(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

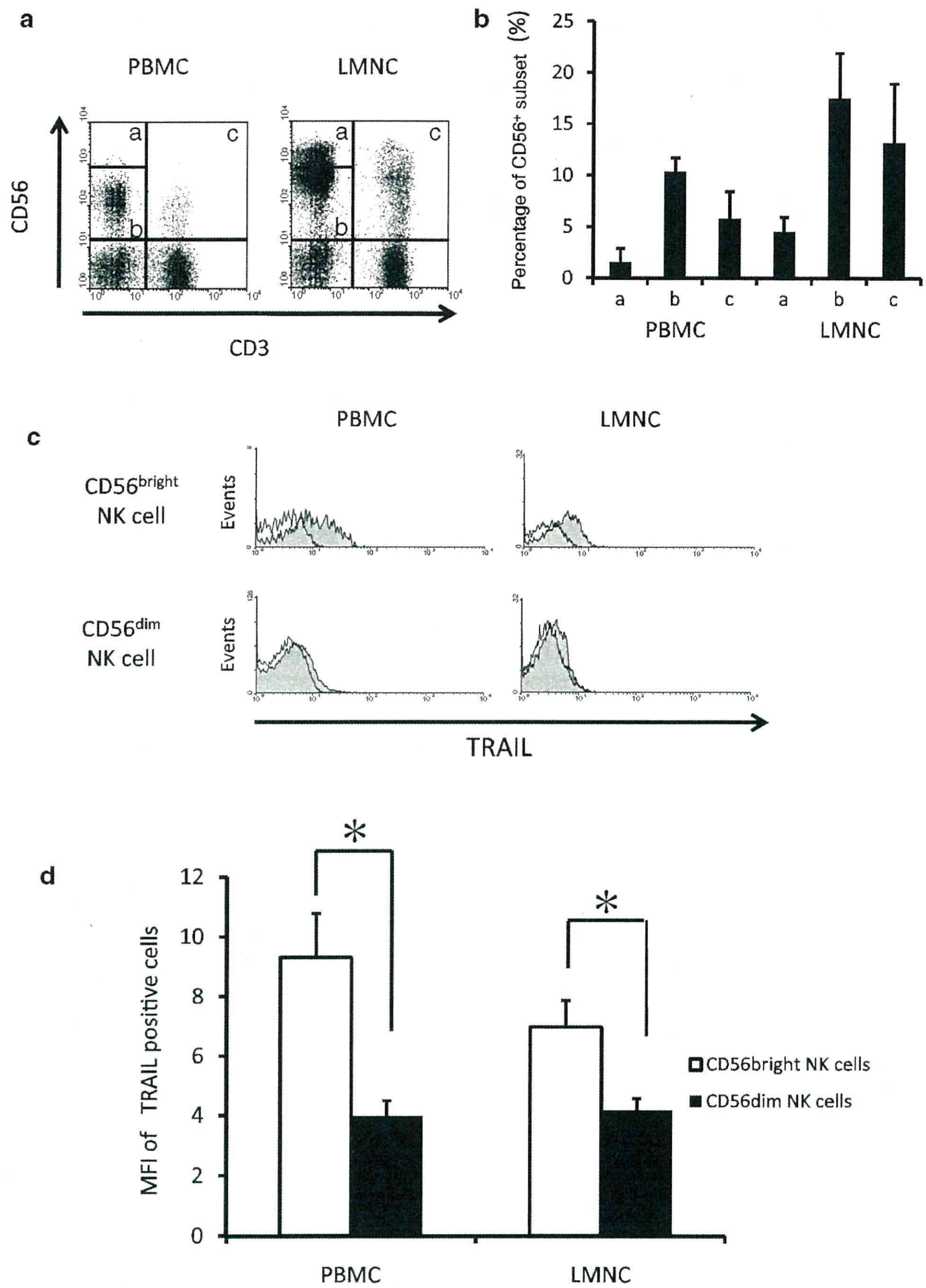


Fig. 1 The proportions of $CD56^+CD3^-$ NK and $CD56^+CD3^+$ NKT cells in LMNCs extracted from liver perfusates were significantly higher than those in PBMCs. **a** Flow cytometric (FCM) analyses of freshly isolated LMNCs obtained from liver perfusates and PBMCs from the corresponding donors were analyzed 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.

