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ORIGINAL ARTICLE

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41.8°C whole body hyperthermia as an adjunct to chemotherapy induces prolonged T cell activation in patients with various malignant diseases

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Abstract Whole body hyperthermia (WBH) has been used as an adjunct to radio-/chemotherapy in patients with various malignant diseases. Although clear evidence is still missing, it has been hypothesized that an activation of the immune system might contribute to the therapeutic effect of WBH. To examine whether a treatment with 60-minute 41.8°C WBH as an adjunct to chemotherapy (WBH-CT) induces an activation of T cells, blood samples were collected at numerous time points before and up to 48 h post-treatment. The aim of this study was to examine the effect of WBH-CT on the expression of a broad range of activation markers on peripheral blood lymphocytes (PBL), on serum cytokines and intracellular cytokine levels in T cells, and the capacity of these cells to proliferate. Immediately after 41.8 °C WBH-CT treatment, a drastic increase in peripheral natural killer (NK) cells ($P < 0.05$) and $CD56^+$ cytotoxic T lymphocytes (CTL; $P < 0.01$) in the patients' peripheral blood was observed. At 5 h post-treatment, the percentages of both effector cell types had returned to baseline levels. This transient phenomenon was accompanied by a short period of reduced T cell activity, indicated by diminished serum levels of soluble interleukin-2 receptors (sIL-2R) at 3 h post-WBH-CT ($P < 0.05$) and decreased lymphocytic proliferation at the same point in time. This first phase was followed by a marked but short-lived increase in the patients' serum levels of interleukin-6 (IL-6; $P < 0.01$) during the first 5 h following treatment, with a subsequent decrease to baseline levels at 24 h and significantly increased serum levels of tumor necrosis factor-α (TNF-α) at 0 h ($P < 0.01$), 3 h ($P < 0.05$), 5 h ($P < 0.05$) and 24 h ($P < 0.01$) post-WBH-CT. The third phase of the immunological consequences of WBH-CT consisted of an increase in the percentage of peripheral cytotoxic T lymphocytes (CTL) expressing CD56, reaching a maximum at 48 h post-WBH ($P < 0.01$). Furthermore, the percentage of $CD4^+$ T cells expressing the T cell activation marker CD69 increased nearly two-fold over time, reaching its maximum at 48 h ($P < 0.05$). As an additional marker for T cell activation, serum levels of sIL-2R increased markedly ($P < 0.01$), reaching maximum levels at the same point in time. Elevated intracellular concentrations of interferon-gamma (IFN-γ) and/or TNF-α in $CD8^+$ T cells were found in 4 out of 5 patients at 24 h post-WBH-CT. Since similar changes were not observed in patients receiving chemotherapy alone, this is the first study to provide evidence for prolonged WBH-CT-induced activation of human T cells.

Keywords Cancer · Immunotherapy · NK T cell · T cell · Tumor immunity

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Introduction

Since the 1970s, systemic whole body hyperthermia (WBH) has been explored in phase I/II studies as a therapeutic option for patients with disseminated malignant disease [16, 44, 60, 61, 62] and has been used as an adjunct to radio-/chemotherapy in recent years [10]. Besides the well-known effects of hyperthermia that enhance the efficacy of certain cytotoxic drugs [17, 42], it has been suggested that an activation of the

immune system might also contribute to its therapeutic effects.

Thus, it has been shown that treatment with WBH as an adjunct to chemotherapy (WBH-CT) causes a marked but short-lived increase in the production of different cytokines. A 60-min, 41.8°C WBH-CT induced elevated plasma levels of granulocyte colony-stimulating factor (G-CSF), interleukin-1 beta (IL-1β), IL-6, IL-8, IL-10, and tumor necrosis factor-alpha (TNF-α) in cancer patients [45]. However, no study has thus far systematically included a control group receiving chemotherapy alone. In addition, it has not been shown whether increased levels of such cytokines have consequences for the function of immunological effector cells in vivo.

Only very few studies have examined the effects of 41.8°C WBH-CT on the cellular immune system in humans. These studies included very small numbers of subjects and focused only on short-term effects of WBH-CT. Nevertheless, results uniformly show an increase in the number of peripheral leukocytes [1, 8], neutrophils [1], and a transient increase in the number and activity of peripheral blood natural killer (NK) cells immediately after treatment with 41.8°C WBH-CT [2, 52].

It is clear that cytotoxic T lymphocytes (CTL) play a crucial role in the induction of an effective immune response against cancer [24]. In this context, it seems surprising that until now only one study has been published describing the effect of WBH-CT on lymphocyte subpopulations. In this study, published as an abstract, Ahlers et al. observed under comparable WBH-CT conditions a short-lasting decrease in peripheral $CD4^+$ and $CD8^+$ T cells, and in the expression of the IL-2 receptor on T lymphocytes [2]. The effect of WBH-CT on other T cell activation markers has not been investigated so far.

We examined the effects of 41.8°C hyperthermia on different immunological parameters in cancer patients undergoing WBH-CT treatment. Our primary aim was to investigate whether hyperthermia in comparison to chemotherapy alone had the potential to induce the production of cytokines related to T cell function, and whether this would be accompanied by changes in the expression of a variety of activation markers on the surface of these cells. Other goals of the study were to investigate WBH-CT-induced changes in the composition of lymphocyte subpopulations and the capacity of T cells to proliferate and to produce certain cytokines.

