Difference in Cytotoxicity Against Hepatocellular Carcinoma Between Liver and Periphery Natural Killer Cells in Humans

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In rodents, liver natural killer (NK) cells have been shown to mediate higher cytotoxic activity against tumor cells than do peripheral blood (PB) NK cells. However, such differences between liver and PB NK cells have not been extensively investigated in humans. The phenotypical and functional properties of NK cells extracted from liver perfusates at the time of living donor liver transplantation were investigated. The tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), a critical molecule for NK cell–mediated anti-tumor cell killing, was not expressed by freshly isolated PB NK cells or by liver NK cells. Stimulation with interleukin (IL)-2, significantly up-regulated the expression of TRAIL on liver NK cells, but this effect was barely observed on PB NK cells. Donor liver NK cells showed the most vigorous cytotoxicity against HepG2, a hepatocellular carcinoma (HCC) cell line, after IL-2 stimulation (90.5% ± 2.2% at E: T = 10:1), compared with donor and recipient PB NK cells and recipient liver NK cells (64.8% ± 8.2%, 56.1% ± 8.9%, and 34.6% ± 7.5%, respectively). IL-2 stimulation resulted in an increased expression of killing inhibitory receptors on liver NK cells in parallel with TRAIL expression. Consistently, the cytotoxicities of IL-2–stimulated donor liver NK cells against self and recipient lymphoblasts were negligible. In conclusion, adoptive transfer of IL-2–stimulated NK cells extracted from donor liver graft perfusate could mount an anti-tumor response without causing toxicity against 1-haplotype identical recipient intact tissues. These findings present a concept to prevent recurrence of HCC after liver transplantation. (HEPATOLOGY 2006;43:362-372.)

Natural killer (NK) cells are thought to provide a first line of defense against invading infectious microbes and neoplastic cells by exerting an effector function without the necessity for priming.[1,2] Given the efficacy of NK cells in selectively killing abnormal cells, a variety of approaches have been taken to try and selectively augment NK cell response to tumors.[3,4] Several therapeutic cytokines primarily act via NK cells [such as interleukin (IL)-2, IL-12, IL-15, and interferons (IFNs)] and many studies have shown that activation of NK cell differentiation and function leads to a more efficient elimination of tumor growth.[5-9] Despite these promising advances, the systemic administration of cytokines such as IL-2, that nonspecifically activate a broad range of different immune cell types, is associated with significant toxicity.[5,10] The adoptive transfer of NK cells further demonstrates the ability of NK cells to mount a therapeutic anti-tumor response and suggests that NK cells can be used in controlling human malignancy.[11,12] In these studies, autologous or even haploidentical lymphokine-activated killer cells obtained from peripheral blood mononuclear cells (PBMCs) have been administered to patients, although their comprehensive role in the treatment of selected malignancies remains to be elucidated.

NK cells are quite abundant in the liver of mice, in contrast to a relatively small percentage in the periph-

Abbreviations: NK; natural killer; IL, interleukin; IFN, interferon; PBMC, peripheral blood mononuclear cell; PB, peripheral blood; LDLT; living donor liver transplantation; HCC; hepatocellular carcinoma; TNF, tumor necrosis factor; TRAIL; TNF-related apoptosis-inducing ligand; DR, death receptor; LMNC; liver mononuclear cell; FCM, flow cytometric; MAb, monoclonal antibody; HLA, human leukocyte antigen; E:T, effector:target; MC, mononuclear cell; KIR, killer cell immunoglobulin-like receptors; MHC, major histocompatibility complex.

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Table 1 Clinical Characteristics of Living-Related Liver Transplant Donors and Corresponding Recipients With Cirrhosis