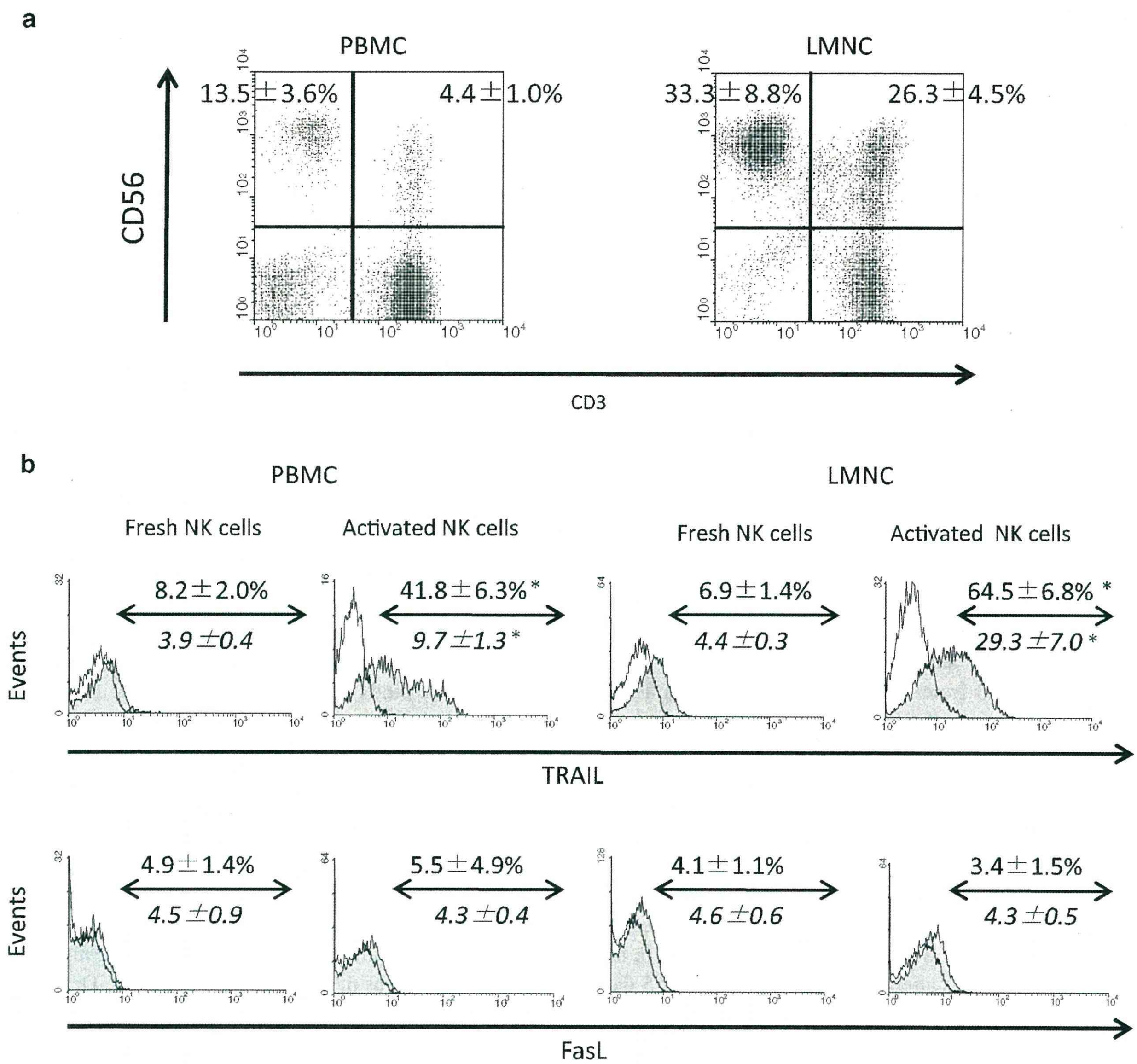


Fig. 2 Phenotypic analyses of human PB and liver NK cells. **a** Flow cytometric (FCM) analyses of PBMCs and LMNCs cultivated with IL-2 for 5 days were performed after staining with mAbs against CD3 and CD56. FCM profiles are representative of five and six independent experiments (using PBMCs and LMNCs, 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

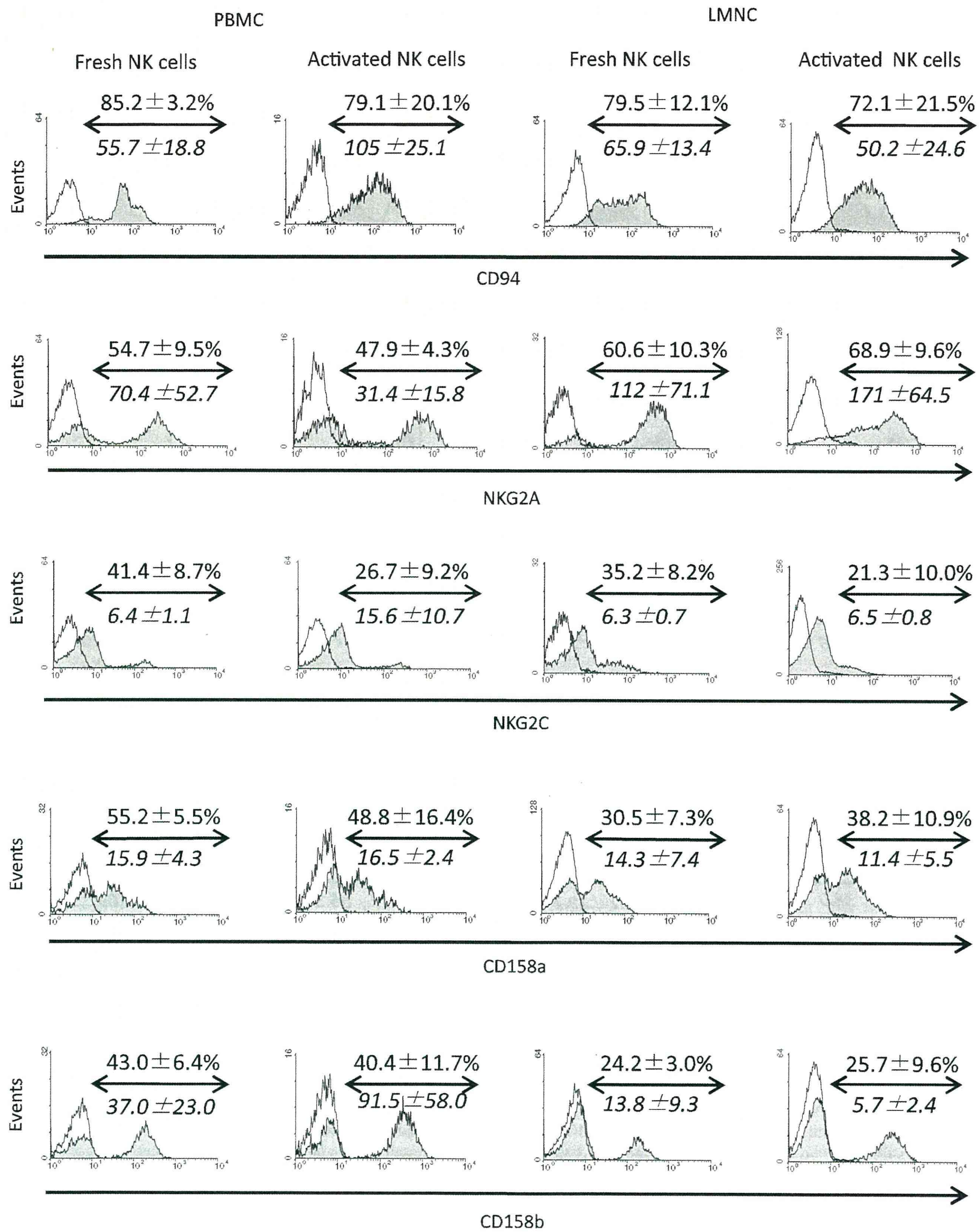
Phenotypic properties of human NK cells

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
Events
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
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
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
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
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
CD158b

