Cytotherapy 000 (2019) 1–9

Contents lists available at ScienceDirect

CYTOTHERAPY

journal homepage: www.isct-cytotherapy.org

Efficacy of cascade-primed cell infusion as an adjuvant immunotherapy with concurrent chemotherapy for patients with non–small-cell lung cancer: A retrospective observational study with a 5-year follow-up

Hong Li[1,*], Zhen Zhang[1,*], Xiaoran Duan[1], Nomathamsanqa Resegofetse Maimela[1], Shuangning Yang[1], Xuan Zhao[1], Jianmin Huang[1], Yi Zhang[1,2,3,4,**]

[1] *Biotherapy Center, the First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan, China*
[2] *Cancer Center, the First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan, China*
[3] *Henan Key Laboratory for Tumor Immunology and Biotherapy, Zhengzhou, Henan, China*
[4] *School of Life Sciences, Zhengzhou University, Zhengzhou, Henan, China*

ARTICLE INFO

Article History:
Received 13 July 2019
Accepted 3 December 2019
Available online xxx

Keywords:
cascade-primed cell
efficacy
malignancies
safety
survival

ABSTRACT

Background: Clinical studies have shown the efficacy of combination therapy for various malignancies. In this study, the characteristics, safety and feasibility of use of cascade-primed (CAPRI) cells for the combination treatment of non–small-cell lung cancer (NSCLC) were evaluated both *in vitro* and *in vivo*.
Methods: Sixty-five patients with stage II–IV NSCLC were recruited. Of these patients, 31 patients received CAPRI cell therapy combined with chemotherapy (CAPRI group), and the other 34 patients constituted the control group and received chemotherapy alone. This study primarily aimed to evaluate the overall survival (OS), progression-free survival (PFS), short-term responses and treatment efficacy.
Results: CD83, CD1a, CD80 and CD86 marker levels were significantly upregulated in CAPRI cells. Interferon-γ expression levels were highest in $CD3^+CD8^+$ cells (33.77% ± 4.40%). Furthermore, interleukin-2 levels were highest in $CD3^+CD56^+$ cells (26.73% ± 6.63%), whereas perforin expression levels were similar in $CD3^+CD8^+$ and $CD3^+CD56^+$ cells. Furthermore, CAPRI cells had a better anti-tumor potential in $CD3^+CD56^+$ cells and displayed the highest expression levels of CD107a to H460 and A549 cell lines. The 5-year OS was significantly greater in the CAPRI group than in the control group ($P = 0.008$), and the PFS of two groups exhibited a significant difference ($P = 0.007$). Median OS (48 versus 31.6 months; $P = 0.004$) and PFS (48 versus 36.4 months; $P = 0.016$) differed between these two groups. Moreover, treatment-associated toxicities were mild and well-tolerated by patients with NSCLC.
Conclusion: CAPRI cell therapy potentially prolongs the survival of patients with NSCLC when combined with chemotherapy.

Introduction

Lung cancer, the most common of the malignant cancers, has the highest morbidity and mortality rates among the various cancer types and has been showing a growing trend. In 2017, 13% of all new diagnoses and 26% of cancer-related deaths were due to lung cancer [1]. Non–small-cell lung cancer (NSCLC) accounts for approximately 75% of all cases of lung cancer, and shows high morbidity, high mortality and low survival rates [2]; furthermore, most patients are diagnosed at advanced stages. Although systemic chemotherapy is the recommended first-line treatment for patients with advanced-stage and metastatic NSCLC, it is often considered ineffective or excessively toxic [3]. Therefore, multidisciplinary approaches toward the development of novel treatments for NSCLC are required to improve the prognosis of lung cancer.

Adoptive cell therapy has been proving to be an effective therapeutic strategy against certain cancers. It involves the reinfusion of autologous cultured immune cells and is considered a viable alternative therapeutic for patients with cancer [4-7]. Various immune cells have been applied in clinical trials, including tumor-infiltrating lymphocytes (TILs), cytokine-induced killer (CIK) cells, natural killer cells and chimeric antigen receptor (CAR) T cells. However, certain tumors lose the expression of the major histocompatibility complex (MHC) and/or antigens, rendering TIL therapy beneficial only for some patients [8]. CAR-T cells have been successfully applied to treat B-cell

** Correspondence: **Yi Zhang,** MD, PhD, No. 1 Jianshe Road, Zhengzhou 450052, Henan, China.
E-mail address: yizhang@zzu.edu.cn (Y. Zhang).
* These authors contributed equally to this work.

https://doi.org/10.1016/j.jcyt.2019.12.002
1465-3249/

malignancies, but have failed to treat solid tumors owing to the tumor immune-suppressive microenvironment and the lack of optimal targets on the tumor surface [9]. CIK cell therapy is recognized as a cell-based therapy, with the $CD3^+CD56^+$ cells being the main effector cells [10]. CIK cells exhibit enhanced tumor cell lysis, higher proliferation and relatively lower toxicity than TILs. Despite their nonspecific cytotoxicity, CIK cells reportedly regulate and enhance the immune functions of patients with cancer [11]. However, the most pressing issues in CIK therapy have been the generation of a sufficient quantity of less-differentiated cells for transfer and the maintenance of long survival time *in vivo*.

Cascade-primed (CAPRI) cells are generated *ex vivo* by incubating peripheral blood (PB) with two reagents (herein named reagent 1 and 2) for 5 days of stimulation. CAPRI cells consist of four cell types (i.e., monocytes, dendritic cells [DCs], $CD4^+$ T cells and $CD8^+$ T cells) that are primed and do not require tumor cells or identifying peptides for their activation. In our study, the antigen-presenting cells (APCs) were stimulated by activating T cells in culture with anti-CD3 antibody (OKT3) to upregulate the expression of MHCs and co-stimulatory molecules. The leukocyte differentiation antigen $CD14^+$ on monocytes plays a critical role in both the inflammatory and immune response. Thus, the tumor-immunogenic information has been derived from $CD14^+$ monocytes [12]. Furthermore, shortening the culture periods with CAPRI cells is reportedly beneficial for combination therapy for various cancers. This study aimed to investigate and report the safety and clinical efficacy of CAPRI cells in combination with traditional therapy for patients with NSCLC. We found that CAPRI cells presented stronger anti-tumor ability by secreting high levels of multiple cytokines, including interferon (IFN)-γ, interleukin (IL)-2 and perforin, and producing a higher expression level of CD107a. Furthermore, treatment-related toxicity was mild and tolerable, and the overall survival (OS) and progression-free survival (PFS) of patients in the CAPRI group were greater than those in the control group.

Materials and Methods

Patients

We conducted this retrospective study with patients with NSCLC who were admitted in the First Affiliated Hospital of Zhengzhou University from January 2011 to December 2016. All eligible patients were 18 years of age and older and their diagnoses were cytologically confirmed. Their Karnofsky performance status was >70, and their expected life expectancy was >3 months. Patients were excluded from the study if they had an active infection, coagulation disorder, immune deficiency or major organ dysfunction. Thirty-one patients with NSCLC received CAPRI cell treatment combined with chemotherapy (CAPRI group). An additional 34 patients with NSCLC receiving chemotherapy alone during the same period were enrolled as the control group. The characteristics of the CAPRI group (median age, 58 years; range, 47–85 years) and the control group (median age, 58 years; range, 48–80 years) are shown in Table 1. Data from all patients with NSCLC with different American Joint Committee on Cancer stages were analyzed to establish baseline parameters. Ten patients at stage IIA or IIB underwent surgery before cell infusion and chemotherapy. Moreover, the age, gender, tumor type, stage and chemotherapy regimens of patients were similar in these two groups (Table 1). This study was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University (Zhengzhou, China; Research-2015-LW-108). All patients provided written informed consent in accordance with the tenets of the Declaration of Helsinki and the study was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University. None of the participants received targeted therapy or checkpoint inhibitors before treatment.

