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Case Report

Autologous *ex vivo* expanded NK cells combined with PD-1 inhibitor improved ascitic fluid immune microenvironment of peritoneal metastatic pancreatic cancer: a case study

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Abstract: The presence of peritoneal metastasis in patients with pancreatic cancer is associated with poor prognosis. Chemotherapy and radiotherapy may result in poor prognosis in patients with pancreatic cancer. However, immunotherapy improves prognosis even at an advanced stage of the disease. The present study reported a case of a combined therapy of autologous *ex vivo* expanded natural killer (NK) cells and programmed cell death 1 (PD-1) inhibitor in a patient with pancreatic cancer and peritoneal metastasis. The NK cells were expanded *ex vivo* and intravenously injected. This was followed by intravenous administration of two dosages of PD-1 inhibitor. Computed tomography and magnetic resonance imaging were performed to assess the size of tumor before and after the combined therapy. In addition, the blood sample and ascites were collected and analyzed before and after the combined therapy. Flow cytometry was carried out to measure the subsets of T cells and macrophages in the collected ascites. Meanwhile, the levels of cytokines in the ascites were quantified through enzyme-linked immunosorbent assay, and Luminex assays were conducted on the supernatant. It was revealed that after the combined therapy, cancer cells disappeared in the ascites, and the T cells were activated, which could be confirmed by the decreased levels of PD-1 and T cell immunoglobulin and mucin domain-containing protein 3. Also, the functioning of macrophages was improved, as shown by the increased level of CD86 and the reduced levels of CD206 and HLA-DR. Notably, the levels of cytokines (transforming growth factor-β, vascular endothelial growth factor, and interleukin-10) in ascites were significantly upregulated after the combined therapy. In conclusion, it was evident that NK cells combined with PD-1 inhibitor improved the immune microenvironment of carcinomatosis in the peritoneal cavity. Therefore, the combined therapy may be beneficial for suppressing pancreatic cancer and the presence of metastases in the peritoneal cavity. However, there is a need for additional randomized studies to confirm the efficacy of combined therapy.

Keywords: Autologous NK cells, PD-1 inhibitor, pancreatic cancer, combined immunotherapy

Introduction

Pancreatic ductal adenocarcinoma (PDAC) accounts for 90% of all pancreatic cancer cases in the world. It is one of the top five leading causes of the global cancer-related death and has a 5-year survival rate of less than 9% [1]. The high mortality of PDAC is partly related to its late diagnosis and aggressive nature, because cancer cells have metastasized before the diagnosis [2]. Patients are assumed to be incurable and with poor prognosis if they are diagnosed at an advanced stage, when cancer cells have already metastasized to peritoneum. Consequently, aggressive treatment for these patients is prohibited, and palliative care is typically recommended.

Infusion of live immune cells is important in immunotherapy. It may enhance the antitumor effect of the innate immune system. Furthermore, employing autologous *ex vivo* expanded natural killer (NK) cells to treat cancers is worthy of assessment in a clinical trial, and some

preclinical trials have achieved promising results [3]. Infusion of NK cells possess multiple advantages compared with other immunotherapies. Identifying tumor antigens for NK cell-based immunotherapy is not necessary compared with the vaccine therapy or antigen-specific adoptive T cell therapy. This makes the infusion of NK cells generally more applicable and effective against solid tumors that frequently lose tumor-associated antigens and/or self-major histocompatibility complex (MHC) molecules [4]. Therefore, NK cell-based immunotherapy has been recommended to treat hematologic malignancies and solid tumors in clinical practice [5-7]. The present study reported a case of pancreatic cancer and peritoneal metastasis treated by infusion of autologous NK cells and PD-1 inhibitor. It was found that the combined therapy improved the immune microenvironment of carcinomatosis in the peritoneal cavity.

