RESULTS

Deceased donor LMNCs contain a large population of NK and NT cells

As an initial step, we compared the characteristics between LMNCs and PBMCs derived from deceased donors to determine whether liver NK cells could be used for clinical immunotherapy. To characterize the donor liver and peripheral NK cells, we collected liver graft perfusate and peripheral blood during regular organ procurement. The liver graft perfusate contained a large number of mononuclear cells ($1.2 \pm 0.2 \times 10^9$ cells), with a viability of 90% ± 3%. The phenotype of these cells was markedly different from that of matched donor PBMCs (Table 1). The proportions of $CD3^-CD56^+$ NK and $CD3^+CD56^+$ natural killer-like T (NT) cells in the LMNCs were significantly higher than those in the PBMCs. In contrast, the LMNCs possessed a smaller number of T cells and B cells than did the PBMCs. There was no significant difference in the number of monocytes or granulocytes. Phenotypical flow cytometry analysis of other surface markers was then performed in a comparative analysis between liver and blood NK cells (Table 1). The CD69 early activation marker was expressed on the majority (75.0%) of liver NK cells, whereas the same subset in the PBMCs showed a significantly lower frequency of expression (9.6%). When the expression of the non-major histocompatibility complex class I-specific-activating NK cell receptors (natural cytotoxicity receptors; NKp44 and NKp46) was examined in both liver and peripheral blood, nearly all NK cells (>90%) expressed NKp46. In agreement with Vitale et al. (40), NKp44 was not detectable in peripheral blood NK cells, while a mean of 3.8% of liver NK cells expressed NKp44. These results indicate a physiological activation status for liver NK cells. The percentage of NK cells expressing CD16, an NK cell lysis receptor (22), was higher in PBMCs than in LMNCs. Both the liver and peripheral blood NK cells expressed the C-type lectin receptor CD94. This molecule binds human leukocyte antigen

(HLA)-E loaded with leader peptides from major histocompatibility complex (MHC) class I molecules (10).

Next, we analyzed the response of NK cells in LMNCs and PBMCs after IL-2 stimulation. 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 (30,43,44). We previously reported that in vitro IL-2 stimulation upregulated the expression of TRAIL and induced strong cytotoxicity for liver NK cells extracted from living donor liver graft perfusate (17). As shown in Figure 1, freshly isolated liver NK cells and peripheral blood NK cells barely expressed TRAIL. Stimulation with IL-2 significantly upregulated the expression of TRAIL in liver NK cells, but this effect was barely observed for peripheral blood NK cells. IL-2 stimulation also resulted in an increased expression of the activation molecule NKp44 and maintained the expression of the inhibitory receptor CD94. These results indicate that cultivated NK cells have a compensatory mechanism to protect the self-MHC class I-expressing cells from NK cell-mediated cell death.

Characteristics of the liver NK cell-enriched product

For determining whether NK cells from deceased donor liver graft perfusate could be processed using cGMP-compliant components, the LMNC cultivation was analyzed. At the start of the culture (pre-culture), the mean percentage of NK cells was 45.0% (range: 21.2–76.2%), whereas T cells constituted 22.8% (range: 6.6–35.2%). After processing, NK cells were enriched to 52.0% ± 5.0%. The viability of the enriched NK cells, as determined by trypan blue staining, remained >90% during the process. No microbial contamination was detected in the final product or in the culture medium. In addition, the cell processing resulted in a significant reduction of T cells in the final product. The percentage of $CD3^{+}CD56^{-}$ T cells decreased to 0.6% ± 0.2% (0.18×10^{5} cells·kg^{-1}). Other CD56-positive

components of the final product included NT cells (0.2% ± 0.1%). Next, we further examined the phenotype of the CD56-negative fraction of the final product. After IL-2 stimulation, the phenotype of the final product was assessed using another T cell marker, CD7, with or without the addition of OKT3. As shown in Table 2, the final product contained $CD7^{+}$T cells at 24.7%. Goat anti-mouse IgG antibody detection of OKT3 (isotype: mouse IgG) on T cells showed that 14.4 % of the final product was bound with OKT3. After administration of the final product to the recipient, these T cells would be depleted by several mechanisms, including T cell opsonization and clearance by mononuclear phagocytic cells, and complement-mediated cell lysis (6,7,39). The remainder of the T cells (10.3% of the final product) is involved in CD3 internalization or modulation, which induce T cell dysfunction (6,36). Other components of the final product are shown in Table 2.

