

Treatment of Colon and Lung Cancer Patients with *ex Vivo* Heat Shock Protein 70-Peptide-Activated, Autologous Natural Killer Cells: A Clinical Phase I Trial

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ABSTRACT

Purpose: The 14 amino acid sequence (aa_{450–463}) TKDNNLLGRFELSG (TKD) of heat shock protein 70 (Hsp70) was identified as a tumor-selective recognition structure for natural killer (NK) cells. Incubation of peripheral blood lymphocyte cells with TKD plus low-dose interleukin 2 (IL-2) enhances the cytolytic activity of NK cells against Hsp70 membrane-positive tumors, *in vitro* and *in vivo*. These data encouraged us to test tolerability, feasibility, and safety of TKD-activated NK cells in a clinical Phase I trial.

Experimental Design: Patients with metastatic colorectal cancer ($n = 11$) and non-small cell lung cancer ($n = 1$) who had failed standard therapies were enrolled. After *ex vivo* stimulation of autologous peripheral blood lymphocytes with Hsp70-peptide TKD (2 $\mu\text{g}/\text{ml}$) plus low-dose IL-2 (100 units/ml), TKD was removed by extensive washing, and activated cells were reinfused *i.v.* The procedure was repeated for up to six cycles, applying a dose escalation schedule in 4 patients.

Results: The percentage of activated NK cells in the reinfused leukapheresis products ranged between 8 and 20% of total lymphocytes, corresponding to total NK cell counts of 0.1 up to 1.5×10^9 . Apart from restless feeling in 1 patient and itching in 2 patients, no negative side effects were observed. Concomitant with an enhanced CD94 cell surface density, the cytolytic activity of NK cells against Hsp70 membrane-positive colon carcinoma cells was en-

hanced after TKD/IL-2 stimulation in 10 of 12 patients. Concerning tumor response, 1 patient was in stable disease during therapy by formal staging criteria and another patient showed stable disease in one metastases and progression in another.

Conclusions: Reinfusion of Hsp70-activated autologous NK cells is safe. Immunological results warrant additional studies in patients with lower tumor burden.

INTRODUCTION

Beside their chaperoning functions (1–3), heat shock proteins (HSPs) have been found to play key roles in tumor immunity (4). Most immunotherapeutical approaches exploit the carrier function of HSP for tumor-specific antigenic peptides (5–7). After receptor-mediated uptake and re-presentation of HSP-chaperoned peptides by antigen-presenting cells, a tumor-specific, CD8-positive T-cell response is inducible (8–13).

We and others (14–17) identified an additional function of the major stress-inducible Hsp70: its immunostimulatory activity upon natural killer (NK) cells. By flow cytometry using an Hsp70-specific antibody (18), membrane-bound Hsp70 was selectively found on tumor cell lines but not on normal cells (19). Screening of freshly isolated tumor biopsy material and the corresponding normal tissues confirmed these results (20). Presently, >700 biopsies have been screened for their Hsp70 cell surface expression. Among different tumor entities, colorectal cancer and lung carcinoma frequently (75%) express Hsp70 on their plasma membrane, whereas the corresponding normal tissues were negative for membrane-bound Hsp70 (20).

The epitope of the antibody detecting membrane-bound Hsp70 on viable tumor cells was found to be localized within the COOH-terminal substrate binding domain by pepscan analysis (21). Antibody blocking studies demonstrated that this sequence is exposed to the extracellular milieu and acts as a recognition structure for human NK cells (22). Incubation of human NK cells with low-dose interleukin 2 (IL-2) and Hsp70-peptide TKD (aa_{450–463}), containing the antibody epitope, stimulated the cytolytic activity against Hsp70 membrane-positive tumors, *in vitro* (23). In contrast, peptides with only two conservative amino acid exchanges were unable to activate NK cells (23). Furthermore, we could show that the cell surface density of the C-type lectin receptor CD94 was up-regulated on NK cells after incubation with TKD plus low-dose IL-2 (14, 23). An association of the expression of the C-type lectin killer cell receptor CD94 and the cytolytic activity against Hsp70-positive tumor cells was characterized as follows: (a) a CD94-specific antibody inhibited the cytolytic response of NK cells against Hsp70 membrane-positive tumor cells (14); (b) adoptive transfer of CD94 positively enriched NK cells resulted in tumor regression of Hsp70 membrane-positive tumors in SCID/beige mice; in contrast, CD94-negative effector cells failed to do so

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Table 1 Patient characteristics, tumor anatomy/histology, previous therapies, and site of disease^a

Code no.	Age (yrs)	Sex	Anatomy/histology	Stage at first diagnosis	Previous therapies ^b	Site of disease
1	67	M	Colon/adeno ca	pT4/pN2/cM1/G3/R2 (4/1999)	CHT × 4	Pulmonal/hepatic/ossary
2	61	M	Rectum/adeno ca	pT3/G3/pN2/M0/R0 (7/1996)	CHT × 4	Pulmonal/hepatic/local relapse
3	64	M	Sigma/adeno ca	pT3/pN2/L1/pM1/R2/G2 (5/1995)	CHT × 3, LITT	Hepatic
4	66	M	Sigma/adeno ca	pT3/pN1/pM1/G2/R0 (5/1995)	CHT × 6	Hepatic/local relapse
5	59	M	Sigma/adeno ca	pT4/pN3/G3/M1/R1 (1/1997)	CHT × 4	Hepatic
6	69	M	Sigma/adeno ca	pT3/pN2/R0/M0/G3 (12/1996)	CHT × 3	Hepatic/pulmonal
7	49	M	Rectum/adeno ca	pT1/pN0/M0/G2 (5/1999)	CHT × 2	Hepatic
8	49	F	NSCLC/adeno ca	cT1/N2/M0/IIIA (9/1998)	CHT, RT	Pulmonal/multiple
9	68	M	Colon/adeno ca	pT4/pN1/M1/G2 (10/1999)	CHT × 5	Hepatic/soft tissue/local relapse
10	59	M	Sigma/adeno ca	pT3/pN0/R0/M1/G2 (10/1998)	CHT × 4	Hepatic
11	65	M	Colon/adeno ca	pT3/pN2/pMx/G2 (7/1995)	CHT	Hepatic/pulmonal
12	41	M	Rectum/adeno ca	PT3/pN2/M2/L1/G2 (12/1999)	CHT × 5	Pulmonal

