Title: 2003–2013, a valuable study: autologous tumor lysate-pulsed dendritic cell immunotherapy with cytokine-induced killer cells improves survival in stage IV breast cancer

Authors: Mao Lin, Shuzhen Liang, Feng Jiang, Jiongyuan Xu, Weibing Zhu, Wei Qian, Yong Hu, Zhanchun Zhou, Jibing Chen, Lizhi Niu, Kecheng Xu, Youyong Lv

PII: S0165-2478(16)30326-1
DOI: http://dx.doi.org/doi:10.1016/j.imlet.2017.01.014
Reference: IMLET 5978

To appear in: *Immunology Letters*

Received date: 7-12-2016
Revised date: 24-1-2017
Accepted date: 24-1-2017

Please cite this article as: Lin Mao, Liang Shuzhen, Jiang Feng, Xu Jiongyuan, Zhu Weibing, Qian Wei, Hu Yong, Zhou Zhanchun, Chen Jibing, Niu Lizhi, Xu Kecheng, Lv Youyong.2003–2013, a valuable study: autologous tumor lysate-pulsed dendritic cell immunotherapy with cytokine-induced killer cells improves survival in stage IV breast cancer.*Immunology Letters* http://dx.doi.org/10.1016/j.imlet.2017.01.014

2003-2013, a valuable study: autologous tumor lysate-pulsed dendritic cell immunotherapy with cytokine-induced killer cells improves survival in stage Ⅳ breast cancer

Mao Lin[a,b], Shuzhen Liang[a,b], Feng Jiang[b], Jiongyuan Xu[a], Weibing Zhu[a], Wei Qian[a], Yong Hu[a], Zhanchun Zhou[a], Jibing Chen[a,b*], Lizhi Niu[b], Kecheng Xu[b], Youyong Lv[c]

[a]Department of Biotherapy Center, Fuda Cancer Hospital, Jinan University School of Medicine, NO.2 TangDe Xi Rd.Tianhe District, Guangzhou, China

[b]Department of Fuda Cancer Institute, Guangzhou, NO.2 TangDe Xi Rd.Tianhe District, Guangzhou, China

[c] Peking University, Central lab of Cancer Hospital, Beijing, China

Corresponding author. Tel: +86 2038993994

Correspondence to: Jibing Chen, E-mail: linmaogz@163.com; Lizhi Niu, E-mail: little.silly.ding@qq.com

Research highlights

Preparation and identification of DC and CIK are showed. A potentially effective approach for the control of tumour growth in stage Ⅳ breast cancer patients is proposed. Chemotherapy combined DC-CIK can markedly improve the DFS and OS in stage Ⅳ breast cancer patients.

Abstract

Dendritic cells (DCs) and cytokine-induced killer (CIK) cells have both shown activity as immunotherapy in some malignancies. Our aim was to prospective assess the effect of this immunotherapy in patients with stage Ⅳ breast cancer**.** Between Aug 2003 and Dec 2013, we collected 368 patients who met inclusion criteria and divided into immunotherapy group (treatment group: 188 patients) and chemotherapy group (control group: 180 patients). DCs were prepared from the mononuclear cells isolated from patients in the treatment group using IL-2/GM-CSF and were loaded with tumour antigens; CIK cells were prepared by incubating peripheral blood lymphocytes with IL-2, IFN-γ, and CD3 antibodies. After the patients had received low-dose chemotherapy, those in the treatment group also received the DC-CIK therapy, which was repeated four times in a fortnight to form one cycle. At least three cycles of DC-CIK therapy were given. Immune function was measured in treatment group patients' sera. Disease-free survival (DFS) and Overall survival (OS) after the diagnosis of stage Ⅳbreast cancer was assessed after a 10-year follow-up. The result demonstrated that immune function is obviously enhanced after DC-CIK therapy. By Cox regression analysis, DC-CIK therapy reduced the risk of disease progression ($p<0.01$) with an increased OS ($p<0.01$). After low-dose chemotherapy, active immunization with DC-CIK immunotherapy is a potentially effective approach for the control of tumour growth in stage Ⅳ breast cancer patients.

