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ORIGINAL ARTICLE

Pilot study to determine the safety and feasibility of deceased donor liver natural killer cell infusion to liver transplant recipients with hepatocellular carcinoma

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Abstract

Liver transplantation (LT) is a viable treatment option for cirrhosis patients with hepatocellular carcinoma (HCC). However, recurrence is the rate-limiting factor of long-term survival. To prevent this, we conducted the phase I study of the adoptive transfer of deceased donor liver-derived natural killer (NK) cells. Liver NK cells were extracted from donor liver graft perfusate and were stimulated in vitro with IL-2. The patient received an intravenous infusion of NK cells 3–5 days after LT. Eighteen LT recipients were treated. There were no severe cell infusion-related adverse events or acute rejection episodes. One patient withdrew from the study because the pathological observation revealed sarcoma instead of HCC. All patients who received this immunotherapy completed the follow-up for at least 2 years without evidence of HCC recurrence (median follow-up, 96 months [range, 17–121 months]). Considering that 9 (52.9%) of the 17 patients pathologically exceeded the Milan criteria, liver NK cell infusion is likely to be useful for preventing HCC recurrence after LT. This is the first-in-human immunotherapy study using deceased donor liver-derived NK cells to prevent HCC recurrence after LT. This treatment was well tolerated and resulted in no HCC recurrence after LT.
Clinical trial registration www.clinicaltrials.gov; NCT01147380; registration date: June 17, 2010.

Keywords Immunotherapy · NK cell · Liver transplantation · HCC

Abbreviations

CIK	Cytokine-induced killer
CTCAE	Common Terminology Criteria for Adverse Events
DDLT	Deceased donor liver transplantation
FCM	Flow cytometry
GVHD	Graft-versus-host disease
HCC	Hepatocellular carcinoma
HLA	Human leukocyte antigen
KIR	Killer cell immunoglobulin-like receptor
LDLT	Living donor liver transplantation

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LMNCs	Liver mononuclear cells
LT	Liver transplantation
MELD	The Model for End-Stage Liver Disease
NK	Natural killer
PBMCs	Peripheral blood mononuclear cells
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
UNOS	The United Network for Organ Sharing

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide and is an indication for liver transplantation (LT). The number of LTs in HCC patients has gradually increased since the Milan criteria have been established for organ allocation in the United States [1, 2]. Among the immunosuppressed conditions, the rate of recurrence of HCC after LT is still 10–20% [3, 4]. However, effective means to prevent HCC recurrence after LT remain unestablished [1, 5–9]. There is an urgent need to develop effective and safe therapeutic interventions that can be used to prevent HCC recurrence, despite the immunosuppressive environment.

Natural killer (NK) cells are a subset of lymphocytes defined by the expression of CD56 and the negative expression of the T cell receptor (CD3), comprising 10–15% of all circulating lymphocytes. NK cells can kill target cells without prior sensitization based on inhibitory receptor recognition (missing-self hypothesis) and activating receptor recognition [10, 11]. It has been demonstrated that the numbers and function of NK cells are attenuated in HCC/cirrhosis patients [12–14], implicating that the deficient function of NK cells might be responsible for the failure of anti-tumor immune responses after LT in HCC patients. Since the immunosuppressive regimen that is currently used after organ transplantation reduces the adaptive immune components but effectively maintains the innate components of cellular immunity [15–17], augmentation of the NK cell response may be a promising immunotherapeutic approach [18, 19]. In an autologous setting, cytokine-induced killer (CIK) cell therapies were well tolerated and could improve the outcomes of patients with HCC after curative treatment [20, 21]. A recent phase-3 trial study revealed that adjuvant immunotherapy using autologous CIK cells increased recurrence-free survival and overall survival after curative HCC treatment [22]. However, there are no reports on the use of allogeneic NK cells for HCC treatment. Previous clinical studies have demonstrated the safety and efficacy of allogeneic NK cell adoptive transfer therapies as a means for the treatment of hematologic malignancies and, to a lesser extent, of solid tumors [17, 23, 24]. In a Phase I/II study of injecting allogeneic NK cells into patients suffering from renal cell carcinoma, the distribution of the inoculation to the whole body, with a preference for the liver, spleen, and bone marrow and their extended survival were observed [24]. In allogeneic NK cell therapy, the mismatch between the killer cell immunoglobulin-like receptor (KIR) and human leukocyte antigen (HLA) can be harnessed to increase anti-tumor activity. We have previously demonstrated that the multiplicity of the functional compound KIR-HLA genotype in patients with HCC who underwent primary curative hepatectomy is associated with the HCC recurrence rate, indicating that the KIR-HLA axis might be involved in NK-mediated killing of HCC [25]. Hence, with less donor/recipient HLA-matching, DDLT might be advantageous over LDLT in terms of the anti-tumor effects of the immunotherapy.

