

Original Article

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

Peng Gao^{1*}, Aili Zhang^{2*}, Zhenhua Sun¹, Yuxi Shan¹

¹Department of Urology, The Second Affiliated Hospital of Soochow University, Suzhou, China; ²Department of Paediatrics, Suzhou Municipal Hospital, Suzhou, China. *Equal contributors.

Received October 30, 2015; Accepted January 15, 2016; Epub February 15, 2016; Published February 29, 2016

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 15×10^9 cells), and the other four patients received four infusions (total dose 20×10^9 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.

NK cells have a therapeutic effect on advanced PCA patients

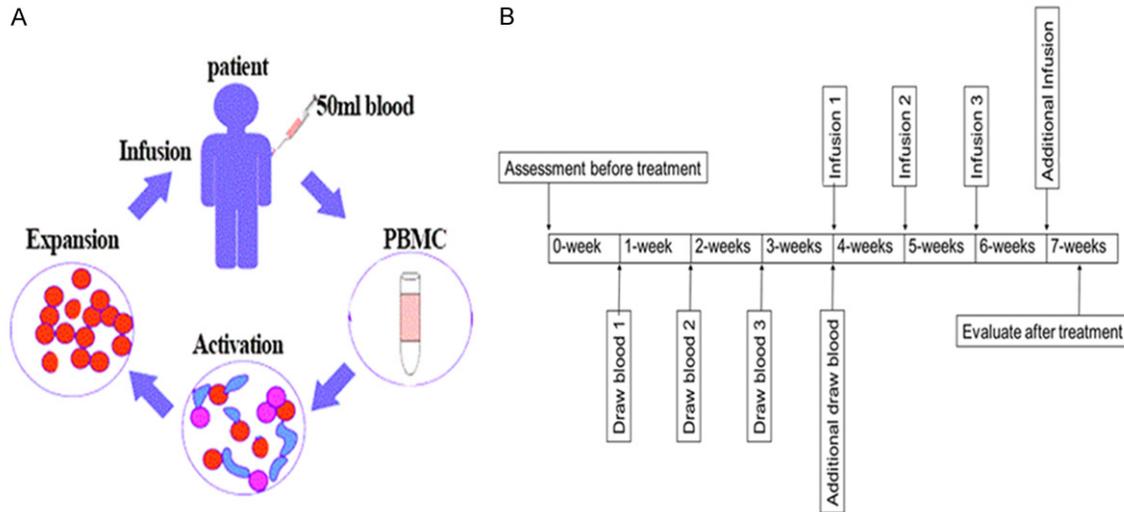


Figure 1. Therapeutic strategy and schedule of NK cells. Laboratory procedures of ex vivo-expanded NK cells (A). Eight patients were enrolled in this clinical study, and four of them received three infusions of autologous expanded NK cells, the other patients received four infusions (B). All patients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

logous NK cells were obtained from the patients themselves. All patients received three to four infusions of autologous activated NK cells (total dose $15-20 \times 10^9$ 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-

