Cancer Immunol Immunother (2010) 59:1781–1789
DOI 10.1007/s00262-010-0904-3

ORIGINAL ARTICLE

A phase I trial of adoptive transfer of allogeneic natural killer cells in patients with advanced non-small cell lung cancer

Eleni G. Iliopoulou · Panteleimon Kountourakis · Michalis V. Karamouzis · Dimitrios Doufexis · Alexandros Ardavanis · Constantin N. Baxevanis · Gerasimos Rigatos · Michael Papamichail · Sonia A. Perez

Received: 22 March 2010 / Accepted: 2 August 2010 / Published online: 12 August 2010

Abstract HLA-mismatched natural killer (NK) cells have shown efficacy in acute myeloid leukemia, and their adoptive transfer in patients with other malignancies has been proven safe. This phase I clinical trial was designed to evaluate safety (primary endpoint) and possible clinical efficacy (secondary endpoint) of repetitive administrations of allogeneic, in vitro activated and expanded NK cells along with chemotherapy in patients with advanced non-small cell lung cancer (NSCLC). Patients with unresectable, locally advanced/metastatic NSCLC receiving 1st/2nd line chemotherapy were eligible to receive 2–4 doses of activated NK cells from two relative donors. Donor's $CD56^+$ cells were cultured for 20–23 days with interleukin-15 (IL-15) and hydrocortisone (HC) and administered intravenously between chemotherapy cycles. Premedication with corticosteroids and/or H1 inhibitors was allowed. Sixteen patients (performance status 0–1) with adenocarcinoma ($n = 13$) or squamous cell carcinoma ($n = 3$) at stage IIIb ($n = 5$) or IV ($n = 11$) receiving 1st ($n = 13$) or 2nd ($n = 3$) line treatment were enrolled. Fifteen patients received 2–4 doses of allogeneic activated NK cells ($0.2–29 \times 10^6$/kg/dose, median 4.15×10^6/kg/dose). No side effects (local or systemic) were observed. At a median 22-month follow-up (range, 16.5–26 months) 2 patients with partial response and 6 patients with disease stabilization were recorded. Median progression free survival and overall survival were 5.5 and 15 months, respectively. A 56% 1-year survival and a 19% 2-year survival were recorded. In conclusion, repetitive infusions of allogeneic, in vitro activated and expanded with IL-15/HC NK cells, in combination with chemotherapy are safe and potentially clinically effective.

Keywords Natural killer cells · Allogeneic · Adoptive transfer · Lung cancer

Electronic supplementary material The online version of this article (doi:10.1007/s00262-010-0904-3) contains supplementary material, which is available to authorized users.

E. G. Iliopoulou · C. N. Baxevanis · M. Papamichail · S. A. Perez (✉)
Cancer Immunology and Immunotherapy Center, Saint Savas Cancer Hospital, 171 Alexandras Avenue, 115 22 Athens, Greece
e-mail: perez@ciic.gr

P. Kountourakis · M. V. Karamouzis · D. Doufexis · A. Ardavanis · G. Rigatos
First Department of Medical Oncology, Saint Savas Cancer Hospital, Athens, Greece

P. Kountourakis
Medical Oncology Department, Bank of Cyprus Oncology Centre, Nicosia, Cyprus

M. V. Karamouzis
Department of Biological Chemistry, Medical School, University of Athens, Athens, Greece

Introduction

The encouraging results demonstrated during the past decade from the use of natural killer (NK) cells in the treatment of hematological malignancies have reestablished their role in the fight against cancer [1–3]. Our current knowledge on the requirements for effective activation and function of NK cells was the basis for the design of new approaches in cancer immunotherapy [4–6].

Data obtained from transplantations of haploidentical hematopoietic stem cells across HLA-I barriers highlighted the graft-versus-leukemia (GVL) effect of alloreactive NK cells in acute myeloid leukemia (AML) patients [7].

Immunotherapy of patients with solid tumors or hematological malignancies with intentionally mismatched IL-2-activated lymphocytes (DLI), including T, NK and NKT cells, has further strengthened the graft-versus-tumor (GVT) effect of alloreactive NK cells [8]. Donor-versus-recipient NK-cell alloreactivity derives from a mismatch between inhibitory receptors for self-MHC class I molecules on donor NK clones and the MHC class I ligands on recipient cells. Although autologous NK cells have been reported to efficiently kill some freshly isolated tumor cells [9], it is generally accepted that allogeneic HLA/killer immunoglobulin-like receptor (KIR) mismatched NK cells may be more potent killers in hematological malignancies [10], as well as for solid tumors [9, 11].

A study by Miller et al. [12] revealed that adoptive transfer of haploidentical NK cells in patients with metastatic melanoma, renal cell carcinoma, Hodgkin's disease, and poor prognosis AML was safe. Furthermore, depending on the preconditioning regimen used before infusion, allogeneic NK cells were detectable for at least 7 days in recipients' peripheral blood (PB). On the other hand, it has recently been documented that in vitro expanded NK cells have increased Natural Cytotoxity Receptors, TRAIL and NKG2D expression, and superior tumor cytotoxicity compared with short-term IL-2-activated NK cells [13]. Moreover, despite extensive ex vivo proliferation these NK cells appeared to maintain in mice similar longevity in vivo as non-expanded short-term IL-2 activated NK cells [14].