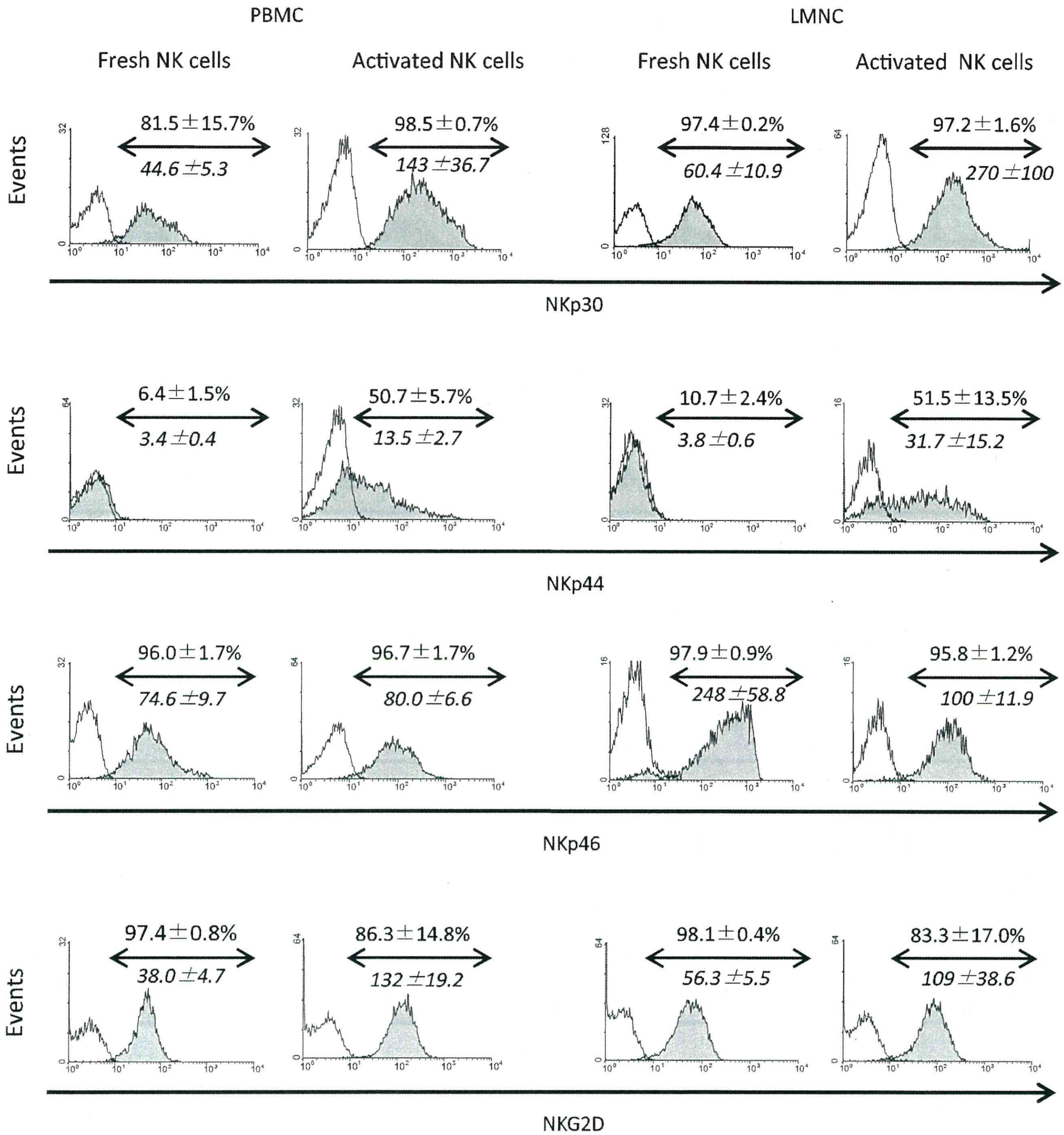


Fig. 4 IL-2 stimulation led to elevated expression of NKp30, NKp44, and NKG2D in both human PB and liver NK cells. Expression of the cytotoxicity-associated receptors, including NKp30, NKp44, NKp46, and NKG2D, in NK cell subsets among LMNCs or PBMCs freshly isolated or cultivated with or without IL-2 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

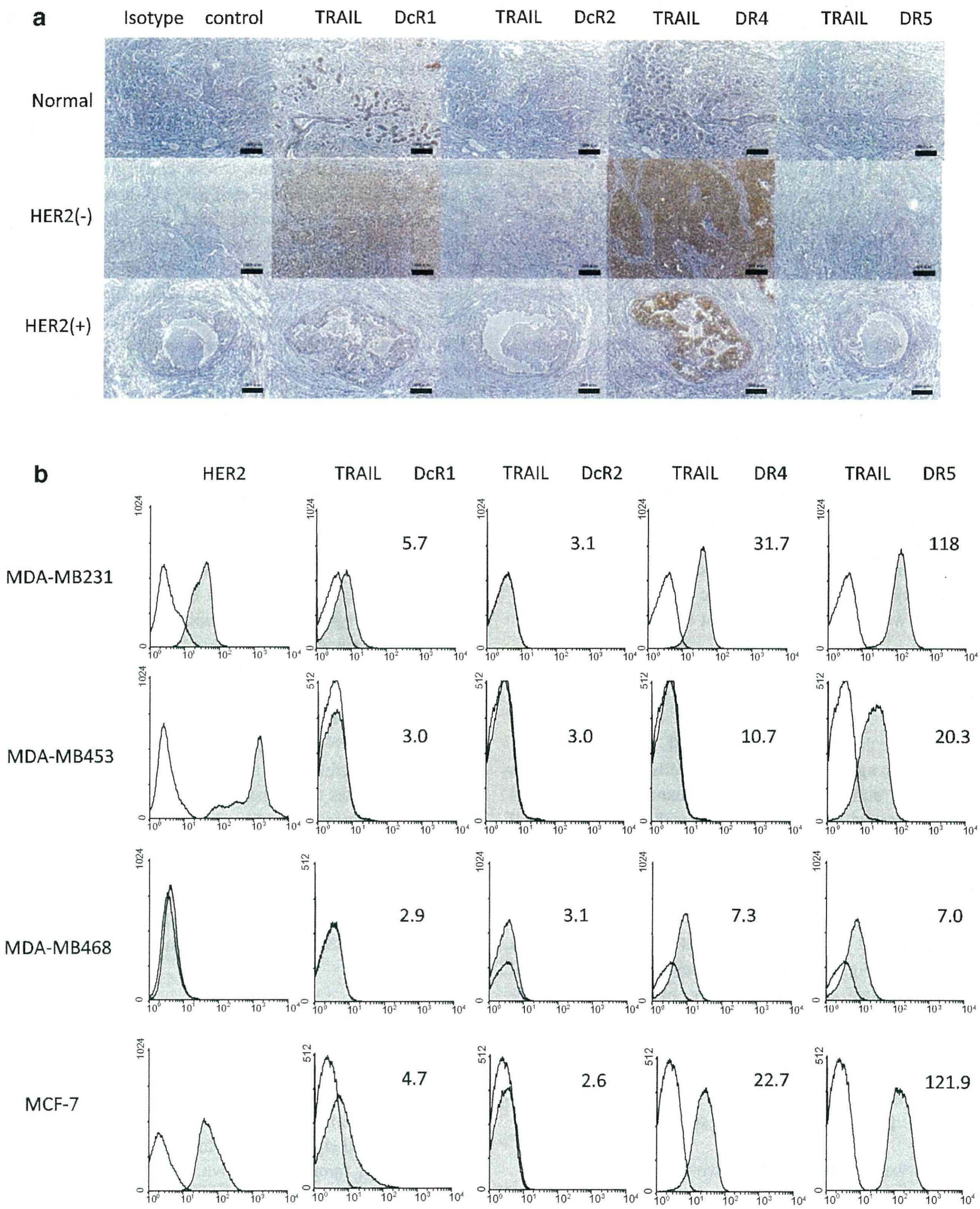
To determine the contribution of TRAIL to the cytotoxicity of NK cells against breast cancer cells, the effect of a neutralizing anti-TRAIL mAb was examined in a cytotoxicity assay with PB and liver NK cells as effectors and MDA-MB231 cells as the target. Both PB and liver NK cell-induced cytotoxicity 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
5.7
3.1
31.7
118
MDA-MB453
3.0
3.0
10.7
20.3
MDA-MB468
2.9
3.1
7.3
7.0
MCF-7
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

