T cells and Tregs between t0 and t5. CD4<sup>+</sup> T helper cells remained stably low between t0 and t5 in patients with locoregional recurrence compared to Ctrl. NKT/NK cell subsets (CD56<sup>+</sup>/CD69<sup>+</sup>, CD3<sup>-</sup>/CD56<sup>+</sup>, CD3<sup>-</sup>/CD94<sup>+</sup>, CD3<sup>-</sup>/NKG2D<sup>+</sup>, CD3<sup>-</sup>/NKp30<sup>+</sup>, CD3<sup>-</sup>/NKp46<sup>+</sup>) increased continuously up to 6 months after RCT (t0-t3) in patients without locoregional recurrence, whereas in patients with locoregional recurrence, these subsets remained stably low until time of recurrence (t5).

**Conclusion:** Monitoring the kinetics of lymphocyte subpopulations especially activatory NK cells before and after RCT might provide a clue with respect to the development of an early locoregional recurrence in patients with SCCHN. However, studies with larger patient cohorts are needed.

## Background

Head and neck cancer is the seventh most common cancer worldwide with an incidence rate of approximately 600.000 new cases in 2012 [1]. Despite progress in radiation oncology over the past decades, the 5 year survival rate of 40–60% of patients with locally advanced SCCHN is still not satisfying [1]. In advanced inoperable stages, SCCHN patients are treated primarily with RCT. Potential prognostic markers for the response to RCT include the human papillomavirus (HPV) infection status, tumor infiltrating lymphocytes (TILs), such as CD8<sup>+</sup> cytotoxic T cells and CD3<sup>-</sup> NK cells, and the Hsp70 and PD-L1 expression of the tumor. These biomarkers are usually assessed in formalin-fixed, paraffin-

embedded (FFPE) tumor specimens of patients who underwent radical surgery [2, 3]. Due to the lack of tumor material after RCT there is an unmet medical need for prognostic biomarkers which can be determined in the peripheral blood of patients. As radiotherapy (RT) as well as chemotherapy (CT) can result in drastic changes in immune-related parameters, such as the composition, phenotype and function of immunocompetent effector cells, the abundance and/or activation status of these markers might provide valuable tools to predict clinical outcome [4]. Therapy-induced modulations in immune cell homeostasis are of great importance as they might interfere with antitumor immune responses [5]. Therefore, we investigated the composition of major lymphocyte subsets in the peripheral blood of SCCHN patients treated with primary RCT before, during and at different timepoints after treatment.

## Methods

### Study collective

Thirty-two patients (n = 32), with histologically confirmed squamous cell carcinoma of the oral cavity, oro- and hypopharynx at UICC stadium III or IV without any remote metastases were included in our evaluation. The patients are a subset of patients recruited into the HNprädBio study (clinicaltrials.gov, NCT02059668) of the German Cancer Consortium Radiation Oncology Group (DKTK-ROG) and were selected by the availability of blood samples. Nine of these patients developed a locoregional recurrence within a median timeperiod of 11 months (range: 3–15 months) after diagnosis (t<sub>0</sub>). All patients received primary state-of-the-art RCT with a total radiation dose of 69–72 Gy to the boost volume, > 49 Gy to the elective volume and a cisplatin-based simultaneous CT. Patients with initial secondary tumors, previous RT and neoadjuvant CT were not enrolled in the HNprädBio study. EDTA-blood (7.5 ml) was collected at five sequential timepoints: before start of RCT (t<sub>0</sub>), after application of 20 to 30 Gy (t<sub>1</sub>), 3 months (t<sub>2</sub>), 6 months (t<sub>3</sub>) after RCT and at time of suspicion of recurrence or metastases, or after their histological examination (t<sub>5</sub>, 3–15 months after t<sub>0</sub>). As a control (Ctrl), blood samples of twenty-two healthy human volunteers (n = 22) with a median age of 64 years (range: 27–80 years) were included into the study. This trial was approved by the ethics committees of all eight DKTK partner sites and all patients and healthy donors gave written informed consent.

### Flow cytometry

The following major lymphocyte subpopulations were analyzed using fluorescence-labeled antibodies (supplementary Table 1) by multiparameter flow cytometry on a FACSCalibur flow cytometer (BD Biosciences):

Leukocytes (CD45)

B lymphocytes (CD3<sup>-</sup>/CD19<sup>+</sup> B cells)

T lymphocytes (CD3<sup>+</sup>/CD56<sup>-</sup> T cells, CD3<sup>+</sup>/CD4<sup>+</sup> T helper cells, CD3<sup>+</sup>/CD8<sup>+</sup> cytotoxic T cells)

T regulatory cells (CD4<sup>+</sup>/CD25<sup>+</sup>/FoxP3<sup>+</sup> Tregs)

Natural killer-like T (NKT) cells (CD3<sup>+</sup>/CD56<sup>+</sup>NKT cells, activated CD56<sup>+</sup>/CD69<sup>+</sup> NKT cells)