Table 1
The clinical parameters of patients.

	CAPRI (n = 31)	Control (n = 34)	χ^2 (*P*)
Gender			
Male	19	26	1.175 (0.185)
Female	12	8	
Age (y)			
≥60	17	21	0.320 (0.571)
<60	14	13	
Previous therapy			
Surgery	6	7	0.639 (0.850)
Chemotherapy	23	23	
Others	2	4	
Pathological stage			
Adenocarcinoma	22	18	2.511 (0.285)
Squamous carcinoma	8	13	
Others	1	3	
Metastasis sites			
Brain	3	3	2.168 (0.729)
Bone	7	5	
Liver	4	2	
Lymph node	11	15	
Others	6	9	
TNM			
IIA/IIB	5	5	0.025 (0.874)
III/IV	26	29	

TNM, Tumor lymph node metastasis.

Generation of CAPRI cells

CAPRI cells were generated *ex vivo* by incubating peripheral blood mononuclear cell (PBMCs) from patients with reagent 1 (300 IU/mL interleukin-2, 500 IU/mL interferon-γ, 200 IU/mL interleukin-18, 700 IU/mL granulocyte-macrophage colony-stimulating factor and 200 IU/mL interleukin-4) and 2 (300 IU/mL interleukin-2, 500 IU/mL interferon-γ and 200 IU/mL interleukin-18) and stimulation for 5 days. First, anti-CD3 antibodies were immobilized at l μg/mL in 0.05 mol/L borate buffer (pH 8.6) as coating solution, following which 60 mL of the coating solution was dispensed into four 175 cm^2 culture flasks (Greiner Bio-One, Frickenhausen, Germany). The coated culture bottles were maintained at 4°C for 24 h. PBMCs from patients were then obtained through Ficoll density gradient centrifugation (Tianjin HY, China). Then the PBMCs were washed twice with phosphate-buffered saline and incubated in 20 mL of GT551 for 3 h. Reagent 1 was then added to stimulate PBMCs for 3 h. To generate CAPRI cells, equal numbers of unstimulated autologous PBMCs were co-cultured with CD3-activated PBMCs for 16 h. The CD3-activated T cells were then transferred to four 175 cm^2 flasks and reagent 2 was added on day 1, and the CAPRI cells were harvested on day 5, whereupon they were washed twice before infusion or cryopreservation. After culturing and expansion, the final number of CAPRI cells was approximately 6×10^8 to 1.2×10^9. Quality control of the CAPRI cells was performed and the absence of bacterial and fungal contamination was confirmed 3 days prior to cell infusion. The dye-exclusion assay was performed to verify cell viability, which was >90%. The CAPRI cell subset was analyzed using a flow cytometer (BD Biosciences, San Jose, CA, USA).

Treatment plan

The chemotherapy regimens for these patients with NSCLC included the following: pemetrexed and cisplatin or carboplatin (PP), paclitaxel and carboplatin (TP), docetaxel and cisplatin (DP), gemcitabine and cisplatin (GP), docetaxel and oxaliplatin (DO) or docetaxel, Nedaplatin and Pemetrexed (DNP). Dosages were based on National Comprehensive Cancer Network (NCCN) guidelines. Apheresis was performed to harvest mononuclear cells before precondition chemotherapy to ensure the reinfusion of an adequate number of the T cells produced herein into each patient. For safety and efficacy, CAPRI cells

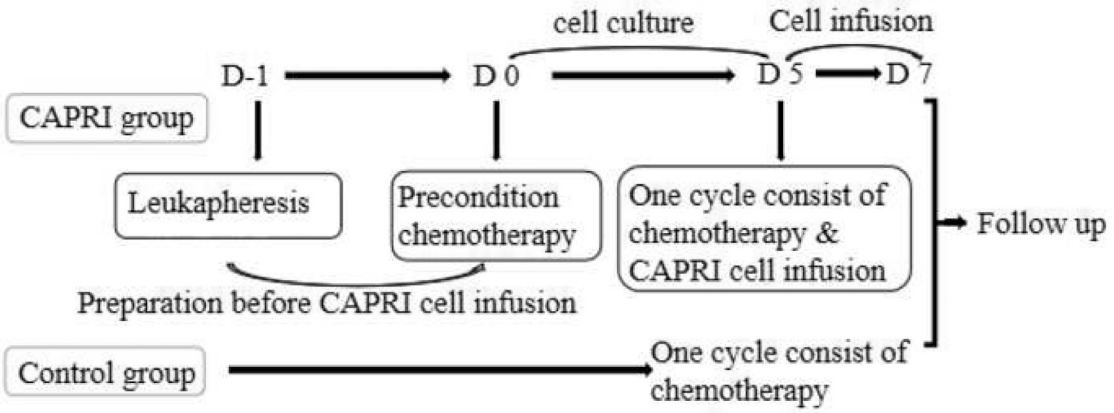

Fig. 1. Clinical protocol design. Autologous cells were obtained via leukapheresis, and T cells were expanded for 5 d. Patients were administered lymphodepleting chemotherapy, with or without CAPRI cell infusions. Subjects were transferred to a destination protocol for long-term follow-up evaluation per the schedule.

