Adoptive Transfer of TRAIL-Expressing Natural Killer Cells Prevents Recurrence of Hepatocellular Carcinoma After Partial Hepatectomy

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Background. Antitumor activity of the liver natural killer (NK) cells reportedly decreases after partial hepatectomy, suggesting that patients with such depressed immune status are susceptible to the recurrence of hepatocellular carcinoma (HCC). We hypothesize that adoptive immunotherapy using activated NK cells can be a novel strategy to improve the depressed immune status in patients with HCC after hepatectomy or partial liver transplantation. In the present study, we have tested this hypothesis by using a mouse model.

Methods. Intraportal injection of $1–5\times10^6$ Hepa1-6 cells (hepatoma cell line) did not result in liver metastases in untreated B6 mice, but led to the growth of liver metastases after extensive partial hepatectomy. Utilizing this murine HCC metastasis model, we investigated the antitumor activity of both remnant liver and exogenously transferred NK cells.

Results. The anti-HCC activity of liver NK cells significantly decreased after partial hepatectomy. The expression of CD69 and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) on liver NK cells was temporarily downregulated. The adoptive transfer of NK cells, including a TRAIL-expressing fraction, extracted from the liver perfusates of poly I:C-stimulated B6 mice inhibited the growth of liver metastasis in B6 or (B6×BALB/c) F1 (B6CF1) mice that underwent hepatectomy and received intraportal Hepa1-6 injection.

Conclusions. These findings indicate that adoptive immunotherapy using activated NK cells extracted from normal liver perfusates may be a novel technique for reconstituting the depressed immune status in cases of living donor liver transplantation involving HCC patients, recipients of a partial liver graft.

Keywords: NK cells, Hepatocellular carcinoma, Partial hepatectomy, Living donor liver transplantation.

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Natural killer (NK) cells are believed to constitute the first line of defense against invading infectious microbes and neoplastic cells by exerting an effector function independent of priming (*1*, *2*). Given the efficacy of NK cells in selectively killing abnormal cells, a variety of approaches have been considered when attempting selective augmentation of NK cell response to tumors (*3–6*).

We have recently determined the functional properties of peripheral blood (PB) NK cells and liver NK cells extracted from liver perfusates of donors and recipients in clinical living donor liver transplantation (*7*). Donor liver NK cells showed maximum vigorous cytotoxicity against a hepatocellular carcinoma (HCC) cell line after in vitro interleukin (IL)-2 stimulation as compared to the donor PB NK cells and recipient liver NK cells. IL-2 stimulation increased tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) expression on liver NK cells; this was critical for mounting an NK cell-mediated antitumor response without affecting normal cells. These findings lead to a novel concept for preventing HCC recurrence after living donor liver transplantation, i.e., the adoptive transfer of IL-2-stimulated NK cells extracted from the donor liver graft into recipients sharing at least one haplotype.

Antitumor activity of liver NK cells reportedly decreases after partial hepatectomy, suggesting that patients with such depressed immune status are susceptible to HCC recurrence after partial hepatectomy or partial liver transplantation (*8–10*). Therefore, adoptive immunotherapy using activated NK cells probably has potential as a strategy to reconstitute the depressed immune status in HCC patients after partial liver transplantation. Here, using mice as a

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model, we investigated the influence of partial hepatectomy on NK cell activity against HCC and the potential of adoptively transferring activated NK cells to prevent HCC recurrence after partial hepatectomy.

MATERIALS AND METHODS

Mice

C57BL/6J (B6) (H-2^b) mice and BALB/c (H-2^d) mice were purchased from CLEA Japan, Inc. (Osaka, Japan). (B6×BALB/c) F1 (B6CF1) (H-2^b×H-2^d) mice were bred in the animal facility at Hiroshima University. The mice were used when they reached an age between eight and 12 weeks. All experiments were performed according to the guidelines of the NIH Guide for the Care and Use of Laboratory Animals.

Partial Hepatectomy of Mice

Mice were anesthetized by an intraperitoneal injection of ketamine/xylazine. The large median lobe of the liver along with the left lateral lobe was securely ligated, and subsequently excised. Portions of the hepatic parenchyma ranging from 65–75% of the total liver were removed in this manner.

