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Clinical Trial Results

Adoptive Transfer of Autologous Invariant Natural Killer T Cells as Immunotherapy for Advanced Hepatocellular Carcinoma: A Phase I Clinical Trial

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Key Words. Hepatocellular carcinoma • Natural killer T cells • Immunotherapy

Trial Information

- **ClinicalTrials.gov Identifier:** NCT03175679
- **Sponsors:** Beijing YouAn Hospital
- **Principal Investigator:** Jun Lu
- **IRB Approved:** Yes

Lessons Learned

- Administration of autologous invariant natural killer T (iNKT) cells was safe and well-tolerated in patients with hepatocellular carcinoma (Barcelona Clinic Liver Cancer stage B/C).
- Expanded iNKT cells produced T-helper 1–like responses with possible antitumor activity.
- No severe adverse events were observed in any of the enrolled patients, including one patient who received 10^{10} in vitro–expanded autologous iNKT cells as a single infusion.

Abstract

Background. Invariant natural killer T cells co-express T-cell antigen receptor and natural killer (NK) cell receptors. Invariant natural killer T (iNKT) cells exhibit antitumor activity, but their numbers and functions are impaired in patients with hepatocellular carcinoma (HCC). The adoptive transfer of iNKT cells might treat advanced HCC.

Methods. This phase I study (NCT03175679) enrolled 10 patients with HCC (Barcelona Clinic Liver Cancer [BCLC] stage B/C) at Beijing YouAn Hospital (April 2017 to May 2018). iNKT cells isolated from peripheral blood mononuclear cells (PBMCs) were expanded and alpha-galactosylceramide (α-GalCer)–pulsed. Dosage escalated from 3×10^7 to 6×10^7 to 9×10^7 cells/m^2 (3+3 design). An exploratory dose trial (1×10^{10} cells/m^2) was conducted in one patient.

Results. Expanded iNKT cells produced greater quantities of T-helper 1 (Th1) cytokines (e.g., interferon-gamma, perforin, and granzyme B) but less interleukin-4 than nonexpanded iNKT cells. Circulating numbers of iNKT cells and activated NK cells were increased after iNKT cell infusion. Most treatment-related adverse events were grade 1–2, and three grade 3 adverse events were reported; all resolved without treatment. Four patients were progression-free at 5.5, 6, 7, and 11 months after therapy, and one patient was alive and without tumor recurrence at the last follow-up. Five patients died at 1.5 to 11 months after treatment.

Conclusion. Autologous iNKT cell treatment is safe and well-tolerated. Expanded iNKT cells produce Th1-like responses with possible antitumor activity. The antitumor effects of iNKT cell infusion in patients with advanced HCC merit further investigation. ***The Oncologist*** 2021;26:e1919–e1930

Discussion

iNKT cells have potent antitumor activities [1–4]. Decreases in iNKT cell number and function correlate with a poor clinical outcome [5, 6]. Therefore, activated iNKT cells could be used therapeutically, either alone or in combination with other treatments, to reverse the defects in iNKT cell number and function [7–10].

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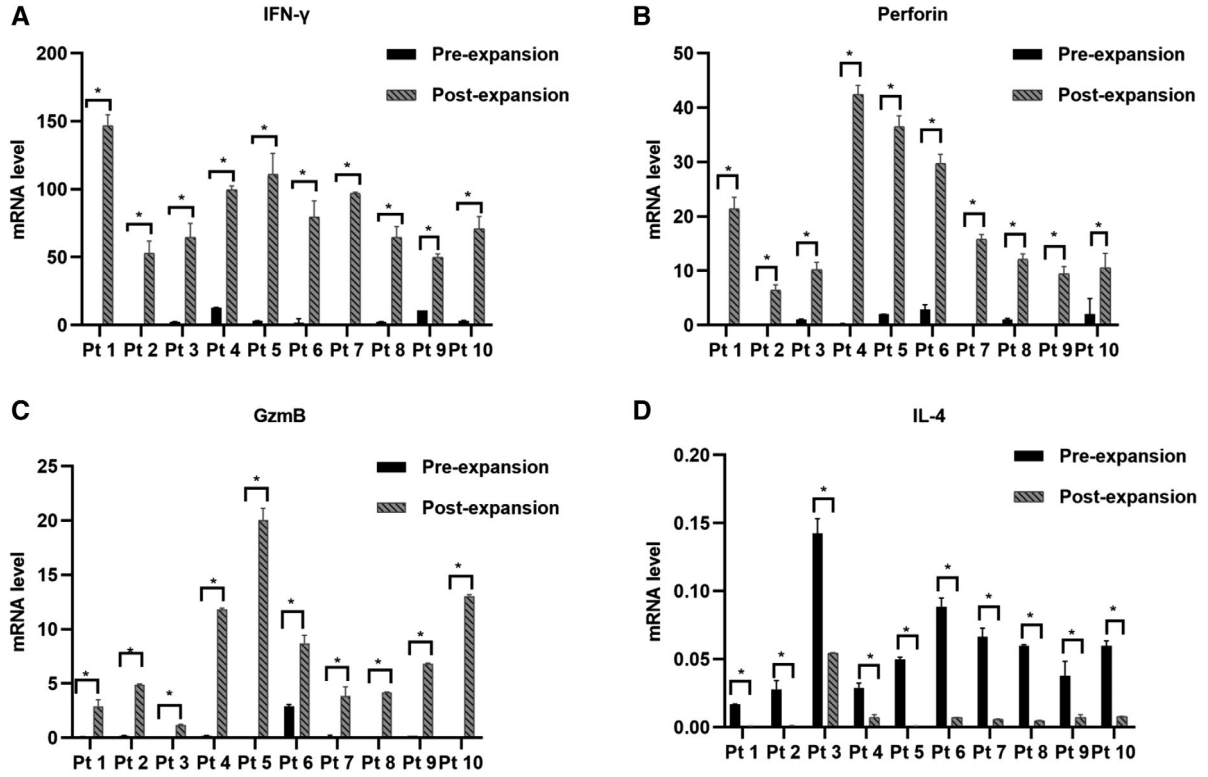

Figure 1. Expression of cytokines by invariant natural killer T (iNKT) cells before and after in vitro expansion. Quantitative real-time polymerase chain reaction was used to quantify the mRNA expression of T-helper (Th) 1 and Th2 cytokines by iNKT cells before and after cultivation and expansion. **(A):** The Th1 cytokine, IFN-γ. **(B):** The Th1 cytokine, perforin. **(C):** The Th1 cytokine, GzmB. **(D):** The Th2 cytokine, IL-4. All data were obtained from three independent experiments ($n = 3$). *$p < .001$.
Abbreviations: GzmB, granzyme B; IFN-γ, interferon-gamma; IL-4, interleukin-4; Pt, patient.