Materials and methods

Ex vivo stimulation of hematopoietic stem-derived (HSC) NK cells

In the present study, 40 ml of peripheral blood was collected and 4.2×10^7 mononuclear cells were isolated for expansion. The initial induction bag of HSC-NK cells was removed with coating antibodies (0.1 μg/mL anti-CD3 mAb (OKT3) and 20 μg/mL anti-CD52 mAb (alemtuzumab)) overnight at 4°C, and then washed with phosphate-buffered saline (PBS). Mononuclear cells were added into the bag and supplemented with 30 ml of natural killer growth medium (NKGM), 500 IU/ML interleukin (IL)-2, as well as 5% autologous plasma. After 3 days of culture, the cells were transferred to T175 culture bottle and supplemented with NKGM and autologous plasma. After 5 days, the cells were transferred to the HSC-NK expansion bag, containing 1 L serum-free medium. The NKGM and IL-2 (1000 IU/ML) were later added according to the density of the cells to form a final volume of 2300 ml, and a liquid whole-cell suspension was then collected. Thereafter, centrifugation was conducted, and the supernatant was removed. The cells were twice washed with saline and then resuspended with saline and albumin. After 3 weeks of expansion, the total number of HSC-NK cells reached 7.4 × 10^9, with a significant purity (83.3%).

Laboratory parameters and flow cytometry analysis of peripheral blood lymphocytes

Routine laboratory parameters (differential blood counts, red blood cell (RBC) count and white blood cell (WBC) count), blood chemistry (creatinine, aspartate transaminase (AST)/SGOT, alanine transaminase (ALT)/SGPT, γ-glutamyl transferase (γ-GT) and lactic dehydrogenase (LDH)) and tumor-related markers (carbohydrate antigen 19-9 (CA199), carcinoembryonic antigen (CEA), carbohydrate antigen 72-4 (CA724), alpha-fetoprotein (AFP), cancer antigen-125 (CA125) and cancer antigen 15-3 (CA15-3)) were assessed before and after each treatment and in the follow-up period. Flow cytometry was conducted to assess the subpopulations of lymphocytes in the ascites cells. The flow cytometry was performed at different time points and with combination of different antibodies ($CD45^+CD3^+$ T cells, $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, $CD3^+CD4^+$PD-1 T cells, $CD3^+CD4^+$TIM-3 T cells, $CD3^+CD8^+$PD-1^+ T cells and $CD3^+CD8^+$TIM-3^+ T cells).

Cytokine profiling of ascites

Samples containing ascites were obtained from patients before and after intravenous injection of the NK cells and administration of the two doses of PD-1 inhibitor. All the samples were stored at -80°C until the analysis. A Luminex Multiplex Cytokine Array system (Luminex X-200; Luminex Corp., Shanghai, China) was used with a panel to quantify the 5 different cytokines (transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF), IL-10, IL-2 and tumor necrosis factor-α (TNF-α)) according to the manufacturer's instructions.

Results

Patient characteristics

The treatment process in the current study is briefly summarized in **Figure 1**. In July 2019, a 79-year-old male patient was diagnosed with histologically confirmed, inoperable, stage IV PDAC with peritoneal metastases. The patient had neither history of smoking or drinking, nor significant family history of the disease. He was admitted to the Changhai Hospital Affiliated to Naval Medical University (Shanghai, China) on July 9, 2019, and he complained of a weight

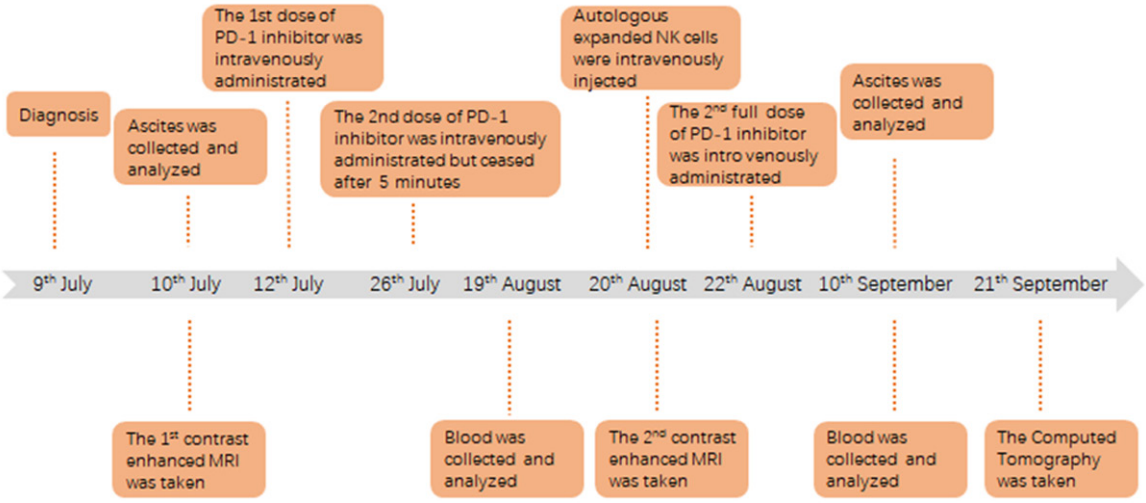

Figure 1. The whole treatment process for the patient.

