(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

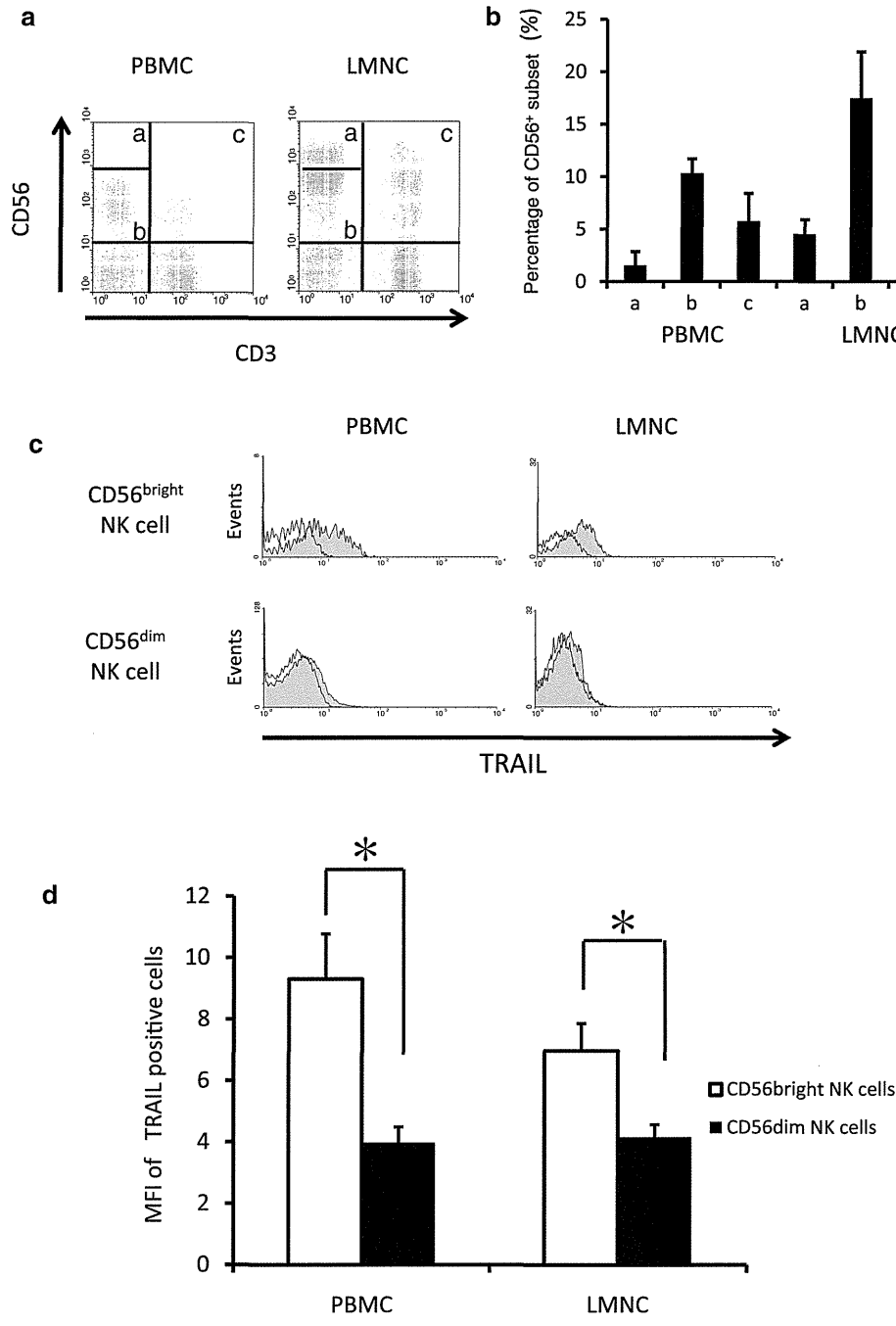


Fig. 1 The proportions of $CD56^{+}CD3^{-}$ NK and $CD56^{+}CD3^{+}$ NKT cells in LMNCs extracted from liver perfusates were significantly higher than those in PBMCs. **a** Flow cytometric (FCM) analyses of freshly isolated LMNCs obtained from liver perfusates and PBMCs from the corresponding donors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

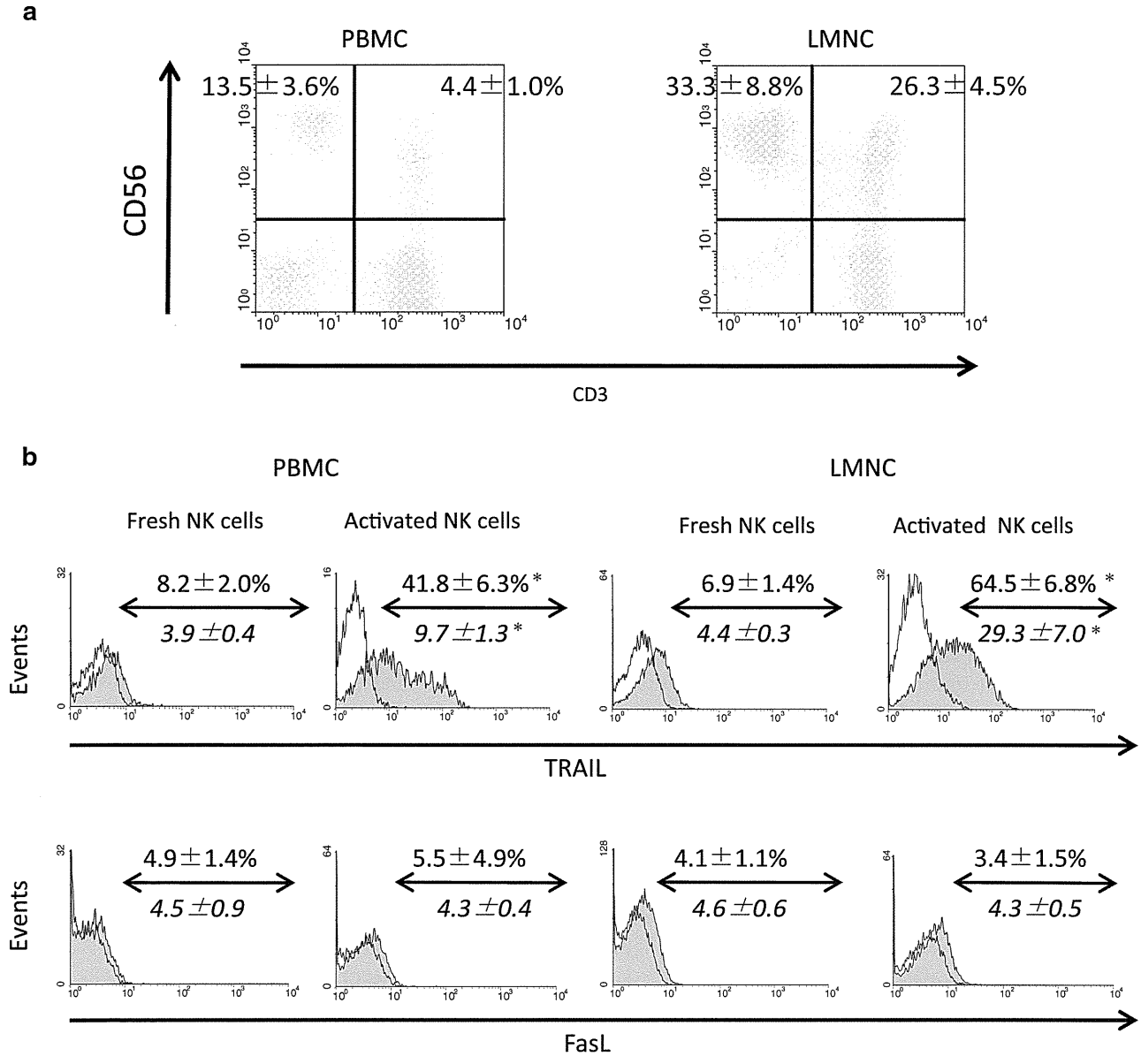


Fig. 2 Phenotypic analyses of human PB and liver NK cells. **a** Flow cytometric (FCM) analyses of PBMCs and LMNCs cultivated with IL-2 for 5 days were performed after staining with mAbs against CD3 and CD56. FCM profiles are representative of five and six independent experiments (using PBMCs and LMNCs,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