^a All patients had undergone previous surgery of primary tumor.

^b Number of different therapies (adjuvant or palliative) applied before study entry.

^c CHT, chemotherapy; adeno ca, adenocarcinoma; LITT, laser-induced thermotherapy; NSCLC, non-small cell lung cancer; RT, radiotherapy.

(24, 25); (c) CD94-positive NK cells were specifically attracted by Hsp70 membrane-positive tumor cells (26); and (d) both Hsp70 protein and Hsp70-peptide TKD have been found to bind to CD94-positive NK cells in a saturable and dose-dependent manner, indicating a receptor-mediated interaction (27).

In the present study, we addressed the question whether an NK cell-mediated Hsp70 reactivity is also inducible in peripheral blood lymphocyte (PBL) derived from tumor patients and whether these cells might confer an antitumor effects, *in vivo*. Tolerability, feasibility, and safety of adoptively transferred, TKD-activated, autologous NK cells, the phenotype, and the antitumor activity of these cells were investigated in a first clinical trial.

PATIENTS AND METHODS

Eligibility Criteria. Patients > 18 years with metastasized, histologically confirmed advanced colorectal carcinoma and non-small cell lung cancer with Karnofsky index > 60% were enrolled in the clinical trial. All patients had progressive tumor disease after surgery and standard chemotherapy (Table 1). Because of the therapy refractory, advanced tumor stages of the patients, it was not possible to obtain fresh tumor biopsies to determine the Hsp70 status. However, screening of >100 tumor biopsies of colorectal and lung cancer patients revealed that a Hsp70 membrane-positive phenotype is frequently (75%) found in these tumor entities (19, 20).

Hsp70-Peptide TKD. The Hsp70-peptide TKDNNLL-GRFELSG (TKD) corresponds to the amino acid sequence aa₄₅₀₋₄₆₃, of the human major stress-inducible Hsp70. It has been shown that TKD is the minimal essential sequence, exhib-

iting identical activating properties such as full-length Hsp70 protein (14) at equimolar concentrations (23). In the present study, GMP-grade Hsp70-peptide TKD was used (purity >96%, lot no. 054 1026; Bachem, Bubendorf, Switzerland) for *ex vivo* stimulation.

Therapy Regimen and *ex Vivo* Stimulation of PBL. At least 4 weeks after the last standard chemotherapy, leukocyte concentrates were obtained from the patients by a 3–4-h leukapheresis (Cobe Spectra, Heimstetten, Germany), and PBLs were additionally purified by Ficoll-Hypaque (Life Technologies, Inc., Paisley, Scotland) density gradient centrifugation in a closed cell culture bag and tubing system (IBM 2997 cell washer). Cells were counted and re-suspended at cell densities of 5–10 × 10⁶/ml in GMP-grade X-VIVO 20 medium (Bio-Whittaker, Walkersville, MD). During the dose escalation part of the study, PBLs were frozen in aliquots after Ficoll separation and thawed sequentially 4 days before stimulation.

After simultaneous addition of Hsp70-peptide TKD (2 μg/ml) and recombinant IL-2 (100 units/ml Aldesleukin; Chiron, Ratingen, Germany), cells were transferred into sterile 250-ml Teflon cell culture bags (VueLife-118; CellGenix, Freiburg, Germany) and cultured in an incubator (B5060; Heraeus, Nuernberg, Germany) under gentle rotation (Cell Shaker; multimune GmbH, Regensburg, Germany) at 37°C in a humidified atmosphere (90%), containing 5% CO₂, for 4 days. Preclinical data demonstrated that an optimal stimulation of NK cells is achieved if 5–10 × 10⁶ PBLs/ml were incubated with Hsp70-peptide TKD, as indicated (unpublished observation); therefore, these culture conditions were also used in the clinical trial. Then cells were harvested and washed twice in sterile physiological

Table 2 Cell dose escalation, reinfusion cycles, and side effects

A. Cell infusions and side effects in the dose escalation part of the study						
Treatment		1 dose	2 doses	3 doses	4 doses	
Code no.	Initial value	Reinfused natural killer cells/total cells ($\times 10^9$) (% natural killer cells)				Side effects
6	(13)	0.006/0.07 (9)	0.001/0.01 (13)	0.001/0.01 (10)	0.14/1.4 (10)	None
8	(12)	0.075/0.5 (15)	0.075/0.5 (15)			None
11	(10)	0.01/0.1 (9)	0.03/0.18 (16)	0.03/0.32 (10)		None
12	(16)	0.3/1.5 (20)				None