NK cells have a therapeutic effect on advanced PCA patients

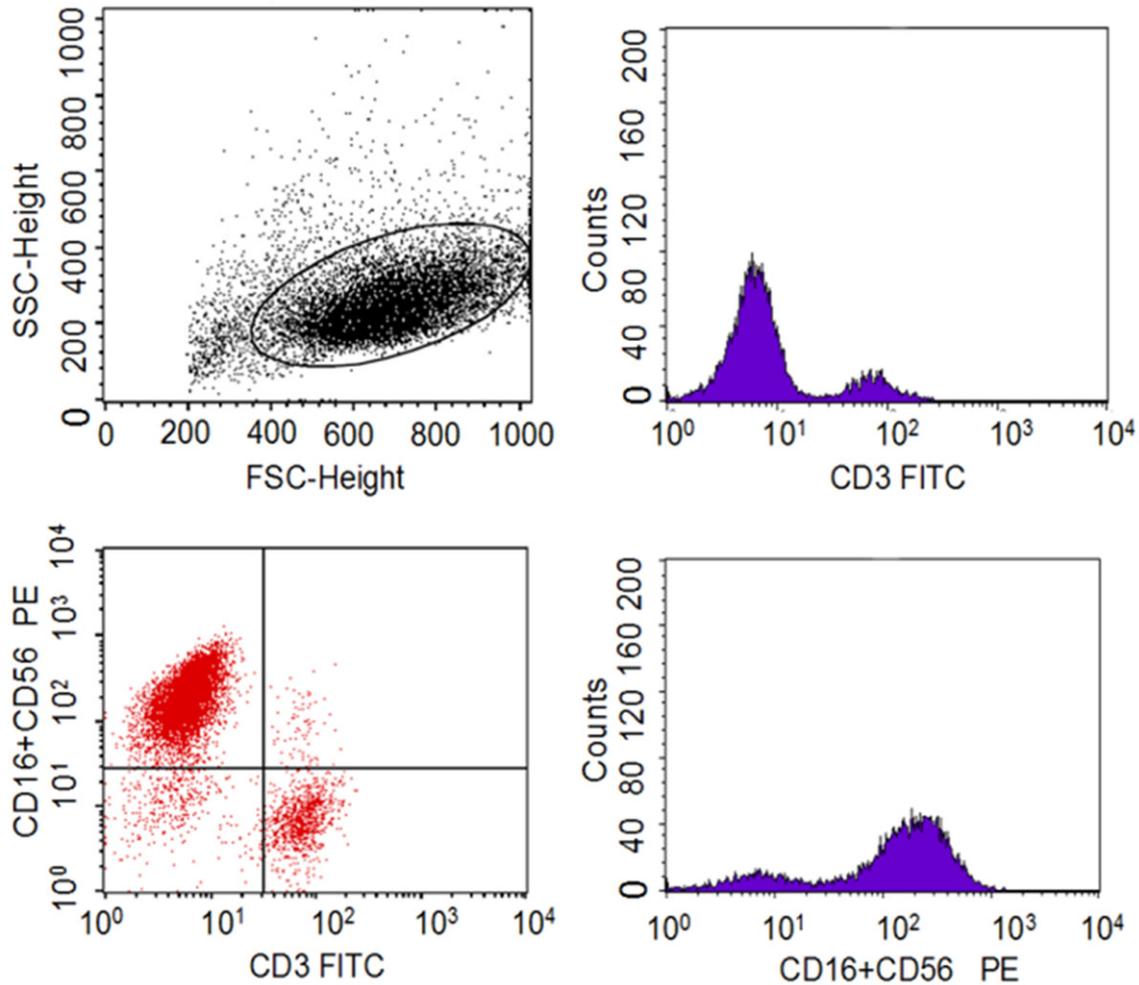


Figure 2. Phenotype of NK cells after 21 days of culture. PBMC isolated from 50 mL blood range from 1×10^7 to 5×10^7 cells. After activation and expansion of NK cells, the final cell population contained about 80% of CD3⁺CD56⁺ NK cells.

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% CO₂. 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 1×10^6 cells were washed and incubated with 20 μ L 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