We have previously reported that liver mononuclear cells (LMNCs) derived from donor liver perfusate contain a large number of NK cells that have vigorous cytotoxicity against hepatoma cells with the expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a critical molecule for NK cell-mediated anti-tumor cell killing, after IL-2 stimulation [26, 27]. As a preliminary proof of concept study, we have undertaken a pilot clinical trial of adoptive immunotherapy approach, using lymphocytes extracted from liver allograft perfusate, which includes an abundance of NK cells that mount an anti-HCC response, in living donor LT (LDLT) recipients with HCC in Hiroshima University in Japan [18]. In this trial, twenty-four LT recipients safely received NK cell infusion with acceptable outcomes, that is, with the absence of any severe adverse events, including graft-versus-host disease (Table S1) and a recurrence-free satisfactory overall survival (Figure S1). Here, we applied NK cell immunotherapy to deceased donor LT (DDLT) patients with HCC, based on the results of another preliminary study showing that the quality and quantity of liver NK cells derived from cadaveric donors were similar to those of living donors [27]. This study aimed to determine the feasibility of adoptive immunotherapy using activated liver NK cells, to assess the toxicity of this regimen and evaluate its ability to exert an anti-tumor effect in recipients of cadaveric liver transplant.

Methods

Patient eligibility

Primary LT recipients with HCC who were 18 years of age or older were eligible for this study. The absence of serious cardiovascular disease and a satisfactory renal function, as indicated by receiving hemodialysis more than twice a week as well as bone marrow competence (WBC > 1000/mm^3, hematocrit > 20%, platelets > 20,000/mm^3) were

prerequisites for patient enrollment. Exclusion criteria included living donor LT, multiple organ transplant, prior solid organ or bone marrow transplant, fulminant hepatic failure, ABO-incompatible transplant, and intercurrent chemotherapy at the time of enrollment. Donors were negative for serology (HCV, HBsAg, HBcAb, HTLV-1, HTLV-3, and EBV IgM) and had satisfactory liver functions, as indicated by a value of total bilirubin < 3.0 mg/dL and prothrombin time < 35 s. In all cases, the pathologist tentatively confirmed the tumors in the liver before cell administration. If there were no tumors, the final product was not administered.

Study design

Twenty patients were enrolled in this study. Eligible patients received standard LT and were infused with enriched donor liver NK cells 3–5 days after LT. The enrolled patients were divided into two groups according to the following cell doses: the low-dose group, $10–100 \times 10^6$ cells; and the high-dose group, $100–1000 \times 10^6$ cells/body, according to the results of our preliminary study [27]. Patients and donors were matched by the United Network for Organ Sharing (UNOS) liver allocation system, based on the Model for End-Stage Liver Disease (MELD). The primary aim of this study was to evaluate the safety of intravenous administration of deceased donor liver NK cells after LT. The possible clinical efficacy of this immunotherapy has also been evaluated as a secondary endpoint. Immunosuppression consisted of a 3- to 6-month course of tapering corticosteroids and a calcineurin inhibitor, usually tacrolimus, for maintenance with or without mycophenolate mofetil.

Cell preparation

The method of cell preparation was previously described [27]. Briefly, after organ recovery, the liver was perfused through the portal vein with 2 L of University of Wisconsin solution on the back table. LMNCs were collected by Ficoll-Hypaque density-gradient centrifugation and were cultured with 1000 U/mL of human recombinant interleukin (IL)-2 (Proleukin, Novartis, Emeryville, CA, USA) in complete medium for 3–5 days to prime NK cells with enhanced anti-tumor properties. To prevent graft-versus-host disease (GVHD), that is, to inactivate $CD3^+$ alloreactive T cells, an anti-CD3 monoclonal antibody (mAb) (Orthoclone OKT3, Ortho Biotech, Raritan, NJ, or anti-CD3 antibody), was added to the culture medium (100 μg/mL) 1 day before the cell harvest. A minimum of 1×10^7 cells with a cell viability of > 80% was required to release the NK cell product for infusion. Following quality control verification, including the Gram Stain, culture, endotoxin, and mycoplasma testing, all unfractionated cells, which were processed as described above and defined as the final NK cell products, were infused into the patients via intravenous administration on the day of cell harvesting.

Flow cytometry analysis

All flow cytometry (FCM) analyses were performed on a BD LSR-II flow cytometer (BD Biosciences, San Jose, CA). To detect the surface phenotype, leukocytes were stained with the following monoclonal antibodies: against CD3, TRAIL, NKG2D, CD69, CD226, and CD56. The data were analyzed using FlowJo software (Tree Star, Inc. Ashland, OR).

Cytotoxic assay

The cell cytotoxicity assay was performed using FCM as described previously [27]. Briefly, the target cells were labeled with 0.1 μM carboxyfluorescein diacetate succinimidyl ester (CFDA SE) Cell Tracer Kit (Invitrogen), and the effector cells were co-incubated at various effector/target ratios of target cells for 1 h at 37 °C in 5% CO_2. The cytotoxic activity was calculated as a percentage using the following formula: % cytotoxicity = [(% experimental $DAPI^+$ dead targets)-(% spontaneous $DAPI^+$ dead targets)]/[(100-(% spontaneous $DAPI^+$ dead targets)] × 100. K562, a human chronic myelogenous leukemia cell line (ATCC #CCl-243), was cultured in Dulbecco' s modified Eagle' s medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (Mediatech, Inc., Manassas, VA, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen) (complete medium) at 37 °C and 5% CO_2.