Natural killer (NK) cells (activated CD56<sup>+</sup>/CD69<sup>+</sup> NK cells, CD3<sup>-</sup>/CD56<sup>+</sup> NK cells, CD3<sup>-</sup>/CD94<sup>+</sup> NK cells, CD3<sup>-</sup>/NKG2D<sup>+</sup> NK cells, CD3<sup>-</sup>/NKp30<sup>+</sup> NK cells, CD3<sup>-</sup>/NKp46<sup>+</sup> NK cells) and NK subsets (CD56<sup>bright/dim</sup>/CD16 NK cells)

Briefly, 100 µl of EDTA-blood was mixed with different combinations of fluorescence-labeled, undiluted antibodies according to the manufacturer's recommendations. After incubation in the dark for 15 minutes, washing in phosphate buffered saline (PBS)/10% heat-inactivated fetal calf serum (Sigma F7524) and erythrocyte lysis buffer (BD, 349202), a total of 100.000 CD45<sup>+</sup> cells were gated and analyzed. In case that CD45 was not in the antibody panel, the data were determined on the basis of at least one additional marker that is in common with a panel that contains the leukocyte marker CD45. To determine the proportion of regulatory T cells, the CD4<sup>+</sup>/CD25<sup>+</sup> gated T cell population was stained with FoxP3 antibody after fixation (BD51-9005451) and permeabilization (BD51-9005450). The data were analyzed by using the software BD CellQuest Pro. The ratio of positively stained cells is defined as the percentage of cells within a defined lymphocyte gate minus the percentage of cells stained with a fluorescence labeled control antibody.

## Statistics

Statistical differences between sets of data were evaluated by IBM SPSS Statistics software (IBM GmbH, Ehningen, Germany). For data sets following a normal distribution the student's t-test was used, for all other data sets the Mann–Whitney Rank Sum Test was used. Values at different timepoints before, during and after RCT (t0, t1, t2, t3, t5) of all patients were considered as dependent samples; values at different timepoints of non-recurrent and locoregional recurrent patients in comparison with healthy controls were considered as independent samples. Data sets were considered as statistically significantly different at  $p \leq 0.05$ .

## Results

Kinetics of the proportion of CD3<sup>-</sup>/CD19<sup>+</sup> B cells before, during RCT and in the follow-up period

As summarized in Fig. 1A and supplementary Table 2, the percentage of CD3<sup>-</sup>/CD19<sup>+</sup> B cells in patients with non-recurring SCCHN (n = 23) was significantly lower compared to that of healthy volunteers (Ctrl) already before start of RCT at t0 (t0 vs. Ctrl: 8.88% vs. 10.83%,  $p \leq 0.05$ ), and remained low until 3 months after RCT at t2. The most striking drop in B cells was observed directly after the application of 20 to 30 Gy at t1 (t0 vs. t1: 8.88% vs. 4.66%,  $p \leq 0.001$ ; supplementary Table 3). A recovery to initial B cell levels was detected 6 months after RCT at t3 (Fig. 1A, supplementary Table 2). Patients with locoregional recurrence did not reach initial levels up to t5 (3–15 months) in case of tumor recurrence (Fig. 1A, supplementary Table 5).

Kinetics of the proportions of CD3<sup>+</sup>/CD4<sup>+</sup> T helper, CD3<sup>+</sup>/CD8<sup>+</sup> cytotoxic and CD4<sup>+</sup>/CD25<sup>+</sup>/FoxP3<sup>+</sup> regulatory T cell subsets before, during RCT and in the follow-up period

The percentage of CD3<sup>+</sup>/CD56<sup>-</sup> T cells in SCCHN patients without locoregional recurrence dropped significantly 3 (t2) and 6 (t3) months after RCT compared to initial levels (t0 vs. t2: 70.37% vs. 60.88%,  $p \leq 0.01$ ; t0 vs. t3: 70.37% vs. 56.05%,  $p \leq 0.001$ ; Fig. 1B). At t2 and t3, the values were also significantly lower than those of controls (Ctrl vs. t2: 67.29% vs. 60.88%,  $p \leq 0.05$ , Ctrl vs. t3: 67.29% vs. 56.05%,  $p \leq 0.001$ ; Fig. 1B, supplementary Tables 2) and to initial levels (supplementary Table 3). In patients with locoregional recurrence T cell ratios showed a slight increase at t1 that dropped below initial levels until t5 (Fig. 1B, supplementary Table 4).

As CD3<sup>+</sup>/CD4<sup>+</sup> T helper cells make up more than 2/3 of the total CD3<sup>+</sup> T cell counts, the kinetics of these cells followed that of the T cells in the course of therapy and in the follow-up period of SCCHN patients without locoregional recurrence. T helper cells dropped significantly at t2 and t3 (t0 vs. t2: 46.7% vs. 25.2%, t0 vs. t3: 46.7% vs. 26.27%,  $p \leq 0.001$ ; Fig. 1C, supplementary Tables 3), and patients without locoregional recurrence had significantly lower percentages of T helper cells compared to healthy controls between t1 and t3 (Ctrl vs. t1: 48.82% vs. 46.7%,  $p \leq 0.05$ ; Ctrl vs. t2: 48.82% vs. 25.2%, Ctrl vs. t3: 48.82% vs. 26.27%,  $p \leq 0.001$ ; Fig. 1C, supplementary Tables 2). Interestingly, the proportion of T helper cells in patients with locoregional recurrence was below that of controls and non-recurrent patients already before start of RCT and remained unaltered low throughout the whole course of therapy (Fig. 1C, supplementary Table 4, 5).