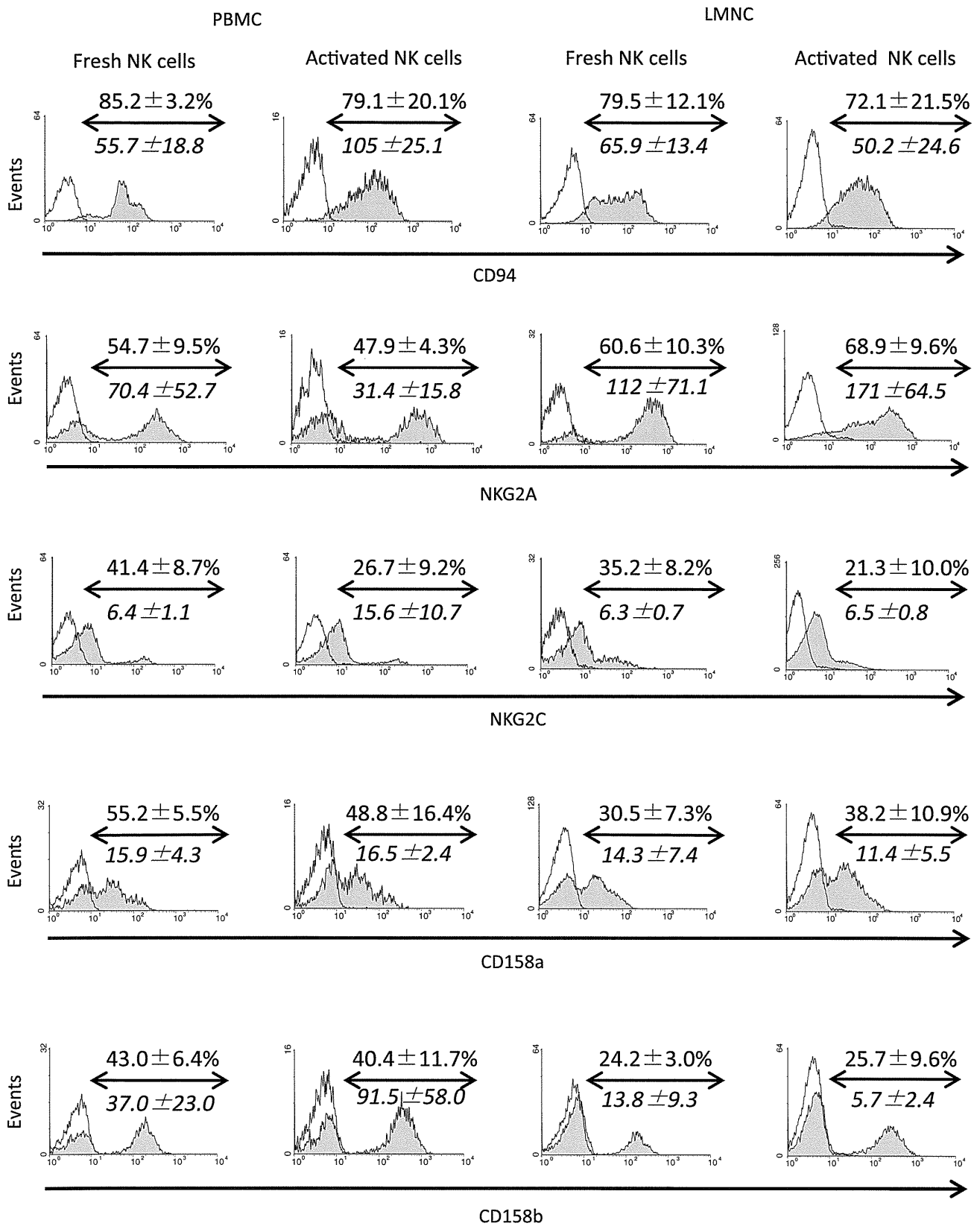
Phenotypic properties of human NK cells

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

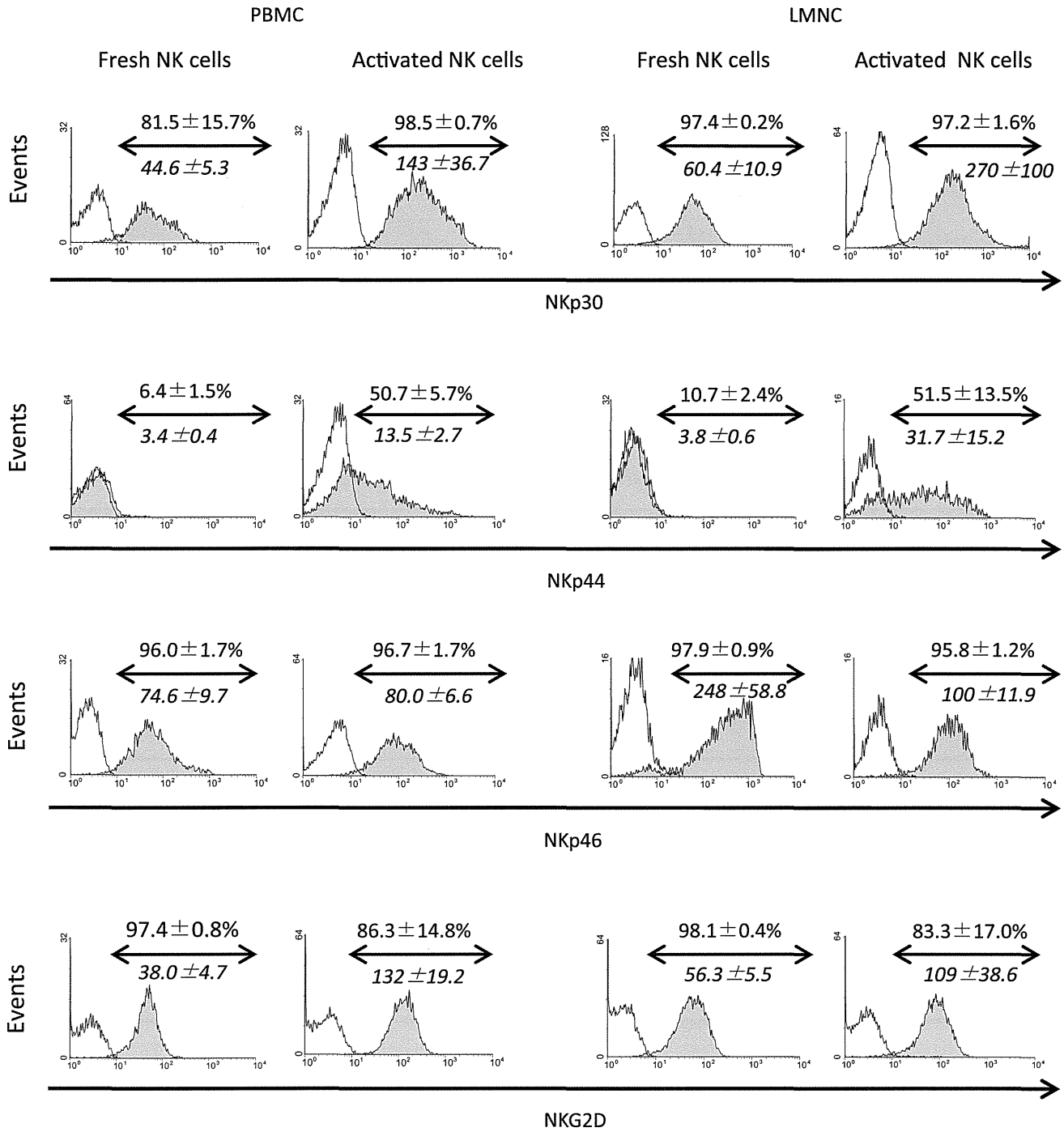


Fig. 4 IL-2 stimulation led to elevated expression of NKp30, NKp44, and NKG2D in both human PB and liver NK cells. Expression of the cytotoxicity-associated receptors, including NKp30, NKp44, NKp46, and NKG2D, in NK cell subsets among LMNCs or PBMCs freshly isolated or cultivated with or without IL-2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