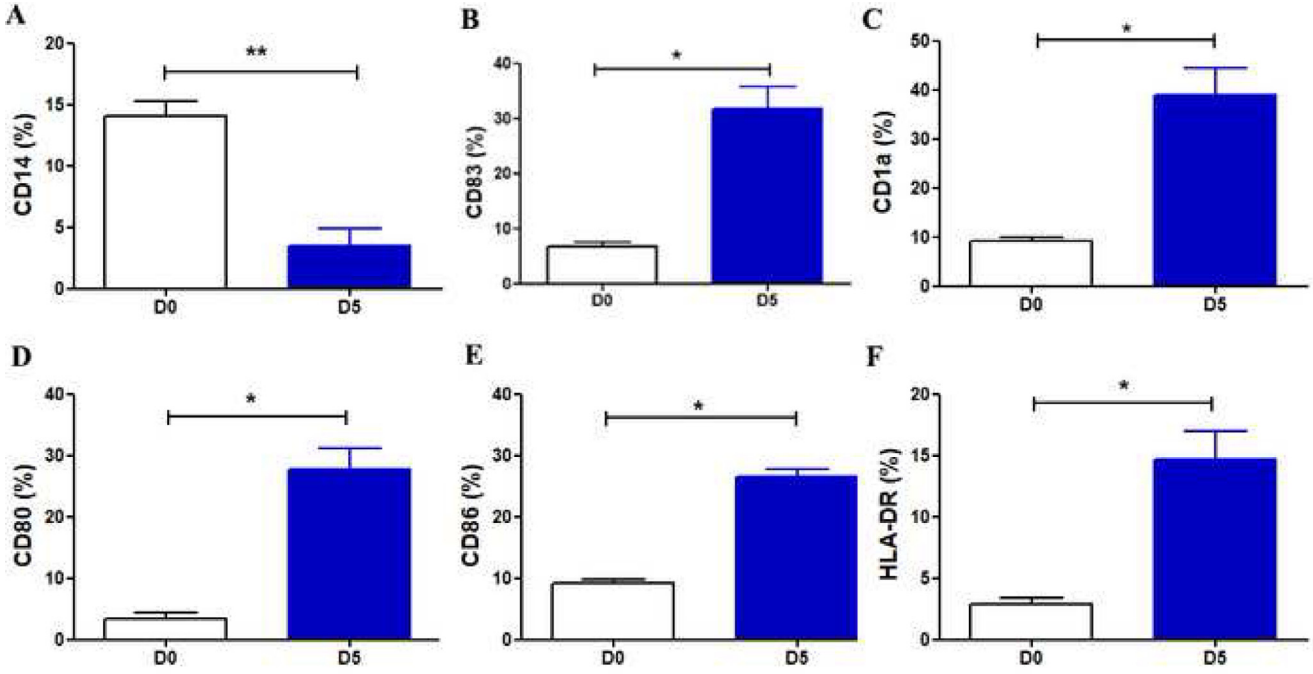

Fig. 2. Expression of cell differentiation and activation markers using fluorescein isothiocyanate (FITC)-labeled $CD14^+$ monocytes during CAPRI cell activation among PBMCs. The CAPRI cells were harvested on day 5 for phenotypic characterization using flow cytometry. (A) Frequencies of CD14 (monocytes), (B) CD83 (dendritic cells) and (C) CD1a CAPRI cells (n = 7 independent CAPRI cultures). Results are represented as mean ± SD. $^*P < 0.05$. (D and E) Frequencies of CD80 and CD86 (activation markers) in CAPRI cells. (F) Proportion of HLA-DR of CAPRI cells (n = 7 independent CAPRI cell cultures). $^*P < 0.05$.

were infused every alternate day for 12 does to the present patients (Figure 1). Each dose comprised 5×10^7 to 1×10^8 cells. Patients received chemotherapy regimens in both study groups until death or the follow-up deadline.

Assay of the phenotypes of CAPRI cells

Phenotypes of CAPRI cells were assessed using the FACSCanto II flow cytometer (Figure 2). In total, 5×10^5 CAPRI cells were harvested and labeled with fluorescence-conjugated antibodies against CD14, CD83, CD1a, CD80, CD86, HLA-DR, CD3, CD4, CD8 and CD56 at 4°C in the dark for 15 min. The cells were suspended in phosphate-buffered saline and analyzed using Diva software (BD Biosciences).

Assay of cytokine production by the CAPRI cells

CAPRI cells were extracted from seven patients on day 5 and screened for functional markers. The secretion of cytokines including interferon-γ, interleukin-2 and perforin from CAPRI cells of these patients was analyzed via flow cytometry. Intracellular staining was performed after stimulating cells with phorbol myristate acetate (PMA; 1 μg/mL, Sigma-Aldrich, St. Louis, MO, USA), ionomycin (1 μg/mL; Sigma-Aldrich, St. Louis, MO, USA) and in the presence of Brefeldin A (BFA; 1 × BFA, BioLegend, San Diego, CA, USA) at 37°C in 5% CO_2 for 5 h. CAPRI cells were fixed with 4% formalin for 15 min and then stained with antibodies against IL-2, IFN-γ and perforin at 4°C in the dark for 30 min. Finally, cells were analyzed using the FACSCanto II flow cytometer.

CD107a degranulation assay

For the degranulation assay, CD107a expression was assessed in seven patients and considered a marker of cytotoxicity. CD107a expression is mediated by $CD3^+CD4^+$, $CD3^+CD8^+$ and $CD3^+CD56^+$ cells in response to incubation with tumor cells [13]. In brief, human colon cancer cell line H460 and human lung cancer cell line A549 were

seeded into separate plates and incubated with 2×10^6 CAPRI cells at an effector cell-to-target cell ratio of 20:1 for 5 h. Thereafter, the BFA and anti-CD107a antibody were added. In this study, PMA was considered as a positive control and unstimulated CAPRI cells were used as the negative control to assess the spontaneous expression of CD107a. Finally, cells were washed and stained with surface antibodies specific for $CD3^+CD4^+$, $CD3^+CD8^+$ and $CD3^+CD56^+$ cells in the dark at 4°C. CD107a levels were determined with the FACSCanto II flow cytometer.

Assessment of clinical responses and toxicity

The clinical response of these patients, including complete remission (CR), partial remission (PR), stable disease (SD) and progressive disease, was assessed according to the Response Evaluation Criteria in Solid Tumors guidelines [14]. The CAPRI group was evaluated at 4 weeks after the final cell infusion and subsequently at the scheduled time until recurrence or disease progression. The control group was assessed after each chemotherapy cycle and in accordance with the recommended schedule until recurrence or disease progression. After each cell infusion, adverse events and safety parameters were evaluated according to the National Cancer Institute Common Terminology Criteria for Adverse Events, Version 4.0.

Follow-up evaluation

The clinical response of each patient was evaluated during a follow-up period of 3–67 months. The recommended schedule for follow-up time was every 3 months within the first year of treatment and every 6 months in the subsequent years. Laboratory tests, computed tomography, X-ray, Karnofsky performance status evaluations, as well as disease-specific death were recorded. Furthermore, patients in the CAPRI group completed questionnaires on changes in their quality of life after the cell infusions (e.g., appetite, sleeping, weight and pain).

Statistical analysis

All statistical analyses were performed using the SPSS 21.0 software package. One-way analysis of variance was used to analyze the difference among multiple groups, whereas the least significant difference test was used to compare the results between the two groups. Differences in clinical parameters between CAPRI group and control group were evaluated using the chi-square test. Survival curves were assessed using Kaplan-Meier method with survival data from 65 patients with lung cancer, and the OS durations were compared using the log-rank test.

Results

Phenotypes of the CAPRI cells

CAPRI cells were assessed via flow cytometry and further analyzed using the Student *t* test (Figure 2). Compared with day 0, CD14 cells (monocytes) were sharply decreased on day 5 (14.03% ± 1.81% versus 3.47% ± 1.95%; $P = 0.0092$), suggesting that more monocytes tend to mature into DCs (Figure 2A). Compared with baseline levels, the expression level of CD83, a unique surface marker of mature DCs, drastically increased on day 5 (6.70% ± 1.22% versus 31.80% ± 5.65%; $P = 0.0162$; Figure 2B). Furthermore, the proportion of cells expressing CD1a, another marker of matured DCs, also displayed an obvious increase on day 5 (9.23% ± 0.87% versus 38.90% ± 7.90%; $P = 0.0298$; Figure 2C). These data indicated a significant increase in the number of mature DCs in the CAPRI cells that might have differentiated from the mononuclear cells. Furthermore, the expression levels of co-stimulatory molecules on mononuclear cells on day 5, including CD80 and CD86 (expressed on DCs), were markedly higher than those on day 0. The percentages of CD80 on day 0 and day 5 are shown in Figure 2D (3.30% ± 1.57% versus 24.37% ± 9.50%; $P = 0.0112$) and these cells expressing CD86 are shown in Figure 2E (9.07% ± 0.87% versus 19.93% ± 5.58%; $P = 0.0107$) respectively. Meanwhile, the frequency of HLA-DR had increased sharply from 2.87% ± 0.76% to 14.67% ± 3.31% ($P = 0.0425$; Figure 2F). Together, these data showed that monocytes had differentiated into mature DCs on day 5 in the culture system.