NK cells have a therapeutic effect on advanced PCA patients

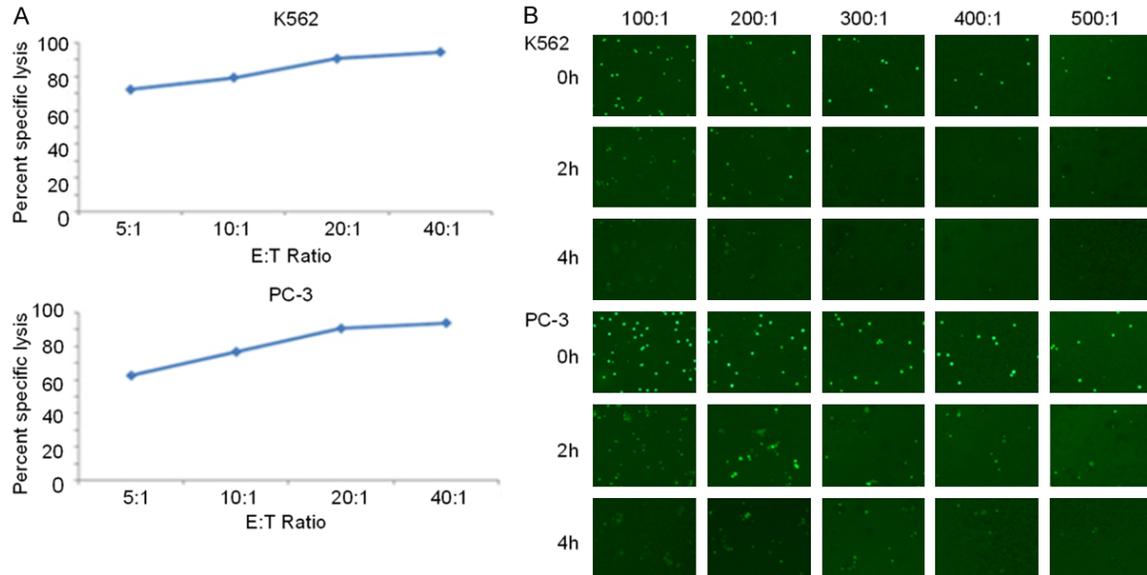


Figure 3. Expanded NK cells had demonstrated cytotoxicity against K562 and PC-3 cell lines. Cultured NK cells were used as effector cells in a cytotoxicity assay against the K562 and PC-3 cell line at effector: target (E:T) ratio of 5:1, 10:1, 20:1 and 40:1 (A). Expanded NK cells cytotoxicity assay was examined using Calcein-AM. The assay used Calcein-AM, a nonfluorescent 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 1×10^6 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 6×10^5 NK cells and 3×10^5 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 5×10^9 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 $5 \times 10^9 \sim 1 \times 10^{10}$ cells, with the purity of over 90%. As illustrated in **Figure 3A**, the cytotoxicity increased when the E:T ratio

NK cells have a therapeutic effect on advanced PCA patients

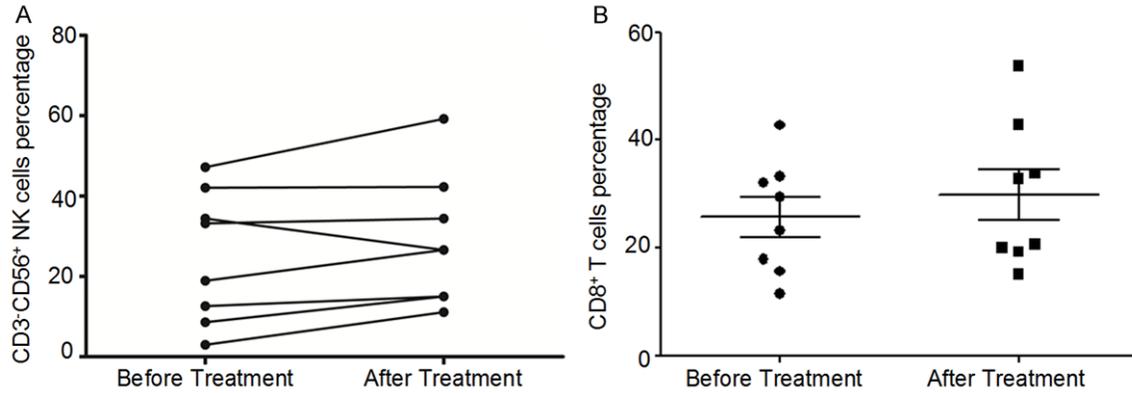


Figure 4. The changes of CD3⁺CD56⁺ NK cells and CD8⁺ T cells before and after treatment. The frequency of peripheral blood CD3⁺CD56⁺ NK cells in all patients was measured by flow cytometry analysis (A). The frequency of peripheral blood CD8⁺ T cells in all patients was measured by flow cytometry analysis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

Address correspondence to: Dr. Yuxi Shan, Department of Urology, The Second Affiliated Hospital of Soochow University, Suzhou 215006, China. Tel: (86) 512-68282030; Fax: (86) 512-68284303; E-mail: yuxishan_su@hotmail.com