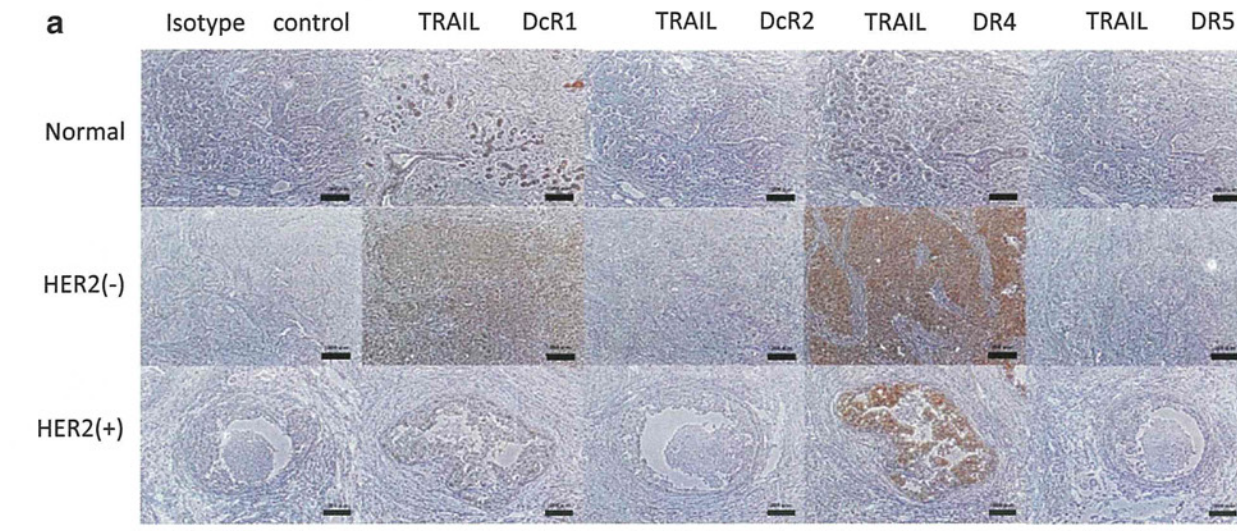
To determine the contribution of TRAIL to the cytotoxicity of NK cells against breast cancer cells, the effect of a neutralizing anti-TRAIL mAb was examined in a cytotoxicity assay with PB and liver NK cells as effectors and MDA-MB231 cells as the target. Both PB and liver NK cell-induced cytotoxicity 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].

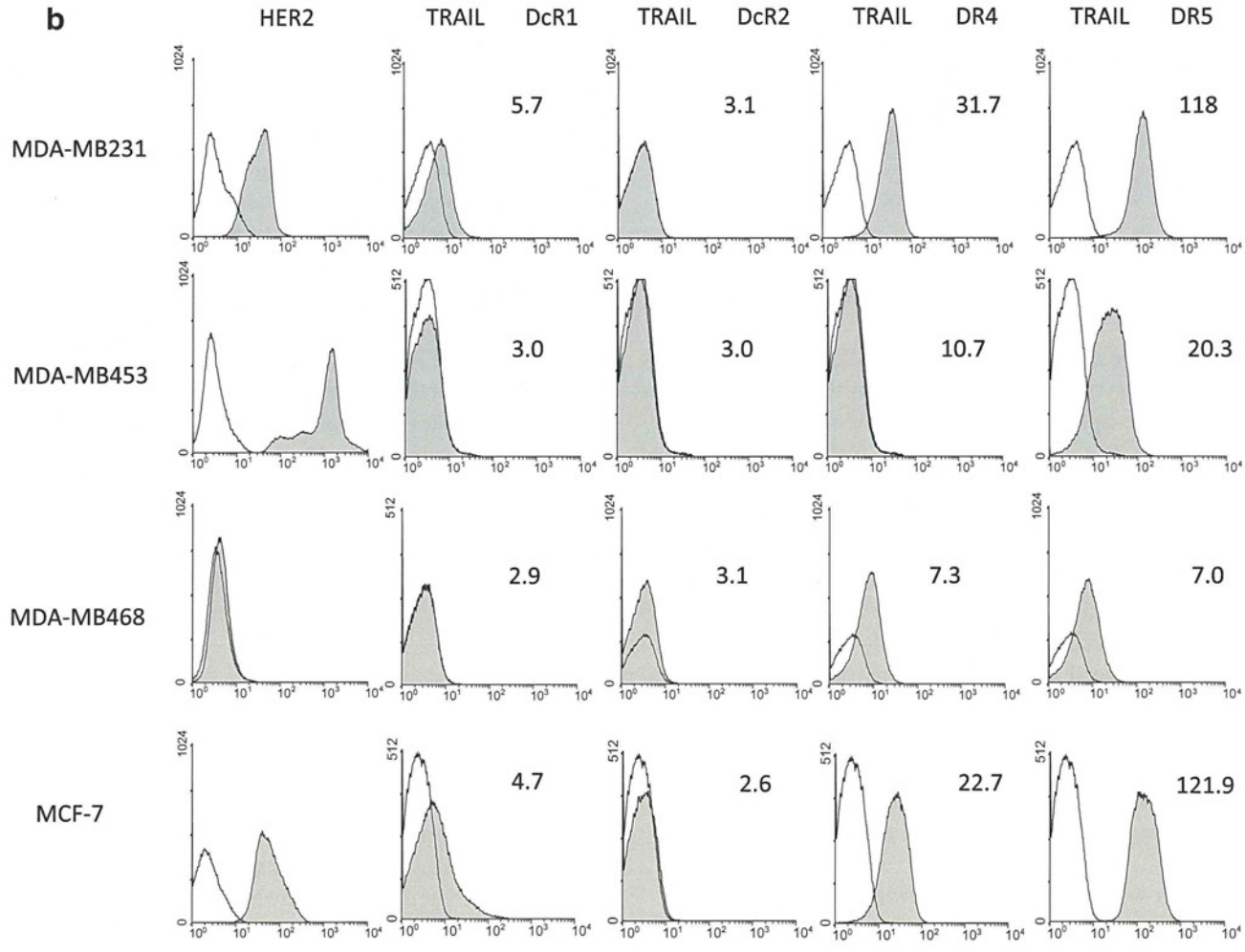
NK cells were preferentially drawn by chemokines secreted from breast cancer cells, presumably through the CXCL10/CXCR3 axis