Initial research evaluating the use of α-GalCer, an agonist of CD1d and an NK T-cell stimulator, showed that α-GalCer–loaded dendritic cells (DCs) evoked significant antitumor immune response [11]. Antigen-presenting cells loaded with α-GalCer showed effective immune responses against tumors [7, 12–14]. The adoptive transfer of activated iNKT cells to restore iNKT cell numbers in patients with cancer might be a more promising approach than the intravenous administration of α-GalCer [15]. The safety, feasibility, and clinical effects of adoptive transfer of iNKT cells have been described for patients with melanoma [15], non-small cell lung cancer [16], and head and neck squamous cell carcinoma [17].

The liver is a unique immunological site. iNKT cells are tissue-homing and enriched in the liver more than any other organ [18]. iNKT cells activated by α-GalCer (KRN7000) exert antitumor immune responses and antimetastatic effects in murine models [19, 20]. Numerical and functional defects in iNKT cells were found in patients with HCC and positively correlated with recurrence [5, 21].

The primary goal of this study was to validate the safety and feasibility of using adoptive transfer of in vitro–expanded and activated autologous iNKT cells as a potential treatment for patients with HCC. Therefore, we conducted a phase I trial of 10 patients with advanced or recurrent HCC. Three grade 3 adverse events were reported, and most adverse events were grades 1–2, such as transitory fever that might be due to the subcutaneous injection of low-dose interleukin (IL)-2, resolved without treatment within 1 week after iNKT infusion. There were no signs of dose-limiting toxicities (DLTs) and graft-versus-host disease for the doses tested, which ranged from 3×10^7 cells/m^2 given as three infusions to a total of 1.0×10^{10} cells administered as a single infusion. Moreover, the cytokine profile of iNKT cells before and after in vitro culture was examined using quantitative reverse-transcription polymerase chain reaction (qRT-PCR). After expansion, the mRNA expression levels increased 4.57–523.77-fold for interferon-gamma (IFN-γ), 3.02–899.48-fold for granzyme B (GzmB), and 5.25–653.51-fold for perforin, but the mRNA expression of IL-4 decreased 2.62–62.85-fold after expansion (Fig. 1). These results indicate that α-GalCer–pulsed in vitro expansion of autologous iNKT cells restores their production of Th1-like cytokines and hence might enhance their antitumor actions. The median progression-free survival (PFS) was 6.5 (1.5–16) months, whereas the median overall survival (OS) was 13 (1.5–16) months.

Therefore, we consider this therapy to be feasible, safe, and easily combined with other antitumor strategies for patients with advanced HCC, allowing for further clinical studies to refine the technique and evaluate its efficacy.

Trial Information	
Disease	Hepatocellular carcinoma
Stage of Disease/Treatment	Metastatic/advanced
Prior Therapy	No designated number of regimens
Type of Study	Phase I, 3+3
Primary Endpoint	Safety
Secondary Endpoint	Tolerability

Additional Details of Endpoints or Study Design

Study design and participants : This prospective clinical trial was a single-center, phase I study (NCT03175679) conducted at Beijing YouAn Hospital, Capital Medical University, Beijing, China, between April 2017 and May 2018. This study was performed in accordance with the Declaration of Helsinki. The protocol and the consent form for the study were approved by the ethics committee of Beijing YouAn Hospital (Jing You Ke Lun [2017] No. 05). All patients provided written informed consent.

The inclusion criteria were as follows: (a) 18–80 years old; (b) HCC of BCLC stage B/C; (c) life expectancy of at least 12 weeks; and (d) adequate hematological and renal function, as indicated by a white blood cell count ≥3 $\times$ 10^9/L, lymphocyte count ≥0.8 $\times$ 10^9/L, platelet count ≥50 $\times$ 10^9/L, hemoglobin concentration ≥ 85 g/L, and serum creatinine concentration ≤ 1.5 times the upper limit of normal. Patients with tumor recurrence who had received previous chemotherapy or local therapy, such as surgery, radiotherapy, hepatic arterial embolization, chemoembolization, radiofrequency ablation, percutaneous injection, or cryoablation, were eligible for enrolment in the study provided the therapy had been performed at least 4 weeks before study entry. The exclusion criteria were (a) known history of syphilis or infection with human immunodeficiency virus, (b) clinically serious infection, (c) history of stem cell transplantation or organ allotransplantation, (d) history of severe hypertension or cardiac disease, (e) central nervous system tumor, (f) autoimmune disease requiring systemic therapy with immunosuppressive agents, (g) history of allergy to immunotherapy or related drugs, and (h) pregnant or breastfeeding.