	Donor								Recipient									
Case No.	Age(y)/ Sex	Graft Liver	Graft Wt(g)	LMNC (×10⁵/ g)	HLA A	HLA B	HLA C	Relation	Age(y)/ Sex	Child Pugh	MELD	Original Disease	HCC	Liver Wt(g)	LMNC (×10⁵/ g)	HLA A	HLA B	HLA C
1	27/F	Left	262	3.7	2,26	51,54	1,–	Offspring	59/F	B	13.4	HCV	-	800	4.4	11,26	54,–	1,–
2	29/F	Left	460	7.8	2,26	56,61	1,8	Offspring	56/F	C	13.6	HCV	-	776	2.7	26,–	61,–	8,–
3	21/M	Right	550	6.4	2,24	7,35	3,7	Offspring	49/M	C	18.9	HCV	+	718	0.4	2,24	13,35	3,–
4	24/M	Right	564	1.2	24,26	62,-	3,4	Offspring	60/M	B	10.4	HCV	+	846	0.6	24,–	60,62	3,4
5	20/M	Right	896	1.9	2,33	37,46	1,3	Offspring	54/M	B	13.3	HBV	+	665	0.2	31,33	37,61	3,4
6	20/M	Right	632	3.2	24,26	52,62	3,12	Offspring	47/M	A	10.6	HCV	+	1150	3.1	24,–	52,–	12,–
7	57/F	Right	678	9.2	2,24	52,54	1,11	Spouse	43/M	C	35.2	HBV	+	810	7.0	24,–	7,52	7,12
8	59/M	Left	398	4.2	11,31	39,61	7,8	Spouse	57/F	C	18.9	HCV	-	684	9.5	24,26	35,39	3,7
9	30/M	Right	550	3.7	26,33	44,62	3,14	Offspring	58/M	C	29.4	HBV	-	820	0.4	2,26	51,62	3,15
10	49/F	Right	660	7.4	2,24	7,61	7,8	Sibling	44/F	C	18.1	AIH	-	753	1.2	2,24	7,61	7,8
11	44/F	Right	576	4.0	26,-	35,62	3,–	Spouse	48/M	C	37.5	HCV	-	410	13.4	24,–	52,–	12,–
12	29/M	Right	900	4.4	2,24	51,54	1,14	Offspring	58/M	C	16.9	HCV	+	714	4.2	24,–	54,–	1,8
13	43/M	Left	350	10.6	24,–	7,46	1,7	Sibling	46/F	C	17.6	HBV	-	425	1.4	24,–	7,46	1,7
14	18/M	Right	630	3.2	2,31	54,61	1,3	Offspring	57/F	A	3.1	HBV	+	932	8.2	24,31	54,61	1,3

Abbreviations: F; female, M; male, Graft wt; graft weight, LMNC; liver mononuclear cell, HLA; human leukocyte antigens, MELD; Model for End-Stage Liver Disease, HCV; hepatitis C virus, HBV; hepatitis B virus, AIH; autoimmune hepatitis, HCC; hepatocellular carcinoma.

eral lymphatics.[13-15] The underlying reason for this anatomically biased distribution has not been fully elucidated. In addition, liver NK cells have been shown to mediate higher cytotoxic activity against tumor cells than spleen or peripheral blood (PB) NK cells in rodents.[13-16] However, such differences between liver and PB NK cells have not been extensively investigated in human because of the limited availability of appropriate human samples.

In the current study, we have determined phenotypical and functional properties of liver NK cells extracted from donor and recipient liver perfusates in clinical living donor liver transplantation (LDLT). Donor liver NK cells showed the most vigorous cytotoxicity against a hepatocellular carcinoma (HCC) cell line after *in vitro* IL-2 stimulation, compared with donor and recipient PB NK cells and recipient liver NK cells. IL-2 stimulation led to an increased expression of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) on liver NK cells, which has been shown to be critical for NK cell–mediated anti-tumor cell killing without affecting normal cells.[17-19] In addition, we have confirmed that HCC expressed the death-inducing TRAIL receptors (TRAIL-Rs), TRAIL-R1/death receptor (DR) 4, and TRAIL-R2/DR5 that contain cytoplasmic death domains and signal apoptosis.[20,21] These findings raise a novel concept to prevent recurrence of HCC after liver transplantation, in other words, adoptive transfer of IL-2–stimulated NK cells extracted from donor liver graft into 1-haplotype identical recipients.

Patients and Methods

. Fourteen patients who underwent adult-to-adult LDLT at The Hiroshima University Hospital (Hiroshima, Japan) were involved in this study. The 14 patients with hepatic cirrhosis included 8 men and 6 women, ranging in age from 43 to 60 years [mean age (in years) ± SD, 52.6 ± 5.5]. Original diseases of the patients are shown in Table 1. Nine of the graft donors were offspring, two were siblings, and three were spouses, with ages ranging from 18 to 59 years (33.6 ± 12.0).

. Donor hepatectomy and the recipient transplantation procedure were performed as described previously.[22] In brief, the right or left lobe was harvested from the donor. After hepatectomy, *ex vivo* perfusion of the liver graft was performed through the portal vein. The initial perfusate consisted of saline solution (500 mL) followed by University of Wisconsin solution (1,000 mL). For the recipient, the implantation was performed after total hepatectomy. *Ex vivo* perfusion of the removed recipient liver was also performed through the portal vein by using the same perfusates. Liver mononuclear cells (LMNCs) were obtained from those perfusate effluents from healthy donor liver grafts and recipient livers with cirrhosis as follows. The effluents were condensed by centrifuging and LMNCs were isolated by gradient centrifugation with Separate-L (Muto Pure Chemicals Co., Ltd, Tokyo, Japan). PBMCs were also isolated by gradient centrifugation with Separate-L from 40 mL heparinized peripheral

blood from donors and recipients. LMNCs and PBMCs were suspended in RPMI 1640 medium that was supplemented with 10% heat-inactivated fetal calf serum (Sanko Chemical Co., Ltd., Tokyo, Japan), 25 mmol/L HEPES Buffer (Gibco, Grand Island, NY), 50 μmol/L 2 mercaptoethanol (Katayama Chemical Co., Osaka, Japan), 50 U/mL penicillin, and 50 μg/mL streptomycin (Gibco) (from hereon we refer to this medium as 10% RPMI). Ethical approval for this study was obtained from the Ethics Committee at The Hiroshima University Hospital.