Statistical analysis

Survival curves were generated using the Kaplan–Meier method and were compared by log-rank tests between the groups. Differences between the results of comparative tests were considered significant if the *P*-values were less than 0.05. Analyses were performed using JMP statistical software (JMP Pro 15.2.1; SAS Institute Inc., Cary, NC, USA).

Results

Patient Characteristics

From June 2010 to April 2013, twenty LT patients with HCC were enrolled in this study. Two patients withdrew from the study and did not receive immunotherapy because the provisional pathological observation revealed no tumors in the resected native livers (Fig. 1). The demographics of patients who received immunotherapy and their clinical characteristics are shown in Table 1. One patient was removed from the study after immunotherapy because the final pathology

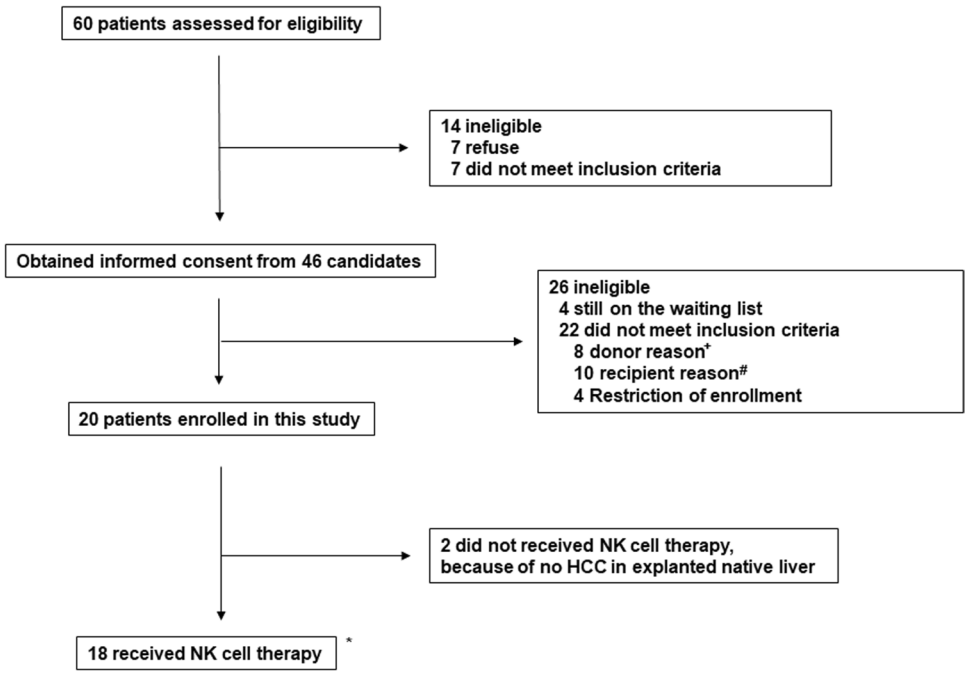

Fig. 1 CONSORT flow diagram. (+) High-risk donor ($n=3$), no research consent ($n=2$), import donor ($n=2$). (#) HCC progression ($n=6$), poor condition ($n=2$), no HCC ($n=1$), expired ($n=1$). (*) One patient received NK cell therapy but withdrew from this study because he was diagnosed with sarcoma based on postoperative pathological findings

report disclosed sarcoma, not HCC (patient 1). The patient's median age was 60 years (range, 20–68 years). Twelve (70.6%) of the 17 patients had undergone pretreatment for HCC before transplantation. Eight patients received transarterial chemoembolization, and six patients underwent radiofrequency ablation. Four (23.5%) of the 17 patients had an AFP value of 200 ng/mL or higher. There were nine patients (52.9%) exceeded the Milan criteria based on the pathology of liver explants.

Characteristics of the NK cells

LMNCs derived from donor liver perfusate contained a median of 57% of NK cells (21–94%) before manipulation. Following ex vivo cultivation with IL-2 and anti-CD3 mAb to prime NK cells and inactivate T cells (processing), the median cell number of the final product was 200×10^6 (range, 18–626×10^6) cells. The viability was 93.3% (range, 86.2–98.1%). Figure 2 shows the representative phenotype of LMNCs before and after processing. The final product contained 64.8% (range, 25.2–98.4%) of $CD3^-CD56^+$NK cells, which expressed a high level of TRAIL (53.5%; range 3.1–84.5%). The TRAIL expression of $CD56^{bright}$ NK cells (72.8%; range 6.4–94.8%) was higher than that of $CD56^{dim}$ NK cells (44.4%; range 2.1–70.5%). Cell processing significantly decreased T cell contamination in the final product from 15.0% (range, 0.3–45.0%) to 0.4% (range, 0.0–2.3%), yielding a final T cell dose of 2.3×10^3 cells/kg. Other components of the final product included B cells, monocytes, and granulocytes, with the following percentages: 6.4% (range, 0.1–20.7%), 1.7% (range, 0.1–3.8%), and 5.2% (range, 0.2–12.7%), respectively. The functional assay revealed vigorous cytotoxicity of the final product as 38.0% (range, 10.3–70.0%) with an effector: target ratio of 10:1. All products tested below the accepted limit for endotoxins and Gram staining was negative on the day of infusion. Subsequent microbiological cultures of all infusions were negative (Table 2).