***Preparation of iNKT cells*:** All procedures were performed according to Good Manufacturing Practice (GMP) standards. Isolation and culture of PBMCs were carried out in a GMP-compliant laboratory (Beijing GeneKey Co. Lab, Beijing, China). Briefly, PBMCs were isolated by leukapheresis using a COM.TEC apheresis system (Fresenius Medical Care, Bad Homburg, Germany) and Lymphoprep density gradient medium (Stemcell Technologies, Cambridge, UK). PBMCs were resuspended in Corning serum-free cell medium KBM581 (Corning Inc., Corning, NY) and stimulated with 100 ng/mL α-GalCer (BioVision, Milpitas, CA) and 100 U/mL animal-free recombinant human interleukin-2 (rhIL-2; BioLegend, San Diego, CA) for 48 hours; the rhIL-2 was replenished every other day. Mature, monocyte-derived DCs were obtained from PBMCs stimulated by 1,000 IU/mL granulocyte-macrophage colony-stimulating factor (BioLegend) and IL-4 (BioLegend) for 1 week. On day 7, iNKT cells were positively sorted using anti-iNKT microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. The sorted iNKT cells were co-cultured with mature DCs for 14 days, and cells were collected for infusion on day 21. An aliquot of cells was used for immunophenotyping and functional testing. The frequency of iNKT cells was determined using a FACSCanto II cytometer (BD Biosciences, San Jose, CA) and expressed as a percentage of lymphocytes. The criteria for iNKT cell administration included a negative bacterial culture, a negative mycoplasma test result, and an endotoxin level < 0.05 units/mL [22].

***Administration of iNKT cells to patients*:** In this phase I dose-escalation study, the patients received different doses of in vitro–expanded autologous iNKT cells through intravenous infusion. The dosage was escalated from 3 $\times$ 10^7 cells/m^2 to 6 $\times$ 10^7 cells/m^2 to 9 $\times$ 10^7 cells/m^2. For each patient, 10%, 30%, and 60% of the total dose of iNKT cells were administered over 3 consecutive days (Table 2). Tegafur (40–60 mg twice a day) was given for 2 weeks before iNKT cell infusion as lymphodepleting chemotherapy. Human IL-2 (25,000 IU/kg per day) was administered for 5 days after iNKT cell infusion to enhance the expansion and persistence of the iNKT cells. Antiviral drugs were administered immediately if a patient tested positive for hepatitis B virus (HBV) DNA or hepatitis C virus (HCV) RNA. DLT was defined as grade ≥ 3 for any adverse event related to the administration of cultured iNKT cells. If none of the first three patients experienced DLT, another three patients were treated at the next higher dose level. However, if one of the first three patients experienced DLT, three more patients were treated at the same dose level. Dose escalation continued until at least two patients among a cohort of three to six patients experienced DLT. The maximum tolerated dose (MTD) was defined as the last dose level, and the dosage would be up to 1 $\times$ 10^{10} cells/m^2 if no MTD was observed after a 3 3 design.

***Outcomes and follow-up*:** During the first 12 weeks, patients were monitored every 2–4 weeks after infusion to evaluate the safety of the therapy. Laboratory tests and physical examinations were performed at weeks 2, 4, 8, and 12 after iNKT infusion, and the clinical characteristics of the patients were recorded, including HBV infection status (detection of HBV s antigen), HCV infection status (detection of HCV RNA), serum alpha-fetoprotein (AFP), Child-Pugh score (to assess hepatic function) [23], and Karnofsky performance status score (to evaluate general well-being and activities of daily living) [24]. The patients were then followed up every 3 months from 12 weeks after infusion (Fig. 2). All laboratory tests were carried out by the Laboratory Department of Beijing YouAn Hospital. Adverse events and changes in laboratory values were graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 4.03. For immunomonitoring, samples of PBMCs were obtained from patients and frozen in Cellbanker2 (ZENOAQ, Fukushima, Japan) with liquid nitrogen until use.

***Flow cytometric analysis of immune cell subtypes in peripheral blood*:** Two samples of peripheral blood (10–15 mL) were collected in two heparin-treated tubes, one of which was used for an automated complete blood count (Sysmex SF-3000 automated hematology analyzer, Toa Medical Electronics, Kobe, Japan) and the other for flow cytometry. PBMCs were

separated using Lymphoprep (Stemcell Technologies). For flow cytometric analysis, PBMCs from all samples were incubated with the following antibodies for 20 minutes at room temperature in the dark: anti-CD3–fluorescein isothiocyanate (FITC), anti–T-cell receptor (TCR) Vα24-Jα18–phycoerythrin (PE), anti-CD8–peridinin chlorophyll protein complex (PerCP)/cyanin (Cy) 5.5, anti-CD4–PE/Cy7, anti-CD56–allophycocyanin (APC), anti-CD69–PB, anti-CD279 (programmed death-1 [PD-1])–PE, anti-CD127–APC, anti-CD25–APC/Cy7, anti-CD4–FITC, anti-CD11b–FITC, anti-CD45RA–PE, and anti–C-C chemokine receptor 7 (CCR7)–PerCP/Cy5.5 (BioLegend). Then, the cells were washed once in phosphate-buffered saline and analyzed using a FACSCanto II flow cytometer (BD Biosciences). The frequencies of iNKT cells ($CD3^+$ TCR $V\alpha24^-$ $J\alpha18^+$), NK cells ($CD3^-$ $CD56^+$), activated NK cells ($CD3^-$ $CD56^+$ $CD69^+$), regulatory T cells ($CD4^+$ $CD25^+$ $CD127^{low}$), programmed death-1^+ $CD8^+$ ($CD8^+$ PD-1^+) T cells, myeloid-derived suppressor cells ($CD11b^+$ $CD33^+$), central memory T cells ($CCR7^+$ $CD45RA^-$), and effector memory T cells ($CCR7^-$ $CD45RA^+$) were measured using FlowJo 7.6.1 software (TreeStar, Ashland, OR). The absolute count of each immune cell type was calculated by multiplying the flow cytometry–derived cell percentage by the absolute lymphocyte count.

***Detection of intracellular IFN-γ by immunohistochemistry*:** PBMCs and cultured iNKT cells were stimulated for 5 hours with phorbol myristic acid (50 ng/mL, Sigma-Aldrich, St. Louis, MO), ionomycin (1 μg/mL, Sigma-Aldrich), and brefeldin A (5 μg/mL, Sigma-Aldrich) at 37°C in 5% CO_2. Cells were stained with anti-CD3–APC (BioLegend), anti-TCR Vα24Jα18–PE (BioLegend), and anti-CD8–PerCP/Cy5.5 (BioLegend) antibodies for 20 minutes at room temperature in the dark. Then, the cells were fixed and permeabilized with fix/perm buffers (BD Biosciences), washed with phosphate-buffered saline, and incubated with anti–IFN-γ–FITC antibody (BioLegend) for 20 minutes. Finally, the cells were washed and analyzed using a FACSCanto II flow cytometer (BD Biosciences). The mean fluorescence intensity (MFI) value for IFN-γ expression by the $CD8^+$ iNKT cell population was obtained using FlowJo 7.6.1 software (TreeStar).