B. Reinfusion cycles, numbers of reinfused cells, and side effects in the second part of the study using complete leukapheresis products							
Treatment		1 cycle	2 cycles	3 cycles	4 cycles	5 cycles	
Code no.	Initial value	Reinfused NK cells/total cells ($\times 10^9$) (% NK cells)					Side effects
1	(13)	0.2/1.7 (12)					None
2	(8)	0.1/1.3 (8)					Itching
3	(9)	0.2/2.3 (9)					None
4	(10)	1.5/8.5 (17)	0.9/7.7 (12)				None
5	(13)	0.2/1.0 (15)	0.3/2.0 (12)				Itching
6	(13)	0.1/0.7 (12)	0.3/2.4 (13)				None
7	(15)	0.5/3.3 (15)	0.1/0.7 (14)	0.1/0.9 (11)			None
8	(12)	0.1/0.7 (20)	0.1/1.5 (7)	0.1/1.1 (9)	0.1/0.9 (11)		None
9	(15)	0.3/1.8 (19)	0.6/3.0 (18)	0.4/2.4 (17)	0.8/4.0 (20)		None
10	(6)	0.7/8.2 (9)	0.3/5.2 (6)	0.8/5.2 (15)	0.5/5.3 (10)	0.4/2.5 (16)	None
11	(10)	0.3/3.1 (10)	0.3/2.1 (14)	0.6/6.2 (10)	0.5/3.6 (14) ^a	0.2/1.7 (12)	Restless feeling, itching for 1 day
12	(16)	0.3/2.3 (13)	0.7/4.3 (16)	0.6/3.8 (16)	0.7/5.0 (14)	0.5/3.1 (16)	None

^a Last two treatment cycles were performed on demand of the patient.

saline (0.9%; Braun, Melsungen, Germany). Sterility tests of the cell products were performed before, on day 3 after stimulation, and directly before re-infusion.

Four patients were treated within an intraindividual and interindividual dose escalation schedule, as described in Table 2A. Because none of the patients receiving either two (patient no. 8), three (patient no. 11), or four (patient no. 6) fractionated doses of *ex vivo*-activated cells or one unfractionated cell dose (complete leukapheresis product; patient no. 12) showed any signs of toxicity, from that point forth, all patients were treated with complete *ex vivo*-stimulated leukapheresis products. These treatment cycles were repeated every fortnight up to five times (Table 2B).

For reinfusion, cells were resuspended in 1000 ml of physiological saline supplemented with 100 units/ml IL-2 and reinfused within 1 h. After adoptive cell transfer, vital parameters of all patients were monitored the following 3 h after the first reinfusion patients were hospitalized over night. To monitor biological parameters, including cell phenotype and cytolytic

function, aliquots of each individual stimulation were cultured in parallel, as described below.

The clinical protocol was approved by the institutional ethical review board of the medical faculty of the University Hospital Regensburg. Signed informed consent was obtained from all patients before entering the study.

Laboratory Follow-Up. Routine laboratory parameters were determined during the treatment cycles at the following time points: before treatment; before leukapheresis; before cell reinfusion; 1 day after cell reinfusion; and 1 week after the last reinfusion. The following parameters were measured: differential blood count; serum alkaline phosphatase; bilirubin; C-reactive protein; γ -glutamine transferase; aspartate amino transferase; creatinine; and lactate dehydrogenase. Additionally, the serum levels of IFN- γ and tumor necrosis factor α (TNF- α) were determined with the OptEIA Test Kits (PharMingen, Heidelberg, Germany). Granzyme B, an apoptosis-inducing enzyme, produced and secreted by activated T and NK cells, was measured in the serum of selected patients before and after

reinfusion, using a standard Granzyme B ELISA-Kit (Hölzel Diagnostika, Köln, Germany).

Measurement of Phenotype and Cytolytical Activity of Patient-Derived PBLs. For *in vitro* analysis, sterile aliquots of PBLs of each patient were incubated under identical culture conditions as the samples intended for reinfusion, either with low-dose IL-2 (100 units/ml) alone or with Hsp70-peptide TKD (2 $\mu\text{g/ml}$) plus low-dose IL-2. On days 0 and 4, cell surface expression of the following markers was determined by flow cytometry on a FACScalibur instrument (Becton Dickinson, Heidelberg, Germany): CD3-FITC (Becton Dickinson); CD16/CD56-PE (Becton Dickinson); CD56-FITC (Becton Dickinson); and CD94-PE (Ancell, Bayport, MN). The mean fluorescence intensity of the CD94 expression was determined within the CD3-/CD56+ NK cell population.

The lytic activity of patient-derived PBLs before and after *in vitro* stimulation with Hsp70-peptide TKD plus low-dose IL-2 or with IL-2 alone and of patient-derived PBLs before and after reinfusion *in vivo* was assessed in a standard 4-h ^{51}Cr -release assay (28) against Hsp70-positive colon carcinoma cells (CX+) as target cells. Antibody blocking studies were performed using antibodies directed against Hsp70 (10 $\mu\text{g/ml}$ cm-Hsp70.1; multimmune GmbH) on CX+ tumor target cells and against the C-type lectin receptor CD94 on NK cells, as described previously (29).

Statistics. Statistical analysis was performed using the Student's *t* test.

RESULTS

Patient Characteristics

From September 2000 to September 2002, 11 male patients with progressive colorectal cancer and 1 female patient with non-small cell lung cancer entered the study. The unequal male to female ratio of the patients was not intended. Patient characteristics, tumor anatomy and histology, stage of disease at first diagnosis, previous therapies, and site of disease were summarized in Table 1. The age of the patients was ranging between 41 and 69 years; data of first diagnosis was July 1995 to December 1999. All patients had received surgery and had been treated with various chemical and physical therapies before entering the study. At time of recruitment, all patients suffered from progressive, metastatic disease. Karnofsky index in all patients at start of therapy was $>60\%$.