Toxicity

There were no fatal adverse events including GVHD during the study trial. Within 3 months after cell infusion, there were some adverse events categorized as grade 3–4 in Common Terminology Criteria for Adverse Events (CTCAE) ver 4.0 (Table 3). There were 13 and 11 grade 3–4 adverse events in the high- and low-dose groups, respectively. No signs of acute rejection were observed in this trial. There were no significant differences between the dose-dependent groups. All events were related to LT rather than cell infusion in the decision of the Data Safety Monitoring Board.

Immunological effects

Laboratory tests were performed every week to monitor immune activation. NK cell number and NK cell activation markers in the peripheral blood of the recipient are shown in Fig. 3. NK cell proportion and NK cytotoxicity gradually increased after immunotherapy in both dose groups, but there were no statistically significant differences. The activation markers on the peripheral blood

Table 1 Baseline characteristics of the patients

No	Age	Sex	Group A: High B: Low	Etiology	Donor age	Pre-Tx	Tumor number	Max tumor diameter (cm)	AFP (ng/ml)	Pathology	Vascular invasion	LN metastasis	Milan criteria	Status (Cause of death)	Follow-up (months)
1	64	M	A	NASH	27	TACE×1, RFA×1	2	5.2	439.0	SARCOMA	+	+	N/A	DEAD (Sarcoma)	3
2	58	M	A	HCV	22	TACE×1, RFA×1	1	4.0	9.4	HCC (WEL)	-	-	IN	ALIVE	121
3	61	M	B	HCV	47	NO	2	2.3	24.3	HCC (WEL)	+	-	OUT	ALIVE	118
4	65	M	A	HCV	57	TACE×1	2	2.9	233.0	HCC (MOD)	-	-	IN	ALIVE	118
5	66	M	B	Laennec's	16	NO	1	3.5	7.9	HCC (MOD)	-	-	IN	DEAD (Sepsis)	49
6	56	M	B	Laennec's	6	RFA×1	2	4.0	2.0	HCC (N/A)	-	-	OUT	DEAD (Graft failure)	72
7	56	F	A	HCV	37	TACE×1	1	5.0	56.4	HCC (MOD)	-	-	IN	ALIVE	114
8	54	M	A	HCV	52	RFA×2	2	1.9	444.0	HCC (MOD)	-	-	IN	ALIVE	110
9	49	M	A	HCV	49	TACE×2, NEX	3	1.5	1.9	HCC (MOD)	-	-	IN	ALIVE	109
10	63	M	A	HCV	40	NO	1	3.6	40.8	HCC (WEL)	-	-	IN	ALIVE	108
11	68	M	A	HCV	25	NO	5	2.2	6.1	HCC (MOD)	+	-	OUT	ALIVE	104
12	60	M	A	HCV	68	NO	2	4.3	3.7	HCC (MOD)	-	-	OUT	ALIVE	25
13	58	M	A	HCV	48	RFA×1	7	2.3	293.0	HCC (MOD)	+	-	OUT	ALIVE	77
14	60	M	B	HBV, HCV	30	TACE×3	4	7.0	25.0	HCC (MOD)	-	-	OUT	ALIVE	33
15	20	F	A	HCV	39	NO	1	2.2	543.0	HCC (WEL)	-	-	IN	ALIVE	97
16	62	M	B	HCV	27	TACE×2	1	6.3	54.0	HCC (WEL)	-	-	OUT	DEAD (HCV)	17
17	52	M	B	Laennec's	21	RFA×2 TACE×1	4	4.0	6.7	HCC (MOD)	-	-	OUT	ALIVE	96
18	53	M	A	HCV	16	TACE×1	2	3.1	23.0	HCC (MOD)	+	-	OUT	ALIVE	91

AFP, α-fetoprotein; *NEX* Nexavar; *TACE* Transcatheter Arterial Chemoembolization; *RFA* radiofrequency ablation; *HCC* hepatocellular carcinoma; *LN* lymph node

