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*Original Article*

# Autologous natural killer cells have a therapeutic effect in advanced prostate cancer patients and can mediate cytotoxicity in vitro

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**Abstract:** Purpose: To evaluate the efficacy and safety of autologous natural killer (NK) cells therapy for patients with advanced prostate cancer (PCA). Methods: Eight patients with advanced PCA were enrolled in this study and received infusions of autologous activated NK cells. Four patients received three infusions (total dose $15\times10^9$ cells), and the other four patients received four infusions (total dose $20\times10^9$ cells). Clinical and immunologic outcomes were evaluated after infusion compared with those before treatment. Results: Ex vivo-expanded NK cells could mediate cytotoxicity, especially when the effector: target (E:T) ratio increased from 5:1 to 40:1, using unsorted cells as effectors. At the E:T ratio of 10:1, the sorted cells exhibited highest cytotoxicity, 78% for PC-3 and 82% for K562. Moreover, reduction of PSA was observed in five out of eight patients, and no adverse events and side effects were found after treatment. In terms of immunoreaction, seven out of eight patients indicated an increase of $CD3^-CD56^+$ NK cells and six patients presented rising proportion of peripheral blood $CD8^+$ T cells. Conclusions: Immunotherapy of ex vivo-expanded autologous NK cells is well tolerated, and it can be carried out safely without obvious adverse events or side effects in the treatment of advanced PCA.

**Keywords:** Cellular immunotherapy, prostate cancer, natural killer cell

## Introduction

The incidence of PCA ranks the second place of male malignant tumors all over the world [1]. There are approximately 32,050 patients dying of this disease in the USA in 2010 [2]. The incidence of PCA in China is rising rapidly in the past decade, it has become one of the most common cancer in elderly men in recent years [3]. Despite some different therapies have provided "weapons" for us in the treatment of early prostate cancer including surgical resection, endocrine therapy, chemotherapy or radiation therapy, there is no effective treatment for advanced PCA, especially if it is hormone-refractory [4]. Therefore, more basic and clinical in-depth researches are an indispensable part of advanced PCA treatment, among which cell therapy using immune cells with antitumor activity is a promising candidate.

NK cells are one of the major lymphocyte subsets and the first-line troops of immune system that can recognize and kill virally infected or malignant cells. In addition to direct cytotoxicity, NK cells secrete cytokines which can also contribute to innate and adaptive immune responses [5, 6] and is involved in cancer immunotherapies [7]. Previous studies demonstrate that NK cells play a critical role in the treatment of some diseases such as acute myeloid leukemia (AML) [8, 9] and viral infections [10-12]. Studies also show that adoptively transferred expanded NK cells can suppress the growth of solid malignant tumors and increased the lymph node homing in vivo [13, 14], and autologous and allogeneic activated NK cells can attenuate chemotherapy-resistant of certain cancer cell lines [15, 16]. Based on the above researches, we found that NK cells have strong killing activity against tumor cells. However, the anti-tumor activity of the expansion NK cells in advanced PCA and NK cells-mediated cytotoxicity in PCA cell lines had been less reported.