In contrast to the T helper cells, non-recurrent SCCHN patients had significantly higher proportions of cytotoxic CD8<sup>+</sup> T cells at all timepoints (t0, t1, t2, t3) compared to controls (Ctrl vs. t0: 10.83% vs. 17.12%, Ctrl vs. t1: 10.83% vs. 19.03%, Ctrl vs. t2: 10.83% vs. 25.44%, Ctrl vs. t3: 10.83% vs. 10.54%,  $p \leq 0.001$ ; Fig. 1D, supplementary Table 2). After RCT, the proportion of CD8<sup>+</sup> T cells in patients without locoregional recurrence increased further (t0 vs. t2: 17.12% vs. 25.44%,  $p \leq 0.001$ ; t0 vs. t3: 17.12% vs. 10.54%,  $p \leq 0.05$ ; Fig. 1D, supplementary Tables 3). Notably, recurrent patients showed a trend towards higher percentages of cytotoxic T cells at all timepoints (Fig. 1D, supplementary Tables 4 and 5), but no significant increase after RCT.

The percentage of regulatory T cells in SCCHN patients without locoregional recurrence at t0 was lower than that in healthy controls and dropped further directly after application of 20 to 30 Gy (t1) (Ctrl vs. t0: 9.92% vs. 6.77%,  $p \leq 0.01$ ; Ctrl vs. t1: 9.92% vs. 5.98%,  $p \leq 0.001$ ; Fig. 1E, supplementary Table 2). However, in the follow-up period (t2, t3) the percentage of Tregs increased significantly above initial levels (t0 vs. t2: 6.77% vs. 10.34%,  $p \leq 0.01$ ; t0 vs. t3: 6.77% vs. 10.06%,  $p \leq 0.05$ ; Fig. 1E, supplementary Table 3). In patients with locoregional recurrence Tregs remained below that of controls throughout the course of therapy (Fig. 1E, supplementary Table 4).

Kinetics of the proportion of CD3<sup>+</sup>/CD56<sup>+</sup> NKT cells before, during RCT and in the follow-up period

Similar to CD8<sup>+</sup> T cells, NK-like T (NKT) cells increased significantly during therapy in patients without locoregional recurrences (t0 vs. t1: 4.41% vs. 7.18%,  $p \leq 0.001$ ; t0 vs. t3: 4.41% vs. 7.07%,  $p \leq 0.01$ ; Fig. 1F, supplementary Table 3). Notably, before start of therapy at t0, the percentage of NKT cells was almost twice as high compared to healthy controls (Ctrl vs. t0: 2.46% vs. 4.41%,  $p \leq 0.05$ , Ctrl vs. t1: 2.46% vs. 7.18%,  $p \leq 0.001$ ; Ctrl vs. t2: 2.46% vs. 6.69%,  $p \leq 0.001$ ; Ctrl vs. t3: 2.46% vs. 7.07%,  $p \leq 0.01$ ; Fig. 1F, supplementary Table 2). Patients with locoregional recurrences showed stably higher proportions of NKT cells than controls throughout the therapy (Fig. 1F, supplementary Table 4).

Kinetics of the proportion of NK cell subsets (CD56<sup>+</sup>/CD69<sup>+</sup>, CD3<sup>-</sup>/CD56<sup>+</sup>, CD3<sup>-</sup>/CD94<sup>+</sup>, CD3<sup>-</sup>/NKG2D<sup>+</sup>, CD3<sup>-</sup>/NKp30<sup>+</sup>, CD3<sup>-</sup>/NKp46<sup>+</sup>) and CD56<sup>bright/dim</sup>/CD16 NK subset analysis before, during RCT and in the follow-up period

Already during RCT the proportion of NK cells continuously increased up to t3 in patients without, but not in patients with locoregional recurrence (Fig. 2B). Before RCT at t0 none of the tested NK cell subpopulations differed between healthy controls and patients without locoregional recurrence (Fig. 2, supplementary Table 6). However, the percentage of CD3<sup>-</sup>/CD56<sup>+</sup> NK cells in patients without locoregional recurrence increased significantly after RCT (t0 vs. t2: 11.1% vs. 15.83%, t0 vs. t3: 11.1% vs. 15.79%,  $p \leq 0.01$ ; Fig. 2B, supplementary Table 7) In contrast, patients with recurrence had lower proportions of all NK cell subpopulations already before RCT than controls that remained at low levels at all other timepoints (Fig. 2B-F, supplementary Table 8, 9).