For phenotypically characterizing the NK cells in the final product relative to those in the starting material, a detailed flow cytometry analysis was undertaken. As shown in Figure 2, freshly isolated liver NK cells barely expressed TRAIL, NKp44, and CD25 (IL-2αR) and produced little cytokines. The cell processing significantly upregulated the expression of TRAIL and NKp44 in liver NK cells, but these changes were not seen in peripheral blood-NK cells. The expression of CD69 and CD25 in liver NK cells also increased, but not significantly. In contrast, NKp46 expression significantly decreased after the cell processing. The activating receptors are defined by their ability to directly mediate the killing of the targets. Nevertheless, recent findings have demonstrated that the activation of some of the NK-triggering receptors requires the synergistic stimulation of more than 1 receptor (3). Our results are compatible with this theory. Intracellular staining flow cytometry showed that IL-2 stimulation induced significant cytokine production (IFN-γ and TNF-α [5.8% to 37.0% and 4.1% to 59.2%, respectively, n = 4, $p<0.01$]) in liver NK cells (Figure 2). These results are similar to those of studies of living donor liver graft perfusate (17,27). Next, NK cell

cytotoxicity assays using LMNCs and PBMCs isolated from the deceased donor as effectors and K562 as targets were performed. Cytotoxicity against the standard NK cell target K562 was markedly elevated using effector cells from the final products relative to those from pre-cultured LMNCs and PBMCs (Figure 3). At a 20:1 effector/target cell ratio, 56.3% of the K562 targets were killed on average by the final products, whereas pre-cultured LMNCs and pre- and post-PBMCs killed only 11.8%, 2.5%, and 23.8% of K562 targets, respectively. We also tested the difference between with and without addition of OKT3 after IL-2 stimulation. The addition of OKT3 did not significantly enhance the NK cytotoxicity of either PBMCs or LMNCs.

DISCUSSION

In this study, we demonstrated the phenotypical and functional properties of NK cells extracted from deceased donor liver graft perfusate under cGMP conditions. Methods for processing allogeneic NK cell products for human use on a clinical scale are limited to FDA-approved selection facilities and devices. The cGMP facility at University of Miami has published methods for processing different products (4,9). Lot release testing is described in "Patients and Methods" section and is as dictated by the FDA guidelines for cellular products. First, LMNCs were shown to contain a large number of NK and NT cells, with both cell types possessing characteristics different from those of PBMCs. Second, in vitro stimulation with IL-2 induced liver NK cells to strongly upregulate activation markers, cytotoxicity, and cytokine production and to maintain the expression of inhibitory receptors. These results were compatible with those for living donor liver graft perfusate (17). Finally, we confirmed that the final product met the lot release criteria and contained low T cell numbers, thereby reducing the possibility of graft vs. host disease (GVHD) in a recipient.

This study demonstrated that deceased donor liver graft perfusate contained an average of 1.2 $\pm$ 0.2 $\times$ 10^9 mononuclear cells and 5.3 $\times$ 10^8 NK cells, whereas living donor liver graft perfusate, which contained 9.1 $\pm$ 0.8 $\times$ 10^8 mononuclear cells (Ohdan H et al., Hiroshima University, Japan, unpublished data). However, this number is several fold higher than the numbers calculated in previous studies on deceased donor liver perfusate (2,18,25). This discrepancy may be due to the fact that we collected the liver perfusate at the time of organ procurement while others did so just before liver transplantation.