Activated NK cells, autologous or allogeneic, are known to be retained in lung tissue within minutes of intravenous (i.v.) injection and their density increases at the tumor sites within 24 h [15] [16], thus implying that adoptive transfer of allogeneic, in vitro activated, and expanded NK cells is a promising therapeutic approach for the treatment of lung cancer.

Recently, the combination of immunotherapy with chemotherapy has proven to be promising [17, 18]. Immunotherapy prior to chemotherapy in the vaccination setting has revealed that tumor cells become more susceptible to chemotherapeutic drugs [17, 19].

Based on the above data, we have designed a phase I clinical trial of adoptive transfer of in vitro activated and expanded allogeneic NK cells in patients with advanced non-small-cell lung cancer (NSCLC) in combination with their standard chemotherapy. One single dose of allogeneic NK cells, even at the highest dose (2×10^7 cells/kg), has been reported to be safe [12]. In this context, we tested if repetitive administrations of allogeneic NK cells, which could increase their in vivo anti-tumor effect, would also be safe, given the pre-sensitization of the recipient to the donors' alloantigens. Repetitive injections of NK cells derived from at least two different relatives, were applied between chemotherapy cycles.

Large-scale expansion of activated effector cells from a relatively small volume of PB, was succeeded by culturing isolated NK cells with IL-15 and hydrocortisone (HC), a combination which has been previously demonstrated by us to enhance the expansion of fully functional NK ($CD56^+CD3^-$) cells [20]. The primary objective of the study was the evaluation of the safety of allogeneic NK cell intravenous administration in combination with chemotherapy. The possible clinical efficacy of repetitive administrations has also been evaluated as a secondary objective.

Patients and methods

Eligibility

Patients with unresectable, locally advanced, and/or metastatic NSCLC receiving 1st/2nd line chemotherapy were eligible. Good performance status [0 or 1 according to Eastern Cooperative Oncology Group (ECOG) scale], absence of serious cardiovascular diseases, satisfactory renal and liver functions, as indicated by creatinine clearance (>50 mL/min), and total bilirubin (<2 mg/dL), respectively, as well as bone marrow competence (leukocytes > 3,000/μl, lymphocytes > 1,000/μl and platelets > 100,000/μl) were prerequisites for patient enrollment. Patients and donors were negative for hepatitis B and C and HIV infection. This phase I clinical trial was approved by the institutional ethics committee of Saint Savas Cancer Hospital and the Greek competent authorities EOF (National Drug Organization) and EED (National Ethics Committee) and registered under code IS89/2006 and EudraCT number: 2005-005125-58. All patients gave written informed consent before enrolling into the study. The trial was designed and conducted in accordance with the Declaration of Helsinki.

Study design

Eligible patients were to receive 2–4 intravenous infusions of activated and expanded NK cells 2 days after previous and 1 week prior to their next chemotherapy. The number of NK cells to be injected was up to 2×10^{11}/dose. Premedication with corticosteroids and/or H1 inhibitors was allowed. NK cells were isolated from the PB of two relative donors. Donor selection was based on their HLA and KIR typing. The primary aim of the study was the evaluation of the safety of allogeneic NK cell intravenous administration in combination with chemotherapy. The possible clinical efficacy of repetitive administrations has also been evaluated as a secondary endpoint.

HLA and KIR typing

Sequence-specific oligonucleotid (SSO)-typing for HLA-A, -B, -C, and KIR (Tepnel Lifecodes Corp, Stamford CT)

was performed for each patient and at least two family members (siblings or children) using the Luminex microbead method (Luminex 100 System; Luminex, Austin, TX).

Most of the donors were haploidentical to the corresponding recipient and at least one of the donors had a KIR mismatch (Supplementary data Table S1).

Isolation and expansion of NK cells

PB mononuclear cells (PBMC) were isolated from 150 ml of selected donor's PB by Ficoll-Hypaque density centrifugation, using standard procedures. $CD56^+$ cells were isolated from PBMC using CliniMACS CD56 microbeads (Miltenyi Biotec, Gladbach, Germany) according to manufacturer's instructions. Isolated $CD56^+$ cells were then cultured in alpha-minimum essential medium (alpha-MEM; Life Technologies, Paisley, Scotland) supplemented with 20% fetal bovine serum (FBS; Hyclone, Logan, UT), 2 mM L-glutamine (Life Technologies), 50 μg/ml gentamicin, 10^{-5} M HC (PFIZER, Belgium NV), and 20 ng/mL IL-15 (specific activity $1.7x10^7$ IU/mg, CellGenix, Freiburg, Germany). Fresh medium was added every 3–4 days. After 19–21 days of culture, phenotypic characteristics of activated cells as well as their cytotoxic activity were evaluated, as described below. Cells were also tested for mycoplasma contamination using the mycoAlert kit (Lonza, Rockland, ME). To exclude endotoxin contamination, the endotoxin concentration was measured with a Limulus Amebocyte Lysate (LAL) test (Cambrex, Walkersville, MD). Sterility testing of culture supernatants was also performed.

On day 21–23 cells were harvested, resuspended in 50 mL of fresh medium containing IL-15 and HC, and incubated for 1 h at 37°C to ensure the appropriate signals for further expansion in vivo, since the IL-15/IL-15Rα complex formed on the cell surfaces has been reported to survive intracellular endosomal processing and to recycle back to the cell surface as a biologically active cytokine [21]. Then cells were washed twice with cold PBS Dulbecco (Biochrom AG, Berlin, Germany)/2% human serum albumin (HSA; ZLB Behring AG, Bern, Switzerland) and diluted in 500 mL saline solution 0.9%/2% HSA. Cells were infused into the patient within 1 h post collection by intravenous administration over 1 h in an outpatient setting.