Cell Infusions and Treatment Effects

PBLs were obtained from patients by leukapheresis followed by Ficoll density gradient centrifugation. All subsequent steps were performed in a closed system using GMP-grade Teflon-coated cell culture bags (250 ml) and tubing sets and media. PBLs were suspended in serum-free X-VIVO 20 medium in the presence of TKD (2 $\mu\text{g/ml}$) plus low-dose IL-2 (100 units/ml). Preclinical data demonstrated that an optimal stimulation of NK cells is achieved if $5\text{--}10 \times 10^6$ PBLs were stimulated with the indicated concentrations of TKD and IL-2 (unpublished observation). On day 4, *ex vivo*-activated, unseparated cells were injected *i.v.* in the patient within ~ 60 min.

Four patients were treated within a intraindividual and interindividual dose escalation schedule as follows: 3 patients

(nos. 8, 11, and 6) receiving either two, three, or four injections of escalating NK cell numbers ranging from 0.001×10^9 up to 0.075×10^9 . Another patient (no. 12) received the complete leukapheresis product containing 0.3×10^9 *ex vivo*-activated, autologous NK cells (Table 2A). The number of reinfused NK cells *versus* total cell numbers was shown in the first line of each column; the percentage of NK cells before start of therapy (initial values) and that of reinfused NK cells are given in parenthesis in the second line (Table 2A). Because none of the patients showed any signs of acute or subacute side effects, from this point on, all patients received infusions consisting of the complete *ex vivo*-stimulated leukapheresis product. As summarized in Table 2B, total lymphocyte numbers after stimulation ranged from 0.7×10^9 to 8.5×10^9 ; the number of activated NK cells, ranged from 0.1×10^9 to 1.5×10^9 , corresponding to 8% up to 20% of total lymphocytes (mean, 14%). These percentages did not differ significantly from that of healthy human individuals (range, 5–20%) and from that of unmanipulated NK cells of the different patients, as shown in parenthesis in the second column of Table 2B.

Even after repeated reinfusion cycles (maximum 6, dose escalation included), the cellular therapy was well tolerated. None of the patients showed any signs of severe toxicity (Table 2B, last column). Patient no. 11 had restless feeling and prickle and itching over the whole body with no obvious association to the cell infusions, and patient nos. 2 and 5 reported feeling itchy, which might be related to the cell infusions but also could be explained by an increasing icterus because of compression of bile ducts.

With respect to laboratory parameters, no treatment associated changes were observed. A gradual deterioration of bilirubin, lactate dehydrogenase, and liver enzymes in some of the patients can be explained by the progressing malignant disease. Irrespectively of the leukapheresis and cell infusion cycles, the total number of leukocytes, thrombocytes, and hemoglobin levels remained unaltered (Table 3).

Concerning clinical tumor response, 1 patient (no. 11) was in stable disease during therapy for 12 weeks, and no additional formal restaging was performed after the last two reinfusion cycles, which were performed on demand of the patient. Another patient (no. 8) showed stable disease in the left lung filiae and progression in the right lung filiae during therapy. This patient died 92 weeks after start of the therapy. As indicated in Table 3, a formal restaging could not be obtained for all patients.

Immunological Responses

IFN- γ , TNF- α , and Granzyme B Serum Levels after NK Cell Infusion. *In vitro* experiments revealed that after contact with TKD and low-dose IL-2, NK cells secreted high amounts of IFN- γ ; in contrast, TNF- α secretion was only marginally induced. The IFN- γ levels increased significantly ($P < 0.05$) in patient nos. 1, 3, 6, 7, 8, 9, 10, 11, and 12, if serum levels were compared before start of the therapy and on day 1 or day 2 after the last treatment cycle (Table 3). A significant increase in TNF- α serum levels was detected only in patient nos. 1 and 2, if compared with initial levels (Table 3). Because of the relatively low patient numbers, no obvious correlation between serum IFN- γ and TNF- α levels and patient characteristics was observed.

Table 3 Laboratory parameters, tumor markers, and clinical outcome before/after last treatment cycle

Code no.	Cycles	Leucocytes/nl	Thrombocytes/nl	Hemoglobin [g/dl]	IFN- γ [pg/ml]	Tumor necrosis factor α [pg/ml]	Tumor marker CA19-9 Carcinoembryonic antigen	Clinical course of disease
1	1	8.6/8.3 (8.0) ^c	317/301 (387)	14/14 (14)	1/18	10/23	nd/nd ^a 396/nd	ne ^b
2	1	8.6/8.1 (9.2)	135/161 (147)	7/8 (8)	7/9	63/114	10,109/nd 580/nd	ne ^d
3	1	6.7/6.2 (7.7)	187/166 (177)	13/12 (12)	8/26	24/2	28,110/nd 93.4/nd	Progression
4	2	13.3/13 (13.3)	239/242 (224)	11/11 (10)	nd	nd	1,216/nd 246/nd	Progression
5	2	9.5/13.5 (11.3)	411/482 (425)	11/12 (11)	28/30	4/5	1,847/nd 100/nd	ne ^e
6	4 + 2	8.6/9.3 (8.8)	239/338 (268)	14/13 (13)	12/25	10/8	437/319 1,449/2,172	Progression
7	3	5.9/8.0 (6.4)	125/228 (167)	15/13 (14)	14/36	9/10	61/252 341/905	Progression
8	2 + 4	5.1/6.0 (5.4)	236/273 (244)	14/13 (13)	20/28	7/7	2.3/0.3 1.8/0.3	Progression ^f
9	4	9.2/10 (9.5)	424/560 (488)	12/9 (10)	11/30	6/2	2.3/2.3 2.5/2.4	Progression
10	5	13/12.7 (12.4)	353/319 (315)	14/11 11	4/43	6/2	2,227/1,732 5,528/5,456	Progression
11	3 + 5	7.6/9.0 (8.3)	374/377 (321)	14/13 (12)	12/86	10/11	1,899/3,634 5,186/6,645	sd during therapy
12	1 + 5	7.3/8.2 (8.1)	271/319 (308)	16/15 (16)	15/20	6/9	2.3/2.3 2.8/2.8	Progression

^a ne, not evaluable; sd, stable disease.