Cytokine production by CAPRI cells

To characterize the functions of CAPRI cells, the expression levels of interleukin-2, interferon-γ and perforin were assessed in $CD3^+CD8^+$, $CD3^+CD56^+$ and $CD3^+CD4^+$ CAPRI cells. The $CD3^+CD8^+$ and $CD3^+CD56^+$ CAPRI cells showed higher IFN-γ levels (33.77% ± 4.40% and 26.90% ± 3.56% versus 16.83% ± 7.85%) than those in $CD3^+CD4^+$ CAPRI cells (Figure 3A). Furthermore, IL-2 expression levels were the highest in the $CD3^+CD56^+$ CAPRI cells (26.73% ± 6.63%), whereas the percentages in the $CD3^+CD8^+$ and $CD3^+CD4^+$ cells were 21.05% ± 3.03% and 14.53% ± 3.50%, respectively (Figure 3B). Moreover, $CD3^+CD56^+$ CAPRI cells expressed the highest level of perforin (20.15% ± 2.10%), followed by $CD3^+CD8^+$ CAPRI cells (18.38% ± 7.15%) and $CD3^+CD4^+$ CAPRI cells (16.88% ± 7.45%; Figure 3C).

Anti-tumor effects through degranulation of CAPRI cells

To further evaluate the anti-tumor effects of CAPRI cells, CD107a expression was assessed to detect the degranulation of $CD3^+CD56^+$, $CD3^+CD8^+$ and $CD3^+CD4^+$ T cells. CAPRI cells were incubated with or without A549 and H460 cells at an effector-target ratio of 20:1 for 5 h. Compared with the negative control cells, $CD3^+CD56^+$ T cells, $CD3^+CD8^+$ T cells and $CD3^+CD4^+$ T cells showed higher CD107a expression levels against H460 cells (38.7% ± 9.17%, 28.1% ± 3.87% and 26.8% ± 5.50%), respectively (Figure 4). Furthermore, the degranulation of $CD3^+CD56^+$ T cells, $CD3^+CD8^+$ T cells and $CD3^+CD4^+$ T cells displayed a similar trend against A549 cells (34.2% ±3.21%, 20.7% ± 5.38% and 24.5% ± 7.67%, respectively; Figure 4). The results demonstrated that CAPRI cells had strong anti-tumor ability for lung cancer cell lines.

Immune status of patients during CAPRI cell treatment

To evaluate the changes in the immune status of patients, peripheral T-cell subsets were detected via flow cytometry immediately before and after CAPRI cell infusion (Table 2). The data showed that CAPRI cell therapy had a slight impact on total $CD3^+$ T cells. The frequency of $CD4^+$ T cells tended to slightly but not significantly increase at 4 weeks and 8 weeks after CAPRI cell infusions. However, the proportion of $CD3^+CD8^+$ T cells gradually decreased at 4 weeks and 8 weeks of CAPRI cell administration. Moreover, the ratio of CD4/CD8 increased after CAPRI cell therapy. These results suggest that CAPRI cell treatment potentially improves the immune status of patients with NSCLC in a short period. This could be explained through infusion of CAPRI cells with a higher frequency of $CD8^+$ T cells, which skewed the CD4/CD8 ratio, indicating a crucial role of CAPRI cell therapy through positive modulation of the immune system.

Short-term clinical response

The short-term clinical response was evaluated in both groups at 4 weeks after treatment. The clinical response rate (CR + PR) was higher among patients in the CAPRI group than in the control group (9.6% versus 2.9%). However, no statistically significant difference was observed in clinical response rates ($P = 0.180$; Table 3). The 1-, 3- and 5-year OS rates were 100% (31/31), 67.7% (21/31) and 22.6% (7/31), respectively, in the CAPRI group and 64.7% (22/34), 32.4% (11/34)

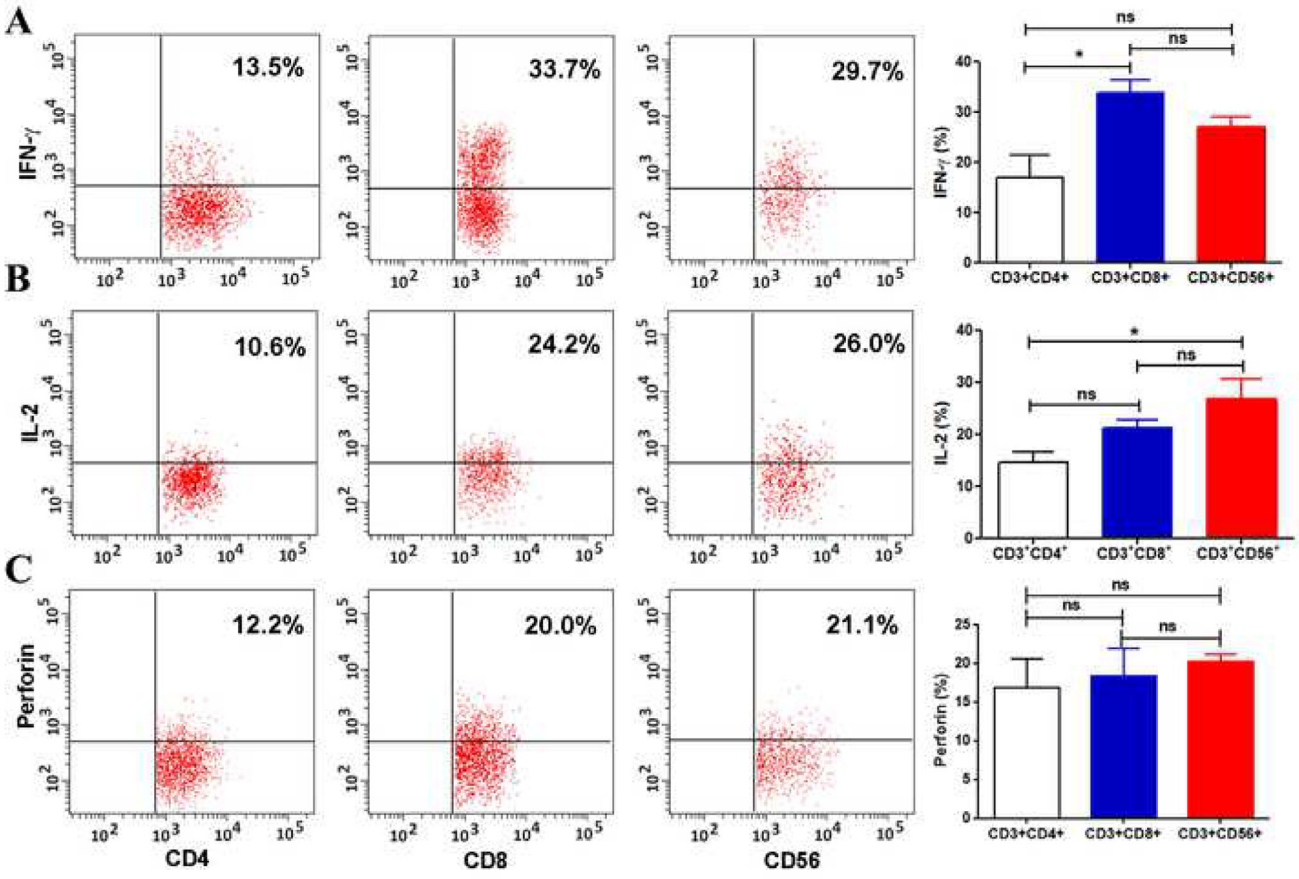

Fig. 3. Cytokine production by CAPRI cells. The cells (1×10^6) were cultured for 5 h with PMA, ionomycin and BFA, and then stained with their surface markers. After cell fixation, the cells were incubated with anti-IFN-γ, anti-IL-2 and anti-perforin monoclonal antibodies. (A) Frequencies of IFN-γ in $CD3^+CD4^+$, $CD3^+CD8^+$ and $CD3^+CD56^+$ subsets of CAPRI cells. (B and C) Frequencies of IL-2 and perforin in $CD3^+CD4^+$, $CD3^+CD8^+$ and $CD3^+CD56^+$ subsets of CAPRI cells. The results are represented as mean ± SD values (n = 7 independent CAPRI cells cultures).