Cell lines

The human cell line K562 was obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI-1640 medium (Life Technologies) supplemented with 10% FBS, 2 mM L-glutamine, and 50 μg/ml gentamycin.

Immunophenotyping

Monoclonal antibodies (mAb) specific for CD3 and CD244, conjugated with fluorescein isothiocyanate (FITC), CD3, CD56, NKp30, NKp44, NKp46, and NKG2D conjugated with phycoerythrin (PE) and isotype-matched controls were purchased from Becton–Dickinson (Mountain View, CA). Samples were analyzed using FACSCalibur (Becton–Dickinson, Heidelberg, Germany) and CellQuest analysis software.

CFSE-based cytotoxicity assay

Cytotoxic activity of cultured $CD56^+$ cells was determined by a fluorescent-based flow cytometric assay, against the NK- sensitive cell line K562. Target cells were washed with PBS, resuspended at $1.5–2 \times 10^6$/mL, and labeled with 1 mM CFSE for 5 min at 37°C. After two washes and a 30-min incubation at 37°C, labeled K562 cells were incubated with effector cells at 1:1 ratio for a time period of 4 h at a 37°C humidified incubator with 5% CO_2. Following incubation, cells were stained with 7-AAD (Becton–Dickinson) for 10 min at 37°C, washed, and fixed with 1% PFA. Flow cytometric analysis of cells was performed within 1 h.

Statistical analysis

GraphPad Prism 4.0 software was used for the statistical analyses. Kaplan–Meier curves and log rank test were used for the statistical analysis of progression-free survival (PFS) and overall survival (OS). A P value <0.05 was considered as significant.

Results

Patient characteristics

Between November 2007 and November 2008 16 NSCLC patients were enrolled. The median age of patients was 65 years (range 50–75 years), and they were diagnosed with adenocarcinoma ($n = 13$) or squamous cell carcinoma ($n = 3$). Five patients had stage IIIb, and 11 stage IV disease. All patients were receiving 1st ($n = 14$) or 2nd ($n = 2$) line chemotherapy. Between their chemotherapy cycles each patient received two ($n = 8$), three ($n = 2$), or four ($n = 5$) doses of allogeneic activated NK cells. One patient did not receive NK cells because of severe disease progression after enrollment. Patient demographics and clinical characteristics are shown in Table 1. Chemotherapeutic drugs included cis-platinum, paclitaxel, docetaxel, gemcitabine, pemetrexed, and vinorelbine. No patient

Table 1 Patients' demographics and clinical characteristics

Patient no	Age	Gender	Histology	Stage	Metastasis	Treatment line	Chemotherapy
1	66	M	A	IV	Bone, Liver	1st	Cisplatin–Paclitaxel
2	74	F	A	IV	Bone	1st	Cisplatin–Docetaxel
3	69	M	A	IIIb	–	1st	Carboplatin–Docetaxel
4	66	M	SCC	IIIb	–	1st	Carboplatin–Paclitaxel
5	60	M	A	IIIb	–	1st	Carboplatin–Paclitaxel
6	61	M	A	IV	Bone	1st	Carboplatin–Paclitaxel
7	63	F	A	IV	Brain	2nd	Gemcitabine–Pemetrexed
8	75	M	SCC	IV	Bone	1st	Carboplatin–Paclitaxel
9	54	M	A	IV	Bone, Brain	1st	Cisplatin–Vinorelbine
10	68	M	SCC	IV	Lung	1st	Cisplatin–Gemcitabine
11	55	F	A	IIIb	–	1st	Cisplatin–Vinorelbine
12	56	M	A	IV	Brain	2nd	Carboplatin–Docetaxel
13	64	F	A	IV	Lymph nodes	1st	Carboplatin–Paclitaxel
14	71	F	A	IV	Bone	1st	Paclitaxel
15	67	M	A	IIIb	–	1st	Docetaxel
16	50	M	A	IV	Bone	1st	Gemcitabine

M male, *F* female, *A* adenocarcinoma, *SCC* squamous cell carcinoma

received therapeutic and/or palliative radiotherapy and no targeted agent (e.g. bevacizumab, cetuximab, erlotinib) was used during the study. Median lymphocyte counts throughout the period of chemotherapy and NK cell infusions were 1.2×10^3 lymphocytes/μL of blood (range 0.4×10^3–2.6×10^3), which is indicative of a relative lymphodepletion.

Characteristics of NK cells

$CD56^+$ cells were isolated from the PB of two or three relative donors for each patient. The number of $CD56^+$ cells acquired from 150 ml of donors' PB varied (median 35.1×10^6, range 9.5×10^6–85.8×10^6). The median purity of $CD56^+$ cells was 92.4% (range, 83.2–98.4%). Within this population the amount of NK ($CD56^+CD3^-$) cells varied among donors (median 67.2%, range 17.8–89.9%) (Fig. 1a). However, as expected [20], after 20 days of culture with IL-15 and HC, the median percentage of NK cells ($CD56^+CD3^-$) was significantly increased (97.9%, range 82.7–99.6%) in all donors (Fig. 1a).

