Liver Sinusoidal Endothelial Cells Tolerize T Cells across MHC Barriers in Mice[1]

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Although livers transplanted across MHC barriers in mice are normally accepted without recipient immune suppression, the underlying mechanisms remain to be clarified. To identify the cell type that contributes to induction of such a tolerance state, we established a mixed hepatic constituent cell-lymphocyte reaction (MHLR) assay. Irradiated C57BL/6 (B6) or BALB/c mouse hepatic constituent cells (HCs) and CFSE-labeled B6 splenocytes were cocultured. In allogeneic MHLR, whole HCs did not promote T cell proliferation. When liver sinusoidal endothelial cells (LSECs) were depleted from HC stimulators, allogeneic MHLR resulted in marked proliferation of reactive $CD4^+$ and $CD8^+$ T cells. To test the tolerizing capacity of the LSECs toward alloreactive T cells, B6 splenocytes that had transmigrated through monolayers of B6, BALB/c, or SJL/j LSECs were restimulated with irradiated BALB/c splenocytes. Nonresponsiveness of T cells that had transmigrated through allogeneic BALB/c LSECs and marked proliferation of T cells transmigrated through syngeneic B6 or third-party SJL/j LSECs were observed after the restimulation. Transmigration across the Fas ligand-deficient BALB/c LSECs failed to render $CD4^+$ T cells tolerant. Thus, we demonstrate that Fas ligand expressed on naive LSECs can impart tolerogenic potential upon alloantigen recognition via the direct pathway. This presents a novel relevant mechanism of liver allograft tolerance. In conclusion, LSECs are capable of regulating a polyclonal population of T cells with direct allospecificity, and the Fas/Fas ligand pathway is involved in such LSEC-mediated T cell regulation. *The Journal of Immunology,* **2005, 175: 139–146.**

Liver allografts are extraordinarily tolerogenic, and stable grafts can be maintained without immunosuppression in some species (1–3). In addition, the presence of a liver allograft can suppress the rejection of other solid tissue grafts from the same donor (4, 5).

The high capacity of the transplanted liver to establish tolerance in an allogeneic host has been attributed to the unique features and architecture of hepatic constituent cells (HCs).[3] The abundant release of allogeneic (donor-type) MHC class I molecules from the liver into the blood (6), the emigration of mobile and surviving passenger lymphocytes and phagocytes from the donor liver (thereby creating microchimerism with a high zone tolerance effect) (7), the regulatory properties of the prevalent NKT cell subset in the liver (8), and the termination of immune responses by intrahepatic entrapment and deletion of activated T cells by resident APCs (9) are some of the multiple factors that are likely to be involved in the tolerogenicity of liver allografts. However, the details of each mechanism remain to be elucidated.

It is generally accepted that the immunogenicity of solid organ allografts is due to the presence of APCs expressing MHC class II within the grafts (10). MHC molecules on these cells are directly recognized by the host $CD4^+$ T cells, which become activated and produce various cytokines that initiate a cascade of alloimmune events. Liver dendritic cells (LDCs) have abnormal APC properties and a low expression of costimulatory molecules, and they preferentially induce Th2 responses, suggesting a dendritic cell-mediated tolerogenicity (11, 12). In contrast, Kupffer cells (KCs), which are also resident APCs in the liver, participate in rejection, rather than tolerance, induction (13).

In addition to LDCs and KCs, liver sinusoidal endothelial cells (LSECs), which constitute the lining of the hepatic sinusoid, are also able to present soluble exogenous Ags to T cells with transgenic T cell receptors (14–17). Although a number of studies have demonstrated the importance of Ag presentation by LDCs for liver allograft tolerance, the role of direct alloantigen presentation by LSECs in such tolerance has not been extensively investigated.

In the present study we investigated the tolerogenicity of LSECs in mice in which liver allografts are normally accepted without recipient immune suppression across MHC barriers. Through the use of a mixed hepatic constituent cell-lymphocyte reaction (MHLR) assay and a transendothelial migration assay, we have demonstrated a novel and surprising effect of LSECs, i.e., naive allogeneic LSECs selectively render reactive $CD4^+$ and $CD8^+$ T cells tolerant at least in part via the Fas/Fas ligand (FasL) pathway. This result provides the first demonstration that LSECs are capable of regulating a polyclonal population of T cells with certain specificity through direct Ag recognition.

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[3] Abbreviations used in this paper: HC, hepatic constituent cell; 7-AAD, 7-aminoactinomycin D; Ac-LDL, acetylated low density lipoprotein; BM, bone marrow; FasL, Fas ligand; FCM, flow cytometry; KC, Kupffer cell; LDC, liver dendritic cell; LNPC, liver nonparenchymal cell; LSEC, liver sinusoidal endothelial cell; MHLR, mixed hepatic constituent cell-lymphocyte reaction; PI, propidium iodide; SA, streptavidin; PD-L1, programmed death ligand-1.

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Materials and Methods

Animals

Eight- to 12-wk-old female wild-type C57BL/6 (B6, H-2^b), BALB/cA (BALB/c, H-2^d), and SJL/jorllco (SJL/j, H-2^s) mice were purchased from Clea Japan. Female Cpt.C3-*Tnfsf6*^{gld} mice (BALB/c-*gld*, H-2^d, BALB/cA background), which were homozygous for the *Fasl*^{gld} mutation, were purchased from The Jackson Laboratory. Age-matched (8- to 12-wk-old) mice were used for the experiments. All animals were maintained under pathogen-free conditions and in compliance with national and institutional guidelines. All protocols were approved by the Hiroshima University animal ethics committee.

Immunohistochemistry

To detect CD105 expressed on LSECs, we fixed murine liver cryosections with formaldehyde and preincubated them with normal rabbit serum to block nonspecific binding. Sections were incubated with anti-CD105-biotin mAb for 1 h at room temperature. Biotinylated mAbs were visualized by a standard avidin-biotin-alkaline phosphatase method.

Isolation of LSECs, whole HCs, and LSEC-depleted HCs

Whole HCs were isolated using the collagenase perfusion technique, as previously described (18, 19). Liver nonparenchymal cells (LNPCs) containing LSECs and KCs were separated from whole HCs by low speed centrifugation, as previously described (18). LNPCs were preincubated with FcγR-blocking mAb (2.4G2; BD Pharmingen) and incubated with anti-CD105-biotin mAb (MJ7/18; eBioscience) and streptavidin (SA) microbeads (Miltenyi Biotec). The LSECs were then isolated from LNPCs by positive selection using auto MACS (Miltenyi Biotec). The purity of LSECs was confirmed by phase contrast microscopy and flow cytometry (FCM) after culture for 12 h in collagen I-coated dishes with Bodipy-labeled acetylated low density lipoprotein (Ac-LDL). LSEC-depleted HCs were obtained directly from whole HCs by negative selection using anti-CD105-biotin mAb, as described above. The obtained whole HCs, LSECs, and LSEC-depleted HCs were used as stimulators in the MHLR.

Construction of bone marrow chimeras

Bone marrow (BM) transplants were performed from B6 mice to BALB/c mice, as previously described (20). In brief, BALB/c recipients were lethally irradiated (13 Gy) and injected with T cell-depleted BM cells (10×10^6 cells/mouse) from B6 mice. T cell depletion was performed by MACS using biotin-conjugated anti-mouse CD4 and CD8 mAbs and streptavidin-conjugated microbeads (Miltenyi Biotec). BM chimeras were used at least 90 days after the BM transplant.

In vitro MHLR

For MHLR using a [^{3}H]thymidine technique, fractions of whole HCs and LSEC-depleted HCs were prepared from B6 and BALB/c mice as described above. After each fraction had been irradiated (30 Gy) for use as stimulator cells, 4×10^6 naive splenocytes isolated from B6 mice were cocultured with 0.8×10^6 whole HCs or LSEC-depleted HCs. Cells were incubated in 24-well, flat-bottom plates (BD Labware) in culture medium at 37°C with 5% CO_2 for 5 days, including a final 12-h pulse with [^{3}H]thymidine (10 μCi/well). Cells were harvested, and the amount of [^{3}H]thymidine was measured using a scintillation counter.

