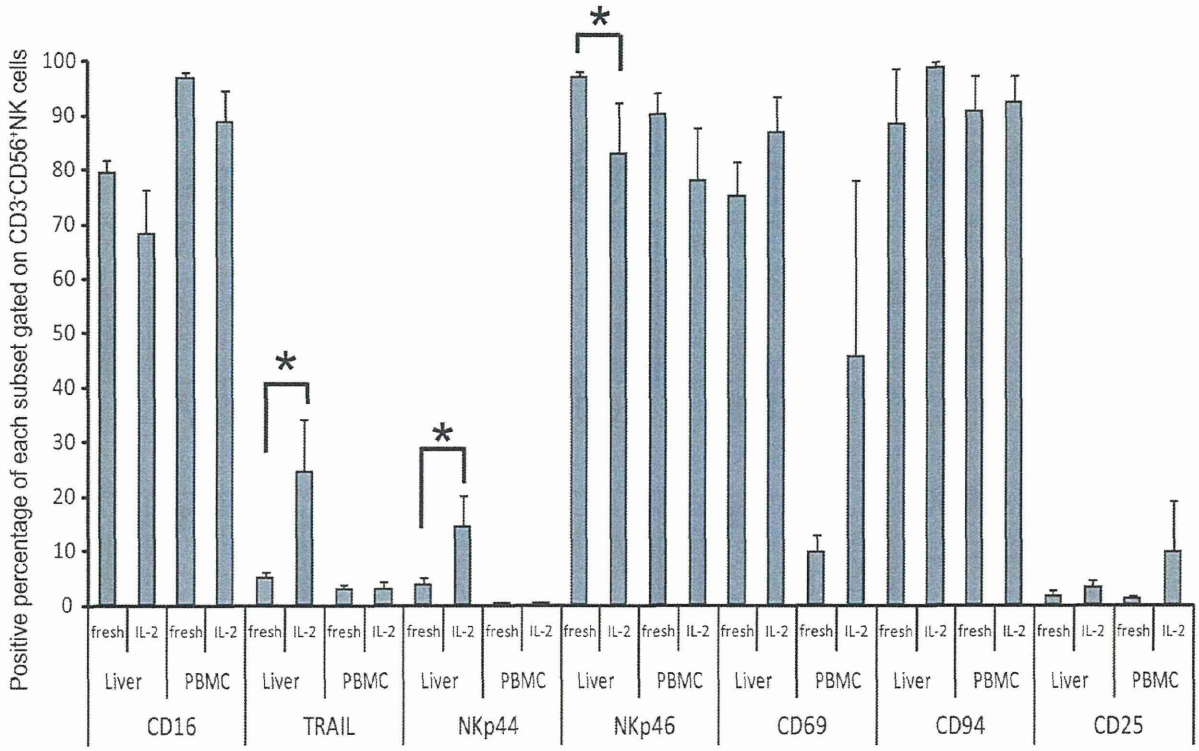

Figure 1. Comparison of surface marker expression patterns in response to interleukin-2 (IL-2) stimulation in liver perfusate and blood natural killer (NK) cells. Flow cytometry (FCM) analysis of freshly isolated or cultured with IL-2 (1,000 U/ml) liver mononuclear cells (LMNCs) and peripheral blood 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).

Table 2. Phenotypical Characteristics of the $CD56^-$ Fraction of the Final Product From the Liver Perfusate

	% of the $CD56^-$ Fraction	% of the Final Product
Goat anti-mouse IgG antibody	—	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*

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.

effector cells from the final products relative to those from precultured LMNCs and PBMCs (Fig. 3). At a 20:1 effector/target cell ratio, 56.3% of the K562 targets were killed on average by the final products, whereas precultured 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 the Patients and Methods

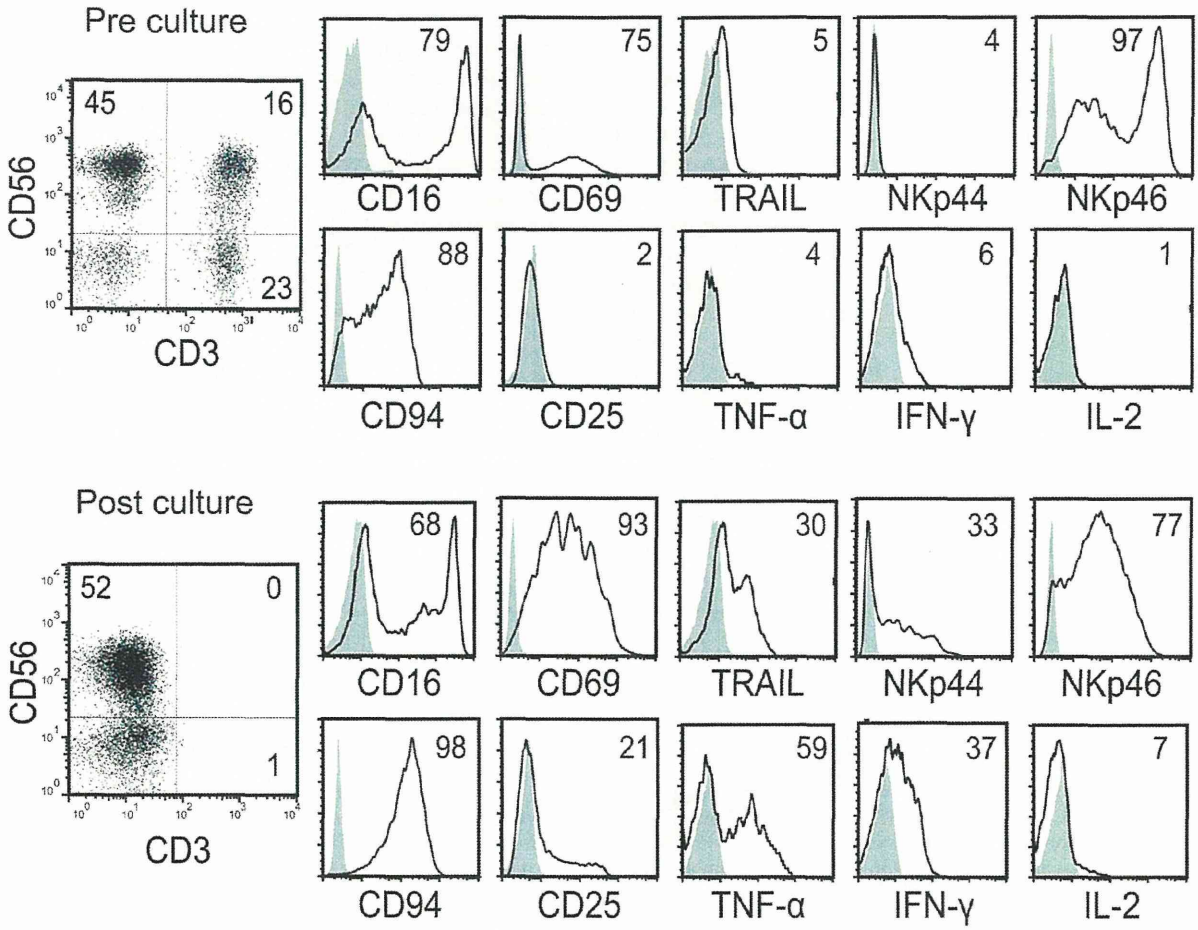

Figure 2. Liver NK cells inductively express significant levels of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and cytokines after cell processing. FCM analysis of LMNCs obtained from deceased donor liver graft perfusate before the culture (upper panel) and after the culture (lower 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.

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 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 5.3×10^8 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 (Fig. 1). Functionally, we also have shown that IL-2-activated liver NK cells were highly cytotoxic against tumors compared with PBMCs (Fig. 3). In addition to having an antineoplastic 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 antiviral and antibacterial 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

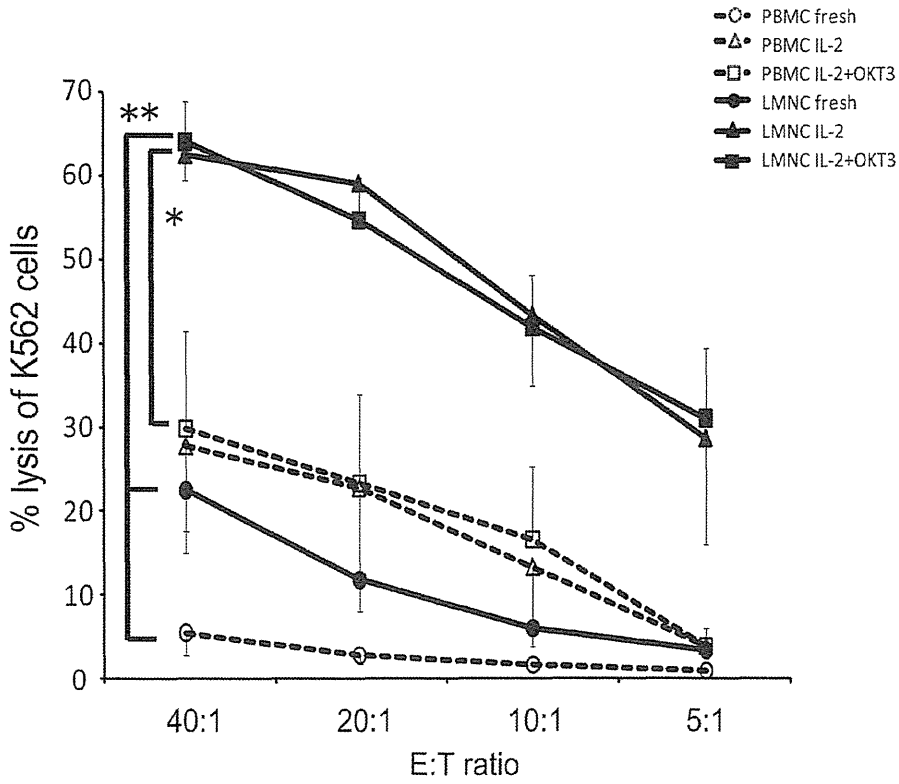

Figure 3. LMNC final products show strong cytotoxicity against NK-susceptible target cells. The NK cytotoxic activities of freshly isolated (circle) and IL-2 stimulated (triangle) LMNCs (black) and PBMCs (white) with or without anti-CD3 monoclonal antibody (OKT3; square) against K562 target cells were 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).

perfusate contained 0.02×10^6 $CD3^+CD56^-$ T cells/kg. 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 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 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 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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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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play an important role in prevention of both early and metastatic cancer, the role of NK cell activity in controlling breast cancer is still controversial and few studies have addressed whether enhancing this activity is of clinical benefit to breast cancer patients.