Key words: dendritic cell; immunotherapy; cytokine-induced killer; stage Ⅳ breast cancer; DFS; OS

Abbreviations: DC, dendritic cell; CIK, cytokine induced killer**;** DFS, disease-free survival, OS, overall survival; MBC, metastatic breast cancer; PBMCs, peripheral blood mononuclear cells; PAP, prostatic acid phosphatase; GM-CSF, granulocyte-macrophage colony-stimulating factor; NKTs, nature killer T cells; IL2, interleukin-2; IFN-γ, interferon-γ; LAK, lymphokine-activated killer cells.

1. Introduction

Breast cancer is the second leading cause of mortality among women. Breast cancer-related mortality is almost invariably due to metastasis. Between 25% and 50% of patients diagnosed with breast cancer will eventually develop deadly metastases, often decades after the diagnosis and removal of the primary tumour. The prognosis for patients with metastatic breast cancer (MBC) is generally unfavourable, with an average 5-year survival rate of only about 25%[1],[2]. The therapeutic alternatives for MBC are mainly based on the systemic administration of cytotoxic chemotherapeutic agents; their long-term impact on survival is, however, only around 20 months and the outcome depends heavily on the nature of the metastases and the tumour biology[3-5].

The dendritic cells (DCs) play a crucial role in the induction of antigen-specific T-cell responses to provide active immunotherapy [6,7]. Clinical studies using specifically designed DC-targeted cancer cell vaccines have demonstrated varying clinical benefits. In patients with lymphoma[8], metastatic melanoma[9], colon cancer and non-small-cell lung cancer [10], studies have shown that vaccination with tumour antigen-pulsed DCs, either isolated directly from blood or generated ex vivo from blood precursors, elicited antigen-specific immune reactions and, in some cases, significant tumour responses. The application of an active immunotherapy regimen Sipuleucel-T (APC8015), which works by activating peripheral blood mononuclear cells (PBMCs) with a recombinant fusion protein consisting of prostatic acid phosphatase (PAP), a prostate cancer antigen, and GM-CSF, resulted in an approximately 4-month prolongation of median survival in prostate cancer patients[11-13], and has been approved by the US Food and Drug Administration (FDA) for the treatment of metastatic prostate cancer[12,14,15].

Cytokine-induced killer (CIK) cells are a subset of natural killer T lymphocytes (NKTs) that are predominantly CD3+CD56+ type II NKT cells [16], and such cells can be generated ex vivo by incubating peripheral blood lymphocytes with an agonistic anti-CD3 monoclonal antibody, interleukin (IL)-2, and interferon (IFN)-γ. CIK cells, supported by encouraging clinical trial results in both autologous and allogeneic contexts, are known to cytolytically eliminate tumour cells. In contrast to lymphokine-activated killer (LAK) cells, which are cytotoxic effector T cells stimulated predominantly in

response to a high concentration of interleukin-2 (IL-2), CIK cells exhibit enhanced tumour cell lytic activity[17,18], a higher proliferation rate [19], and relatively lower toxicity [20].

Although passive immunotherapy by adoptive transfer of T cells is believed to be effective in the control of primary tumours, it is unclear whether passive immunotherapy is effective in the long-term control of tumour relapse. Active immunotherapy using tumour-specific vaccines, such as DC vaccines, has the potential to significantly enhance the effect of tumour-specific effector and memory T cells. Anti-tumour responses triggered by DC-CIK therapy have been reported in a number of ex vivo [21-24] and in vivo studies, as well as in preliminary clinical trials in patients with non-Hodgkin's and Hodgkin's lymphoma[25,26], and non-small-cell lung cancer with few side effects [27].

Here, we prospectively analysed the data of patients admitted to our cancer hospital in order to screen the best treatment method. To specifically determine the survival time of breast cancer patients, the overall survival (OS) after the diagnosis of metastatic disease was used as the main evaluation index.

2. Methods

2.1 Study design.

This study was a retrospective observational study performed at the Guangzhou Fuda Cancer Hospital. The study protocol received ethical approval from the Regional Ethics Committee of Guangzhou Fuda Cancer Hospital.