For MHLR using a CFSE-labeling technique (CFSE-MHLR), naive splenocytes from B6 mice were labeled with 5 μM CFSE (Molecular Probes), as previously described (21), and resuspended in culture medium as responder cells. Fractions of whole HCs, LSEC-depleted HCs, and isolated LSECs were prepared from B6, BALB/c, and BM chimeric mice. After each fraction had been irradiated (30 Gy) for use as stimulator cells, the stimulator (0.8×10^6 cells) and responder (4×10^6 cells) cells were cocultured in 24-well, flat-bottom plates at 37°C in a 5% CO_2 incubator in the dark for 5 days. In some experiments isolated LSECs were returned to the LSEC-depleted MHLR culture wells using culture inserts that contained polycarbonate filters of 0.45 μm pore size (Kurabou) to separate LSECs from MHLR. Control inserts contained an equivalent volume of medium without isolated LSECs.

FCM analyses

The following reagents were used for surface staining: anti-CD4-PE (GK1.5), anti-CD8-PE (53-6.7), anti-CD11b-PE (M1/70), anti-I-A/I-E-PE (M5/114.15.2), anti-CD40-FITC (3/23), anti-CD80-FITC (16-10A1), anti-CD86-FITC (GL1) mAbs, annexin V-allophycocyanin, SA-PE, and SA-allophycocyanin. These were purchased from BD Pharmingen. Anti-CD105-biotin, anti-CD45-FITC (30-F11), and anti-FasL-PE (MFL3) mAbs were purchased from eBioscience. All analyses were performed on a FACSCalibur (BD Biosciences). Nonspecific FcγR binding of labeled mAbs was blocked by 2.4G2. Dead cells were excluded from the analysis by forward scatter and propidium iodide (PI). To detect T cell apoptosis in some experiments, annexin V-allophycocyanin and 7-aminoactinomycin D (7-AAD) (BD Pharmingen) stainings were performed in accordance with the manufacturer's instructions.

Quantification of T cell proliferation

Stimulation indexes as alloreactivities of responder $CD4^+$ and $CD8^+$ T cells were quantified by their CFSE fluorescence intensities, modified as previously described (22, 23). On CFSE fluorescence histograms, $CD4^+$ and $CD8^+$ T cells were selected by gating and were analyzed for CFSE fluorescence. Theoretically, the CFSE fluorescence intensity of cells that have divided once shows half the value of CFSE fluorescence intensity of nondivided cells. According to this theory, the number of divisions of alloreactive T cells could be mathematically determined by the logarithmic CFSE intensities on the basis of the peak at the extreme right (the peak of undivided cells). The limit of detection is eight division cycles caused by the compression of peaks as the CFSE intensity approaches autofluorescence levels. Thus, the numbers of divisions beyond seven cycles are indistinguishable and are collectively referred to as division 7+. A single cell dividing n times will generate 2^n daughter cells. Using this mathematical relationship, the number of division precursors was extrapolated from the number of daughter cells of each division, and mitotic events were calculated in each $CD4^+$ and $CD8^+$ T cell subset. Using these values, mitotic indexes were calculated by dividing the total mitotic events by the total precursors. Stimulation indexes of allogeneic combinations were calculated by dividing the mitotic indexes of allogeneic combination by those of the control syngeneic combination.

Transendothelial migration assay

The transmigration experiments were conducted as follows. LSECs (1×10^5 cells/200 μl) were grown on culture inserts containing polycarbonate filters, with a pore size of 8 μm (Costar), precoated with 100 μl of recombinant human fibronectin (50 μg/ml; Sigma-Aldrich), and left overnight to form a monolayer. Monolayers were rinsed, and the inserts were transferred into wells of 24-well plates (800 μl/well). CFSE-labeled, nonadherent lymphocytes (10×10^6 cells/200 μl) from B6 mice were added to each insert and were left for 12 h to migrate through the monolayer. The migrated lymphocytes were subsequently collected from the lower chambers. Control inserts contained an equivalent number of lymphocytes. These transmigrated B6 lymphocytes were cocultured with irradiated (30 Gy) BALB/c splenocytes in a total volume of 2 ml of medium in 24-well, flat-bottom plates (BD Biosciences) for 5 days (subsequent MLR).

Statistical analysis

Statistical analysis among experimental groups was performed by ANOVA, and Tukey's test was used to compare individual groups. A value of $p < 0.05$ was considered statistically significant.

Results

Naive LSECs constitutively express all molecules necessary for Ag presentation, but do not induce proliferation of allogeneic T cells

To characterize the phenotype of naive LSECs in mice, we first developed a method to isolate LSECs. As observed by immunohistochemical studies, LSECs highly express CD105 molecules (endoglin), which have been used as a marker for vessel endothelia (24, 25), compared with the endothelia of central veins or other vessels in the liver (Fig. 1*A*). Therefore, $CD105^+$ cells were positively selected by MACS to isolate LSECs from the LNPC fraction of disaggregated HCs. The mean yield of $CD105^+$ sorted cells from a mouse liver was $3.4 \times 10^6 \pm 8.5 \times 10^5$ cells ($n = 10$). The appearance of $CD105^+$ sorted cells was consistent with that of LSECs (Fig. 1*B*). To analyze the purity of LSECs, we cultured aliquots of the sorted fractions in the presence of Ac-LDL-Bodipy (this fluorescence-labeled lipoprotein is exclusively taken up by endothelial cells, such as LSECs). The $CD105^+$ sorted cells always contained >95% of $CD11b^-$ cells that had taken up Ac-LDL-Bodipy, which represented the LSECs, and were not contaminated with CD45 (leukocyte common Ag)-positive cells