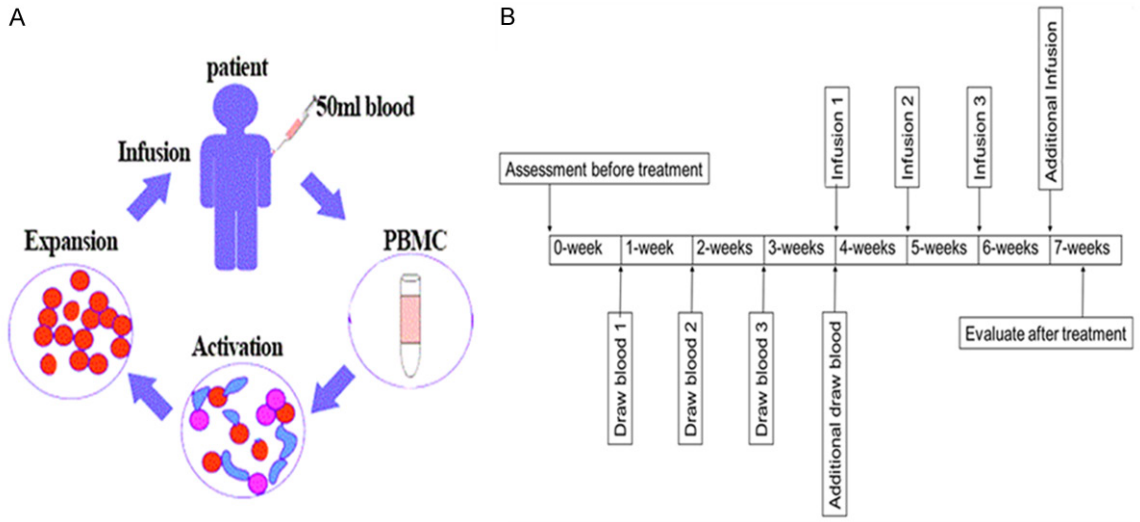


**Figure 1.** Therapeutic strategy and schedule of NK cells. Laboratory procedures of ex vivo-expanded NK cells (A). Eight patients were enrolled in this clinical study, and four of them received three infusions of autologous expanded NK cells, the other patients received four infusions (B). All patients finished this trail in two months.

**Table 1.** Characteristics of advanced prostate cancer patients

| Patient | Age (years) | Prior therapy | Gleason | Total NK cell dose ($10^9$) | Change of PSA |
|---|---|---|---|---|---|
| 1 | 68 | EN, CH, RA | 9 | 15 | Upregulated |
| 2 | 77 | EN, CH, RA | 8 | 15 | Downregulated |
| 3 | 70 | EN, CH | 9 | 20 | Unable to evaluate |
| 4 | 73 | SU, EN, CH, RA | 9 | 15 | Upregulated |
| 5 | 66 | EN, CH | 7 | 15 | Downregulated |
| 6 | 66 | SU, EN, CH, RA | 7 | 20 | Downregulated |
| 7 | 54 | EN, CH | 7 | 20 | Downregulated |
| 8 | 73 | SU, EN, CH, RA | 8 | 20 | Downregulated |

SU, surgical therapy; EN, endocrine therapy; CH, chemotherapy; RA, radiation therapy; PSA, prostate specific antigen.

In the present study, peripheral blood mononuclear cells (PBMCs) from advanced PCA patients were obtained to stimulate the selective expansion of human NK cells, and autologous expanded NK cells were injected to observe the anti-tumor activity in advanced PCA patients, and NK cells-mediated cytotoxicity in K562 and PC-3 cell lines was investigated.

## Patients and methods

### *Patients*

All patients signed informed consent before enrollment. Inclusion criteria: advanced PCA patients aged between 50 and 80 years, with histologic or cytologic diagnosis. Patients did not have other vital organ dysfunction and no history of endocrine therapy, chemotherapy or radiotherapy within four weeks. Eight patients with advanced PCA were enrolled in this study from Jan 2013 to Jun 2014.

### *Study design*

The protocol was approved by the Medical Ethics Committee of the Second Affiliated Hospital of Soochow University in 2012. Autologous NK cells were obtained from the patients themselves. All patients received three to four infusions of autologous activated NK cells (total dose 15-20×$10^9$ cells). The NK cells therapy was schematically presented in **Figure 1**. Adverse effects were recorded in accordance with the National Cancer Institute Common Toxicity criteria Version 2.0.