2.2 Patients.

Between August 2003 and December 2013, breast cancer patients who were admitted to our hospital and satisfied our inclusion criteria were enrolled in this study. Patients were assigned to one of two groups: a treatment group that received immunotherapy after their chemotherapy and a control group that simply chemotherapy. Ideal patients for DC-CIK therapy were those who satisfied the following criteria: 18 years and older, 2) pathologically confirmed breast cancer, 3) with hormone receptor negative, 4) with symptomatic visceral metastatic, 5) with hormone receptor positive but insensitive to endocrine therapy, 6) having completed chemotherapy for at least 1 month, 7) the Karnofsky performance status score was >70, and 8) signing the consent form.

Written informed consent was obtained from each participant in accordance with the Declaration of Helsinki. Tumours were staged according to the Union for International Cancer Control (UICC) classification based on pTNM subsets

2.3 Antigen preparation.

Tumor antigen was prepared by following the established protocol [28] from patient 's tumor biopsy tissue. Tumour cells were cultured for two to three passages ($1–2\times10^8$), then collected and washed three times with normal saline, before being lysed by freezing and thawing three times, and analysed with ultrasonic cell disruption. Lysates from the tumour cells were then fractionated by centrifugation (1200 rpm for 5 minutes), and the supernatant was collected and filtered with a 0.22 μm filter (Carrighwohill, Co. Cork, Ireland), with the protein concentration in the supernatant being measured before storage at −80°C.

2.4 Preparation of DC and CIK cells.

For DC culture, peripheral blood mononuclear cells from samples drawn from the patients in the treatment group were separated and resuspended in DC medium, which consisted of X-VIVO 15 (Lonza, Basel, Switzerland), 25 ng/ml recombinant human interleukin [rhIL]-4 (Peprotech), and 30 ng/ml rhGM-CSF (Peprotech), at a concentration of between 1×10^6/ml and 2×10^6/ml. The prepared tumour antigen combined with above-mentioned DC medium was added to the cultured cells to give a final concentration of 50 mg/ml, and the cells were cultured for 7 days. The presence of mature DCs was assessed using the DC marker CD83 and cell morphology, with the percentage of CD83+ cells being measured by flow cytometry every day and the morphological cell type being observed using an inverted optical microscope on days 5 and 7.

To culture CIK cells, the non-adherent cells from the DC culture were suspended in CIK medium, which consisted of X-VIVO 15 (Lonza), 1000 U/ml rhIL-2 (Peprotech), 2.5 μg/ml monoclonal antibody to CD3 (OKT-3; Jansen-Kyowa, Tokyo, Japan), 25μg/ml phytohemagglutinin (Peprotech), and 1000 U/ml recombinant human IFN-γ (Peprotech). The CIK cells were allowed to grow and then were continuously passaged. After approximately 7 days of culture, the CIK cells were passaged to fourteen T225 flasks. Cells adhering to the flasks were removed with a cell spatula, centrifuged, and

resuspended in DC-CIK medium, consisting of X-VIVO 15 (Lonza), 400 U/ml IL-2, and 0.5μg/ml monoclonal antibody to CD3. The DCs were distributed evenly in the fourteen T225 flasks containing CIK cells (approximately 10^8 DCs per flask).

After co-culture for 48 hours, the DC/CIKs were harvested and suspended in 100 ml saline for intravenous injection (cells were collected on four consecutive days, with 6×10^9 to 10×10^9 cells being collected each day). Flow cytometric analysis showed that the final cell products included 4% ± 2% CD19+ cells, 87% ± 8% CD3+ cells, and 9% ± 4% CD16+CD56+ cells (NK cells). The final cell products were assessed for viability by the dye-exclusion test and checked twice for possible contamination by bacteria, fungi, and endotoxins.

2.5 DC vaccine and CIK cell administration.

Patients in the treatment group received one cycle of low-dose chemotherapy with Carmofure (100 mg, po, bid) for 5 or 6 days. The infusion of DCs and CIK cells was started at 7-day intervals after chemotherapy. DC cells were mixed with CIK cells in 250 ml normal saline containing 1500 U/ml IL-2 and 1% human albumin, and were infused into the patients intravenously. The treatment was repeated four times in a fortnight, which formed one cycle. All patients received at least three cycles of the treatment. The time schedules of DC and CIK preparation and infusion are shown in Figure 1.