C ***A*** **.** All flow cytometric (FCM) analyses were performed on a FACS Calibur dual-laser cytometer (BD Biosciences, Mountain View, CA). For phenotyping of NK cells, LMNCs and PBMCs were stained with fluorescein isothiocyanate–conjugated anti-CD3 (BD Pharmingen, San Diego, CA), phycoerythrin-conjugated anti-CD56 (B159) (BD Pharmingen), and biotin-conjugated anti-TRAIL monoclonal antibodies (MAbs) (RIK-2) (e Bioscience, Oxford, U.K.). For analyzing inhibitory receptors on NK cells, LMNCs and PBMCs were stained with peridin chlorophyll protein–conjugated anti-CD3 (SP34-2) (BD Pharmingen), phycoerythrin-conjugated anti-CD56, biotin-conjugated anti-TRAIL and fluorescein isothiocyanate–conjugated anti-CD-158a (HP-3E4) (BD Pharmingen), anti-CD-158b (CH-L) (BD Pharmingen), or anti-CD94 MAbs (HP-3D9) (BD Pharmingen). For analyzing TRAIL receptors on the HCC cell line, HepG2 cells were stained with biotin-conjugated anti-TRAIL-R1/DR4 (DJR1), anti-TRAIL-R2/DR5 (DJR2-4), anti-TRAIL-R3/decoy receptor (DcR) 1 (DJR3), or anti-TRAIL-R4/DcR2 (DJR4-1) MAbs (all MAbs from eBioscience). All the biotinylated MAbs were visualized with allophycocyanin-streptavidin (BD Pharmingen). Dead cells were excluded from the analysis by light-scatter and propidium iodide staining.

C ***C*** **.** LMNCs and PBMCs were cultured with or without human recombinant IL-2 (100 U/mL) (Takeda, Tokyo, Japan) in 10% RPMI at 37°C in a 5 % CO_2 incubator. After 4 days in culture, cells were harvested for further analyses.

C ***A*** **.** HepG2 cells established from HCC tissue from a hepatitis B virus– and hepatitis C virus–negative HCC patient (HLA; human leukocyte antigens: −A02,24, −B35,51 −CO,4) were purchased from The Japanese Cancer Research Resources Bank and were maintained in 10% RPMI.[23] HepG2 were labeled with 100 μCi Na2 (^{51}Cr) O4 for 60 minutes at 37°C in 5% CO_2 in 10% RPMI, washed 3 times with medium, and then subjected to the cytotoxicity assay. The labeled HepG2 cells were adjusted to 1×10^6 cells in 10 mL volumes (1×10^4/well) and were incubated in a total volume of 200 μL with effector cells in 10% RPMI in round-bottomed 96-well microtiter plates (Nunclon; Inter Med, Denmark). LMNCs or PBMCs from healthy donors or recipients with cirrhosis were used as effectors at effector-target (E:T) ratios of 2.5:1 to 40:1. When indicated, LMNCs and PBMCs were cultured *in vitro* with IL-2 for 4 days before using as effectors. As a control, the target cells were incubated either in culture medium alone to determine spontaneous release, or in a mixture of 2% Nonidet P-40 (Nacalai Tesque, Inc., Kyoto, Japan) to define the maximum ^{51}Cr release. The plates were centrifuged at 1,000 rpm for 3 minutes to pack the cell layer at the end of the reaction, after which the cell-free supernatants were carefully harvested, and its radioactivity was measured with a gamma counter. The percentage of specific ^{51}Cr release was calculated by the following formula: % cytotoxicity = [(cpm of experimental release − cpm of spontaneous release)]/[(cpm of maximum release − cpm of spontaneous release)] × 100. The spontaneous release was less than 20% of the maximum release. All assays were performed in triplicate.

C **.** LMNCs and PBMCs were separated into a $CD3^-CD56^+$ NK cell fraction and a non-NK cell fraction (T cells, NKT cells, B cells, and monocytes/macrophages) by magnetic cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany) using the human NK cell isolation Kit II (Miltenyi Biotec) according to the manufacturer's instructions. The purity of isolated fractions was assessed by FCM, and only preparations whose purities were greater than 95% were used for functional studies. Using a similar method as described, NK and non-NK cells isolated from IL-2–stimulated donor LMNCs and PBMCs were subjected to the cytotoxicity assay against HepG2 cells. When indicated, the isolated NK cells were pre-incubated for 30 minutes at 4°C with neutralizing Abs against TRAIL (purified anti-TRAIL MAbs; eBioscience) (final concentration 20 μg/mL) or with isotype-matched non-reactive Abs before the cytotoxicity assay. In addition, a similar cytotoxicity assay with those donor NK cells was performed against autologous and allogenic lymphoblasts, which had been prepared from PBMCs of donors and corresponding recipients by cultivating with 5 μg/mL PHA (Sigma, Poole, UK) for 4 days.