There were variations in the expansion rate of NK cells among donors. The median expansion (fold increase from day 0) of $CD56^+CD3^-$ cells was 23-fold (range, 3.2–131.3) (Fig. 1c). Due to these variations, the median number of NK cells administered at each dose was 4.15×10^6/kg (range, 0.2–29 $\times 10^6$/kg) (Table 2). Accordingly, the median number of T cells (mostly $CD3^+CD56^+CD28^-$) in each inoculation was 0.12×10^6/kg (range, 0.009–1.8 $\times 10^6$/kg).

The majority (>95%) of IL-15 plus HC-activated NK cells in all donor preparations expressed activating NK receptors including NKp30, NKp44, NKp46, NKG2D, and 2B4 (Fig. 1b). The cytotoxic capacity of activated NK cells against K562 leukemic cells was examined 2 days prior injection day (day 19–21 of culture). Activated NK cells from all donors were cytotoxic. However, there were also variations in the killing potential of NK cells among donors with a median cytotoxicity of 23.2% (range, 7.0–54.7%) at effector/target ratio 1:1 (Fig. 1c).

Toxicity

During and after injection of NK cells no side effects were observed, locally or systemically, independently of the number of cells or the number of infusions administered. No signs of graft-versus-host disease (GVHD) were reported during the whole follow-up period. Therefore, no maximum tolerated dose of the total amount or number of infusions of administered allogeneic NK cells could be determined.

Efficacy

After a median follow-up of 22 months (range, 16.5–26 months), 4 out of 15 patients (27%) who received NK cells were alive. According to Response Evaluation Criteria in Solid Tumors (RECIST,) two patients (13.3%) achieved partial clinical response (PR), six (40%) had disease stabilization (SD), and seven (46.6%) experienced progression of their disease (PD) (Table 3). Noteworthy is that among PD patients three had local regression and one

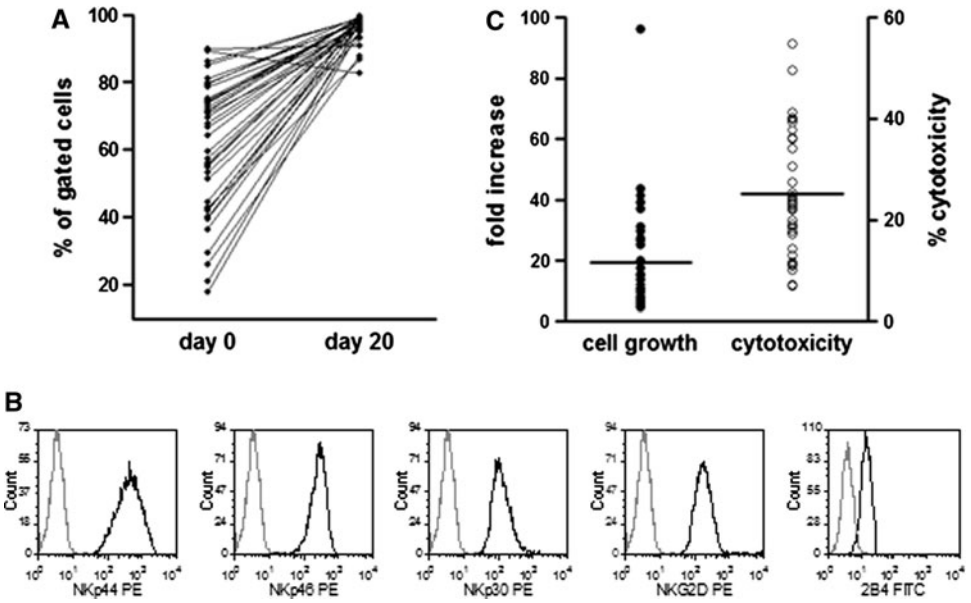

Fig. 1 **a** Percentages of $CD56^{+}CD3^{-}$ cells among total $CD56^{+}$ gated cells isolated from donors' peripheral blood on day 0 and after 18–21 days of culture with 20 ng/mL IL-15 and 10^{-5} M HC. **b** Expression of activating NK receptors (NKp30, NKp44, NKp46, NKG2D and 2B4) on donor's $CD56^{+}$ cells expanded for 18–21 days of culture with 20 ng/mL IL-15 and 10^{-5}M HC. *Gray lines* indicate isotype matched controls. Histograms from a representative donor. All donors gave similar results. **c** Cell growth (fold increase) and cytotoxic activity against K562 cells of donors' $CD56^{+}$ cells after 21–23 days of culture with 20 ng/mL IL-15 and 10^{-5}M HC. Cytotoxicity assay was performed at an effector: target ratio 1:1. *Horizontal bars* indicate mean values

Table 2 Number of doses and NK cells administered to each patient

Patient no	Doses administered	10^6 cells/kg			
1	2	1.1	0.9	–	–
2	2	5.6	3.1	–	–
3	4	0.3	0.4	0.7	19
4	2	0.2	2.9	–	–
5	2	0.4	5.7	–	–
6	4	0.6	2.5	4.7	3.2
7	4	10.1	3.9	4.1	7.1
8	3	2.4	6.6	1.2	–
9	0	–	–	–	–
10	2	2.5	1.8	–	–
11	4	23.1	11.9	26.3	13.4
12	3	13.5	7.2	11.9	–
13	2	4.2	3.2	–	–
14	2	17.8	11.5	–	–
15	2	29	21	–	–
16	4	2.2	9.8	9.7	1.3