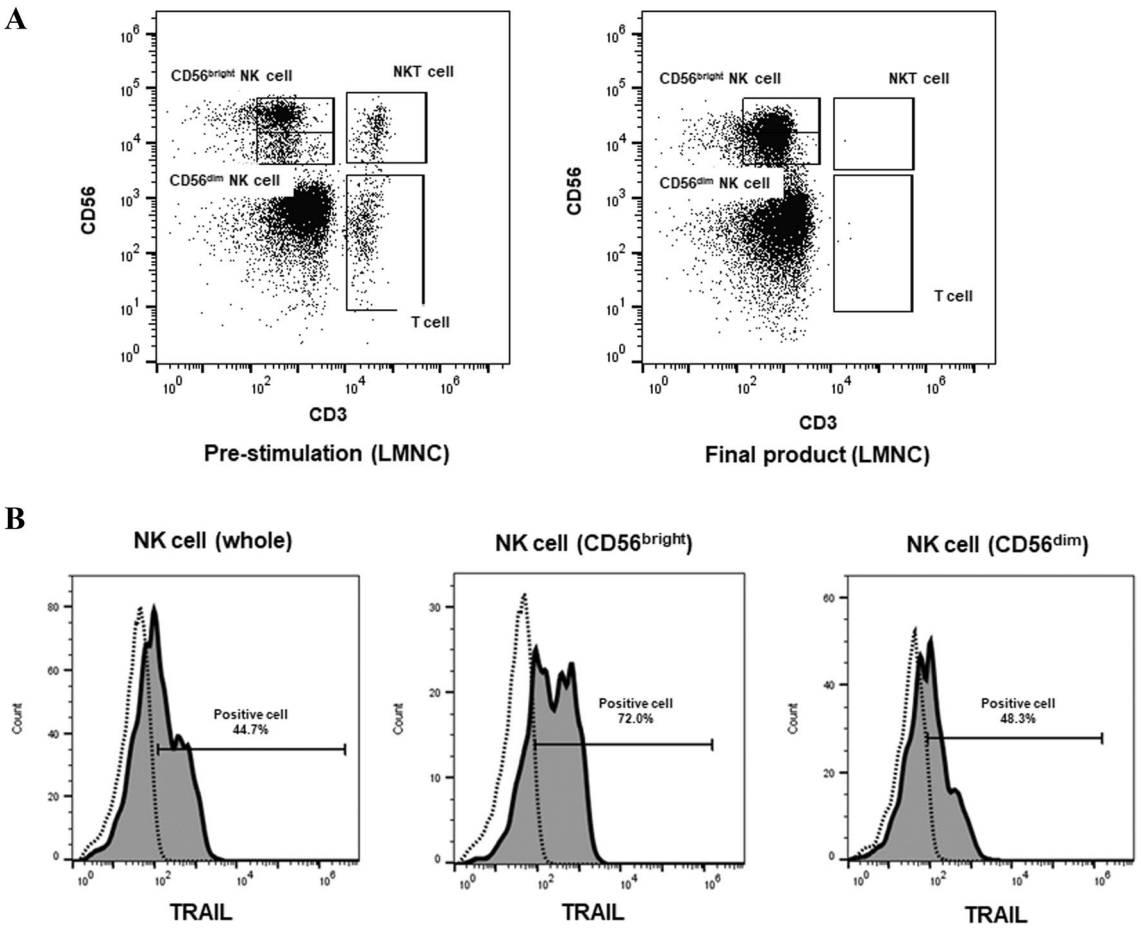

Fig. 2 Immunological assessment of the administrated NK cells. (**a**) The representative phenotype of LMNCs that was obtained from deceased donor liver perfusate before the culture (left) and after the culture (right) was depicted. (**b**) Histograms show the logarithmic fluorescence intensities of TRAIL after gating of the $CD3^{-}CD56^{+}$ NK cells (left), $CD3^{-}CD56^{bright}$ NK cells (middle), and $CD3^{-}CD56^{dim}$ NK cells (right). The dotted lines indicate a negative control staining with isotype-matched mAbs. Abbreviations: NK cell, natural killer cell; NKT cell, natural killer T cell; LMNCs, liver mononuclear cells; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand

NK cells did not change significantly. Each group had no significant differences in all categories.

Clinical efficacy

All participants were eligible for survival analysis and completed a follow-up period of 2 years. At the time of the data cutoff, the median follow-up duration was 96 months (range, 17–121 months). Nine of 17 patients had to exceed the Milan criteria diagnosed based on pathological findings. Among the 17 patients, no patients experienced HCC recurrence, even though 4 patients died during follow-up. Regarding overall survival, the high-dose group had significantly better survival than the low-dose group (log-rank; $p = 0.0064$) (Fig. 4).