Materials and methods

Patients

A total of 28 consecutive patients undergoing WBH-CT in the Department of Oncology and Hematology at the University Hospital Eppendorf in Hamburg were included in the study. The majority of patients were male, and suffered from metastatic colorectal carcinoma ($N=16$). Other diagnoses included cholangiocellular carcinoma ($N=5$), pleural mesothelioma ($N=3$), soft tissue sarcoma ($N=2$), ovarian carcinoma ($N=1$), and non-small-cell lung cancer ($N=1$). All patients received 60 min of 41.8°C WBH generated by a radiant heat device (Enthermics Medical Systems) in combination with intravenous platinum-containing chemotherapy. One session lasted for about 4 h (90 min heating time, 60 min plateau at 41.8°C, 60–80 min cooling). For control experiments, blood was drawn from 5 comparable patients receiving platinum-containing chemotherapy alone. Cisplatin or its derivatives, carboblatin or oxaliplatin, whose action is known to be potentiated by hyperthermia [17, 42], were given on day 1 to all patients examined. This was followed by either ifosfamide or 5-fluorouracil. Dosages and schedule of administration were comparable in both groups.

Because of the excessive amount of blood that would have been needed to perform all the assays for every single patient, we had to limit the number of tests per patient. Therefore, it was possible that the numbers of patients assigned to each test might vary.

Absolute leukocyte numbers and white blood cell differential

Absolute leukocyte numbers and differential white blood cell (WBC) count were determined from 2 ml blood and ethylenediamine tetraacetic acid (EDTA) before WBH-CT treatment was started, immediately after the 60-min plateau at 41.8°C, and 3 h, 5 h, 24 h and 48 h after WBH using a VCS-based hematological counter (Beckman Coulter, Krefeld, Germany).

Peripheral blood mononuclear cell preparation

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by Biocoll (Biochrom, Berlin, Germany) density gradient centrifugation and washed two times in RPMI 1640 culture medium (Life Technologies, Paisley, U.K.).

Flow cytometry and cell surface marker analysis

All samples were stained and analyzed immediately after the blood had been drawn. Cell fluorescence was measured using a FACSCalibur cytometer (Beckton Dickinson, Heidelberg, Germany). Data analysis was performed using CellQuest software (Beckton Dickinson, Heidelberg, Germany). Lymphocyte subpopulations of patients were determined before WBH was started and at different time points thereafter. In a first group of patients, an analysis of the "early" effects of WBH was performed immediately following the 60-min plateau at 41.8°C (pre-WBH and 0 h, 3 h, and 5 h post-WBH). In a second group of patients, the"late" effects of WBH were examined (pre-WBH and 0 h, 3 h, 5 h, 24 h, and 48 h post-WBH). For the analysis of lymphocyte subpopulations, the phycoerythrin (PE) or fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (mAb) CD3, CD4, CD8, and CD56 (Becton Dickinson, San Jose, Calif.) were used. To investigate cellular activation in the second group of patients, we used PE-conjugated CD69, CD25, CD38, CD45RO, CD45RA, and HLA-DR antibodies (Becton Dickinson, San Jose, Calif.). IgG isotype controls were used in all experiments. A total of 1×10^6 PBMC were washed in phosphate-buffered saline (PBS; Life Technologies, Paisley, U.K.) and resuspended in 80 µl PBS, 10 µl of FITC-conjugated antibody, and 10 µl of PE-conjugated antibody according to the manufacturer's instructions. After incubation on ice for 30 min, the cells were washed in PBS and resuspended in 500 µl PBS. Data concerning activation markers are presented as the proportion of $CD4^+$ or $CD8^+$ lymphocytes expressing the marker relative to lymphocytes of the same sample not expressing the marker.

Intracellular cytokines

Intracellular cytokines in $CD8^+$ T cells were stained before WBH-CT treatment and 5 h and 24 h post-WBH-CT using the Cytodetect kit (IQ Products, Groningen, Netherlands) containing antibodies against several intracellular cytokines, wash solution, fixative solution and permeabilization reagent. Staining of intracellular cytokines was carried out according to the manufacturer's

instructions. Briefly, PBMC were prepared from heparinized patient blood as described above: 500 µl of the fixative solution (4°C) was added to 1×10^6 cells, and the cells were incubated for 10 min at room temperature. Afterwards cells were washed and permeabilized using 1.5 ml of the permeabilization solution. Intracellular antigens were incubated in the dark for 20 min (4°C) with 10 µl of PE-conjugated antibodies against TNF-α and interferon-gamma (IFN-γ). Surface antigens on lymphocytes were stained using CD8-FITC. Expression of antigens was analyzed using flow cytometry gating on the morphologically defined lymphocyte population.

Lymphocyte proliferation assay

The effect of WBH-CT on the proliferation rate of lymphocytes was assessed in 2 patients before WBH treatment was started and 0 h, 24 h, and 48 h post-treatment. PBMC were prepared from the peripheral blood of patients as described above, and cells were suspended in RPMI with 10% fetal calf serum (FCS) at a concentration of 1×10^6 cells/ml; 100 µl of cell suspension was placed in each well of a round-bottomed 96-well cell culture plate (Becton Dickinson, Lincoln Park, N.J.) and 100 µl complete medium with 2 ng OKT3 (Cymbus Biotechnology, Hants, U.K.) and 10 U IL-2 (Strathmann Biotech, Hannover, Germany) was added to each well. Controls without OKT3 and IL-2 were used in all experiments. Cells were incubated for 72 h at 37°C and 5% CO_2, then 1 µCi of methyl-[^{3}H]thymidine (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) was added to each well, and cells were again incubated for 24 h. DNA was then precipitated onto glass fiber filters by means of an automatic cell harvester, and the amount of incorporated [^{3}H]thymidine was determined in a liquid scintillation counter (Beckman Instruments, Fullerton, Calif.), and expressed as counts per minute (cpm).