NK cells can destroy many solid tissue-derived malignant cells through death receptor-ligand interactions (42). Previously, we found that normal hepatocytes express TRAIL-DR4 and

TRAIL-DR5 together with TRAIL-DcR1 and TRAIL-DcR2, but that moderately or poorly differentiated HCCs highly express TRAIL-DR4 and TRAIL-DR5 but do not express TRAIL-DcR1 and TRAIL-DcR2, which indicates a susceptibility to TRAIL-expressing NK cell-mediated activity toward HCC (17,28). We have now shown that IL-2 stimulation significantly increases the expression of TRAIL in liver NK cells that are extracted from deceased donor liver graft perfusate (Figure 1). Functionally, we also have shown that IL-2 activated liver NK cells were highly cytotoxic against tumors compared with PBMCs (Figure 3). In addition to having an anti-neoplastic effect, NK cells are important components of the innate immune response due to their ability to lyse virus-infected cells and to recruit cells involved in adaptive immune responses. IFN-γ is a known host mediator that shapes the tumor phenotypes in a broader process known as "immunoediting" (19). Mice that lack either IFN-γ or its functional receptor are more susceptible to both viral and bacterial infections, indicating that IFN-γ plays an important role in anti-viral and anti-bacterial responses (33,47). It is possible that these liver NK cells can prevent the replication of viruses including hepatitis C virus through an IFN-γ-dependent mechanism. Further studies are required to address this possibility.

The induction of GVHD is a major risk factor associated with the use of lymphocyte infusions from unrelated or haploidentical family donors (8,15). For clinical-scale experiments, OKT3 was added to the culture media 1 day prior to cell harvesting. The administration of OKT3-coated T cells in vivo has been shown to result in the opsonization and subsequent trapping or lympholysis of cells by the reticuloendothelial system (6,7,39). This method has been performed for clinical NK therapy in Japan, with no GVHD cases reported (27). Our final product from the cadaveric donor liver perfusate contained 0.02×10^6 $CD3^+CD56^-$ T cells·kg^-

[1]. There are some clinical studies regarding T cell contamination. For example, Miller et al. reported that the final T-cell dose was 0.18×10^6 cells·kg^{-1} and that GVHD did not occur after haploidentical NK cell infusion (24). Schulze et al. reported that T cell contamination was 0.01×10^6 cells·kg^{-1} in allogeneic stem cell transplantation and that no GVHD occurred (34). Frohn et al. performed allogeneic NK cell infusion for renal cell carcinoma. The T cell contamination was 1.0% (0–7%) in their study (11). Passweg et al. defined the upper limit of acceptable T cell contamination as 0.1×10^6·kg^{-1} BW for allo-NK cell infusion in stem cell transplantation. No patients developed clinical signs of GVHD after NK cell infusion (29). Compared with other clinical studies, our final product contains an acceptable level of T cell contamination. NK cells exert alloreactivity after mismatched haploidentical transplantations due to an incompatibility between killer cell inhibitor receptors of donor NK cells and the recipient HLA type C (31). However, there is no known evidence of NK-mediated GVHD in humans.

In conclusion, liver NK cells derived from deceased donor liver graft perfusate inductively expressed TRAIL and secreted IFN-γ. IL-2 stimulated liver NK cells showed strong cytotoxicity against NK-susceptible K562 targets. Hence, these cells are potentially useful for the immunotherapy of LT recipients with HCC. This study is the first attempt to apply cadaveric donor liver NK cells to clinical cell transplantation. Our results will have a positive effect on adoptive immunotherapy using liver NK cells. However, further clinical studies are needed to elucidate the role played by donor liver NK cell infusions in the treatment of HCC patients after LT.

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Authors declare no conflicts of interest.

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Table 1

	LMNC	PBMC	*p* value
$CD3^-CD56^+$ NK	45.0 ± 4.0	21.8 ± 5.2	0.001
$CD3^+CD56^+$ NT	16.0 ± 1.8	3.3 ± 1.3	0.00001
$CD3^+CD56^-$ T	22.8 ± 2.4	37.0 ± 4.7	0.014
$CD3^+CD4^+$ T	7.1 ± 0.8	20.7 ± 3.3	0.002
$CD3^+CD8^+$ T	28.5 ± 2.8	17.4 ± 3.4	0.017
$CD19^+$ B	3.8 ± 1.3	9.8 ± 3.0	0.085
$CD14^+$ mono	16.7 ± 7.5	18.2 ± 12.5	0.909
$CD15^+$ Gran	13.6 ± 6.2	17.0 ± 13.3	0.806
	Liver NK cells	PB NK cells	
$CD16^+$	79.3 ± 2.4	96.8 ± 1.2	0.000001
$TRAIL^+$	5.1 ± 1.0	2.8 ± 0.9	0.089
$NKp44^+$	3.8 ± 1.1	0.2 ± 0.1	0.011
$NKp46^+$	96.8 ± 0.9	90.1 ± 3.7	0.045
$CD69^+$	75.0 ± 6.3	9.6 ± 3.0	0.00002
$CD94^+$	88.1 ± 10.3	90.6 ± 6.5	0.828
$CD25^+$	1.7 ± 1.1	1.1 ± 0.7	0.618