***Measurement of the mRNA expressions of IFN-γ, GzmB, IL-4, and perforin by qRT-PCR*:** iNKT cells were sorted from PBMCs by flow cytometry. Total RNA was isolated from iNKT cells using the Mini BEST Universal RNA Extraction kit (TakaraBio, Shiga, Japan). cDNA was synthesized according to the manufacturer's protocol. qRT-PCR was performed on a StepOnePlus Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Waltham, MA) using SYBR Premix Ex Taq (Clontech Laboratories, Mountainview, CA). The primers for IFN-γ, GzmB, IL-4, and perforin are shown in Table 3 . The polymerase chain reaction conditions were as follows: 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Data were normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase, which was used as the reference (endogenous control). The $2^{-\Delta\Delta}$CT method was used for relative quantification of mRNA level.

***Statistical analysis*:** Data were analyzed using SPSS 17.0 software (SPSS Inc., Chicago, IL) and are presented as means ± SD. Comparisons between two groups were made using two-tailed unpaired Student's *t* tests. A value of $p < .05$ was considered significant.

Investigator's Analysis	Active and should be pursued further

Drug Information

Generic Name	Autologous iNKT cells
Company Name	GeneKey GMP Lab for iNKT Cell Culture
Drug Type	Immune cells
Drug Class	Immune therapy
Dose	Escalation from 3×10^7 cells/m^2 to 6×10^7 cells/m^2 to 9×10^7 cells/m^2
Route	Intravenous infusion
Schedule of Administration	10%, 30%, and 60% of the total dose of iNKT cells were administered over 3 consecutive days for each patient; see Table 2 for details.

Dose-Escalation Table

Dose level	Dose of drug: Autologous iNKT cells	Number enrolled	Number evaluable for toxicity
1	3×10^7 cells/m^2	3	3
2	6×10^7 cells/m^2	3	3
3	9×10^7 cells/m^2	3	3
Exploratory	1×10^{10} cells/m^2	1	1

Patient Characteristics

Number of Patients, Male	9
Number of Patients, Female	1
Stage	At study entry: BCLC stage B: two patients (20%); stage C: eight patients (80%)
Age	Median (range): 51.5 (37–60) years
Number of Prior Systemic Therapies	Median (range): 2(0–5)

Other	Performance status (Child-Pugh): A, seven (70%) patients; B, three (30%) patients Virus infection: HBV, eight (80%) patients HCV: one (10%) patient None: one (10%) patient See also Table 1 for detailed patient characteristics.
Cancer Types or Histologic Subtypes	Hepatocellular carcinoma: 10

Primary Assessment Method	
Title	Phase I Clinical Trial of iNKT Cells for HCC
Number of Patients Screened	12
Number of Patients Enrolled	10
Number of Patients Evaluable for Toxicity	10
Number of Patients Evaluated for Efficacy	10
Evaluation Method	Survival (PFS and OS); immune Response Evaluation Criteria In Solid Tumors
(Median) Duration Assessments PFS	6.5 months, confidence interval: 1.5 to >16
(Median) Duration Assessments OS	13 months, confidence interval: 1.5 to >16

Outcome Notes	
Evaluation Method	Survival (PFS and OS)
Duration assessments PFS	Median (range): 6.5 (1.5 to >16) months
Duration assessments OS	Median (range): 13 (1.5 to >16) months
Expression of cytokines after iNKT therapy	IFN-γ in CD8-positive cells (MFI values:): increased from 146–231 to 293–458; IFN-γ: increased 4.57–523.77-fold; GzmB: increased 3.02–899.48-fold; Perforin: increased 5.25–653.51-fold; IL-4: decreased 2.62–62.85-fold
AFP levels after iNKT therapy	Maintained or decreased
Others	For details, see Figs. 2, 3 and supplemental online Figs. 1, 2.

Adverse Events

All Dose Levels, All Cycles

Name	NC/NA	1	2	3	4	5	All Grades
Chills	80%	20%	0%	0%	0%	0%	20%
Fever	70%	30%	0%	0%	0%	0%	30%
Pain	90%	10%	0%	0%	0%	0%	10%
Fatigue (asthenia, lethargy, malaise)	80%	20%	0%	0%	0%	0%	20%
Anemia	80%	20%	0%	0%	0%	0%	20%
Platelet count decreased	60%	10%	30%	0%	0%	0%	40%
Neutrophil count decreased	90%	10%	0%	0%	0%	0%	10%
White blood cell decreased	70%	30%	0%	0%	0%	0%	30%
Blood bilirubin increased	30%	50%	0%	20%	0%	0%	70%
Alanine aminotransferase increased	80%	20%	0%	0%	0%	0%	20%
Aspartate aminotransferase increased	60%	30%	0%	10%	0%	0%	40%
Induration/fibrosis (skin and subcutaneous tissue)	90%	10%	0%	0%	0%	0%	10%

Adverse Events Legend
Summary of adverse events in all 10 patients for entire time on study (all grades).
Abbreviation: NC/NA, no change from baseline/no adverse event.

Serious Adverse Events		
Name	**Grade**	**Attribution**
Cirrhosis and upper gastrointestinal bleeding	5	Unrelated
Refractory cholangitis/toxic megacolon	5	Unrelated
Rupture of the tumor	5	Unrelated

Serious Adverse Events Legend
Patients 4, 8, and 9 died due to complications associated with severe cirrhosis and upper gastrointestinal bleeding; patient 2 died due to refractory bile duct infection and toxic megacolon; and patient 10 died due to spontaneous rupture of the tumor. These were not considered related to the iNKT cell therapy.

