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Adoptive Transfer of NKG2D CAR mRNA-Engineered Natural Killer Cells in Colorectal Cancer Patients

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By fusing the extracellular domain of the natural killer (NK) cell receptor NKG2D to DAP12, we constructed a chimeric antigen receptor (CAR) to improve NK cell tumor responses. An RNA electroporation approach that provides transient expression of the CAR was adopted as a risk mitigation strategy. Expression of the NKG2D RNA CAR significantly augmented the cytolytic activity of NK cells against several solid tumor cell lines *in vitro* and provided a clear therapeutic benefit to mice with established solid tumors. Three patients with metastatic colorectal cancer were then treated with local infusion of the CAR-NK cells. Reduction of ascites generation and a marked decrease in number of tumor cells in ascites samples were observed in the first two patients treated with intraperitoneal infusion of low doses of the CAR-NK cells. The third patient with metastatic tumor sites in the liver was treated with ultrasound-guided percutaneous injection, followed by intraperitoneal infusion of the CAR-NK cells. Rapid tumor regression in the liver region was observed with Doppler ultrasound imaging and complete metabolic response in the treated liver lesions was confirmed by positron emission tomography (PET)- computed tomographic (CT) scanning. Our results highlight a promising therapeutic potential of using RNA CAR-modified NK cells to treat metastatic colorectal cancer.

INTRODUCTION

Natural killer (NK) cells are innate lymphocytes that recognize and lyse transformed cells and virally infected cells without prior activation. Adoptive cancer therapy with NK cells is increasingly being tested in clinical trials due to the capability of NK cells in inducing immune responses against malignancies in an antigen-independent and non-major histocompatibility complex (MHC) restricted manner.[1] The use of NK cells for cancer immunotherapy was given a new lease of life when allogeneic NK cells were found to treat leukemia patients effectively. Importantly, transplantation from NK cell alloreactive donors has produced beneficial graft-versus-leukemia outcome and protected patients against graft rejection and detrimental graft-versus-host disease (GVHD).[2] The favorable therapeutic and safety profile of allogeneic NK cell therapy is considered as an important practical advantage over adoptive therapy of autologous T cells.[3,4] Clinically, the use of allogeneic NK cells from healthy donors offers the attractive potential to overcome cancer-treatment-caused immune defects associated with the use of patient immune cells. Significant efforts are currently undertaken to fully exploit the anti-tumor effect of NK cells in the clinic, but failures to achieve primary clinical end points, especially during the treatment of solid tumors, are still common.[1,5–8]

The cytotoxicity potential of NK cells against cancer is regulated by a panel of endogenous activating and inhibitory receptors.[9] Among the activation receptors is the natural killer group 2 member D (NKG2D) receptor. The NKG2D receptor, expressed by human NK cells, $CD8^+$ T cells, γδ T cells, and NKT cells, can interact with eight NKG2D ligands (NKG2DLs) belonging to two families: two MHC class I

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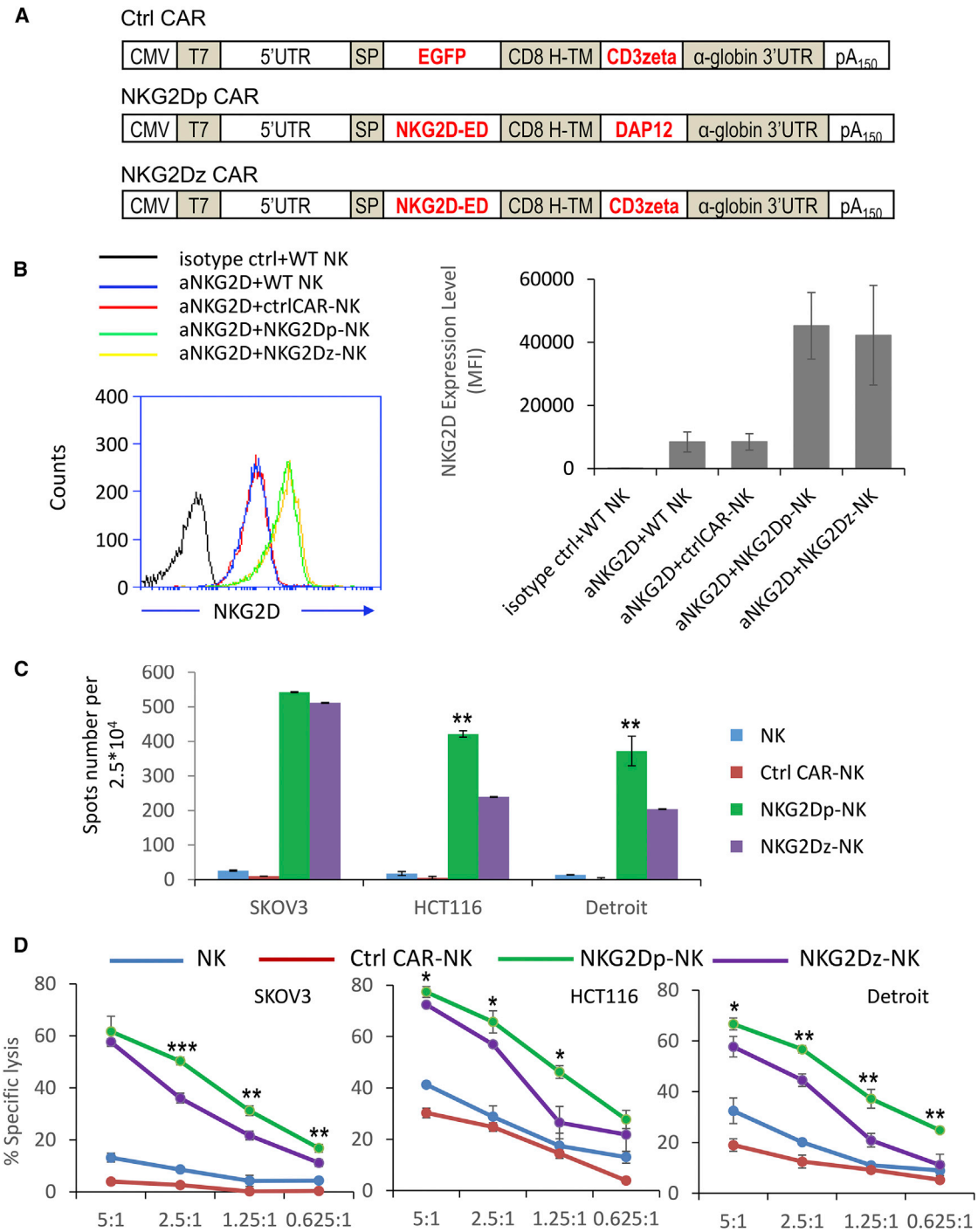