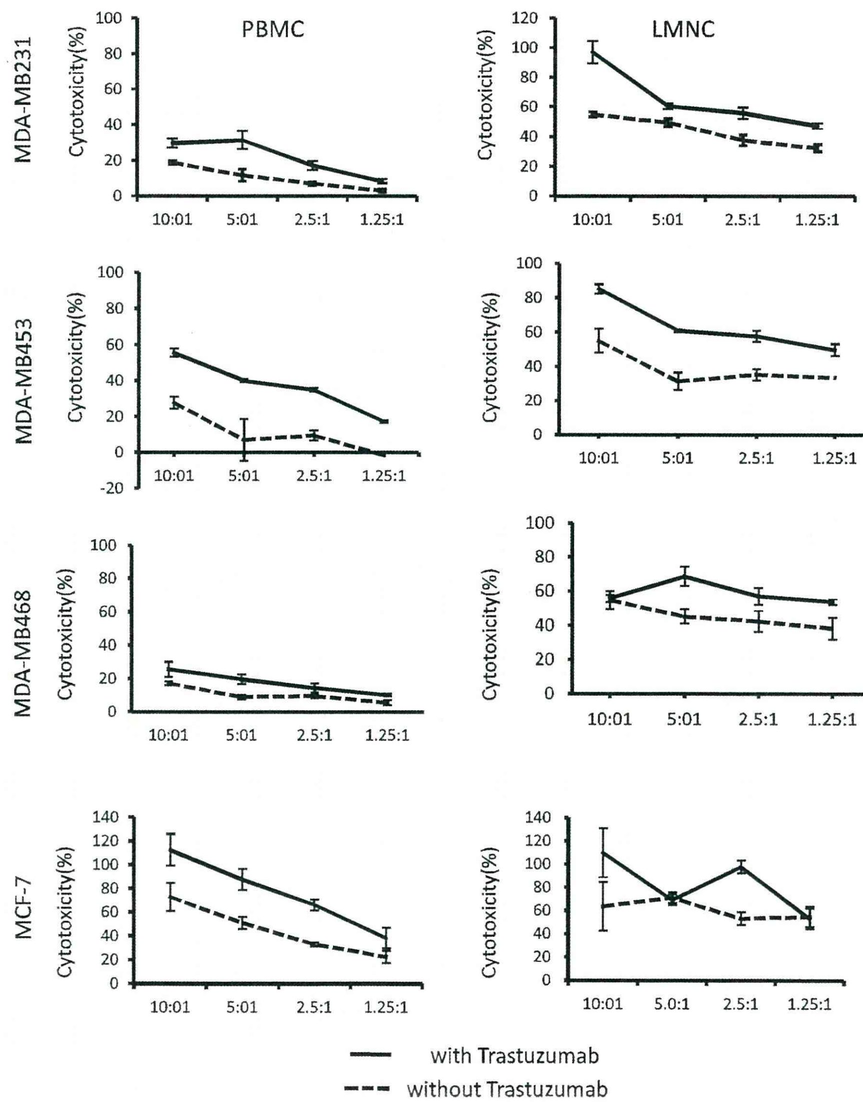


Fig. 6 Liver NK cells showed more vigorous cytotoxicity against breast cancer cell lines compared with PB NK cells. Cytotoxic activities of NK cells isolated from IL-2-stimulated PBMCs and LMNCs with or without trastuzumab against target cells (MDA-MB231, MDA-MB453, MDA-MB468, and MCF-7) were analyzed 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

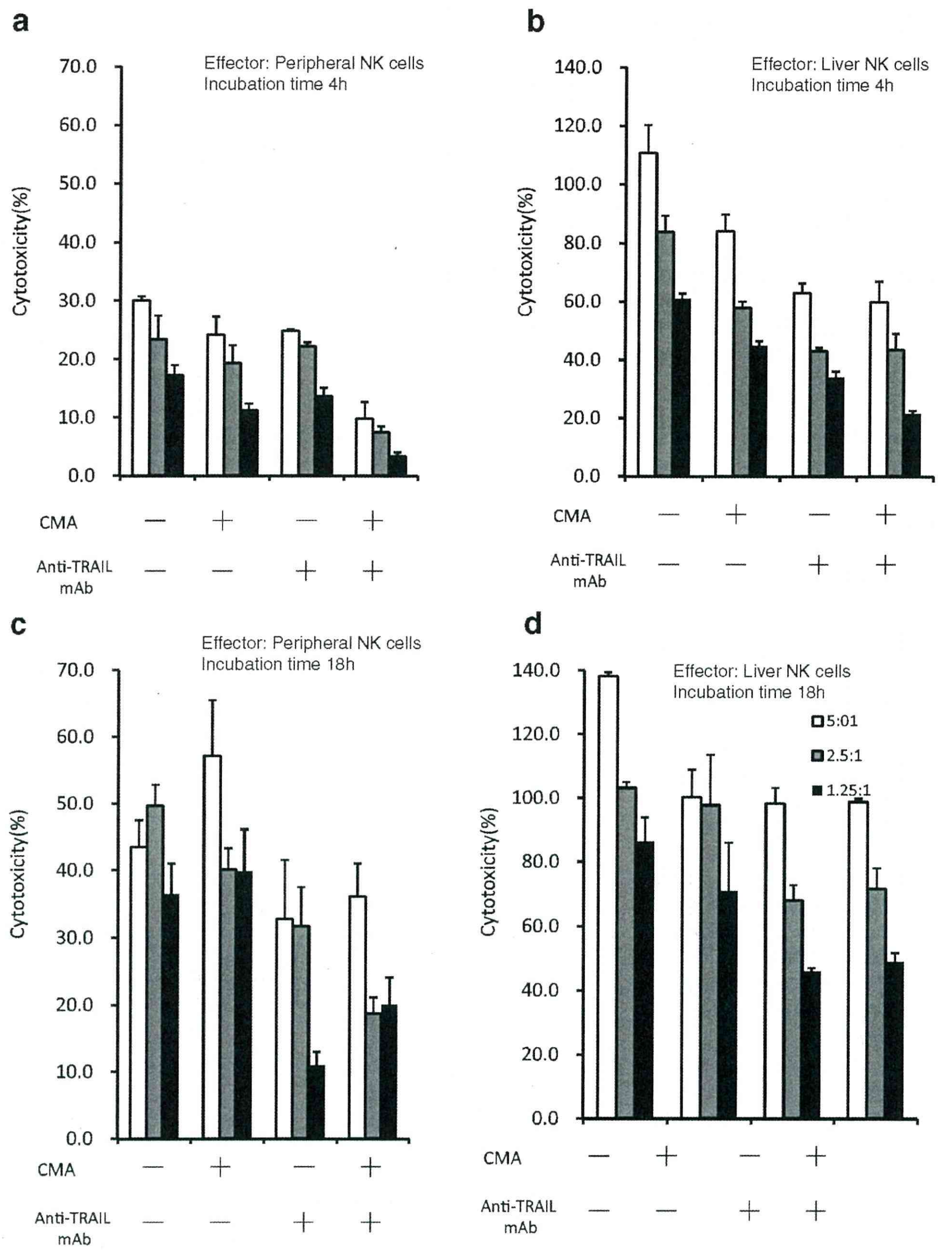


Fig. 7 NK cell-induced cytotoxicity was inhibited partially by the anti-TRAIL mAb alone and more profoundly by the combination of anti-TRAIL mAb and CMA. Isolated PB and liver NK cell populations were used as effector cells (*E*) in assays of cytotoxicity against the target (*T*) MDA-MB231 human breast 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