A variety of mechanisms are involved in controlling neoplastic cells by NK cells, one of which is the direct release of cytolytic granules that contain perforin, granzymes, and granulysin by exocytosis to kill target cells (i.e., the granule exocytosis pathway) [3, 4]. Most mature human NK cells in peripheral blood (PB) constitutively express granzyme B and perforin, and have basal cytotoxicity against NK-sensitive targets. Cytokine exposure with interleukin (IL)-2 or IL-15 is known to increase the baseline granzyme B and perforin abundance and cytotoxic activity of NK cells, and also converts basal NK cytotoxicity to lymphokine-activated killing. Another mechanism is mediated by death-inducing ligands such as Fas ligand (FasL) and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL). Fas, a TNF family protein, is expressed on breast cancer cell membranes [5, 6], suggesting that activation of the Fas/FasL pathway induces apoptosis mediated by caspase activation. An additional mechanism is involved when HER2-overexpressing breast cancer cells are targeted because differential levels of HER2 expression in normal versus HER2-overexpressing tumor cells, together with the clear involvement of HER2 in tumor progression, make HER2 an ideal target for therapeutic approaches. NK cell-mediated antibody-dependent cell-mediated cytotoxicity (ADCC) is thought to contribute to the therapeutic effects of monoclonal antibodies (mAbs) specifically directed against the extracellular domain of HER2 (trastuzumab).

NK cells are abundant in the liver in contrast to their relatively small percentage in the peripheral lymphatics and other lymphatic organs in rodents [7–9] and humans [10]; however, the underlying reason for the anatomically biased distribution of NK cells has not been elucidated. In addition, liver NK cells have been shown to mediate higher cytotoxic activity against tumor cells than PB NK cells do in rodents [7–9, 11]. However, these functional differences between PB and liver NK cells have not been extensively investigated in humans because of the limited availability of appropriate samples. In this study, we extracted NK cells from allograft liver perfusates in clinical liver transplantation and examined the quantitative and qualitative cytotoxic functions of those liver NK cells targeting various breast cancer cell lines in comparison with PB NK cells. Through the experiments, we attempted to define whether PB NK cells can recognize and kill breast cancer cells, and whether liver resident NK cells can hinder metastasis of breast cancer to the liver, to assess the potential therapeutic use of NK cells, i.e., by either activation of endogenous NK cells or adoptive transfer of in vitro-activated autologous NK cells. As the therapeutic efficacy of endogenous or exogenous NK cells likely depends on their migration and accumulation at tumor metastasis sites, we further analyzed the expression of receptors and ligands for chemokines secreted from breast cancer cells on PB and liver NK cells.

Materials and methods

Isolation of liver and PB lymphocytes

Liver mononuclear cells (LMNCs) were obtained from liver perfusates in clinical living donor liver transplantation as previously described [10]. LMNCs were isolated by gradient centrifugation with Separate-L (Muto Pure Chemicals Co., Ltd, Tokyo, Japan). PB mononuclear cells (PBMCs) were also isolated by gradient centrifugation with Separate-L from heparinized PB from healthy volunteers and liver transplant donors. LMNCs and PBMCs were suspended in DMEM (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Sanko Chemical Co., Tokyo, Japan), 25 mmol/l HEPES buffer (Gibco), 50 μmol/l 2-mercaptoethanol (Katayama Chemical Co., Osaka, Japan), 50 U/ml penicillin, and 50 μg/ml streptomycin (Gibco) (10% DMEM). The ethics committee at Hiroshima University Hospital approved this study.

Cell culture

LMNCs and PBMCs were cultured with human recombinant IL-2 (100 Japanese reference U/ml; Takeda, Tokyo, Japan) in DMEM at 37°C in a 5% CO_2 incubator. Cells were harvested for further analyses after 5 days in culture. Cell viability was assessed by the dye-exclusion test.

Isolation of NK cells

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 (Miltenyi Biotec) according to the manufacturer's instructions. The purity of isolated fractions was assessed by FCM, and only preparations with purities >90% were used for functional studies.

Cell lines

The human breast cancer cell lines were obtained as follows: MDA-MB-231 and MDA-MB-468 were from ATCC

(Manassas, VA), MDA-MB-453 was from Riken Cell Bank (Saitama, Japan), and MCF-7 was kindly provided by Dr. A. Kikuchi (Osaka University, Suita, Japan). MDA-MB-231, MDA-MB-453, and MDA-MB468 cells were cultured in Leibovitz's L-15 medium (Invitrogen) supplemented with 10% FCS at 37°C in a 0% CO_2 incubator. MCF-7 cells were cultured in DMEM supplemented with 10% FCS at 37°C in a 5% CO_2 incubator. The phenotypic features of those cell lines were well documented [12, 13].

Flow cytometric analyses

Flow cytometric analyses were performed using a FACSCalibur® cytometer (BD Biosciences, San Jose, CA). The following mAbs were used for surface staining of the lymphocytes: anti-CD3-fluorescein isothiocyanate (FITC), HIT3a; anti-CD56-phycoerythrin (PE), B159; anti-NKG2D-allophycocyanin (APC), 1D11; anti-CD183-APC, CXCR3 (1C6/CXR3); and anti-FasL-biotin, NOK-1. The biotinylated mAb was visualized using APC-streptavidin (all mAbs from BD Biosciences, Franklin Lakes, NJ).

To analyze activating receptors or TRAIL expression on NK cells, LMNCs and PBMCs were stained with anti-CD3-FITC, anti-CD56-APC, anti-TRAIL-PE, RIK-2; anti-NKp30-PE, P30-15; anti-NKp44-PE, P44-8.1; and anti-NKp46-PE, 9E2/NKp46; mAbs (all mAbs from BD Biosciences). To analyze inhibitory receptors on NK cells, LMNCs and PBMCs were stained with anti-CD3-APC, SP34-2; anti-CD56-PE, anti-CD-158a-FITC, HP-3E4; anti-CD-158b-FITC, CH-L; or anti-CD94-FITC, HP-3D9; mAbs (all mAbs from BD Biosciences). To analyze the expression of CD94/NKG2A or CD94/NKG2C on NK cells, LMNCs and PBMCs were stained with anti-CD3-PerCP, SK7; (BD Biosciences), anti-CD56-APC, anti-CD94-FITC, and anti-NKG2A-PE, z199; (Beckman Coulter), or anti-NKG2C-PE, 134591; (R&D Systems, Minneapolis, MN) mAbs. To analyze TRAIL receptors on breast cancer cell lines, they 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). To analyze HER2 expression on breast cancer cell lines, breast cancer cells were stained with PE-conjugated anti-HER2 mAb, Neu 24.7; (Becton–Dickinson). All biotinylated mAbs were visualized with APC-streptavidin (BD Biosciences). Dead cells were excluded from analysis by light-scatter and propidium iodide staining.

Immunohistochemistry

Surgically resected breast specimens were obtained from breast cancer patients who had undergone curative tumor resection at Hiroshima University Hospital. Breast specimens that had been pathologically proven to be normal (i.e., excluding mastopathic and mastitic tissues) were used as normal controls. Each tumor section (4-μm thickness) was deparaffinized and subjected to antigen retrieval by incubation in 10 mM citrate buffer (sodium citrate, pH 6.0) at 99°C for 25 min. To block non-specific antibody binding, sections were incubated with blocking solution (PBS containing Sanglopor I.V., 1 mg/ml; CSL Behring AG, Bern, Switzerland) for 20 min. Sections were then incubated overnight at 4°C in blocking solution in the presence of the first antibody (biotin-conjugated anti-TRAIL-R1/DR4, anti-TRAIL-R2/DR5, anti-TRAIL-R3/DcR1, and anti-TRAIL-R4/DcR2) (5 μg/ml) (all mAbs from eBioscience) or biotin-conjugated mouse IgG1, κ isotype control (P3) at the same concentration (eBioscience). Sections were washed twice in PBS, and primary antibody binding sites were visualized using the Dako EnVision Kit (Dako, Copenhagen, Denmark) according to the manufacturer's instructions. Sections were faintly counterstained with Harris' hematoxylin and mounted with glycerol gelatin.

Cytotoxicity assay

The ^{51}Cr-labeled breast cancer cells were incubated in a total volume of 200 μl with effector cells in L-15 medium in round-bottomed 96-well microtiter plates (BD Falcon) for 4 h. IL-2-stimulated NK cells were used as effectors at an effector-to-target (E:T) ratio of 1.25:1–10:1. To evaluate the ADCC of trastuzumab, the assay was performed in the presence of 0.1 μg/ml trastuzumab (kindly provided by Genentech Inc., San Francisco, CA). Target cells were incubated either in culture medium alone to determine spontaneous ^{51}Cr release or in a mixture of the culture medium and 2% Nonidet P-40 (Nakalai Tesque Inc., Kyoto, Japan) to determine the maximum ^{51}Cr release for controls. The radioactivity of the cell-free supernatants was measured in a gamma counter. The percentage of specific ^{51}Cr release was calculated as % cytotoxicity = ([cpm of experimental release − cpm of spontaneous release]/[cpm of maximum release − cpm of spontaneous release]) × 100. In some experiments, the ^{51}Cr-labeled breast cancer cells were incubated with effector cells either for 4 or 18 h in the presence of 10 μg/ml of anti-TRAIL (N2B2) mAb, 10 μg/ml of anti-FasL (MFL3) mAb (both from BD Pharmingen), and/or 50 nmol/l of concanamycin A (CMA) (Wako Chemicals, Osaka, Japan), which inhibits perforin-mediated cytotoxicity [14].