Serum cytokines

Serum was prepared at the given time points from 7 ml of freshly drawn heparinized blood, and stored at –80°C until analysis. Analysis was performed using the Immulite system (DPC Biermann, Bad Neuheim, Germany), a fully automatic random access chemiluminescence-enhanced enzyme immunoassay system. The Immulite assays for sIL-2R, TNF-α, and IL-6 are based on a solid-phase sandwich chemiluminescence-enhanced enzyme immunoassay technique [30]. A polystyrene bead coated with murine mAb specific to the molecule to be measured serves as the solid phase. Enzyme-labeled polyclonal (rabbit) antisera for TNF-α, sIL-2R, and IL-6 are used as detection antibodies. The samples were incubated for 30 min (sIL-2R) or 60 min (TNF-α, IL-6) at 37°C with intermittent agitation. Unbound components were removed after 30 min using a patented centrifugal washing technique. Automatically added chemiluminescence substrate, AMPPD [3-(2'-spirodamantane)-4-methoxy-4-(3'-phosphoryloxy) phenyl-1,2-dioxetane] is converted by the bound enzyme during the following 10-min incubation period to an unstable intermediate. The resulting light emission is directly proportional to the concentration of the analyte in the samples.

Statistical analysis

Data concerning measurement of WBH, differential, intracellular calcium, lymphocyte surface markers, and serum cytokines were analyzed statistically. All other results were analyzed descriptively. To avoid multiple testing, variation across the consecutive blood samples was first tested using the Friedman's test for related variables. In case at least a trend ($P<0.1$) towards a significant variation was found, the Wilcoxon's test for paired samples was used to locate differences from pre-WBH values. For analysis of activation marker expression at the same time points on lymphocytes of healthy donors incubated at different temperatures, the Kruskall-Wallis test was used. Values were considered significant when $P<0.05$. The values given represent means plus standard error of the means in parentheses.

Results

Absolute leukocyte numbers and differential WBC

Absolute numbers of peripheral WBC increased following WBH-CT and remained elevated during the following 48 h (Table 1). The elevated leukocyte counts were mainly due to a significant and prolonged increase in the percentage and number of neutrophils. Absolute numbers of lymphocytes remained stable, although there was a relative decrease in relation to absolute leukocyte numbers following WBH-CT treatment. No changes were detected regarding percentages or numbers of monocytes. In patients receiving platinum-containing chemotherapy alone, no changes in any of the above-mentioned cell types were observed (data not shown).

Lymphocyte subpopulations

To limit the amount of blood needed per patient, we divided the participants into two subgroups. Within the first group of patients, we focused on the effects of WBH-CT treatment on the distribution of lymphocyte subpopulations during the first 5 h following treatment. The first remarkable finding during this initial phase followingg WBH treatment consisted of a drastic increase in the percentage and the absolute number of peripheral NK cells. Absolute numbers per microliter increased from baseline values 57.3 (±13.7) to 165.2 (±38.3) immediately after WBH treatment ($P<0.05$)

Table 1 Differential WBC count in patients receiving 41.8°C WBH as an adjunct to chemotherapy (WBH-CT) before and 5 h, 24 h, and 48 h post-treatment. Percentages determined in 11 patients, absolute numbers determined in 8 patients

	Pre-WBH		5 h post-WBH		24 h post-WBH		48 h post-WBH	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
WBC/µl	4,625	419	8,388[a]	1,148	8,188[a]	1,748	7,250[a]	870
Neutrophils (%)	71.3	3.3	81.5[b]	2.3	81.5[a]	2.3	83.7[b]	1.8
Neutrophils/µl	3,297	462	7,151[a]	1,204	6,900[a]	1,550	6,119[a]	882
Lymphocytes (%)	20.1	2.8	8.0[b]	1.5	10.0[b]	1.9	9.3[b]	1.4
Lymphocytes/µl	944	153	700	129	740	251	764	136
Monocytes (%)	8.5	1.1	8.5	1.8	7.4	1.7	5.5	1.5
Monocytes/µl	354	32	522	84	539	98	376	99

Significant difference from pre-WBH value: [a]$P<0.05$; [b]$P<0.01$

and were back to baseline values 3 h later. In contrast, no significant changes occurred in patients receiving chemotherapy alone. NK T cells are phenotypically and functionally diverse [14]. Initially, NK T cells were described as cells that express an invariant TCR V-alpha 14 in mouse and V-alpha 24 in humans [23]. Recently, the existence of NK T cells expressing a variety of TCR has been demonstrated [9]. Cytotoxic T cells expressing the NK cell marker CD56 represent one of these NK T cell subpopulations. We observed a WBH-CT-induced increase in $CD56^+$ CTL that was even more pronounced than the increase in NK cells. Percentages and absolute numbers of cytotoxic T cells expressing the NK marker CD56 were markedly elevated ($P < 0.01$) at the end of the 60-min plateau at 41.8°C (Fig. 1A and B). At 3 h post-WBH-CT, the absolute numbers and percentages of this cell type had nearly returned to baseline levels. Again, no significant changes were observed in the patients receiving chemotherapy alone (Fig. 1A and B).