Overall, the percentages of all NK cell subsets increased significantly after RCT in patients without locoregional recurrences (Fig. 2A-2F, supplementary Table 7). At t3, values of all NK cell subsets including those bearing activating receptors such as NKG2D, NKp30 and NKp46 were significantly higher than initial levels and those of healthy controls (Fig. 2A-2F, supplementary Tables 6, 7). In contrast, all NK cell subgroups in recurrent patients remained below that of patients without locoregional recurrence (Fig. 2A-2F, supplementary Table 8). Furthermore, recurrent patients had lower NK cell counts at time of locoregional recurrence (t5) compared to patients without recurrence at t3 (Fig. 2A-2F, supplementary Table 8).

A subgroup analysis of CD56<sup>bright</sup>/CD16<sup>+</sup> NK cells in SCCHN patients without locoregional recurrence revealed significantly higher proportions of CD56<sup>bright</sup>/CD16<sup>+</sup> NK cells compared to healthy controls, which dropped after application of 20 to 30 Gy at t1, but increased until t3 (Table 1A). A similar course was observed with respect to the CD56<sup>bright</sup>/CD16<sup>-</sup> NK cell subset in patients without locoregional recurrence. In recurrent patients both CD56<sup>bright</sup> NK cell subsets remained below that of patients without recurrence throughout the whole course of therapy (t0–t5) (Table 1B). In contrast, values of the CD56<sup>dim</sup> NK cell subset appeared to be elevated in recurrent patients (Table 1B).

## Discussion

It has been reported that the amount of B cells that contribute to the humoral immune response are lower in SCCHN patients than in healthy individuals [6–8]. Belka et al. were among the first to describe B cells as the most sensitive lymphocyte subpopulation towards radiation since they rapidly undergo radiation-induced apoptosis. However, the drop in B cells was not associated with a decline in the immunoglobulin levels [9], which might provide a hint that B cell activity is not impaired by radiotherapy. Our data are in line with these previous findings showing a significant drop in B cells after application of 20 to 30 Gy during RCT and a recovery to initial levels within a timeperiod of 6 months. The recovery might be mediated by B cell precursor cells originating from non-irradiated bone marrow [10] or by a retranslocation of B cells into the periphery. However, further studies are needed to address the question whether B cell functions such as antibody production or antigen presenting capacity in secondary lymphoid organs are impaired in SCCHN patients following RCT.

A long-lasting T cell imbalance with diminished T cell counts can be caused by immunosuppressive effects of the tumor and its microenvironment or by treatment-related effects [11]. Abnormalities in the T cell repertoire have been described for patients with myeloma, breast-, ovarian- and liver cancer [12–15]. Similar to our data, Nollert et al. demonstrated a significant drop in total CD3<sup>+</sup> T cell counts in stage IV SCCHN patients by RT combined with a platinum-based chemotherapy [16]. Since cisplatin is known to cause a systemic toxicity which is associated with a reduced T cell proliferation, the drug (prescribed cumulative total dose of cisplatin was not less than 180 mg/m<sup>2</sup> body surface area) might be responsible for the steady decrease in CD3<sup>+</sup> T cells during and after RCT in our cohort [17, 18].

Other studies have reported that the CD4<sup>+</sup> T cell counts in SCCHN patients, especially in advanced UICC stages III and IV, are significantly lower than in healthy subjects [19, 20]. Melioli et al. observed a severe proliferative defect especially in the CD4<sup>+</sup> T helper subset [21]. As T helper cells are the dominant T cell subset (up to 2/3 of the total T cell counts) and cisplatin's systemic toxicity results in a reduced T cell proliferation [17, 18], the drop in CD3<sup>+</sup> T cells is reflected in the CD4<sup>+</sup> T helper subpopulation upon RCT. In line with our findings, others also observed a decrease in the T helper subset in head and neck cancer patients during and after definite and adjuvant RT [11, 15, 22]. As patients with recurrent SCCHN are more likely to display abnormalities in their T cell development [23], the drop in CD4<sup>+</sup> T cells before start of therapy (t<sub>0</sub>) appeared to be more pronounced in patients with locoregional recurrence compared to patients who remained free of recurrence [22–24]. Therefore, we hypothesize that low initial T helper counts might provide a potential predictive marker for patients that more likely will develop a recurrent disease.

As cytotoxic T cells are potent mediators of protective antitumor immunity [25], a significant increase in the percentage of CD8<sup>+</sup> T cells in SCCHN patients without locoregional recurrence upon RCT might be indicative for the induction of an antitumor immune response induced by RCT. Wolf et al. also demonstrated a weak increase in CD8<sup>+</sup> T cells in head and neck cancer patients during and after definite and adjuvant RT [15]. Moreover, Balermipas et al. reported that elevated numbers of CD8<sup>+</sup> tumor infiltrating lymphocytes (TILs) in SCCHN patients treated with adjuvant RCT serve as a prognostic marker

for improved clinical outcome [2]. Compared to controls, SCCHN patients showed significantly increased activation status of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, as well as a higher migratory potential of these lymphocyte populations [24], which might explain an increased infiltration into the tumor tissue. However, an expansion of CD8<sup>+</sup> effector cells was accompanied by a rapid demise through apoptosis [26, 27]. In line with our findings, Johnson et al. observed that patients with recurrent SCCHN tended to have higher proportions of circulating CD8<sup>+</sup> cytotoxic T cells at diagnosis than patients who remained disease-free [23]. In addition, increased CD8<sup>+</sup> T cells in patients at diagnosis directly correlated with the level of tumor cell differentiation and histological grading [8]. However, we could demonstrate that the proportion of CD8<sup>+</sup> T cells did not increase significantly upon therapy in patients with locoregional recurrence.