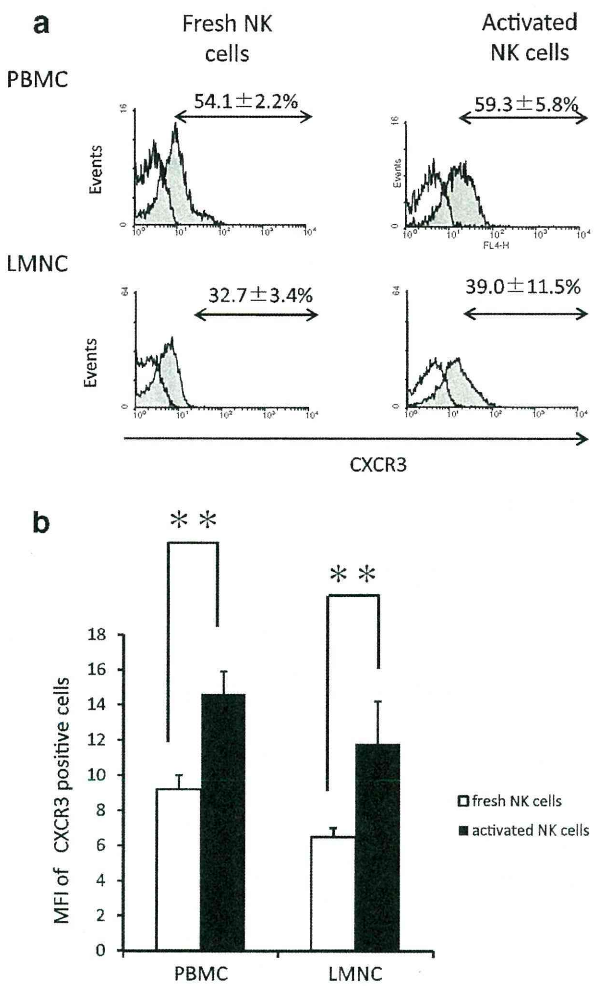


Fig. 8 CXC chemokine receptor 3 (CXCR3) expression was significantly upregulated on both liver and PB NK cells after cultivation with IL-2. **a** *Histograms* representing the log fluorescence intensities for CXCR3 expression on freshly isolated and IL-2-stimulated liver and PB NK cells. *Dotted lines* 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

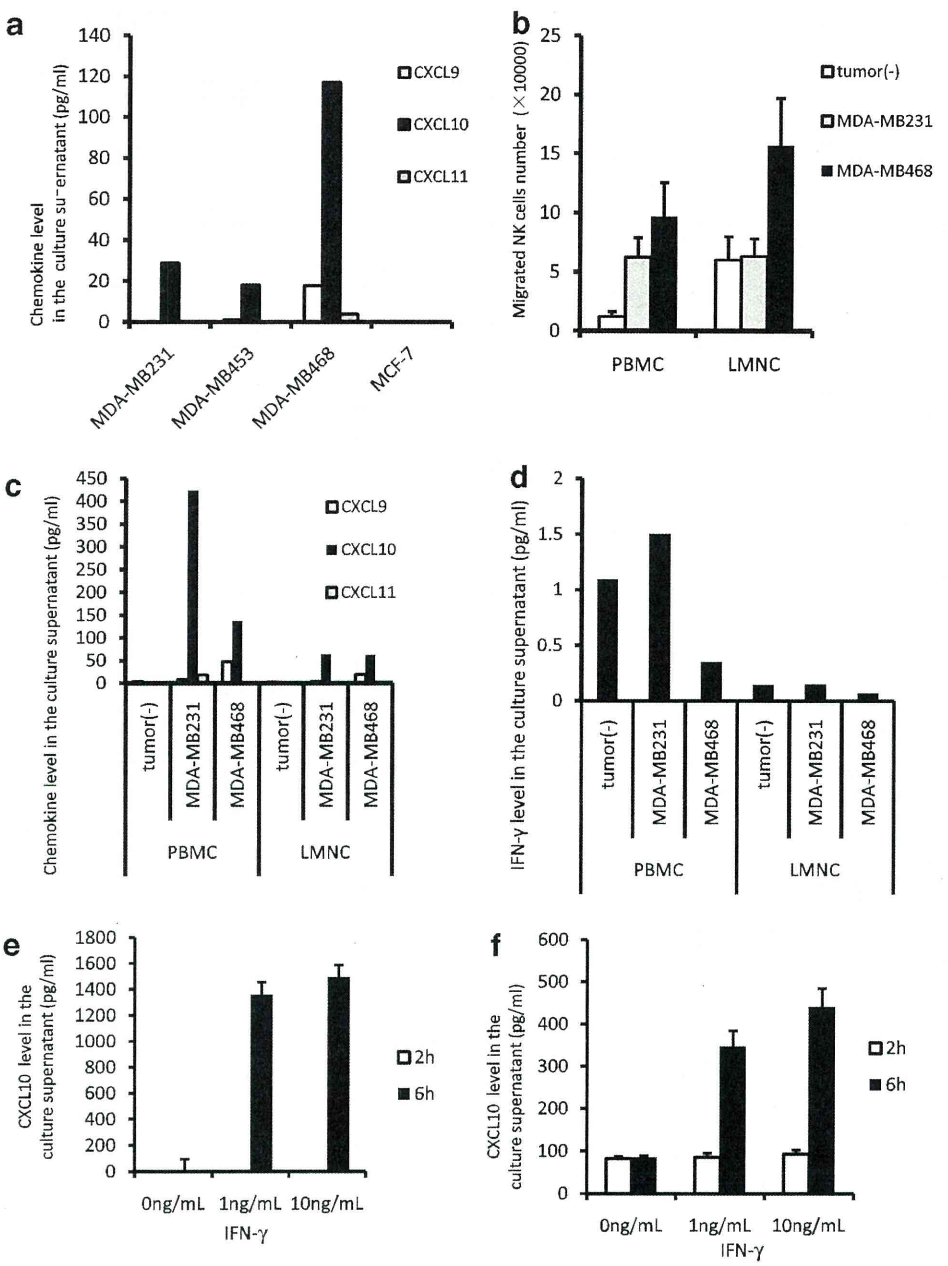


Fig. 9 NK cells were preferentially drawn by chemokines secreted from breast cancer cells, presumably through the CXCL10/CXCR3 axis. **a** Levels of various chemokines (CXCL9, CXCL10, and CXCL11) in the culture supernatants of breast cancer cell lines (MDA-MB231, MDA-MB453, MDA-MB468, and MCF-7) were 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