Immunophenotypical comparison of NK cells in liver perfusate and peripheral blood. The value indicate the percentage of cell types after Ficoll density-gradient centrifugation (mean ± SEM, $n = 4–14$). Statistical analysis was performed using Student's *t*-test. Abbreviations; LMNC, liver mononuclear cells; PBMC, peripheral blood mononuclear cells; NK, natural killer cells; NT, natural killer-like T cells; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; mono, monocyte; Gran, granulocyte.

Table 2

	% of the $CD56^-$ fraction	% of the final product
goat aMIgG	-	14.4 ± 5.6
$CD56^-$	-	43.4 ± 11.8
$CD7^+$	56.8 ± 14.5	24.7 *
$CD4^+$	10.5 ± 3.9	4.6 *
$CD8^+$	27.5 ± 7.7	12.0 *
$CD19^+$	27.1 ± 11.0	11.8 *
$CD14^+$	0.4 ± 0.1	0.2 *
$CD15^+$	2.6 ± 0.3	1.1 *
$CD11b^+$	4.6 ± 3.2	2.0 *
$CD11c^+$	4.6 ± 1.8	2.0 *

Phenotypical characteristics of the $CD56^-$ fraction of the final product from the liver perfusate.

The values indicate the percentage of each marker (mean ± SEM, n = 5). *The percentage of the final product was calculated as follows: % of $CD56^-$ fraction × $CD56^-$ percentage (43.4)/100. Abbreviation: goat aMIgG, goat anti-mouse IgG antibody.

Figure legends

Fig. 1

Comparison of surface marker expression patterns in response to interleukin (IL)-2 stimulation in liver perfusate and blood natural killer (NK) cells.

Flow cytometry (FCM) analysis of freshly isolated or cultured with IL-2 (1000 $U \cdot mL^{-1}$) liver mononuclear cells (LMNCs) and peripheral blood mononuclear cells (PBMCs) obtained from deceased donor after staining with anti-CD3 and anti-CD56 mAbs. The numbers indicate the mean percentage of each positive subset on electronically gated $CD3^-CD56^+$ NK cells (mean ± SEM, n = 4–14). Statistical analyses were performed using Student's *t*-test (*$p < 0.01$ vs. fresh control).

Fig. 2

Liver NK cells inductively express significant levels of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and cytokines after cell processing.

FCM analysis of LMNCs obtained from deceased donor liver graft perfusate before the culture (upper panel) and after the culture (lower panel), after staining with mAbs against CD3 and CD56. Lymphocytes were gated by forward and side scatter. The FCM dot plot profiles are representative of 14 independent experiments. The percentages of $CD3^+CD56^-$ (T), $CD3^-CD56^+$ (NK), and $CD3^+CD56^+$ (NT) cells are indicated at each quadrant. Histograms show the logarithmic fluorescence intensities obtained on staining for each surface marker or intracellular protein after gating on the $CD3^-CD56^+$ NK cells. Shaded regions indicate negative control staining with isotype-matched mAbs. The numbers indicate the mean percentages of positive cells in each group (n = 4-14). The histogram profiles are representative of 14 independent experiments.

Fig. 3

LMNC final products show strong cytotoxicity against NK-susceptible target cells.

The NK cytotoxic activities of freshly isolated (circle) and IL-2 stimulated (triangle) LMNCs (black) and PBMCs (white) with or without anti-CD3 monoclonal antibody (OKT3; square) against K562 target cells were analyzed by a FCM-based cytotoxic assay. All data are expressed as the mean ± SEM (n = 5). Statistical analysis was performed using one-way ANOVA followed by Student-Newman-Keuls *post hoc* analysis (*$p < 0.01$ vs. PBMC fresh and LMNC fresh, **$p < 0.01$ vs. PBMC IL-2 and PBMC IL-2 + OKT3).