Isolation of Liver, Spleen, and Lung Leukocytes

We isolated leukocytes from the liver, spleen, and lung of either untreated or hepatectomized B6 mice. When indicated, liver leukocytes were isolated from B6 mice receiving an intraperitoneal injection of polyinosinic-polycytidylic acid (poly I:C; 150 μg/mouse) (Sigma, St. Louis, MO), 24 hr prior to harvesting. Poly I:C activates NK cells primarily by inducing the production of type I (α, β) IFN and IL-12 from a wide variety of cell types (*11*). Liver leukocytes were prepared as described previously (*12*). In brief, after preperfusion with 1 ml phosphate-buffered saline (PBS) supplemented with 10% heparin via the portal vein, the liver was perfused with 50 ml PBS supplemented with 0.1% ethylenediamine tetraacetic acid (EDTA), and the perfusate was collected and subjected to erythrocyte lysis, using ammonium chloride/potassium solution.

Flow Cytometry

All flow cytometric (FCM) analyses were performed on a FACS Calibur cytometer (BD Biosciences, Mountain View, CA). For phenotyping NK cell surface markers, the leukocytes were stained with the following monoclonal antibodies (mAbs) from BD Pharmingen (San Diego, CA): fluorescein isothiocyanate (FITC)-conjugated anti-NK1.1 (PK136), phycoerythrin (PE)-conjugated anti-T cell receptor (TCR)-β (H57-597), and biotin-conjugated anti-CD69 (H1.2F3) or unconjugated anti-TRAIL mAb (N2B2). Staining with unconjugated anti-TRAIL mAb was followed by staining with biotin-conjugated antirat immunoglobulin (Ig)G2a mAb (RG7/1.30). The biotinylated mAb was visualized using allophycocyanin-streptavidin (BD Pharmingen). Nonspecific FcγR binding of labeled Abs was blocked by anti-CD16/32 (2.4G2) (BD Pharmingen). Dead cells were excluded by light scatter and propidium iodide staining.

Isolation of NK Cells

Liver leukocytes were obtained from B6 mice administered an injection of poly I:C one day before harvesting. NK cells were separated by autoMACS (Miltenyi Biotec, Auburn, CA) (*13*). In brief, leukocytes were magnetically labeled using a mouse NK cell isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. TRAIL$^-$ NK cells were further magnetically sorted using unconjugated anti-TRAIL mAb, biotin-conjugated antirat IgG2a mAb, and streptavidin microbeads in the negative fraction. Whole NK, TRAIL$^-$ NK, and non-NK cells were used in the subsequent cytotoxic assay.

Hepatoma Cell Line

The mouse hepatoma cell line Hepa1-6 (derived from H-2^b mice) was purchased from RIKEN Cell Bank (Tsukuba, Japan).

Cytotoxicity Assay

The $Na_2[^{51}Cr]O_4$-labeled Hepa1-6 cells were incubated with effector cells in DMEM supplemented with 10% fetal calf serum in round-bottomed 96-well microtiter plates. As the control, target cells were incubated either in the culture medium alone to determine spontaneous release or in a mixture of 2% Nonidet P-40 to define the maximum ^{51}Cr release. The cell-free supernatants were carefully harvested, and its radioactivity was measured using a gamma counter. The percentage of specific ^{51}Cr release was calculated as percent cytotoxicity=[(cpm of experimental release – cpm of spontaneous release)]/[(cpm of maximum release – cpm of spontaneous release)]×100. All assays were performed in triplicate.

Induction of Liver Metastasis

Under anesthesia, Hepa1-6 tumor cells (5×10^5) in 0.5 ml of medium 199 (Sigma, St. Louis, MO) were injected slowly into the portal vein.

Adoptive Transfer of Activated NK Cells

Liver NK cells were obtained from B6 mice treated with poly I:C 1day before harvesting. Three days after tumor cell injection, the tumor-bearing mice were randomly assigned to the group receiving NK cell treatment or the control group receiving the medium alone. The former received 5×10^5 NK cells in 0.5 ml of medium 199 and the latter, the same volume of medium 199 alone.

Histological Evaluation of Metastatic Growth in the Liver

The mice were sacrificed seven days after tumor cell injection, and the liver was removed and fixed overnight in 10% formalin. For studies using a BZ-8000 microscope (KEYENCE, Osaka, Japan), 4-μm tissue sections from the right lobe were stained with hematoxylin-eosin (HE). The relative areas occupied by the tumors were calculated as the percentage of the total scanned liver area by using a BZ-H1M3 program, image analyzer (KEYENCE, Osaka).