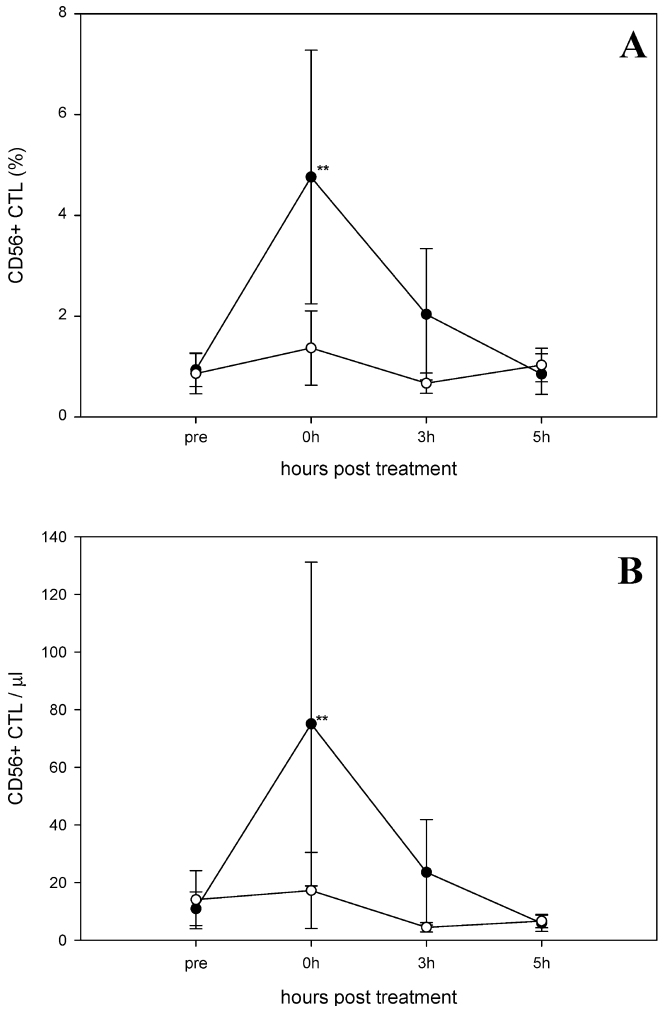

Fig. 1 Percentages (**A**) and absolute numbers (**B**) of peripheral $CD3^+/CD8^+/CD56^+$ cytotoxic T cells in patients ($N = 9$) undergoing 41.8°C WBH-CT (*filled circle*) and controls ($N = 5$) receiving platinum-containing chemotherapy alone (*open circle*) before treatment and 0 h, 3 h, and 5 h post-treatment. *Asterisks* indicate significant differences from baseline value (**$P < 0.01$)

In the second group of patients receiving WBH-CT treatment, we examined lymphocyte subpopulations before and 5 h, 24 h, and 48 h post-treatment. In this "late" phase of WBH-CT-induced immunological changes, we observed a transient and significant decrease in the percentage of $CD8^+$ lymphocytes at 5 h post-WBH (Table 2). $CD4^+$ lymphocytes also decreased, but this did not reach statistical significance. No significant changes were observed for $CD19^+$ B cells (data not shown) and for the $CD4^+/CD8^+$ ratio, but the percentage of $CD4^+/CD8^+$ double-positive cells decreased during the first 24 h following WBH-CT. Towards the end of the observation period, we found an increase in the percentage of $CD56^+$ cytotoxic T cells in each patient examined. This increase was more than 30% at 48 h post-treatment as compared to baseline ($P < 0.01$). Noticeably, in individual patients we found an up to 5-fold increase in the percentage as well as in the absolute numbers of the same cells at 48 h post-WBH-CT. None of the above-mentioned changes occurred in patients receiving chemotherapy alone. The only significant change in these patients was a slight decrease in the percentage of NK cells at 5 h post-treatment (Table 3).

Lymphocyte activation markers

Lymphocyte activation markers were assessed in 11 patients. The expression of the "early" activation

Table 2 Lymphocyte subpopulations (percentages) in patients receiving 41.8°C WBH-CT before and 5 h, 24 h, and 48 h after treatment

	Pre-WBH		5 h post-WBH		24 h post-WBH		48 h post-WBH	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
$CD4^{+c}$	38.0	3.4	31.5	4.0	33.3	4.2	24.3	2.1
$CD8^{+c}$	23.4	1.5	17.2[b]	1.8	21.4	2.1	24.3	2.1
CD4/CD8 ratio[c]	1.6	0.1	1.9	0.2	1.6	0.2	1.6	0.2
$CD4^+/CD8^{+c}$	1.5	0.3	1.1[a]	0.3	1.3*	0.3	1.6	0.3
$CD3^-/CD56^{+d}$	7.8	1.5	6.6	1.8	7.9	2.6	6.6	1.4
$CD3^+/CD8^+/CD56^{+d}$	1.5	0.3	0.9	0.3	1.4	0.3	2.0[b]	0.4

Significant difference from pre-WBH value: [a]$P < 0.05$; [b]$P < 0.01$
[c]Parameters determined in 18 patients
[d]Parameters determined in 11 patients

Table 3 Lymphocyte subpopulations (percentages) in patients ($N = 5$) receiving cisplatinum-based chemotherapy without WBH treatment before and 5 h, 24 h, and 48 h after treatment

	Pre-treatment		5 h post-treatment		24 h post-treatment		48 h post-treatment	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
$CD4^+$	28.6	7.0	24.9	7.2	27.4	8.6	27.2	7.5
$CD8^+$	24.3	4.7	18.6	2.3	23.2	2.6	20.0	1.1
CD4/CD8 ratio	1.3	0.3	1.3	0.5	1.3	0.5	1.3	0.3
$CD4^+/CD8^+$	1.2	0.4	0.8	0.3	1.1	0.2	0.9	0.3
$CD3^-/CD56^+$	10.4	2.2	6.6[a]	1.4	10.8	3.6	10.1	3.3
$CD3^+/CD8^+/CD56^+$	0.9	0.4	1.0	0.3	0.9	0.4	1.2	0.3

[a]Significant difference from pre-treatment value: $P < 0.05$

antigen CD69 on $CD4^+$ lymphocytes continuously increased following WBH-CT. At 48 h post-WBH, nearly twice as many $CD4^+$ lymphocytes expressed CD69 than before treatment ($P < 0.05$) (Table 4) and the intensity of CD69 expression on a per-cell level had also increased (Fig. 2A). Noteworthy, in single patients we noticed an up to 4-fold increase in the percentage of $CD4^+$ lymphocytes expressing CD69. In the majority of patients, there was also an increase in the percentage of $CD8^+$ lymphocytes expressing CD69 and in the intensity of CD69 expression on a per-cell level (Fig. 2B).