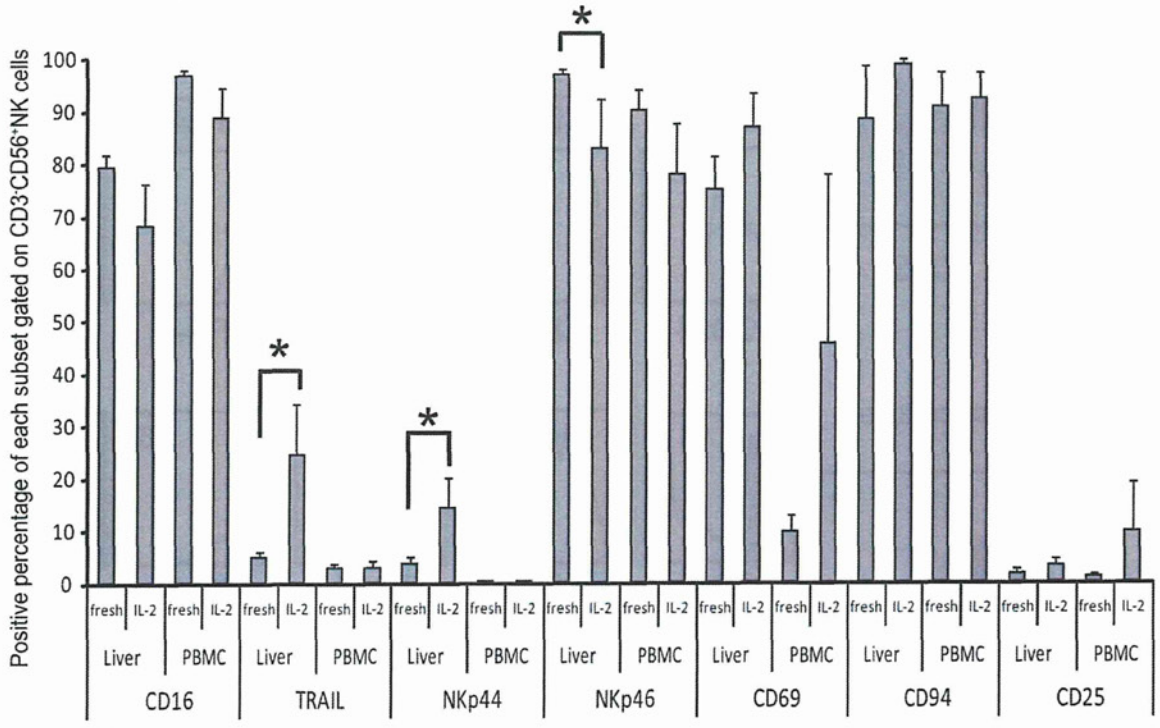

Figure 1

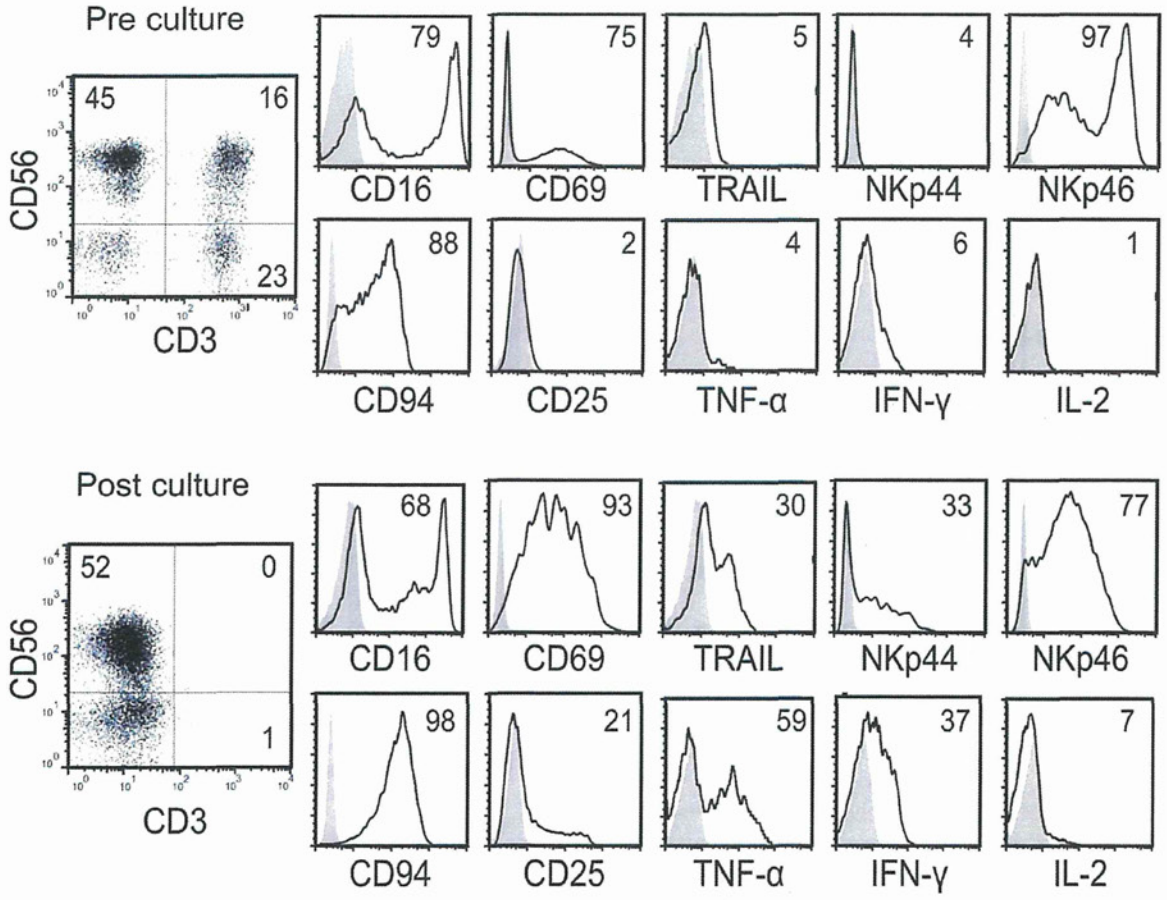

Figure 2

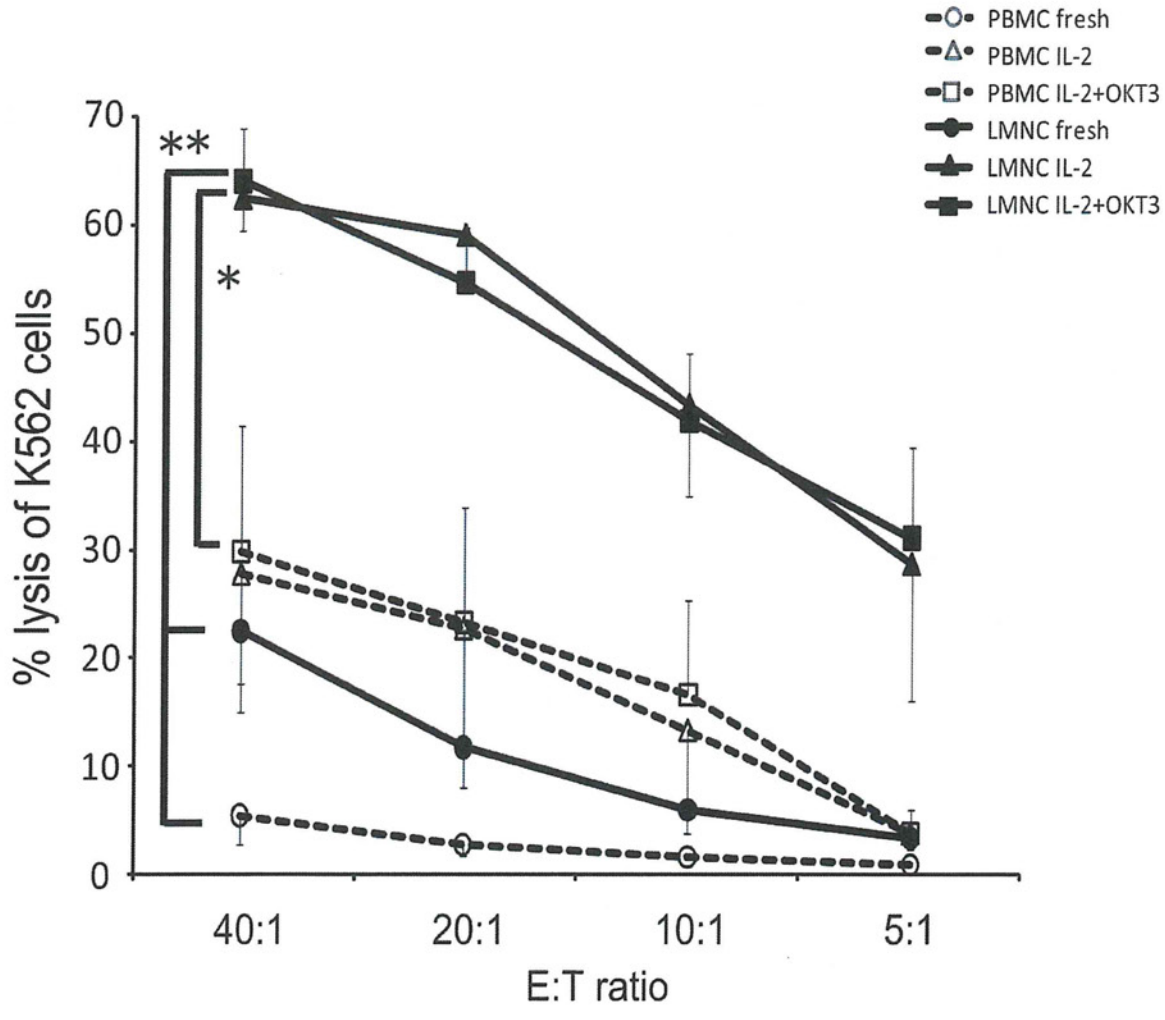

Figure 3

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PRECLINICAL STUDY

Mechanistic analysis of the antitumor efficacy of human natural killer cells against breast cancer cells

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Abstract We investigated the role of human natural killer (NK) cells in the peripheral blood (PB) and liver in controlling breast cancer. The proportion of NK cells among liver mononuclear cells was significantly higher than among PB mononuclear cells. Liver NK cells inductively expressed higher levels of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) than PB NK cells in response to interleukin-2 (IL-2). Liver NK cells displayed higher cytotoxicity against various breast cancer cell lines (MDA-MB231, MDA-MB453, MDA-MB468, and