Cytometric bead assay

Chemokine production in the cell culture supernatants was analyzed by the cytometric bead array (CBA) using Human MIG (CXCL9), Human IP-10 (CXCL10), and Human I-TAC (CXCL11) Flex Sets (BD Bioscience), according to

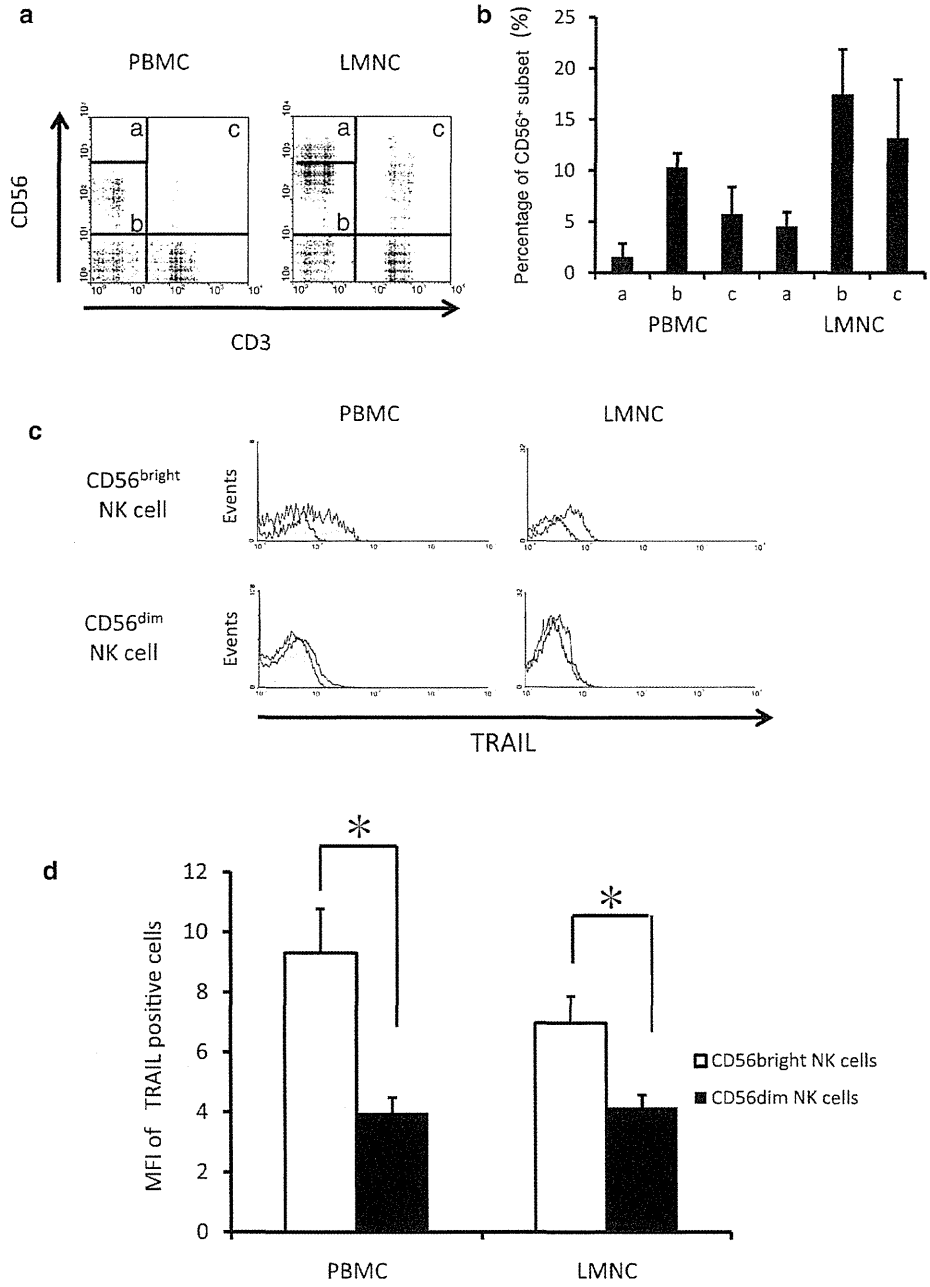


Fig. 1 The proportions of $CD56^+CD3^-$ NK and $CD56^+CD3^+$ NKT cells in LMNCs extracted from liver perfusates were significantly higher than those in PBMCs. **a** Flow cytometric (FCM) analyses of freshly isolated LMNCs obtained from liver perfusates and PBMCs from the corresponding donors were analyzed after staining with mAbs against CD3 and CD56. Lymphocytes were gated by forward scatter and side scatter. FCM profiles are representative of six and five independent experiments (using PBMCs and LMNCs, respectively). *a* $CD3^-CD56^{bright}$ NK cell subset, *b* $CD3^-CD56^{dim}$ NK cell subset, *c* $CD3^+CD56^+$ (NKT) cell subset. **b** Percentages of $CD3^-CD56^{bright}$ NK, $CD3^-CD56^{dim}$ NK, and $CD3^+CD56^+$ NKT cells among mononuclear cells (mean ± SEM, PBMCs; $n = 6$, LMNCs; $n = 5$). **c** Histograms showing the log fluorescence intensities obtained for TRAIL staining after gating $CD3^-CD56^{bright}$ NK and $CD3^-CD56^{dim}$ NK cell subsets of LMNCs and PBMCs from the corresponding donors. Data are shown as overlays with comparison against isotype controls. Histogram profiles are representative of independent experiments (PBMCs; $n = 6$, LMNCs; $n = 5$). **d** Mean fluorescence intensities (MFI) of TRAIL staining on NK cells freshly isolated from LMNCs and PBMCs ($CD56^{bright}$ NK cells; *open column*, $CD56^{dim}$ NK cells; *closed column*). Data represent mean ± SEM (PBMCs; $n = 6$ LMNCs; $n = 5$). Statistical analyses were performed using the paired Student's *t* test ($^*P < 0.05$). *NK* natural killer, *TRAIL* TNF-related apoptosis-inducing ligand, *LMNC* liver mononuclear cells, *PBMC* peripheral blood mononuclear cells, *mAb* monoclonal antibody

the manufacturer's instructions, for the production of CXCL9, CXCL10, and CXCL11, respectively.

Cell migration assay

Migration assays were performed in transwell culture inserts (BD Falcon) of 6.4-mm diameter and 3-μm pore filters. MDA-MB231 and MDA-MB468 cells (1×10^6/well) were cultured in the lower chamber of a 24-well plate (BD Falcon) in 0.5 ml L-15 medium. After 2 days, IL-2-activated NK cells derived from PBMCs and LMNCs in 0.2 ml L-15 medium were added to the upper chamber (1×10^6/well), and cells were allowed to migrate for 2 h.