Immune function detection. The immune responses of patients were monitored by measuring serum levels determined by flow cytometry (FACSCanto™ II; BD, Franklin Lakes, NJ, USA). We examined serum levels of cytokines for each patient in treatment group at three time points: 1 day before chemotherapy, 1st day after chemotherapy and 1st day after DC-CIK therapy.Peripheral blood (2 ml) was used for detecting non-specific immunity by flow cytometry (FACSCanto™ II). Multitest six-color TBNK Reagent (no. 644611; BD) was used to detect the number of cells in the various lymphocyte subsets; Cytometric Bead Array (CBA) Human Th1/Th2 Cytokine Kit II (no. 551809; BD) was used to detect cytokine expression levels. All steps were performed in accordance with the corresponding instructions.

2.6 Outcomes.

After cell-based immunotherapy in the treatment group and after chemotherapy in the control group, all patients continued to be followed up with hospital visits and/or

telephone interviews at least once every 6 months. Local tumour recurrence and distant metastasis were examined by imaging. Disease-free survival (DFS; the time interval between surgery and tumour recurrence) and OS (the time between surgery and last follow-up) were collected in the project research database. Complications were recorded and classified in accordance with the Common Terminology Criteria of Adverse Events v4.0.

2.7 Statistical analysis.

Data are presented as a percentage, mean with standard deviation (mean ± SD), or median with 95% confidence interval (95% CI). Treatment outcomes were analysed by Kaplan–Meier survival curves with log rank test, and multivariate Cox proportional hazards regression tests. Statistical significance was set at $p<0.05$. All analyses were conducted using GraphPad software (San Diego, CA, USA).

3. Results

3.1 The study design and patients characteristic.

A total of 368 patients with histologically confirmed stage Ⅳ breast adenocarcinoma were recruited into this study between August 2003 and December 2013, and between the treatment and control groups. The demographic and clinicopathological data of the patients in the treatment and control groups are presented in Table 1. All of the patients in both groups had received chemotherapy before enrolment. At least three cycles of immunotherapy were initiated for patients in the treatment group after chemotherapy had been completed. The follow-up time was up to 10 years for all patients in both the treatment and control groups.

3.2 *DC and CIK cells culture and identification*.

The cell culture was examined for the presence of mature DCs by cell morphology (Figure 2A, B, C) and by flow cytometry for CD83. DCs prepared from patients were 87% for CD83+ on day 7 and the percentage was rising day by day (Figure 2D). CIK cells prepared from patients showed 79%–82% for CD3+CD8+ cells, 11%–13% for CD3+CD4+ cells, 3%–5% for CD16+CD56+ cells, and 2%–4% for CD19+ cells over the 7 days of culture (Figure 3). These results indicate that the majority of the DCs and CIK cells prepared from patients were mature and functional.

3.3 Immune function detection.

Lymphocyte counts and function were compared before chemotherapy, after chemotherapy and after DC-CIK therapy. In terms of lymphocyte count, all subsets were significantly lower after chemotherapy than before chemotherapy, but they became higher again after DC-CIK immunotherapy (Figure 4A, * $P < 0.05$; ** $P < 0.01$); the same to lymphocyte function, Th1-type cytokines (IL-2, TNF-β, and IFN-γ) were decreased after chemotherapy but they became higher again post DC-CIK therapy (Figure 4B, * $P < 0.05$; ** $P < 0.01$).

3.4 OS and DFS.

As shown in Figure 5, DFS and OS were both significantly prolonged in patients in the DC-CIK treatment group (5-year DFS 42%, OS 44%) compared with the patients in control groups (30% and 29%, respectively, $p<0.01$). In addition, analysis with multivariate Cox proportional regression confirmed that DC-CIK therapy significantly and independently reduced the risk of post-operative disease progression (odds ratio [OR] 0.09, 95% CI 0.02–0.42, $p<0.01$) and patient death (OR 0.05, 95% CI 0.01–0.37, $p<0.01$) after adjusting for age, tumour grade, TNM stages, and previous chemotherapy treatments.