Table 3 Clinical response of patients

Patient	First injection time	Last injection time	Clinical Response by RECIST	Lung disease best response
1	11/2007	1/2008	PD	D
2	12/2007	3/2008	SD	S
3	1/2008	6/2008	PR	D
4	1/2008	3/2008	PD	D
5	2/2008	4/2008	SD	S
6	2/2008	4/2008	SD	S
7	3/2008	7/2008	PD	D
8	3/2008	6/2008	PD	I
9	–	–	PD	I
10	4/2008	5/2008	PD	S
11	4/2008	8/2008	PR	D
12	4/2008	8/2008	SD	S
13	5/2008	6/2008	SD	S
14	9/2008	10/2008	PD	NE
15	9/2009	11/2008	PD	NE
16	10/2009	2/2009	SD	D

PD progressive disease, *SD* stable disease, *PR* partial response, *D* decrease, *S* stable, *I* increase, *NE* not evaluated

stabilization of their lung disease after receiving NK cells along with chemotherapy.

The median PFS of enrolled (intended to treat) patients was 5.5 months (range, 1–22 months), while their median OS was 15 months (range, 2–26 months). The one-year survival was 56% (9/16) and the two-year survival 19% (4/16). In the treated group median PFS and OS were 7 (range 2–22) and 15.5 (range 3-26) months, respectively. Their one- and two-year survival rates were 60% (9/15) and 27% (4/15), respectively. Despite the small number of patients enrolled, among patients exhibiting local disease stabilization (S) or

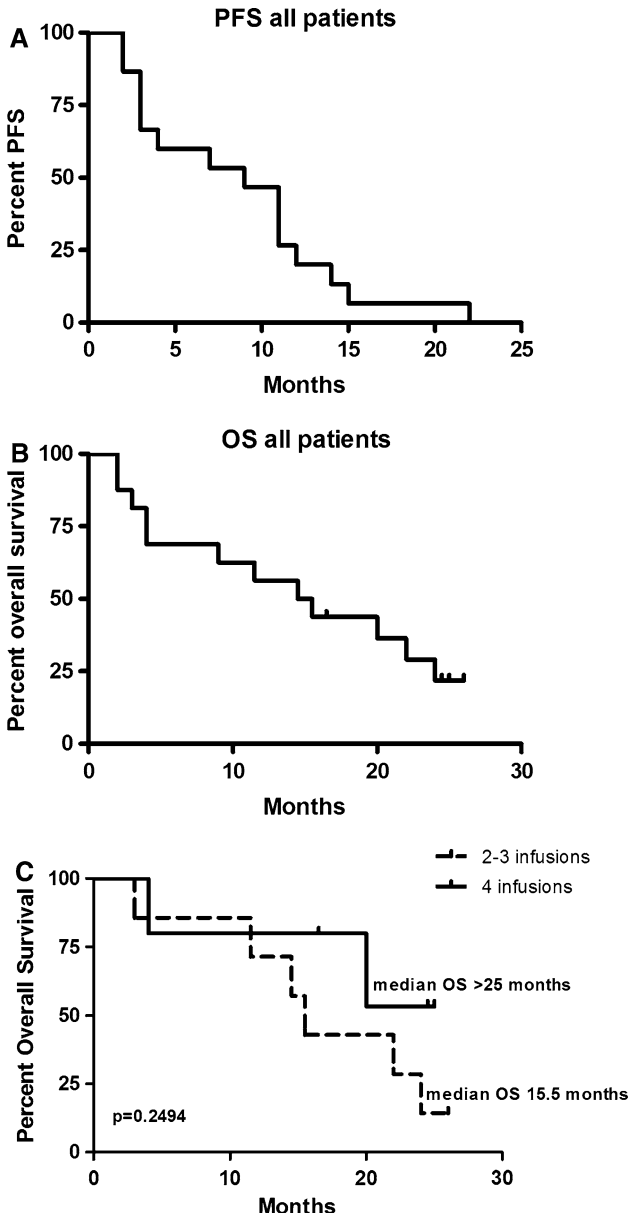

Fig. 2 Effect of number of infusions of allogeneic activated NK cells on progression-free survival (**a**) and overall survival (**b**) of all intended to treat non-small-cell lung cancer patients. **c** Overall survival of patients with stable or decreased pulmonary disease, receiving 2–3 and 4 NK cell infusions, respectively

decrease (D), a tendency for better OS was recorded among patients receiving four doses of NK cells ($n = 5$, median OS > 25 months) compared with those receiving two or three doses ($n = 7$, median OS 15.5 months) although not statistically significant ($p = 0.2494$) (Fig. 2).

Discussion

Lung cancer is a major cause of mortality worldwide. Furthermore, the majority of patients (80%) are diagnosed having locally advanced or metastatic disease with dismal prognosis [22]. This group of patients is ineligible for potential curative surgery and although research efforts are intensive, new agents and treatment approaches are needed. To the best of our knowledge, herein we report the results of the first phase I clinical trial of NK-cell based immunotherapy in patients with advanced NSCLC.

The rationale for the design of this trial was (1) to use ex vivo activated and expanded allogeneic NK cells in order to benefit from their ability to overcome inhibitory signals from the tumor itself and its microenvironment; (2) to achieve longer exposure of tumor cells to NK cells by repetitive infusions; (3) to ensure the standard treatment of patients taking advantage of the relative lymphodepletion achieved by chemotherapy, without applying further myeloablative conditioning regimens, which would be more toxic and not necessarily more efficacious [23].