Table 1. Tumor related parameters before and after the combined treatment

Parameters	19th Aug	10th Sep
CA199 (U/ml)	>1200	>1200
CA724 (U/ml)	6.23	2.28
CEA (ng/ml)	2.71	2.53
AFP (ng/ml)	3.79	1.84
CA125 (U/ml)	81	226.1
CA15-3 (U/ml)	6.5	9

Tumor-related markers (Carbohydrate Antigen 19-9 (CA199), Carcinoembryonic Antigen (CEA), Carbohydrate Antigen 72-4 (CA724), Alpha-Fetoprotein (AFP), Cancer Antigen-125 (CA125) and Cancer Antigen 15-3 (CA15-3)).

loss of 10 kg and abdominal distension in the past three months.

The B-mode ultrasound was performed in the hospital and revealed that the patient had a thickened echo in the right lobe of his liver, a rough gallbladder wall, and peritoneal effusion in abdominal cavity (6.4 cm). On July 10, 2019, the results of computed tomography (CT) scan showed that the tail of his pancreas was slightly enlarged, the peripancreatic fat space was not clear, the omentum was diffusely nodular and flocculent, and there were several enlarged lymph nodes behind the hepatic portal as well as retroperitoneum. Contrast-enhanced magnetic resonance imaging (MRI) of the pancreas was conducted and a pancreatic tumor (pancreatic tail, 10.4 × 26.1 mm) was identified. The contrast-enhanced MRI image also revealed a group of lymph nodes around the hepatic artery, and it was suspected that the omental nodules had been metastasized. In addition, it was found that his CA199 level was higher than 1200 U/ml (**Table 1**).

On July 12, 2019, the first dose of PD-1 inhibitor was intravenously administrated. High fever occurred 2 days after the intravenous infusion of 200 mg PD-1 inhibitor. An intramuscular injection of dexamethasone was carried out to treat developed pyrexia without extra discomfort. On July 26, 2019, the second dose of PD-1 was intravenously administrated. However, 5 min after the second administration of PD-1, the patient developed chest tightness and chills, while the body temperature remained at 37.5°C. Therefore, administration of PD-1 was stopped, and the chills were effectively treated through intravenous administration of 5 mg dexamethasone sodium phosphate. In addition, rectal administration of indomethacin was given to treat pyrexia, and his body temperature dropped to 36.8°C. On August 20, 2019, the autologous expanded HSC-NK cells were intravenously injected, and a second dose of PD-1 inhibitor was intravenously administrated on August 22, 2019. It was evident that the patient effectively tolerated the combined therapy, with no occurrence of noticeable adverse events (**Figure 1**).

The patient was 165 cm in height, 43 kg in weight, and had body mass index of 15.8 kg/m^2. It was found that his nutritional risk screen-