### *Autologous NK cells preparation*

PBMCs of each patient were obtained by Ficoll density gradient (1.077 ± 0.002 g/ml, Jinmei, China) and washed twice with PBS, and resuspended at 1×$10^6$ cells/ml in Takara GT-T551 medium. For the first 5 days, the medium was supplemented with NK cell expansion kit (sup-

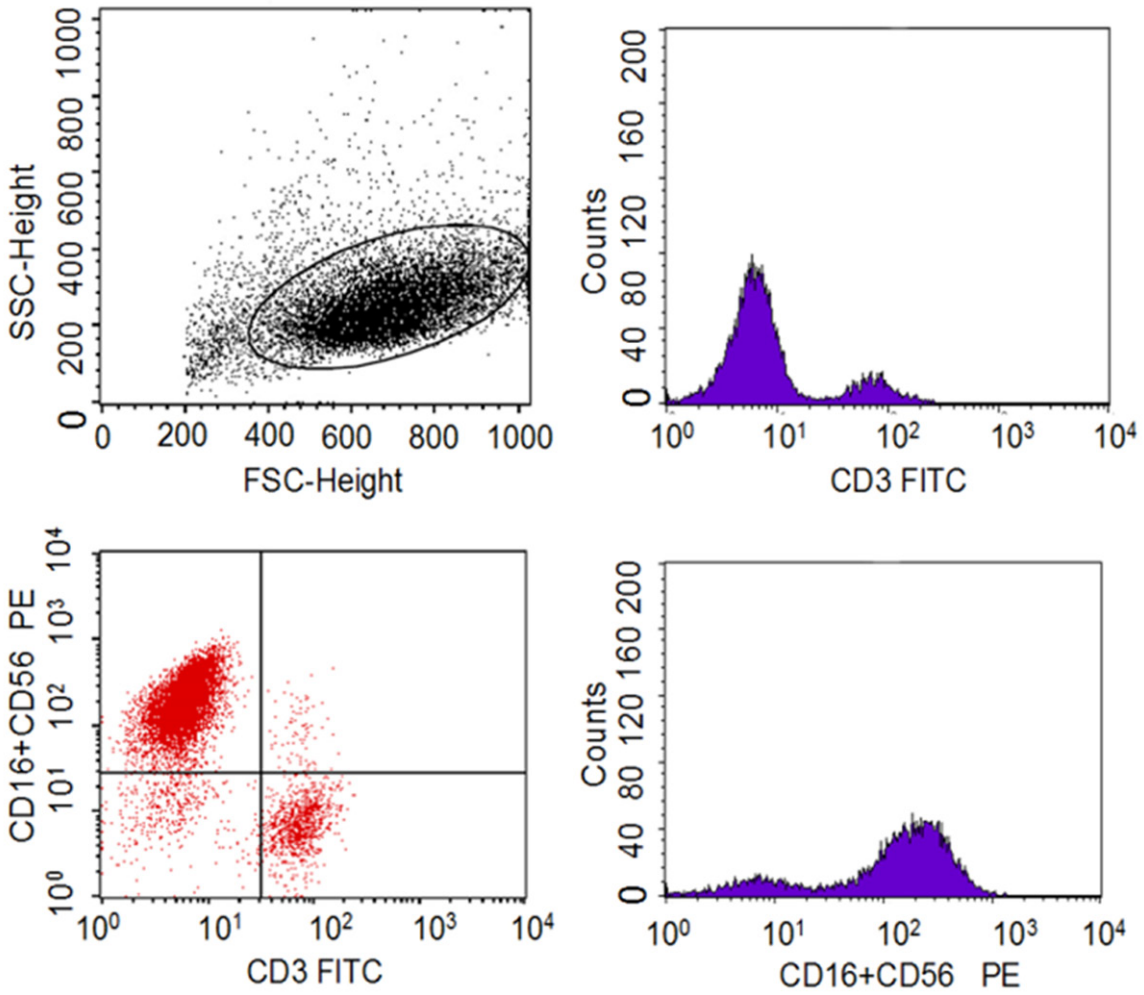


**Figure 2.** Phenotype of NK cells after 21 days of culture. PBMC isolated from 50 mL blood range from $1\times10^7$ to $5\times10^7$ cells. After activation and expansion of NK cells, the final cell population contained about 80% of $CD3^-CD56^+$ NK cells.

plied by Hope Bio-technology Limited Company). On day 5, the medium was replaced by fresh medium with IL-2 (500 U/mL) and 5% human serum. Cells were incubated at 37°C in a humidified atmosphere of 5% $CO_2$. The medium was changed every three days. The NK cell purity and cytotoxicity were tested following activation and expansion to ensure the quality and capability of cells.