MCF-7) after IL-2 stimulation than did PB NK cells. Anti-HER2 monoclonal antibody (mAb) promoted the cytotoxicity of both the types of NK cells toward HER2-expressing cell lines. All breast cancer cell lines highly expressed death-inducing TRAIL receptors, death receptor 4, but did not express death-inhibitory receptors (DcR1 and DcR2). Both PB and liver NK cell-induced cytotoxicity was inhibited partially by anti-TRAIL mAb and more profoundly by the combination of anti-TRAIL mAb and concanamycin A, indicating that TRAIL and perforin are involved. IL-2-stimulated liver and PB NK cells exhibited upregulated expression of CXCR3, which bind to the chemokines CXCL9, CXCL10, and CXCL11 secreted by breast cancer cells. We also found that IFN-γ promoted the production of CXCL10 from breast cancer cells. The results of this study show that IFN-γ secreted from NK cells likely promotes the production of CXCL10 from breast cancer cells, which in turn accelerates the migration of CXCR3-expressing NK cells into the tumor site. These findings suggest the possibility of a therapeutic approach by either activation of endogenous PB and liver NK cells or adoptive transfer of in vitro-activated autologous NK cells.

Keywords NK cells · TNF-related apoptosis-inducing ligand (TRAIL) · TRAIL-receptors · ADCC · Chemokine · Breast cancer

Abbreviations

NK	Natural killer
TRAIL	TNF-related apoptosis-inducing ligand
PBMC	Peripheral blood mononuclear cell
LMNC	Liver mononuclear cell
mAbs	Monoclonal antibodies

Introduction

Natural killer (NK) cells, the frontline defense in cellular immunity, exert an effector function on neoplastic cells, modified cells, and invading infectious microbes without the necessity for priming [1, 2]. Although, NK cells might

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