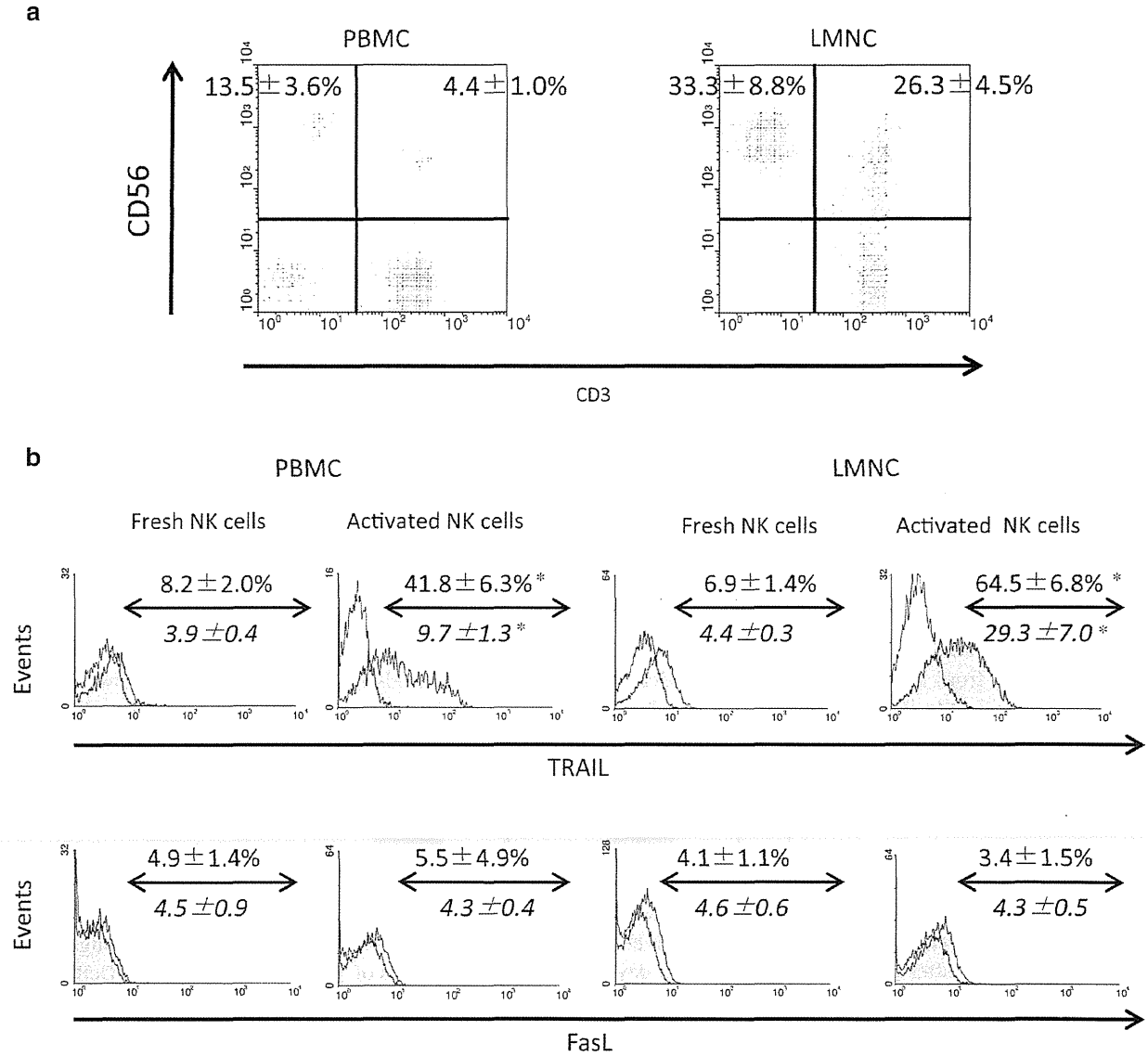


Fig. 2 Phenotypic analyses of human PB and liver NK cells. **a** Flow cytometric (FCM) analyses of PBMCs and LMNCs cultivated with IL-2 for 5 days were performed after staining with mAbs against CD3 and CD56. FCM profiles are representative of five and six independent experiments (using PBMCs and LMNCs, respectively). Percentages of NK and NKT cells are indicated (mean ± SEM, PBMC; $n = 5$, LMNC; $n = 6$). **b** Expression of cytotoxic effector molecules on NK cell subsets among LMNCs or PBMCs freshly isolated or cultivated with or without IL-2 was analyzed. Expression of TRAIL and FasL on electronically gated $CD3^-CD56^+$ NK cells was analyzed by FCM. *Numbers* above the lesion marker line indicate the percentages of cells expressing TRAIL and FasL, and *numbers* below the *line* indicate the median florescence intensity of expression of whole NK cells (mean ± SEM, $n = 4$ each). PBMCs were obtained from the corresponding LMNC donor. *Histogram* profiles are representative of independent experiments. *Dotted lines* represent negative control staining with isotype-matched mAbs. *$P < 0.05$ PB NK cells versus liver NK cells

Enzyme-linked immunosorbent assay

The supernatants from the cell migration assay and coculture of MDA-MB231 or MDA-MB468 with IFN-γ for 2 or 6 h were used. IFN-γ and CXCL10 levels in the cell culture supernatants were determined by an enzyme-linked immunosorbent assay (ELISA) with the Quantikine kit (R&D Systems), according to the manufacturer instructions. Absorbance was measured at 492 nm on a microplate reader (MTP-300; CORONA Electric, Ibaraki, Japan).

Statistical analysis

Data are presented as mean ± SEM. The statistical differences of the results were analyzed by the 2-tailed, paired *t* test and Mann–Whitney *U* test, using Excel. *P* values of <0.05 were considered statistically significant.

Results

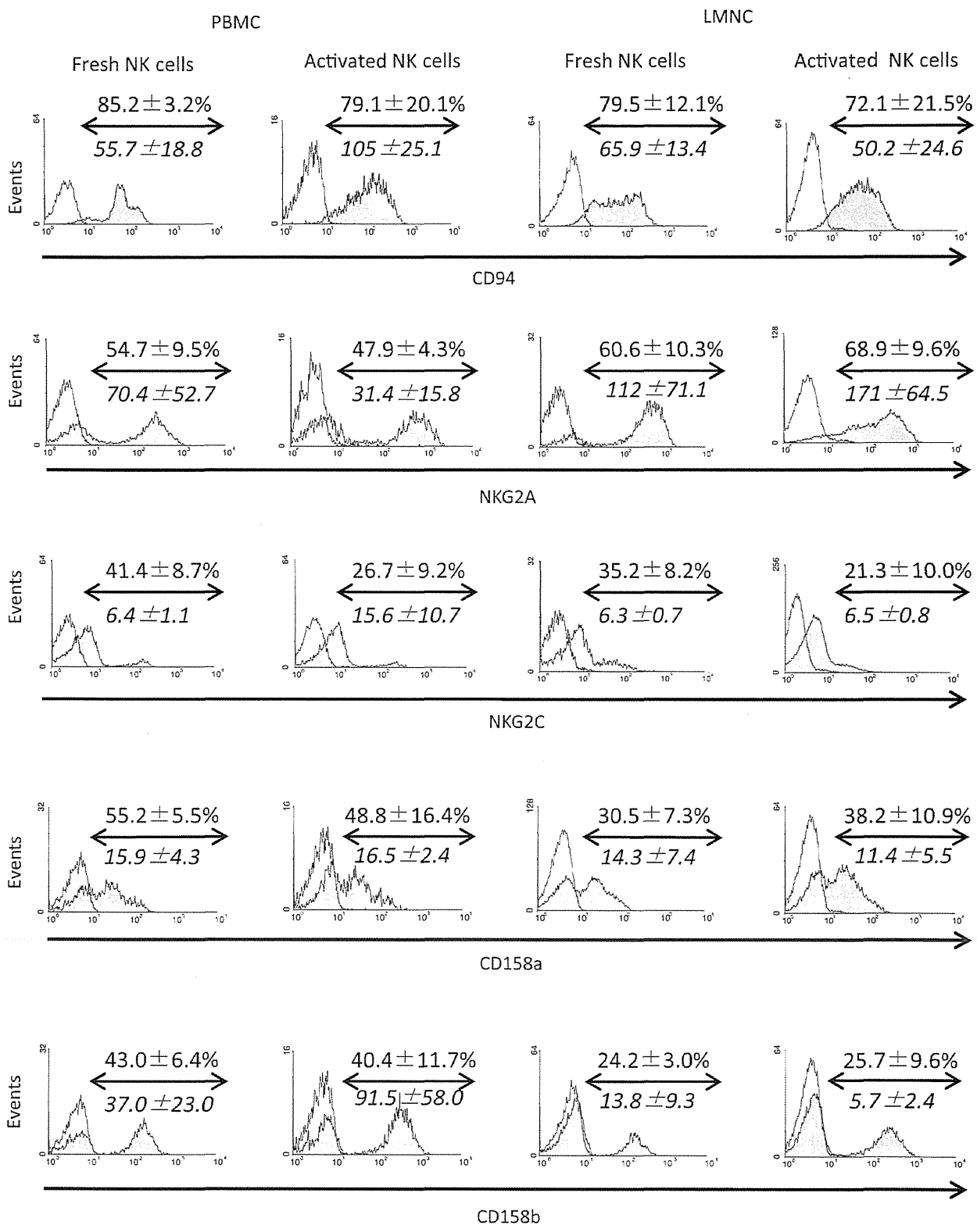
Phenotypic properties of human NK cells

NK cells are abundant in the liver in contrast to their relatively small percentage in the peripheral lymphatics and other lymphatic organs in humans [10]. While NK cells in circulating lymphocytes have been phenotypically and functionally defined, those that reside in the liver remain to be characterized. We phenotypically analyzed the LMNCs that were extracted from the perfusates of allograft livers during liver transplantation surgery. The proportions of $CD56^+CD3^-$ NK and $CD56^+CD3^+$ NKT cells in the LMNCs extracted from liver perfusates were significantly higher than those in the PBMCs (Fig. 1a). Although this non-destructive method might allow some extent of contamination with circulating mononuclear cells, these data were consistent with previous reports using the enzymatic dissociation method [15]. Among $CD56^+CD3^-$ NK cells, $CD56^{bright}$ cells, which constitutively expressed TRAIL, were abundant in LMNCs but were almost undetectable in PBMCs (Fig. 1b–d). On effector molecule analyses, the expression of TRAIL was significantly upregulated in both liver and PB NK cells after cultivation with IL-2 for 5 days. Both the proportion of the $TRAIL^+$ fraction and the staining intensity of liver NK cells were significantly higher than those of PB NK cells (Fig. 2a). Neither PB nor liver NK cells expressed FasL even after IL-2 stimulation (Fig. 2b). We further analyzed the C-type lectin-like receptors CD94, NKG2A, and NKG2C and killer cell immunoglobulin-like receptors (KIR) such as CD158a and CD158b (Fig. 3). CD94 recognizes the non-classical MHC class Ib molecule HLA-E, whereas KIRs are MHC class I-restricted molecules that recognize HLA-A, HLA-B, HLA-C, and HLA-G molecules. All freshly isolated PB and liver NK cells expressed CD94, and cell subpopulations expressed CD158a/CD158b (Fig. 3). No statistically significant differences were observed in the expression of CD94, NKG2A, NKG2C, CD158a, and CD158b between PB and liver NK cells. IL-2 stimulation maintained the expression of these molecules in both liver and PB NK cells, indicating that these cells retain their ability to protect self-MHC class I-expressing cells from NK cell-mediated death. On the analyses of the cytotoxicity-associated receptors, including NKp30, NKp44, NKp46, and NKG2D, no statistically significant differences were found between PB and liver NK cells even after IL-2 stimulation (Fig. 4). Although liver NK cells tended to express higher levels of NKp44 and NKp46 than did PB NK cells, the differences did not reach statistical significance.