Discussion

Immunotherapy with donor liver-derived NK cells has been originally applied to LDLT at Hiroshima University, Japan since 2006 [18]. Although the long-term benefits of this approach for the control of HCC recurrence after LT remain to be elucidated, the successful administration of this trial raised a further challenge of expanding the liver-derived NK cell immunotherapy to DDLT recipients. With more ischemia and less controlled procedures, DDLT might in theory lead to more adverse outcomes in immunotherapy than LDLT. Despite such potential concerns, immunotherapy was well tolerated and induced significant objective responses in DDLT patients with HCC. The procedure itself was not associated with serious adverse

Table 2 Characteristics of final product

No	Group A: High B: Low	Cell Dose (×108)	Viability (%)	Cytotoxicity (%)	Purity NK cell (%)	TRAIL on NK cell (%)	Purity T cell (%)	Purity B cell (%)	Endotoxin (EU/kg)	Culture test
1	A	362	89.4	55.9	25.2	3.1	0.30	12.2	0.610	Negative
2	A	200	95.2	22.7	70.2	68.2	0.53	4.0	0.528	Negative
3	B	18	87.2	64.4	98.4	79.2	0.05	0.5	0.382	Negative
4	A	200	93.2	70.0	86.1	62.9	0.03	3.6	0.540	Negative
5	B	34	92.8	46.9	78.2	84.5	0.10	15.9	0.614	Negative
6	B	21	86.2	36.4	67.6	79.1	0.08	20.7	0.800	Negative
7	A	111	90.6	16.4	55.0	57.6	2.12	4.9	0.430	Negative
8	A	390	91.8	19.8	71.5	53.4	0.01	4.3	0.590	Negative
9	A	150	94.1	13.1	63.5	34.5	0.32	5.0	0.700	Negative
10	A	260	93.1	46.1	71.6	46.5	0.06	7.0	0.630	Negative
11	A	279	96.2	16.2	59.8	53.5	0.05	3.3	0.590	Negative
12	A	352	95.2	23.0	55.7	44.7	0.13	18.0	0.580	Negative
13	A	263	97.1	26.3	72.2	20.5	0.02	2.0	0.810	Negative
14	B	40	95.7	54.2	56.7	23.2	0.28	0.5	0.580	Negative
15	A	626	98.1	65.2	60.6	8.6	1.95	2.2	0.790	Negative
16	B	35	91.9	10.3	68.0	51.2	0.11	1.0	0.880	Negative
17	B	58	94.8	69.1	54.3	68.2	0.36	1.0	0.800	Negative
18	A	198	88.1	16.6	79.8	66.0	0.89	5.1	0.249	Negative

NK cell; Natural killer cell, TRAIL; TNF-related apoptosis-inducing ligand

events and there were no treatment-related deaths. No HCC recurrence was observed in this study, with a follow-up period of over 24 months. The outcomes of this study are especially favorable considering the study population, which included advanced HCC patients (52.9% of them exceeding the Milan criteria); the anticipated incidence of HCC recurrence in similar LT recipients is typically 10–20% within 24 months [3, 4].

The arguments stating that immunosuppression increases the risk of recurrence of malignancy, including HCC, has been accepted. Supporting this, regression of HCC has been described following the discontinuation of immunosuppressive therapy [28]. While immunosurveillance against cancer and microbes is exerted by orchestrating innate and acquired immunity, immunosuppressive drugs currently used after LT preferentially suppress acquired components such as T and B cells [15]. Hence, in the LT setting, innate immunity, including NK cells, plays a major role in prophylactic activity. Consistently, we have previously demonstrated that polymorphisms of genes encoding FcγRIIIa, which is expressed by NK cells and mediates natural antibody-directed activity in innate immunity, can be a predisposing factor for severe bacterial infections and to predict mortality after LT [29]. Despite the important role of NK cells, functional impairment and a decreased number of NK cells have been observed in patients with end-stage liver disease requiring LT [12–14]. In LT recipients with HCC, who might be in such an immunological predicament, the augmentation of functionally activated liver NK cells could be a promising approach.

Challenges associated with sourcing allogeneic NK cells have given rise to controversy over the contribution of NK cells to GVHD. LMNCs derived from donor liver perfusate contain contaminating T cells, whose activation with NK-stimulating cytokines has been known to lead to an increased release of proinflammatory cytokines and to trigger the onset of GVHD in vivo. To avoid such an unfavorable response, in this study, anti-CD3 mAb was used during in vitro cell processing to inactivate T cells. As a result, the final product contained 0.002×10^6 $CD3^+CD56^-$T cells/kg, a seemingly acceptable level by comparison with the previous clinical trials reporting that the alloreactive T cells were contaminated with $0.01–0.18 \times 10^6$ $CD3^+CD56^-$T cells/kg [17, 30–32]. Therefore, the risk of GVHD would be minimal in this immunotherapy. Regarding regulatory T (Treg) cells, stimulation with IL-2 activated $CD4^+$ T cells in liver-derived lymphocytes, and it is possible that Treg cells were induced. However, to prevent GVHD, T cells were depleted by adding an anti-CD3 mAb prior to cell infusion, and thus, very few Treg cells were transferred to the patients (0.002×10^6 $CD3^+CD56^-$ T cells/kg). Further research is needed for understanding the merits and demerits of transferring activated lymphocytes without depleting activated T cells including Treg cells.