Figure 1. Construction, Expression, and Effects of NKG2D Ligand-Specific CARs

(A) Schematic diagrams of mRNA CAR constructs used in this study, including the control vector mGFP CAR, the first-generation CAR NKG2D-DAP12 (NKG2Dp), and the first-generation CAR NKG2D-CD3ζ (NKG2Dz). The DNA templates of the CARs were PCR amplified using a CMV forward primer and reverse primer with 150 Ts. The PCR amplicons were then used for RNA transcription to generate mRNA CARs for NK cell electroporation. (B) Flow cytometric analysis with an anti-NKG2D antibody to detect the surface expression of NKG2D CARs on NK cells. The analysis was performed 24 h after mRNA electroporation. Mean fluorescence intensities (MFIs) are shown on the right (n = 3). (C) IFNγ secretion as determined by an IFNγ ELISPOT assay. Human SKOV3 ovarian cancer cell line, HCT116 colon cancer cell line, and Detroit562 pharyngeal carcinoma cell lines were used. NK cells were electroporated with various NKG2D RNA CARs and co-cultured with target tumor cells at an E:T ratio of 5:1 overnight before

(legend continued on next page)

chain-related proteins MICA and MICB and six human cytomegalovirus (HCMV) UL16-binding proteins (ULBP1–6). NKG2DLs are not usually expressed on healthy tissues[10–14] but can be upregulated upon DNA damage, infection, and transformation of cells, thus being commonly detected on carcinomas.[15–18] Because of their tumor-associated overexpression, NKG2DLs have been a favorable therapeutic target for anticancer strategies.[15–18]

To improve the efficacy of adoptive NK cell therapy, chimeric antigen receptors (CARs) have been developed to modify the immune effector cells by gene transfer.[1,19–22] CARs can redirect the specificity of immune cells to cancer cell surface antigens, including NKG2DLs.[23–34] We hypothesized that after introducing a CAR specific to NKG2DLs into NK cells, the binding of the CAR to the ligands expressed on cancer cells could activate the NK cells directly, thus significantly enhancing the antitumor immunity of the cells. To test the hypothesis, we have constructed a CAR that use the extracellular domain (ED) of the human NKG2D receptor fused with the cytoplasmic domain of DAP12, an adaptor molecule associated with activating NK cell receptors and crucial in signal transduction of activated NK cells.[35,36] In order to address the concern of on-target and off-tumor toxicity of an NKG2D CAR against non-tumor tissues,[37–39] we adopted an RNA CAR approach to transiently enhance the specificity of NK cells toward NKG2DLs and their tumor-cell-killing activity. We further hypothesized that the local treatment of intraperitoneal tumors with the CAR-NK cells would be a safe and effective way. The present study aims to test the hypotheses.

RESULTS

NK Cells Expressing NKG2D CAR with DAP12 Resulted in Strong Cytotoxic Immune Responses *In Vitro*

We designed two NKG2D RNA CARs consisting of the ED of NKG2D, the CD8α hinge and transmembrane region, and the intracellular signaling domain CD3zeta (NKG2Dz) or DAP12 (NKG2Dp) (Figure 1A) and compared the expression and activity of the two constructs in NK cells *in vitro*. We adopted an mRNA electroporation approach to facilitate fast evaluation of the two constructs. RNA electroporation was optimized in NK cells using EGFP mRNA, which provided almost 99% transfection efficiency (Figure S2). While the electroporation of NK cells with the EGFP fused with CD3 zeta (mGFP), a control construct with EGFP to replace the ED of NKG2D, did not increase NKG2D expression, the increased NKG2D expression was observed after the electroporation of NK cells with NKG2Dz or NKG2Dp (Figure 1B). Time-lapse analysis of NKG2Dp CAR expression after electroporation demonstrated that RNA CAR expression maintained for at least 5 days (Figures S2 and S3).

To compare cytotoxic immune responses induced by NK cells electroporated with NKG2Dz and NKG2Dp mRNA CARs, NK cells expanded from the same donors and under the same controlled conditions were electroporated with the same amount of mRNA CARs. We first performed interferon (IFN)γ ELISPOT assays using human SKOV3, HCT116, and Detroit562 cancer cells as targets (Figure 1C). Analysis with a spot-counting machine indicated that there was stronger development of spots with CAR-equipped NK cells than with the control NK cells ($p < 0.001$). Comparing the two different NKG2D RNA CAR-modified NK cells, the IFNγ secretion levels of NKG2Dz CAR-equipped NK cells were relatively lower than those induced by NKG2Dp CAR-equipped NK cells. *In vitro* cytotoxicity assays using the Delfia cytotoxicity kit further confirmed that NKG2Dp CAR-modified NK cells were more effective in cancer cell killing as compared with NKG2Dz CAR-modified NK cells (Figure 1D). Based on these results, we selected NKG2Dp CAR-modified NK cells for downstream studies.

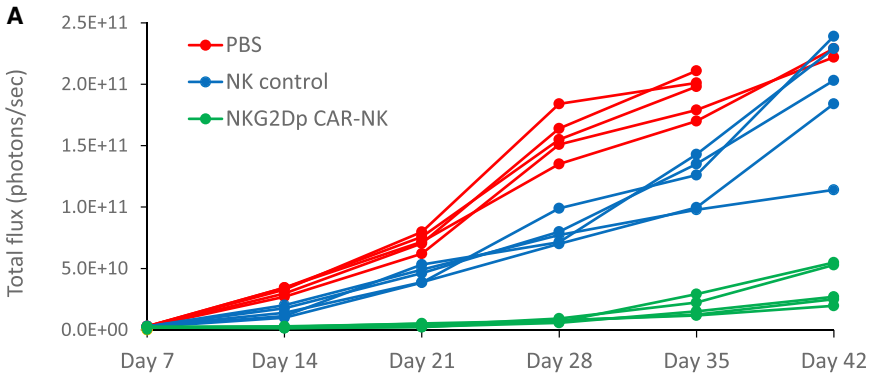
Multiple Injections of CAR-NK Cells Delay Disease Progression in Tumor-Bearing Mice