^b Patient off study on his own demand due to decreasing performance status.

^c Numbers in parenthesis indicate mean values of laboratory parameters after each treatment.

^d Rapid clinical deterioration and death, cholestasis already increasing between recruitment to study and first infusion, then further rapid deterioration.

^e No formal restaging performed but increasing compression of bile duct documented.

^f Stable disease in one pulmonary metastasis and progression in another.

The apoptosis-inducing enzyme granzyme B was not detectable in the serum of healthy human individuals and in patients before start of the cellular therapy. However, on day 1 after reinfusion of TKD-activated cells, in 4 of 4 patients tested (nos. 2, 4, 7, and 10) granzyme B levels (9.1, 4.6, 0.4, and 231 pg/ml) were found to be elevated in the serum. Granzyme B could be identified most recently as the key mediator for the initiation of apoptosis in Hsp70 membrane-positive tumors, as described by biochemical and immunological studies (30), and thus might provide a useful surrogate marker for determination of the Hsp70 reactivity in NK cells.

CD94 Expression on NK Cells after TKD Stimulation.

Previously, we reported on increased CD94 expression levels on purified NK cells after incubation either with Hsp70 protein or Hsp70-peptide TKD (14, 23). It is important to note that the expression density of the C-type lectin receptor CD94 is associated with the capacity of NK cells to bind Hsp70 protein or TKD (27). Concomitantly, an increase in the fluorescence intensity of the partner receptor of the NKG2 family was detected, however, not as pronounced as CD94. In accordance to our previous findings with NK cells of healthy human donors, a significant increase in the mean fluorescence intensity of CD94 on CD3-negative and CD16/CD56-positive gated NK cells of tumor patients was also measured after incubation with TKD. The CD94 mean fluorescence intensity was up-regulated >3-fold, between days 0 and 4 (Fig. 1), from 94 ± 22 to 392 ± 189 .

Regarding the percentages of CD94-positive, CD3-negative, CD16/CD56-positive NK cells before (Table 2A, second column) and after TKD stimulation, only slight changes were observed (Table 2A, third to eighth column).

With respect to these findings, the CD94 mean fluorescence intensity values were determined in PBL of patient nos. 2, 4, 5, 9, 10, 11, and 12 on day 0 (control) and on day 4 after the first treatment cycle (one cycle). All patients (nos. 9, 10, 11, and 12) receiving four and more than four treatment cycles with TKD/IL-2-activated PBLs were also tested for their CD94 mean fluorescence intensity. After the first TKD treatment cycle, all tested patients (nos. 2, 4, 5, 9, 10, 11, and 12) revealed a significant increase in the cell surface density of CD94 (Fig. 2; left graph). Due to lack of material, the mean fluorescence values on PBLs of patient nos. 1, 3, 6, 7, and 8 could not be evaluated. After four and more than four treatment cycles, CD94 expression was also up-regulated in patient nos. 9, 10, and 12 as compared with initial levels, whereas patient no. 11 exhibited only a very weak increase (Fig. 2; middle graph). If CD94 mean fluorescence intensity values before and after the first treatment cycle were compared, a significant increase ($P = 0.02$) was detected. Also, after four and more than four therapy cycles, an increase in CD94 mean fluorescence intensity was detected; however, this increase was not significant if compared with initial values ($P = 0.21$; Fig. 2, right graph).

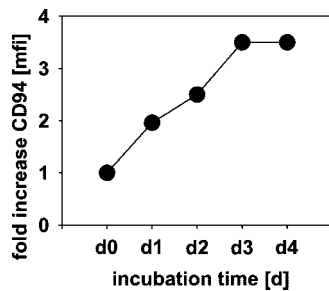


Fig. 1 Fold increase in CD94 mean fluorescence intensity (mfi) during TKD stimulation. PBLs derived from tumor patients were incubated with TKDNNLLGRFELSG (TKD; 2 $\mu\text{g}/\text{ml}$) plus low-dose interleukin 2 (100 units/ml) for 4 days. On days 0, 1, 2, 3, and 4, cell aliquots were harvested and analyzed by flow cytometry with respect to the mfi of CD94. The percentage of CD94-positive cells did not increase significantly between 4 days of stimulation. The percentage of reinfused CD94-positive cells in the different patients ranged between 8 and 20%. Data show one representative experiment derived from patient no. 12.