Table 3 Summary of adverse event within 3 months after liver transplantation

Adverse events n (%)	High dose ($n=12$)		Low dose ($n=6$)	
	All Grade	Grade III, IV	All Grade	Grade III, IV
Alkaline Phosphatase increase	4 (33)	2 (17)	2 (33)	1 (17)
Aspartate aminotransferase increase	4 (33)	2 (17)	4 (67)	0
Alanine aminotransferase increase	7 (58)	1 (8)	5 (83)	0
Blood bilirubin increase	9 (75)	0	3 (50)	0
Hyponatremia	4 (33)	2 (17)	3 (50)	1 (17)
Hyperkalemia	5 (42)	0	6 (100)	0
Hypoalbuminemia	5 (42)	1 (8)	4 (67)	1 (17)
Anemia	5 (42)	1 (8)	6 (100)	0
Platelet count decrease	5 (42)	0	4 (67)	1 (17)
Pneumonitis	0	0	1 (17)	1 (17)
Small intestine obstruction	0	0	1 (17)	1 (17)
Hepatic artery thrombosis	0	0	1 (17)	1 (17)
Liver abscess	0	0	1 (17)	1 (17)
Diarrhea	1 (8)	0	1 (17)	1 (17)
Pericardial tamponade	0	0	1 (17)	1 (17)
Dehydration	0	0	1 (17)	1 (17)
Colitis	1 (8)	1 (8)	0	0
Pulmonary embolism	1 (8)	1 (8)	0	0
Abdominal pain	1 (8)	1 (8)	0	0
Tacrolimus encephalopathy	1 (8)	1 (8)	0	0
White blood cell decrease	2 (17)	0	1 (17)	0
White blood cell increase	1 (8)	0	1 (17)	0
Creatinine increase	1 (8)	0	3 (50)	0
BUN increase	1 (8)	0	3 (50)	0
Pleural effusion	1 (8)	0	0	0
Dyspnea	1 (8)	0	0	0
Wound infection	1 (8)	0	1 (17)	0
Catheter related infection	1 (8)	0	0	0
Ascites	1 (8)	0	1 (17)	0
PT prolonged	0	0	1 (17)	0
PT shortened	1 (8)	0	0	0
APTT prolonged	1 (8)	0	1 (17)	0
Edema limbs	1 (8)	0	1 (17)	0
Fever	1 (8)	0	0	0

Data are presented as No. (%). Data include Adverse Events reported within 3 months after transplantation
All events were not study related in the decision of Data Safety Monitoring Board

Various protocols have been used to isolate and preferentially expand primary NK cells from peripheral blood mononuclear cells (PBMCs) because of their ease and convenience for collection [33, 34]. We have previously shown that liver NK cells are more abundant and mediate a higher cytotoxic activity against HCC peripheral blood (PB) NK cells [12–14]. NK cells are a heterogeneous population consisting of different subsets with unique phenotypic and functional features. Tissue-resident NK cells, which are distinct from conventional NK cells, are preferentially distributed throughout non-lymphoid tissues, such as the liver, uterus, salivary gland, and adipose tissues. As an organ with predominant innate immunity, the liver is enriched with NK cells, which are phenotypically distinct from PB NK cells [12–14]. One of the hallmarks of liver NK cells is the expression of TRAIL. TRAIL binds to at least four receptors. Two of these death-inducing receptors (TRAIL-R1/DR4 and TRAIL-R2/DR5) contain cytoplasmic death domains and signal apoptosis, whereas two other death-inhibitory receptors (TRAIL-R3/DcR1 and TRAIL-R4/DcR2) lack a functional death domain and do not mediate apoptosis, all having similar affinities, and the latter acts as a decoy [35, 36]. We have demonstrated that moderately/poorly differentiated HCCs express remarkable levels of TRAIL-DR4 and -DR5 but do