*Flow cytometry method*

On day 21, NK cell densities were determined with a hemocytometer, the phenotypes of the cells from each patient were identified by flow cytometry using $CD3^+$FITC/$CD16^+$56PE antibodies (BD science, USA). A total of $1\times10^6$ cells were washed and incubated with 20 μL of monoclonal antibody for 20 min at 4°C. After incubation, the cells were washed twice and resuspended in 0.5 ml of assay buffer. Mouse immunoglobulin isotypes IgG1-FITC and IgG1-PE were used as negative control. The fluorescence was analyzed by a FACSCalibur flow cytometer and CellQuest software was used for data acquisition (BD Biosciences, Mountain View, CA, USA).

*Tumor killing in vitro*

The cytotoxic activity of NK cells was determined by using Calcein-AM (Sigma, Cat#17783) according to the manufacturer's instructions. Briefly, CAM media was prepared by diluting Calcein-AM stock solution (1 mg/mL in DMSO) with medium. Prewashed K562 and PC-3 cells

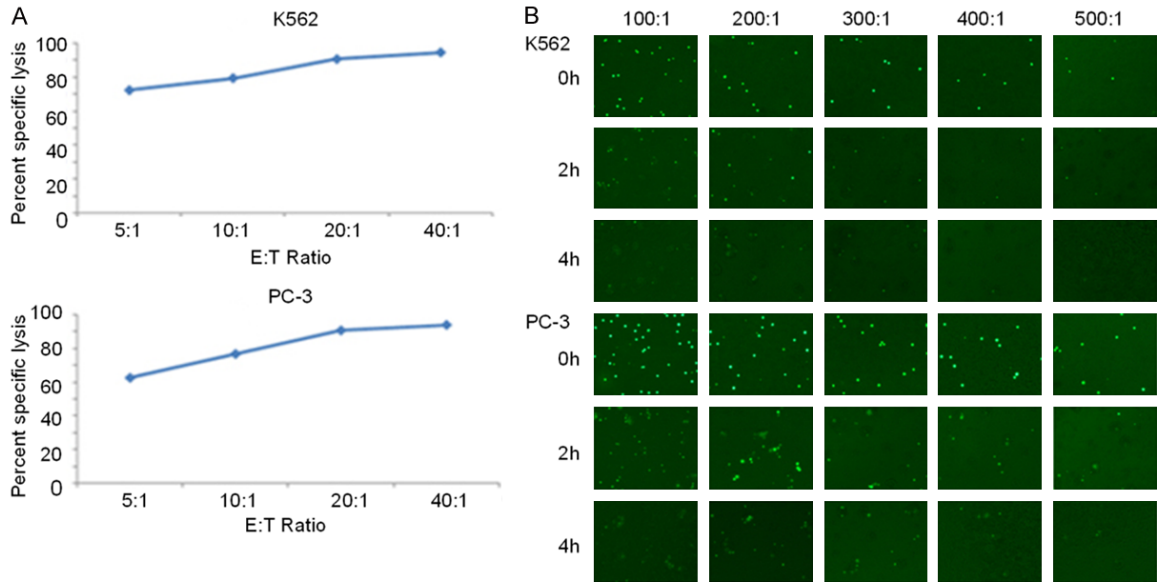


**Figure 3.** Expanded NK cells had demonstrated cytotoxicity against K562 and PC-3 cell lines. Cultured NK cells were used as effector cells in a cytotoxicity assay against the K562 and PC-3 cell line at effector: target (E:T) ratio of 5:1, 10:1, 20:1 and 40:1 (A). Expanded NK cells cytotoxicity assay was examined using Calcein-AM. The assay used Calcein-AM, a nofluorescent esterase substrate, which inside living cells with bright-green fluorescence at excitation/emission wavelengths of 495/515 nm (B).