In Vitro Cytolytic Activity against Hsp70 Membrane-Positive Tumor Targets after TKD Stimulation. In NK cells of healthy human volunteers, a high CD94 cell surface density correlated with a strong lytic activity against Hsp70-positive tumor target cells. Therefore, in addition to the determination of the CD94 expression, the lytic activity of patient-derived PBLs was assessed against Hsp70 membrane-positive colon carcinoma target cells (CX+). For comparative reasons, all cytotoxicity data were performed using PBLs stimulated either with TKD plus IL-2 (TKD/IL-2; Fig. 3A) or with IL-2 alone (IL-2; Fig. 3B), at a defined E:T cell ratio of 20:1 after the first stimulation round. In total, 10 of 12 patients (nos. 2, 4, 5, 6, 7, 8, 9, 10, 11, and 12) showed a significantly enhanced cytolytic activity against Hsp70 membrane-positive CX+ cells after stimulation with TKD plus IL-2; 2 patients (nos. 1 and 3) did not respond (nonresponder; Fig. 3A) to this stimulation. Even if the results of responder and nonresponder patients were taken together, the cytolytic activity after stimulation with TKD plus low-dose IL-2 was significantly increased ($P = 0.02$). In contrast, no significant increase in cytotoxicity ($P = 0.87$) was detected if patient-derived PBLs were stimulated with low-dose IL-2 alone (Fig. 3B). Lysis of Hsp70 membrane-negative CX- tumor cells, as a control, was identical to that mediated by IL-2-stimulated PBLs (data not shown).

To demonstrate that indeed Hsp70 is the recognition site for TKD-activated PBL, blocking studies were performed using Hsp70-specific monoclonal antibody (mAb) cmHsp70.1 (10 $\mu\text{g}/\text{ml}$). Compared with IL-2 alone (Fig. 4, left graph, *filled circles*), addition of TKD resulted in a significantly increased lytic activity against CX+ target cells (Fig. 4, middle graph, *filled circles*). Preincubation of tumor target cells with Hsp70-specific mAb completely abrogated this lysis (Fig. 4, middle graph, *open circles*). To confirm our results that CD94 is a relevant receptor for the interaction with Hsp70 (27), CD94 mAb was used for additional blocking studies. As shown in the right panel of Fig. 4, Hsp70-positive CX+ tumor target cell lysis was indeed reduced in the presence of CD94 mAb. However, compared with the Hsp70 antibody blocking, inhibition of lysis by CD94 mAb was incomplete. This finding might be

because CD94 is known to be associated with different coreceptors of the NKG2 family, triggering either inhibitory or activating signals.

In Vivo Cytolytic Activity against Hsp70-Positive Tumor Cells. Stimulated PBLs were reinfused into patients after a 4-day *ex vivo* incubation period with TKD plus low-dose IL-2. As indicated in Table 2B, the amount of reinfused NK cells ranged from 0.1 to 1.5×10^9 . The *in vitro* lytic activity was strongly enhanced against Hsp70 membrane-positive tumor target cells after stimulation with Hsp70-peptide TKD plus low-dose IL-2, as shown in Fig. 3A. To evaluate the cytolytic response of TKD-stimulated NK cells *in vivo*, peripheral blood was taken from patients before start of the therapy and on day 1 after the first cell reinfusion. Before starting therapy, the lytic capacity of patient-derived PBLs against Hsp70 membrane-positive CX+ colon carcinoma cells was always $<10\%$ (data not shown). As summarized in Fig. 5, after the first treatment cycle, the lytic activity of PBLs derived from patient nos. 6, 7, 8, 10, 11, and 12, tested against CX+ target cells, was significantly enhanced as compared with initial levels. No significant change in the capacity to lyse CX+ tumor cells was observed with PBLs of patient nos. 1, 2, 3, and 5, after one treatment cycle. Data derived from patient nos. 4 and 9 could not be evaluated because of technical problems.

In a next step, the cytolytic activity of PBLs derived from patient nos. 10, 12, and 11, after receiving four cell infusion cycles, was tested against CX+ tumor cells. As illustrated in Fig. 6, the cytolytic response mediated by these PBLs was again significantly enhanced (Fig. 6, *closed circles*), if compared with that mediated by unstimulated PBL of the same donor (Fig. 6, *open circles*). Interestingly, the strongest cytolytic activity was detected with PBLs derived from patient no. 11 (Fig. 6, left graph), who also showed clinical response during therapy. It is important to note that PBLs derived from all tested patients after

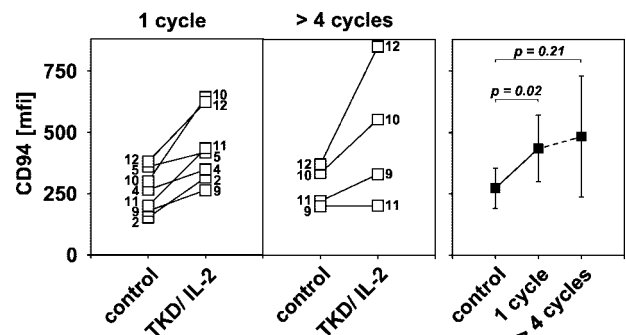


Fig. 2 Comparison of the increase in CD94 mean fluorescence intensity (mfi) after one and more than four treatment cycles. In one treatment cycle, PBLs were incubated with TKDNNLLGRFELSG (TKD; 2 $\mu\text{g}/\text{ml}$) plus low-dose interleukin 2 (IL-2; 100 units/ml) for 4 days. CD94 mfi was determined comparatively in patient nos. 2, 4, 5, 9, 10, 11, and 12 on day 0 (control) and on day 4 after the first treatment cycle (one cycle). Patient nos. 9, 10, 11, and 12, receiving four and more than four treatment cycles were analyzed identically. Patient codes are given as numbers in each graph. The graph on the right side shows mean values of the CD94 mfi \pm SD of patients after the first treatment cycle (one cycle); the *dashed line* summarizes mfi \pm SD of patients after four and more than four treatment cycles. Significant differences as compared with initial levels are indicated as *Ps* in this graph.

the first and after four treatment cycles were cultured overnight in cytokine-free medium without any additional *in vitro* restimulation.