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
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

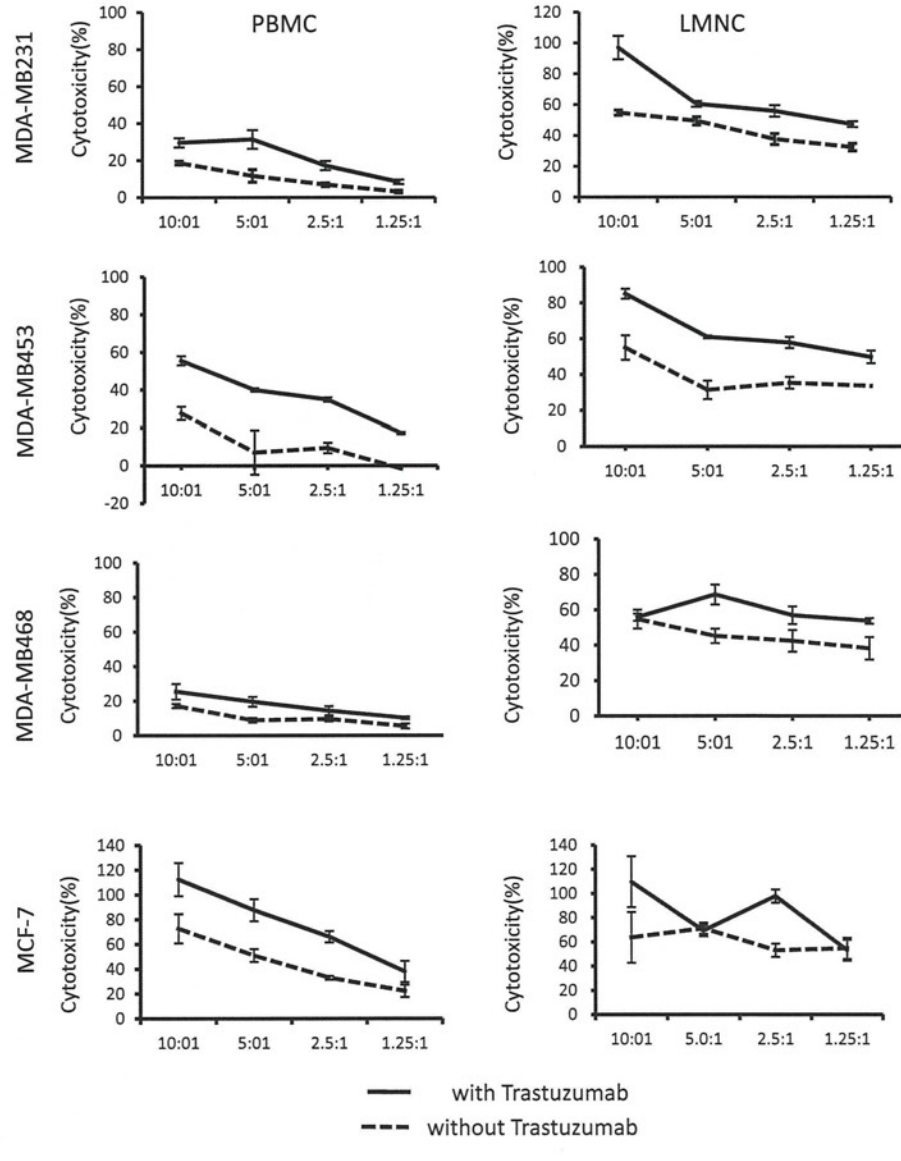


Fig. 6 Liver NK cells showed more vigorous cytotoxicity against breast cancer cell lines compared with PB NK cells. Cytotoxic activities of NK cells isolated from IL-2-stimulated PBMCs and LMNCs with or without trastuzumab against target cells (MDA-MB231, MDA-MB453, MDA-MB468, and MCF-7) were analyzed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