were resuspended in CAM media ($10^6$ cells/mL) and incubated at 37°C for 1 h with occasional shaking. NK cells were resuspended at $1\times10^6$ cells/mL and 200 μL of NK cells was added into each well of K562 or PC-3 cells in a U-bottom 96-well plate. The effector: target (E:T) ratio ranged from 5:1 to 100:1. Each run of cytotoxicity assay used $6\times10^5$ NK cells and $3\times10^5$ cells of K562 or PC-3.

*Autologous NK cells infusion*

In this study, we assessed clinical and laboratory condition of patients. A volume of 50 ml blood once every other week for three or four times during the period of treatment. After expansion *in vitro*, NK cells were infused into the corresponding patient. Then the responses of the patients were evaluated at three days after the final infusion of NK cells.

*Post-infusion monitoring*

The clinical manifestation and immunological response of all patients were evaluated at the end of autologous NK cell infusions. The chest x-ray and bone scan, computed tomography (CT) or magnetic resonance imaging (MRI) were carried out on each patient. Meanwhile, serum levels of PSA, lymphocyte and cytokines were detected. The efficacies such as complete remission, partial response, stable disease and progressive disease were recorded following the Immune-Related Response Criteria 9 (irRC) [17].

## Results

*The characteristics of advanced prostate cancer patients*

All PCA patients had multiple sites bone metastases without treatment before NK cell therapy. The characteristics of eight advanced PCA patients (aged from 54 to 77 years) in this study were summarized in **Table 1**.

*Ex vivo-expanded NK cells and NK-mediate cytotoxicity*

The NK cells isolated from 50 ml peripheral blood reached $5\times10^9$ cells, more than 250-folds to the initial number after 21 days expansion. The final cell population contained about 80% of $CD3^-CD56^+$ NK cells (**Figure 2**).

Using large scale culture system, we could obtain more than $5\times10^9$~$1\times10^{10}$ cells, with the purity of over 90%. As illustrated in **Figure 3A**, the cytotoxicity increased when the E:T ratio

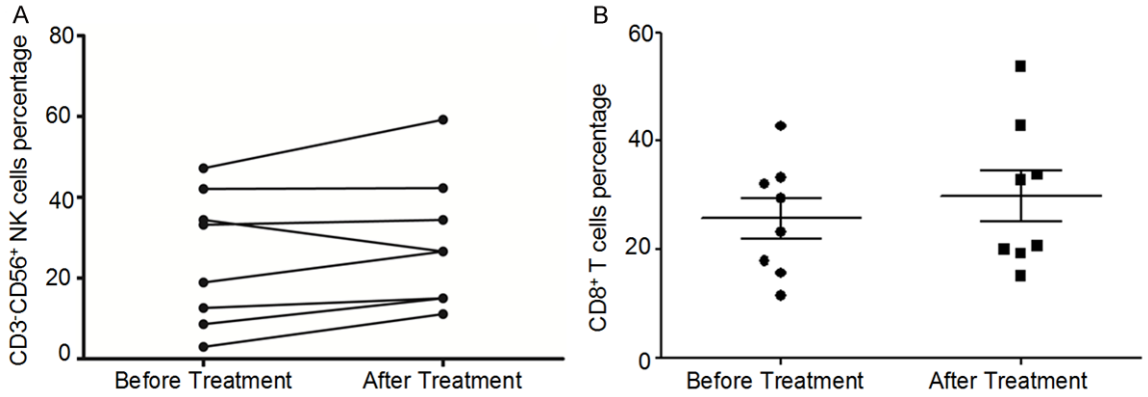


**Figure 4.** The changes of $CD3^-CD56^+$ NK cells and $CD8^+$ T cells before and after treatment. The frequency of peripheral blood $CD3^-CD56^+$ NK cells in all patients was measured by flow cytometry analysis (A). The frequency of peripheral blood $CD8^+$ T cells in all patients was measured by flow cytometry analysis (B).

increased from 5:1 to 40:1, using unsorted cells as effectors. At the E:T ratio of 10:1, the sorted cells exhibited highest cytotoxicity, 78% for PC-3 and 82% for K562. As shown in **Figure 3B**, we observed obvious cell killing activation at an E:T ratio of 100:1 after coculture with K562 and NK cells for 4 h. In addition, we observed the same phenomenon in PC-3 cell line.