Histological Assessment of Hepatocyte Proliferation

Bromodeoxyuridine (BrdU) immunostaining was performed as described previously (*14*). In brief, BrdU (Sigma Chemical Co., St Louis, MO) was administered intraperitoneally at a dose of 30 mg/kg, 60 min before sacrificing the mice to measure DNA synthesis in the regenerating liver. Deparaffinized liver sections were incubated with anti-BrdU antibody (Becton Dickinson Immunocytometry Systems, Mountain View, CA) for 60 min. Immunostaining for BrdU was performed by the avidin-biotin-immunoperoxidase method using a Vectastain ABC kit (Vector Laboratories Inc., Burlingame, CA).

Statistical Analysis

The results were analyzed by Student's *t* test. $P<0.05$ was considered statistically significant.

RESULTS

The Intensities of CD69 and TRAIL-Expression on Liver NK Cells Were Temporarily Downregulated After Extended Partial Hepatectomy

Resident leukocytes were extracted from the liver, spleen, and lung of B6 mice that were untreated or underwent partial hepatectomy. As shown in Figure 1, liver NK cells comprised cells that expressed CD69—one of the earliest expressed activating markers—at a greater frequency than spleen and lung NK cells. After three days of partial hepatectomy, the number of CD69-expressing NK cells was significantly reduced in the liver; however, the number remained constant in the spleen and lung. Thus, liver NK cells are naturally activated but extended hepatectomy probably deactivates them. Providing efficient cytotoxicity against trans-