DISCUSSION

Knowledge on NK cells and their critical role in the innate immune system has increased enormously since their discovery as large granular lymphocytes with broad cytolytic activity toward virally infected and tumor cells (31, 32). Recent advances in the understanding of physiological factors orchestrating NK cell activation provide the basis for innovative therapeutic approaches. Lytic activity against target cells has been found to be determined by the expression of specialized NK cell surface receptors and their specific ligands, predominantly MHC class I molecules on the target cells (33, 34). The balance between activating and inhibitory receptors of the immunoglobulin (killer cell immunoglobulin-like receptors), immunoglobulin-like transcripts, or C-type lectin family regulates activity of human NK cells (35–38). A strong MHC class I expression has

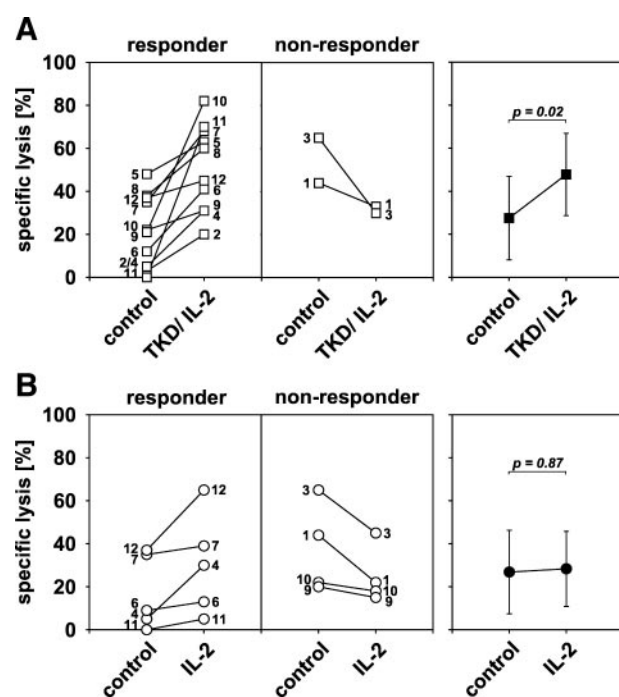


Fig. 3 Comparison of the *in vitro* cytolytic activity of peripheral blood lymphocytes (PBLs) derived from patient nos. 1–12, stimulated either with TKDNNLLGRFELSG/interleukin 2 (TKD/IL-2; **A**) or with IL-2 (**B**) alone, against heat shock protein 70 membrane-positive colon carcinoma cells (CX+) after the first treatment cycle. **A**, lytic activity of untreated (control) and TKD-stimulated (TKD/IL-2) PBLs of indicated patients was determined against CX+ tumor cells at a defined effector to target cell ratio of 20:1, *in vitro*. Results derived from patients with increased ($n = 10$) and decreased ($n = 2$) lytic activity were shown in two separate graphs. Mean cytotoxicity values and P of all patients are depicted in the right graph. **B**, lytic activity of untreated (control) and IL-2-stimulated PBLs of patients was determined against CX+ tumor cells at a defined E:T cell ratio of 20:1, *in vitro*. Results derived from patients with increased ($n = 5$) and decreased ($n = 4$) lytic activity were shown in two separate graphs. Mean cytotoxicity values and P of all patients are depicted in the right graph.

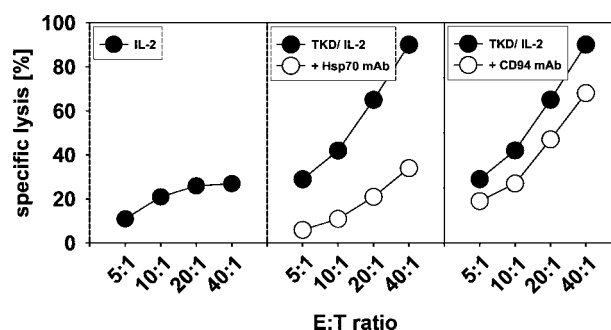


Fig. 4 Blocking studies using Hsp70 and CD94-specific monoclonal antibodies (mAbs) for inhibition of lysis of CX+ tumor cells. Lytic activity against heat shock protein 70 (Hsp70) membrane-positive CX+ tumor cells was significantly stronger by peripheral blood lymphocytes (PBLs) stimulated with TKDNNLLGRFELSG/interleukin 2 (TKD/IL-2; **middle graph**, ●), as compared with IL-2 (**left graph**, ●) alone. Cytolytic activity was completely abrogated by the addition of Hsp70 mAb (10 μ g/ml; **middle graph**, ○) and inhibited to a lower extent by CD94 mAb (10 μ g/ml; **right graph**, ○).