Fig. 3 IL-2 stimulation maintained the expression of C-type lectin-like receptors and killer cell immunoglobulin-like receptors (KIR) in both human PB and liver NK cells. Expression of the C-type lectin-like receptors CD94, NKG2A and NKG2C, and KIR such as CD158a and CD158b on NK cell subsets among LMNCs or PBMCs freshly isolated or cultivated with or without IL-2 was analyzed. Expression on electronically gated $CD3^-CD56^+$ NK cells was analyzed by FCM. *Numbers* above the lesion marker *line* indicate the percentages of cells expressing each molecule, and *numbers* below the *line* indicate the median florescence intensity of expression of whole NK cells (mean ± SEM, $n = 4$ each). PBMCs were obtained from the corresponding LMNC donor. *Histogram* profiles are representative of independent experiments. *Dotted lines* represent negative control staining with isotype-matched mAbs. $^*P < 0.05$ PB NK cells versus liver NK cells

Breast cancer cells express the death-inducing receptor

Susceptibility to TRAIL-induced apoptosis may be related to the expression levels of multiple receptors on target cells. TRAIL binds to at least four receptors: two of these are death-inducing receptors (TRAIL-R1/DR4 and TRAIL-R2/DR5) containing cytoplasmic death domains and signal apoptosis, whereas the other two are death-inhibitory receptors (TRAIL-R3/DcR1 and TRAIL-R4/DcR2) that lack a functional death domain and do not mediate apoptosis; all have similar affinities for TRAIL and the latter two may act as decoys [16, 17]. The susceptibility to TRAIL-induced apoptosis is related to the expression levels of those receptors in tumor cells. We investigated the expression patterns of TRAIL-DR and TRAIL-DcR in both normal mammary gland and breast cancer tissue samples. Ductal cells in normal mammary gland tissues expressed TRAIL-DR4 together with TRAIL-DcR1 (Fig. 5a). Breast cancer cells showed a much higher expression of TRAIL-DR4 than did normal mammary gland cells, but little TRAIL-DcR1, regardless of the HER2 type. Similar to the clinical breast cancer tissues, all the tested breast cancer cell lines expressed high TRAIL-DR4 together with TRAIL-DR5, but no TRAIL-DcR1 and TRAIL-DcR2, regardless of their HER2 status (Fig. 5b).


PBMC
LMNC
Fresh NK cells
Activated NK cells
Fresh NK cells
Activated NK cells
85.2±3.2%
55.7±18.8
79.1±20.1%
105±25.1
79.5±12.1%
65.9±13.4
72.1±21.5%
50.2±24.6
Events
CD94
54.7±9.5%
70.4±52.7
47.9±4.3%
31.4±15.8
60.6±10.3%
112±71.1
68.9±9.6%
171±64.5
Events
NKG2A
41.4±8.7%
6.4±1.1
26.7±9.2%
15.6±10.7
35.2±8.2%
6.3±0.7
21.3±10.0%
6.5±0.8
Events
NKG2C
55.2±5.5%
15.9±4.3
48.8±16.4%
16.5±2.4
30.5±7.3%
14.3±7.4
38.2±10.9%
11.4±5.5
Events
CD158a
43.0±6.4%
37.0±23.0
40.4±11.7%
91.5±58.0
24.2±3.0%
13.8±9.3
25.7±9.6%
5.7±2.4
Events
CD158b

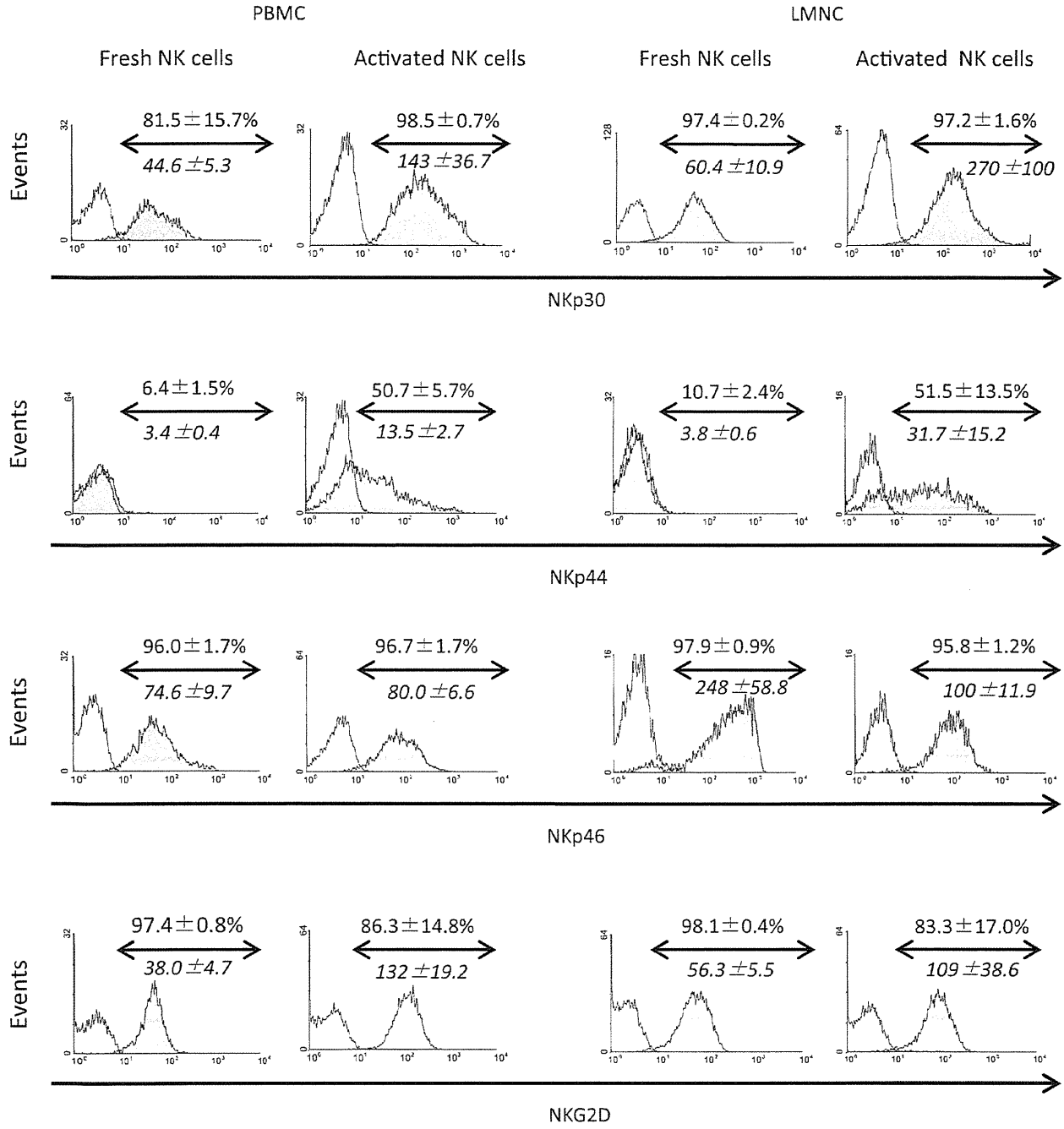


Fig. 4 IL-2 stimulation led to elevated expression of NKp30, NKp44, and NKG2D in both human PB and liver NK cells. Expression of the cytotoxicity-associated receptors, including NKp30, NKp44, NKp46, and NKG2D, in NK cell subsets among LMNCs or PBMCs freshly isolated or cultivated with or without IL-2 was analyzed. Expression in electronically gated $CD3^{-}CD56^{+}$ NK cells was analyzed by FCM. *Numbers* above the lesion marker *line* indicate the percentages of cells expressing each molecule, and *numbers* below the *line* indicate the median florescence intensity of expression of whole NK cells (mean ± SEM, $n = 4$ each). PBMCs were obtained from the corresponding LMNC donor. *Histogram* profiles are representative of independent experiments. *Dotted lines* represent negative control staining with isotype-matched mAbs.

IL-2-stimulated NK cells showed significant cytotoxicity against breast cancer cells

Cytotoxicity assays of NK cells isolated from LMNCs and PBMCs as effectors and various breast cancer cell lines as targets were performed. Cells were stimulated by 5-day culture with IL-2 before use in the cytotoxicity assays. Liver NK cells showed more vigorous cytotoxicity against all tested cell lines (MDA-MB231, MDA-MB453, MDA-MB468, and MCF-7) compared with PB NK cells (Fig. 6). Addition of trastuzumab enhanced the cytotoxicity of both liver and PB NK cells toward MDA-MB231, MDA-MB453, and MCF-7, which express HER2. Although MDA-MB468 has been reported to be a triple-negative breast cancer cell line [12], it seemed to express dim HER2 on phenotypic analysis of the breast cancer cell lines in this study (Fig. 5b). This may explain why trastuzumab did not promote the cytotoxicity of PB NK cells but somewhat enhanced the cytotoxicity of liver NK cells toward MDA-MB468. Nevertheless, these observations suggest the involvement of HER2/trastuzumab-mediated ADCC. Despite the strong cytotoxicity exhibited by IL-2-stimulated donor liver NK cells, their cytotoxicities toward one-haplotype identical allogeneic and autologous lymphoblasts were negligible (data not shown).