Dose-Limiting Toxicities Table				
Dose level	**Dose of Drug: Autologous iNKT cells**	**Number enrolled**	**Number evaluable for toxicity**	**Number with a DLT**
1	3×10^7 cells/m^2	3	3	0
2	6×10^7 cells/m^2	3	3	0
3	9×10^7 cells/m^2	3	3	0
Exploratory	1×10^{10} cells/m^2	1	1	0

Assessment, Analysis, and Discussion	
Completion	Study completed
Investigator's Assessment	Active and should be pursued further

Invariant natural killer T (iNKT) cells are a unique lymphocyte subpopulation [25]. iNKT cells are CD1d-restricted, are characterized by an invariant T-cell receptor (TCR) α-chain, and express several receptors in common with natural killer (NK) cells [25, 26]. These lymphocytes share distinct recognition systems with T cells or NK cells [27]. iNKT cells recognize glycolipid ligands presented by CD1d [28–30], which is a monomorphic major histocompatibility complex class-I–like glycoprotein that binds distinct lipid-based antigens [28].

After activation, iNKT cells respond very rapidly to TCR and/or cytokine signals with immediate and copious production of cytokines such as interferon-gamma (IFN-γ) and interleukin-4 (IL-4), which transactivate other immune cells such as NK cells, T cells, B cells, and dendritic cells (DCs) [25, 31]. Beside the production of cytokines, iNKT cells also have potent antitumor activity mediated through Fas ligand and perforin-dependent mechanisms [11, 32]. Therefore, iNKT cells exert potent antitumor and immunoregulatory effects by bridging the innate and adaptive immune responses.

Early systemic administration of alpha-galactosylceramide (α-GalCer) or α-GalCer–loaded DCs generated in vitro can generate immunological responses against various solid tumors, and therapeutic effects have been observed in patients with cancer through a reversal of NK T-cell deficiency [7, 12, 33, 34]. Recent clinical studies of α-GalCer–loaded monocyte-derived antigen-presenting cells, peripheral blood mononuclear cells (PBMCs) cultured with α-GalCer, and iNKT cells isolated with a monoclonal antibody (6B11) have described objective immune responses against solid tumors [7, 12, 15]. In this study, patients underwent leukapheresis to isolate PBMCs, which were first pulsed with α-GalCer and then subjected to positive selection using anti-iNKT microbeads to increase the purity of Vα24 NK T cells (up to 90%). The purified cells were then co-cultured with α-GalCer–loaded mature antigen-presenting cells, which present glycolipid antigen to the iNKT cells and thereby activate them.

The frequency of iNKT cells among freshly isolated PBMCs was analyzed by flow cytometry. Representative data for one patient are shown in Figure 3A and B: iNKT cell purity was increased from 0.16% to 90.24% after in vitro expansion. The median iNKT cell expansion rate for the 10 patients was 63,500-fold (range, 13,500-fold to 135,300-fold) after 21 days of cultivation, and the purity of the iNKT cells increased from 0.05%–1.48% to 85.66%–94.55% (Table 2). The expression of IFN-γ in $CD8^+$ iNKT cells was upregulated after expansion in all 10 patients, with mean fluorescence intensity values increasing from 146–231 (range among the 10 patients) to 293–458 (Fig. 3C). After expansion, the mRNA expression levels increased 4.57–523.77-fold for IFN-γ, 3.02–899.48-fold for granzyme B (GzmB), and 5.25–653.51-fold for perforin, but the mRNA expression of IL-4 decreased 2.62–62.85-fold after expansion (Fig. 1).

Hence, successful activation of iNKT cells was demonstrated by significant increases in the mRNA levels of T-helper 1 (Th1) cytokines (IFN-γ, perforin, and GzmB; Fig. 1A–C). The human $CD8^+$ iNKT subset is similar to NKT1 cells in mice, showing increased IFN-γ secretion and cytotoxic function when activated [33, 35]. We also detected an increase in the expression of IFN-γ protein in $CD8^+$ iNKT cells after in vitro expansion (Fig. 3C). A restoration of iNKT cell immune function after in vitro expansion has also been reported in the setting of non-small cell lung cancer [16]. iNKT cells are noncirculating, tissue-resident lymphocytes. Previous studies have reported that the frequency of circulating Vα24 NK T

cells before the expansion is low and shows a substantial variation (0.01%–2%) [36, 37], and the fold-increase in Vα24 NK T cell number after in vitro expansion varies among patients [33]. The protocol described in this study (which involves microbead-based isolation) provides a reproducibly high number of iNKT cells for adoptive infusion and thus will greatly facilitate the future assessment of the efficacy of this therapeutic approach in patients.

In this study, the number of iNKT cells (baseline, 4.15–30.80 $\times 10^4$/L) rapidly increased 1.5–13.0-fold (to 13.13–60.81 $\times 10^4$/L) at week 2 and then decreased to 7.60–37.8 $\times 10^4$/L at week 4 and to baseline levels from week 8 onward (Fig. 4). A previous study of patients with melanoma found that circulating levels of NK cells and $CD69^+$ NK cells increased after iNKT cell infusion [15]. Since central memory T cells have potent antitumor activity characterized by memory and persistence [38, 39], this raises the possibility that antigens specific to hepatocellular cancer (HCC) may have activated an antitumor response.

Survival is typically short (<6 months) in patients with advanced HCC. Although none of the patients in this study exhibited a complete response or partial response to iNKT cell infusion, five patients were still alive at the end of follow-up, one of whom had no tumor recurrence and good quality of life. In addition, serum alpha-fetoprotein (AFP) value decreased in three patients within 2 weeks of iNKT cell administration (Fig. 4), directly suggesting an antitumor effect. Therefore, the adoptive transfer of in vivo–expanded iNKT cells to patients with HCC is well tolerated and associated with only minor adverse events.

Data for overall survival and progression-free survival are presented in Table 4. Patient 3 was still alive without tumor recurrence. Patients 1, 5, 6, and 7 exhibited tumor progression at 11, 6, 5.5, and 7 months after iNKT infusion, respectively, and were alive without extrahepatic metastasis. Tumor progression was treated by transcatheter arterial chemoembolization and radiofrequency ablation in patients 5 and 6 and by surgical resection in patients 1 and 7.