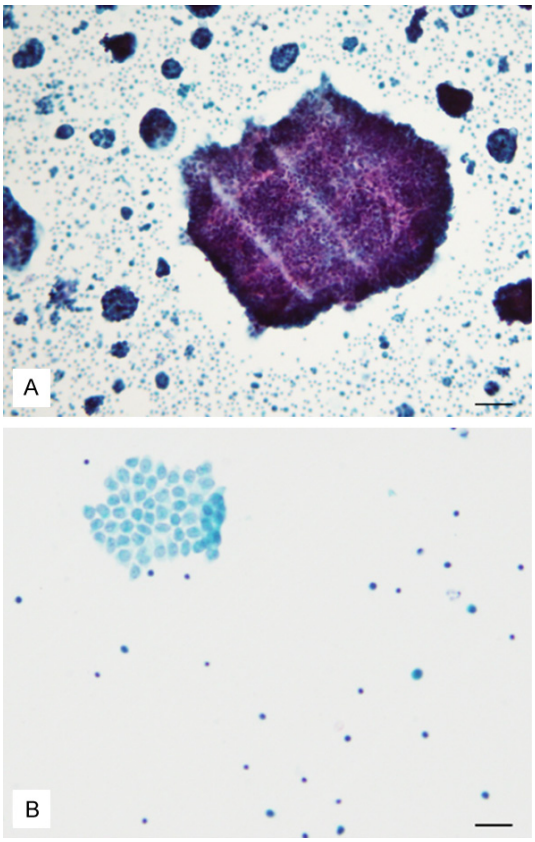

Figure 2. The Thinprep cytologic test results of ascites before (A) and after (B) the combined therapy. Bar = 100 μm.

ing-2002 score was greater than 3, and the nutritional status was severely insufficient. Thus, a diet including oral nutritional supplements was recommended to the patient. Eventually, the patient died from cachexia in November 2019. A written informed consent for the current case study was obtained from the patient, and the clinical trial protocol was approved by the Institutional Review Board of the Changhai Hospital Affiliated to Naval Medical University.

Safety of infusion of HSC-NK cells

It was found that there was no obvious sign of graft-versus-host disease (GVHD) after the infusion of HSC-NK cells. Furthermore, no observable clinical sign of cytokine release syndrome or other unexpected toxicities was identified. In addition, it was noted that there was no significant increase in levels of IL-6 or ALT. However, a short-term fever was observed, which was alleviated after treatment. Moreover, there were no complications, such as rash and reduction in WBC count, were detected after infusion of HSC-NK cells.

Clinical outcomes and the effects of the combined therapy on pancreatic cancer

CT and MRI were performed before and after the combined therapy (infusion of HSC-NK cells) to investigate the clinical outcome and the effects of the combined therapy on pancreatic cancer. The imaging results revealed that there was a slight enlargement in tumor size after the administration of the first dose of PD1 inhibitor (Figure S1A, S1B), which might be related to immune cell infiltration rather than tumor progression. The CT scan revealed a mild swelling of the pancreas tail after the combined therapy, while the patient reported a relieved abdominal distension, a reduced abdominal circumference as well as increased physical activity, thus, contrast-enhanced MRI was not performed after the combined therapy. The patient's blood sample and ascites were collected and centrifuged. It was found that, although NK cells and PD-1 inhibitor were intravenously administrated, the cancer cells in ascites (**Figure 2A**), which were revealed by the ThinPrep cytologic test before the treatment, could not be detected (**Figure 2B**). In addition, T cells and macrophages were significantly activated. Therefore, we draw the conclusion that live immune cells can reach the omentum through blood circulation and modulate the immune microenvironment of carcinomatosis in the peritoneal cavity.

Overall, it was evident that tumor cells, which spread to the omentum, were eradicated. Furthermore, there was an increase in the number of T cells and macrophages, as well as an activation of their function. In addition, it was revealed that the expression levels of PD-1 and T cell immunoglobulin and mucin domain-containing protein 3 (TIM-3), which are ICIs that are mainly expressed on T cells, were reduced in both $CD4^+$ T cells and $CD8^+$ T cells (**Figures 3A**, S2A-D). The results of the present case study showed that there was an increase in the level of CD86 and a decrease in the levels of both CD206 and HLA-DR in macrophages, demonstrating that there was transition of M1 macrophages (**Figures 3B**, S2E-G). Moreover, it was found that among the 5 cytokines (TGF-β, VEGF, IL-10, IL-2 and TNF-α) that were assessed, the