References

- [1] Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D. Global cancer statistics. *CA Cancer J Clin* 2011; 61: 69-90.
- [2] Jemal A, Siegel R, Xu J and Ward E. Cancer statistics, 2010. *CA Cancer J Clin* 2010; 60: 277-300.
- [3] Zhang L, Yang BX, Zhang HT, Wang JG, Wang HL and Zhao XJ. Prostate cancer: an emerging threat to the health of aging men in Asia. *Asian J Androl* 2011; 13: 574-578.
- [4] Oikawa T, Kawai K, Ishiwata I, Ohno T and Akaza H. Induction of potent antitumour natural-killer cells from peripheral blood of patients with advanced prostate cancer. *BJU Int* 2003; 92: 1009-1015.
- [5] Klingemann HG. Cellular therapy of cancer with natural killer cells-where do we stand? *Cytotherapy* 2013; 15: 1185-1194.
- [6] Wu Z, Sinzger C, Reichel JJ, Just M and Mertens T. Natural killer cells can inhibit the transmission of human cytomegalovirus in cell culture by using mechanisms from innate and adaptive immune responses. *J Virol* 2015; 89: 2906-2917.
- [7] Nayar S, Dasgupta P and Galustian C. Extending the lifespan and efficacies of immune cells used in adoptive transfer for cancer immunotherapies-A review. *Oncoimmunology* 2015; 4: e1002720.
- [8] Miller JS, Soignier Y, Panoskaltis-Mortari A, McNearney SA, Yun GH, Fautsch SK, McKenna D, Le C, Defor TE, Burns LJ, Orchard PJ, Blazar BR, Wagner JE, Slungaard A, Weisdorf DJ, Okazaki IJ and McGlave PB. Successful adoptive transfer and *in vivo* expansion of human haploidentical NK cells in patients with cancer. *Blood* 2005; 105: 3051-3057.
- [9] Rubnitz JE, Inaba H, Ribeiro RC, Pounds S, Rooney B, Bell T, Pui CH and Leung W. NKAML: a pilot study to determine the safety and feasibility of haploidentical natural killer cell transplantation in childhood acute myeloid leukemia. *J Clin Oncol* 2010; 28: 955-959.
- [10] Alter G, Rihn S, Walter K, Nolting A, Martin M, Rosenberg ES, Miller JS, Carrington M and Altfeld M. HLA class I subtype-dependent expansion of KIR3DS1⁺ and KIR3DL1⁺ NK cells during acute human immunodeficiency virus type 1 infection. *J Virol* 2009; 83: 6798-6805.
- [11] Carrington M, Martin MP and van Bergen J. KIR-HLA intercourse in HIV disease. *Trends Microbiol* 2008; 16: 620-627.
- [12] Vidal-Castineira JR, Lopez-Vazquez A, Diaz-Pena R, Alonso-Arias R, Martinez-Borra J, Perez R, Fernandez-Suarez J, Melon S, Prieto J, Rodrigo L and Lopez-Larrea C. Effect of killer immunoglobulin-like receptors in the response to combined treatment in patients with chronic hepatitis C virus infection. *J Virol* 2010; 84: 475-481.
- [13] Wennerberg E, Kremer V, Childs R and Lundqvist A. CXCL10-induced migration of adoptively transferred human natural killer cells toward solid tumors causes regression of tumor growth *in vivo*. *Cancer Immunol Immunother* 2015; 64: 225-235.
- [14] Somanchi SS, Somanchi A, Cooper LJ and Lee DA. Engineering lymph node homing of ex vivo-expanded human natural killer cells via trogo-