We observed a transient decrease in the expression of HLA-DR on $CD4^+$ lymphocytes 24 h post-WBH-CT ($P < 0.05$). Regarding the expression of all other surface markers (CD25, CD38, HLA-DR, CD45RO, CD45RA) we found no significant WBH-CT-induced changes. Pretreatment or the number of WBH-CT cycles the patient had received before the analysis was performed had no effect on the extent of the increase in CD69 expression on $CD4^+$ lymphocytes. Treatment with chemotherapy alone did not lead to an increase in CD69 expression on $CD4^+$ or $CD8^+$ lymphocytes (data not shown).

Lymphocyte proliferation

Additional experiments were performed in 2 patients to examine WBH-CT-induced changes in the proliferative potential of PBL. Immediately after the 41.8°C WBH-CT treatment, T lymphocyte proliferation after stimulation with anti-CD3/IL-2 in both patients was markedly reduced, but returned to baseline values 24 h post-WBH (Fig. 3). Importantly, 41.8°C hyperthermia did not cause increased cell death, as viability indicated by trypan blue exclusion always exceeded 95%.

Serum cytokines

WBH-CT-induced changes in serum cytokine levels (IL-6, TNFα, sIL-2R) were assessed in 11 patients. Five patients receiving chemotherapy alone served as controls. The serum concentration of IL-6 increased significantly directly after the treatment and reached its maximal concentration at 3 h post-WBH-CT (Fig. 4A). Twenty four hours after the WBH-CT treatment, the serum concentration of this cytokine was still slightly elevated. In 7 patients, we also evaluated the IL-6 concentration as late as 48 h after the treatment. At that point in time, the concentration of IL-6 had returned to baseline levels in all patients examined (data not shown). Patients receiving chemotherapy alone showed no changes in serum IL-6 concentrations.

Serum concentration (pg/ml) of TNF-α (Fig. 4B) was found to increase immediately after treatment ($P < 0.01$) in contrast to patients treated with chemotherapy alone. The concentration remained significantly elevated 3 h, 5 h, and 24 h post-hyperthermia when compared to baseline values.

Serum activity of sIL-2R (Fig. 4C) decreased immediately after treatment ($P < 0.01$). Subsequently, sIL-2R activity increased continuously from 3 h post-WBH to 24 h post-treatment ($P < 0.01$) compared to baseline values. In fact, in every single patient sIL-2R activity at 24 h post-WBH was higher than at all previous time points. In 7 of these patients, we also determined sIL-2R values at 48 h post-WBH-CT treatment. In all these cases, IL-2R activity at 48 h post-WBH was even higher than at 24 h post-treatment. Again, no such changes were observed for patients receiving chemotherapy alone.

Table 4 Percentage of $CD4^+$ or $CD8^+$ lymphocytes expressing CD69 in patients receiving 41.8 °C WBH-CT before and 5 h, 24 h, and 48 h after treatment ($N = 11$)

	Pre-WBH		5 h post-WBH		24 h post-WBH		48 h post-WBH	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
CD4/CD69	5.7	1.1	7.2	1.5	14.2	4.8	9.9[a]	2.0
CD8/CD69	23.3	3.5	22.8	4.0	31.4	4.2	30.3	3.8

[a]Significant difference from pre-treatment value: $P < 0.05$

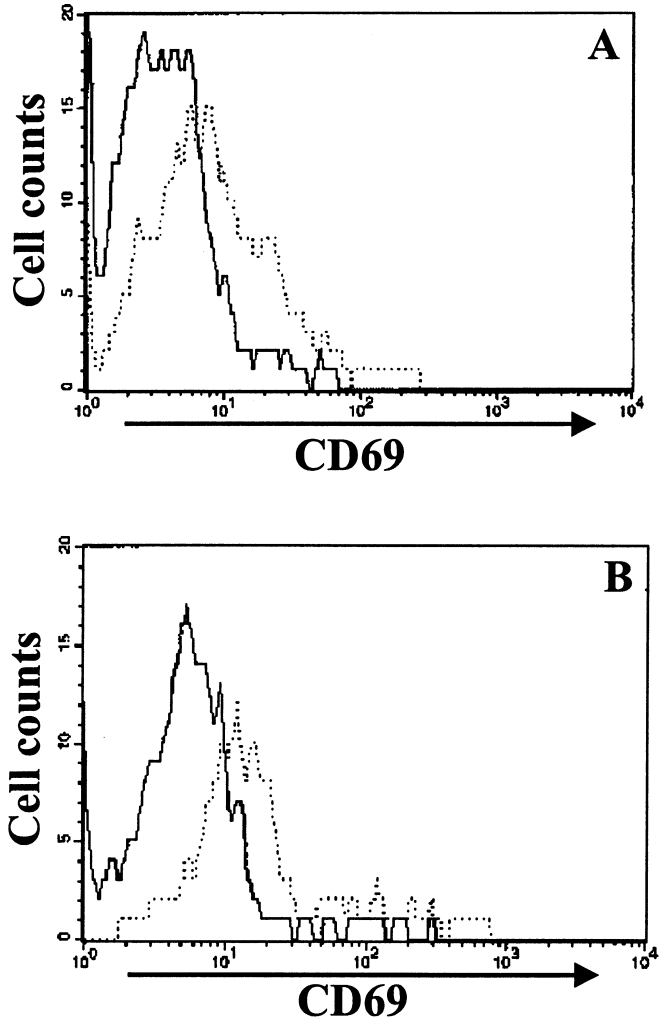

Fig. 2 Representative example of CD69 expression on $CD4^+$ (**A**) and $CD8^+$ (**B**) lymphocytes at baseline (*unbroken line*) and 48 h (*dotted line*) post-41.8°C WBH-CT treatment. Samples were stained and analyzed immediately after blood had been drawn

Intracellular cytokines

We examined intracellular cytokines in $CD8^+$ lymphocytes of 5 patients. At 5 h post-WBH-CT treatment, no changes were observed in the concentration of intracellular cytokines in $CD8^+$ lymphocytes (data not shown). At 24 h post-hyperthermia, we observed an increase in intracellular IFN-γ (Fig. 5A) and/or TNF-α (Fig. 5B) in $CD8^+$ lymphocytes in 4 out of 5 patients.