Currently, functions of Tregs are not completely understood. While a high infiltration of FoxP3<sup>+</sup> Tregs at the tumor site correlates with an impaired overall survival in patients with melanomas, cervical-, renal- and breast cancers an opposite effect was observed in colorectal, head and neck as well as esophageal cancers [28]. As head and neck cancer, colon cancer and hematologic malignancies are among those tumors which are heavily infiltrated by immune cells that facilitate tumor progression by producing growth factors and/or proinflammatory cytokines, Tregs might limit tumor-promoting inflammation by the release of immunosuppressive cytokines [29–32]. Similar to our results, Lee et al. observed a weak decline in CD4<sup>+</sup>/CD25<sup>+</sup>/FoxP3<sup>+</sup> Tregs in patients with oral squamous cell carcinoma compared to healthy controls [24], while a rise in the frequency of Tregs was found in patients with SCCHN treated with adjuvant RCT [5, 33]. Finally, Kachikwu et al. observed an increase in Tregs after radiation which was associated with a more radioresistant phenotype [34].

As NKT cells are sharing some receptors and common features with T lymphocytes and NK cells they provide a functional link between the innate and adaptive antitumor immunity [35, 36] and play crucial roles in immune regulation and surveillance against cancer [37]. Kobayashi et al. described the ability of radiotherapy to improve proliferation and antitumor immunity of NKT cells [38]. In our SCCHN cohort, especially patients with locoregional recurrences, exhibited increased NKT cell levels at diagnosis which further increase throughout therapy as compared to controls. These data suggest the presence of a predefined immune imbalance which correlates with treatment response and the ability of RCT to boost SCCHN patients' immunocompetence. Additionally, the particularly high NKT cell value in recurrent patients at t0 could provide a hint for an increased risk for an early recurrence.

NK cells are important effector cells of the innate immune system and their ability to attack tumor cells have been considered to be of great importance in the fight against cancer [39]. In our study, a significant increase in the percentage of NK cells with activating receptors such as NKG2D, NKp30 and NKp46 might reflect the effect of RCT on patients' immunocompetence to improve antitumor responsiveness.

Moreover, it also has been reported that cytotoxic lymphocytes, including CD8<sup>+</sup> T cells and NK cells, contribute to the efficacy of certain chemotherapeutic drugs [4], such as cisplatin, paclitaxel and doxorubicin, which have been shown to enhance the NK and CD8<sup>+</sup> T cell-mediated killing as they increase the permeability of tumor cells towards the apoptosis inducing serine protease granzyme B [40].

Recurrent patients, in particular at diagnosis, tended to have much lower NK cell counts than healthy controls and patients without recurrence at all timepoints. An impaired first line defense might indicate an imbalance of the innate immune cell function in patients with recurrence.

NK cell function is dependent on a vast network of inhibitory and activatory signals and receptors. To avoid autoimmunity, the expression of these receptors is tightly regulated and balanced. As soon as stimulatory signals overrule inhibitory signals by a critical threshold, expression of activating receptors is induced which mediate direct cytotoxicity without further priming. Several of these activating receptors, including CD94/NKG2C, NKG2D and the natural cytotoxicity receptors NKp30 and NKp46, have been characterized to be particularly important for the recognition of tumor cells [41–44]. The expression density of the neuronal adhesion molecule-1 (NCAM-1) CD56 as a classical NK cell marker discriminates the two major NK cell subsets CD56<sup>dim</sup>/CD16<sup>+</sup> (90–95%) and CD56<sup>bright</sup>/CD16<sup>+</sup> (5–10%) which differ with respect to their functional characteristics including cytolytic activity, response to activating agents, cytokine profile and trafficking towards secondary lymphoid organs [45]. In general, CD56<sup>bright</sup>/CD16<sup>+</sup> NK cells are considered to have a low cytotoxic activity and a high cytokine/chemokine production capacity, whereas the CD56<sup>dim</sup>/CD16<sup>+</sup> NK cell population is associated with a high cytotoxic potential [45–47]. In accordance with this hypothesis, Mamessier et al. observed an increased proportion of CD56<sup>bright</sup>/CD16<sup>+</sup> circulating NK cells in advanced breast cancer patients and patients with metastases [48]. However, more recent data by the group of Wagner et al. revealed that upon stimulation CD56<sup>bright</sup>/CD16<sup>+</sup> NK cells can also exert direct antitumor activity [49]. CD56<sup>bright</sup>/CD16<sup>+</sup> NK cells mediate strong cytotoxicity against MHC class I negative as well as MHC class I positive tumors [47]. Our findings indicate significant higher percentages of CD56<sup>bright</sup>/CD16<sup>+</sup> NK cells in patients without locoregional recurrence compared to healthy controls and patients with recurrence. These values remained elevated in patients without locoregional recurrence throughout the course of therapy and in the follow-up period. Activation of NK/NKT cells is associated with an increase in the early activation marker CD69 in patients without locoregional recurrence. A high NK cell activity has been shown to correlate with lower incidence of tumors and thereby improves, alongside with NK cell infiltration in SCCHN, patients' clinical outcome [50, 51]. Similar to our findings, Takeuchi et al. demonstrated that a low NK cell activity leads to a higher incidence of tumor occurrence and metastasis, and its degree correlates with an increased invasiveness of the tumor [52]. In line with these findings, impairment of NK cell function is greater in patients with advanced tumors, as primary squamous cell carcinoma patients in stages T3-T4 showed lower NK cell activity than patients in earlier stages T1-T2 [53, 54]. Ye et al. observed a significant decrease in CD56<sup>+</sup>/CD16<sup>+</sup> circulating NK cells in oral cancer patients after surgery [55], indicating that not only RCT, but also inflammatory effects induced by surgery may have an impact on the NK cell hemostasis.