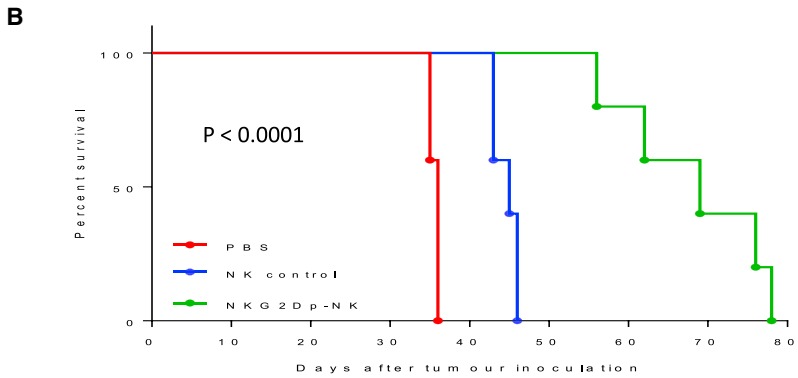
To obtain a proof of concept of the *in vivo* tumor killing effect of the NKG2D RNA CAR-modified NK cells, we established a xenograft mouse model by intraperitoneal (i.p.) injection of 1×10^7 human colorectal HCT116-Luc cancer cells into NSG mice. Seven days post-tumor-cell injection, the tumor-bearing mice were treated with i.p. injection of PBS, mock NK cells, or NK cells modified with NKG2Dp CAR (1×10^7 cells per injection) twice a week for 3 weeks. Tumor growth was monitored with non-invasive whole-body bioluminescent imaging (BLI) of HCT116-Luc cells from day 7 to day 42 (Figure 2A). BLI demonstrated that HCT116 tumors progressed aggressively in the two control groups treated with PBS and mock NK cells. In the group of mice treated with NKG2Dp CAR-modified NK cells, the tumor burdens were reduced relative to the initial tumor burdens during the 3-week treatment period but tumor regrowth was noticed after termination of the treatment. Mice in the two control groups were euthanized by day 37 and day 47, respectively, due to rapid disease progression (Figure 2B). In contrast, mice receiving NKG2Dp CAR-modified NK cells were significantly protected from rapid tumor progression and the median survival time of the mice was prolonged by 92% as compared to the PBS group and 53% as compared to the mock NK group ($p < 0.0001$; Figure 2B).

Characterization of CAR-NK Cells Generated with Blood Samples Collected from a Patient and Haploidentical Family Donors

We conducted a pilot clinical trial study in three patients with chemotherapy-refractory metastatic colorectal cancer to evaluate the safety and feasibility of adoptive cell therapy with NK cells modified by electroporation of mRNA encoding NKG2Dp CAR. The production and characterization of CAR-NK cells used for patient treatment,

assay. Mock NK cells and mGFP RNA CAR-transfected NK cells served as negative controls. Mean IFNγ spots per 2.5×10^4 cells ± SD from triplicate cultures are shown. (D) Delfia EuTDA cytotoxicity assay (2 h EuTDA culturing) to assess tumor cell lysis efficiency. The cytotoxicity of CAR-NK cells against tumor cells were observed after NKG2D CAR mRNA electroporation, but not after mGFP mRNA electroporation. The results of one representative experiment out of three are shown. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, between NKG2Dp-NK and NKG2Dz-NK.





	Median Survival (days)
PBS	36
NK	45
NKG2Dp-NK	69

Figure 2. Effects of NK Cells Electroporated with NKG2D CAR mRNA in Mouse Tumor Models

NSG mice (n = 5 per group) were i.p. injected with the HCT116-Luc human cancer cells, 1×10^7 cancer cells per mouse. CAR-NK cell treatment started 7 days after tumor cell inoculation, twice a week for 3 weeks, 1×10^7 CAR-NK cells per injection. The mice were followed with serial weekly imaging to assess the tumor burden. (A) Effects of NK cells electroporated with NKG2Dp mRNA on tumor burden over time in mice with HCT116-Luc xenografts. Tumor burden over time by BLI is shown. Each mouse is represented by one line. (B) Kaplan-Meier analysis of survival in the HCT116-Luc tumor model. Statistical analysis of survival between groups was performed using the log-rank test. Medium survival days are shown below.

including CAR-NK cell release criteria and the cell viability of the CAR-NK cells after electroporation are summarized in Table 1 and Figures S4 and S5. Autologous NK cells were prepared with 100 mL of blood from the first patient (1001), and allogeneic NK cells were prepared with 200 mL of blood from haploidentical family donors for the second and third patients (1002 and 1003). NK cell expansion was performed with K562 feeder cells expressing mbIL-15, 4-1BBL, and mbIL-21. Flow cytometry analysis was carried out before and after NK cell expansion and before and following transduction with NKG2Dp CAR construct. For the three patients, the mean percentage of $CD56^+$ $CD3^-$ cells increased from initial 11.7% to 35.1% to 96.9% (±2.83%) after expansion and $CD3^+$ T cell depletion. Electroporation was used to transfect NKG2Dp CAR mRNA into NK cells. Successful transfection was demonstrated by increase of NKG2D median fluorescent intensity (MFI) (Table 1) and *in vitro* cytotoxicity assays that confirmed the robust lysis of NKG2D ligand-positive HCT116 human colorectal carcinoma cells by CAR-NK cells (Table 1). Cells in the final products were above 90% viable before infusion. The treatment scheme for the three patients is detailed in Table 1 and illustrated in Figure S6.

Adverse Events Experienced from CAR-NK Cell Therapy

We evaluated adverse events of any grade attributable to any cause in the three patients according to Common Terminology Criteria for Adverse Events (CTCAE) v4.0 (Table 2). No CAR-NK dose reduction was required and, therefore, all patients received scheduled doses. There were no dose-limiting toxicities. No serious adverse effects (≥grade 3 adverse events) were noted in any of the three patients. Only grade 1 cytokine release syndrome was observed, and none of the patients experienced neurologic symptoms. The most common treatment-related adverse events reported included fever (Figures S7–S9), fatigue, and anorexia. Patient 1003 developed fever after the second, fourth, fifth, and sixth infusions that peaked at 40.0°C (Figure S9), associated with increased plasma interleukin (IL)-6 levels peaked at 224 pg/mL and C-reactive protein (CRP) levels peaked at 117.6 mg/L (Figure S10). The fever resolved after ice-pack application. GVHD was not observed in the two patients treated with haploidentical NK cells.