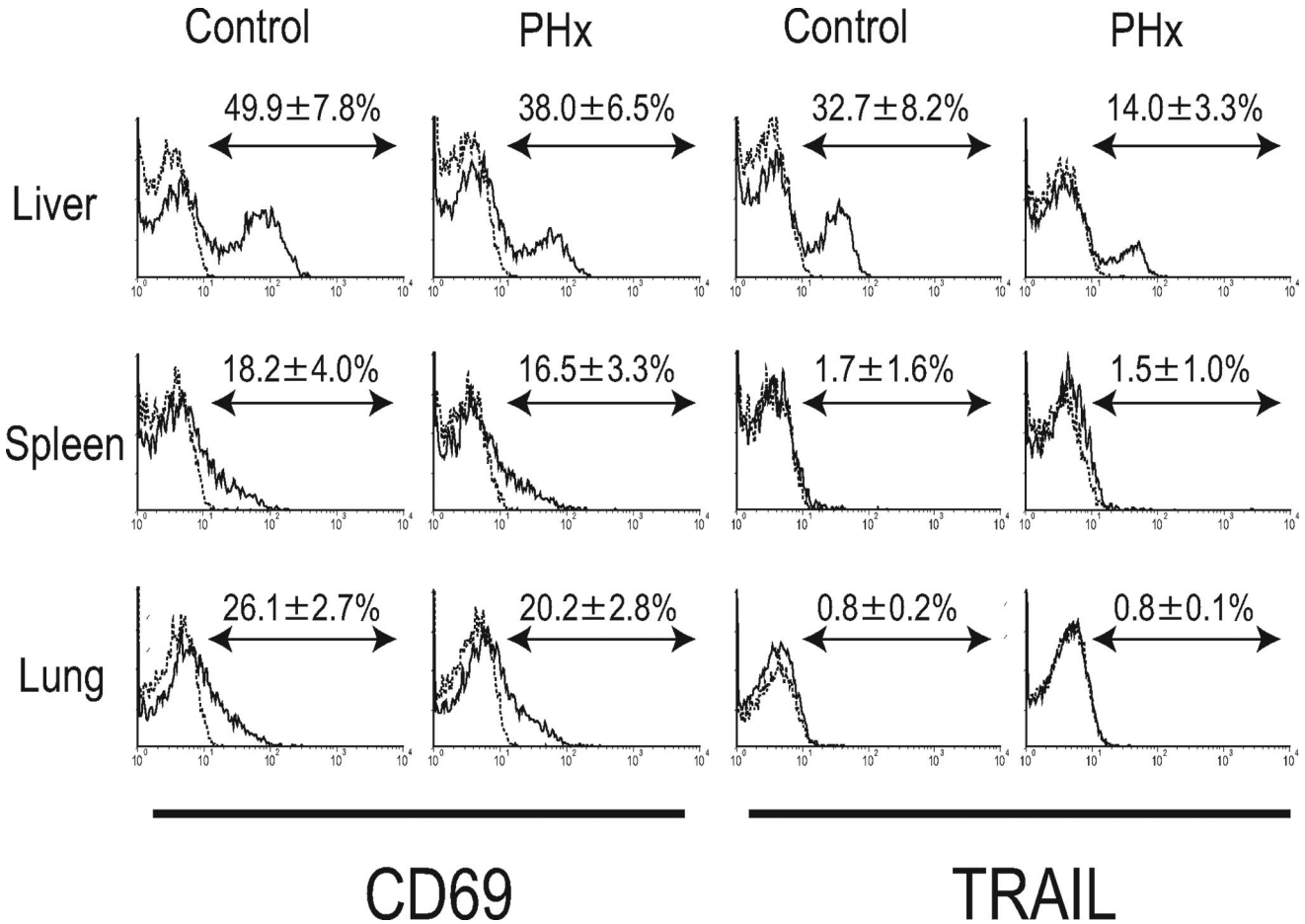

FIGURE 1. The intensities of CD69 and TRAIL-expression on liver NK cells were downregulated three days after partial hepatectomy. Liver, spleen, and lung leukocytes were isolated from untreated or partial hepatectomized B6 mice. These leukocytes were stained with FITC-conjugated anti-NK1.1, PE-conjugated anti-TCRβ, and biotin-conjugated anti-CD69 (a very early activation marker), or anti-TRAIL (and biotin-conjugated antirat IgG2a) and then with allophycocyanin-streptavidin. The expression of CD69 and TRAIL on electronically gated NK1.1$^+$TCRβ^- NK cells was analyzed by multi-color FCM (solid line). The dotted lines represent negative control staining with isotype-matched mAbs. The percentage of positive subsets is indicated (mean±SEM of values; n=17, n=13, and n=4 for the liver, spleen, and lung specimens, respectively). The absolute number of liver NK cells isolated from untreated mice and partial hepatectomized mice were 3.6×10^5 and 2.9×10^5 cells/liver, respectively.

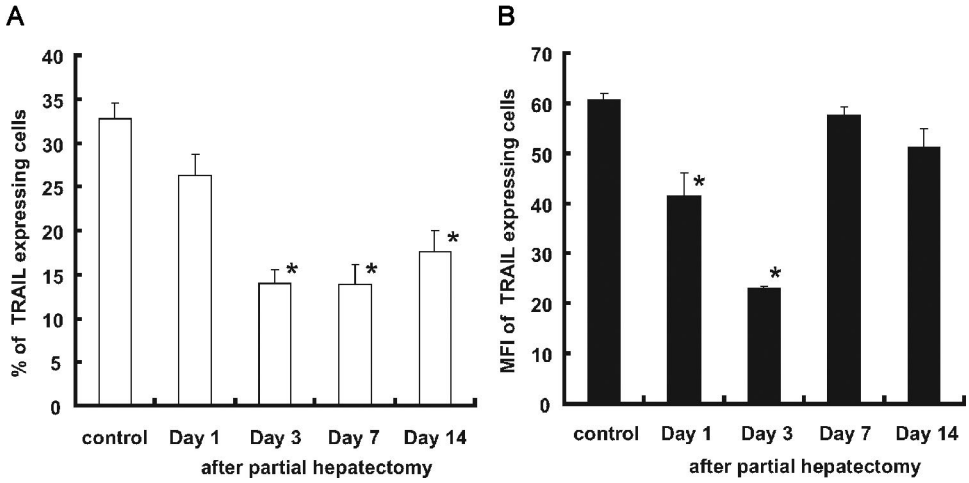

FIGURE 2. The kinetics of TRAIL-expression on liver NK cells after the hepatectomy. (A) The numbers represent the mean±SEM of the TRAIL$^+$ cell populations in the NK1.1$^+$TCRb$^-$ NK cell subsets (n=7). *$P<0.01$ for Day three, Day seven, and Day 14 vs. control. (B) The numbers indicate the mean fluorescence intensity (MFI) of cells that stained positive for TRAIL (mean±SEM, n=7). *$P<0.01$ for Day one and Day three vs. control.