In our study, the proportion of all activated NK cell subsets was lower in the recurrent patient group compared to healthy individuals and patients without locoregional recurrence. Activatory NK cell subsets increased significantly in the course of therapy in non-recurrent patients, but remained nearly unaltered until t5 in patients with locoregional recurrence. Since NK cell responses occur fast this finding might be of relevance for predicting a potential recurrence and to adjust the treatment at an earlier timepoint. Our

findings demonstrated the ability of RCT in enhancing antitumor immune responses mediated by NK cells in patients with SCCHN. Therefore, we speculate that a combination of RCT with immunotherapy such as immune checkpoint inhibitors might provide a promising strategy to increase antitumor activity of NK cells in patients with SCCHN after RCT who are on high risk to develop recurrences.

## Conclusions

We could show altered lymphocyte compositions in patients with non-locoregional recurrences and patients with locoregional recurrences after primary RCT. These changes already existed before start of therapy and persisted for months in the follow-up period. B cells, T cells, T helper cells and regulatory T cells dropped significantly after RCT, but unlike T and T helper cells, B cells and regulatory T cells recovered to initial levels 6 months after RCT. Inversely, cytotoxic T cells, NKT and especially activating CD3<sup>+</sup> NK cell subsets gradually increased in the same timeperiod in non-recurrent patients, but remained nearly unaltered in patients with recurrent tumors. Following validation of our data in a larger patient cohort, monitoring cellular immunity in the peripheral blood of SCCHN patients during therapy might offer an opportunity for an early evaluation of treatment outcome and might help to stratify for patients that most likely will benefit from an additive immunotherapy.

## List Of Abbreviations

SCCHN squamous cell carcinoma of the head and neck

RCT radiochemotherapy

CT chemotherapy

RT radiotherapy

NK natural killer cells

NKT natural killer-like T cells

MHC major histocompatibility complex

## Declarations

### Acknowledgements

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### Ethical Approval and Consent to participate

This study was approved by ethical committees of all DKTK partner sites and informed consent to participate has been given by patients included into this trial.

### **Consent for publication**

All authors have given consent for publication.

### **Availability of supporting data**

Data and material are available by the corresponding author.

### **Competing interests**

No competing interest exists by any of the authors.

### **Funding**

Funding statement has been given.

### **Authors' contributions**

Patients included in this current study were treated within the HNprädBio trial at 5 DKTK partner sites. MN has performed the experiments, analyzed the data and has written the Ms. MB has initiated the DKTK trial. GM has designed this study and proof-read the Ms together with MK and AL. SEC, IT, VB, FR, JG, MK, AL, MB, NE, FL, ALG have provided blood samples and/or clinical data.

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# Table

Table 1A

Significantly different values in NK cell subsets in controls (Ctrl) and non-recurrent SCCHN patients

%	Ctrl	t0	t1	t2	t3
CD56 <sup>bright</sup> /CD16 <sup>-</sup> NK cells	1.83 ± 1.14	2.48 ± 1.94	1.78 ± 1.65*	2.11 ± 1.55	2.25 ± 1.62
CD56 <sup>bright</sup> /CD16 <sup>+</sup> NK cells	2.52 ± 1.34	4.72 ± 3.98*	3.72 ± 3.32	3.08 ± 2.15	4.09 ± 2.68
CD56 <sup>dim</sup> /CD16 <sup>-</sup> NK cells	2.8 ± 1.8	3.98 ± 2.82	4.42 ± 3.53	2.32 ± 1.33	7.5 ± 8.53
CD56 <sup>dim</sup> /CD16 <sup>+</sup> NK cells	87.27 ± 5.97	83.10 ± 10.29	84.93 ± 10.49	89.3 ± 5.46*	82.8 ± 12.27
CD56 <sup>-</sup> /CD16 <sup>+</sup> NK cells	0 ± 0	0.01 ± 0.02	0.14 ± 0.27	0.05 ± 0.09	0.09 ± 0.16
<p>Composition of NK cell subsets (CD56/CD16) in % (mean value ± standard deviation) in healthy controls (Ctrl, n = 22) and non-recurrent patients (n = 23) before (t0), after application of 20 to 30 Gy (t1), 3 months (t2) and 6 months (t3) after RCT. Significantly different values (t0 vs. t1; Ctrl vs. t0; t0 vs. t2) are indicated in bold with an asterisk (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).</p>					