NK cells have a therapeutic effect on advanced PCA patients

- cytosis of the chemokine receptor CCR7. *Blood* 2012; 119: 5164-5172.
- [15] Buddingh EP, Schilham MW, Ruslan SE, Berghuis D, Szuhai K, Suurmond J, Taminiau AH, Gelderblom H, Egeler RM, Serra M, Hogendoorn PC and Lankester AC. Chemotherapy-resistant osteosarcoma is highly susceptible to IL-15-activated allogeneic and autologous NK cells. *Cancer Immunol Immunother* 2011; 60: 575-586.
- [16] Frings PW, Van Elssen CH, Wieten L, Matos C, Hupperets PS, Schouten HC, Bos GM and van Gelder M. Elimination of the chemotherapy resistant subpopulation of 4T1 mouse breast cancer by haploidentical NK cells cures the vast majority of mice. *Breast Cancer Res Treat* 2011; 130: 773-781.
- [17] Wolchok JD, Hoos A, O'Day S, Weber JS, Hamid O, Lebbe C, Maio M, Binder M, Bohnsack O, Nichol G, Humphrey R and Hodi FS. Guidelines for the evaluation of immune therapy activity in solid tumors: immune-related response criteria. *Clin Cancer Res* 2009; 15: 7412-7420.
- [18] Oh WK and Kantoff PW. Management of hormone refractory prostate cancer: current standards and future prospects. *J Urol* 1998; 160: 1220-1229.
- [19] Heidenreich A, von Knobloch R and Hofmann R. Current status of cytotoxic chemotherapy in hormone refractory prostate cancer. *Eur Urol* 2001; 39: 121-130.
- [20] Di Lorenzo G, Buonerba C, Autorino R, De Placido S and Sternberg CN. Castration-resistant prostate cancer: current and emerging treatment strategies. *Drugs* 2010; 70: 983-1000.
- [21] Kantoff PW, Higano CS, Shore ND, Berger ER, Small EJ, Penson DF, Redfern CH, Ferrari AC, Dreicer R, Sims RB, Xu Y, Frohlich MW, Schellhammer PF and Investigators IS. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *N Engl J Med* 2010; 363: 411-422.
- [22] Nowak M and Schmidt-Wolf IG. Natural killer T cells subsets in cancer, functional defects in prostate cancer and implications for immunotherapy. *Cancers (Basel)* 2011; 3: 3661-3675.
- [23] Liu X, Chen Q, Yan J, Wang Y, Zhu C, Chen C, Zhao X, Xu M, Sun Q, Deng R, Zhang H, Qu Y, Huang J, Jiang B and Yu J. MiRNA-296-3p-ICAM-1 axis promotes metastasis of prostate cancer by possible enhancing survival of natural killer cell-resistant circulating tumour cells. *Cell Death Dis* 2013; 4: e928.
- [24] Hallett WH and Murphy WJ. Positive and negative regulation of Natural Killer cells: therapeutic implications. *Semin Cancer Biol* 2006; 16: 367-382.
- [25] Romagne F and Vivier E. Natural killer cell-based therapies. *F1000 Med Rep* 2011; 3: 9.
- [26] McKenna DH Jr, Sumstad D, Bostrom N, Kadidlo DM, Fautsch S, McNearney S, Dewaard R, McGlave PB, Weisdorf DJ, Wagner JE, McCullough J and Miller JS. Good manufacturing practices production of natural killer cells for immunotherapy: a six-year single-institution experience. *Transfusion* 2007; 47: 520-528.
- [27] Arai S, Meagher R, Swearingen M, Myint H, Rich E, Martinson J and Klingemann H. Infusion of the allogeneic cell line NK-92 in patients with advanced renal cell cancer or melanoma: a phase I trial. *Cytotherapy* 2008; 10: 625-632.
- [28] Berg M, Lundqvist A, McCoy P Jr, Samsel L, Fan Y, Tawab A and Childs R. Clinical-grade ex vivo-expanded human natural killer cells up-regulate activating receptors and death receptor ligands and have enhanced cytolytic activity against tumor cells. *Cytotherapy* 2009; 11: 341-355.
- [29] Siegler U, Meyer-Monard S, Jorger S, Stern M, Tichelli A, Gratwohl A, Wodnar-Filipowicz A and Kalberer CP. Good manufacturing practice-compliant cell sorting and large-scale expansion of single KIR-positive alloreactive human natural killer cells for multiple infusions to leukemia patients. *Cytotherapy* 2010; 12: 750-763.
- [30] Lundqvist A, Berg M, Smith A and Childs RW. Bortezomib Treatment to Potentiate the Antitumor Immunity of Ex-vivo Expanded Adoptively Infused Autologous Natural Killer Cells. *J Cancer* 2011; 2: 383-385.
- [31] Spanholtz J, Preijers F, Tordoir M, Trilsbeek C, Paardekooper J, de Witte T, Schaap N and Dolstra H. Clinical-grade generation of active NK cells from cord blood hematopoietic progenitor cells for immunotherapy using a closed-system culture process. *PLoS One* 2011; 6: e20740.
- [32] Parkhurst MR, Riley JP, Dudley ME and Rosenberg SA. Adoptive transfer of autologous natural killer cells leads to high levels of circulating natural killer cells but does not mediate tumor regression. *Clin Cancer Res* 2011; 17: 6287-6297.
- [33] Krause SW, Gastpar R, Andreesen R, Gross C, Ullrich H, Thonigs G, Pfister K and Multhoff G. Treatment of colon and lung cancer patients with ex vivo heat shock protein 70-peptide-activated, autologous natural killer cells: a clinical phase I trial. *Clin Cancer Res* 2004; 10: 3699-3707.
- [34] Motohashi S, Ishikawa A, Ishikawa E, Otsuji M, Iizasa T, Hanaoka H, Shimizu N, Horiguchi S, Okamoto Y, Fujii S, Taniguchi M, Fujisawa T and

NK cells have a therapeutic effect on advanced PCA patients

Nakayama T. A phase I study of in vitro expanded natural killer T cells in patients with advanced and recurrent non-small cell lung cancer. *Clin Cancer Res* 2006; 12: 6079-6086.

[35] Drake CG. Immunotherapy for prostate cancer: an emerging treatment modality. *Urol Clin North Am* 2010; 37: 121-129.