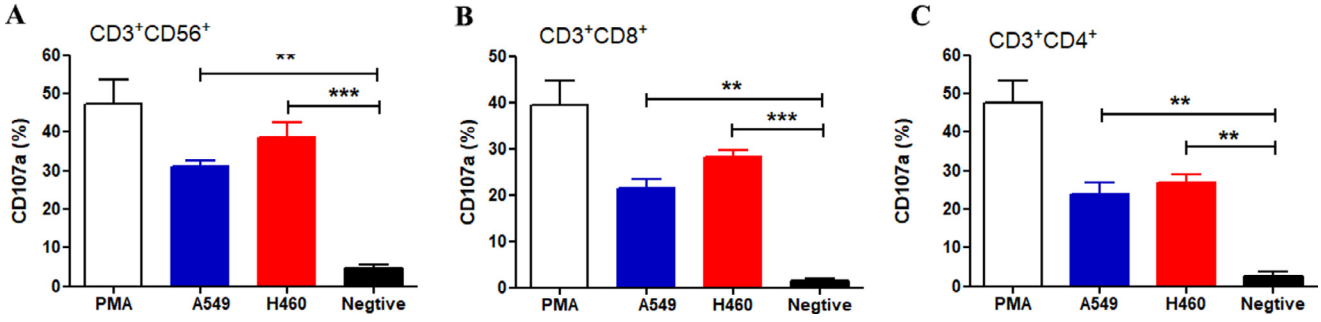

Fig. 4. Anti-tumor effects of CAPRI-CIK cells. CAPRI cells were incubated with A549 or H460 at a ratio of 20:1 for 5 h. CD107a expression was quantified via flow cytometry for degranulation of CAPRI cells in $CD3^+CD4^+$, $CD3^+CD8^+$ and $CD3^+CD56^+$ cell subsets. The results are represented as mean ± SD values. $^*P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$.

and 14.7% (5/34), respectively, in the control group (Table 4). Furthermore, the 1-, 3- and 5-year PFS rates are shown in Table 4. The 1- and 3-year OS and PFS data show a clear significant difference between the two groups. Representative computed tomography scans (before and after treatment) for one of the patients with CR are shown in Figure 5.

Table 2
Dynamics of peripheral T-cell subsets after the start of CAPRI cell treatment.

	0 Weeks (%)	4 Weeks (%)	8 Weeks (%)
$CD3^+$	70.5 ± 14.1	70.7 ± 14.7	81.6 ± 12.3
$CD3^+CD4^+$	36.7 ± 13.4	40.5 ± 10.6	48.9 ± 9.1
$CD3^+CD8^+$	31.3 ± 10.9	29.1 ± 10.1	28.8 ± 9.9
$CD4^+/CD8^+$	1.4 ± 0.9	1.6 ± 0.6	1.9 ± 0.8

Table 3
Clinical response of patients in CAPRI group and control group.

Response	CAPRI n = 31(%)	Control n = 34 (%)	χ^2 (P)
CR	1 (3.2)	0 (0)	4.885 (0.180)
PR	2 (6.4)	1 (2.9)	
SD	26 (83.8)	24 (70.5)	
PD	3 (9.7)	9 (26.4)	

PD, progressive disease.

Table 4
OS and PFS rates of patients over different years.

Response		CAPRI n = 31 (%)	Control n = 34 (%)	χ^2 (P)
1 y	OS rate	31 (100)	22 (64.7)	13.418 (<0.001)
	PFS rate	31 (100)	22 (64.7)	13.418 (<0.001)
3 y	OS rate	21 (67.7)	11 (32.4)	8.125 (0.004)
	PFS rate	25 (80.6)	18 (52.9)	5.558 (0.018)
5 y	OS rate	7 (22.6)	5 (14.7)	0.668 (0.414)
	PFS rate	8 (25.8)	4 (11.8)	2.124 (0.145)

Long-term response and follow-up evaluation

Follow-up evaluation of patients was initiated 4 weeks after the first cycle of chemotherapy had been completed. The PFS and OS data of all patients were analyzed. The PFS data indicated a significant difference between the two groups (P = 0.007; Figure 6A). To further explore the long-term clinical efficiency of the combination treatment, the OS of the patients in the two groups was compared. Consequently, the CAPRI group had a significantly prolonged OS in comparison with the control group (P = 0.008; Figure 6B). As shown in Table 5, the median OS and PFS were 48 months in the CAPRI group, and 31.6 and 36.4 months in the control group, respectively (P = 0.004 and P = 0.016). These results indicate that patients with lung cancer may benefit from adjuvant CAPRI cell therapy, both in terms of OS and PFS.

Side effects

Before and during the CAPRI cell infusion, evaluation and assessment of vital signs, especially body temperature and function of major organs including the heart and liver, were performed. All patients completed the treatment course and only mild side effects were observed in patients. No severe adverse effects were experienced. Compared with the control group, fever occurred in three patients with a maximum temperature of 38.9°C, and these patients

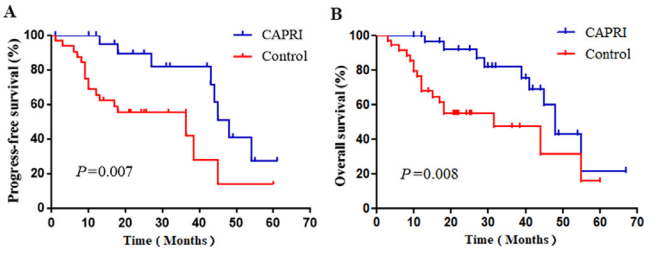

Fig. 6. PFS and OS. Kaplan-Meier survival curves were generated using a log-rank test among patients with lung cancer in the CAPRI and control groups. (A) A significant difference was observed in the PFS between the CAPRI group and control group (P = 0.007). (B) The OS of patients in the CAPRI group was significantly higher than that of the control group (P = 0.008).

responded well to antipyretic medication. Four patients experienced nausea or vomiting and two patients experienced rashes (Table 6). However, no significant difference was observed between the two groups in terms of side effects.