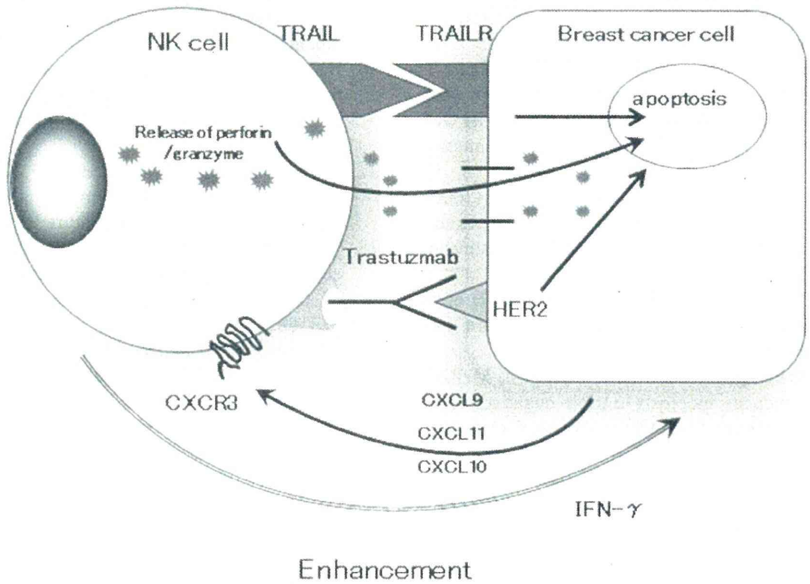


Fig. 10 Mechanistic paradigm of interaction between NK and breast cancer cells. IFN-γ secreted from NK cells promotes the production of CXCL10 from breast cancer cells, which in turn further accelerates migration of CXCR3-expressing NK cells into the tumor site. Migrated NK cells kill breast cancer cells by either of the two major mechanisms that require direct contact between NK cells and target cells. In the first, cytoplasmic granule toxins, perforin, and granzymes are secreted by exocytosis and together induce apoptosis of the target cell. The second mechanism involves the engagement of death receptors on target cells by expressing of their cognate ligands (TRAIL) on NK cells, resulting in apoptosis of the target cells

function leads to more efficient elimination of tumor growth 9 [40–44]. Despite these promising advances, the systemic administration of cytokines, such as IL-2, which non-specifically activate a broad range of immune cell types, is associated with significant toxicity [40, 45]. Recent animal experiments have demonstrated the ability of adoptive transfer of NK cells to mount a therapeutic antitumor response [46, 47], and translational clinical research suggests that NK cells are useful for controlling human malignancy [48–50]. Our results have proven that PB NK cells can kill breast cancer cells and liver NK cells can hinder metastasis of breast cancer to the liver, which suggests 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. Although liver NK cells displayed higher cytotoxicity than PB NK cells, no clinically applicable method for obtaining liver NK cells from patients with breast cancer has yet been established. Alternatively, locally infusing IL-2 into the liver through the portal vein likely activates endogenous liver NK cells, which in turn might infiltrate or accumulate to the tumor site probably through the CXCL10/CXCR3 axis.

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Conflict of interest None.

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特集 肝癌の診療 Up to date

肝癌再発予防

Prevention for recurrence after curative treatment for hepatocellular carcinoma

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KOBAYASHI Tsuyoshi　OHDAN Hideki

肝細胞癌の高率な再発には癌転移再発と多中心性発癌という再発形式が複雑に関与している．肝細胞癌の根治療法後の再発を予防する目的で行われる補助療法として，インターフェロン療法は一定のサブグループの予後に寄与すると考えられるが，進行肝細胞癌に対して転移再発を抑制する有効な治療法はいまだ確立されていない．肝移植後には転移再発が重要だが，免疫抑制状態や肝炎ウィルスへの対策も重要である．分子生物学的あるいは免疫学的機序を応用した新たな治療戦略を期待する．

はじめに

肝細胞癌は根治療法を施しても再発の多い癌である．肝細胞癌の特殊性として，癌転移再発に加えて背景に高頻度に存在するB型肝炎ウィルスやC型肝炎ウィルス感染による障害肝からの多中心性発癌がある．このことが肝細胞癌の初期治療ならびに再発予防戦略を複雑にしていると考えられる．

肝細胞癌に対する治療法の選択に関して，科学的根拠に基づく肝癌診療ガイドライン(2009年版)によると肝障害度，腫瘍数，腫瘍径の3因子を基に設定され，肝切除，局所療法，肝動脈塞栓療法，肝動注化学療法，肝移植，緩和ケアが推奨されている[1]．これらの治療法のうち根治が期待できる治療法には肝切除，局所療法，肝移植が位置づけられるが，根治療法後の再発率は高く，再発を予防する目的で行われる補助療法として確立されたものはない．再発を予防する目的で行う補助療法は，再発高危険群に対して行うべきであり，補助療法にはそれぞれの危険因子に応じた戦略が必要と考えられる．

本稿では，肝細胞癌に対する根治療法後の再発予防の現状と今後の展望について述べる．

I. 肝細胞癌の再発危険因子

肝細胞癌根治療法後の再発危険因子としては，Stage分類，脈管侵襲，腫瘍数，腫瘍径，被膜形成，肝機能などがあげられる[2][3]．これらのうちStage分類，脈管侵襲，腫瘍数，腫瘍径，被膜形成は主に転移再発に関与していると考えられるが，肝機能に関しては主に多中心性発癌に関与し

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Key words：肝細胞癌／術前補助療法／術後補助療法

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ていると考えられる．補助療法にはそれぞれの危険因子に応じた戦略が必要であり，高率に転移再発が疑われる症例に対しては転移再発を予防可能な治療が必要である．またHCVやHBV陽性肝細胞癌のように多中心性発癌による再発が予想される症例には，積極的なウィルス駆除療法を含めた肝庇護療法が合理的と考えられる．

一方で肝細胞癌に対する肝移植術後の再発には，脈管侵襲と腫瘍分化度が重要な位置を占める[4)5)]．肝移植は背景肝と肝癌を同時に治療できる治療法であり，背景肝因子は再発に大きく影響しないと考えられる．肝移植後早期の再発はほとんどの場合，術前にすでに存在した微小転移の増大，あるいは循環血液中の腫瘍細胞が着床して生じる転移再発と考えられ，抗腫瘍効果を期待した治療戦略が求められる．一方で免疫抑制剤の使用が必須でありまた肝炎ウィルス陽性例では肝移植後の肝炎ウィルス再燃が危惧されるため，これらに対する対策も重要となる．

II. 術前補助療法

1．術前肝動脈（化学）塞栓療法（transcatheter arterial embolization/chemoembolization：TAE/TACE）

経肝動脈的に塞栓物質を投与するあるいは抗癌剤を併用する方法が肝動脈（化学）塞栓療法として切除不能肝細胞癌に対して行われてきた．2002年に報告された2件のRCTの結果から，切除不能な肝細胞癌に対してTAE/TACEは対症療法と比較して抗腫瘍効果および生存率向上に寄与するとされる[6)7)]．肝癌診療ガイドラインでは，「肝障害度A，Bの進行肝細胞癌（手術不能で，かつ穿刺局所療法の対象とならないもの）に対する治療法として推奨」されている．切除可能な肝細胞癌に対しては，TAE/TACEを術前化学療法として肝切除や穿刺局所療法の前に，最近では肝移植の術前に行われている．