Discussion

Although many investigators in recent years have suggested a stimulatory effect of 41.8°C WBH-CT on the human immune system, clear evidence has still been missing. The findings described herein, based on extensive correlative laboratory studies, provide evidence for a prolonged T cell activation induced by WBH as an adjunct to chemotherapy (WBH-CT).

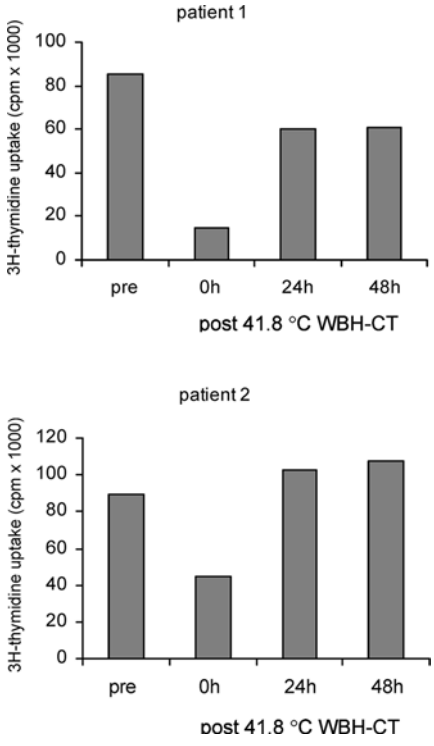

Fig. 3 OKT 3 and IL-2-induced [^{3}H]thymidine incorporation of PBL of 2 patients before 41.8°C WBH-CT and 0 h, 24 h and 48 h post-treatment

In previous studies, we as well as others have mainly focused on short-term effects of WBH-CT treatment on the immune system. One of the most obvious findings was a marked increase in peripheral NK cell numbers immediately after the treatment was finished, and a return to baseline values only a few hours later [52]. In this study, we were able to confirm these findings in a broader setting of patients and demonstrate that the transient increase in peripheral NK cells is accompanied by an even more pronounced increase in cytotoxic T cells expressing the NK cell marker CD56. Most importantly, no significant immunological changes were observed immediately after administration of chemotherapy alone.

Interestingly, during the increase of NK cells and $CD56^+$ CTL in the peripheral blood, a downregulation of the capacity of peripheral T cells to proliferate seemed to take place, which was also of short duration. To examine T cell proliferation, we stimulated unseparated PBMC with anti-CD3 antibody and IL-2. Therefore, an increase in peripheral NK cells and a proportional decrease in peripheral T cells could at least in part be responsible for the reduced lymphocytic proliferation rate immediately after WBH treatment. On the other hand, the treatment with hyperthermia itself might cause a state of transient cellular exhaustion in T cells.

Concerning the effect of WBH on the composition of peripheral leukocytes, other groups have shown an increase in the number of peripheral leukocytes [1, 8] and neutrophils [1] immediately after treatment with