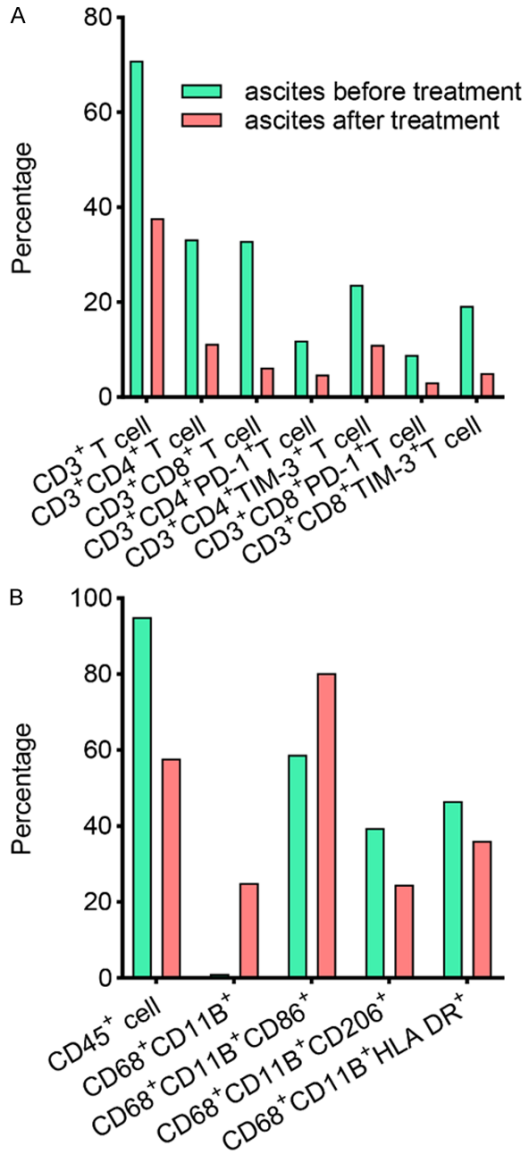

Figure 3. Molecules expressed in ascites T cells and macrophage before and after the combined therapy. A. PD-1 and TIM expression in CD4 T cells and CD8 T cells before and after the combined therapy. B. CD86, CD206 and HLA-DR expression in macrophages before and after the combined therapy.

expression levels of TGF-β, VEGF and IL-10 were significantly upregulated, the level of IL-2 was slightly elevated, whereas the level of TNF-α was downregulated (**Figure 4**). However, other tumor-related markers in blood such as CA-199, CA724, CEA, AFP, CA125 and CA153 did not show significant changes (**Table 1**).

Our results showed that the combined therapy of NK cells and PD-1 inhibitor significantly improved the immune microenvironment of carcinomatosis in the peritoneal cavity. However, further research is required to understand the mechanism and to explore the complex metastatic immune microenvironment of the peritoneal cavity.

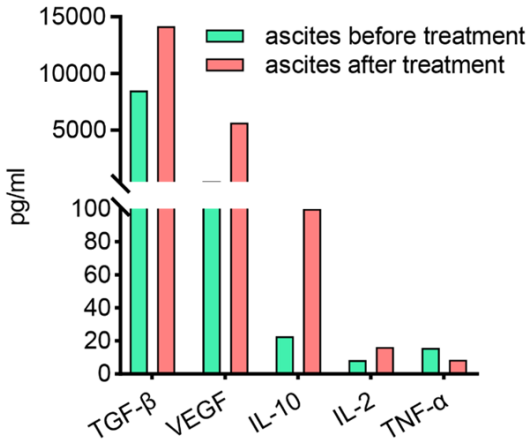

Figure 4. The concentration of cytokines (TGF-β, VEGF, IL-10, IL-2, and TNF-α) in ascites before and after the combined therapy. TGF-β: Transforming Growth Factor-β, VEGF: Vascular Endothelial Growth Factor, IL: Interleukin, TNF-α: Tumor Necrosis Factor-α.

Discussion

A patient was diagnosed with advanced-stage pancreatic cancer, peritoneal carcinomatosis and severe malnutrition in the Changhai Hospital Affiliated to Naval Medical University on July 9, 2019. After treatment, it was revealed that intravenously administration of *ex vivo* expanded autologous NK cells and PD-1 inhibitor significantly improved the immune microenvironment of carcinomatosis in the peritoneal cavity. Flow cytometry, enzyme-linked immunosorbent assay, and Luminex assays were performed to analyze the patient's ascites before and after the treatment.