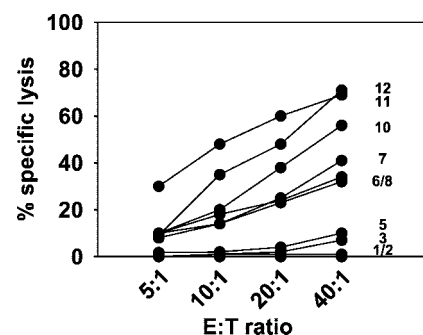


Fig. 5 Comparison of the *in vivo* heat shock protein 70 (Hsp70) reactivity in patient-derived peripheral blood lymphocytes (PBLs) after one treatment cycle. The cytolytic activity of PBLs of patient nos. 1, 2, 3, 5, 6, 7, 8, 10, 11, and 12 derived on day 1 after one reinfusion cycle of Hsp70-peptide-activated, autologous NK cells was tested against Hsp70 membrane-positive CX+ tumor cells. E:T cell ratios were ranging between 40:1 and 5:1, and percentage spontaneous release was <20% for the tumor target cell line. It is important to note that patient-derived PBLs used in the cytotoxicity assays were not restimulated *in vitro*.

been found to inhibit the lytic activity of NK cells because of the expression of inhibitory killer cell immunoglobulin-like receptors (39). In contrast, target cells with a down-modulated or missing MHC class I expression provide ideal targets for the lytic attack mediated by these NK cells. Although the molecular basis for the inhibitory function of autologous MHC molecules is well characterized, less is known about MHC-independent activating ligands for killer cell immunoglobulin-like receptor, immunoglobulin-like transcripts, and C-type lectin receptors.

Recently, our group has shown that lysis of target cells with surface expression of Hsp70 is strongly associated with the expression of the C-type lectin receptor CD94 on NK cells (26, 27). Depending on the NKG2 coreceptors, CD94 either functions as an activating or inhibitory receptor (40). CD94 is known to interact with nonclassical HLA-E molecules, presenting leader peptides of classical MHC class I molecules (41). Binding studies indicated that apart from nonclassical MHC mole-

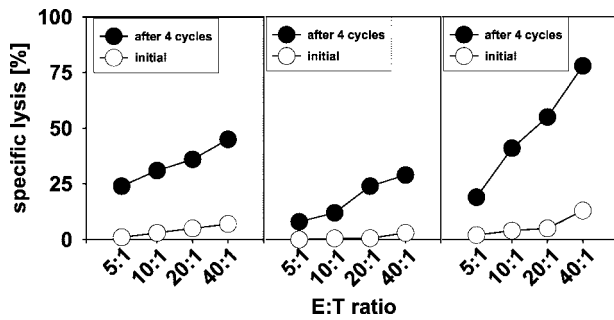


Fig. 6 Comparison of the *in vivo* Hsp70 reactivity in patient-derived peripheral blood lymphocytes (PBLs) after four treatment cycles. PBLs of patient nos. 10, 12, and 11 were derived either before start of immunotherapy (initial, ○) or on day 1 after four injection cycles with TKDNNLLGRFELSG/interleukin 2-activated cells (after four cycles, ●). The cytolytic activity of PBL was tested against Hsp70 membrane-positive CX+ tumor cells at E:T cell ratios ranging from 40:1 to 5:1; percentage spontaneous release of the target cell line was <20%. It is important to note that patient-derived PBLs used in the cytotoxicity assays were not restimulated *in vitro*.

cules, Hsp70 protein and a 14-mer peptide derived thereof (TKD) provide a stimulatory signal for activating CD94 receptors (27, 29, 30). Furthermore, we could show that the protein density of CD94 and, to a weaker extent, also of members of the NKG2 family, were up-regulated on NK cells after incubation with Hsp70 protein or Hsp70 peptide. In contrast, the expression of NK cell cytotoxicity receptors Nkp30 and Nkp46 remained unaffected.

In the present study, we demonstrated that PBL of heavily pretreated, therapy refractory tumor patients responded to TKD with an up-regulated mean fluorescence intensity of CD94 on NK cells. In addition, the *in vitro* cytolytic activity against Hsp70-positive colon carcinoma cells of pretreated PBLs was significantly increased in comparison to PBL incubated with low-dose IL-2 alone. These data emphasized the immunostimulatory capacity of Hsp70-peptide TKD also for patient-derived NK cells. The Hsp70 reactivity was also detectable *in vivo* in PBLs derived from patients after one and four reinfusion cycles of *ex vivo*-activated cells. Without additional *in vitro* stimulation, PBLs showed an increased cytolytic activity against Hsp70-positive tumor targets. Future clinical studies will address the question about the optimal number of stimulation rounds.

With respect to the clinical response, 1 patient receiving five Hsp70-activated, complete leukapheresis products showed stable disease during therapy, and another patient receiving four Hsp70-activated, complete leukapheresis products showed stable disease in one metastases and progression in another. Because all patients were in a progressive disease stage when they entered the study and because all patients were heavily pretreated, this outcome was not too astonishing. The major aim was to test feasibility, safety, and toxicity of adoptively transferred *ex vivo*-stimulated autologous NK cells. Our data clearly demonstrated that none of the patients showed severe signs of toxicity. *In vitro*, an increased lytic activity against Hsp70 membrane-positive tumor target cells, corresponding to an increased CD94 expression density, was shown in 10 of 12

patients. This is in line with previous findings showing a correlation of the cytolytic activity and the CD94 expression on NK cells of healthy human donors (42). Also, *in vivo*, a Hsp70-specific immune response against Hsp70 membrane-positive tumor cells could be demonstrated by testing patient-derived PBLs after injection of *ex vivo*-stimulated NK cells. Future clinical studies on patients with lower tumor burden will provide additional insights to the clinical value of our NK cell-based cellular immunotherapy. A Hsp70 membrane-positive tumor phenotype will be a prerequisite for patients entering this trial. To follow the route of NK cells after injection, immunocytochemistry studies of NK cells in patient-derived tumors are planned. *In vitro* migration assays already have shown that Hsp70-activated, CD94-positive NK cells specifically migrate toward Hsp70 membrane-positive tumor cells (26).

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