3.5 Adverse events.

Throughout the study, all adverse events experienced by the patients were recorded and included local (mainly injection site reaction or localized edema) and systemic (mainly chills, fatigue, or fever) reactions (Table 2). Other possible side effects such as blood or bone marrow changes were not detected. After symptomatic treatment, all symptoms were relieved within the day and did not reappear. Among the patients, the most common reaction was fever (34.6 %); chill was the least common reaction (2.1 %).

4. Discussion

Cancer immunotherapy has shown potential efficacy in tumour growth control and patient survival [12,29,30] with news articles stating ‘Instead of using surgery, chemotherapy, or radiotherapy, researchers from the National Institutes of Health are finding so far limited but inspiring success in a new approach for fighting cancer, using the immune system to attack the tumors the way it would be a cold or flu. Although extensively studied in cells and animal models, the clinical data regarding the exact

benefit of immunotherapy in terms of patient survival and disease progression require further investigation[15]. Despite the limited size of the cohorts, this study demonstrates a remarkable enhancement in post-surgical control of tumour recurrence and survival rates in MBC patients treated with combined DC-CIK therapy.

It has been well established that DCs prime naïve T cells and DC vaccine combined with CIK cell therapy has achieved encouraging results as a novel therapeutic approach for disease control in specific cancers [31]. DCs [7,32] have the capability to present tumour antigens to T lymphocytes and induce the specific cytotoxic T cells against those tumour antigens [33]. Sipuleucel-T, the first DC vaccine, was approved for clinical application by the FDA in the USA to treat asymptomatic metastatic castrate-resistant prostate cancer, having improved patients' OS in a phase III trial [12]. Additional promising results were reported in recent phase III trials using tumour vaccine to treat various late-stage cancers [34], including melanoma, follicular lymphoma, colorectal cancer, and non-small-cell lung cancer. Clinical studies have established that DCs capture and process tumour-associated antigens and secrete cytokines to initiate an immune response.

As well as DC vaccines, CIKs are also induced by cytokines and possess non-specific cytotoxicity against tumours. CIK cells can kill tumour cells directly, but have very short-term anticancer efficacy and are less likely to control tumour growth in the long-term. In contrast, DC vaccines have been shown to induce tumour-specific effector and memory T cells. Therefore, the combination of DC vaccination with CIK treatment may potentially have higher cytotoxic activity and specificity in the effector T cells, which show both short-term and long-term anti-tumour efficacy. In agreement with the clinical benefits observed in other clinical trials[34], our study suggests that DC-CIK treatment can significantly enhance patient survival, and supports its further clinical investigation.

Although a number of pro-inflammatory cytokines were elevated in the DC-CIK-treated patients, only three Th1 cytokines (IL-2, TNF-β, and IFN-γ) showed a significant increase in patients' sera in our study. Other studies have demonstrated that IL-12 also plays a critical role in immunotherapy using DC and CIK [23,27]. Tumour cells are highly heterogeneous [35] and a specific tumour may contain cells with both high and low major histocompatibility complex class I (MHC-I) populations

[36,37] .Interestingly, MHC-I expression appears heterogeneous among tumour cells and radiation promotes the immunological recognition of the tumour cells by immune cells via MHC-I. However, probably because of the heterogeneity in a given tumour, a single type of immune therapy may only be effective in a subpopulation of cancer patients. Similarly, tumour cells with higher MHC-I expression may be more sensitive to DC vaccine therapy, while cells with lower MHC-I expression may be killed by CIK cells. Our results suggest that combined DC-CIK therapy may promote tumour cell cytotoxicity by targeting different populations of tumour cells, such as those with various levels of MHC-I.