. Surgically resected liver specimens were obtained from patient with HCC who had undergone potentially curative tumor resection at The Hiroshima University Hospital. All HCC tissues were pathologically confirmed. Normal liver samples obtained from patients with metastatic liver tumors were used as control. Informed consent was obtained from all

patients. To localize TRAIL receptors *in situ* in the liver, immunohistochemistry was performed on frozen tissue sections of normal liver tissues and carcinoma cases as described previously.[24] For protein detection by immunohistochemistry, 4-μm frozen sections from representative tumor tissue of at least 1 cm^2 were fixed in acetone for 15 minutes. Sections were incubated for 60 minutes with the following MAbs: biotin-conjugated anti-TRAIL-DR4, anti-TRAIL-DR5, anti-TRAIL-DcR1, and anti-TRAIL-DcR2 at appropriate concentrations in phosphate-buffered saline. Binding sites of primary antibodies were visualized using the Dako EnVision kit (Dako, Copenhagen, Denmark) according to the manu-

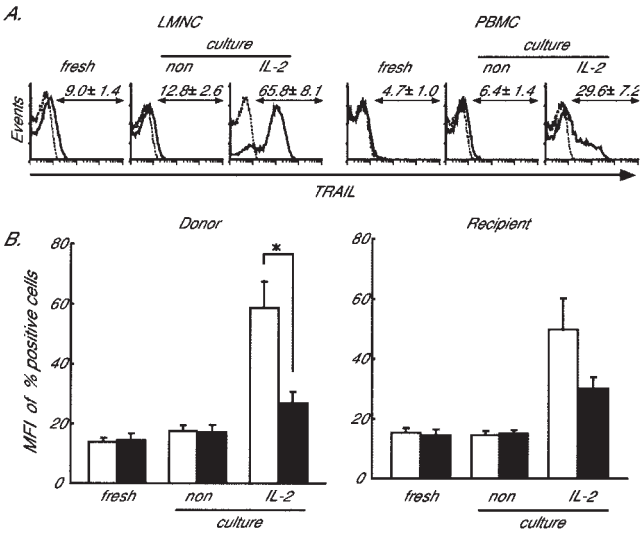

Fig. 2. Liver NK cells inductively express remarkable levels of TRAIL, but PB NK cells do not. Freshly isolated or cultivated with or without IL-2 LMNCs and PBMCs obtained from healthy donors and corresponding recipients were stained with CD3 and CD56 MAbs together with TRAIL MAb. (A) Histograms represent the log fluorescence intensities obtained on staining for TRAIL after gating on the $CD3^{-}CD56^{+}$ NK cells subsets obtained from healthy donors. Dotted lines represent negative control staining with isotype-matched MAbs. The numbers (mean ± SEM) indicate the percentages of cells in each group that were positive for TRAIL expression (n = 7 each). Histogram profiles shown are representative of 7 independent experiments. (B) The numbers indicate the mean fluorescence intensity (MFI) of cells in each group that were staining positively for TRAIL on freshly isolated, or cultivated with/without IL-2 NK cells (LMNC; open column, PBMC; closed column). The data represent mean ± SEM (n = 7). Statistical analyses were performed using ANOVA (*$P < 0.05$). NK, natural killer; TRAIL, TNF-related apoptosis-inducing ligand; LMNC, liver mononuclear cell; PBMC, peripheral blood mononuclear cell; MAb, monoclonal antibody.

portion between LMNCs and PBMCs was not conspicuous in recipients with cirrhosis, because of a relatively reduced proportion of NK cells in LMNCs from livers with cirrhosis. In LMNCs from donors and recipients, the $CD3^{-}CD56^{high}$ NK cell subpopulation, which is known to produce preferentially large amounts of cytokines,[28,29] was easily detectable, whereas it was undetectable in PBMCs (Fig. 1C).

C ***E*** ***A*** **.** TRAIL is a type II transmembrane protein that belongs to the TNF family, which preferentially induces apoptotic cell death in a wide variety of tumor cells but not in most normal cells.[17-19] We and others have previously reported that a subpopulation of NK cells in adult mouse liver, unlike other tissues, constitutively express TRAIL, and these liver NK cells were partially responsible for the natural anti-tumor function against TRAIL-sensitive tumor cells.[9,16,17] As shown in Fig. 2, freshly isolated liver NK cells from normal liver and liver with cirrhosis barely expressed TRAIL, whereas freshly isolated PB NK cells completely lacked TRAIL-expression. *In vitro* stimulation with IL-2 significantly up-regulated the expression of TRAIL on liver NK cells (the expression of TRAIL on liver NK cells from donor normal livers was somewhat higher than that on liver NK cells from recipient livers with cirrhosis). Conversely, even after IL-2 stimulation, PB NK cells expressed little TRAIL. Thus, liver NK cells inductively expressed remarkable levels of TRAIL, but PB NK cells did not.