It was evident that the combined therapy eradicated tumor cells that were metastasized to the omentum. In addition, the number of T cells and macrophages in the ascites had significantly risen (about 100-fold) and were notably activated after the combined therapy. ICIs, such as PD-1 and TIM-3, are regarded as the "brake" of the immune system, inhibiting the function of the activated immune cells. The present case study showed that the expression levels of PD-1 and TIM-3 were significantly

reduced in CD8 T cells and CD4 T cells. Lower expression of PD-1 in CD8 T cells usually indicates a better prognosis for cancer patients due to a less exhausted immune phenotype. Though PD-1 expression in CD4 T lymphocytes was rarely reported, a preliminary investigation reported that a group of tumor-infiltrating $PD1^+$ $CD39^+$ CD4 T lymphocytes were terminally exhausted, and blockade of PD-1 restored CD4 T helper activity and further boosted CD8 T and Dendrite cell function [8]. In this study, a lower PD-1 expression in $CD4^+CD25^-$ T cells was revealed after the combined therapy, which indicated that this subset was not Tregs but CD4 T helper cells that might contribute to CD8 T cell function. In addition, the M2-like macrophages were remarkably converted to M1-like macrophages. This was confirmed by the increased expression level of CD86 and the decreased level of CD206, and HLA-DR showed an antitumor phenotype.

Several studies have previously identified the role of cytokines in remodeling the tumor microenvironment. The present study measured the expression levels of some cytokines and the changes in the levels before and after the combined therapy. It was found that the expression levels of IL-10, TGF-β and VEGF were significantly upregulated among the 5 cytokines that were assessed (TNF-α, IL-2, IL-10, TGF-β and VEGF). TGF-β, VEGF and IL-10 were usually defined as inhibitory cytokines secreted by tumor cells. The increased levels of TGF-β, VEGF and IL-10 in ascites after the combined therapy may be due to the rapid eradication of tumor cells as well as the fast decrease of ascites volume in the peritoneum cavity. Based on the facts that tumor cells were eradicated in the peritoneum immune microenvironment and that CD8 T cells and macrophages were significantly activated, we made the conclusion that the combined therapy significantly improved the immune microenvironment of carcinomatosis in the peritoneal cavity.

Utilization of expanded and activated NK cells for pancreatic cancer is still under investigation, and several relevant clinical trials have been carried out [9]. A study assessed the safety of autologous NK cell therapy, while showing that the therapy has no or limited antitumor effect [3]. Furthermore, a number of studies have previously concentrated on only a small proportion of patients [10]. Multiple studies have explored the combined therapy of NK cells with chemotherapy [11], radiotherapy, and/or PD-1 inhibitor [12] in other solid tumors to further improve the NK cell infusion therapy. However, it is essential to explore the mechanism of NK cells to improve the outcome of malignancies after combined therapies including infusion of NK cells.

It was noted that PD-1 expression level was upregulated by a population of NK cells in the tumor microenvironment. The PD-1 suppressed degranulation and cytotoxic functions of NK cells *in vitro*, and hence generated more aggressive tumors *in vivo*. In addition, it was indicated that the blockade of PD-1 by its inhibitor significantly improved the activation of NK cells to kill tumor cells [13].

The results of the present case study showed the importance of the PD-1/PD-L1 axis in inhibiting NK cell responses *in vivo*. Moreover, it was evident that the effect of the blockade of PD-1/PD-L1 immunotherapy was mediated by NK and T cells. It was found that in addition to the role of NK cells in direct tumor surveillance, NK cells also contribute to T cell anti-tumor immunity. Previous studies on mouse models have shown that NK cells in mice could facilitate the accumulation of T-bet + $CD4^+$ T cells in the tumor region [14], promote the production of effector molecules, TNF-α and interferon gamma (IFN-γ), by tumor-infiltrating CD8 T cells, and suppress the expression level of exhaustion marker PD-1 in CD8 T cells [15].

On the contrary, a study conducted using mouse models also showed that depletion of NK cells abrogated the efficacy of the blockade of PD-L1 immunotherapy [14]. It was noted that the presence of NK cells prevented formation of a more exhausted status of tumor-infiltrating $CD8^+$ T cells even under PD-L1 blockade. This was evidenced by the decreased expression levels of degranulation marker, CD107a, effector cytokines, TNF-α and IFN-γ, as well as increased expression level of exhaustion marker PD-1 in $CD8^+$ T cells, after depletion of NK cells [15].