Table 1B

Significantly different values in NK cell subsets in controls (Ctrl) and recurrent SCCHN patients

%	Ctrl	t0	t1	t2	t5
CD56 <sup>bright</sup> /CD16 <sup>-</sup> NK cells	1.83 ± 1.14	0.92 ± 0.7*	0.75 ± 0.63*	2.07 ± 1.52	1.6 ± 1.52
CD56 <sup>bright</sup> /CD16 <sup>+</sup> NK cells	2.52 ± 1.34	2.88 ± 1.85	1.47 ± 0.7*	5.76 ± 4.85	1.54 ± 1.32
CD56 <sup>dim</sup> /CD16 <sup>-</sup> NK cells	2.8 ± 1.8	10.19 ± 11.85	6.08 ± 5.3	4.3 ± 3.97	2.25 ± 2.05
CD56 <sup>dim</sup> /CD16 <sup>+</sup> NK cells	87.27 ± 5.97	81.4 ± 13.9	88.23 ± 7.52	84.78 ± 11.56	92.88 ± 4.26*
CD56 <sup>-</sup> /CD16 <sup>+</sup> NK cells	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Composition of NK cell subsets (CD56/CD16) in % (mean value ± standard deviation) in healthy controls (Ctrl, n = 22) and recurrent patients (n = 9) before (t0), after application of 20 to 30 Gy (t1), 3 months (t2) after RCT and at time of locoregional recurrence (t5, 3–15 months after t0). Significantly different values (Ctrl vs. t0, t1; t0 vs. t1; Ctrl vs. t5) are indicated in bold with an asterisk (\*p ≤ 0.05; \*\*p ≤ 0.01; \*\*\*p ≤ 0.001).