TRAIL and perforin are involved in the cytotoxicity of NK cells against breast cancer cells

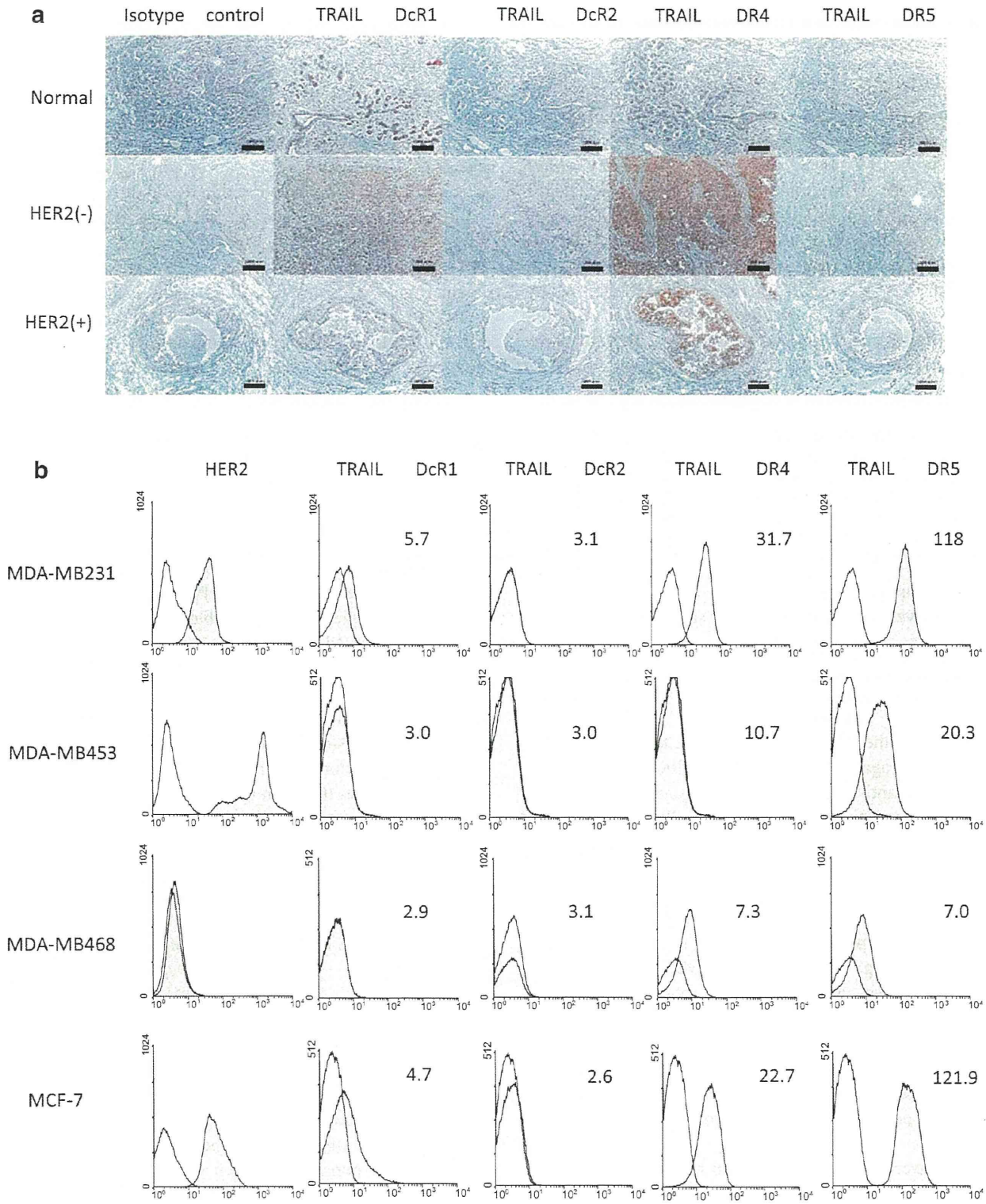
To determine the contribution of TRAIL to the cytotoxicity of NK cells against breast cancer cells, the effect of a neutralizing anti-TRAIL mAb was examined in a cytotoxicity assay with PB and liver NK cells as effectors and MDA-MB231 cells as the target. Both PB and liver NK cell-induced cytotoxicity was inhibited partially by the anti-TRAIL mAb alone and more profoundly by the combination of the anti-TRAIL mAb and CMA, indicating that TRAIL and perforin are involved in NK cell-mediated cytotoxicity (Fig. 7). Remarkable levels of inhibition of NK cell-induced cytotoxicity were observed with anti-TRAIL mAb at 18-h culture when compared with those at 4 h. This finding is consistent with the results of the previous study with a mouse model demonstrating that death receptor-mediated NK cell kill needs longer incubation times than perforin-mediated NK cell kill [18].

NK cells were preferentially drawn by chemokines secreted from breast cancer cells, presumably through the CXCL10/CXCR3 axis

The distribution of NK cells is known to be associated with their expression of receptors and ligands for chemokines secreted from infectious or neoplastic sites [19–21]. We found that NK cells freshly isolated from PBMCs and LMNCs highly expressed CXCR3, which binds to the chemokines CXCL9, CXCL10, and CXCL11 secreted by breast cancer cells. IL-2 activation increased the levels of CXCR3 expression on both NK cell types (Fig. 8a, b). We further investigated the secretion activities of these various chemokines from the breast cancer cell lines. Significant levels of CXCL10 were detected in the culture supernatants of three of four breast cancer cell lines: MDA-MB231, MDA-MB453, and MDA-MB468 (Fig. 9a). PB or liver NK cells activated with IL-2 were cultured in the upper compartment of transwell tissue culture plates in the presence or in the absence of MDA-MB231 or MDA-MB468 cells in the lower compartment for 2 h. The migration of PB and liver NK cells through the membrane was markedly promoted by the presence of tumor cells in the lower compartment, suggesting that NK cells are preferentially drawn by chemokines secreted from tumor cells (Fig. 9b). Significant levels of CXCL10 were detected only in the culture supernatants in the lower compartment with MDA-MB231 (Fig. 9c), suggesting that the CXCL10/CXCR3 axis plays an important role in the accumulation of NK cells in tumor sites. The MDA-MB231 cell line produced a lot more CXCL10 when PB NK cells were added (Fig. 9a, c), suggesting that soluble factors secreted from NK cells promoted the production of CXCL10 from this cell line. Taken together with the fact that CXCL10 is an IFN-γ-inducible protein [22] and that IL-2 augments the active production of IFN-γ from NK cells, we could assume that IFN-γ secreted from NK cells promotes CXCL10 production from the breast cancer cell line, which in turn accelerates the migration of CXCR3-expressing NK cells into the tumor site. Consistent with this hypothesis, the CXCL10 levels were well correlated with IFN-γ levels in the culture supernatants of the cell migration assay (Fig. 9d). In addition, we directly confirmed that IFN-γ promoted the production of CXCL10 from MDA-MB231 and MDA-MB468 in a dose-dependent manner (Fig. 9e, f).

Discussion

Human NK cells can be divided into the $CD56^{bright}$ and $CD56^{dim}$ subsets. These subsets have different phenotypic expression and may have different functions, although the direct functional significance of the expression levels of the CD56 antigen remains unknown. We previously demonstrated that $CD56^{bright}$ NK cells, which constitutively express low levels of TRAIL, are abundant in the liver [10]. $CD56^{bright}$ NK cells also constitutively express the high-affinity heterotrimeric IL-2R (IL-2R$\alpha\beta\gamma$) [23, 24]; hence, this subset has a high proliferative response to IL-2 and expand and survive through the upregulation of bcl-2 in


a
Isotype control
TRAIL DcR1
TRAIL DcR2
TRAIL DR4
TRAIL DR5
Normal
HER2(-)
HER2(+)
b
HER2
TRAIL DcR1
TRAIL DcR2
TRAIL DR4
TRAIL DR5
MDA-MB231
MDA-MB453
MDA-MB468
MCF-7
5.7
3.1
31.7
118
3.0
3.0
10.7
20.3
2.9
3.1
7.3
7.0
4.7
2.6
22.7
121.9

◀ **Fig. 5** Breast cancer cells express death-inducing TRAIL-DR4 but lack death-inhibitory TRAIL-DcR1 and TRAIL-DcR2. **a** Immunohistochemical expression of TRAIL-DcR1, TRAIL-DcR2, TRAIL-DR4, and TRAIL-DR5 in normal breast tissue and tumor sites of HER2 (+)- and HER2 (−)-type breast cancer tissues. Immunopathological findings are representative of three individual samples in each breast cancer category. Magnification ×200. *Scale bar* 100 μm. **b** Surface expression of HER2 and TRAIL receptors on the surface of MDA-MB231, MDA-MB453, MDA-MB468, and MCF-7 was analyzed by FCM. *Dotted lines* represent negative control staining with isotype-matched mAbs. *Numbers* indicate the mean fluorescence intensity (MFI) of cells that stained positively for HER2 and TRAIL receptors. TRAIL, TNF-related apoptosis-inducing ligand; *FCM* flow cytometric, *mAb* monoclonal antibody, *TNF* tumor necrosis factor

vitro in response to IL-2 [25, 26]. In contrast, resting $CD56^{dim}$ NK cells, which express IL-2R$\beta\gamma$ only, show almost no proliferation in response to even high doses of IL-2 in vitro [23, 26]. In this study, $CD56^{bright}$ NK cells exclusively survived and significantly upregulated TRAIL expression after in vitro cultivation of both PBMCs and LMNCs with IL-2 (Fig. 2). IL-2 stimulation also increased the surface expression of inhibitory receptors such as the KIR, including CD158a/158b and C-type lectin-like receptors (the CD94/NKG2 complex). CD94, which is expressed on essentially all NK cells, uses HLA-E expression as a sensor for the overall HLA class I level of a cell. In contrast, individual KIR family members are expressed on certain NK cell subsets and exhibit finer specificity for HLA class I allotypes and can distinguish between groups of HLA-A, HLA-B, and HLA-C allotypes. Ligation of such KIRs/CD94 to HLA class I molecules on self cells results in inhibition of NK cell cytotoxic activity, as originally predicted by the "missing-self" hypothesis [1, 27]. This regulation ensures