formed targets is a feature of activated NK cells—a property believed to be attributable to the elevated expression of TNF family members, including the Fas ligand (FasL), and the increased production of perforin, granzymes, and cytokines by these cells (*15–19*). Among the TNF family members, TRAIL has recently been shown to be critical for NK cell-mediated antitumor functions (*20–22*). Although FasL expression was not detected on any of the liver, spleen, and lung NK cells (data not shown), approximately 30–40% of the liver NK cells constitutively expressed TRAIL molecules unlike other tissues (Fig. 1). It is noteworthy that the expression of TRAIL was markedly reduced on the liver NK cells after partial hepatectomy. The kinetics of TRAIL-expression on liver NK cells after the hepatectomy are shown in Figure 2. Both the proportion of TRAIL-expressing cells and the expression levels of TRAIL were progressively reduced until three days of hepatectomy (Fig. 2A and B). Thereafter, by two weeks after hepatectomy, these levels gradually improved. Such a change in the expression level of TRAIL on liver NK cells was well correlated with that in the cytotoxicity of liver NK cells against the Hepa1-6 hepatoma cell line (data not shown).

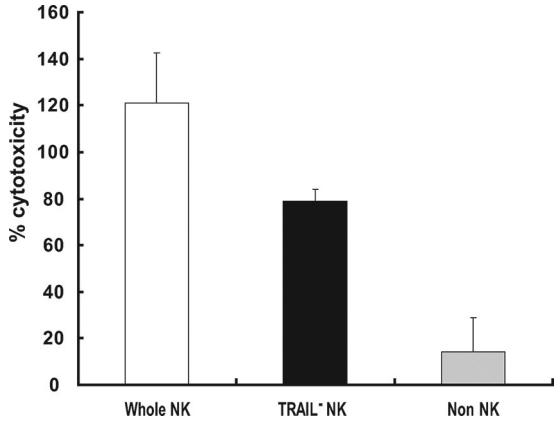

FIGURE 3. Cytotoxicity against the Hepa1-6 hepatoma cell line was mediated predominantly by TRAIL$^+$ liver NK cells. Unlabeled NK cells and non-NK cells were isolated from the liver leukocytes of poly I:C-treated B6 mice (n=25) by negative selection using magnetic sorting as described in Materials and Methods. TRAIL$^-$ NK cells were further sorted from the isolated liver NK cells. The isolated NK cell populations were used as effector cells in the assays for cytotoxicity against the indicated target cells at an E/T ratio of 20. Comparative cytotoxicity was calculated with the following formula: percent of cytotoxicity=(cytotoxicity of fractionated liver leukocytes)/(cytotoxicity of nonfractionated liver leukocytes)×100%. The data are represented as the average±SEM of values from triplicate samples.

TRAIL-Expressing Liver NK Cells Show Cytotoxicity Against the Hepatoma Cell Line

We attempted to determine whether TRAIL$^+$ NK cells mediate cytotoxicity against the Hepa1-6 hepatoma cell line. By magnetic sorting, NK cells were isolated from the liver leukocytes of poly I:C-treated B6 mice. TRAIL$^+$ and TRAIL$^-$ NK cells were further sorted from the isolated liver NK cells, and the resulting populations were then analyzed for cytotoxicity against Hepa1-6. As shown in Figure 3, liver NK cells mediated significantly higher cytotoxicity against hepatoma cells when compared to the non-NK cell fraction. The liver NK cells comprising both TRAIL$^+$ and TRAIL$^-$ cells mediated a significantly higher antitumor activity than the TRAIL$^-$ NK cells. This finding indicates that TRAIL$^+$ liver NK cells efficiently mediate cytotoxicity against the hepatoma cell line.

The Remnant Liver After Extended Partial Hepatectomy Was Susceptible to the Growth of Hepatoma Metastases

Based on our results that the number of TRAIL-expressing NK cells that provided significant antihepatoma activity decreased after extended partial hepatectomy, we assumed that partial hepatectomy promotes susceptibility to the engraftment of intraportally injected hepatoma cells. To verify this, Hepa1-6 cells were intraportally injected in either