However, there are some obstacles that need to be resolved during exploration of the use of NK cells in cancer treatment. First, the source of NK cells needs to be expanded. A major

advance of using the cells from patients is that the mentioned method does not require non-myeloablative chemotherapy before infusion of NK cells and there is a low risk of GVHD. However, there is a need for a prolonged period that may last for at least two weeks [5]. Patients in the late stage of the disease cannot afford the time of processing. Furthermore, it was reported that the inhibitory effects of interactions between the autologous NK cells and MHC I molecules, which are expressed in tumor cells, may limit the role of autologous NK cells [16].

Other sources include the peripheral blood of haploidentical donors or unrelated donors, HSCs, umbilical cord blood, embryonic stem cells, induced pluripotent stem cells and NK cell lines [17-19]. The key role of NK cells in the hematopoietic stem cell transplantation setting was elucidated by Ruggeri *et al.* in 2002 [20]. They found that the mismatches between inhibitory killer Ig-like receptors expressed by NK cells, in haploidentical grafts and the host human leukocyte antigen ligands, unleashed the alloreactive cytotoxicity of NK cells against acute myeloid leukemia cells in mouse models, as well as patients in a setting of T cell depletion and high doses of $CD34^+$ cells.

Different protocols were also proposed to obtain a large number of NK cells *ex vivo* for cellular immunotherapy. However, the functional differences between NK cells from different sources remain to be elusive [7], and allogeneic NK cells were mainly rejected at 2-3 weeks after infusion. One approach is to further intensify lymphodepletion before infusion of NK cells, including total body irradiation or anti-T cell agents, while this approach might increase toxicity and limit the applicability of NK cells.

The pre-activation of NK cells needs to be further improved, and this improvement is worthy of further assessment. Combination of different cytokines, such as IL-2, IL-15 and IL-18, with or without the K562 feeder cells, may be advantageous for activating NK cells from the resting state [21, 22]. It has been previously suggested that there is a need for a more efficient activation method [23]. The present case study showed the promising efficacy of infusion of NK cells in cancer treatment. However, additional well-designed trials should be conducted to further examine the efficacy of the infusion of NK cells.

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Disclosure of conflict of interest

None.

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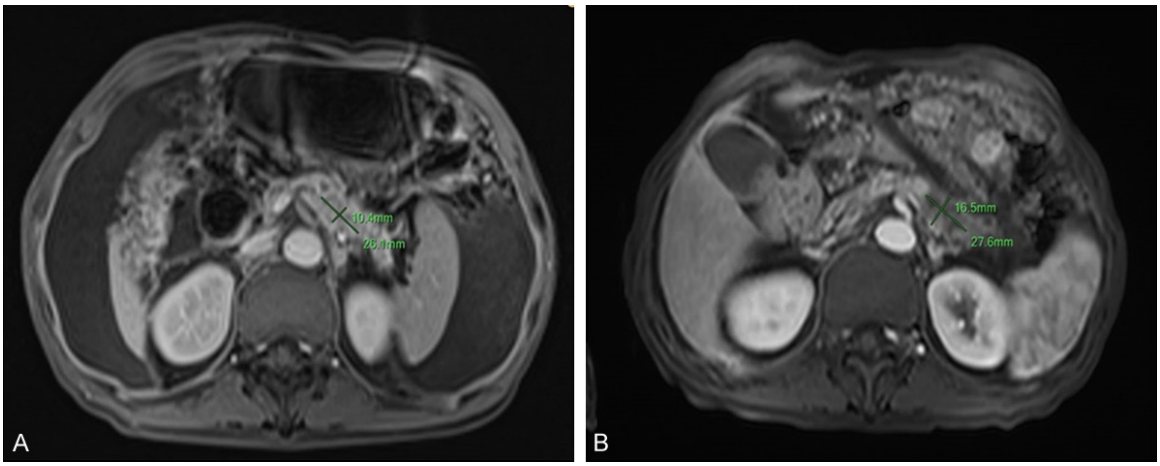

Figure S1. The representative images of contrast enhanced magnetic resonance imaging before (A) and after (B) the combined therapy.