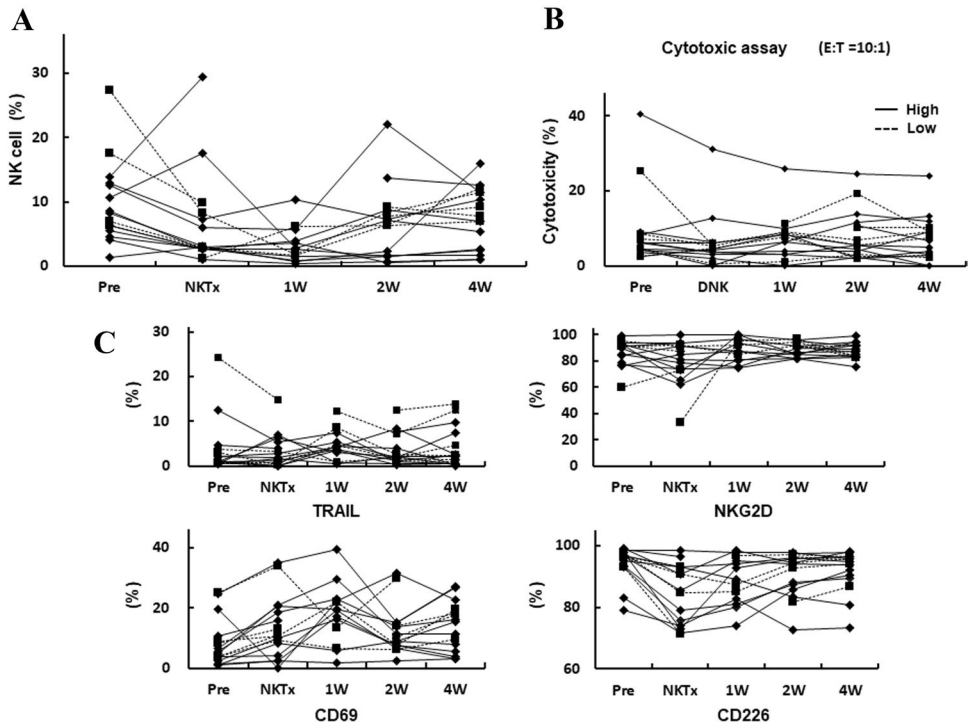

Fig. 3 Immunological assessment of the recipient. (**a**) The dot shows the percentage of $CD3^{-}CD56^{+}$ NK cells isolated from the peripheral blood of the recipient. (**b**) Cytotoxicity of PBMCs against K562 with an effector:target cell ratio of 10:1 and (**c**) each molecule of NK cells in the peripheral blood of the recipient. The solid line indicates the high-dose group ($n=11$) and the dotted line indicates the low-dose group ($n=5$)

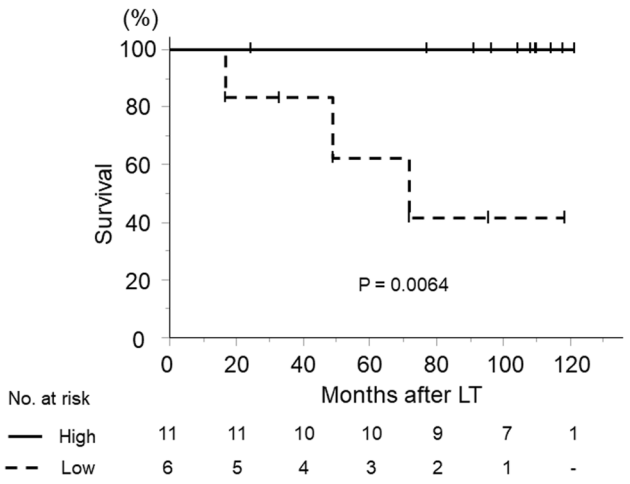

Fig. 4 Kaplan–Meier estimates of overall survival between the high-dose and the low-dose groups. Symbols indicate censored observations. The recipient survival of the high-dose group was significantly higher than the low-dose group (Log-rank: $P=0.0064$)

not express TRAIL- DcR1 and -DcR2, indicating susceptibility to TRAIL-expressing NK cell-mediated cell killing [12–14]. TRAIL expression was also clearly confirmed in the activated NK cells obtained from deceased donor liver perfusates in this study.

In order to investigate whether the frequency and type of adverse events were affected by the dosage of transferred immune cells, the subjects were divided into two groups: high-dose and low-dose groups. There was no difference in either the incidence or type of adverse events between the two groups. Surprisingly, with respect to the survival rates, the high-dose group showed significantly better survival than the low-dose group. Of note, the survival curves of the two groups dissociated more than one year after LT. Considering the lifespan of the transferred immune cells in vivo, it is possible that the difference in the survival rates of the two groups may be due to the difference in the dose of transferred cells. This said, in the study of LDLT (Hiroshima University), in which donor-derived NK cell chimerism in the peripheral blood was followed by flow cytometry using donor HLA as a marker, the period of detection was restricted to up to one month after LT (data not shown). Importantly the cause of death in the low-dose group was either infectious diseases or the recurrence of diseases other than HCC. It is possible that the administered NK cells might have stimulated liver immunity and maintained it for a long time [37]. As a result, patients in the high-dose group might have maintained their biological defenses and acquired resistance to infection. Additionally, the number of NK cells collected at the time of organ procurement may reflect the graft function. Since all of the collected graft-derived immune cells were cultured and administered, the patients in the high-dose group were transplanted with liver grafts with a higher volume of immune cells. Hence, the survival rate of patients transplanted with better-functioning liver grafts might have been better. In any case, future studies should be designed to determine the appropriate dose of immune cells for immunotherapy.

In conclusion, we can feasibly isolate, activate the liver NK cells from a deceased donor liver graft perfusate, and safely administer them to HCC recipients. This immunotherapy does not increase the risk of GVHD or graft rejection. This immunotherapy could be a potential therapeutic tool for preventing the recurrence of HCC after LT. Larger controlled studies are required in future.

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Declarations

Conflict of interest The authors of this manuscript have no conflict of interest to disclose as described by the Cancer Immunology, Immunotherapy.

Ethical approval This phase I clinical trial was approved by the Institutional Review Board of the University of Miami Miller School of Medicine (IRB#20100344) and the Food and Drug Administration, and was registered with ClinicalTrials.gov (NCT01147380). The trial was designed and conducted according to the Declaration of Helsinki.

Informed consent All patients provided written informed consent before enrolling in the study.

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