## Figures

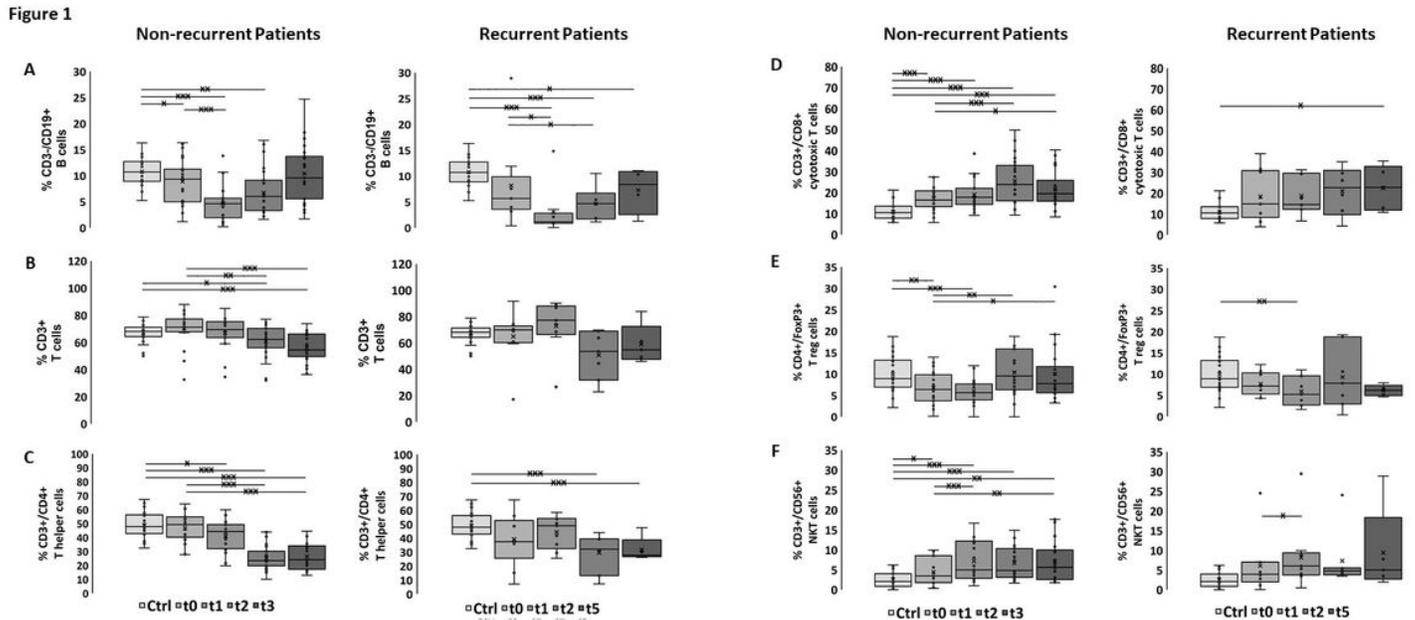
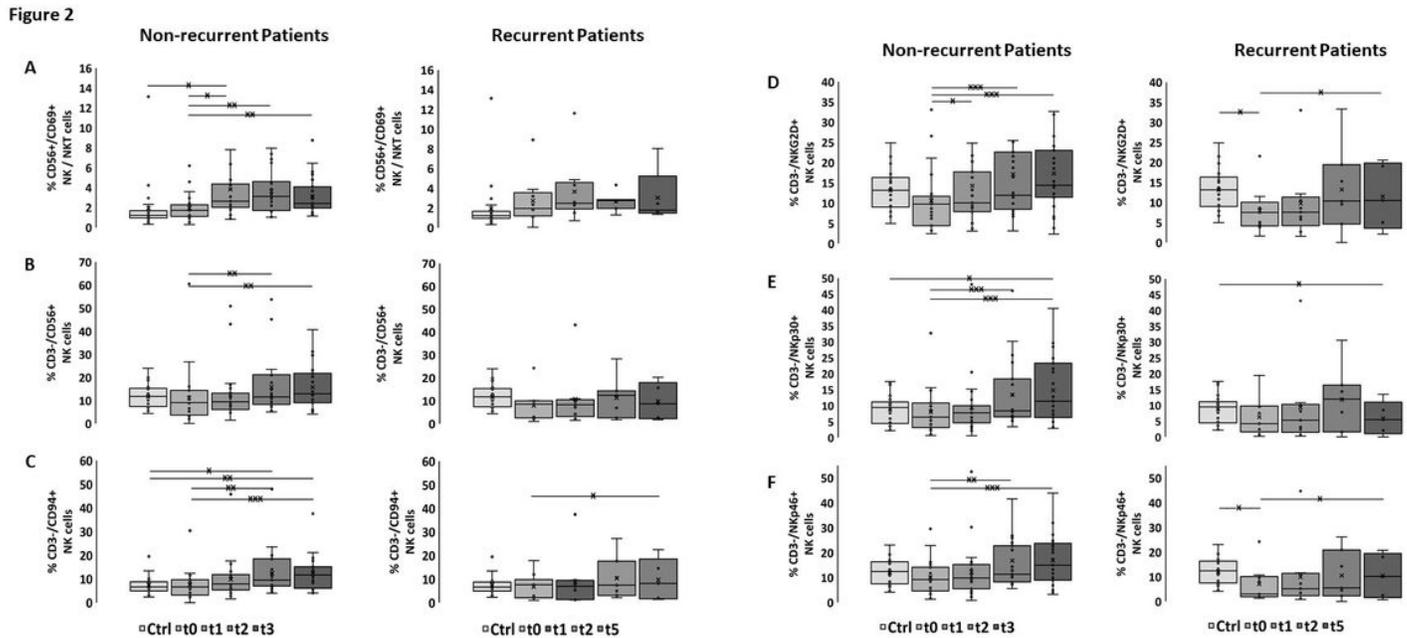


Figure 1

Immunophenotyping of major lymphocyte subpopulations. Percentages of A) CD3-/CD19+ B cells, B) CD3+ T cells, C) CD3+/CD4+ T helper cells, D) CD3+/CD8+ cytotoxic T cells, E) CD4+/CD25+/FoxP3+ Tregs, F) CD3+/CD56+ NK-like T cells in healthy controls (Ctrl, n=22), non-recurrent (n=23) and recurrent patients (n=9) with SCCHN before (t0), after application of 20 to 30 Gy (t1), 3 months (t2), 6 months (t3) after treatment and at time of locoregional recurrence (t5, 3-15 months after t0). The data show mean values  $\pm$  standard deviation of the percentage of positively stained cells. Significances are illustrated between t0 and other timepoints (tx) after start of RCT as well as between controls (Ctrl) and all timepoints of RCT (\* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$ ).



**Figure 2**

Immunophenotyping of NK cell subpopulations. Percentages of A) CD56+/CD69+ NKT/NK cells, B) CD3-/CD56+ NK cells, C) CD3-/CD94+ NK cells, D) CD3-/NKG2D+ NK cells, E) CD3-/NKp30+ NK cells, F) CD3-/NKp46+ NK cells in healthy controls (Ctrl, n=22), non-recurrent (n=23) and recurrent patients (n=9) with SCCHN before (t0), after application of 20 to 30 Gy (t1), 3 months (t2), 6 months (t3) after treatment and at time of locoregional recurrence (t5, 3-15 months after t0). The data show mean values  $\pm$  standard deviation of the percentage of positively stained cells. Significances are illustrated between t0 and other timepoints (tx) after start of RCT as well as between controls (Ctrl) and all timepoints of RCT (\* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$ ).

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