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# Comparative analysis of the effects of radiotherapy versus radiotherapy after adjuvant chemotherapy on the composition of lymphocyte subpopulations in breast cancer patients

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ABSTRACT

*Background:* Breast cancer is the most common cancer in women worldwide and surgery, radiotherapy (RT) and chemotherapy (ChT) are frequently used to treat this cancer. Adjuvant RT has been shown to cause long-term changes in lymphocyte counts in the peripheral blood. Herein, the time course of changes in lymphocyte subpopulations upon RT was studied in patients with and without adjuvant ChT in order to explore its potential clinical impact.

*Materials and methods:* Total lymphocyte counts and the composition of lymphocyte subpopulations before RT (t0), after 30 Gy (t1), at the end of RT (t2), and 6 weeks (t3), 6 months (t4), and 1 year (t5) after RT were studied by flow cytometry.

*Results:* Absolute lymphocyte counts were significantly lower in all breast cancer patients ($n = 40$) before and also 1 year after RT compared to healthy controls. The percentage of $CD3^+/CD4^+$ helper T cells and $FoxP3^+$ regulatory T cells increased significantly in patients without adjuvant ChT. Different NK cell subpopulations dropped during RT in patients with and without ChT, but recovered to initial levels 6 months after RT (t4). During RT (t0–t2) the percentage of $CD19^+$ B cells significantly dropped in patients without ChT, but gradually increased in patients with adjuvant ChT. Both patient groups reached initial levels 6 months after RT (t4).

*Conclusion:* Different lymphocyte subpopulations respond differently to RT with and without adjuvant ChT. $CD4^+$ T cells increase during RT, whereas NK cells and B cells decrease in patients without ChT, but recover within 6 months after RT. Treg cells gradually increase in patients without ChT from t0 to t5, but not in patients with adjuvant ChT.

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Breast cancer is the most common cancer in females worldwide with approximately 1.7 million newly diagnosed cases in 2012 (World Cancer Research Fund). Postoperative radiotherapy (RT) that is given to abolish remaining tumor stem cells after surgical resection is one of the most important treatment options to improve local tumor control and overall survival in breast cancer patients [1]. Although the main effect of RT is the direct killing of tumor cells, non-targeted radiation effects that can modulate antitumor immune responses may also affect clinical outcome. Different mechanisms are presently discussed that impair white blood cell counts and affect the composition of lymphocyte subpopulations. Stjernsward et al. [2] described long-term changes in lymphocyte counts induced by RT of breast cancer patients and related those to tumor responses. Immune effects have been shown to influence clinical outcome and prognosis [3]. Cho et al. claim that the nadir of lymphocyte counts has prognostic value in patients with head and neck cancer [4], while others have shown that white blood cell counts and serum markers did not correlate with prognosis in breast cancer patients treated with surgery, RT and ChT [5]. Varying numbers of dendritic cells (DCs), regulatory T cells, and tumor infiltrating lymphocytes (TILs) in patients suffering from head and neck [6] or colorectal cancer [7] have been described to have prognostic value and a correlation of these cell types and tumor stage was found in gastric or colorectal carcinoma patients [8,9].

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Although a highly suppressive microenvironment which impairs cellular immune responses has been shown to support tumor progression and metastatic spread [10,11], little is known about how cancer therapies directly impact on immune cells. A potential augmentation of antitumor immunity through chemotherapy [12] and radiotherapy [13] has been described. For a better understanding of frequently used tumor therapies on immune cells we investigated the effects of local RT and adjuvant ChT on lymphocyte composition in two breast cancer patient cohorts before, during and after RT.

## Material and methods

### *Study collective*

Female patients with unilateral invasive breast cancer, who received breast-conserving surgery, were recruited into the study which was approved by the local ethics committee of the Medical Faculty at TUM. Patients with distant metastasis, neoadjuvant ChT, mastectomy, previous RT, and secondary tumors were excluded. All patients ($n = 40$) received standard tangential chestwall RT with a median total dose of 60 Gy in daily 2 Gy fractions to the planning target volume (PTV). Nine patients received additional adjuvant ChT (5-fluorouracil (5-FU), epirubicin, cyclophosphamide, FEC) before RT. ChT was completed two to six weeks prior initiation of RT (Table 1). Blood (9.5 ml EDTA blood) was collected at six different time points: before RT (t0), after 30 Gy (t1), after RT (t2), 6 weeks (t3), 6 months (t4), and 1 year (t5) after the end of RT. Blood samples of twenty healthy female blood donors with a median age of 61.5 years (range: 41–77 years) were collected as a control collective.