In addition, patients also received one cycle of low-dose chemotherapy prior to the DC and CIK infusion, which may also affect the overall effectiveness of DC-CIK therapy. We hypothesised that a 7-day gap is critical for sufficient 'washout' of chemotherapeutic agent, so as not to affect the effectiveness of the infused DCs and CIK cells. At the same time, chemotherapy can severely reduce the number of lymphocytes and the level of cytokines, although DC-CIK infusion clearly appears to improve immune function (Figure 3). Patients received a varying number of cycles of chemotherapy depending on their condition, but all received at least three cycles. The potential effects of low-dose chemotherapy before cell infusion [23] may also induce tumour cell killing, up-regulate the expression of tumour antigen, and contribute to the composition of host immune cells. In conclusion, this research indicates that the combination of DC vaccine and CIK therapy can significantly improve DFS and OS in patients with stage Ⅳ breast cancer who only rely on chemotherapy. There is no doubt that DC-CIK is a boon to stage Ⅳ breast cancer patients.

Conflict of interest

The authors declare that there are no conflicts of interest

Acknowledgments

We would like to thank the native English speaking scientists of Elixigen Company for editing our manuscript. This research is supported by International Scientific Fund of Fuda Cancer Hospital, Guangzhou (No. Y2016-ZD-006).

References

[1] Rabbani SA, Mazar AP. Evaluating distant metastases in breast cancer: from biology to outcomes. Cancer metastasis reviews 2007;26:663-74.

[2] Valastyan S, Weinberg RA. Tumor metastasis: molecular insights and evolving paradigms. Cell 2011;147:275-92.

[3] Andre F, Slimane K, Bachelot T, Dunant A, Namer M, Barrelier A, et al. Breast cancer with synchronous metastases: trends in survival during a 14-year period. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 2004;22:3302-8.

[4] Chia SK, Speers CH, D'Yachkova Y, Kang A, Malfair-Taylor S, Barnett J, et al. The impact of new chemotherapeutic and hormone agents on survival in a population-based cohort of women with metastatic breast cancer. Cancer 2007;110:973-9.

[5] Gennari A, Conte P, Rosso R, Orlandini C, Bruzzi P. Survival of metastatic breast carcinoma patients over a 20-year period: a retrospective analysis based on individual patient data from six consecutive studies. Cancer 2005;104:1742-50.

[6] Frankenberger B, Schendel DJ. Third generation dendritic cell vaccines for tumor immunotherapy. European journal of cell biology 2012;91:53-8.

[7] Melief CJ. Cancer: immune pact with the enemy. Nature 2007;450:803-4.

[8] Timmerman JM, Czerwinski DK, Davis TA, Hsu FJ, Benike C, Hao ZM, et al. Idiotype-pulsed dendritic cell vaccination for B-cell lymphoma: clinical and immune responses in 35 patients. Blood 2002;99:1517-26.

[9] Nestle FO, Alijagic S, Gilliet M, Sun Y, Grabbe S, Dummer R, et al. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nature medicine 1998;4:328-32.

[10] Fong L, Hou Y, Rivas A, Benike C, Yuen A, Fisher GA, et al. Altered peptide ligand vaccination with Flt3 ligand expanded dendritic cells for tumor immunotherapy. Proceedings of the National Academy of Sciences of the United States of America 2001;98:8809-14.

[11] Higano CS, Schellhammer PF, Small EJ, Burch PA, Nemunaitis J, Yuh L, et al. Integrated data from 2 randomized, double-blind, placebo-controlled, phase 3 trials of active cellular immunotherapy with sipuleucel-T in advanced prostate cancer. Cancer 2009;115:3670-9.

[12] Nabhan C. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. The New England journal of medicine 2010;363:1966-7; author reply 8.

[13] Kantoff PW, Higano CS, Small EJ, Whitmore JB, Frohlich MW, Schellhammer PF. Re: interdisciplinary critique of sipuleucel-T as immunotherapy in castration-resistant prostate cancer. Journal of the National Cancer Institute 2012;104:1107-9; author reply 9-12.

[14] Huber ML, Haynes L, Parker C, Iversen P. Interdisciplinary critique of sipuleucel-T as immunotherapy in castration-resistant prostate cancer. Journal of the National Cancer

Institute 2012;104:273-9.

[15] Frohlich MW. Sipuleucel-T for the treatment of advanced prostate cancer. Seminars in oncology 2012;39:245-52.

[16] Gutgemann S, Frank S, Strehl J, Schmidt-Wolf IG. Cytokine-induced killer cells are type II natural killer T cells. German medical science : GMS e-journal 2007;5:Doc07.