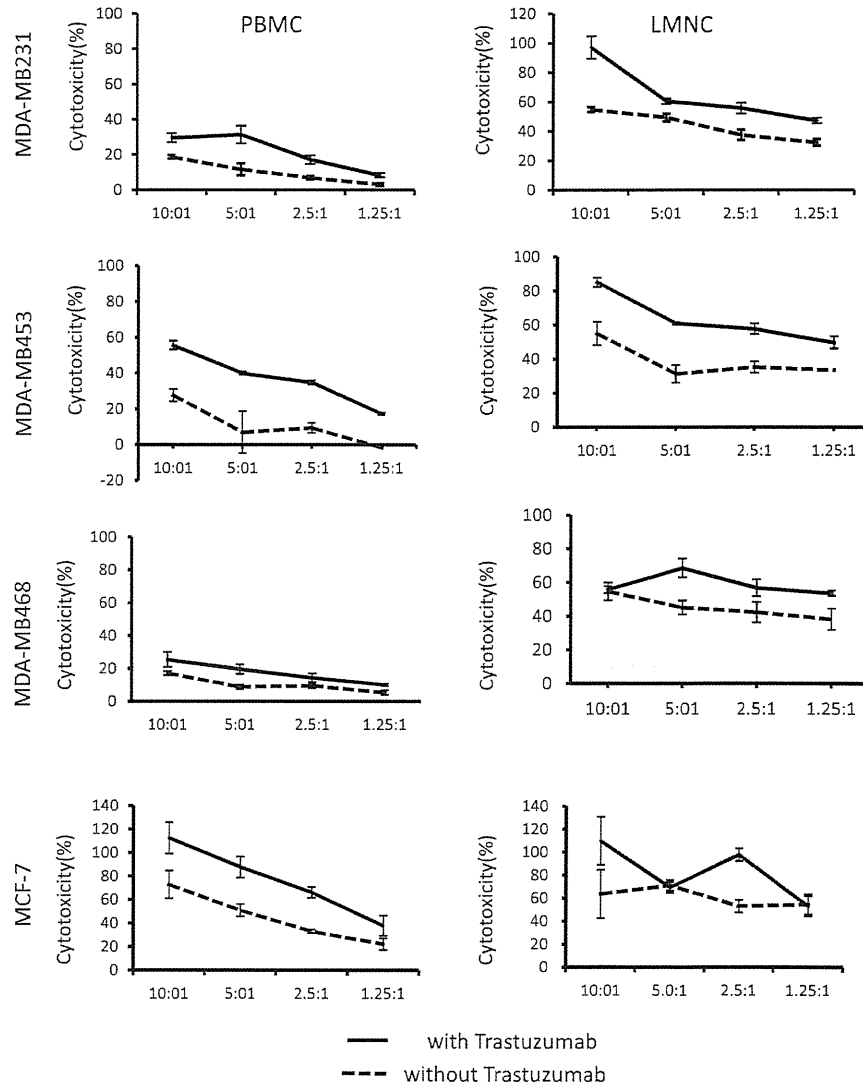


Fig. 6 Liver NK cells showed more vigorous cytotoxicity against breast cancer cell lines compared with PB NK cells. Cytotoxic activities of NK cells isolated from IL-2-stimulated PBMCs and LMNCs with or without trastuzumab against target cells (MDA-MB231, MDA-MB453, MDA-MB468, and MCF-7) were analyzed by the ^{51}Cr release assay. NK cells were isolated from PBMCs and LMNCs after stimulation with IL-2 for 5 days by magnetic sorting (purity > 90%). Data represent the mean ± SEM of values from triplicate samples and represent four similar experiments

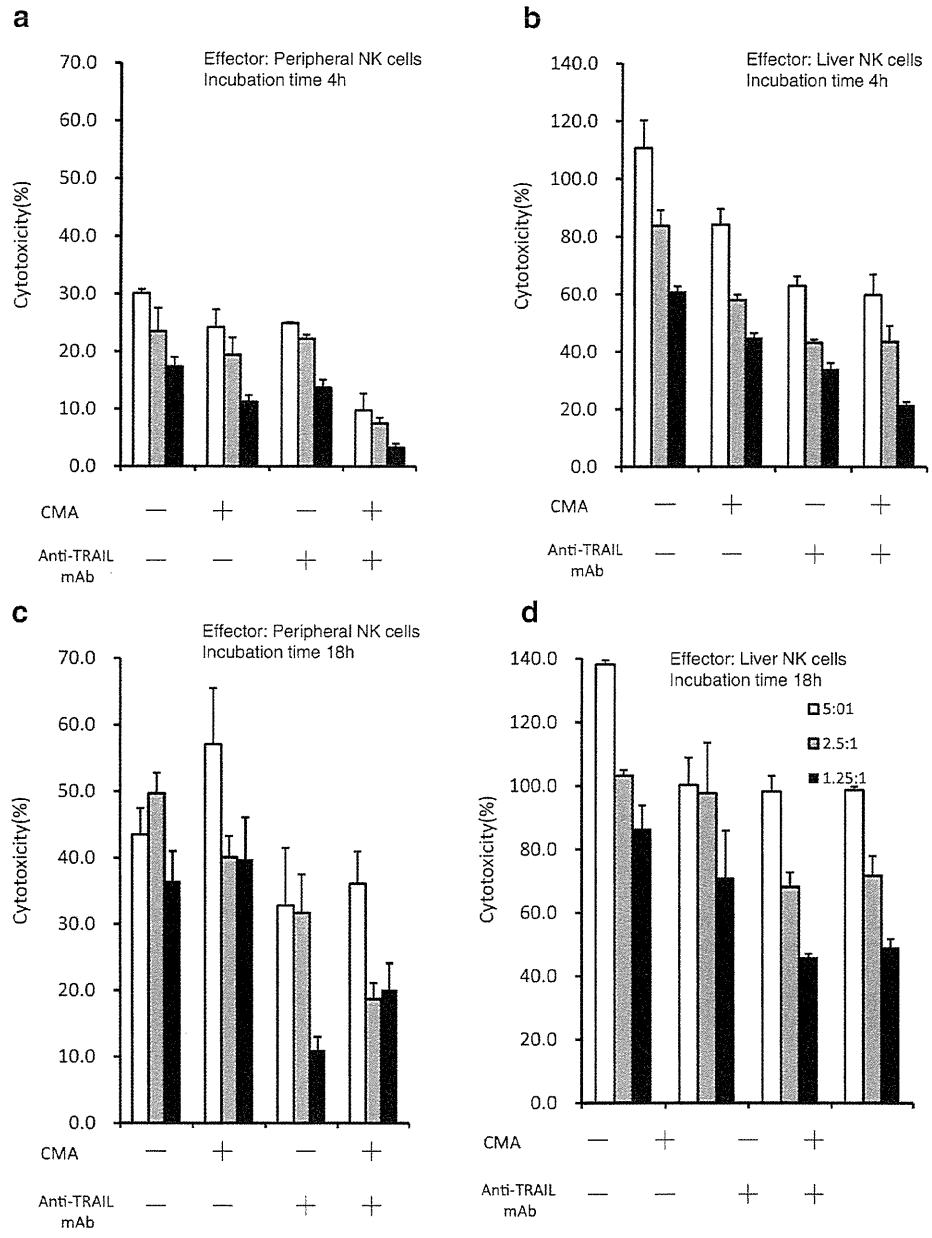


Fig. 7 NK cell-induced cytotoxicity was inhibited partially by the anti-TRAIL mAb alone and more profoundly by the combination of anti-TRAIL mAb and CMA. Isolated PB and liver NK cell populations were used as effector cells (*E*) in assays of cytotoxicity against the target (*T*) MDA-MB231 human breast cancer cell line. Cytotoxicity assays were performed at an *E*/*T* ratio of 10:1, 5:1, or 2.5:1 in the presence or in the absence of anti-TRAIL (N2B2) mAb (10 μg/ml) and/or concanamycin A (CMA) (50 nmol/l). Data are the average ± SEM values from triplicate samples and represent four similar experiments, in which five different donor individuals were used (the results of four other experiments are shown in Supplementary Figures 1 and 2). *Error bars* not shown appear within the data point. *NK* natural killer, *TRAIL* tumor necrosis factor-related apoptosis-inducing ligand, *mAb* monoclonal antibody, *FasL* Fas ligand, *E*/*T* effector-to-target, *CMA* concanamycin A

that cells expressing none, altered, or reduced MHC-I molecules, such as malignant or virus-infected cells, are eliminated by NK cells. The modulated expression of KIRs/CD94 by IL-2 is likely associated with the changed cytotoxic target-discriminating ability of NK cells upon their exposure to IL-2.

The significantly upregulated TRAIL expression on the IL-2-stimulated NK cells implies that they have the ability to target cancer cells expressing death-inducing receptors. TRAIL is a member of the TNF superfamily, which includes TNF and FasL [28]. The expression of TNF and FasL leads to damage of normal tissues in addition to their proapoptotic effect on transformed cells [29, 30], limiting their clinical applications. Conversely, TRAIL selectively induces apoptosis in transformed cells but not in most normal cells [28, 31, 32], making it a promising candidate for tumor therapy. However, intravenous delivery of recombinant TRAIL has met with problems, including a short pharmacokinetic half-life [32], necessitating frequent and high doses to produce the desired effect. The use of TRAIL-expressing NK cells as a delivery vector might promise both targeted and prolonged delivery of this death ligand.

TRAIL binds DR4 and DR5, leading to the formation of the death-inducing signaling complex and the

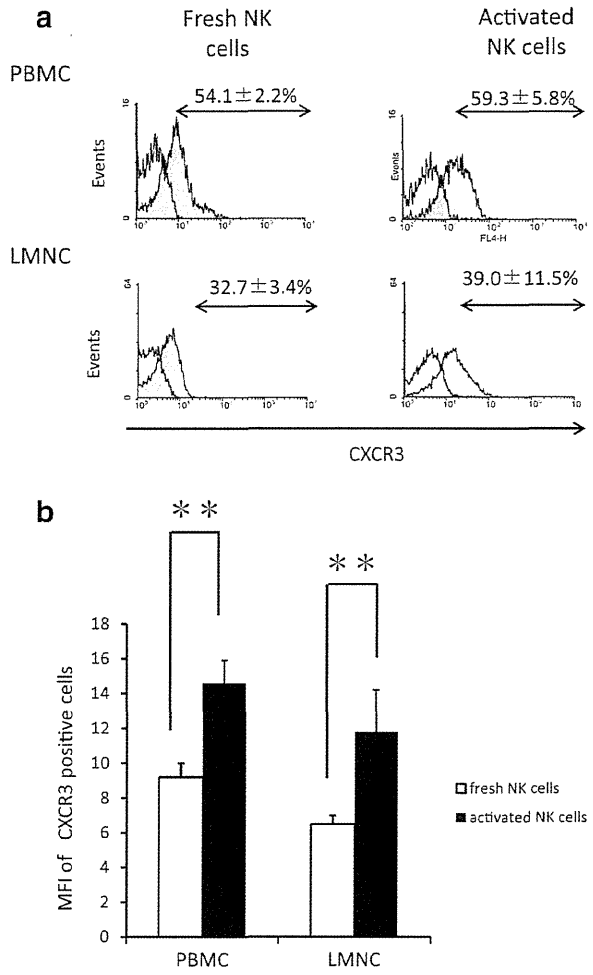


Fig. 8 CXC chemokine receptor 3 (CXCR3) expression was significantly upregulated on both liver and PB NK cells after cultivation with IL-2. **a** *Histograms* representing the log fluorescence intensities for CXCR3 expression on freshly isolated and IL-2-stimulated liver and PB NK cells. *Dotted lines* represent negative control staining with isotype-matched mAbs. *Numbers* (mean ± SEM) indicate the percentages of cells in each group that were positive for CXCR3 expression (PBMCs; $n = 6$, LMNCs; $n = 7$). *Histogram* profiles are representative of independent experiments. **b** *Numbers* indicate the mean fluorescence intensity (MFI) of cells that stained positively for CXCR3 on liver and PB NK cells (fresh NK cells *open column*, activated NK cells *closed column*). Data represent mean ± SEM (PBMCs; $n = 6$, LMNCs; $n = 7$). Statistical analyses were performed using the paired Student's t test (**$P < 0.01$). *NK* natural killer, *LMNC* liver mononuclear cell, *PBMC* peripheral blood mononuclear cell, *mAb* monoclonal antibody