Discussion

Recent studies have reported advancements in adoptive cell therapy in the field of cancer immunotherapy [15], and the safety and efficacy of immunotherapy have been improved in a number of clinical trials [16], particularly novel immunotherapies presented as CAR-T cell therapy. CD19 CAR-T cells are potent T cells because of their specific functional receptor, which is able to recognize B-cell leukemia and lymphoma cells that specifically express CD19. However, CD19 CAR-T cells have not shown much success against solid tumors for the following reasons. On the one hand, they lack the specific antigens of the solid tumors that are recognized by the CAR-T cells. On the other hand, the immunosuppressive microenvironment can cause T-cell dysfunction, thus limiting the clinical application and anti-tumor efficiency of CAR-T cells. Regarding TIL therapy,

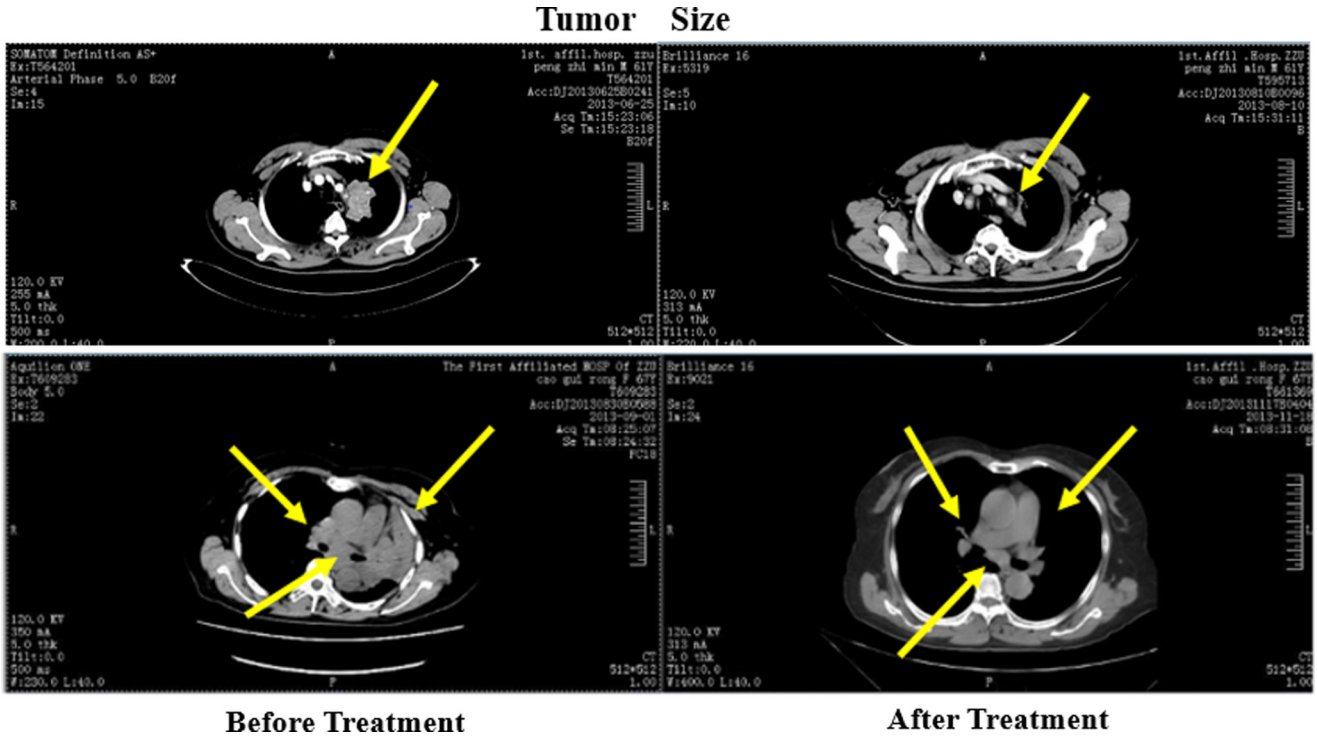

Fig. 5. Representative computed tomography scans of patients experiencing CR before and after treatment.

Table 5
Median survival rates of patients.

Response		CAPRI n = 31	Control n = 34	χ^2 (*P*)
Median time (mo)	OS	48.0	31.6	8.118 (0.004)
	PFS	48.0	36.4	5.835 (0.016)
(95% CI)	OS	43.3–52.7	9.1–54.1	
	PFS	39.1–50.9	6.1–66.7	

CI, confidence interval.

Table 6
Side effects of CAPRI cell infusion (N [%]).

Group	N	Fever	Gastrointestinal reactions	Rash	Tumor lysis syndrome
CAPRI	31	3 (9.6)	4 (12.9)	2 (6.4)	0 (0)
Control	34	8 (25.8)	10 (29.4)	3 (8.8)	1 (2.94)
χ^2		2.378	2.821	0.156	0.956
P		0.123	0.093	0.693	0.328

difficulties in the acquisition of tumor tissue and proliferation of TILs can affect the clinical implementation of TIL therapy among patients with cancer. Thus, compared with CAR-T and TIL therapy, CAPRI therapy has several advantages, as was presented in this study.

The combination of chemotherapy with immune cell infusions has become popular in cancer treatments, showing improved clinical efficacy in several types of malignant tumors [17–24]. There are several possible explanations for this phenomenon. First, low-dose chemotherapy or radiotherapy has sensitizing effects on tumor cells, thereby improving the efficiency of cytotoxic T lymphocytes. Second, chemotherapeutic agents decrease the number of immunosuppressive cells, such as myeloid-derived suppressor cells and regulatory T cells, which may alter the microenvironment into an immunosuppressive status. Finally, chemotherapy further promotes the release of tumor-related antigens from tumors to stimulate T-cell activity, thus improving their anti-tumor potential.

CAPRI immune cell therapy is an effective adjuvant therapy in various cancers and can be implemented through different effector cells including cytotoxic T lymphocytes, helper T cells, DCs, natural killer–like T cells and monocytes [14]. The biological characteristics of CAPRI cell therapy differ from those of other adoptive cell therapies in that the infusion of CAPRI cells does not require gene manipulation or cancer-related peptides identification. Herein, CAPRI cells were cultured with the anti-CD3 antibody OKT3 to enhance the expression of MHCs and co-stimulatory molecules within a short period. Therefore, the infusion of CAPRI cells could be initiated within a few days after the isolation of immune cells from PBMCs.

In CAPRI cell therapy, monocytes served as the primary cells, stimulating naïve cells into effector cells, which then rapidly differentiated into DCs within a short period. In other words, autologous monocytes presented the best cancer-immunogenic peptides among different individuals. In this study, the proportion of monocytes with the CD14 marker decreased sharply on day 5, which illustrated that a large number of these cells had potentially differentiated into mature DCs during CAPRI cell therapy. Meanwhile, the expression level of CD1a and CD83, classic markers of mature DCs, was drastically increased. DCs are APCs that link the innate immune system with the adaptive immune system to facilitate an efficient immune response. DCs play an essential role in stimulating T-cell activation, thus facilitating anti-tumor activity [25]. Another valuable approach is co-culturing with antigen-presenting DCs because this interaction helps CAPRI cells more aggressively enhance the expansion of the primary effectors. Obleukhova *et al*. observed impaired migration among DCs in NSCLC and detected low levels of circulating immature and mature DCs in PBMCs [24]. Herein, the increased proportion of DCs *in vitro* indicates the effective induction and co-stimulation of T lymphocytes by the generated DCs, potentially improving the cytotoxic ability of these monocytes. Because DCs are considered the most suitable vehicles in presenting microbial and tumor antigens to T lymphocytes, antigen-activated DCs generated from mononuclear cells could stimulate a specific immune response when exposed to autologous tumor lysates or tumor-genetic peptides. Furthermore, upregulation of activation markers CD80 and CD86 suggests that stimulated APCs and co-stimulatory molecules potentially have a synergistic effect on the activation and differentiation of naïve T cells into effector cells that may recognize and eliminate cancer cells [12,26].