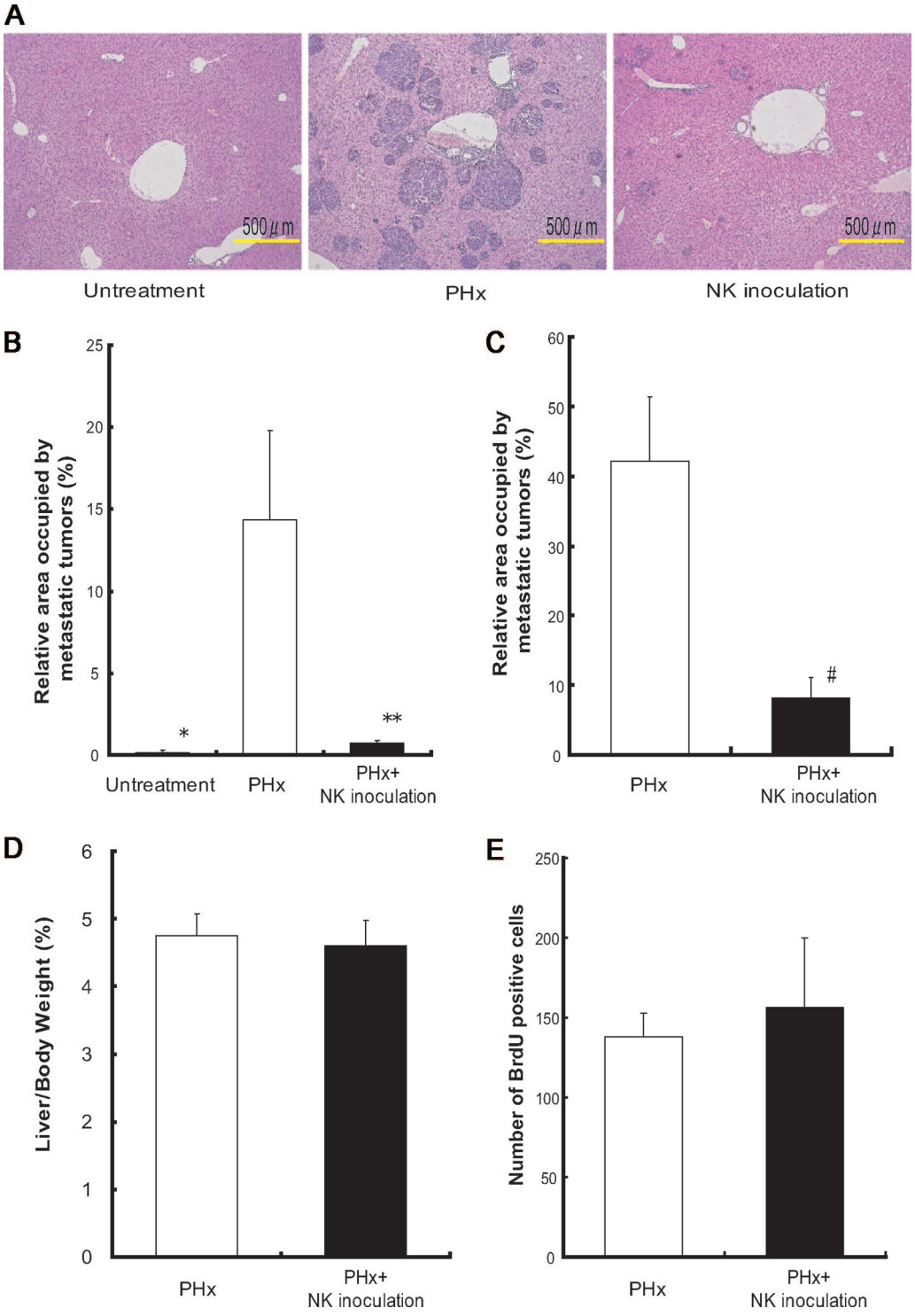

FIGURE 4. The adoptive transfer of activated liver NK cells inhibited the growth of liver metastasis induced by a portal venous injection of hepatoma cells in extensively hepatectomized mice. (A) Representative histopathological findings of the liver specimen (HE, ×4 objective). Specimens from the untreated (left), partially hepatectomized (PHx; middle), and NK cell-receiving groups were inoculated after partial hepatectomy (right). (B) The adoptive transfer of activated B6 liver NK cells inhibited the growth of liver metastasis induced by a portal venous injection of hepatoma cells in extensively hepatectomized B6 mice. B6 mice were that either underwent or did not undergo partial hepatectomy and injected with tumor cells on Day 0. On Day three, the NK inoculation group was administered an intravenous injection of 5×10^5 B6 liver NK cells. On Day seven, the relative areas occupied by the tumors were calculated as the percentage of the total liver area. Data are represented as the mean±SEM of values from four untreated mice, 12 hepatectomized mice without NK-inoculation, and nine hepatectomized mice with NK-inoculation. *$P<0.01$ for untreated mice vs. PHx mice; **$P<0.01$ for PHx mice vs. NK-inoculated mice. (C) The adoptive transfer of activated B6 liver NK cells inhibited the growth of liver metastasis induced