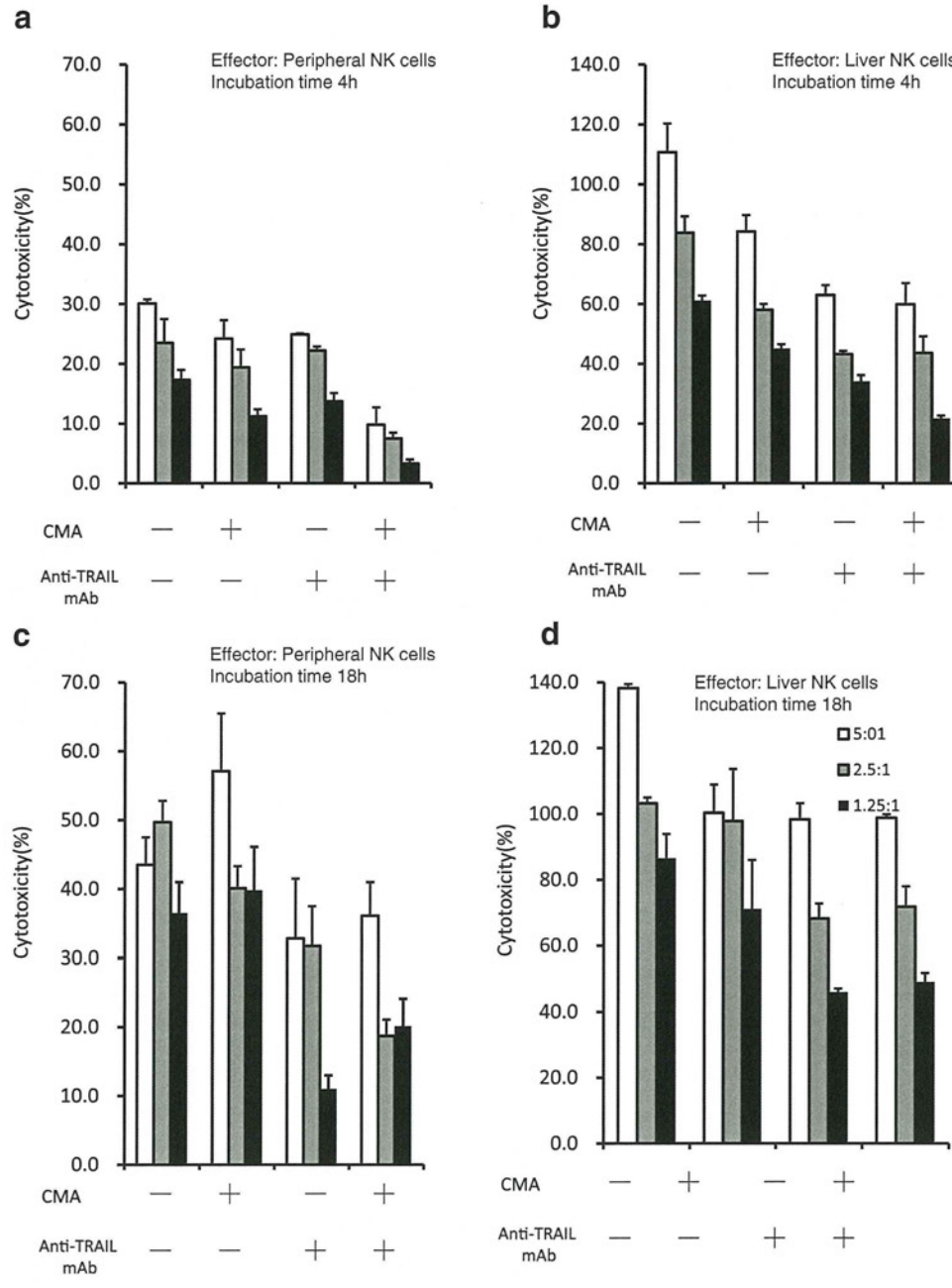


Fig. 7 NK cell-induced cytotoxicity was inhibited partially by the anti-TRAIL mAb alone and more profoundly by the combination of anti-TRAIL mAb and CMA. Isolated PB and liver NK cell populations were used as effector cells (*E*) in assays of cytotoxicity against the target (*T*) MDA-MB231 human breast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

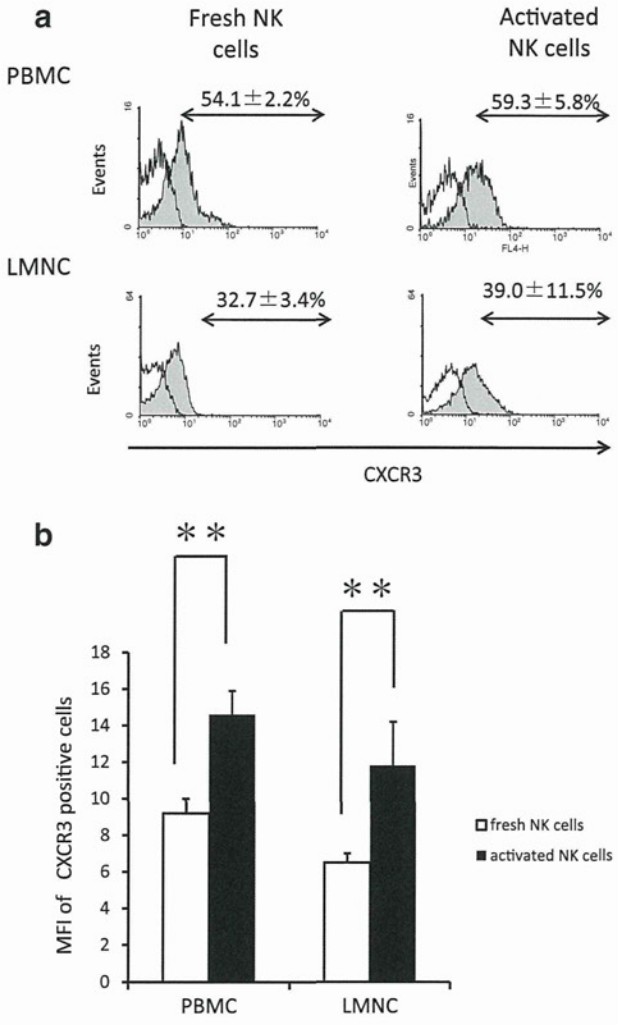


Fig. 8 CXC chemokine receptor 3 (CXCR3) expression was significantly upregulated on both liver and PB NK cells after cultivation with IL-2. **a** *Histograms* representing the log fluorescence intensities for CXCR3 expression on freshly isolated and IL-2-stimulated liver and PB NK cells. *Dotted lines*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

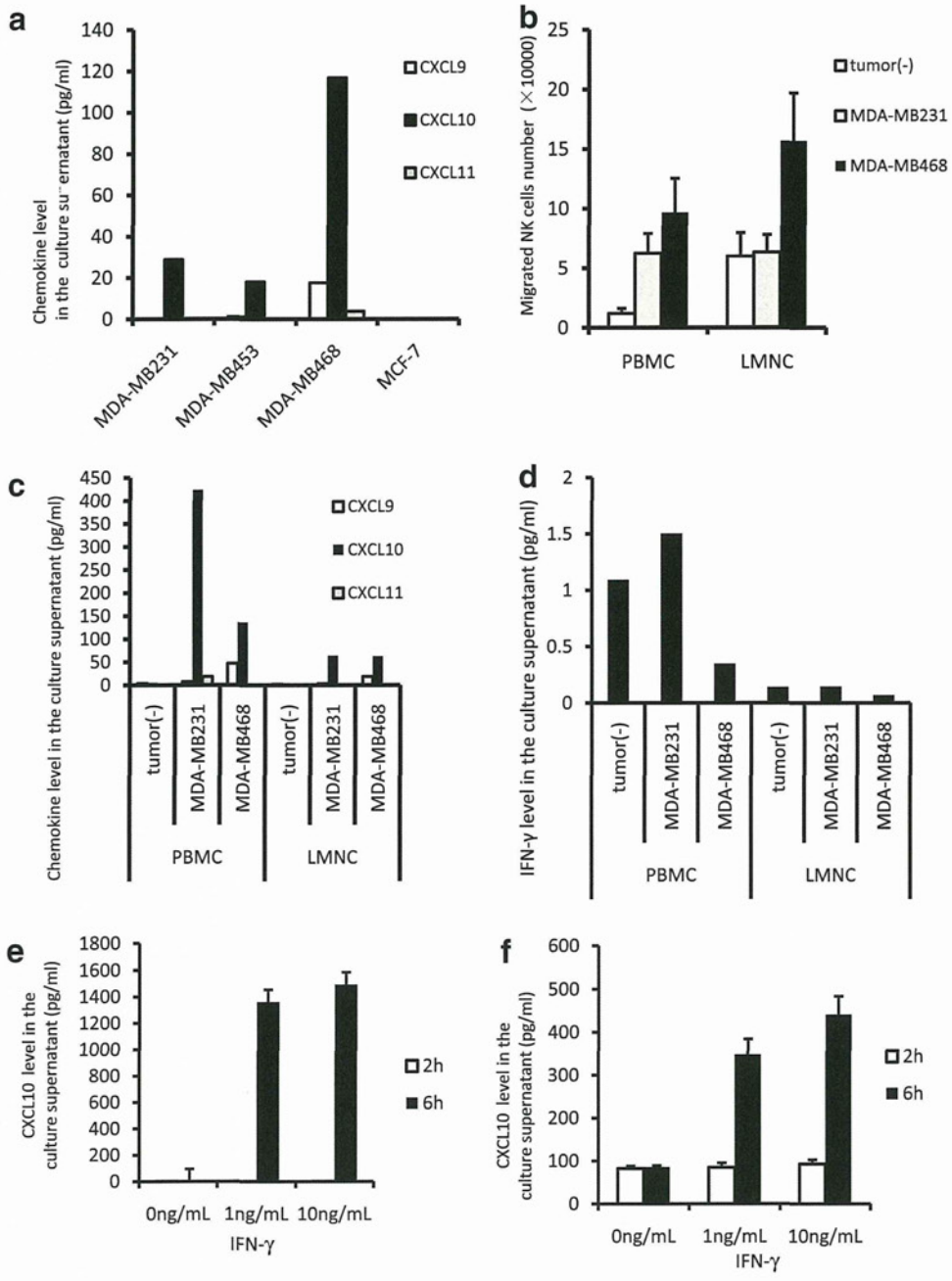


Fig. 9 NK cells were preferentially drawn by chemokines secreted from breast cancer cells, presumably through the CXCL10/CXCR3 axis. **a** Levels of various chemokines (CXCL9, CXCL10, and CXCL11) in the culture supernatants of breast cancer cell lines (MDA-MB231, MDA-MB453, MDA-MB468, and MCF-7) were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