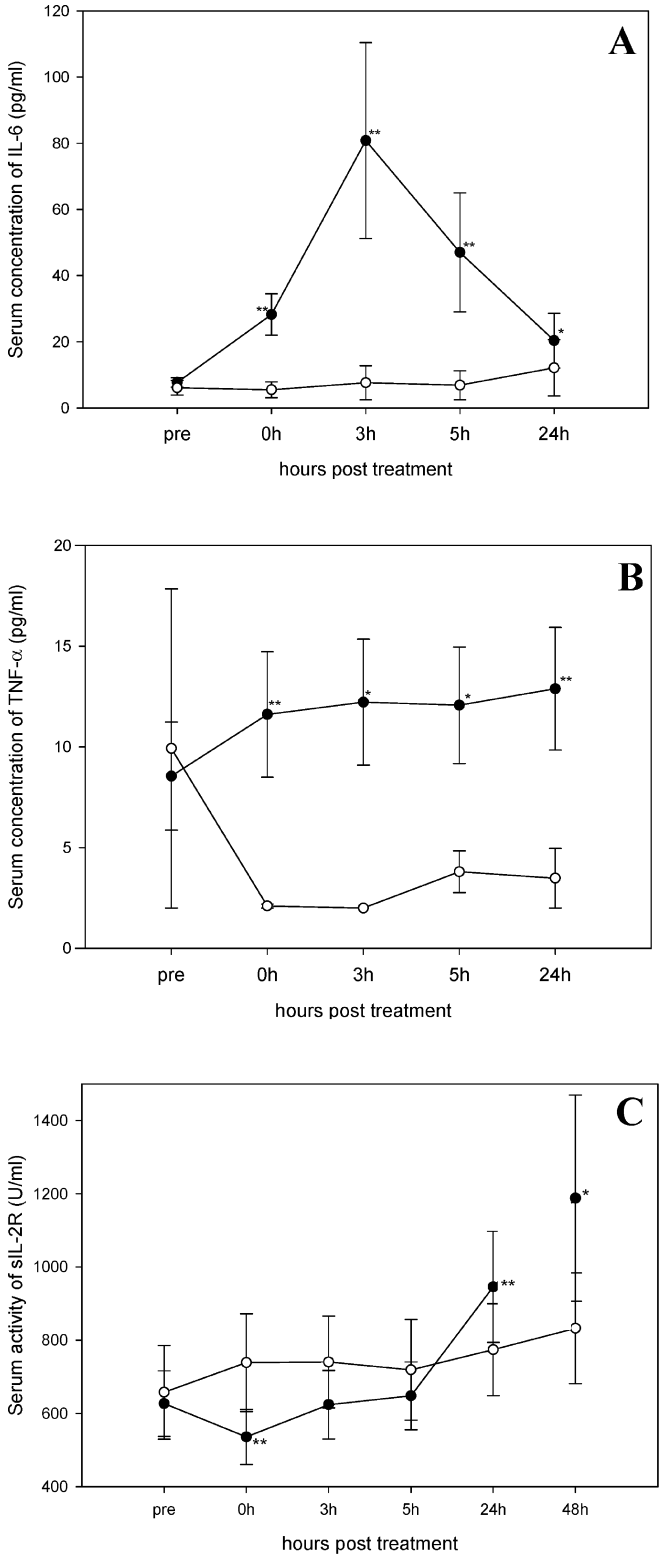

Fig. 4 Serum concentration (mean ± SEM) of IL-6 (**A**), TNF-α (**B**), and sIL-2R (**C**) in patients ($N = 11$) undergoing 41.8°C WBH-CT (*filled circles*) and in patients ($N = 5$) receiving platinum-containing chemotherapy alone (*open circles*) before treatment and 0 h, 3 h, 5 h, and 24 h post-treatment. In 7 of the patients receiving WBH-CT and in all patients receiving chemotherapy alone, sIL-2R levels were also measured at 48 h post-treatment. *Asterisks* indicate significant differences from baseline value (*$P < 0.05$; **$P < 0.01$)

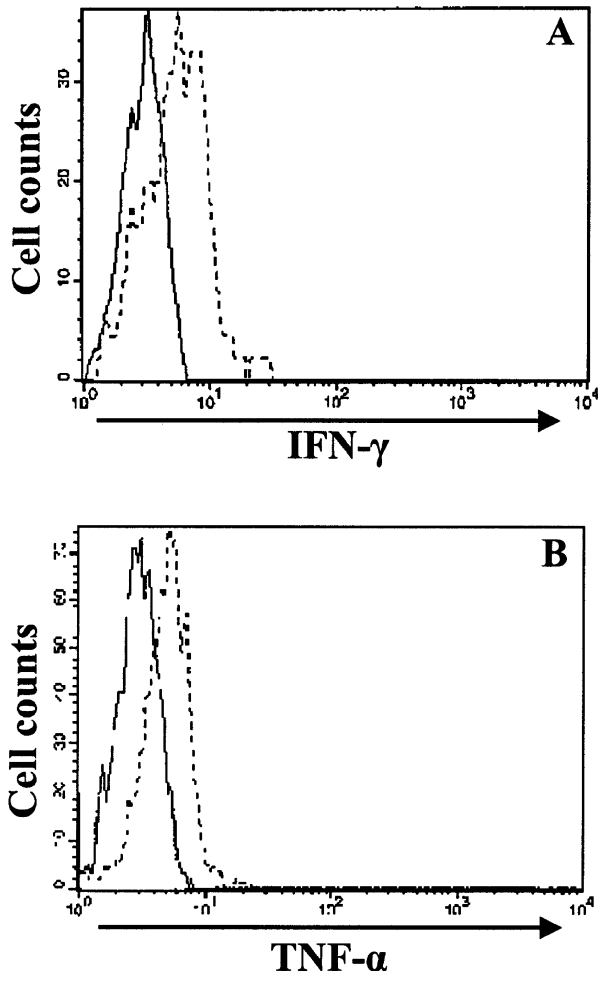

Fig. 5 Representative example of intracellular staining for IFN-γ (**A**) and TNF-α (**B**) in $CD8^+$ lymphocytes at baseline (*unbroken line*) and 24 h (*dotted line*) post-41.8°C WBH-CT treatment. Samples were stained and analyzed immediately after blood had been drawn

WBH-CT. We could demonstrate that this increase lasted at least for 2 days following the treatment. We did not observe any significant changes in the absolute numbers of peripheral monocytes and lymphocytes.

This is the first study to examine the effects of WBH on the percentages of cytotoxic T cells expressing the NK cell marker $CD56^+$ ($CD3^+/CD8^+/CD56^+$). Here, we also found a short-lived but marked increase immediately after the treatment followed by an equally rapid decrease below baseline. However, in contrast to peripheral NK cells, the percentage of cytotoxic T cells had significantly increased compared to baseline levels as late as 48 h post-WBH in all patients examined. Like NK cells, $CD56^+$ cytotoxic T cells have long been thought to mediate only major histocompatiblity (MHC)-unrestricted cytotoxicity, e.g. against tumor cell targets [21, 38]. Therefore, $CD56^+$ CTL have been referred to as NK T cells, and it has been shown that these cells are very potent mediators of cytotoxicity [35]. Only very recently, it has been demonstrated that $CD56^+$ CTL also provide MHC-restricted specific

cytotoxicity [50], and it has been suggested that $CD56^+$ CTL in the peripheral blood represent the currently effector-circulating lymphocytes [39]. CTL expressing NK cell markers are preferentially present among tumor-infiltrating lymphocytes (TIL) and PBL in cancer patients [36, 57]. Interestingly, recent studies have shown that a great number of $CD56^+$ T cells may represent CTL specific for certain antigens [20, 53], e.g. tumor antigens [56].

There are practically no studies examining the effect of 41.8°C WBH-CT treatment on surface activation markers on human lymphocyte subpopulations. One group recently reported a decrease in IL-2 receptor expression on T lymphocytes following WBH-CT [2]. We also observed a slight decrease in the expression of CD25 on $CD4^+$ lymphocytes in our patients, but this did not reach statistical significance and was only short-lived. In contrast, we found a continuous, nearly 2-fold increase in the expression of CD69 on peripheral $CD4^+$ lymphocytes and an increase of the same activation marker on $CD8^+$ lymphocytes in the majority of our patients.