untreated mice or mice that underwent partial hepatectomy; the mice were then sacrificed seven days after the inoculation to evaluate metastatic growth in the liver. In the control untreated mice, limited numbers of metastatic lesions were detected in the liver, indicating their natural defensive activity against invading hepatoma cells. Even when 5×10^6 Hepa1-6 cells (10-fold higher than the number used in the present study) were intraportally injected into the untreated control mice, metastatic lesion were barely detected (data not shown). However, after extensive hepatectomy, significant growth of liver metastases was detected in the liver, suggesting the breakdown of such natural defensive activities (Fig. 4A and B).

The Adoptive Transfer of Activated Liver NK Cells Inhibited the Growth of Liver Metastasis Induced by Portal Venous Injection of Hepatoma Cells in Extensively Hepatectomized Mice

We next investigated whether the adoptive transfer of liver NK cells, including the TRAIL-expressing fraction, in the hepatectomized mice could reconstitute the defensive activities in intraportally injected hepatoma cells. After three days of administering the portal injection of Hepa1-6 cells in the hepatectomized B6 or B6CF1 mice, we intravenously administered NK cells extracted from the livers of poly I:C stimulated B6 mice (5×10^5 cells/mouse). Both B6 and B6CF1 mice receiving activated liver NK cells showed significantly suppressed levels of metastases seven days after the inoculation (Fig. 4B and C).

It has been previously speculated that the activity of liver NK cells is relevant to regenerating the activity of the liver (*13*, *23*, *24*). In the present study, however, NK cell inoculation influenced neither the liver mass to body weight ratios nor the number of BrdU-positive cells in the remnant liver seven days after the partial hepatectomy (Fig. 4D and E). This indicates that the adoptive transfer of activated liver NK cells did not prevent the regeneration of the remnant liver after partial hepatectomy.

FIGURE 4. Continued by a portal venous injection of hepatoma cells in extensively hepatectomized B6CF1 mice. B6CF1 mice were partially hepatectomized and injected with tumor cells on Day 0. On Day three, the NK-inoculation group was administered an intravenous injection of 5×10^5 B6 liver NK cells. On Day seven, the relative areas occupied by the tumors were calculated as the percentage of the total liver area. Data are represented as the mean±SEM of values from four mice without NK cell inoculation and five mice with NK cell inoculation. #$P<0.01$ for PHx mice vs. NK-inoculated mice. (D) Comparison of liver weight to body weight ratio seven days after partial hepatectomy. These values were obtained from B6 mice partially hepatectomized on Day 0 (n=4 in each group). On Day three, the NK inoculation group was administered an intravenous injection of 5×10^5 B6 liver NK cells. On Day seven, the body weight and liver weight were measured. Data are represented as the mean±SEM. There was no difference in the liver weight to body weight ratio ($P=0.75$ for untreated mice vs. NK-inoculated mice). (E) For the bromodeoxyuridine (BrdU) incorporation assay, 60 min before sacrificing the B6 mice, BrdU was intraperitoneally injected at a concentration of 30 mg/kg of the body weight. The number of cells per field that stained positive was calculated seven days after partial hepatectomy (n=4 in each group). Data are shown as mean±SEM. There was no difference in the number of BrdU-positive hepatocytes in either group ($P=0.7$ for untreated mice vs. NK-inoculated mice).

DISCUSSION

Liver transplantation is one of the few curative treatment modalities for patients with unresectable HCC (*25*, *26*). Managing the prevention of graft rejection requires the use of immunosuppressive therapy after liver transplantation; however, this therapy poses a serious problem in that immunosuppressants increase the incidence of recurrence of HCC and induce HCC progression. It has been well accepted that the immunosuppressive regimen that is currently being used after liver transplantation, which uses tacrolimus/cyclosporine and methylprednisolone and reduces adaptive components of cellular immunity (predominantly T cell-mediated immune responses) while maintaining the innate components of cellular immunity (*3*, *27*, *28*). Because immune surveillance against tumors is mediated by both innate and adaptive components of cellular immunity, augmentation of NK cell responses to tumors, which has been believed to play a central role in innate immunity against tumors, might be a promising immunotherapy approach.