We have recently demonstrated that most murine TRAIL-expressing liver NK cells lack expression of Ly-49 inhibitory receptors, which recognize self-MHC class I.[16] To address whether the same is true in humans, we have analyzed inhibitory receptors on human liver NK cells. Inhibitory receptors on human NK cells can be subdivided into 2 groups: killer cell immunoglobulin-like receptors (KIRs) (belonging to the immunoglobulin superfamily) including CD158a and CD158b; and C-type lectin-like receptors, CD94/NKG2. KIRs are major histocompatibility class (MHC) class I–restricted molecules that recognize HLA-A, -B, -C, and -G molecules, whereas CD94 recognizes the nonclassical MHC class Ib molecule HLA-E. CD94 is expressed essentially on all NK cells, and uses HLA-E expression as a sensor for the overall MHC class I level of a cells.[30,31] In contrast, individual KIR family members express on certain NK cell subsets, exhibit finer specificity for HLA class I allotypes, and can distinguish between groups of HLA-A, -B, and -C allotypes. Ligation of such KIRs/CD94 to HLA class I molecules on self cells results in inhibition of NK cytotoxic activity, as originally predicted by the "missing-self" hypothesis.[32,33] This regulation ensures that cells expressing none, altered, or reduced MHC-I molecules, such as malignant or virus-infected cells, can be killed by NK cells. All freshly isolated liver NK cells expressed CD94 and subpopulations of those cells expressed CD158a/CD158b (Fig. 3A). Cultivation of liver NK cells with no stimulants resulted in reduced expressions of CD158a, CD158b and CD94 (Fig. 3B). However, IL-2 stimulation led to the maintenance of those expressions even on TRAIL expressing liver NK cells (Fig. 3A-B). Thus, as opposed to TRAIL-expressing NK cells in mice, those cells in humans equip a compensatory mechanism to protect the self-MHC class I–expressing cells from NK cell-mediated cell killing.

CC ***E*** ***D*** **-** ***A*** **-D 4** **-D 5.** The susceptibility to TRAIL-induced apoptosis may be related to the expression levels of multiple receptors on target cells. Recent molecular cloning of the TRAIL-receptors elucidated that TRAIL binds to at least four receptors, two of these death-inducing receptors (TRAIL-R1/DR4 and TRAIL-R2/DR5) contain cyto-

plasmic 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 have similar affinities, and the latter may act as decoys.[20,21] Many cancer cell lines preferentially express TRAIL-DR4 and -DR5, suggesting differential regulation of the death and decoy receptors.[24] The preferential expression of these decoy receptors in normal tissue suggests that TRAIL may be useful as an