Adoptive Transfer of CAR-NK Cells into the Patient's Peritoneal Cavity Shows a Clinical Benefit in Controlling Malignant Ascites

Two patients, 1001 and 1002, with substantial disease burdens and malignant ascites (Table 3) received multiple rounds of intraperitoneal NK cell infusions in a dose-escalation manner (Table 1; Figure S6). Patient 1001 received two infusions of CAR-NK cells (2×10^7 and 1×10^8) prepared with autologous NK cells, while patient 1002 was treated with four infusions of CAR NK cells (1×10^8, 3×10^8, 5×10^8, and 7×10^8) prepared with allogeneic haploidentical NK cells. Direct local injection into the peritoneal cavity allows the quick access of CAR-NK cells to tumor sites, facilitating the interaction between

Table 1. NKG2Dp CAR-NK Cells Used in the Clinical Trial

NK Cell	Specification	Patient 1001	Patient 1002	Patient 1003
Cell Expansion				
Autologous or haploidentical	N/A	autologous	haploidentical	haploidentical
Blood vol. collected (mL)	N/A	100	200	200
% $CD56^+CD3^-$ cells	initial	11.7	13.2	35.1
% $CD56^+CD3^-$ cells	before T cell depletion	N/A	91.9	96
% $CD56^+CD3^-$ cells	after T cell depletion	N/A	93.3	96.6
% $CD56^+CD3^-$ cells	before infusion	93.7 ± 0.49	98.6 ± 0.25	98.6 ± 0.32
NK cell expansion fold	before infusion	590.8	624.8 ± 132.1	601.1 ± 95.5
CAR-NK Cell Release Criteria				
% $CD56^+CD3^-$ cells	>90	93.7 ± 0.49	98.6 ± 0.25	98.6 ± 0.32
% $CD3^+$ cells	auto <10; allo <5 × 10^5/kg	5.7 ± 0.45	7.7 × 10^4 ± 7.8 × 10^4/kg	1.7 × 10^5 ± 9.9 × 10^4/kg
Endotoxin (EU/mL)	<3.5	<0.25	<0.25	<0.25
Mycoplasma[a]	negative	negative	negative	negative
Sterility (bacterial)[a]	negative	negative	negative	negative
NKG2D MFI after transfection	>1.2-fold of the original level	2.25 ± 0.46	1.87 ± 0.59	2.52 ± 0.43
% $NKG2D^+$ cells	>95	99.3 ± 0.71	99.9 ± 0.18	97.9 ± 2.0
Cell viability after transfection	>80%	93.6% ± 1.15%	94.2% ± 3.18%	94.6% ± 1.50%
Cancer cell killing activity	>60%, CAR-NK:HCT116[b] = 10:1	96.2% ± 5.4%	86.6% ± 11.9%	93.8% ± 7.4%
Treatment Scheme				
Treatment frequency	N/A	once a week	once a week	twice a week
Total infusion/injection times	N/A	2	4	6
Dose level (E8 cells per infusion)	N/A	0.2, 1	1, 3, 5, 7,	5, 5, 10, 10, 20, 20

MFI, median fluorescent intensity.
[a]Results were not available at the time of infusion.
[b]HCT116 human colorectal carcinoma cell line.

transferred cells and cancer cells and limiting the potential on-target and off-tumor risks associated with systemic exposure to the CAR-NK cells.

We observed a significant reduction in the number of epithelial cell adhesion molecule (EpCAM)-positive cancer cells in ascites fluid in both patients (Figure 3A; Figure S11). The cytotoxicity of CAR-NK cells against cancer cells become obvious right after the infusion of the first dose of 2 × 10^7 cells. The number of cancer cells in ascites fluid continued to decrease during the course of treatment and became almost undetectable at the end of treatment. A significant reduction in volume of ascites was also observed in both patients (Figure 3B). Patient 1002 was evaluated for ascites volume change by computed tomographic (CT) imaging, which confirmed ascites reduction (Figure 3C). While the appearance of ascites was initially opalescent in patient 1002, it became clear after i.p. treatment of CAR-NK cells (Figure 3D), a clear indication of clinical benefit in controlling malignant ascites. CT scans were performed in the two patients at baseline and 2 weeks (patient 1001) or 4 weeks (patient 1002) after the initiation of the CAR-NK treatment. Both were found to have stable disease in peritoneal target lesions by CT scanning (Figure S12). A summary of clinical characteristics of the patients treated with NKG2Dp CAR-NK cells is provided in Table 3.

Ultrasound-Guided Percutaneous Injection of CAR-NK Cells Effectively Reduce Tumor Burden in a Patient with Metastatic Colon Cancer in the Liver

Patient 1003 presented with unresectable metastatic colorectal cancer that progressed after three lines of conventional systemic therapy (Table 3) and was treated with six infusions of allogeneic haploidentical CAR-NK cells: 5 × 10^8 CAR-NK cells administered 2×/week for the first week, 1 × 10^9 CAR-NK cells 2×/week in the second week, and 2 × 10^9 CAR-NK cells 2×/week in the third week. Starting from the second infusion and guided by ultrasound (Video S1), half of each dose of CAR-NK cells were percutaneously injected into cancer lesions with high metabolic activity in the liver segment VI, and the rest of the CAR-NK cells were i.p. infused. Measurable clinical responses were observed in the liver region. Doppler ultrasound imaging detected tumor area change from 66.0 mm × 28.5 mm to 46.9 mm × 24.0 mm in the liver region 4 days after one injection of 2.5 × 10^8 CAR-NK cells (Figure 4A). CT and positron emission tomography (PET) scans were performed at baseline and 4 weeks after

Table 2. Adverse Event Grading Based on CTCAE v4.0

Adverse Event (AE)[a]	Patient 1001	Patient 1002	Patient 1003
Treatment-Related AE			
Fever	1	1	2
Hypotension	1	0	0
Fatigue	1	1	1
Anorexia	1	1	1
Respiratory distress	2	1	0
Tachycardia	2	1	0
Non-Treatment-Related AE			
Nausea	0	1	0
Vomiting	0	2	0
Constipation	1	0	0
Diarrhea	0	1	0
Bloating	2	2	0
Abdominal pain	2	1	0
Back pain	0	1	0
Tinnitus	1	0	0

[a]AEs labeled as possibly/probably/definitely related were considered as treatment-related AEs and not related or unlikely related as non-treatment-related AEs for this analysis.