**Table 1**
Patient characteristics.

| Characteristics | *N* | % |
|---|---|---|
| Age (years) | | |
| 30–39 | 1 | 2.5 |
| 40–49 | 9 | 22.5 |
| 50–59 | 11 | 27.5 |
| 60–69 | 13 | 32.5 |
| ⩾70 | 6 | 15 |
| Chemotherapy | | |
| Yes | 9 | 22.5 |
| No | 31 | 77.5 |
| Hormone therapy | | |
| Beginning before RT | 16 | 40.0 |
| Beginning after RT | 22 | 55.0 |
| No hormone therapy | 2 | 5.0 |
| Antibody therapy | | |
| Yes | 1 | 2.5 |
| No | 39 | 97.5 |
| Size of tumor (cm) | | |
| <1 | 15 | 37.5 |
| 1–2 | 21 | 52.5 |
| >2 | 4 | 10 |
| pTNM | | |
| T1(a–c) | 34 | 85 |
| T2 | 6 | 15 |
| N0 | 35 | 87.5 |
| N1 | 5 | 12.5 |
| M0 | 40 | 100 |
| G1 | 8 | 20 |
| G2 | 29 | 72.5 |
| G3 | 3 | 7.5 |
| Hormone receptor status | | |
| Estrogen receptor positive | 40 | 100 |
| Progesterone receptor positive | 39 | 97.5 |
| Her2-neu (DAKO 3+) | 1 | 2.5 |
| Total dose (Gy) | | |
| 40* | 3 | 7.5 |
| 60 | 29 | 72.5 |
| 66 | 8 | 20 |
| Total dose of boost (Gy) | | |
| 10 | 29 | 72.5 |
| 16 | 8 | 20 |

*Abbreviations:* TNB, tumor, nodes, metastases; T1a = ⩽0.5 cm (including micro-invasion); T1b = 0.5 cm and ⩽1 cm; T1c = 1 cm and ⩽2 cm; T2 = 2 cm and ⩽5 cm.
* Two patients were participants in a hypofractionation study and received 16 × 2.5 Gy to the whole breast and a simultaneously integrated boost (SIB) to the tumor bed (16 × 3 Gy). One patient was treated with hypofractionated radiotherapy of 15 × 2.67 Gy without a subsequent boost analog to the START-B trial.

### *Flow cytometry*

Lymphocyte subpopulations (T cells, regulatory T cells, NK cells, B cells) were analyzed by flow cytometry on a FACSCalibur flow cytometer (BD Biosciences). A representative instrument setting of the FACSCalibur for the analysis of lymphocyte subpopulations is summarized in Supplementary Table 1A. Briefly, 100 µl of EDTA-blood was mixed with the fluorescence-labeled, undiluted antibody combinations (Supplementary Table 1B) and incubated for 15 min in the dark. After washing in Flow-Cytometry buffer (PBS buffer with 10% heat-inactivated fetal calf serum, Sigma F7524) and lysis (BD, 349202) 50,000 to 100,000, CD45 positively-gated cells were analyzed. Regulatory T cells were determined in the $CD4^+CD25^+$ T cell population using FoxP3-PE-antibody after fixation (BD51-9005451) and permeabilization (BD51-9005450). The respective percentage of lymphocyte subpopulations is defined as the proportion of cells within the lymphocyte gate.

### *Statistics*

Statistical differences between sets of data were evaluated by using either the two-sided or one-sample student's t-test where the samples followed a normal distribution according to SigmaPlot software (Systat GmbH, Erkrath, Germany) using the Shapiro–Wilk test. Where the samples did not follow a normal distribution the Mann–Whitney Rank Sum Test was used. Data sets were considered as statistically significantly different at a $p \leqslant 0.05$.

## Results

### *Kinetics of lymphocyte counts and subpopulation composition before, during and after RT*

As shown in Fig. 1, breast cancer patients with (ChT+) and without (ChT−) adjuvant chemotherapy had significantly lower absolute lymphocyte counts compared to healthy controls ($^{***}p \leqslant 0.001$), before RT (t0) and after RT (t2–t5). This finding is in line with published data [14]. Patients with ChT (ChT+) revealed significantly lower lymphocyte counts than patients without ChT (ChT−, $^{***}p \leqslant 0.001$; Supplementary Fig. 1). With respect to major lymphocyte subpopulations the most striking drop was observed with respect to $CD19^+$ B cells in patients after adjuvant ChT ($^{***}p \leqslant 0.001$; Supplementary Fig. 2). In patients without ChT (ChT−) lymphocyte counts dropped significantly after RT from 1.6 ± 0.2 G/l (t0) to 1.1 ± 0.1 G/l (t2) ($^{***}p \leqslant 0.001$, Fig. 1), but returned to initial levels 6 months after RT (t4). In patients with adjuvant ChT (ChT+), RT did not decrease lymphocyte counts further and lymphocyte counts remained nearly unaltered during the whole therapy (Fig. 1).

The percentage of $CD3^+$ T cells significantly increased in patients without ChT (ChT−, t0 vs t2: 68.4 ± 1.2% vs 71.9 ± 1.4%, $^{*}p \leqslant 0.05$), whereas no significant change was observed in patients with ChT (ChT+, Fig. 2A). A subpopulation analysis revealed an