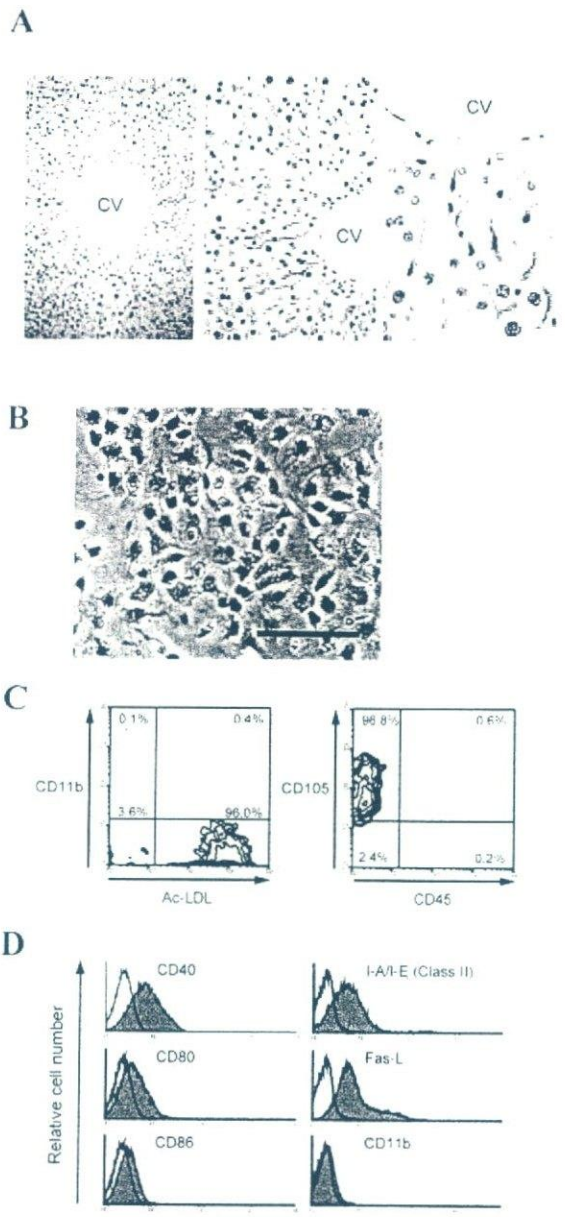

FIGURE 1. LSECs exclusively express CD105 molecules and all molecules necessary for Ag presentation. *A*, CD105 molecule expression on LSECs in normal murine livers (original magnification: *left*, ×100; *middle*, ×200; *right*, ×400). Cryosections of normal murine liver were immunostained with anti-CD105 mAb (red). In normal liver tissue, LSECs express CD105 molecules, whereas endothelia of central veins do not. CV, central vein. *B*, Phase contrast image of $CD105^+$ LNPCs separated from whole HCs by MACS. $CD105^+$ LNPCs were cultured on collagen-coated dishes to confirm morphological character. The cells are characterized by their polygonal shape and cobblestone appearance. Scale bar indicates 100 μm. *C*, Representative FCM results of sorted $CD105^+$ LNPCs. Sorted $CD105^+$ LNPCs were stained for the expression of CD45 as a marker for hemopoietic cells (*right panel*), were cultured and analyzed for purity in the presence of Ac-LDL-Bodipy (this fluorescence-labeled lipoprotein is exclusively taken up by endothelial cells such as LSECs), and were stained for the expression of CD11b as a marker for Kupffer cells (*left panel*). The percentages shown are of total sorted $CD105^+$ LNPCs. The FCM profiles shown are representative of two independent experiments. *D*, Freshly isolated LSECs constitutively express all molecules necessary for Ag presentation and FasL. ■, Staining with anti-CD40, anti-CD80, anti-CD86, anti-I-A/I-E, anti-FasL, or anti-CD11b mAbs; □, staining with isotype-matched control Abs. Dead cells were excluded from the analysis by forward scatter and PI staining. The FCM profiles shown are representative of three independent experiments.

(Fig. 1*C*). The $CD105^+$ cells that were freshly isolated from BALB/c mice expressed MHC class II, CD40, CD80, and CD86; these are surface molecules necessary for the efficient Ag presentation to T cells (Fig. 1*D*). Notably, these cells also expressed FasL. To define the immunological properties of LSECs in liver allografts, we performed a one-way MLR assay using irradiated LSECs as stimulators and splenocytes labeled with the intracellular fluorescent dye, CFSE, as responders in either syngeneic (B6 vs B6) or allogeneic (BALB/c vs B6) combinations. Despite the phenotypical properties of LSECs as APCs, the LSECs failed to induce proliferation of both allogeneic $CD4^+$ and $CD8^+$ T cells (Fig. 2).

LSECs inhibit proliferation, whereas hepatic BM-derived cells promote proliferation of alloreactive T cells

The inability of LSECs to induce proliferation of allogeneic T cells might be merely due to their poor alloantigen-presenting capacity or might be a consequence of their tolerogenic capacity. To address these possibilities, we established an MHLR assay in which irradiated HCs and CFSE-labeled splenocytes were cocultured. In the syngeneic combination (B6 vs B6), T cell proliferation was undetectable, as expected (Fig. 3, *A* and *E*). Even in the fully allogeneic combination (BALB/c vs B6), the MHLR assay revealed minimal proliferation of both allogeneic $CD4^+$ and $CD8^+$ T cells (Fig. 3, *B* and *E*). When LSECs were depleted from whole HC stimulators, the MHLR assay resulted in a marked proliferation of both alloreactive $CD4^+$ and $CD8^+$ T cells (Fig. 3, *B* and *E*), indicating that LSECs might have the capacity to inhibit the proliferation of alloreactive T cells. Consistent results were observed in an MHLR assay using the [^{3}H]thymidine incorporation technique, although this conventional method did not allow phenotypic analysis of proliferating T cells and was less sensitive than the CFSE-MHLR (26) (Fig. 3*C*). These results also indicate that an immunogenic population promoting alloimmune responses should be included in the LSEC-depleted HC fraction. To investigate this issue, we created BM chimeras by lethally irradiating BALB/c mice ($H\text{-}2^d$) and reconstituting them with BM from B6 mice ($H\text{-}2^b$). Thus, we refer to BALB/c mice receiving B6 BM as BALB/c(B6). The chimeric mouse livers expressed $H\text{-}2^d$ on tissue parenchyma and endothelial cells and expressed $H\text{-}2^b$ on hemopoietic resident cells. Even in the case where LSECs were depleted from whole HCs, the MHLR assay, using BALB/c(B6) HCs as stimulators and B6 splenocytes as responders, resulted in limited proliferation of both alloreactive $CD4^+$ and $CD8^+$ T cells (Fig. 3, *D*

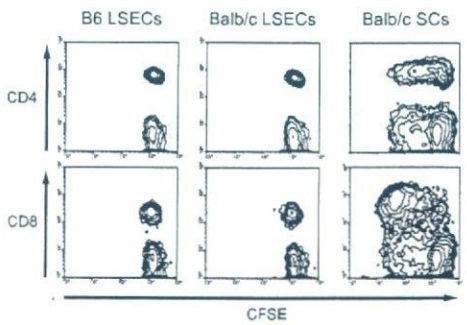

FIGURE 2. LSECs failed to induce the proliferation of allogeneic T cells. Representative FCM results of CFSE-labeled $CD4^+$ and $CD8^+$ T cell division in the MLR using isolated LSECs as stimulator and splenocytes as responder. CFSE-labeled splenocytes from B6 mice were cocultured with irradiated (30 Gy) splenocytes (SCs) from BALB/c mice (positive control) or irradiated LSECs from either B6 or BALB/c mice for 5 days. Using FCM analysis following MLR, the proliferation of reactive T cells can be visualized as the serial halving of the CFSE intensity. The FCM profiles shown are representative of three independent experiments.