Previously published data redefine NK cells as potent constitutive immune effectors; these cells can utilize not only the perforin-mediated secretory/necrotic mechanism to kill rare leukemia cell targets but also the powerful TNF family ligand-mediated nonsecretory apoptotic mechanism to destroy most solid tumor cell targets (*29*). TRAIL is highly expressed on most NK cells after stimulation with IL-2, IFNs, or IL-15 in mice (*21*, *22*, *30*). Neutralization of TRAIL additively enhanced liver metastasis in perforin-deficient mice but not in IFN-γ-deficient mice (*22*). These findings clearly indicate that the two key cytotoxic effector pathways used by NK cells are those of perforin and TRAIL. Consistently, the liver $TRAIL^+$ NK cells mediated cytotoxicity against hepatoma cells more efficiently than the liver $TRAIL^-$ NK cells in the present study (Fig. 3). These $TRAIL^+$ NK cells were temporarily but markedly reduced after extensive partial hepatectomy (Fig. 2). This might be one of the causative mechanisms for a previously reported fact that the level of cytotoxic activity against various tumor cells of liver NK cells decreased after partial hepatectomy (*8–10*).

The first step in the development of experimental liver metastases is believed to involve tumor cells mechanically trapped in the portal sinuses, where they adhere to sinusoidal endothelial cells before extravasation (*31*, *32*). Since liver NK cells are located predominantly within the sinusoidal lumen in close contact with sinusoidal endothelial cells (*33*, *34*), it is possible that intraportally injected hepatoma cells neighboring on the liver sinusoid are targeted by liver NK cell-mediated cytotoxicity. Although whether tumor cells come in contact with $TRAIL^+$ NK cells before or after extravasation remains unknown, the results from the present study suggest that $TRAIL^+$ NK cells play a critical role in preventing tumor cell invasion into the parenchyma.

It is a generally accepted fact that healthy cells are protected by their MHC class I molecules that bind to the corre-

sponding inhibitory receptors on NK cells; therefore, cells that lack or have altered MHC class I molecules are killed due to the absence of inhibitory mechanisms (*35–38*). The only requirement for NK cell receptor repertoire development appears to be that every NK cell should express at least one inhibitory receptor that is specific for autologous HLA class I, thereby ensuring tolerance against healthy cells that share one-haplotype MHC molecules (*39*, *40*). It is well known that irradiated F1 hybrid mice reject bone marrow transplants from either inbred parent. This resistance is mediated by the host NK cells. We are not certain whether a similar hybrid resistance takes place during the engraftment of NK cells in one-haplotype mismatched living liver transplantation in humans. Since the liver is replaced by a donor liver graft after transplantation, the donor-type resident NK cells in the liver cannot reject the inoculated donor-type NK cells extracted from the donor liver grafts. Considering these facts together with the results of the present study, adoptive transfer of activated NK cells extracted from the donor liver graft into one-haplotype matched recipients suffering from HCC would hold potential as a strategy for preventing HCC recurrence.

Previously, we have reported that the majority of the $TRAIL^+$ NK cells lack the expression of Ly-49 inhibitory receptors that are responsible for recognizing self-major histocompatibility complex (self-MHC) class I molecules, thereby indicating a propensity toward cells that share self-MHC class I (*13*) Poly I:C treatment significantly upregulated the expression of Ly-49 receptors on the $TRAIL^-$ NK cells. This might be a compensatory mechanism to protect the self-MHC class I-expressing cells from activated NK cell-mediated cytotoxicity. However, such a compensatory alteration in the $TRAIL^+$ NK cell fraction was not observed at all. Thus, the liver $TRAIL^+$ NK cells are less capable of self-recognition, and this might be involved in the NK cell-mediated self-hepatocyte toxicity. We assumed that a similar mechanism is involved in the $TRAIL^+$ NK cell-mediated effects against Hepa1-6 cells, which expressed syngeneic $H\text{-}2D^b$ (data not shown).

The mechanism by which the TRAIL expression of liver NK cells is down-regulated during the early phase following partial hepatectomy is currently unknown. Considering the previously reported finding that IL-12 induces TRAIL expression in the NK cells (*22*) as well as our unpublished finding that liver mRNA expression of IL-12 is significantly reduced after partial hepatectomy, it is possible that the IL-12 produced by different cells in the liver, such as dendritic cells, Kupffer cells or endothelial cells, plays a critical role in the regulation of TRAIL expression of liver NK cells. This hypothesis is currently being investigated.

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