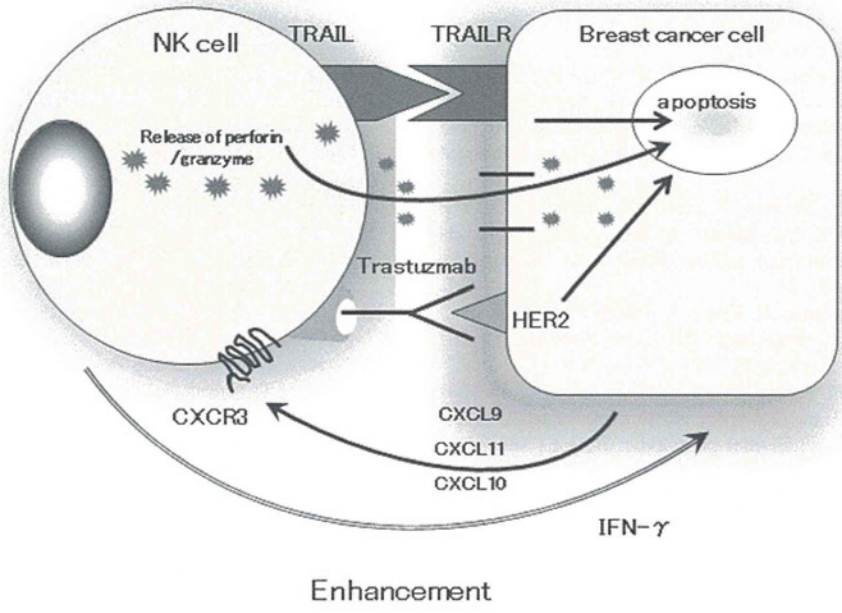


Fig. 10 Mechanistic paradigm of interaction between NK and breast cancer cells. IFN-γ secreted from NK cells promotes the production of CXCL10 from breast cancer cells, which in turn further accelerates migration of CXCR3-expressing NK cells into the tumor site. Migrated NK cells kill breast cancer cells 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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REVIEW ARTICLE

Prevention of recurrence after curative treatment for hepatocellular carcinoma

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Abstract Hepatocellular carcinoma often recurs even after curative treatment. In addition to its high frequency of metastasis, hepatocellular carcinoma recurrence is characterized by multicentric carcinogenesis arising in the liver damaged by viral infection with the hepatitis B or hepatitis C virus. This is considered to complicate the initial treatment and recurrence prevention strategy for hepatocellular carcinoma, and accordingly, there is no established adjuvant therapy to prevent recurrence. Preventive adjuvant therapy should be administered to high-risk patients, and should be optimized based on individual risk factors. This review will summarize the current status and future prospects of preventive therapy for the recurrence of hepatocellular carcinoma after curative treatment. Although transcatheter arterial embolization/chemoembolization prior to curative treatment can induce tumor necrosis in some patients, several studies have failed to show any improvement in survival. Postoperative interferon therapy may contribute to prolonging the survival in specific groups of patients. No established adjuvant therapy against advanced hepatocellular carcinoma that prevents metastasis has been established so far. Novel treatment strategies incorporating molecular and immunological mechanisms are expected in the future.

Keywords Hepatocellular carcinoma · Adjuvant therapy · Recurrence

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Introduction

Hepatocellular carcinoma (HCC) often recurs even after curative treatment. In addition to its high frequency of metastasis, HCC recurrence is characterized by multicentric carcinogenesis arising in the liver damaged by infections with hepatitis B virus (HBV) and hepatitis C virus (HCV). This is considered to complicate the initial treatment and recurrence prevention strategies for HCC.

According to the Clinical Practice Guidelines for Hepatocellular Carcinoma—The Japan Society of Hepatology 2009 update, the choice of treatment for HCC is based on three factors: the degree of liver damage, the number of tumors and the tumor diameter [1]. Treatments include liver resection, local ablation therapy, transcatheter arterial embolization/chemoembolization (TAE/TACE), hepatic arterial infusion chemotherapy, liver transplantation and palliative care. Among these treatments, liver resection, local ablation therapy and liver transplantation are considered to be curative treatments. However, HCC frequently recurs even after curative treatment, and there is no established adjuvant therapy to prevent recurrence. Such preventive adjuvant therapy should be administered to high-risk patients, and the administration of such treatments should be optimized based on individual risk factors. This review will summarize the current status and future prospects of preventive therapy for HCC recurrence after curative treatment.

Risk factors for the recurrence of hepatocellular carcinoma

The risk factors for HCC recurrence after curative treatment include the tumor stage, vascular invasion, number of tumors, tumor size, capsular formation and liver function [1, 2].

Among these factors, the stage, vascular invasion, number of tumors, tumor size and capsular formation are considered to be related to metastasis. Liver function, however, is likely to be primarily related to the multicentric nature of carcinogenesis. The strategy selected for adjuvant therapy should be based on an individual's risk factors; patients at a high risk of metastasis are good candidates for metastasis-preventing therapies. In high-risk patients with multicentric carcinogenesis, such as those with HCV- or HBV-positive HCC, preventive therapy should include aggressive antiviral therapy.

The vascular invasion and the degree of tumor differentiation play important roles in tumor recurrence after liver transplantation in patients with HCC [2]. Liver transplantation simultaneously treats both the background liver disease and liver tumors; therefore, factors related to the background liver disease are not likely to influence recurrence after transplantation. Cases of early recurrence after transplantation are often caused by the progression of pre-existing micro-metastases, or the implantation of tumor cells circulating in the peripheral blood. Therefore, an effective preventive strategy should focus on providing antitumor effects against these remaining cells. In addition, after liver transplantation, patients must be treated with immunosuppressive agents. Accordingly, it is important to take preventive measures against the reactivation of hepatitis viruses in hepatitis virus-infected patients.

Adjuvant therapy prior to curative treatment

Transcatheter arterial embolization/chemoembolization (TAE/TACE) is a procedure wherein embolic material is introduced into the hepatic artery with or without an antitumor agent. This treatment has been administered to patients with unresectable HCC. Two randomized controlled trials (RCT) demonstrated that TAE/TACE improved the antitumor effect and survival rate compared with conservative treatment in patients with unresectable HCC [3, 4]. TAE/TACE is recommended for patients with advanced hepatocellular carcinoma, which is inoperable, and who are not candidates for local ablation therapy in the Clinical Practice Guidelines for Hepatocellular Carcinoma—The Japan Society of Hepatology 2009 update [1]. TAE/TACE is also administered as a preoperative chemotherapy for resectable HCC prior to hepatic resection or local ablative therapy. More recently, it has been administered prior to liver transplantation.