The human CD69 antigen (AIM, Leu-23) is a phosphorylated 28/32 kDa disulfide-linked homodimer [15, 22]. It is a type II transmembrane protein [31] belonging to the family of C-type lectin receptors [26, 49, 63]. However, the identification of the physiological ligand for CD69 remains to be established [28]. CD69 is virtually undetectable on resting lymphocytes [48], and consequently only an extremely small number of circulating NK and T cells express low levels of this marker in normal individuals [22]. Whereas in vitro human T cells show a marked increase in the expression of CD69 as early as 1–2 h after stimulation with anti-CD3 antibody [43, 58] or mitogen [5, 15, 43, 48], it takes 48–72 h after stimulation with antigens before an increase of CD69 is measurable under in vitro conditions [11, 27, 59]. The increase in the expression of CD69 on $CD4^+$ lymphocytes of our patients reached statistical significance 48 h post-WBH-CT treatment. Thus, the increase in CD69 expression could very well be the result of a specific in vivo stimulation of T cells by certain antigens during or shortly after WBH-CT treatment. The activation of $CD4^+$ T cells described herein may play a central role in WBH-CT-induced immunological effects since, at least in animal models, a critical role for $CD4^+$ cells in inducing and maintaining anti-tumor immunity has clearly been demonstrated [12, 37].

CD69 expression on lymphocytes can also be induced by cytokines like IFN-α, IFN-γ [7], IL-2 [22] or TNF-α [25]. In this study, we also observed a long-lasting increase in the serum levels of sIL-2R and TNF-α following hyperthermic treatment. Thus, the increase in lymphocytic expression of CD69 could, at least in part, also be cytokine-induced.

CD69 is widely used as a marker for T cell activation, but it might also have an important biological role [4, 51]. Cytolytic function of NK cells and TCR γ/δ^+ T cells can be triggered through CD69 signaling [31]. Stimulation of CD69 augments, if protein kinase C is simultaneously stimulated, T cell proliferation [32], the expression of different lymphokine genes [6] and their gene products [32]. The pro-inflammatory cytokine TNF-α is one of those lymphokines [51]. Thus, it is feasible that a positive feedback loop is established between the expression of CD69 and the production of TNF-α, and that this contributed to the long-lasting increase in serum TNF-α levels in our patients.

Whereas others have also described an increase in the serum levels of IL-6 following 41.8°C WBH-CT, data concerning TNF-α have been inconsistent [45]. Here, we show that the serum levels of TNF-α are significantly elevated for up to 24 h following WBH-CT treatment. So far, no data have been available concerning the effect of WBH treatment on peripheral levels of sIL-2R. We observed a distinct increase in the serum level of sIL-2R 24 h and 48 h following WBH-CT. The IL-2 receptor is a complex of 3 non-covalently associated polypeptide chains, designated IL-2Rα, IL-2Rβ and IL-2Rγ. IL-2Rβ and IL-2Rγ are expressed constitutively on the surface of T lymphocytes, whereas IL-2Rα (CD25) is expressed at significant levels only after the activation of T cells [33]. Activated human lymphoid cells also release a serum-soluble form of IL-2Rα in vitro [46], and the quantification of this serum-soluble IL-2R has been used as a reliable index of in vivo T cell immune activation [47]. Thus, the increase of sIL-2R 24 h and 48 h following WBH-CT parallels the increase in the expression of the lymphocytic activation marker CD69 during the same period of time. Both parameters point in the direction of a "late" T cell activation in our patients induced by WBH. It seems interesting that although we found elevations in sIL-2R, we did not observe an increase in the expression of membrane-bound CD25 on $CD4^+$ or $CD8^+$ lymphocytes. However, a number of investigations on patients with immune activating diseases found an elevation of serum sIL-2R levels without a concomitant increase in CD25 expression [18, 34, 41]. One possible explanation for the lack of correlation between serum levels of sIL-2R and expression of CD25 in our patients could be that sIL-2R was not produced by PBL, but was released by TIL.

To examine whether T cells also contribute to the increased cytokine levels following WBH-CT treatment, we studied the expression of intracellular cytokines in the lymphocytes of some of our patients. In $CD8^+$ T cells we observed a marked increase in intracellular IFN-γ and TNF-α following WBH in most cases. Since both cytokines play an important role in the effector function of cytotoxic T cells [19, 54, 55], especially in the elimination of tumor cells [3, 29, 40], this finding, which needs to be confirmed in a larger number of patients, may be of clinical significance.

One could argue that factors other than the elevated body temperature might have contributed to the immunological phenomena we observed following WBH-CT, e.g. the simultaneously administered chemotherapy. In contrast to the numerous studies dealing with immunological recovery following chemotherapy-induced

cytopenia, only very limited information is available concerning the immunological changes immediately after administration of chemotherapy. In our control group receiving chemotherapy alone, we observed no significant effects on the distribution of lymphocytes subpopulations, expression of CD69 on T cells, and serum cytokine levels during the first 48 h following application of intravenous chemotherapy.

Taken together, we assume that the immunological consequences of 41.8°C WBH-CT concerning T cells may consist of three phases. Immediately after treatment, the phenomenon of a drastic but short-lived increase in peripheral NK cells and NK T cells might be accompanied by a short period of cellular exhaustion resulting in a reduced proliferative capacity. Reduced levels of sIL-2R represent an additional sign of suppressed T cell activity at this point in time. Following this first phase, cytokines like TNF-α and IL-6 are secreted into the peripheral blood and elevated serum levels of these cytokines are detectable for several hours. Possibly induced by these cytokines, T lymphocytes become more and more activated throughout the third phase of WBH-induced immunological changes. As a consequence, CD69 expression by lymphocytes and serum sIL-2R levels increase. However, activation of T lymphocytes might not only be induced by increased serum levels of certain cytokines but may itself lead to an increased production of lymphokines such as TNF-α or IFN-γ. Thus, a positive feedback loop concerning the production of cytokines favoring an immunological response by cytotoxic T cells might exist. Further studies are in progress in our laboratory to determine whether these WBH-CT-induced effects may lead to an increased number and efficacy of tumor-specific T cells.

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