Fas-associated protein with death domain. In turn, these complexes recruit caspase-8 (or caspase-10), which plays an important role in apoptosis induction either by direct activation of downstream effector caspases (caspase-3, caspase-6, and caspase-7) or by cleaving apoptotic molecules (Bcl-2 and Bcl-xL), resulting in further activation of the caspase-9 complex [33]. In this study, breast cancer cells of clinical samples showed much higher expression of TRAIL-DR4 than normal mammary glands but exhibited little TRAIL-DcR1, regardless of HER2 type. Similarly, all the tested breast cancer cell lines expressed TRAIL-DR4 but not TRAIL-DcR1 and TRAIL-DcR2, regardless of their HER2-status, suggesting that they are susceptible to TRAIL-induced apoptosis.

We tested various breast cancer cell lines to evaluate their susceptibility to NK cell-mediated cytotoxicity. Notably, liver NK cells showed more vigorous cytotoxicity against all the tested cell lines than did PB NK cells (Fig. 6), although the underlying mechanism remains unclear. The contribution of TRAIL to NK cell cytotoxicity was determined using the neutralizing anti-TRAIL mAb (Fig. 7). Trastuzumab addition remarkably enhanced the cytotoxicity of both NK cell types toward HER2-overexpressing breast cancer cell lines, indicating that HER2/trastuzumab-mediated ADCC was involved. As ADCC requires the activation and engagement of the CD16 FcγR on NK cells by Ab-coated targets, $CD56^{dim}$ NK cells, which highly express CD16 (Fcγ receptor III), are generally thought to exhibit greater levels of ADCC than do the $CD56^{bright}$ subset [34]. On the other hand, the majority of $CD56^{bright}$ NK cells expanded after activation with IL-2 expressed CD16 and efficiently mediated ADCC [20], explaining the HER2/trastuzumab-mediated ADCC observed in this study.

The cytotoxic ability of NK cells against cancer cells presumably requires contact between NK cells and their target cells. In general, NK cells are detected infrequently in tumors and their presence in the infiltrate consistently correlates with a good prognosis and increased patient survival [35, 36]. Chemokines acting on CXCR3 and CX3CR1 are considered major determinants of NK cell infiltration. CX3CR1 expression in gastric adenocarcinoma samples directly correlates with the number of NK cells infiltrating the tumor, and patients with higher CX3CL1 levels had a significantly better prognosis than patients with low CX3CL1 levels [37]. Similarly, our in vitro demonstration that the CXCL10/CXCR3 axis plays a role in the attraction between activated NK cells and breast cancer cells suggests that this chemokine system recruits NK cells to cancer cell sites and elicits antitumoral responses. In addition, we proposed a novel mechanistic paradigm in which IFN-γ secreted from NK cells promotes the production of CXCL10 from breast cancer cells, which in turn further accelerates the migration of CXCR3-expressing NK cells into the tumor site (Fig. 10).

Given the efficacy of NK cells to selectively eliminate abnormal cells, a variety of approaches have been taken to selectively augment NK cell response to tumors [38, 39]. Several therapeutic cytokines primarily act through NK cells (e.g., IL-2, IL-12, IL-15, and IFNs), and many studies have shown that activation of NK cell differentiation and

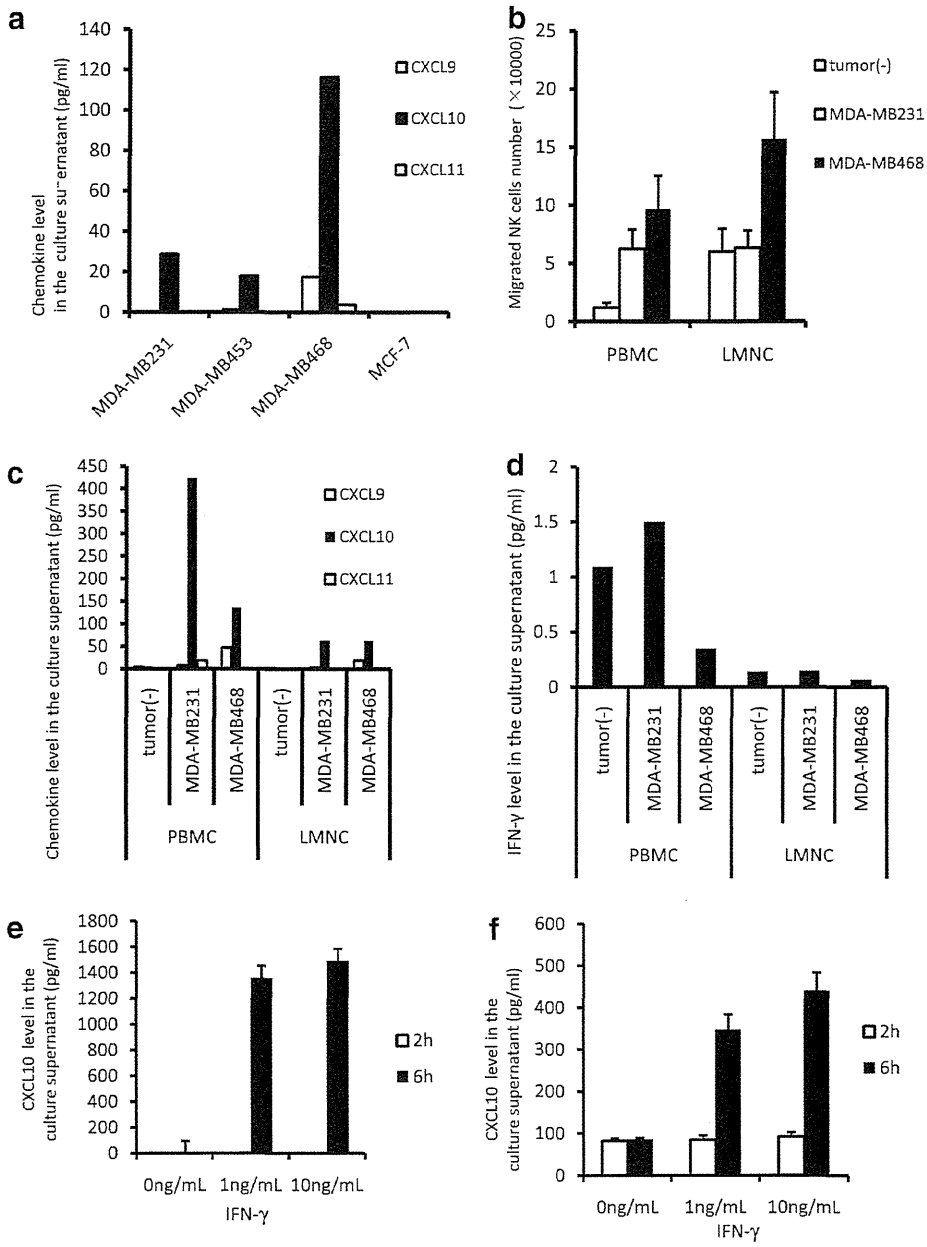


Fig. 9 NK cells were preferentially drawn by chemokines secreted from breast cancer cells, presumably through the CXCL10/CXCR3 axis. **a** Levels of various chemokines (CXCL9, CXCL10, and CXCL11) in the culture supernatants of breast cancer cell lines (MDA-MB231, MDA-MB453, MDA-MB468, and MCF-7) were analyzed using CBA Flex Sets. Supernatants were collected after 2 days of cultivation. **b** Migration assays were performed in transwell culture inserts with 3-μm pore filters. MDA-MB231 and MDA-MB468 cell lines were cultured in the lower chamber of the plate for 2 days and IL-2-activated NK cells from PBMCs and LMNCs were added to the upper chamber. After 2 h, the migrated NK cells were counted. Results are presented as mean migrated cell numbers ± SEM ($n = 3$). **c** Levels of chemokine in the medium of lower chambers in the migration assays described above were measured by CBA assay. Results are the average ± SEM values from triplicate samples and represent three individual experiments. **d** Levels of IFN-γ in the medium of lower chambers in the migration assays described above were measured by ELISA. The results are the average ± SEM values from triplicate samples and represent three individual experiments. **e** MDA-MB231 cells were cultured with various doses of IFN-γ for 2 and 6 h, and the levels of CXCL10 in the medium were measured by CBA assay. Results are the average ± SEM values from triplicate samples and represent three individual experiments. **f** MDA-MB468 cells were cultured with various doses of IFN-γ for 2 and 6 h, and the levels of CXCL10 in the medium were measured by CBA assay. Results are the average ± SEM values from triplicate samples and represent three individual experiments