the initiation of the CAR-NK treatment. By fludeoxyglucose (FDG) PET/CT imaging, a decrease in the maximum standardized uptake value (SUVmax) from 8.2 to 0.14 was seen in the liver segment VI after the completion of the treatment (Figures 4B and 4C). Histological examination with H&E staining of specimens collected by needle biopsy (Figure S13) revealed that necrotic tissues with a few intestinal adenocarcinoma cells were seen in the specimens collected from the CAR-NK injected site, whereas intact adenocarcinoma tissues were clearly visible in the specimens collected from a noninjected tumor site (Figure 4D). We further performed immunohistochemistry (IHC) analysis with antibodies against NKG2DL MICA/B and two markers for adenocarcinomas of intestinal origin, Villin, and CDX2. Loss of the expression of NKG2DL MICA/B, Villin, and CDX2 was observed in the biopsy samples collected from the CAR-NK-treated regions (Figure S14). Overall, these findings suggest a therapeutic role for image-guided percutaneous injection of CAR-NK cells, a minimally invasive and repeatable procedure, in inducing a robust local antitumor effect in the patient.

DISCUSSION

The current study tested NK cells modified with a new type of NKG2D CAR, a CAR composed of the NKG2D ED and DAP12. Different from CD3ζ containing three immunoreceptor tyrosine-based activation motifs (ITAMs), DAP12 contains a single ITAM capable of binding Syk and ZAP70 kinases, providing an alternative signaling pathway involved in the antitumor activity of NK cells. Previous studies have demonstrated the functionality of DAP12-containing CARs in NK cell line YTS[40] and in human peripheral blood NK cells.[36] As demonstrated in the current study, compared to human NK cells modified with the CD3ζ-containing NKG2D CAR, NK cells expressing the DAP12-containing NKG2D CAR provided an enhanced *in vitro* cytotoxicity and increased IFNγ release. Thus, CAR design with DAP12 signaling domain offers new opportunities to enhance the antitumor activity and specificity of engineered NK cells.

Almost all the previous studies using NKG2D CARs were pursued with immune effector cells transduced with integrating viral vectors. When applied clinically, permanent DNA CAR expression mediated by integrating viral vectors cannot be simply shut off when severe toxicity associated with cytokine storm or on-target and off-tumor toxicity occurs. VanSeggelen et al.[37] have reported a strain- and preconditioning-dependent lethal toxicity in mice treated with T cells transduced with retroviral vectors encoding NKG2D DNA CARs, especially when a second-generation NKG2D CAR (NKG2D28ζ) and a NKG2D CAR co-expressed with DAP10 (NKG2Dζ10) were used.[38] CARs can also be expressed by transfection through mRNA electroporation. Using short-lived CAR-expressing cells modified with this non-integrating technology, the duration and potency of CAR effects can be controlled by different dosing and infusion schemes. Understandably, the transient expression of CARs on immune cells requires multiple infusions to achieve antitumor effects. It, however, provides good opportunity to test CAR clinical safety. Through discontinuing the infusion of mRNA CAR-modified immune effector cells, an excessive response caused by the toxicity related to recognition of normal tissues and/or cytokine storms can be stopped. Moreover, transfection of mRNA encoding a CAR is more economical in testing new CARs, particularly when performing an initial clinical trial of a novel CAR. There were two previous studies that assessed *in vitro* effects of NKG2D CAR mRNA transfected αβ T cells and NK cells, respectively,[27,29] but the impact of these RNA CARs has not been evaluated previously in *in vivo* models. Expression of the NKG2Dp RNA CAR in the current study significantly augmented the tumoricidal activity of NK cells against several solid tumor cell lines *in vitro* and displayed significant anti-tumor effects in a mouse model with established tumors.

After confirming the effectiveness of NKG2D ligand-targeting mRNA CAR NK cells *in vitro* and in animal studies, we initiated a pilot clinical trial (NCT03415100) based on the hypothesis that the local treatment of metastatic colorectal cancer by intraperitoneal infusion and intratumoral injection of the CAR-NK cells would be safe and effective. This clinical trial was performed with three vital safety considerations: (1) human primary NK cells were used as immune effector cells for CAR therapy. Given the limited lifespan of the cytotoxic cells, especially when haploidentical NK cells were used in patients,[5] there will be little concern about persistent CAR-associated side effects; (2) using a non-integrating mRNA electroporation technology for CAR gene transfection further ensures the transient nature of CAR expression; and (3) local treatment instead of systemic delivery of CAR NK cells was performed to limit systemic exposure to CAR cells.

In the current study, clinical-grade CAR-NK cell products that met release criteria were successfully manufactured for all three patients.

Table 3. Summary of Clinical Characteristics of Three Patients Treated with NKG2Dp CAR-NK Cells

Subject ID	Age/Sex	Disease	Prior Therapies	CAR-NK Treatment	GVHD	CT/PET in Target Sites	Status
1001	51/F	sigmoid colon cancer + extensive liver metastases, bone and lung metastasis + peritoneal carcinomatosis + malignant ascites	2016: 8 cycles of capecitabine 2017: 3 cycles of oxaliplatin and capecitabine (XELOX) 2017: 2 cycles of capecitabine 2017: paclitaxel liposome chemotherapy	2 i.p. infusions	–	SD	DOD
1002	48/F	sigmoid colon cancer + extensive liver metastases + peritoneal carcinomatosis + malignant ascites	2014: 8 cycles of irinotecan +fluorouracil+cetuximab 2014: surgical resection of liver metastases 2015: 4 cycles of irinotecan+fluorouracil +cetuximab 2017: 8 cycles of capecitabine	4 i.p. infusions	negative	SD	DOD
1003	51/M	rectal cancer + subcapsular liver metastases	2017: surgical resection of metastases in right testicle and sigmoid colostomy 2017: 2 cycles of oxaliplatin+capecitabine 2017: 1 cycle of irinotecan+fluorouracil 2018: 3 cycles of irinotecan+fluorouracil +bevacizumab	6 i.p. infusions + 5 local injections	negative	CMR/SD	DOD

M, male; F, female; i.p., intraperitoneal; SD, stable disease in target site; CMR, complete metabolic response; DOD, death of disease.

This technical implantation success rate is the most important feasibility endpoint. However, due to transient CAR expression feature, we were unable to evaluate the *in vivo* fate of adoptively transferred CAR-NK cells. All three patients met the safety endpoint, as the CAR-NK cells were well tolerated with no serious adverse events, including high-grade cytokine release syndrome. Multiple dose levels of CAR-NK cells were tested, but no dose-limiting toxicities occurred.