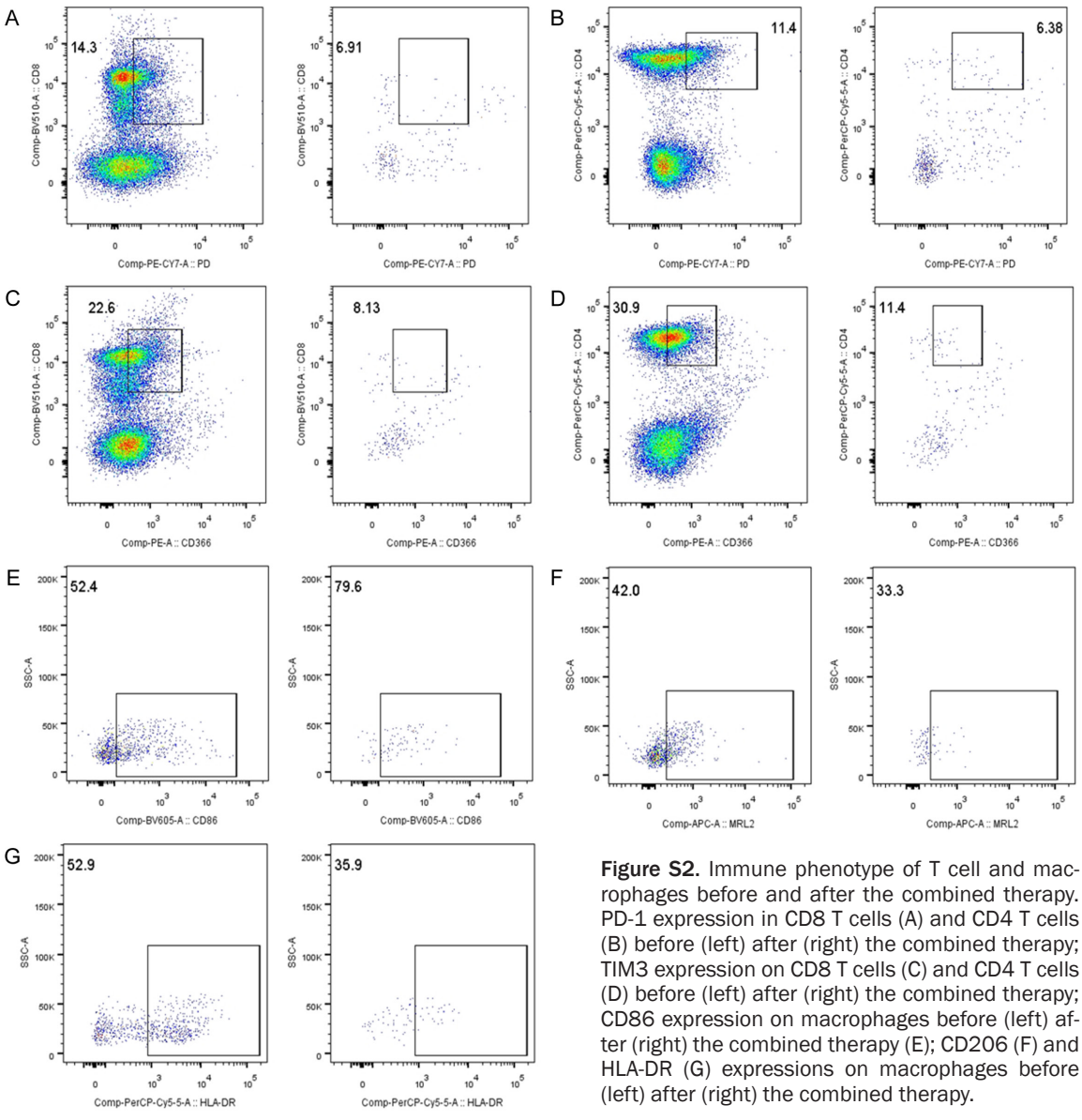

Figure S2. Immune phenotype of T cell and macrophages before and after the combined therapy. PD-1 expression in CD8 T cells (A) and CD4 T cells (B) before (left) after (right) the combined therapy; TIM3 expression on CD8 T cells (C) and CD4 T cells (D) before (left) after (right) the combined therapy; CD86 expression on macrophages before (left) after (right) the combined therapy (E); CD206 (F) and HLA-DR (G) expressions on macrophages before (left) after (right) the combined therapy.