AFP is a frequently used marker for the diagnosis of HCC and monitoring of therapeutic effect, and its level is elevated in around 50% of patients with HCC. Four patients (1, 3, 5, and 7) had AFP levels within the normal range (<7 ng/mL) before treatment, and serum AFP levels in these patients were maintained within the normal range following iNKT cell therapy (Fig. 5). Six patients (2, 4, 6, 8, 9, and 10) had abnormal levels of AFP (Fig. 5).

Patients 2, 4, 8, and 9 died without the occurrence of tumor progression. Patients 4, 8, and 9 were confirmed to have severe cirrhosis, multiple diffuse tumors, portal vein tumor thrombosis, and high-risk esophageal varices (grade 3 on gastroscopic examination) and gastric varices before iNKT cell therapy. These three patients died of upper gastrointestinal bleeding at 11, 1.5, and 1.5 months, respectively, after treatment. Patient 2 was initially diagnosed with cholecystitis and cholestasis caused by biliary bacterial infection before iNKT therapy. Patient 2 died due to refractory bile duct infection and toxic megacolon at 1.5 months. Patient 10 died of spontaneous rupture of the tumor at 7 months after iNKT cell therapy (Table 4). Although these were serious adverse events, they were considered unrelated to treatment and instead related to underlying disease.

All adverse events occurring within 4 weeks of therapy are documented in Table 5. Most adverse events were of grade 1 to 3, grade 4 and 5 were not observed. ALL adverse events resolved without treatment within several days. The common adverse events were fever, elevated TBIL, elevated AST and decreased platelet count. Although the increases in serum AST and/or TBIL in patients 2 and 9 were of grade 3, the levels in both patients recovered within one week without treatment.

This study has some limitations. First, this was a single-center study, so the generalizability of the findings is not known. Second, the sample size was small, limiting the strength of the conclusions that may be drawn. Third, the cohort of 10 patients included only one woman, so possible gender-related differences could not be evaluated. Fourth, as this was a phase I trial focusing on dosage and safety, comparator groups (such as a control group) were not included. Finally, the final dose was only tried exploratively in the 10th patient. No maximum tolerated dose was found, and the safety was very good. Considering that the first three dosages are commonly used, the study was stopped.

In conclusion, the administration of in vitro–expanded autologous iNKT cells to patients with HCC is safe and well tolerated. There were no severe adverse events related to the infusion of iNKT cells, and most of the adverse events that occurred were of grades 1–2 and resolved within a week without treatment. Notably, the expanded iNKT cells produced Th1 cytokines that might enhance their antitumor activity. Further studies are merited to investigate the antitumor effects of iNKT cell therapy in patients with HCC.

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Disclosures

The authors indicated no financial relationships.

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Figures and Tables

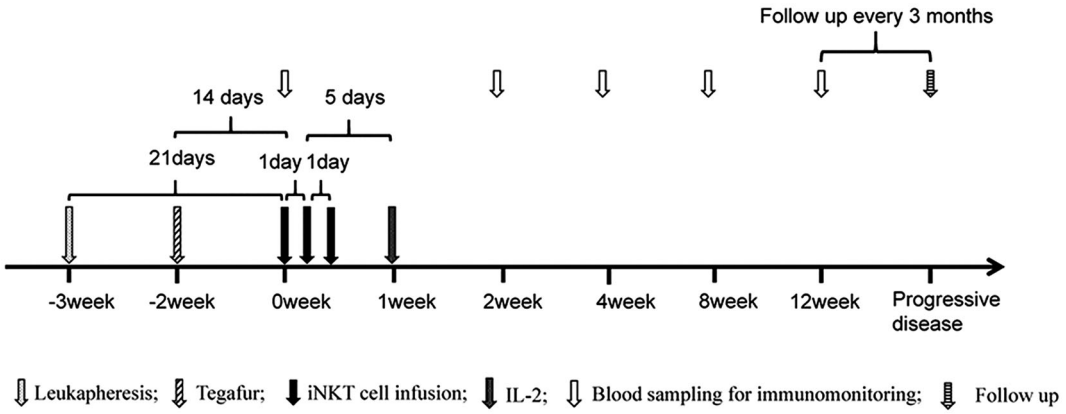

Figure 2. Clinical trial protocol.
Abbreviations: IL-2, interleukin-2; iNKT, invariant natural killer T cells.

Table 1. Clinical characteristics of the 10 patients with hepatocellular carcinoma

Patient no.	Age, yr	Sex	KPS	Viral infection	Child-Pugh	Prior treatment (times)	Vascular invasion	Metastasis	BCLC stage
1	60	M	1	HCV	A	TACE (2)/RFA (1)	N	N	C
2	50	M	2	HBV	B	non treatment	Y (PVTT)	N	C
3	50	M	1	HBV	A	TACE (3)/RFA (2)	Y	N	C
4	51	M	1	HBV	A	Surgery (1)	N	Y	C
5	59	M	1	HCV	A	TACE (1)	N	Y	C
6	44	M	1	HBV	A	TACE (2)/RFA (1)	N	Y	C
7	52	M	1	HBV	A	TACE (2)/RFA (1)	N	N	B
8	37	M	2	HBV	A	non treatment	Y (PVTT)	N	C
9	59	F	2	HBV	B	TACE (1)/RFA (1)	Y	N	C
10	53	M	1	non HBV/ HCV	B	TACE (1)/RFA (1)	N	N	B

Abbreviations: BCLC, Barcelona Clinic Liver Cancer; F, female; HBV, hepatitis B virus; HCV, hepatitis C virus; KPS, Karnofsky performance status score; M, male; N, no; PVTT, portal vein tumor thrombosis; RFA, radiofrequency ablation; TACE, transcatheter arterial chemoembolization; Y, yes.