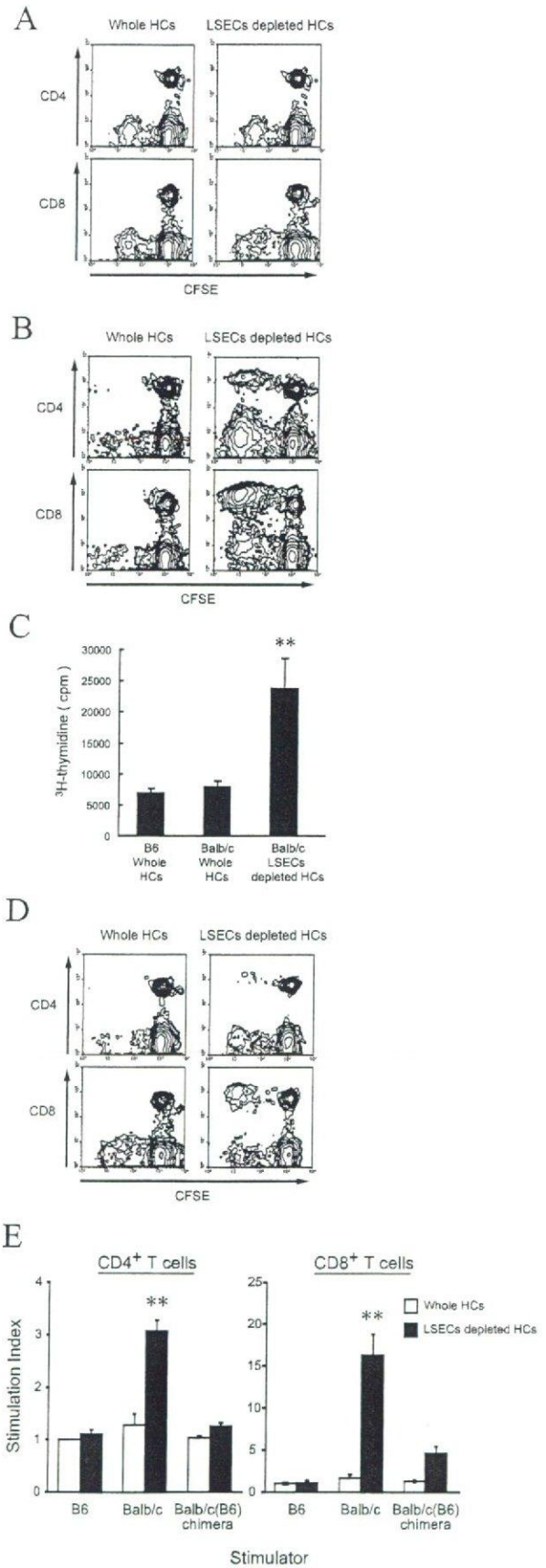

FIGURE 3. LSECs inhibit the proliferation of alloreactive T cells, whereas hepatic BM-derived cells promote their proliferation. *A* and *B*, Representative FCM results of the MHLR using CFSE-labeled splenocytes from B6 mice as responders and irradiated whole HCs or LSEC-depleted HCs from B6 (*A*) and BALB/c (*B*) as stimulators are shown. *C*, Mean proliferation ([^{3}H]thymidine incorporation) of splenocytes isolated from B6 in MHLR are shown. Whole HCs (n = 4) from B6 (syngeneic) mice and whole HCs (n = 4) or LSEC-depleted HCs (n = 4) from BALB/c (allogeneic) mice as stimulators were used. [^{3}H]Thymidine incorporation of splenocytes from B6 mice was estimated with a scintillation counter as described in *Materials and Methods*. The mean ± SEM of four independent experiments are shown. **, $p < 0.01$ vs all groups. *D*, Representative FCM results of the MHLR using CFSE-labeled splenocytes from B6 mice as responders and irradiated whole HCs or LSEC-depleted HCs from BALB/c(B6) BM chimeric mice as stimulators are shown. LSECs were depleted from whole HCs by magnetic cell sorting, as described in *Materials and Methods*. *E*, Stimulation indexes of alloreactive T cells in MHLR are shown. Whole HCs (□; n = 8) or LSEC-depleted HCs (■; n = 8) from B6, BALB/c, or BALB/c(B6) BM chimeric mice as stimulators were used. Stimulation indexes of alloreactive T cells were estimated by FCM analysis of CFSE intensity, as described in *Materials and Methods*. The mean ± SEM of eight independent experiments are shown. **, $p < 0.01$ vs all groups.

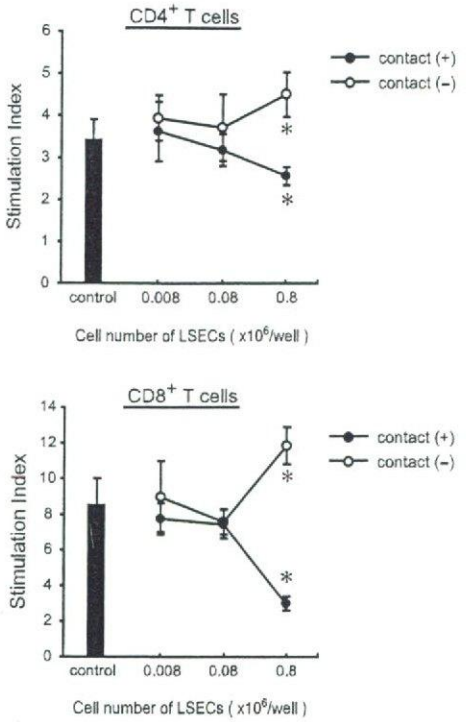

FIGURE 4. LSECs inhibit the proliferation of alloreactive T cells, and cell-cell contact is necessary for their inhibitory effects. Stimulation indexes of alloreactive T cells are shown. CFSE-labeled splenocytes from B6 mice were cocultured with irradiated LSEC-depleted HCs from BALB/c mice in the absence (control; ■) or the presence of increasing numbers of LSECs from BALB/c mice (○ and ●). Isolated LSECs were returned to allogeneic LSEC-depleted MHLR with (●; n = 5) or without (○; n = 5) cell contact. The mean ± SEM of five independent experiments are shown. *, $p < 0.05$ vs control.

and *E*), indicating that BM-derived cells are an alloimmune-stimulating population in liver allografts.

LSECs inhibit proliferation of alloreactive T cells by cell-cell contact

To determine whether cell-cell contact is necessary for the inhibitory effects of LSECs on the proliferation of alloreactive T cells,

we returned the isolated LSECs to the LSEC-depleted MHLR culture wells with or without cell-cell contact using a Transwell culture system. When cell-cell contact occurred, LSECs inhibited the proliferation of alloreactive $CD4^+$ and $CD8^+$ T cells, whereas the physical separation of LSECs from the responder and stimulator cells in the MHLR abrogated the LSEC-induced inhibitory effects on alloreactive T cell proliferation (Fig. 4). Even in the presence of cell-cell contact, when MHC class II on the isolated LSECs were blocked with anti-I-A/I-E mAb before the MHLR culture, LSEC-induced inhibitory effects on alloreactive T cell proliferation were also abrogated (data not shown). These results indicate that the alloreactive T cells are tolerized via direct recognition of MHC class II expressed on LSECs. Interestingly, the enhanced proliferation of both $CD4^+$ T cells and $CD8^+$ T cells was observed in the contact-independent condition compared with LSEC-depleted conditions (Fig. 4). It has been previously reported that LSECs have the capacity to produce proinflammatory cytokines and chemokines, including IL-1, IL-6, TNF-α, and interferon-γ inducible protein-10 (27–29), which can promote T cell activation/proliferation. Such soluble mediators that can pass through the Transwell membrane may enhance the proliferation of alloreactive T cells in the absence of contact-dependent, LSEC-induced inhibition.

LSECs induced apoptosis of alloreactive T cells in the allogeneic MHLR

Several groups have reported that T cells undergo apoptosis in liver allografts (30–32). Based on this fact and the FasL expression on LSECs (Fig. 1*D*), we assumed that LSECs inhibited alloreactive T cell proliferation via apoptosis. To investigate this possibility, the cells harvested after allogeneic MHLR culture, using whole HC stimulators, including LSECs, were stained with annexin V to detect apoptotic cells along with 7-AAD to exclude necrotic cells (33, 34). Four-color FCM revealed that the proliferation of 7-AAD-negative alloreactive $CD4^+$ and $CD8^+$ T cells was detectable (Fig. 5). These proliferating T cells during the early stage of cell division were bound to annexin V with a higher frequency compared with the alloreactive T cells proliferating in response to LSEC-depleted HCs (Fig. 5, *A* and *B*). These findings are consistent with a model in which the apoptosis of alloreactive T cells activated in response to LSECs is a critical event in the induction of liver allograft tolerance.

In the FCM shown in Fig. 3, demonstrating that LSECs inhibit the proliferation of alloreactive T cells, PI was used for the exclusion of nonviable cells. Only PI-negative viable cells were subjected to additional analyses, so that proliferating apoptotic cells as well as necrotic cells would be excluded from the analyses. Hence, proliferation was not observed in Fig. 3.

Fas/FasL signal plays a significant role in specific tolerance among alloreactive T cells induced by transmigration across naive allogeneic LSECs

Blood passes through the liver in a meshwork of sinusoids formed by LSECs. Circulating leukocytes are forced into frequent contact with LSECs due to the small diameter (7–12 μm) of the sinusoids (35). To investigate the possibility that the sinusoidal architecture promotes the immunomodulatory activity of LSECs toward alloreactive T cells, we performed a T cell transendothelial migration assay to mimic the anatomical features of the interaction between LSECs and T cells (Fig. 6*A*). Nonadherent B6 lymphocytes that had transmigrated through a monolayer (with pores 8 μm in diameter) of B6, BALB/c, or SJL/j LSECs were subsequently stimulated with irradiated BALB/c splenocytes. Nonresponsiveness of both $CD4^+$ and $CD8^+$ T cells transmigrated through allogeneic BALB/c LSECs and marked proliferation of those T cells transmigrated through syngeneic B6 and third-party SJL/j LSECs were observed upon the subsequent stimulation (Fig. 6, *B* and *C*). The transmigration of B6 T cells (in particular, $CD4^+$ T cells) across