We clearly show that the repetitive administration of up to 2.9×10^7 NK cells/kg/dose and up to 4.84×10^9 in total, was safe without any sign of GVHD or any other local or systemic side effect. Maximum tolerated dose in terms of NK cell number and/or number of infusions was not reached. It is important to note that the median number of NK cells/kg/dose administered ($4.15 \times 10^{6+}$ NK cells/kg) was comparable to that achieved by Miller's group [12], and it was derived from only 150 ml of donor PB instead of a, cumbersome, 3 to 5-h lymphapheresis which, furthermore, precludes repetition of the procedure in a relatively short period of time. Thus, the protocol applied here offers the possibility of future improvements by obtaining higher NK cell numbers from larger blood volume and by applying higher concentrations/specific activity of IL-15 in combination with HC.

The use of HC along with IL-15 for the ex vivo activation and expansion of NK cells, applied for the first time in this clinical trial, confers long-term survival by inhibition of cytokine-induced cell death and accelerated NK cell expansion without loss of their functional capacity, as previously demonstrated in in vitro experiments [20]. IL-15 is considered to be critical for NK cell development, survival, expansion, and function in vivo [24], although high concentrations of IL-2 in the presence of HC could also be used for their in vitro *expansion* [20]. In a preclinical therapeutic model of pre-existent lung metastases, we observed that syngeneic as well as allogeneic NK cells, ex-vivo activated and expanded with IL-15 in the presence of HC, massively infiltrate the lung and significantly prolong the survival of CT26 tumor-bearing Balb/c mice (unpublished data). Importantly, expansion of NK cells with IL-15 in the presence of pharmacological doses of glucocorticoids (GCs), compared with NK cells grown with IL-15 alone, makes them more resistant to subsequent exposure to the same pharmacological dose of GC even in

the absence of IL-15 (Perez S. et al. unpublished observations). Thus, NK expansion under the conditions described might render them also resistant to the in vivo effects of endogenous cortisol, usually increased in cancer patients due to stress [25] as well as to exogenously administered corticosteroids.

It has been reported [12] that intravenously administered allogeneic NK cells in patients with several malignancies, not including lung cancer, were detectable in the peripheral blood of hosts for at least 7 days after infusion, when methyl-prednisolone and/or low-dose cyclophosphamide was used for pre-conditioning of the patients. High-dose cyclophosphamide plus fludarabine conferred significantly longer persistence (up to 28 days) of NKs attributed to the induction of the endogenous levels of IL-15. It is not known if chemotherapeutic schedules used in our study share similar properties. The presence of circulating allogeneic NK cells in patients with lung cancer would be indicative of their in vivo persistence. However, their absence from the circulation may point to the possibility that upon injection they massively infiltrate lung tissue and are possibly retained within the tumor bed, with very few remaining in, or reentering the circulation [15, 26]. Brand et al. [16] have conducted a study where transfused allogeneic, in vitro expanded, NK cells radiolabeled with ^{111}In were followed by whole body scintigraphy and were detectable up to 6 days.

Sequential lung biopsies would be an alternative option, but could not be routinely applied. Whether the repetitive infusions used in the present protocol might overcome the probable lower time of in vivo expansion and persistence of infused NK cells is not clear.

Although applied in a small number of patients, most of which were receiving first line chemotherapy, the clinical efficacy of the combinatorial protocol used in our trial seems to produce slightly better results compared with those reported for patients with advanced NSCLC receiving similar chemotherapeutic regimens [27, 28]. Noteworthy, there were patients with documented progression, according to RECIST criteria, showing an improvement of their primary lung disease. Furthermore, although not statistically significant, there seems to be a tendency of increased PFS and OS in patients receiving four doses of NK cells compared with patients receiving less (2–3) infusions. The mechanisms underlying this effect, apart from direct killing of tumor cells, could be attributed to the generation of tumor-specific adaptive immune responses triggered by NK cells by cytokine secretion [29], cross talk with resident antigen presenting cells (APC) [30–33] or direct antigen presentation by the activated NK cells themselves [34], and/or even by induction of allo-restricted immune responses [35]. Data from a preclinical study, in a therapeutic metastatic lung cancer model, revealed that the repetitive infusions of syngeneic in vitro activated and expanded with IL-15 plus HC NK cells, could induce adaptive anti-tumor responses (submitted for publication).

The combination of chemotherapy with immunotherapy is currently a challenging approach, since it has been documented that immunotherapy can render tumor cells more susceptible to chemotherapeutic drugs [36] and inversely chemotherapy can potentiate subsequently induced adaptive immune responses [37].

However, there are certain limitations in our study. The small number of enrolled patients could not clearly suggest differences in PFS and OS regarding the number of NK infusions and clarify whether the observed responses were due to chemotherapy or the NK cells injected. Despite the fact that the heterogeneity in the patient cohort regarding chemotherapy regimens could also be an issue, several large randomized trials that compared commonly used platinum doublets have not fully supported the superiority of any of them [27, 38, 39]. Furthermore, there are studies suggesting that non-platinum doublets can also provide similar clinical benefit [40].

In conclusion, the data reported in the present study show that repetitive infusions of allogeneic NK cells, in vitro expanded with IL-15 in the presence of HC along with standard chemotherapy in patients with NSCLC are safe and potentially, clinically effective. The design of a randomized phase II clinical study in a better selected cohort of NSCLC patients with earlier-stage disease to evaluate clinical efficacy is further justified.