Coulie *et al.* first reported that cytotoxic T lymphocytes recognized a mutated intron sequence [27]. Subsequently, studies have reported that MHC class I and II play an important role in specific interactions with immune cells [28]. CAPRI cells required synergistic human leukocyte antigen (HLA) expression for successful tumor cell lysis. We found a significant HLA-DR increase in HLA-DR expression on day 5 after priming compared with that on day 0. Upregulation of this specific HLA class of molecules likely improved the anti-tumor capacity of the immune system, because cancer cell lysis was more efficient when CAPRI cells shared the same HLA class antigens with cancer cells. Moreover, studies have reported a change in the antigenic profile of malignant cells in the blood plasma after cancer treatment [29,30]. Therefore, naïve T cells from the PBMCs can recognize mutations in antigenic peptides, infiltrate a recurring tumor and target peptides of malignant tumors or newly mutated proteins [31]. The stimulated APCs recognized the tumor-associated antigens and then presented them to effector cells. Most significantly, the CAPRI procedure does not require genetic modifications or identification of cancer-immunogenic peptides. Therefore, CAPRI cells may safely and effectively digest tumor cells in patients with cancer receiving other therapies.

IFN-γ and IL-2 are used to enhance the cytotoxicity of cell therapy in the clinical setting [32,33]. Therefore, we quantified the expression of the two cytokines and indirectly the tumoricidal ability of CAPRI cells via flow cytometery. The present findings showed the levels of IFN-γ and perforin were the highest in $CD3^+CD8^+$ T cells, and a significant level of IL-2 was produced in $CD3^+CD56^+$ T cells. These results confirmed that CAPRI cells produce cytokines with oncolytic effects [34,35]. Furthermore, the data show that CAPRI cells induced potent cytotoxicity toward A549 cells but reduced cytotoxic activity toward H460 cells. Therefore, it is important to select appropriate clinical applications for various patients with cancer.

Most patients have been reportedly administered CAPRI cells in accordance with a recommended a schedule of $60–80 \times 10^6$ CAPRI cells thrice a week combined with chemotherapy for 6 months [26]. For most patients, therapy was continued for the next several years. Notably, the recommended modality of treatment was continuous treatment thrice per week at least for 6 months. Patients were treated using an adjuvant strategy, which introduces variables of chemotherapy [26]. In our study, patients with NSCLC recruited in the CAPRI group were treated with at least 6×10^8 CAPRI cells. Moreover, chemotherapy combined with CAPRI cell treatment improved the clinical outcome and safety of patients.

Follow-up evaluation revealed a significant difference in the median OS between the CAPRI group and the control group (48 months versus 31.6 months, respectively). The median PFS in the CAPRI group was greater than that of the control group [48 months versus 36.4 months ($P = 0.004$)]. Furthermore, we reported that CAPRI cell infusion plus chemotherapy was associated with a greater PFS and OS than chemotherapy alone and was well-tolerated. Pan et al. reported the long-term efficacy of patients with breast cancer receiving CIK treatment with chemotherapy. Compared with those in the control group, the OS and PFS of patients were greater in the CIK group, suggesting that CIK treatment may help prolong patient survival [36]. As indicated herein, patients in the CAPRI group received cell infusion and chemotherapy rather than chemotherapy alone,

thus resulting in immune activation and boosting and reconstructing the immune system. Furthermore, the present results were entirely consistent with our previous findings [23,37]. No severe adverse effects were observed during the CAPRI therapy. Only mild side effects were experienced, and they were well tolerated and easy to control. Three patients developed mild fevers, which eventually subsided after 24 h or responded well to antipyretic treatment. Four patients experienced nausea and/or vomiting, and they responded well to clinical intervention. Additional side effects were similarly mild and easily treatable. These results show that CAPRI cell therapy is safe and effective among patients with cancer. Moreover, CAPRI cell therapy may lead to the destruction of large tumors after conventional therapy, thus potentially explaining the encouraging clinical outcomes in the CAPRI group rather than the control group.

Together, our results suggest that CAPRI cells are a feasible and effective alternative for adoptive immunotherapy in different cancers. The favorable biological features of CAPRI cell therapy include potent expansion and wide MHC-unrestricted tumor elimination, which can fulfil various clinical requirements. Furthermore, obtaining "clinically adequate" amounts of CAPRI cells does not require complicated procedures such as genetic engineering. The simplicity of CAPRI cell therapy may facilitate the transition of CAPRI cell therapy into an affordable clinical alternative for many cancer centers.

Conclusion

The vital goals of adoptive cell immunotherapy are to safely increase the quantities and to improve the functions of cytotoxic immune cells for cancer treatment. The features of CAPRI cell therapy include a short production period, PBMC stimulation of cytotoxic T lymphocytes and continuous therapy, thus rendering CAPRI cell therapy superior to other adoptive cell therapy (ACT). This study highlights the therapeutic advantage of using combination chemotherapy and CAPRI adoptive T-cell therapy to treat lung cancer. We observed synergistic interactions between these two therapies, and we propose that such combination therapy may help treat other cancers. However, further studies and clinical trials are required to confirm the efficacy of combination therapy.

Declaration of Competing Interest

This study was supported by the National Natural Science Foundation of China (grant numbers U1804281, 81771781, 81702810 and 81773060) and the Henan Provincial Health Department of Science and Technology Research Project (grant number 201702018). The authors have no commercial, proprietary or financial conflict of interest with the products or companies described in this article.

Author Contributions

Conception and design of the study: YZ, HL and ZZ. Acquisition of data: HL and ZZ. Analysis and interpretation of data: XD, NRM, SY, XZ and JH. Drafting or revising the manuscript: HL, ZZ and YZ. All authors have approved the final article.