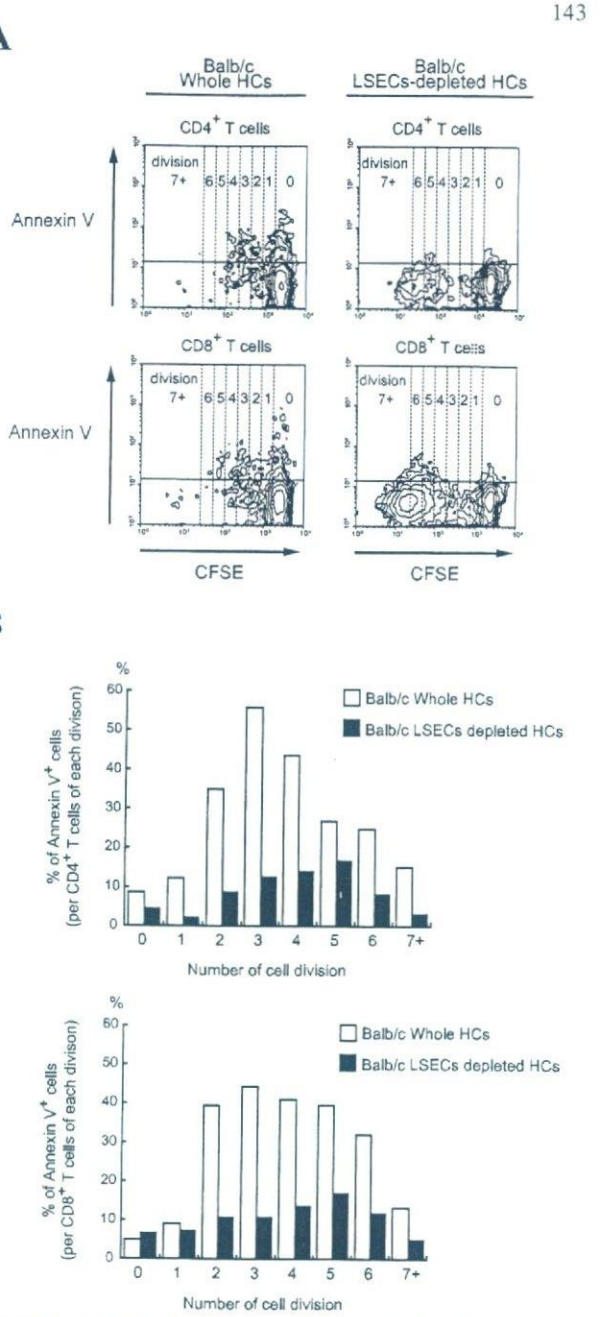

FIGURE 5. LSECs induce apoptosis of alloreactive T cells at the early period of cell division. *A*, Representative FCM results of CFSE-labeled B6 T cell divisions in the allogeneic MHLR using either irradiated whole HCs or LSEC-depleted HCs as stimulators from BALB/c mice. Phosphatidylserine expression of gated T cells in the allogeneic MHLR was visualized by annexin V. The expression of phosphatidylserine is an indicator of early cell apoptosis. Divisions of alloreactive T cells were mathematically determined by the logarithmic CFSE intensities on the basis of the peak at the extreme *right*. The FCM profiles shown are representative of two independent experiments. The numbers of divisions representing dividing times are shown. Necrotic cells were excluded from the analysis by forward scatter and 7-AAD. *B*, Rates of annexin V-positive $CD4^+$ (*upper*) and $CD8^+$ (*lower*) T cells in each cell division in the allogeneic MHLR with (■) or without (□) LSEC depletion are shown. Gating on $CD4^+$ or $CD8^+$ T cells was allowed for analysis of alloreactive T cells. Cells were scored based on their CFSE fluorescence intensities, thus permitting analysis of the number of annexin V^+ cells in each cell division.

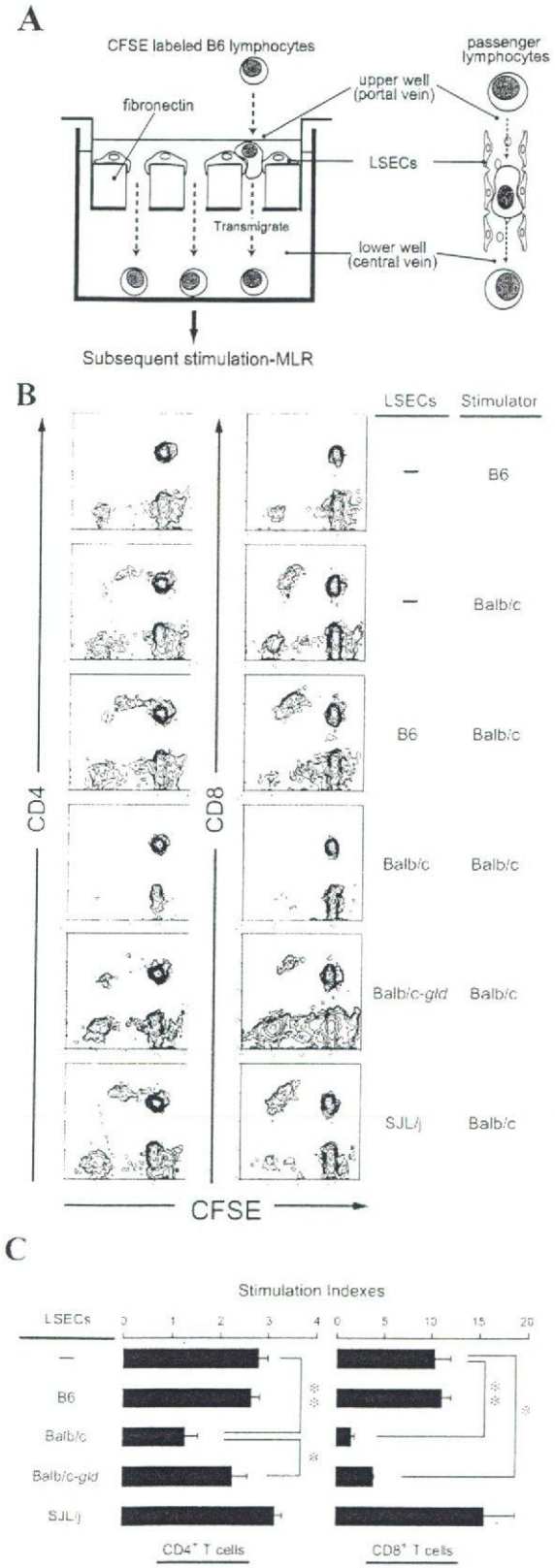

FIGURE 6. T cells that transmigrated across allogeneic LSECs are rendered specifically tolerant to alloantigens by a mechanism involving the Fas-FasL pathway. CFSE-labeled nonadherent B6 lymphocytes that transmigrated across the LSEC monolayer from mice of various strains were subsequently stimulated with irradiated BALB/c splenocytes. *A*, Schemas of transendothelial migration assay are shown. CFSE-labeled nonadherent lymphocytes (10×10^6 cells) from B6 mice were added to each insert and were left for 12 h to migrate through the monolayer of various LSECs. The migrated lymphocytes were cocultured with irradiated (30 Gy) splenocytes from BALB/c mice in subsequent MLR. *B*, Representative FCM results of CFSE-labeled $CD4^+$ and $CD8^+$ T cell division in the subsequent MLR are shown. LSECs from B6, BALB/c, BALB/c-*gld*, or SJL/j mice were used to form a monolayer. *C*, Stimulation indexes of alloreactive T cells in the subsequent MLR using transmigrated B6 lymphocytes as responder and irradiated BALB/c splenocytes as stimulator are shown. The mean ± SEM of four independent experiments are shown. *, $p < 0.05$; **, $p < 0.01$.

the FasL-deficient BALB/c (BALB/c-*gld*) LSECs failed to induce such nonresponsiveness on subsequent stimulation with the irradiated BALB/c splenocytes, including professional APCs. Thus, T cells that transmigrated across the allogeneic LSECs were rendered tolerant to alloantigens at least in part via the Fas/FasL pathways.