Acknowledgments We are indebted to our patients and their relatives for their voluntary participation in this study as well as to the nurses at the Outpatient Unit of Saint Savas Cancer Hospital for taking care of patients. We also thank all the staff of the Biochemistry, the Microbiology, and the Hematology Departments of our Hospital for helping in the accomplishment of this trial and Ms Joanne Kalogeropoulou for excellent technical assistance. Supported by Regional Operational Program Attika no. 20 MIS code 59605 G.R. to M.P.

Conflict of interest None.

References

1. Ruggeri L, Mancusi A, Capanni M, Martelli MF, Velardi A (2005) Exploitation of alloreactive NK cells in adoptive immunotherapy of cancer. Curr Opin Immunol 17:211–217
2. Ruggeri L, Capanni M, Mancusi A, Perruccio K, Burchielli E, Martelli MF, Velardi A (2005) Natural killer cell alloreactivity in haploidentical hematopoietic stem cell transplantation. Int J Hematol 81:13–17
3. Moretta A, Locatelli F, Moretta L (2008) Human NK cells: from HLA class I-specific killer Ig-like receptors to the therapy of acute leukemias. Immunol Rev 224:58–69

4. Gill S, Olson JA, Negrin RS (2009) Natural killer cells in allogeneic transplantation: effect on engraftment, graft- versus-tumor, and graft-versus-host responses. Biol Blood Marrow Transplant 15:765–776
5. Ljunggren HG, Malmberg KJ (2007) Prospects for the use of NK cells in immunotherapy of human cancer. Nat Rev Immunol 7:329–339
6. Malmberg KJ, Bryceson YT, Carlsten M, Andersson S, Bjorklund A, Bjorkstrom NK, Baumann BC, Fauriat C, Alici E, Dilber MS, Ljunggren HG (2008) NK cell-mediated targeting of human cancer and possibilities for new means of immunotherapy. Cancer Immunol Immunother 57:1541–1552
7. Ruggeri L, Mancusi A, Burchielli E, Capanni M, Carotti A, Aloisi T, Aversa F, Martelli MF, Velardi A (2008) NK cell alloreactivity and allogeneic hematopoietic stem cell transplantation. Blood Cells Mol Dis 40:84–90
8. Slavin S, Ackerstein A, Or R, Shapira MY, Gesundheit B, Askenasy N, Morecki S (2010) Immunotherapy in high-risk chemotherapy-resistant patients with metastatic solid tumors and hematological malignancies using intentionally mismatched donor lymphocytes activated with rIL-2: a phase I study. Cancer Immunol Immunother 59:1511–1519
9. Carlsten M, Malmberg KJ, Ljunggren HG (2009) Natural killer cell-mediated lysis of freshly isolated human tumor cells. Int J Cancer 124:757–762
10. De Santis D, Bishara A, Witt CS, Nagler A, Brautbar C, Slavin S, Christiansen FT (2005) Natural killer cell HLA-C epitopes and killer cell immunoglobulin-like receptors both influence outcome of mismatched unrelated donor bone marrow transplants. Tissue Antigens 65:519–528
11. Re F, Staudacher C, Zamai L, Vecchio V, Bregni M (2006) Killer cell Ig-like receptors ligand-mismatched, alloreactive natural killer cells lyse primary solid tumors. Cancer 107:640–648
12. Miller JS, Soignier Y, Panoskaltsis-Mortari A, McNearney SA, Yun GH, Fautsch SK, McKenna D, Le C, Defor TE, Burns LJ, Orchard PJ, Blazar BR, Wagner JE, Slungaard A, Weisdorf DJ, Okazaki IJ, McGlave PB (2005) Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. Blood 105:3051–3057
13. Berg M, Lundqvist A, McCoy P Jr, Samsel L, Fan Y, Tawab A, Childs R (2009) Clinical-grade ex vivo-expanded human natural killer cells up-regulate activating receptors and death receptor ligands and have enhanced cytolytic activity against tumor cells. Cytotherapy 11:341–355
14. Berg M, Lundqvist A, Betters D, Childs RW (2009) In vitro expanded NK cells have increased natural cytotoxity receptors, TRAIL and NKG2D expression, and superior tumor cytotoxicity compared to short-term IL-2-activated NK cells. Blood 114:463 (ASH Annual Meeting Abstracts)
15. Albertsson PA, Basse PH, Hokland M, Goldfarb RH, Nagelkerke JF, Nannmark U, Kuppen PJ (2003) NK cells and the tumour microenvironment: implications for NK-cell function and anti-tumour activity. Trends Immunol 24:603–609
16. Brand JM, Meller B, Von Hof K, Luhm J, Bahre M, Kirchner H, Frohn C (2004) Kinetics and organ distribution of allogeneic natural killer lymphocytes transfused into patients suffering from renal cell carcinoma. Stem Cells Dev 13:307–314
17. Zhang T, Herlyn D (2009) Combination of active specific immunotherapy or adoptive antibody or lymphocyte immunotherapy with chemotherapy in the treatment of cancer. Cancer Immunol Immunother 58:475–492