Table 2. Summary of invariant natural killer T-cell purity before and after expansion and the infusion dosages used

Patient no.	Pre-expansion iNKT cell purity, %	Post-expansion iNKT cell purity, %	iNKT cell dosage, cells/m^2	Total iNKT cell dose	iNKT cell dose, infusion 1	iNKT cell dose, infusion 2	iNKT cell dose, infusion 3
1	0.16	90.24	3×10^7	5.88×10^7	5.88×10^6	1.76×10^7	3.50×10^7
2	1.48	88.91	3×10^7	5.04×10^7	5.04×10^6	1.51×10^7	3.02×10^7
3	0.15	85.66	3×10^7	5.58×10^7	5.58×10^6	1.67×10^7	3.35×10^7
4	0.31	94.55	6×10^7	1.06×10^8	1.06×10^7	3.18×10^7	6.36×10^8
5	0.65	90.01	6×10^7	1.02×10^8	1.02×10^7	3.06×10^7	6.12×10^8
6	0.22	86.94	6×10^7	1.13×10^8	1.13×10^7	3.39×10^7	6.78×10^8
7	0.06	86.63	9×10^7	1.75×10^8	1.75×10^7	5.25×10^7	1.05×10^8
8	0.16	89.72	9×10^7	1.62×10^8	1.62×10^7	4.86×10^7	9.72×10^7
9	0.05	89.12	9×10^7	1.67×10^8	1.67×10^7	5.01×10^7	1.00×10^8
10	0.12	88.3	1.0×10^{10}	1.0×10^{10}	1.0×10^{10}	0	0

Abbreviation: iNKT, invariant natural killer T.

Table 3. Primers used for the quantitative real-time polymerase chain reaction

Gene product	Sequence (5' → 3')
Interleukin-4	Forward: CCGTAACAGACATCTTTGCTGCC
	Reverse: GAGTGTCCTTCTCATGGTGGCT
Interferon-gamma	Forward: TGAATGTCCAACGCAAAGC
	Reverse: CGACCTCGAAACAGCATCTGA
Granzyme B	Forward: CCCTGGGAAAACACTCACACA
	Reverse: GCACAACTCAATGGTACTGTCG
Glyceraldehyde 3-phosphate dehydrogenase	Forward: TGTGGGCATCAATGGATTTGG
	Reverse: ACACCATGTATTCCGGGTCAAT

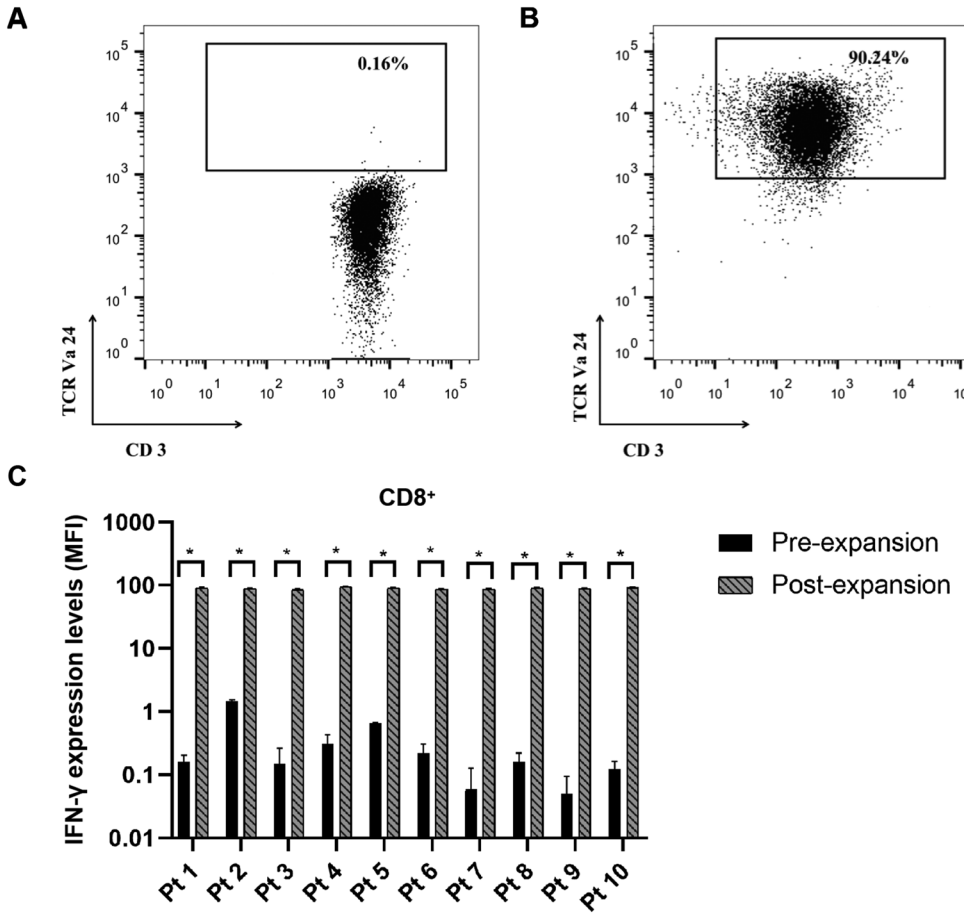

Figure 3. Characteristics of the invariant natural killer T (iNKT) cells. Cells were stained with monoclonal antibodies specific for CD3 and Vα24Jα18 and analyzed by flow cytometry. **(A):** Flow cytometric dot plot showing CD3 and Vα24Jα18 expression in peripheral blood mononuclear cells isolated from one patient. iNKT cell purity was 0.16%. **(B):** Flow cytometric dot plot showing the purity of the expanded iNKT cells (90.24%) in the same patient as above. **(C):** Comparison of IFN-γ expression levels in CD8-positive iNKT cells before and after expansion. IFN-γ expression was evaluated using immunohistochemistry with a calculation of the mean fluorescence intensity. All data were obtained from three independent experiments ($n = 3$). *$p < .001$.
Abbreviations: IFN-γ, interferon-gamma; MFI, mean fluorescence intensity; Pt, patient; TCR Va 24, T-cell receptor Vα24.