Discussion

Although the immunomodulatory activity of LSECs is currently noticed, even its phenotypic property remains controversial. The conflicting results regarding the expression of the molecules necessary for Ag presentation (CD80, CD86, CD40, and MHC classes I and II) on LSECs have been reported (36, 37). This may be attributed to the difference in the methods of preparation of LSECs in the previous studies, i.e., contamination with other cells or degeneration of surface molecules by excessive enzymatic digestion, or in vitro subculture for purity possibly affects their phenotypic profile results. Because the specific surface marker for LSECs remains to be defined, previous methods of LSEC isolation do not directly confirm the presence of LSECs with regard to their anatomical distribution (16, 37, 38). Instead of the previously reported methods, i.e., counterflow elutriation (16, 38) or negative selection using mAbs against molecules that are not expressed on LSECs (37), we used positive selection of $CD105^+$ cells using MACS to isolate LSECs. CD105 is known to be predominantly expressed on endothelial cells (25, 39–41), and its promoter is strongly and selectively active in endothelial cells (42, 43). Elevated levels of CD105 expression were detected on microvascular endothelium and on vascular endothelial cells in regenerating tissue undergoing active angiogenesis (39, 40, 44). Similarly, LSECs, which have an exceptional capacity for angiogenesis, highly expressed CD105 compared with endothelia of other vessels in the livers, as observed in the present immunohistochemical studies (Fig. 1*A*). We cannot rule out the possibility that the $CD105^+$ cell fraction isolated from livers contaminates with primitive hemopoietic cells, which has been also shown to express CD105 Ags (45). However, we confirmed complete absence of contamination with $CD45^+$ hemopoietic cells, which might have the capacity for Ag presentation, in the isolated $CD105^+$ cells, indicating the absence of hepopoietic APCs (Fig. 1*C*). The freshly isolated $CD105^+$ cells expressed MHC class II, CD40, CD80, and CD86, reflecting their potential role as APCs (Fig. 1*D*). A symmetrical histogram with a single peak obtained in the analysis of the expression of such molecules on isolated $CD105^+$ cells proved minimal contamination with cells other than LSECs.

It has been reported that naive $CD4^+$ T cells primed by Ag-presenting LSECs (MHC class II-restricted presentation of soluble Ags) fail to differentiate toward the effector Th1 cells, but express high levels of immune-suppressive mediators (IL-4 and IL-10) (16). It has also been reported that the stimulation of naive $CD8^+$ T cells by LSECs presenting exogenous Ags (cross-presentation) results first in the proliferation of T cells and the release of cytokines and finally leads to Ag-specific tolerance, as demonstrated by a loss of cytokine expression simultaneously with the failure of

$CD8^+$ T cells to develop into cytotoxic effector T cells. The LSEC has been described as a new type of APC that induces immune tolerance in naive T cells in the context of both MHC class I and II restriction (17, 36, 38, 46). However, such immune regulatory effects of LSECs have been observed only in a model in which the interaction of soluble exogenous Ags and respective transgenic T cell receptors takes place; thus, the capacity of LSECs to regulate a polyclonal population of nontransgenic T cells with allogeneic specificity remained to be elucidated. In addition, despite the progress in elucidation of immune functions of LSECs to autologous T cells, the manner in which host T cells respond to allogeneic LSECs in liver allografts has not been determined. In the present study, LSECs failed to induce the proliferation of both allogeneic $CD4^+$ and $CD8^+$ T cells (Fig. 2). The inability of LSECs to induce the proliferation of allogeneic T cells was not merely due to their poor Ag-presenting capacity, but was a consequence of their tolerogenic capacity, as proven by results showing that the presence of LSECs in an allogeneic MHLR assay led to inhibition of alloreactive T cells, and T cells transmigrated across the allogeneic LSECs were rendered tolerant to subsequent stimulation with alloantigens. In the presence of LSECs, the cytokine analyses of the allogeneic MHLR supernatants revealed a remarkable reduction of IL-2 and IFN-γ, rather than the increase in IL-4 and IL-10 (data not shown), suggesting that the tolerogenicity of LSECs toward alloreactive T cells is not predominantly due to the release of such immune-suppressive cytokines. As shown in Fig. 4, the proliferation of alloreactive $CD4^+$ and $CD8^+$ T cells was inhibited when in contact with LSECs, whereas the physical separation of LSECs using a dual chamber Transwell culture system abrogated the LSEC-induced inhibitory effects on alloreactive T cell proliferation. The inhibition of $CD4^+$ T cells in contact with LSECs was less significant compared with that of $CD8^+$ T cells. The inhibition of alloreactive $CD4^+$ to the same degree as that of $CD8^+$ T cells apparently requires a greater number of LSECs. Such a difference between alloreactive $CD4^+$ and $CD8^+$ T cells in susceptibility to inhibition when in contact with LSECs might be caused by the differences in their LSEC-induced inhibition mechanisms. Using FasL-deficient mice, we have demonstrated that the deficiency of FasL on LSECs significantly abrogated their tolerogenicity toward $CD4^+$ T cells, but only partially toward $CD8^+$ T cells (Fig. 6, *B* and *C*), suggesting that FasL on LSECs is more important for the inhibition of $CD4^+$ T cells than that of $CD8^+$ T cells. Consistently, it has been reported that FasL engagement inhibits $CD4^+$ T cell proliferation, cell cycle progression, and IL-2 secretion, but $CD8^+$ T cells are inherently resistant to the FasL-mediated regulation (47, 48). Mechanisms other than the Fas/FasL pathway would also be involved in the LSEC-induced tolerance of alloreactive $CD8^+$ T cells. It has been recently reported that LSECs constitutively expressed programmed death ligand-1 (PD-L1) (the ligand for the immunoinhibitory receptor PD-1) and inhibited the proliferation of effector T cells expressing PD-1 (49). There is a possibility that the PD-1/PD-L1 pathway also plays an important role in the LSEC-induced tolerance of alloreactive $CD8^+$ T cells. Additional studies are required to address this possibility.

Nevertheless, our results indicating the critical role of FasL on LSECs in liver allograft-induced T cell tolerance are consistent with two previously reported findings: 1) that extensive apoptosis of infiltrating cells was observed in the portal areas of liver allografts (30); and 2) that liver allografts from FasL-deficient mice were eventually rejected, whereas those from wild-type mice were spontaneously accepted (50). FasL-mediated tolerization by LSECs resembles the previously reported model in which FasL present in nonlymphoid tissue deletes reactive lymphoid cells during viral infections and is responsible for protecting immune-privileged sites from cellular immune-mediated damage (51, 52). The constitutive expression of FasL on LSECs might be induced by the unique liver microenvironment, i.e., endotoxin, as a physiological constituent of portal venous blood, induces release of the anti-inflammatory mediators from various liver resident hemopoietic cells (i.e., intrahepatic macrophage, dendritic, NK, and NKT cells), which, in turn, may promote FasL on naive LSECs (53). Such a hypothesis is consistent with the earlier findings of hepatic transplantation tolerance being due to a radiosensitive population that include intrahepatic hemopoietic cells (54). Elucidation of the immune mechanism underlying tolerogenicity of LSECs might lead to the establishment of novel means to promote acceptance even of transplant of organs other than liver.

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Disclosures

The authors have no financial conflict of interest.

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Excerpta Medica

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Vascular closure staples for portal vein reconstruction in living-donor liver transplantation

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Abstract

Background: Portal vein reconstruction is still a crucial problem in living-donor liver transplantation. Vascular closure staples (VCS) have been applied for small peripheral and large vessels because of the technical ease with which they can be employed. We describe here our experience with portal vein reconstruction in living donor-liver transplantation and compare VCS with conventional sutures in portal vein reconstruction.

Methods: The anastomosis between the donor portal vein and recipient portal vein or the right external iliac vein graft was created using either VCS or conventional sutures.

Results: The stenotic ratios were .51 ± .15 and .79 ± .25 for the conventional sutures and VCS, respectively. The stenotic ratio was significantly lower in VCS compared with conventional sutures.

Conclusions: VCS compared with conventional sutures has the advantage of low risk of anastomotic stenosis.

Keywords: Living-donor liver transplantation; Portal vein reconstruction; Vascular closure staples

Portal vein reconstruction is a crucial factor for successful liver transplantation. In liver transplantation, stenosis and occlusion of the recipient portal vein have been often observed in liver cirrhosis, and variations of portal vein branches present specific issues regarding surgical techniques for portal vein reconstruction. Techniques using an interposition vein graft—consisting of a donor ovarian, inferior mesenteric, or recipient iliac vein graft—have been reported [1,2]. The reconstruction of a dual portal vein in living-doner transplantation using a right lobe graft having a separate origin of the posterior right portal vein from the main portal vein has also been reported [3].