This study further provides the direct evidence that adoptively transferred NKG2Dp CAR-NK cells did recognize tumor cells and display antitumor effector functions in patients with metastatic colorectal cancer. Notably, our small patient population consisted of heavily treated, refractory patients at a late stage of colorectal cancer with multifocal metastasis and were expected to have poor survival outcomes. In two patients with malignant ascites and treated with intraperitoneal infusion of the CAR-NK cells, the number of tumor cells in ascites was apparently reduced even after infusion of a single low dose of cells and to an almost undetectable level at the end of the treatment (Figure 3A). In the third patient who was treated with ultrasound-guided percutaneous injection of CAR-NK cells in the liver, complete metabolic response was detected in the injection site (Figures 4B and 4C), demonstrating the direct effects of CAR-NK cells on metastatic colorectal cancer. The on-target argument is further supported by the findings that there was a loss of NKG2DL expression in the biopsy samples collected from the injected site (Figure S14) and that necrosis was found in the same biopsy samples (Figure 4D). In combination with the findings in the mouse tumor model shown in Figure 2 that the growth of NKG2DL-positive tumors was effectively inhibited by NKG2Dp CAR-NK cells but not by NK cells expressing a non-relevant CAR or NK cells alone, we concluded that the observed complete metabolic response was likely to be attributable, at least in part, to specific cancer cell lysis effects mediated by NKG2Dp CAR-NK cells.

NK cells are a promising alternative to T cells for CAR-based therapies owing to a number of reasons.[1,19,22] First, NK cells express an array of endogenous activating receptors that can recognize cancer cells and trigger tumor lysis via natural cytotoxicity. Even if a CAR target antigen was downregulated on cancer cells during treatment, CAR-expressing NK cells would still be able to function. Second, unlike T cells, NK cells are unable to produce autocrine growth factors such as IL-2, and this will limit their lifespan. As such, NK cells are expected to disappear relatively rapidly from the circulation, reducing the risk of long-term adverse events and eliminating the need for introducing "suicide genes" to remove CAR-expressing cells. Third, it is generally accepted that NK cells do not induce GVHD or other alloimmune or autoimmune toxicities, permitting "off-the-shelf" cellular immunotherapy with allogeneic NK cells from healthy donors. It is foreseeable that an allogeneic cell approach allows large-scale mass production of cell therapeutics associated with reduced laboriousness and simplified logistics of cell culture operations, thus significantly increasing cost effectiveness. As an allogeneic cell approach allows standardized manufacture, it would certainly be helpful to eliminate variability in the quality of cellular products, thus facilitating reliable comparative analysis of clinical outcomes.

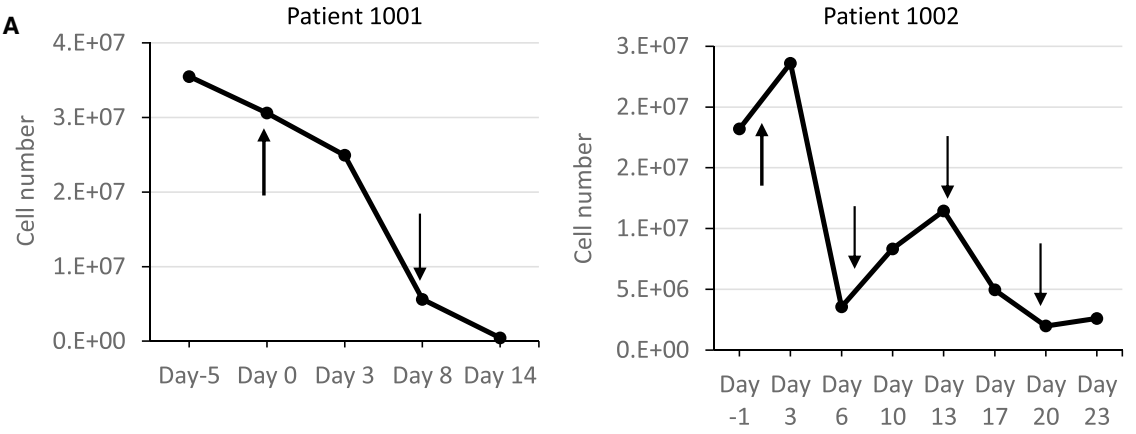
In conclusion, adoptive transfer of NKG2D CAR-expressing NK cells as reported in the current study provides a new option to improve the outcome of cancer immunotherapy and is especially attractive in developing safe "off-the-shelf" CAR therapeutics for allogeneic cell therapy.

MATERIALS AND METHODS

Cells and Cell Culture Conditions

Human peripheral blood mononuclear cells (PBMCs) used for pre-clinical studies were isolated from fresh buffy coats by density

A



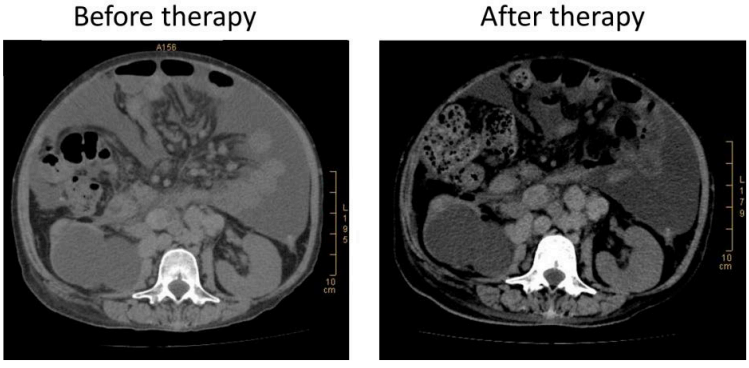
B

Patient 1001			
Ascites collection time	Total volume (ml/days)	Daily Average Vol (ml)	% change*
Before Infusion (INF)	5800/6	967	-
Post-1st INF	5500/8	688	-30%
Post-2nd INF	4850/7	693	-30%

* Percentages based on daily average volume before CAR-NK infusion

Patient 1002			
Ascites collection time	Total volume (ml/days)	Daily Average Vol (ml)	% change*
Before Infusion (INF)	28400/23	1235	-
Post-1st INF	6350/7	907	-27%
Post-2nd INF	4850/7	693	-44%
Post-3rd INF	4850/7	693	-44%
Post-4th INF	5300/9	589	-52%