1）肝切除術前

肝切除術前TAE/TACEに関する論文は多数みられ，予後改善効果があるとするものと無効とするものが複数存在する．これまでに3件のランダム化比較試験（RCT）が報告されたが[8)-10)]，いずれの試験でも主要評価項目である無再発生存を延長する結果は得られなかった（表1）．対象や方法が異なってはいるが，これまでの報告では否定的な結果に終わっている．

2）穿刺局所療法前

肝癌診療ガイドラインでは，「Child分類のAまたはBの肝機能，腫瘍径3 cm以下，腫瘍数3個以下」を穿刺局所療法のよい適応としている．従来は経皮的エタノール注入療法（PEI）が，最近ではラジオ波焼灼療法（RFA）が中心に行われているが，肝切除に比較していずれも局所再発が多い傾向にある．穿刺局所療法の前にTAE/TACEを組み合わせた併用療法が試みられており，穿刺局所療法単独と比較したRCTがこれまでに2件報告されている[11)12)]．1件は併用療法とPEI単独療法の比較[11)]，1件は併用療法とPEIあるいはRFA単独療法の比較であり[12)]，併用療法はPEI単独に対しては局所再発を有意に抑制したが，RFA単独に対しては局所再発に有意な差はみられなかった．いずれの試験も生存率の改善には寄与せず，ガイドラインで推奨される

表1　肝切除術前TACEに関するランダム化比較試験

著　者	Year	症例	腫瘍径	抗癌剤	5年生存率（vs control）	5年無再発生存率（vs control）	結果
Wu	1995	52	10 cm以上	EPI	40% vs 50%(3yr)	32% vs 60%(3yr)	無効
Yamasaki	1996	97	2 cm≦，≦5 cm	CDDP	63% vs 62%	39% vs 31%	無効
Zhou	2009	108	5 cm 以上	CDDP，MMC，FU	30.7% vs 21.1%	12.8% vs 8.9%	無効

EPI：epirubicin　CDDP：cisplatin　MMC：mitomycin C　FU：fluorouracil

には至っていない．

3）肝移植術前

肝移植後の肝細胞癌再発を予防する目的で，術前に肝細胞癌に対する治療を行うことが予後を改善するのかどうかは結論が得られていない．これまでに肝移植前のTACEが肝細胞癌の再発を抑制するのかを論じた報告が複数みられるが，いずれもレトロスペクティブな検討であり，RCTはまだ報告されていない．Majnoらは，肝細胞癌に対する肝移植患者111例の検討で，術前TACE群(54例)と術前無治療群(57例)の無再発生存率に差を認めなかったと報告している[13]．Decaensらは多施設共同症例対照研究により，術前TACE群100例と術前無治療群100例を比較したが，5年生存率でそれぞれ59.4%，59.3%，5年無再発生存率でそれぞれ69.3%，64.1%と有意な差を認めなかったと報告している[14]．TACEによく反応した症例では肝移植後の予後も良いという報告や，肝移植待機中のドロップアウトを減らすために術前にTACEを行うことは有用であるという報告もあるが，肝細胞癌の無再発生存率や生存率に寄与するエビデンスはない[15][16]．

III．術後補助療法

1．ウィルス性肝炎合併例に対する抗ウィルス療法

C型慢性肝炎，代償性C型肝硬変患者の発癌予防にはインターフェロン療法によるウィルス駆除が有用であることが示されている．肝細胞癌は高率にウィルス性肝炎を合併しており，根治療法後にウィルス排除ないし炎症の改善，さらに肝細胞癌再発抑制を目的としてインターフェロン療法が行われ，これまでに肝細胞癌根治療法後のインターフェロン療法に関するRCTが7件報告されている[17)-23)](表2)．Shiratoriらは74例のHCV陽性でかつ腫瘍個数3個以下の肝細胞癌患者に対してPEIを行った後に，49例にインターフェロン療法を行った．再発率に差はみられなかったが，インターフェロン療法は有意に生存率を改善させた[19]．しかしMazzaferroらは150例のHCV陽性肝細胞癌に肝切除を行った後に，76例にインターフェロン療法を行ったが，5年無再発生存率が24.3%とコントロール群の5.8%と有意な差がなかったと結論している[22]．それぞれのRCTからは肝細胞癌根治療法後のインターフェロン療法は再発抑制効果や生存率の改善がみられたとする報告や，一定のサブグループにのみ効果がみられたとする報告がある．しかし複数のメタアナリシス論文でいずれもインターフェロン療法が再発抑制および予後延長に効果があるとする結果であった[24][25]．

これらの報告はインターフェロン単剤療法によるものであるが，現在ではC型肝炎に対してはペグインターフェロン・リバビリン併用療法が標準治療となっている．ペグインターフェロン・リバビリン併用療法は単剤療法に比べてより高い著効率を示しており，根治療法後の補助療法として

表2　肝細胞癌根治療法後インターフェロン療法に関するランダム化比較試験

著　者	Year	症例	対　象	IFN	5年生存率 (vs control)	5年再発率 (vs control)	結　果
Ikeda	2000	20	HCV	IFN beta	ND	0% vs 100%(2yr)	有効(再発)
Kubo	2002	30	HCV	IFN alpha	ND	ND	有効(再発)
Shiratori	2003	74	HCV	IFN alpha	68% vs 48%	82% vs 92%	有効(生存)
Lin	2003	30	HBV，HCV	IFN alpha	ND	40% vs 90%(4yr)	有効(再発)
Sun	2006	236	HBV	IFN alpha	63.8 m vs 38.8 m*	31.2 m vs 17.7 m*	有効(生存，再発)
Mazzaferro	2006	150	HCV	IFN alpha	ND	24.3% vs 5.8%§	無効
Lo	2007	80	HBV	IFN alpha	79% vs 61%	ND	無効

IFN：インターフェロン　ND：not described　*生存期間中央値　§無再発生存率

の成績も報告がみられる．広島大学病院では現在HCV 陽性肝細胞癌に対して肝切除後にペグインターフェロン・リバビリン併用療法を積極的に行っており，5年生存率で91.7%とヒストリカルコントロールの50.6%に比べて有意に良好であった[26]．また最近 HCV genotype 1のタンパク分解酵素阻害薬である Telaprevir に関する RCT の結果が2件報告された．ADVANCE 試験では，治療歴のない HCV genotype 1の肝炎患者1,088例に対してペグインターフェロン・リバビリン療法（PR 群）に対する Telaprevir の上乗せ効果を検討している．ウィルス消失率（Sustained virological response：SVR）においてPR 群44%に対して Telaprevir 併用群（T12PR 群：Telaprevir 12週併用，T8PR 群：Telaprevir 8週併用）では75%，69%と著明な上乗せ効果を認めた[27]．一方，REALIZE 試験では，治療歴のある HCV genotype 1の肝炎患者663例に対してペグインターフェロン・リバビリン療法（PR 群）に対する Telaprevir の上乗せ効果を検討している．PR 群17%に対して Telaprevir 併用群（T12PR 群：Telaprevir12週併用，lead-in T12PR48群：PR 4週後に Telaprevir12週併用）では64%，66%と著明な SVR の改善を認めた[28]．今後 HCV genotype 1型に対して Telaprevir 併用療法が標準治療となる可能性があり，肝細胞癌根治療法後にも応用されることが期待される．