In the 1980s, nonpenetrating arcuate-legged titanium vascular closure staples (VCS) were developed by Kirsch et al [4,5] for brain vascularization, and they have subsequently been used to perform anastomoses of small and large vessels. The use of VCS has several potential benefits: VCS cinch the vessel wall, everting but not penetrating the endothelium yet grasping the adventitia firmly without crushing it, and the time required for VCS closure is shorter than that required for suture closure [6,7]. Here we describe the surgical techniques for portal vein reconstruction, focusing on portal vein reconstruction using VCS, in a series of 30 adult patients who underwent living-donor liver transplantation.

Patients and Methods

Between January 2001 and September 2003, 30 adult patients underwent living-donor liver transplantation at Hiroshima University Hospital. Those 30 patients were enrolled in this study. Patient characteristics are listed in Table 1.

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Surgical techniques for portal vein reconstruction

As shown in Fig. 1, we divided the surgical techniques used for portal vein reconstruction into 2 major groups: single–portal vein anastomosis (group 1) and dual–portal vein anastomosis (group 2). Group 1 was further divided into 2 subgroups: anastomosis without (group 1a) and with a vein graft (group 1b). Group 1a included trunk anastomosis between the donor right or left portal vein and recipient portal vein, and group 1b included the interposition graft technique. In the interposition graft technique, a vein graft is interposed between the graft's portal vein and recipient superior mesenteric vein (SMV)–splenic vein (SP) confluence. The vein grafts used were right external iliac veins from recipients. The vein graft was anastomosed to the recipient's SMV-SP confluence using 6-0 polypropylene running sutures with growth factor.

VCS clip techniques

The anastomosis between the donor portal vein and recipient portal vein or the right external iliac vein graft was created using either VCS or sutures. For VCS anastomosis, four 6-0 polypropylene stay sutures were placed in equally opposite positions, and the vessel edges were held with specially designed VCS-approximating forceps to evert the vessel walls. Medium-sized VCS were then applied at approximately .5-mm intervals between stay sutures to complete the anterior wall. By rotating the vessel with the stay sutures, the posterior wall was exposed and completed identically with VCS. VCS were not applied in dual–portal vein anastomosis because of the difficulty in rotating the vessels. Patients were randomly allocated to either the suture technique or the VCS technique.

Evaluation of anastomotic stenosis in the portal vein

To evaluate the portal anastomotic stenosis, we measured the portal vein diameter of the liver allograft and anastomotic diameter using 3-dimensional computed axial tomography (CAT) imaging [8]. The stenotic ratio is calculated thusly: $\pi r(\text{anastomotic site})^2/\pi r(\text{donor portal vein})^2$. The donor portal vein diameter of the liver allograft was measured ex vivo. The diameter of the anastomotic site was calculated from 3-dimenstional CAT images.

Table 1
Profiles of clinical data of recipients

Age (y)	51
Sex (men:women)	20:10
Indication of liver transplantation	
Cirrhosis	
Hepatitis C virus–positive	14
Hepatitis B virus–positive	6
Hepatocellular carcinoma	14
Primary biliary cirrhosis	2
Autoimmune hepatitis	2
Fulminant hepatic failure	4
Alcohol-induced cirrhosis	2
Liver graft	
Right lobe	26
Left lobe	4

N = 3D.

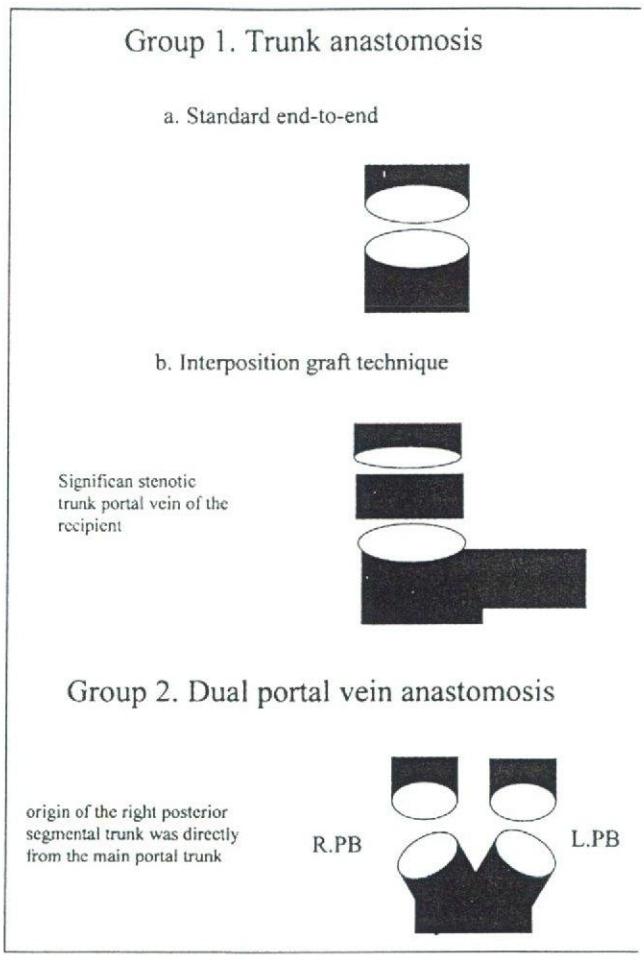

Fig. 1. Portal vein reconstruction was classified into two major groups: (1) anastomosis without vein graft including trunk anastomosis (n = 22) and anastamosis with vein graft including interposition technique (n = 5) (group 1) and (2) dual vein anastomosis (group 2). The origin of the right posterior segmental trunk in right lobe graft was direct from the main portal trunk (n = 3).

Statistics

The differences between means in each group were tested using analysis of variance, and $P < .05$ was considered significant.

Results

Portal vein reconstruction

Three different patterns of anastomosis (Fig. 1) were used for portal vein reconstruction. Modalities for recon-

struction were selected according to the wall status of the recipient's portal vein and anatomic variations of the graft portal vein. The standard end-to-end trunk anastomosis of portal vein reconstruction (type 1a) was used in 22 patients. In 5 patients, the recipient's trunk portal vein was significantly stenotic, and the native trunk was removed to obtain adequate portal flow. The recipient's right external iliac vein graft was interposed between the graft portal vein and the recipient's SMV-SP confluence. The dual–portal vein anastomosis method (type 2) was used in 3 patients. The origin of the right posterior segmental trunk in right lobe graft was direct from the main portal trunk.

Complications occurring after portal vein reconstruction

Postoperative echography and CAT studies showed no early thrombosis or stenosis of the anastomosed portal vein. Protocol CAT studies and echographies were performed to examine the portal vein at 1 month, 6 months, and 1 year after surgery. Portal vein stenosis was found in only 1 patient after trunk anastomosis. This patient had liver dysfunction that was probably caused by portal vein stenosis and had undergone angioplasty using a 10-mm balloon by way of the percutaneous transhepatic approach.

VCS clip anastomosis

The VCS technique was used in 15 patients with trunk anastomosis including 2 with vein graft anastomosis. The conventional suture technique was used in 12 patients with trunk anastomosis including 3 with vein graft anastomosis. The reconstruction times were 22 ± 8 minutes for conventional sutures and 18 ± 5 minutes for VCS (P = .15). The stenotic ratios were .51 ± .15 and .79 ± .25 in the conventional suture and VCS groups, respectively. The stenotic ratio was significantly lower in the VCS than the conventional-suture group (Table 2). Protocol CAT showed several patients having portal vein anastomosis with mild or moderate deformity at the anastomotic site in the conventional suture group compared with normal portal vein anastomosis in the VCS group (Fig. 2).