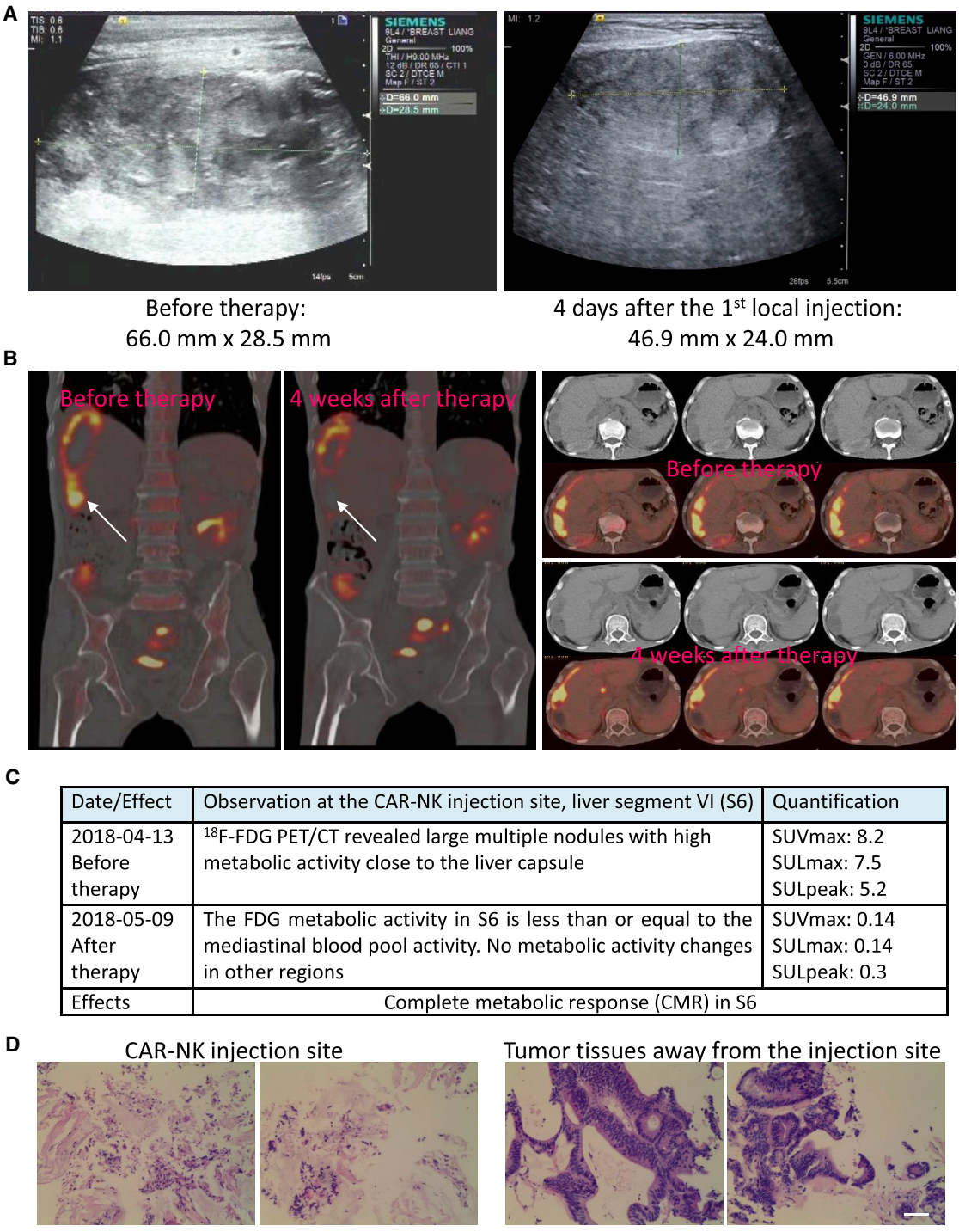
C



D

Figure 3. Effects of Intraperitoneal Injection of CAR-NK Cells on Malignant Ascites

(A) The number of EpCAM-positive cancer cells in ascites fluid following treatment. Two and four injections of CAR-NK cells were given to patients 1001 and 1002, respectively, as indicated by arrows. (B) Changes in ascites generation in patients ID001 and ID002. (C) Computed tomography (CT) findings in patient 1002. While a large volume of ascites was observed before treatment (left), the amount of ascites was reduced significantly after four courses of CAR NK-cell injections. (D) The appearance of ascites from patient 1002 before and after four courses of CAR-NK cell injections.



Date/Effect	Observation at the CAR-NK injection site, liver segment VI (S6)	Quantification
2018-04-13 Before therapy	^{18}F-FDG PET/CT revealed large multiple nodules with high metabolic activity close to the liver capsule	SUVmax: 8.2 SULmax: 7.5 SULpeak: 5.2
2018-05-09 After therapy	The FDG metabolic activity in S6 is less than or equal to the mediastinal blood pool activity. No metabolic activity changes in other regions	SUVmax: 0.14 SULmax: 0.14 SULpeak: 0.3
Effects	Complete metabolic response (CMR) in S6	

Figure 4. Effects of Ultrasound-Guided Local Injection of CAR-NK Cells on Metastatic Colon Cancer

(A) Effects of local injection of CAR-NK cells in the liver region as demonstrated with ultrasound imaging in patient 1003. (B) Positron emission tomography-computed tomography (PET-CT) images of cancer lesions. Left: Coronal PET-CT scan with evidence of CAR-NK effects in S6. Right: cross-section images of CT and PET/CT to show changes in S6 after CAR-NK therapy. (C) Quantification of the metabolic activity of tumor lesions in S6. (D) Histological examination of needle biopsy specimens. Left: specimens collected from the CAR-NK injected site. Right: specimens collected from a tumor site. Scale, 2 cm.

gradient centrifugation using Ficoll-Paque (GE Healthcare, Milwaukee, WI, USA). NK cell expansion was carried out using NK cell growth media composing of GMP serum-free AIM V (Life Technologies, Gaithersburg, MD, USA) supplemented with 1% human AB serum (Gemini Bio-Products, Calabasas, CA, USA) and 50 IU/mL IL-2 (PeproTech, Rocky Hill, NJ, USA). PBMCs were co-cultured with gamma-irradiated K562 feeder cells at a 1:1 ratio in NK cell growth media. In summary, 2×10^6 PBMCs were co-cultured with 2×10^6 K562 feeder cells expressing mbIl15, 4-1BBL, and mbIL21 in 10 mL of NK media in standing T75 flasks. Half media change was carried out every 2 days for 10 days. Then 2×10^6 expanded cells were further co-cultured with 2×10^6 K562 feeder cells in 10 mL of NK cell media in standing T75 flasks. Half media change was carried out every 2 days for 7 days. By using this method, NK cells, with an initial frequency of approximately 10% in PBMCs, could be enriched to 90% in 17 days and undergo around 7000-fold expansion. NK cells can possibly be cryopreserved, but recovery was poor upon thawing. Human solid tumor cell lines SKOV3-luc and Detroit562 were purchased from ATCC (Manassas, VA, USA) and HCT116-luc was purchased from Perkin Elmer (Norwalk, CT, USA). Detroit562 was cultured in Eagle's minimal essential medium (EMEM) (ATCC) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA). SKOV3-luc and HCT116-luc were cultured in McCoy's 5A supplemented with 10% FBS.