References

[1] Siegel RL, Miller KD, Jemal A. Cancer Statistics, 2017. CA: a cancer journal for clinicians 2017;67:7–30.
[2] Chang A. Chemotherapy, chemoresistance and the changing treatment landscape for NSCLC. Lung cancer (Amsterdam, Netherlands) 2011;71:3–10.
[3] Choi MK, Hong JY, Chang W, Kim M, Kim S, Jung HA, et al. Safety and efficacy of gemcitabine or pemetrexed in combination with a platinum in patients with non-small-cell lung cancer and prior interstitial lung disease. Cancer chemotherapy and pharmacology 2014;73:1217–25.
[4] Dudley ME, Yang JC, Sherry R, Hughes MS, Royal R, Kammula U, et al. Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens. Journal of clinical oncology: official journal of the American Society of Clinical Oncology 2008;26:5233–9.
[5] Gammaitoni L, Giraudo L, Leuci V, Todorovic M, Mesiano G, Picciotto F, et al. Effective activity of cytokine-induced killer cells against autologous metastatic melanoma including cells with stemness features. Clinical cancer research: an official journal of the American Association for Cancer Research 2013;19:4347–58.
[6] Zhang J, Zhu L, Du H, He X, Yin Y, Gu Y, et al. Autologous cytokine-induced killer cell therapy in lung cancer patients: a retrospective study. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie 2015;70:248–52.
[7] Khammari A, Knol AC, Nguyen JM, Bossard C, Denis MG, Pandolfino MC, et al. Adoptive TIL transfer in the adjuvant setting for melanoma: long-term patient survival. Journal of immunology research 2014;2014:186212.
[8] Dudley ME, Gross CA, Somerville RP, Hong Y, Schaub NP, Rosati SF, et al. Randomized selection design trial evaluating CD8+-enriched versus unselected tumor-infiltrating lymphocytes for adoptive cell therapy for patients with melanoma. Journal of clinical oncology: official journal of the American Society of Clinical Oncology 2013;31:2152–9.
[9] June CH, Sadelain M. Chimeric Antigen Receptor Therapy. N Engl J Med 2018;379:64–73.
[10] Wei C, Wang W, Pang W, Meng M, Jiang L, Xue S, et al. The CIK cells stimulated with combination of IL-2 and IL-15 provide an improved cytotoxic capacity against human lung adenocarcinoma. Tumour biology: the journal of the International Society for Oncodevelopmental Biology and Medicine 2014;35:1997–2007.
[11] Mesiano G, Todorovic M, Gammaitoni L, Leuci V, Giraudo Diego L, Carnevale-Schianca F, et al. Cytokine-induced killer (CIK) cells as feasible and effective adoptive immunotherapy for the treatment of solid tumors. Expert opinion on biological therapy 2012;12:673–84.
[12] Laumbacher B, Gu S, Wank R. Activated monocytes prime naive T cells against autologous cancer: vigorous cancer destruction *in vitro* and *in vivo*. Scandinavian journal of immunology 2012;75:314–28.
[13] Tang Y, Li X, Wang M, Zou Q, Zhao S, Sun B, et al. Increased numbers of NK cells, NKT-like cells, and NK inhibitory receptors in peripheral blood of patients with chronic obstructive pulmonary disease. Clinical & developmental immunology 2013;2013:721782.
[14] Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). European journal of cancer (Oxford, England: 1990) 2009;45:228–47.
[15] Yee C. Adoptive T-cell therapy for cancer: boutique therapy or treatment modality? Clinical cancer research: an official journal of the American Association for Cancer Research 2013;19:4550–2.
[16] Khatami F, Torabi-Rahvar M, Kiani J, Naderi M, Ahmadbeigi N. Addressing cancer immunotherapy research in Iran: adoptive cell therapy on the horizon. Cytotherapy 2018;20:1227–37.
[17] Guo Z, Liu H, He XP, Tan XH, Zhou Y, Chen X, et al. A clinical study of cytokine-induced killer cells for the treatment of refractory lymphoma. Oncology letters 2011;2:531–6.
[18] Hontscha C, Borck Y, Zhou H, Messmer D, Schmidt-Wolf IG. Clinical trials on CIK cells: first report of the international registry on CIK cells (IRCC). Journal of cancer research and clinical oncology 2011;137:305–10.
[19] Joseph RW, Peddareddigari VR, Liu P, Miller PW, Overwijk WW, Bekele NB, et al. Impact of clinical and pathologic features on tumor-infiltrating lymphocyte expansion from surgically excised melanoma metastases for adoptive T-cell therapy. Clinical cancer research: an official journal of the American Association for Cancer Research 2011;17:4882–91.
[20] Liu L, Zhang W, Qi X, Li H, Yu J, Wei S, et al. Randomized study of autologous cytokine-induced killer cell immunotherapy in metastatic renal carcinoma. Clinical cancer research: an official journal of the American Association for Cancer Research 2012;18:1751–9.
[21] Shi L, Zhou Q, Wu J, Ji M, Li G, Jiang J, et al. Efficacy of adjuvant immunotherapy with cytokine-induced killer cells in patients with locally advanced gastric cancer. Cancer immunology, immunotherapy: CII 2012;61:2251–9.
[22] Liu J, Li H, Cao S, Zhang X, Yu J, Qi J, et al. Maintenance therapy with autologous cytokine-induced killer cells in patients with advanced epithelial ovarian cancer after first-line treatment. Journal of immunotherapy 2014;37:115–22.
[23] Li H, Huang L, Liu L, Wang X, Zhang Z, Yue D, et al. Selective effect of cytokine-induced killer cells on survival of patients with early-stage melanoma. Cancer immunology, immunotherapy: CII 2017;66:299–308.
[24] Obleukhova I, Kiryishina N, Falaleeva S, Lopatnikova J, Kurilin V, Kozlov V, et al. Use of antigen-primed dendritic cells for inducing antitumor immune responses *in vitro* in patients with non-small cell lung cancer. Oncology letters 2018;15:1297–306.
[25] Chakraborty S, Kloos B, Roetz N, Schmidt S, Eigenbrod T, Kamitani S, et al. Influence of Pasteurella multocida Toxin on the differentiation of dendritic cells into osteoclasts. Immunobiology 2018;223:142–50.
[26] Wank R, Song X, Gu S, Laumbacher B. Benefits of a continuous therapy for cancer patients with a novel adoptive cell therapy by cascade priming (CAPRI). Immunotherapy 2014;6:269–82.
[27] Coulie PG, Lehmann F, Lethe B, Herman J, Lurquin C, Andrawiss M, et al. A mutated intron sequence codes for an antigenic peptide recognized by cytolytic T lymphocytes on a human melanoma. In: In: Proceedings of the National Academy of Sciences of the United States of America, 92; 1995. p. 7976–80.
[28] Garrido F, Ruiz-Cabello F. MHC expression on human tumors—its relevance for local tumor growth and metastasis. Seminars in cancer biology 1991;2:3–10.
[29] Diehl F, Schmidt K, Choti MA, Romans K, Goodman S, Li M, et al. Circulating mutant DNA to assess tumor dynamics. Nature medicine 2008;14:985–90.
[30] Forshew T, Murtaza M, Parkinson C, Gale D, Tsui DW, Kaper F, et al. Noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA. Science translational medicine 2012;4:136ra168.
[31] Guo L, Xu D, Lu Y, Peng J, Jiang L. Detection of circulating tumor cells by reverse transcriptionquantitative polymerase chain reaction and magnetic activated cell

sorting in the peripheral blood of patients with hepatocellular carcinoma. Molecular medicine reports 2017;16:5894–900.
[32] Yang J, Lemas VM, Flinn IW, Krone C, Ambinder RF. Application of the ELISPOT assay to the characterization of CD8(+) responses to Epstein-Barr virus antigens. Blood 2000;95:241–8.
[33] Shirai A, Holmes K, Klinman D. Detection and quantitation of cells secreting IL-6 under physiologic conditions in BALB/c mice. Journal of immunology 1993;150:793–9.
[34] Linn YC, Wang SM, Hui KM. Comparative gene expression profiling of cytokine-induced killer cells in response to acute myloid leukemic and acute lymphoblastic leukemic stimulators using oligonucleotide arrays. Experimental hematology 2005;33:671–81.
[35] Li H, Yu JP, Cao S, Wei F, Zhang P, An XM, et al. CD4 +CD25 + regulatory T cells decreased the antitumor activity of cytokine-induced killer (CIK) cells of lung cancer patients. Journal of clinical immunology 2007;27:317–26.
[36] Pan K, Guan XX, Li YQ, Zhao JJ, Li JJ, Qiu HJ, et al. Clinical activity of adjuvant cytokine-induced killer cell immunotherapy in patients with post-mastectomy triple-negative breast cancer. Clinical cancer research: an official journal of the American Association for Cancer Research 2014;20:3003–11.
[37] Huang J, Kan Q, Lan Zhao X, Zhang Z, Yang S, et al. Chemotherapy in combination with cytokine-induced killer cell transfusion: An effective therapeutic option for patients with extensive stage small cell lung cancer. International immunopharmacology 2017;46:170–7.