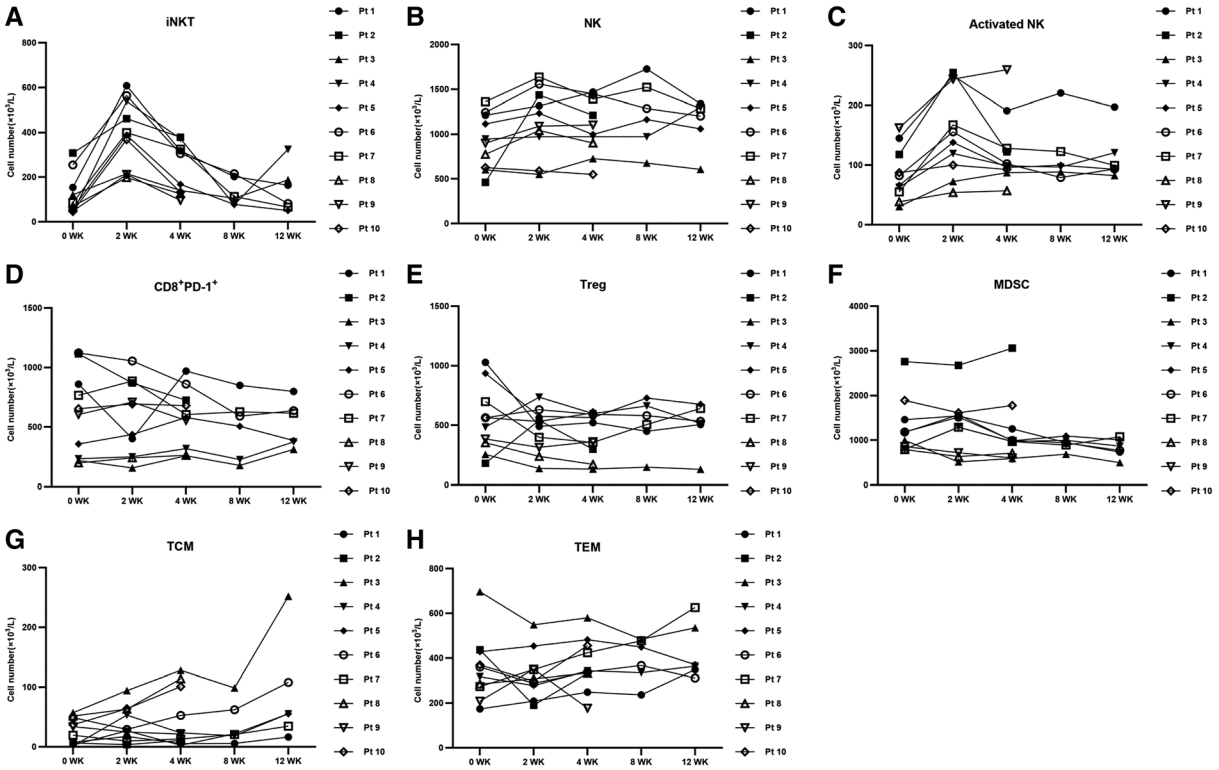

Figure 4. Changes in the numbers of immune cells after the infusion of iNKT cells. The absolute cell count ($\times 10^3$/L) for each type of immune cell was obtained by multiplying the immune cell percentage (measured by flow cytometry) by the absolute lymphocyte count (measured with a hematology analyzer). **(A):** iNKT cells. **(B):** NK cells. **(C):** Activated NK cells. **(D):** CD8-positive PD-1–positive cells. **(E):** Regulatory T cells. **(F):** Myeloid-derived suppressor cells. **(G):** Central memory T cells. **(H):** Effector memory T cells. All data were obtained from three independent experiments ($n = 3$).

Abbreviations: iNKT, invariant natural killer T cells; MDSC, myeloid-derived suppressor cells; NK, natural killer cells; PD-1, programmed death-1; Pt, patient; TCM, central memory T cells; TEM, effector memory T cells; Treg, regulatory T cells; WK, week.

Table 4. Overall survival and progression-free survival

Patient	PFS, months	OS, months	Cause of death
1	11	>16	
2	1.5	1.5	Intra-abdominal bacterial infection
3	>16	>16	
4	11	11	Gastric variceal bleeding
5	6	>16	
6	5.5	>15	
7	7	>15	
8	1.5	1.5	Gastric variceal bleeding
9	1.5	1.5	Gastric variceal bleeding
10	7	7	Spontaneous rupture of hepatocellular carcinoma

Abbreviations: OS, overall survival; PFS, progression-free survival.

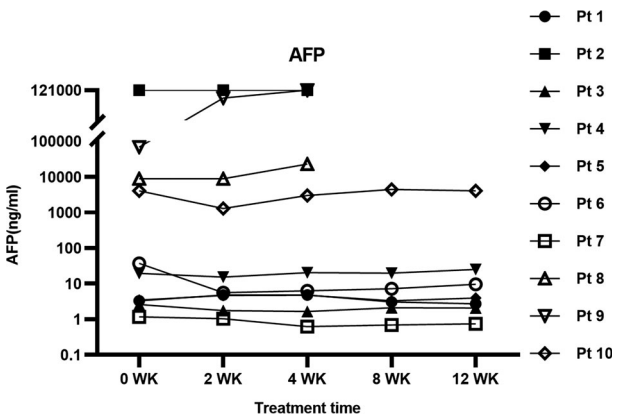

Figure 5. Alpha-fetoprotein levels before and after the infusion of invariant natural killer T cells.
Abbreviations: AFP, alpha-fetoprotein; Pt, patient; WK, week.

Table 5. Adverse events

Adverse event	Grade 1, *n*	Grade 2, *n*	Grade 3, *n*	Total, *n*
Chills	2	0	0	2
Fever	3	0	0	3
Pain	1	0	0	1
Fatigue	2	0	0	2
Anemia	2	0	0	2
Decreased platelet count	1	3	0	4
Decreased neutrophil count	1	0	0	1
Decreased white blood cell count	3	0	0	3
Increased blood bilirubin level	5	0	2	7
Increased alanine transaminase level	2	0	0	2
Increased aspartate transaminase level	3	0	1	4
Subcutaneous induration	1	0	0	1
Total	26	3	3	32