Construction of Chimeric NKG2D CAR Vectors and Generation of CAR NK Cells

To construct NKG2D RNA CAR vectors, the ED of human NKG2D was amplified by PCR from a PBMC cDNA library. NKG2D CAR vectors were generated by fusing NKG2D-ED to the CD8α hinge and transmembrane region and DAP12 or CD3ζ signaling moiety. The mGFP control vector was generated by replacing the NKG2D ED part with the GFP encoding sequence. PCR was performed to generate mRNA template encoding the NKG2D CARs, which were then transcribed *in vitro* to mRNA molecules and electroporated into NK cells. For details, see the Supplemental Information.

Flow Cytometric Analysis, Cytotoxicity Assay, and Animal Experiments

Flow cytometric analysis was performed with Accuri C6 cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The cytolytic activity of CAR-modified NK cells was examined with a non-radioactive method (DELFIA EuTDA cytotoxicity reagents kit, Perkin Elmer, MA, USA).

NSG mice were inoculated via i.p. injection of 1×10^7 HCT116-luc or SKOV3-Luc cells to generate tumor models. To investigate *in vivo* anti-tumor effects of CAR-NK cells, 1×10^7 of human NK cells electroporated with NKG2Dp RNA CAR were i.p. injected into tumor-bearing mice twice a week for 3 weeks, starting from 7 days after tumor cell inoculation.

For details, see the Supplemental Information.

Patient Eligibility and Clinical Study Design

With an IRB approval from the Third Affiliated Hospital of Guangzhou Medical University (Guangzhou, China), an open label pilot study (NCT03415100) was initiated to evaluate the safety and feasibility of NKG2D CAR-NK cell therapy in three patients with chemotherapy-refractory metastatic colorectal cancer. The clinical study was performed in accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained from each patient before they entered the study. Eligible patients were diagnosed with colorectal cancer via pathological examinations and with metastatic disease that was confirmed via adequate radiological imaging. The patients failed at least two lines of systemic therapy. Exclusion criteria (Supplemental Information) were as follows: acute or chronic infections, relevant concomitant hematological, cardiovascular, pulmonary, hepatic, renal, pancreatic, or endocrinal disease, with immune deficiency, autoimmune diseases, severe allergic disorders, or exposure to cell-based therapy in the preceding 3 months. Those receiving systemic steroid therapy or who were pregnant or lactating were also excluded.

The clinical trial scheme is shown in Table 1 and Figure S6. Patients received multiple rounds of CAR-NK cell infusions. The primary endpoint was to determine the feasibility and safety of the treatments. Severity of adverse events was graded using the National Cancer Institute CTCAE v4.0. The secondary endpoints included assessment of clinical responses to NKG2D CAR-NK cell therapy. CT and PET examinations were performed at baseline and 2 weeks (patient 1001) or 4 weeks (patient 1002 and 1003) after the initiation of the CAR-NK treatment. The study radiologists (Tianfa Dong, Jinshan Zhang) graded responses according to modified RECIST (mRECIST).

To prepare CAR-NK cells for the clinical study, blood samples were collected from a patient (100 mL) or haploidentical family donors (200 mL) to isolate PBMCs. NK cells were expanded from PBMCs as described above in G-Rex 100 vessels (Wilson Wolf Manufacturing), supplemented with 1% human AB serum (Gemini Bio-Products, Calabasas, CA, USA) and 50 IU/mL human recombinant IL-2 (Beijing Four Rings Biopharmaceutical, China). NKG2Dp CAR NK cells were prepared as described above and in the Supplemental Information (Figure S1) and administered i.p. via a peritoneal access catheter to patients. Percutaneous injection of CAR-NK cells was performed under real-time ultrasound (SIEMENS Acuson S2000) guidance with a 3 to 6 MHz probe. The patient was placed in the left lateral position, and the ultrasound scan was performed on the right anterior iliac crest. Two tumor sties without major blood vessels were selected for the injection, 5×10^8 to 2×10^9 CAR NK cells in 50–70 mL saline each time, through the seventh and eighth rib intercostal space with a 19G disposable puncture needle. To evaluate the antitumor effects of NKG2D CAR-NK cell therapy on malignant ascites, peritoneal lavage fluid was collected through a catheter. Tumor cells in ascites were analyzed by flow cytometry for the presence of EpCAM-positive cells.

Statistical Analysis

Data are presented as mean ± standard deviation (SD). All statistics were performed with GraphPad Prism 7. p values < 0.05 were considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.ymthe.2019.03.011.

AUTHOR CONTRIBUTIONS

L.X. and X.H.X. conceived and designed the research. L.X., H.G., and X.L. performed the preclinical experiments. L.X., D.C., H.G., Y.S., N.H., H.X., Q.J., L.S., and W.L. performed the clinical trial experiments. L.X., D.C., H.G., K.W., G.Y., T.D., S.W., P.Z., J.Z., W.L., J.R., Y.T., C.C., and X.H.X. analyzed data. L.X. and X.H.X. wrote the manuscript.

CONFLICTS OF INTEREST

The costs of the study were partially covered by Youshan Biomedical Co., Ltd. (Hangzhou, China). The study sponsors had no involvement in study design, collection and interpretation of data, writing the report, and the decision to submit the report for publication. All other authors in the Third Affiliated Hospital of Guangzhou Medical University and Guangzhou Regenerative Medicine, Health-Guangdong Laboratory (GRMH-GDL), Guangzhou, and the Affiliated Hangzhou First's People Hospital, Zhejiang University School of Medicine, Hangzhou, China, have declared that there are no financial conflicts of interest related to this work.

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