*Clinical outcomes and immunologic monitoring*

Ostealgia subsided in different degrees after NK cells infusions. Five out of eight patients indicated a PSA decline, among them three patients received four infusions. The examination of chest X-ray, bone scan, CT or MRI was evaluated. All patients reached "stable disease" according to irRC.

Immunological indexes were detected for eight patients who completed the study. Seven out of eight patients indicated an increase of $CD3^-CD56^+$ NK cells, while one patient (case 1) showed decreased $CD3^-CD56^+$ NK cells (**Figure 4A**). The proportion of peripheral blood $CD8^+$ T cells increased in six patients, except for two patients (case 3, 4) (**Figure 4B**). Cytokines varied from patient to patient. It is interesting to note that there were no obvious increase in the expression of peripheral blood IL2, IL12, IFN and TNF in all patients (data not shown).

*Adverse events*

All infusions were tolerable for patients. No infusion-associated adverse events or side effects were observed. Patients' vital organs function did not change during and post therapy. No anomalous changes were identified in any patient with blood cell counts and hemoglobin levels.

## Discussion

PCA is one of the most common malignant tumors in men. For advanced PCA, androgen deprivation therapy (ADT) is usually effective [18, 19], but most of patients would develop resistance to ADT therapy within two years. If PCA is not cured at early stage, it will evolve from androgen-dependent to castration-resistant prostate cancer (CRPC) [20]. So far, immunotherapy has a great development, for example, Sipuleucel-T has been allowed immunotherapy for CRPC [21]. Emerging data strongly implicates that the activity of NK cells from PBMCs is reduced in patients with prostate cancer as compared to those of healthy controls, and this reduced NK activity levels may correlate with the presence of tumor cells in the circulatory system [4, 22]. Therefore, an efficient method to promote the selective expansion of potent NK cells is needed to overcome the immunosuppressive state in advanced PCA. Here we revealed that ex vivo-expanded NK cells could mediate cytotoxicity *in vitro*, and reduction of PSA was observed in five out of eight patients. Intriguingly, no adverse events and side effects were found in advanced PCA patients treated with ex vivo-expanded NK cells.

NK cells are important in host to eliminate circulating tumor cells in turn preventing the

development of tumor cells into metastasis [23]. Regulating of NK-cell activation is critical for positive immune response [24, 25]. NK cell could be prepared through current good manufacturing process (cGMP), which provides a new choice for advanced PCA clinical treatment except for traditional methods [26-31]. Some previous studies indicates that infusion of autologous NK cells were clinically ineffective [32-34]. We prepared autologous NK cells from PBMC and expanded it using large scale culture system *in vitro*. Subsequently, the anti-cancer efficacy and bone pain alleviation in all patients were observed after injection with autologous NK cells. In addition, PSA, a biomarker for PCA progression [35], was decreased in five patients with a Gleason score under 9 compared with the patients with Gleason score of 9. Except for only one case, the percentage of $CD3^{-}CD56^{+}$ NK cells of other seven patients all increased after treatment with autologous NK cells. The percentage of $CD8^{+}$ T cells increased in six patients after immunotherapy. Based on these data, we drew a limited conclusion that immunotherapy of NK cells could evoke cellular immune response against PCA, and further data were required to verify this finding.

In summary, immunotherapy of NK cells is well tolerated, and can be carried out safely without obvious adverse events or side effects. Although somewhat controversial, treatment of NK cells has been shown to be advantageous in advanced PCA patients.

## Disclosure of conflict of interest

None.

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