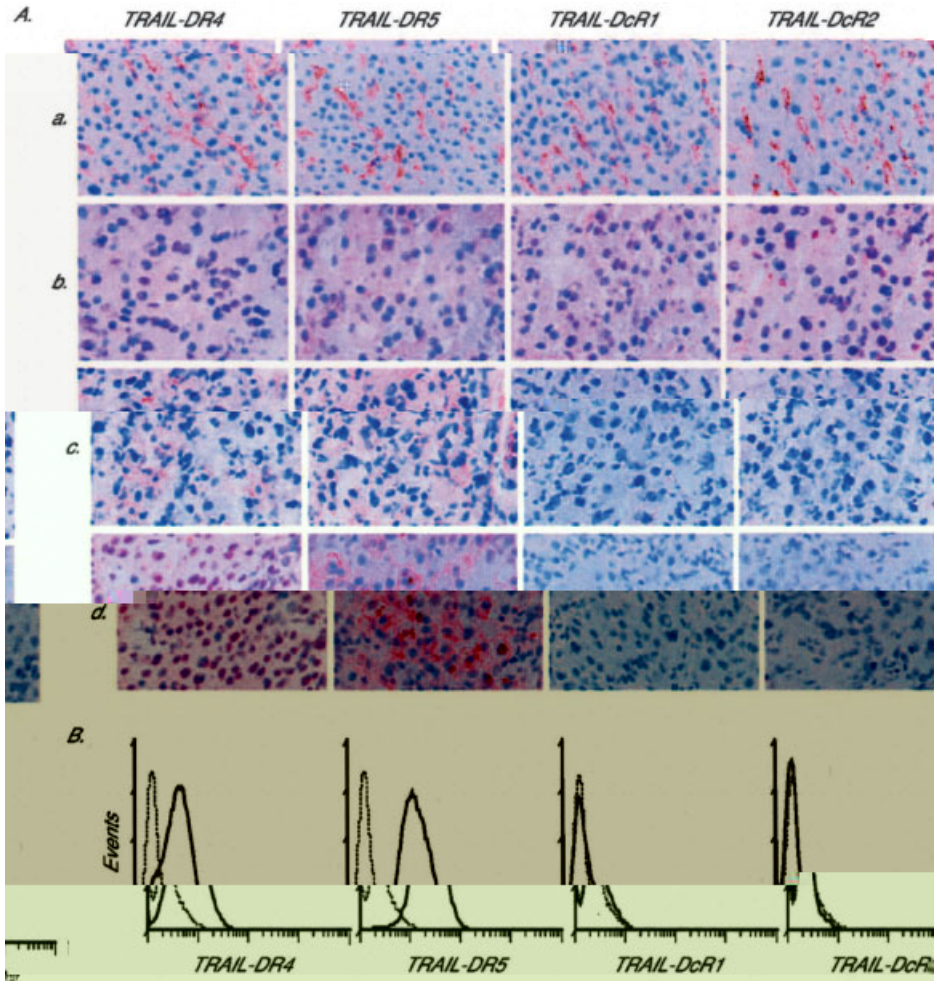

Fig. 4. Differential expression of TRAIL receptors in normal liver tissue and HCC tissue. (A) Immunohistochemical expression of TRAIL-DR4, -DR5, -DcR1, and -DcR2 in normal liver tissue (a), tumor site of well-differentiated HCCs (b), moderately differentiated HCCs (c), and poorly differentiated HCCs (d). Magnification ×400. Immunopathological findings shown are representative of 3 individual samples in each categorized HCCs. (B) Surface expression of TRAIL receptors on the surface of HepG2 was analyzed by FCM. Dotted lines represent negative control staining with isotype-matched MAbs. HepG2 expressed high TRAIL-DR4 and -DR5 but no TRAIL-DcR1 and DcR2, resembling poorly differentiated HCCs. TRAIL, TNF-related apoptosis-inducing ligand; HCC, hepatocellular carcinoma; FCM, flow cytometric; MAb, monoclonal antibody; TNF, tumor necrosis factor.

(64.8% ± 8.2%, 56.1% ± 8.9%, and 34.6% ± 7.5%, respectively) (Fig. 5B). By magnetic sorting, NK and non-NK cells were isolated from donor LMNCs and PBMCs and resulting populations were then analyzed for cytotoxicity against HepG2. As expected, the non-NK cell fraction did not mediate cytotoxicity. The higher cytotoxicity of NK cells isolated from LMNCs was observed than that of NK cells from PBMCs (Fig. 5C). Addition of neutralizing anti-TRAIL MAb partially reduced the cytotoxicity of IL-2–stimulated donor NK cells toward HepG2 cells (24.2% ± 9.1% reduction at E:T = 8:1, data not shown), indicating that TRAIL-mediated NK cell cytotoxicity was involved. Despite strong cytotoxicity of IL-2–stimulated donor liver NK cells, the cytotoxicities of those cells toward 1-haplotype identical allogeneic recipient- and autologous donor-derived lymphoblasts were negligible, indicating their capacity to distinguish tumor cells from normal cells (Fig. 5D).

To determine whether liver non-NK cells are responsible for the increased anti-tumor activity of liver NK cells, we performed additional experiments mixing PB NK and non-NK cells isolated from either LMNCs or PBMCs. Before IL-2 stimulation, NK and non-NK cells were isolated from donor LMNCs and same donor PBMCs. Then, for subsequent cytotoxic assays, PB NK cells were cultured with either liver non-NK or PB non-NK cells at the same ratio in the presence of IL-2. Even when PB NK cells were cultured with liver non-NK cells in the presence of IL-2, PB NK cells expressed little TRAIL (Fig. 6A). No difference in cytotoxicity against HepG2 was observed between a mixture of PB non-NK and PB NK cells and one of liver non-NK and PB NK cells (Fig. 6B).