一方，B 型肝炎においては核酸アナログ製剤の有効性が明らかとなり，RCT でラミブジンがB型慢性肝炎からの発癌を抑制することが証明されている[29]．核酸アナログ製剤は，B 型肝炎ウィルスの増殖を抑制し，肝の炎症を沈静化させ，肝線維化を寛解させる．根治療法後の補助療法としてのエビデンスはないが，今後治療後の再発抑制を目的とした使用法も注目される．

2．術後補助化学療法

一般に肝細胞癌は抗癌剤の感受性が低く，切除不能な進行肝細胞癌に対する全身化学療法の奏効率は20%以下と報告されている．加えて背景に障害肝が併存することが多く，十分な投与量が確保できない，あるいは肝機能増悪のために予後を悪化させるなど複雑な側面を持つ．したがって切除不能な肝細胞癌においても，ソラフェニブの登場までは有効性が証明された標準治療といえるものはなかった．一方，肝動注化学療法は，肝細胞癌局所に高濃度の抗癌剤を投与できることと，全身への抗癌剤濃度が低く抑えられるために全身の副作用が低く抑えられると考えられている．

肝細胞癌根治療法後に転移再発を抑制する目的で多種多様な補助療法が考案され，これまでに小規模ながら多数の RCT の結果が報告されている[30)～41)]（表3）．経口投与，経静脈投与，経肝動脈投与の単独あるいは複数の投与経路の併用など多様な方法が試みられている．経口投与に関しては2つの RCT で有効性が否定されたが，最近小規模な RCT ではあるが Capecitabine が再発を抑制する可能性が示された[30)～32)]．経静注投与として有効性が示されたレジメンはなく，肝機能不良例ではむしろ予後を悪化させたとの報告もある[35)36)]．最も多く用いられているのは経肝動脈経路であり，メタアナリシス論文では肝動注化学療法の有効性が示されている[42)]．それぞれが単一施設からの少数例の報告で，レジメンがさまざまであることから十分なエビデンスはないが，その中でも門脈腫瘍栓を伴う進行肝細胞癌に対象を絞った RCT では，再発を抑制あるいは生存率に寄与したとする結果がみられる[33)39)41)]．肝細胞癌は経門脈的に進展すると考えられており，門脈腫瘍栓合併例は肉眼的に根治切除したとしても高率に転移再発する症例が多いため，積極的な肝動注化学療法の意義がより大きいと考えられる．このように転移再発の高リスク群に対して適切にデザインされた多施設共同研究により，さらなる検討が必要である．

分子標的薬ソラフェニブは，血管新生因子受容体型チロシンキナーゼである VEGF レセプターや PDGF レセプター，MAP キナーゼカスケードのセリンスレオニンキナーゼ Raf を選択的に抑制する．欧米を中心に行われた多施設共同大規

表3 肝細胞癌根治療法後化学療法に関するランダム化比較試験

著 者	Year	症例	対 象	治療内容	5年生存率 (vs control)	5年無再発生存率 (vs control)	結 果
経口抗癌剤							
Yamamoto	1996	76	StageII	経口 HCFU vs 無治療	ND	ND	肝機能良好例で有効
Hasegawa	2006	159	Child A/B, TT(−)	経口 UFT vs 無治療	58% vs 73%	29% vs 29%	無効
Xia	2010	60	Child A, 3個以内, TT(−)	経口 capecitabine vs 無治療	62.5% vs 39.8%	46.7% vs 23.3%	有効(再発抑制)
静注・肝動注							
Izumi	1994	50	TT(+) and/or IM(+)	肝動注(DXR, MMC) vs 無治療	50.3% vs 28.8%	32.0% vs 11.7%	有効(再発抑制)
Kohno	1996	88	治癒切除	経口 UFT+肝動注 EPI vs 経口 UFT	30% vs 35%	17% vs 14%	無効
Ono	1997	58	Child A/B, 治癒切除	肝動注 EPI+静注 EPI+経口 HCFU vs 無治療	31.5% vs 57.1%	32.0% vs 22.5%	無効
Lai	1998	66	治癒切除	静注 EPI+肝動注 CDDP vs 無治療	ND	18% vs 48%	有害(再発増加)
Kwok	2003	40	Child A/B	肝動注 CDDP 4 コース vs 1 コース	40% vs 55%(3yr)	40% vs 44%(3yr)	無効
Shuqun	2004	57	治癒切除	肝動注(CDDP, EPI, MMC)+皮下注 thymosin alpha1 vs 肝動注 vs 無治療	10 m vs 7 m vs 8 m*	7 m vs 5 m vs 4 m*	有効
Tanaka	2005	15	Vp4 or IM3	肝動注(CDDP, FU) vs 無治療	75% vs 25%(3yr)	19% vs 12.5%(2yr)	有効(生存延長)
Zhong	2009	115	StageIII	肝動注 vs 無治療	22.8% vs 17.5%	9.3% vs 1.7%	有効
Peng	2009	126	Child A, 3個以内, Vp4	肝動注(FU, DXR) vs 無治療	21.5% vs 8.5%	ND	有効(生存延長)

TT : tumor thrombus IM : intrahepatic metastasis ; ND : not described *生存期間中央値
HCFU : 1-hexylcarbamoyl-5-fluorouracil UFT : uracil-tegafur DXR : doxorubicin MMC : mitomycin C EPI : epirubicin
CDDP : cisplatin FU : fluorouracil

模臨床試験であるSHARP試験において，ソラフェニブは進行肝細胞癌の予後を有意に延長することが2008年に発表された[43]．ソラフェニブは大規模な第III相試験で肝細胞癌の生存期間を有意に延長することが示された初めての薬剤である．現在，肝細胞癌根治療法後の再発予防としてソラフェニブを投与するという大規模臨床試験が症例登録を終了し，結果が期待される(STORM trial)．

その他の補助療法として，Takayamaらは肝細胞癌患者150例に対して肝切除術後の養子免疫療法に関するランダム化比較試験を行い，再発率を有意に抑制したと報告している[44]．またレチノイドが二次発癌を抑制することが示され，小規模なRCTではあるが術後補助療法として有効性が報告されている[45]．Lauらは放射性同位元素を用いて ^{131}I-リピオドールを経肝動脈的に投与し，無再発生存率，生存率ともに有意に延長したと報告している[46]．しかしいずれも単一施設からの少数例の報告であり，推奨されるには至っていない．ビタミンKが二次発癌を抑制するとの報告があり，RCTが行われたが有意な再発抑制効果を証明できなかった[47]．