[17] Lu PH, Negrin RS. A novel population of expanded human CD3+CD56+ cells derived from T cells with potent in vivo antitumor activity in mice with severe combined immunodeficiency. J Immunol 1994;153:1687-96.

[18] Margolin KA, Negrin RS, Wong KK, Chatterjee S, Wright C, Forman SJ. Cellular immunotherapy and autologous transplantation for hematologic malignancy. Immunological reviews 1997;157:231-40.

[19] Linn YC, Hui KM. Cytokine-induced killer cells: NK-like T cells with cytotolytic specificity against leukemia. Leukemia & lymphoma 2003;44:1457-62.

[20] 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.

[21] Gonzalez-Carmona MA, Marten A, Hoffmann P, Schneider C, Sievers E, Schmidt-Wolf IG, et al. Patient-derived dendritic cells transduced with an a-fetoprotein-encoding adenovirus and co-cultured with autologous cytokine-induced lymphocytes induce a specific and strong immune response against hepatocellular carcinoma cells. Liver international : official journal of the International Association for the Study of the Liver 2006;26:369-79.

[22] Marten A, Greten T, Ziske C, Renoth S, Schottker B, Buttgereit P, et al. Generation of activated and antigen-specific T cells with cytotoxic activity after co-culture with dendritic cells. Cancer immunology, immunotherapy : CII 2002;51:25-32.

[23] Marten A, Ziske C, Schottker B, Weineck S, Renoth S, Buttgereit P, et al. Transfection of dendritic cells (DCs) with the CIITA gene: increase in immunostimulatory activity of DCs. Cancer gene therapy 2001;8:211-9.

[24] Su X, Zhang L, Jin L, Ye J, Guan Z, Chen R. Coculturing dendritic cells with zoledronate acid efficiently enhance the anti-tumor effects of cytokine-induced killer cells. Journal of clinical immunology 2010;30:766-74.

[25] Sun Y, Chen J, Cai P, Hu YH, Zhong GC, Feng HZ, et al. [Therapy of relapsed or refractory non-Hodgkin's lymphoma by antigen specific dendritic cells-activated lymphocytes]. Zhongguo shi yan xue ye xue za zhi / Zhongguo bing li sheng li xue hui = Journal of experimental hematology / Chinese Association of Pathophysiology 2010;18:219-23.

[26] Leemhuis T, Wells S, Scheffold C, Edinger M, Negrin RS. A phase I trial of autologous cytokine-induced killer cells for the treatment of relapsed Hodgkin disease and non-Hodgkin lymphoma. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation 2005;11:181-7.

[27] Li H, Wang C, Yu J, Cao S, Wei F, Zhang W, et al. Dendritic cell-activated cytokine-induced killer cells enhance the anti-tumor effect of chemotherapy on non-small cell lung cancer in patients after surgery. Cytotherapy 2009;11:1076-83.

[28] Pan Y, Zhang J, Zhou L, Zuo J, Zeng Y. In vitro anti-tumor immune response induced by dendritic cells transfected with EBV-LMP2 recombinant adenovirus. Biochemical and biophysical research communications 2006;347:551-7.

[29] Morse MA, Deng Y, Coleman D, Hull S, Kitrell-Fisher E, Nair S, et al. A Phase I study of active immunotherapy with carcinoembryonic antigen peptide (CAP-1)-pulsed, autologous human cultured dendritic cells in patients with metastatic malignancies expressing carcinoembryonic antigen. Clinical cancer research : an official journal of the American Association for Cancer Research 1999;5:1331-8.

[30] O'Neill D, Bhardwaj N. Exploiting dendritic cells for active immunotherapy of cancer and chronic infection. Methods in molecular medicine 2005;109:1-18.

[31] O'Neill DW, Bhardwaj N. Exploiting dendritic cells for active immunotherapy of cancer and chronic infections. Molecular biotechnology 2007;36:131-41.

[32] Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998;392:245-52.

[33] van Broekhoven CL, Parish CR, Demangel C, Britton WJ, Altin JG. Targeting dendritic cells with antigen-containing liposomes: a highly effective procedure for induction of antitumor immunity and for tumor immunotherapy. Cancer research 2004;64:4357-65.