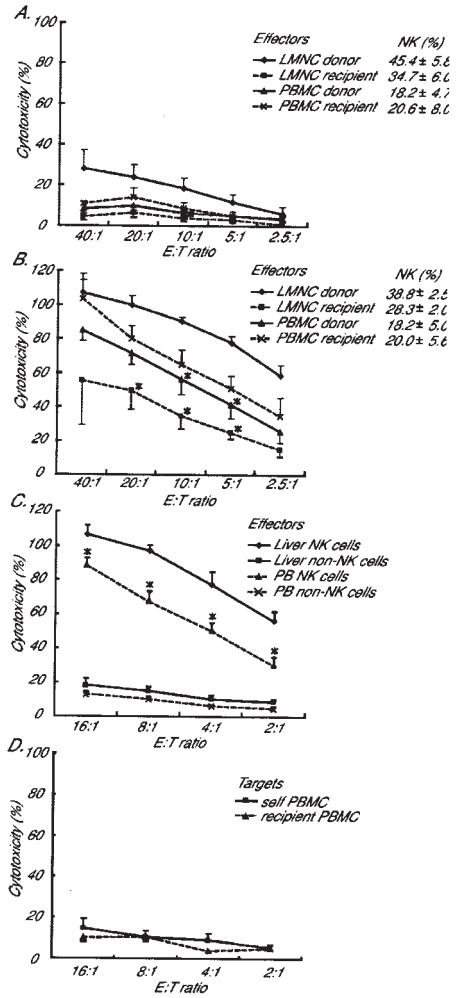

Fig. 5. IL-2–stimulated donor liver NK cells show vigorous cytotoxicity against HepG2. NK cytotoxic activities of indicated effectors against indicated target cells were analyzed by ^{51}Cr release assay. All data are represented as the mean ± SEM of assays, each set up in triplicate. Statistical analyses were performed using ANOVA (*$P < .05$). (A) NK cytotoxic activities of freshly isolated LMNCs and PBMCs obtained from liver transplant donors and recipients against HepG2 target cells (n = 4 each). Percentages of $CD3^-CD56^+$ NK cells in LMNCs or PBMCs obtained from 4 adult healthy donors and 4 corresponding recipients with cirrhosis are shown in the upper right corner (mean ± SEM). (B) NK cytotoxic activities of IL-2–stimulated LMNCs and PBMCs obtained from liver transplant donors and recipients against HepG2 target cells. LMNCs and PBMCs were cultivated for 4 days in the presence of IL-2 before the cytotoxicity assay (n = 4 each). Percentages of $CD3^-CD56^+$ NK cells in LMNCs or PBMCs obtained from 4 adult healthy donors and 4 corresponding recipients with cirrhosis are shown in the upper right corner (mean ± SEM). (C) NK cytotoxic activities of NK and non-NK cells isolated from IL-2–stimulated donor LMNCs and PBMCs against HepG2 target cells (n = 4 and 5, respectively). (D) NK cytotoxic activities of NK cells isolated from IL-2–stimulated donor LMNCs against autologous and allogeneic lymphoblasts, which had been prepared from PBMCs of donors and corresponding recipients by cultivating with PHA for 4 days (n = 4). IL-2, interleukin-2; NK, natural killer; LMNC, liver mononuclear cell; PBMC, peripheral blood mononuclear cell.

These findings are consistent with a model whereby donor LMNCs have greater NK activity because NK cells from LMNCs are more functional on a per cell basis than those from PBMCs, and that liver non-NK cells are not responsible for such altered function of liver NK cells in our experimental system.

Discussion

The role of liver transplantation in patients with HCC has evolved over the past 2 decades, and transplantation has become one of the few curative treatment modalities for patients with unresectable HCC.[35,36] LDLT has become an acceptable therapy for patients with HCC in response to the pervasive shortage of deceased donor livers. In addition, waiting time for HCC patients to receive a deceased donor has decreased significantly, and the number of patients dropping out from the waiting list has decreased because of a decrease of advanced-stage disease. As a result, this may decrease the progression of disease so

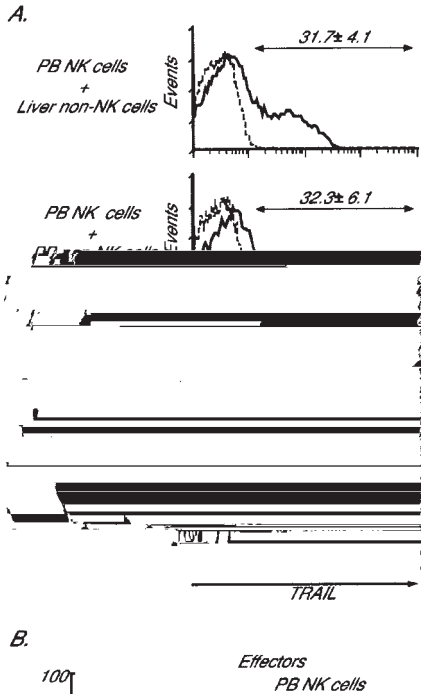

Fig. 6. Liver non-NK cells are not responsible for the increased anti-tumor activity of NK cells. Before IL-2 stimulation, NK and non-NK cells were isolated from donor LMNCs and same donor PBMCs. Then, NK cells from PBMCs were cultured with either liver non-NK or peripheral blood (PB) non-NK cells at the same ratio (3: 7) in the presence of IL-2 for subsequent NK cytotoxic assays. (A) Histograms represent the log fluorescence intensities obtained on staining for TRAIL after gating on the $CD3^-CD56^+$ NK cells subsets obtained from each group. Dotted lines represent negative control staining with isotype-matched MAbs. The numbers indicate the percentages of cells in each group that were positive for TRAIL expression (mean ± SEM, n = 3 each). Histogram profiles shown are representative of 3 independent experiments. (B) NK cytotoxic activities of IL-2–stimulated PB NK cells cultured with either liver non-NK or PB non-NK cells against HepG2 target cells. Data are represented as the mean ± SD of triplicate samples and similar results were obtained in 3 independent experiments. IL-2, interleukin-2; NK, natural killer; LMNC, liver mononuclear cell; PBMC, peripheral blood mononuclear cell; TRAIL, TNF-related apoptosis-inducing ligand.