TAE/TACE prior to hepatic resection

A number of studies have reported inconsistent results on the effect of TAE/TACE prior to hepatic resection. Some reports suggest that TAE/TACE improves the prognosis, while others do not. To date, three RCTs have been reported, and none has shown an improvement in the recurrence-free survival, which was the primary endpoint (Table 1) [5–7]. Although the trials differed in terms of their patient inclusion criteria and embolization methods, the results have been uniformly negative.

TAE/TACE prior to local ablative therapy

The Clinical Practice Guidelines for Hepatocellular Carcinoma—The Japan Society of Hepatology 2009 update recommend that good candidates for local ablation therapy are patients with liver function graded Child–Pugh class A or B, and three or fewer tumors measuring 3 cm or less in diameter [1]. Conventionally, percutaneous ethanol injection (PEI) has been administered; more recently, radiofrequency ablation (RFA) has become popular. However, the

Table 1 Randomized clinical trials about TAE/TACE before curative treatment for hepatocellular carcinoma

References	Cases	Inclusion criteria	Regimen	Treatment	5-year survival (vs. control)	5-year disease-free survival (vs. control)	Result
Wu et al. [5]	52	Larger than 10 cm	TACE (EPI)	RES	40 vs. 50 % (3 years)	32 vs. 60 % (3 years)	Not effective
Yamasaki et al. [6]	97	Solitary, 2 to 5 cm	TAE	RES	62.7 vs. 61.7 %	39.1 vs. 31.1 %	Not effective
Koda et al. [8]	52	Smaller than 3 cm	TACE (EPI)	PEI	40.4 vs. 37.7 %	19.3 vs. 80.1 %[a] (3 years)	Effective (recurrence)
Akamatsu et al. [9]	42	Uninodular	TAE	PEI, RFA	82.4 vs. 82.2 % (3 years)	33.8 vs. 34.3 % (3 years)	Not effective
Zhou et al. [7]	108	Larger than 5 cm	TACE (CDDP, MMC, FU)	RES	30.7 vs. 21.1 %	12.8 vs. 8.9 %	Not effective

TAE transcathether arterial embolization, *TACE* transcathether arterial chemo-embolization, *EPI* epirubicin, *CDDP* cisplatin, *MMC* mitomycin C, *FU* fluorouracil, *RES* resection, *PEI* percutaneous ethanol injection, *RFA* radiofrequency ablation

[a] Recurrence rates

rate of local recurrence after these procedures is high. The use of local ablative therapy preceded by TAE/TACE has been compared to local ablative therapy alone in two RCTs (Table 1) [8, 9]. One RCT compared combination therapy with PEI alone, and the other compared combination therapy with PEI or RFA alone. The results of these studies showed that combination therapy significantly reduced the local recurrence compared with PEI alone, but not with the RFA alone [9]. Neither of the studies found an improvement in the survival rate; therefore, this approach is not recommended in the various treatment guidelines [1, 2, 10].

TAE/TACE prior to liver transplantation

It is not yet clear whether preoperative preventive therapy in patients with HCC improves their prognosis after liver transplantation [11]. Several studies have investigated whether preoperative TACE reduces the recurrence of HCC after liver transplantation, but these were retrospective studies, and no RCT has been reported. Decaens et al. [12] reported no significant difference in the 5-year survival rates (59.4 vs. 59.3 %) and 5-year disease-free survival rates (69.3 vs. 64.1 %) between patients treated with preoperative TACE ($n = 100$) and those without adjuvant therapy ($n = 100$) in a multicenter case–control study. Although other studies have shown a favorable prognosis after transplantation in patients who responded well to TACE, and have demonstrated the efficacy of preoperative TACE in reducing the dropout rate while patients were waiting for liver transplantation, there is no evidence demonstrating that TACE improves the overall or recurrence-free survival [13–15].

Adjuvant therapy after curative treatment

Antiviral therapy in patients with viral hepatitis after curative treatment

Virus eradication by interferon therapy is effective against chronic hepatitis C and compensated cirrhosis type C. Because patients with HCC frequently have viral hepatitis, interferon therapy is administered after curative treatment to eradicate the virus and to repress inflammation. Eight RCTs have investigated the effectiveness of interferon therapy after curative treatment of HCC (Table 2) [16–23]. Shiratori et al. administered PEI to 74 HCV-positive HCC patients with three or fewer tumors; 49 of these patients were subsequently treated with interferon. Interferon therapy did not change the rate of recurrence, but significantly improved the survival [18]. On the other hand, Mazzaferro et al. [21] performed hepatic resection in 150 HCV-positive patients with HCC; 76 of these patients were subsequently treated with interferon. The 5-year recurrence-free survival rate in those treated with interferon (24.3 %) was not significantly different from those not treated with interferon (5.8 %). In summary, one of the RCTs found interferon to be effective in reducing recurrence and improving survival after the curative treatment for HCC, while the other showed a limited effect of interferon in a selected subgroup. However, several meta-analyses of multiple studies have shown that interferon therapy reduced the recurrence and prolonged the survival rate [24–29].

Although interferon was used alone in these studies, the current standard of care for treating chronic hepatitis C is the use of pegylated-interferon in combination with

Table 2 Randomized clinical trials about adjuvant interferon therapy after curative treatment for hepatocellular carcinoma

References	Cases	Inclusion criteria	IFN	Treatment	5-year survival (vs. control)	5-year recurrence rates (vs. control)	Result
Ikeda et al. [16]	20	HCV	IFN beta	RES, PEI	ND	0 vs. 100 % (2 years)	Effective (recurrence)
Kubo et al. [17]	30	HCV	IFN alpha	RES	ND	ND	Effective (survival)
Shiratori et al. [18]	74	HCV	IFN alpha	PEI	68 vs. 48 %	80 vs. 92 %	Effective (survival)
Lin et al. [19]	30	HBV, HCV	IFN alpha	PAI	ND	47 vs. 90 % (4 years)	Effective (recurrence)
Sun et al. [20]	236	HBV	IFN alpha	RES	63.8 vs. 38.8 m[a]	31.2 vs. 17.7 m[b]	Effective (survival)
Mazzaferro et al. [21]	150	HCV	IFN alpha	RES	63.6 vs. 52.4 %	24.3 vs. 5.8 %[c]	Not effective
Lo et al. [22]	80	HBV	IFN alpha	RES	79 vs. 61 %	ND	Not effective
Chen et al. [23]	268	HBV, HCV	IFN alpha	RES	75.4 vs. 72.5 %	42.7 vs. 45.5 %[c]	Not effective

HCV hepatitis C virus, *HBV* hepatitis B virus, *IFN* interferon, *RES* resection, *PEI* percutaneous ethanol injection, *PAI* percutaneous acetic acid injection, *ND* not described