[34] Schlom J. Recent advances in therapeutic cancer vaccines. Cancer biotherapy & radiopharmaceuticals 2012;27:2-5.

[35] Duru N, Fan M, Candas D, Menaa C, Liu HC, Nantajit D, et al. HER2-associated radioresistance of breast cancer stem cells isolated from HER2-negative breast cancer cells. Clinical cancer research : an official journal of the American Association for Cancer Research 2012;18:6634-47.

[36] Li CD, Zhang WY, Li HL, Jiang XX, Zhang Y, Tang PH, et al. Mesenchymal stem cells derived from human placenta suppress allogeneic umbilical cord blood lymphocyte proliferation. Cell research 2005;15:539-47.

[37] Beutler N, Hauka S, Niepel A, Kowalewski DJ, Uhlmann J, Ghanem E, et al. A natural tapasin isoform lacking exon 3 modifies peptide loading complex function. European journal of immunology 2013;43:1459-69.

Figure Legends

Figure 1. **The study treatment schedule.** The timing of the preparation and infusion of the dendritic cell (DC) and cytokine-induced killer (CIK) cell preparation in the treatment group, while Patients in the control group received chemotherapy then proceeded straight to the period of follow-up.

Figure 2. DC identification. The maturity of dendritic cells was determined by assessing the cell morphology and measuring the percentage of expressing CD83+ cells in blood samples collected from treated breast cancer patients. (A) Monocytes from human peripheral blood observed by invert optical microscope (×100); (B) Dendritic cells (DCs) cultured on the 5th day, observed by invert optical microscope (×100); (C) DCs cultured on the 7th day, observed by scanning electron microscope (×2000); (D) The percentage of expressing CD83+ cells on each day.

Figure 3. CIK identification. The function of CIK cells was determined by measuring the percentage of expressing CD3+CD8+, CD3+CD4+, CD16+CD56+, and CD19+ cells in blood samples collected each day from treated breast cancer patients.

Figure 4. Comparison of lymphocyte function before and after treatment. (A) Numbers of the different lymphocytes types measured by flow cytometry. (B) Serum levels of the Th1-type cytokines (IL-2, TNF-β, and IFN-γ) and the Th2-type cytokines (IL-4, IL-6, and IL-10) in peripheral blood. IL, interleukin; TNF, tumour necrosis factor; IFN, interferon.* $P < 0.05$; ** $P < 0.01$.

Figure 5. Comparison of survival rates at 1, 3, and 5 years (marked by the red boxes) for breast cancer patients in the treatment and control groups. (A) The overall survival rates were 93%, 65%, and 44%, respectively, for the treatment group compared with 79%, 46%,and 29% for the control group. (B) The disease-free survival rates were 86%, 66%, and 42%, respectively, for the treatment group compared with 79%, 51%, and 30% for the control group. The cumulative survival curves in A and B were analysed by the Kaplan–Meier method.

Tables

Table 1 Baseline characteristics

Patient characteristics before enrollment	Total, n	Treatment group	Control group	P-value
Median age, years	60	58	64	P>0.05
Karnofsky performance status				
70	172	94	78	P>0.05
80	161	78	83	P>0.05
90	35	13	22	P>0.05
Lymph node metastasis	368	188	180	P>0.05
Tumor free(Ⅳ)	368	188	180	P>0.05
ER-/PR-	336	164	172	P>0.05
Chemotherapy	368	188	180	P>0.05
Surgery	0	0	0	P>0.05

***Baseline characteristics were well balanced between the two groups.** Factors of treatment group and control group were compared by two-way analysis of variance. ER-, no estrogen receptor expression; PR-, no progesterone receptor expression. All patients were recruited as control and treatment groups following the protocol approved by Ethics Committee at Fuda Cancer Hospital Informed Consent.

Table 2 Adverse events

Symptoms	Patients number (n=188)	Percentage (%)
Local		
Injection site reaction	52	27.7
Localized edema	41	21.8
Systemic		
Fever	65	34.6
Fatigue	16	8.5
Chill	4	2.1