that the recurrence rate should be lower than for recipients who wait for an organ from a deceased donor.[37] When liver transplantation is performed as a therapy for HCC, recurrent HCC is nevertheless one of the most fatal complications. The management for the prevention of organ rejection requires the use of postoperative immunosuppressive therapy; however, immunosuppressants increase the incidence of recurrence or metastasis of cancer and induce cancer progression. The immunosuppressive regimen currently used after liver transplantation, consisting of tacrolimus/cyclosporine and methylprednisolone, reduces adaptive components of cellular immunity (predominantly T cell–mediated immune responses), while maintaining innate components of cellular immunity.[3,38,39] Because immune surveillance against tumors is mediated by both innate and adoptive components of cellular immunity, augmentation of NK cell responses to tumors, which have been thought to play a central role in innate immunity against tumors, might be a promising immunotherapy approach. Several therapeutic cytokines including IL-2 and IFNs primarily act through NK cells, and many studies have shown that activation of NK cell differentiation and function leads to a more efficient elimination of tumor growth[5-9]; however, the systemic administration of those cytokines is likely associated with an acceleration of alloimmune responses leading to liver allograft rejection. Hence, the adoptive transfer of cytokine-modulated NK cells might be a reasonable approach in preventing recurrence of HCC after liver transplantation, while minimizing effects on alloimmune responses.

NK cells in the blood stream are ready to kill any cell.[1,2] Healthy cells are spared by their MHC class I molecules, which bind to corresponding inhibitory receptors on NK cells so that only cells with altered or lacking MHC class I molecules, a mechanism to escape recognition by MHC-restricted $CD8^+$ T lymphocytes, are killed because of missing inhibitory mechanisms.[32,33,40,41] The cytotoxic activities of NK cells are controlled by a variety of receptors including CD94/NKG2 and KIRs (CD158a/CD158b), which bind to respective MHC class I molecules on target cells. NK cells can discriminate not only between different class I molecules but also between certain allotypes that differ in single amino acid substitutions at positions 77 and 80 in the 1 domain of the 2 HLA-C groups.[42,43] This process can contribute to detection of transformed cells because downregulation of selected allotypes is an event often occurring in the progression of some tumor types. The group of inhibitory receptors is reported to have higher affinity of ligand binding than the activating group, and thus the inhibitory receptors may play a more dominant role in regulating the cytotoxic activities of NK cells.[44] HCC cell lines, such as HepG2 and HuH-7, have been reported to lose or decrease the expression of HLA-B and -C alleles on the cell surface.[23,45] The only requirement for NK cell receptor repertoire development appears to be that every NK cell express at least one inhibitory receptor specific for autologous HLA class I, thereby ensuring tolerance against healthy cells sharing 1-haplotype MHC molecules.[46,47]

In the current study, we have determined functional properties of liver NK cells extracted from donor and recipient liver perfusates in clinical LDLT. Liver NK cells have never been used for the adoptive transfer to mount anti-tumor activity, because of the limited availability of liver NK cells in a clinical setting. In liver transplantation, *ex vivo* perfusion of the liver through the portal vein should inevitably be done for flushing blood from the liver graft before implantation to avoid intragraft coagulation. We have demonstrated that liver perfusates, which usually are thrown away, contain large amounts of NK cells. Donor liver NK cells showed the most vigorous cytotoxicity against a HCC cell line after *in vitro* IL-2 stimulation, when compared with donor and recipient PB NK cells and recipient liver NK cells. The higher antitumor activity of liver NK cells than PB NK cells has been well demonstrated in mice, although mechanisms underlying this fact remain unclear.[15,16] The consistent results were observed in healthy donors, but not in recipients with cirrhosis. The cytotoxicity of PB NK cells against HepG2 per se did not differ between donors and recipients, but that of liver NK cells from livers with cirrhosis were significantly impaired, regardless of the presence or absence of IL-2 stimulation.

Previously published data redefine NK cells as potent constitutive immune effectors, which are able to use not only the perforin-mediated secretory/necrotic mechanism to kill rare leukemia cell targets, but also a powerful TNF family ligand-mediated nonsecretory apoptotic mechanism to destroy most solid tumor cell targets.[34] TRAIL is highly expressed on most NK cells after stimulation with IL-2, IFNs, or IL-15 in mice.[9,19,48] Neutralization of TRAIL additively enhanced liver metastasis in perforin-deficient mice but not in IFN-γ–deficient mice.[9] These findings clearly place perforin and TRAIL as the 2 key cytotoxic effector pathways used by NK cells. From the current study in human, we now appreciate that freshly isolated liver NK cells barely express a detectable level of TRAIL on their surface, but a remarkable level of TRAIL expression can be induced, preferentially on liver NK cells, by stimulation with IL-2. Taken together with the finding that poorly differentiated HCCs highly express the death-inducing TRAIL receptors (DR 4 and DR5), which contain cytoplasmic death domains and signal ap-

optosis, as well as HepG2, adoptive transfer of IL-2–stimulated NK cells extracted from donor liver grafts are likely to mount an anti-tumor response without causing toxicity

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