[a] Median survival time

[b] Median disease-free survival time

[c] Recurrence-free survival rates

ribavirin. This combination therapy has demonstrated a higher rate of sustained virological response (SVR) than interferon monotherapy. Some studies have also evaluated its effectiveness as an adjuvant therapy after curative treatment for HCC [30, 31]. Our recent report showed that postoperative administration of pegylated-interferon plus ribavirin in patients with HCV-positive HCC resulted in a 5-year survival rate of 91.7 %, which was significantly higher than that of the historical control group (50.6 %) [31]. In addition, two recent RCTs have investigated telaprevir, an HCV genotype 1 protease inhibitor. The ADVANCE trial investigated the effect of adding telaprevir to the pegylated-interferon plus ribavirin combination therapy (PR group) in 1,088 untreated genotype 1 hepatitis C patients [32]. Telaprevir was used with the combination therapy for either 12 weeks (T12PR) or 8 weeks (T8PR). The SVR was 44 % in the PR group, compared with 75 and 69 % in the T12PR and T8PR groups, respectively. This demonstrates a significant additive effect of the combination therapy. The REALIZE trial investigated the effect of adding telaprevir to pegylated-interferon plus ribavirin combination therapy (PR group) in 663 treated genotype 1 hepatitis C patients [33]. The telaprevir combination therapy was used either for 12 weeks (T12PR) or for 12 weeks after an initial 4 weeks of PR and was followed by 32 weeks of PR (lead-in T12PR48). The SVR was 17 % in the PR group as compared with 64 and 66 % in the T12PR and lead-in T12PR48 groups, respectively, demonstrating a significant improvement. Based on these positive outcomes, telaprevir combination therapy is likely to become a standard therapy used for HCV genotype 1 hepatitis. Although telaprevir is not recommended for elderly patients, patients with thrombocytopenia or with low hemoglobin, this drug is expected to be useful as an adjuvant therapy for selected patients after curative treatment for HCC.

Nucleoside analogues are effective against hepatitis B. The results of an RCT demonstrated that lamivudine suppressed carcinogenesis arising from hepatitis B [34]. Nucleoside analogues suppress the replication of HBV, repress inflammation and reverse liver fibrosis. Although nucleoside analogues are expected to be used an adjuvant therapy in patients with hepatitis B virus, no prospective randomized studies have demonstrated its efficacy. It may be difficult to perform an RCT with or without nucleoside analogues after curative treatment for HCC, because these drugs are already recommended for patients with HBV, especially patients with a high viral load.

Adjuvant chemotherapy after curative treatment

HCC is generally insensitive to anti-cancer drugs, and the response rate to systemic chemotherapy against unresectable advanced HCC is less than 20 %. In many cases, HCC develops on a background of chronic liver disease; accordingly, worsening liver function can lead to insufficient dosing or a deteriorated prognosis. Until the introduction of sorafenib, there was no standard therapy with proven efficacy for unresectable HCC [35]. Hepatic arterial infusion chemotherapy is considered to result in a high local concentration and have fewer adverse systemic effects, because the systemic concentration of the anti-cancer drug is reduced. A number of small RCTs have investigated the use of various adjuvant chemotherapies for reducing the rates of metastasis and recurrence after curative treatment of HCC (Table 3) [36–47]. Oral, intravenous and intrahepatic arterial administration routes have all been investigated individually or in combination. Although two previous RCTs have reported that neither 1-hexylcarbamoyl-5-fluorouracil nor uracil-tegafur could reduce the recurrence of HCC, a recent small RCT reported that oral administration of capecitabine reduced the recurrence rate [36–38]. There is currently no evidence that intravenous chemotherapy is effective. The most frequent route of administration is intrahepatic arterial administration, the efficacy of which was demonstrated in a meta-analysis [48]. However, the included studies were single center experiences, with a small number of patients, and incorporated various treatment regimens. As such, there is no established evidence for a single treatment protocol. Still, some RCTs showed reduced recurrence of HCC with portal vein tumors and improved survival in a population with limited inclusion criteria [39, 45–47]. Because HCC is considered to progress via the portal vein, and patients with complicating portal vein tumors have an increased risk of metastasis and recurrence, hepatic arterial infusion chemotherapy is considered to be important in these patients. Therefore, multicenter studies appropriately designed to target patients with a high risk of metastasis are needed for further investigations.

Sorafenib is a molecule that selectively suppresses receptor tyrosine kinases, including the VEGF receptor and PDGF receptor, as well as the serine/threonine kinase Raf in the MAP kinase cascade [49, 50]. In 2008, the SHARP trial, a large multicenter trial conducted primarily in the US and Europe demonstrated that sorafenib significantly prolonged the survival in patients with advanced hepatocellular carcinoma [35]. Sorafenib is the first agent proven to improve the survival in HCC patients in a large phase III trial. A recent, large clinical trial investigated the efficacy of sorafenib for preventing recurrence after curative therapy for HCC; the results are forthcoming (STORM trial).

Other studies have investigated various adjuvant therapies. In a randomized study, Takayama et al. [51] demonstrated that adoptive immunotherapy after hepatic resection significantly reduced the recurrence rate in 150

Table 3 Randomized clinical trials about postoperative chemotherapy after curative resection for hepatocellular carcinoma

References	Cases	Inclusion criteria	Regimen	5-year survival (vs. control)	5-year disease-free survival (vs. control)	Result
Oral regimens						
Yamamoto et al. [36]	76	Stage II	HCFU vs. observation	ND	ND	Effective (clinical stage I)
Hasegawa et al. [37]	159	Child A/B, without VI	UFT vs. observation	58 vs. 73 %	29 vs. 29 %	Not effective
Xia et al. [38]	60	Child A, within three number of tumors, without VI	Capecitabine vs. observation	62.5 vs. 39.8 %	46.7 vs. 23.3 %	Effective (DFS)
Intravenous or intra-arterial regimens						
Izumi et al. [39]	50	With VI and/or IM	Intra-arterial DXR and MMC vs. observation	50.3 vs. 28.8 %	25.6 vs. 5.9 % (4 years)	Effective (DFS)
Kohno et al. [40]	88	No residual disease	Oral UFT and intra-arterial EPI vs. oral UFT	30 vs. 35 %	17 vs. 14 %	Not effective
Ono et al. [41]	57	Child A or B	Intra-arterial and intravenous EPI and oral HCFU vs. observation	31.5 vs. 57.1 %	32.0 vs. 22.5 %	Not effective
Lai et al. [42]	66	No residual disease	Intra-arterial CDDP and intravenous EPI vs. observation	ND	18 vs. 48 %	Worse outcome
Kwok et al. [43]	40	Child A or B	Intra-arterial CDDP 4 dose vs. 1 dose	40 vs. 55 % (3 years)	40 vs. 44 % (3 years)	Not effective
Shuqun et al. [44]	57	No residual disease	Intra-arterial CBDCA, EPI and MMC with/without thymosin α1 vs. observation	10 vs. 7 vs. 8 m[a]	7 vs. 5 vs. 4 m[a]	Effective
Tanaka et al. [45]	15	With VI and/or IM	Intra-arterial CDDP and FU vs. observation	75 vs. 25 % (3 years)	19 vs. 12.5 % (2 years)	Effective (survival)
Zhong et al. [46]	115	Stage III A, Child A or B	Intra-arterial CBDCA, EPI and MMC vs. observation	22.8 vs. 17.5 %	9.3 vs. 1.7 %	Effective
Peng et al. [47]	126	With VI, within three number of tumors, Child A or B	Intra-arterial FU and DXR vs. observation	21.5 vs. 8.5 %	ND	Effective (survival)

VI vascular invasion, *IM* intrahepatic metastasis, *ND* not described, *MST* median survival time, *HCFU* 1-hexylcarbamoyl-5-fluorouracil, *UFT* uracil-tegafur, *DXR* doxorubicin, *MMC* mitomycin C, *EPI* epirubicin, *CDDP* cisplatin, *FU* fluorouracil, *CBDCA* carboplatin

[a] Median survival time