18. Baxevanis CN, Perez SA, Papamichail M (2009) Combinatorial treatments including vaccines, chemotherapy and monoclonal antibodies for cancer therapy. Cancer Immunol Immunother 58:317–324
19. Ramakrishnan R, Assudani D, Nagaraj S, Hunter T, Cho HI, Antonia S, Altiok S, Celis E, Gabrilovich DI (2010) Chemotherapy enhances tumor cell susceptibility to CTL-mediated killing during cancer immunotherapy in mice. J Clin Invest 120:1111–1124
20. Perez SA, Mahaira LG, Demirtzoglou FJ, Sotiropoulou PA, Ioannidis P, Iliopoulou EG, Gritzapis AD, Sotiriadou NN, Baxevanis CN, Papamichail M (2005) A potential role for hydrocortisone in the positive regulation of IL-15-activated NK-cell proliferation and survival. Blood 106:158–166
21. Sato N, Patel HJ, Waldmann TA, Tagaya Y (2007) The IL-15/IL-15Ralpha on cell surfaces enables sustained IL-15 activity and contributes to the long survival of CD8 memory T cells. Proc Natl Acad Sci USA 104:588–593
22. Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ (2007) Cancer statistics, 2007. CA Cancer J Clin 57:43–66
23. Crivellari G, Monfardini S, Stragliotto S, Marino D, Aversa SM (2007) Increasing chemotherapy in small-cell lung cancer: from dose intensity and density to megadoses. Oncologist 12:79–89
24. Becknell B, Caligiuri MA (2005) Interleukin-2, interleukin-15, and their roles in human natural killer cells. Adv Immunol 86:209–239
25. Reiche EM, Nunes SO, Morimoto HK (2004) Stress, depression, the immune system, and cancer. Lancet Oncol 5:617–625
26. Nannmark U, Hokland ME, Agger R, Christiansen M, Kjaergaard J, Goldfarb RH, Bagge U, Unger M, Johansson BR, Albertsson PA, Basse PH (2000) Tumor blood supply and tumor localization by adoptively transferred IL-2 activated natural killer cells. In Vivo 14:651–658
27. Schiller JH, Harrington D, Belani CP, Langer C, Sandler A, Krook J, Zhu J, Johnson DH (2002) Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. N Engl J Med 346:92–98
28. Sculier JP, Moro-Sibilot D (2009) First- and second-line therapy for advanced nonsmall cell lung cancer. Eur Respir J 33:915–930
29. Andoniou CE, Coudert JD, Degli-Esposti MA (2008) Killers and beyond: NK-cell-mediated control of immune responses. Eur J Immunol 38:2938–2942
30. Cooper MA, Fehniger TA, Fuchs A, Colonna M, Caligiuri MA (2004) NK cell and DC interactions. Trends Immunol 25:47–52
31. Moretta L, Ferlazzo G, Bottino C, Vitale M, Pende D, Mingari MC, Moretta A (2006) Effector and regulatory events during natural killer-dendritic cell interactions. Immunol Rev 214:219–228
32. Ghiringhelli F, Apetoh L, Housseau F, Kroemer G, Zitvogel L (2007) Links between innate and cognate tumor immunity. Curr Opin Immunol 19:224–231
33. Krebs P, Barnes MJ, Lampe K, Whitley K, Bahjat KS, Beutler B, Janssen E, Hoebe K (2009) NK-cell-mediated killing of target cells triggers robust antigen-specific T-cell-mediated and humoral responses. Blood 113:6593–6602
34. Hanna J, Gonen-Gross T, Fitchett J, Rowe T, Daniels M, Arnon TI, Gazit R, Joseph A, Schjetne KW, Steinle A, Porgador A, Mevorach D, Goldman-Wohl D, Yagel S, LaBarre MJ, Buckner JH, Mandelboim O (2004) Novel APC-like properties of human NK cells directly regulate T cell activation. J Clin Invest 114:1612–1623
35. Barber LD, Madrigal JA (2006) Exploiting beneficial alloreactive T cells. Vox Sang 91:20–27
36. Ramakrishnan R, Antonia S, Gabrilovich DI (2008) Combined modality immunotherapy and chemotherapy: a new perspective. Cancer Immunol Immunother 57:1523–1529
37. Schlom J, Arlen PM, Gulley JL (2007) Cancer vaccines: moving beyond current paradigms. Clin Cancer Res 13:3776–3782
38. Kosmidis PA, Kalofonos HP, Christodoulou C, Syrigos K, Makatsoris T, Skarlos D, Bakogiannis C, Nicolaides C, Bafa-

loukos D, Bamias A, Samantas E, Xiros N, Boukovinas I, Fountzilas G, Dimopoulos MA (2008) Paclitaxel and gemcitabine versus carboplatin and gemcitabine in patients with advanced non-small-cell lung cancer. A phase III study of the Hellenic Cooperative Oncology Group. Ann Oncol 19:115–122

39. Scagliotti GV, De Marinis F, Rinaldi M, Crino L, Gridelli C, Ricci S, Matano E, Boni C, Marangolo M, Failla G, Altavilla G, Adamo V, Ceribelli A, Clerici M, Di Costanzo F, Frontini L, Tonato M (2002) Phase III randomized trial comparing three platinum-based doublets in advanced non-small-cell lung cancer. J Clin Oncol 20:4285–4291
40. D'Addario G, Pintilie M, Leighl NB, Feld R, Cerny T, Shepherd FA (2005) Platinum-based versus non-platinum-based chemotherapy in advanced non-small-cell lung cancer: a meta-analysis of the published literature. J Clin Oncol 23:2926–2936