Table 2
Comparison of stenotic ratio and anastomotic time between conventional method and VCS clip

Suture method	Conventional method (N = 12)	VCS clip (N = 15)
Rate of stenosis		
Area of anastomosis/ area of graft portal vein	.51 ± .15	.79 ± .25 (P = .025)
Reconstruction time	22 ± 11 min	16 ± 5 min (P = .35)

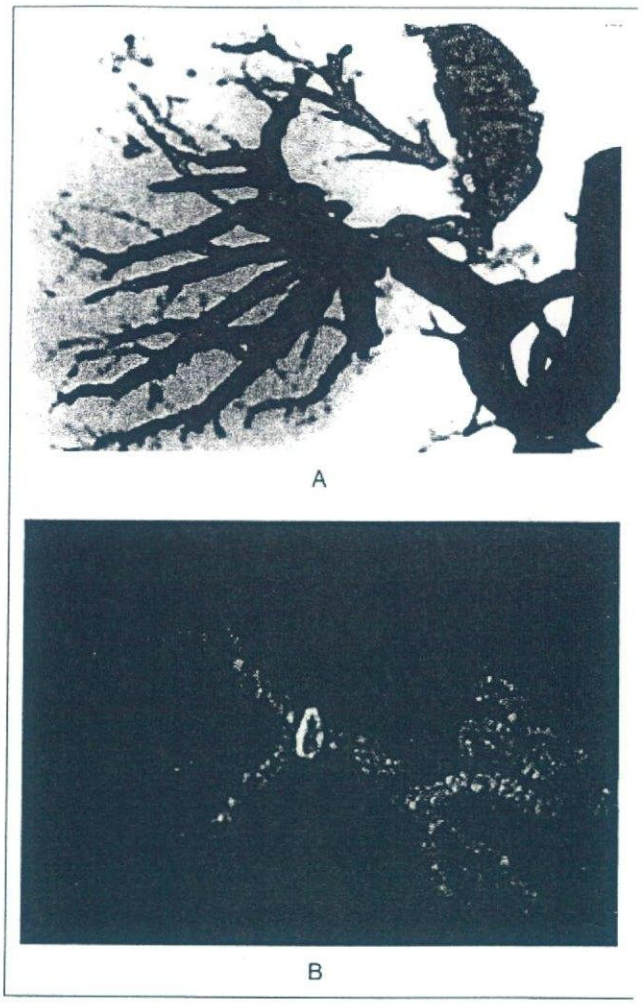

Fig. 2. 3-dimensional images of portal vein after reconstruction by (**A**) conventional sutures and (**B**) VCS. Arrows show anastomotic sites. VCS = vascular closure staples.

Comments

Extrahepatic portal vein complications are uncommon in cadaveric liver transplantation. Portal vein stenosis is rare, and portal vein thrombosis occurs in only 1% to 2% of liver transplants with whole-liver grafts [9]. In living-donor liver transplantation, complications related to portal vein reconstruction have been reported to occur in 5% of recipients [1]. Portal vein complications occur more frequently in living-doner than in cadavaric liver transplantation. Protocol CAT showed several patients having portal vein anastomosis with mild or moderate deformity at the anastomotic site in the conventional suture group but normal portal vein anastomosis in the VCS. Despite having everted the sutures with clips, we noted no narrowing of the vessel diameter. The inner diameter at the VCS site was significantly larger than that of the conventional suture sites. We believe that the intermittent sutures prevented the anastomotic site from narrowing. The most important advantage of the VCS technique was that anastomotic stricture was avoided owing to

interrupted clipping without the use of any special surgical techniques.

In this study, the time required for anastomsis using VCS tended to be shorter than the time required using continuous suture closure, although the difference was not significant. No tears or complications prolonged the procedures. VCS could possibly make vascular anastomosis, in which length of clamp time is critical, more safe. In liver transplant surgery with multiple anastomoses, VCS would offer a definite advantage. The learning curve for the VCS anastomosis technique is short, and with a little practice even a nonvascular surgeon can soon master the process. The critical step in this anastomosis technique is the use of specially designed forceps for accurate approximation of vessel edges before application of the clips.

Although we had 3 patients with the posterior segmental trunk originating from the main portal trunk, reconstruction using bifurcation of the recipient portal vein was feasible and safe in the 3 patients with duplicated portal branches close to each other. Five patients had stenosis of the portal trunk but had patent vessels at the SMV-SP confluence. To obtain efficient size and length to allow for adequate portal flow, we placed an interposed vein graft using the recipient's external iliac vein between the confluence and the graft portal vein. These dual reconstructions of the portal vein and interposed vein grafts enabled good patency to be maintained after reconstruction. In conclusion, VCS compared with conventional suturing has the advantage of low risk of anastomotic stenosis.

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Difference in Cytotoxicity Against Hepatocellular Carcinoma Between Liver and Periphery Natural Killer Cells in Humans

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In rodents, liver natural killer (NK) cells have been shown to mediate higher cytotoxic activity against tumor cells than do peripheral blood (PB) NK cells. However, such differences between liver and PB NK cells have not been extensively investigated in humans. The phenotypical and functional properties of NK cells extracted from liver perfusates at the time of living donor liver transplantation were investigated. The tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), a critical molecule for NK cell–mediated anti-tumor cell killing, was not expressed by freshly isolated PB NK cells or by liver NK cells. Stimulation with interleukin (IL)-2, significantly up-regulated the expression of TRAIL on liver NK cells, but this effect was barely observed on PB NK cells. Donor liver NK cells showed the most vigorous cytotoxicity against HepG2, a hepatocellular carcinoma (HCC) cell line, after IL-2 stimulation (90.5% ± 2.2% at E: T = 10:1), compared with donor and recipient PB NK cells and recipient liver NK cells (64.8% ± 8.2%, 56.1% ± 8.9%, and 34.6% ± 7.5%, respectively). IL-2 stimulation resulted in an increased expression of killing inhibitory receptors on liver NK cells in parallel with TRAIL expression. Consistently, the cytotoxicities of IL-2–stimulated donor liver NK cells against self and recipient lymphoblasts were negligible. In conclusion, adoptive transfer of IL-2–stimulated NK cells extracted from donor liver graft perfusate could mount an anti-tumor response without causing toxicity against 1-haplotype identical recipient intact tissues. These findings present a concept to prevent recurrence of HCC after liver transplantation. (HEPATOLOGY 2006;43:362-372.)

Natural killer (NK) cells are thought to provide a first line of defense against invading infectious microbes and neoplastic cells by exerting an effector function without the necessity for priming.[1,2] Given the efficacy of NK cells in selectively killing abnormal cells, a variety of approaches have been taken to try and selectively augment NK cell response to tumors.[3,4] Several therapeutic cytokines primarily act via NK cells [such as interleukin (IL)-2, IL-12, IL-15, and interferons (IFNs)] and many studies have shown that activation of NK cell differentiation and function leads to a more efficient elimination of tumor growth.[5-9] Despite these promising advances, the systemic administration of cytokines such as IL-2, that nonspecifically activate a broad range of different immune cell types, is associated with significant toxicity.[5,10] The adoptive transfer of NK cells further demonstrates the ability of NK cells to mount a therapeutic anti-tumor response and suggests that NK cells can be used in controlling human malignancy.[11,12] In these studies, autologous or even haploidentical lymphokine-activated killer cells obtained from peripheral blood mononuclear cells (PBMCs) have been administered to patients, although their comprehensive role in the treatment of selected malignancies remains to be elucidated.

NK cells are quite abundant in the liver of mice, in contrast to a relatively small percentage in the periph-

Abbreviations: NK; natural killer; IL, interleukin; IFN, interferon; PBMC, peripheral blood mononuclear cell; PB, peripheral blood; LDLT; living donor liver transplantation; HCC; hepatocellular carcinoma; TNF, tumor necrosis factor; TRAIL; TNF-related apoptosis-inducing ligand; DR, death receptor; LMNC; liver mononuclear cell; FCM, flow cytometric; MAb, monoclonal antibody; HLA, human leukocyte antigen; E:T, effector:target; MC, mononuclear cell; KIR, killer cell immunoglobulin-like receptors; MHC, major histocompatability complex.

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Table 1 Clinical Characteristics of Living-Related Liver Transplant Donors and Corresponding Recipients With Cirrhosis

	Donor								Recipient									
Case No.	Age(y)/ Sex	Graft Liver	Graft Wt(g)	LMNC ($\times 10^5$/ g)	HLA A	HLA B	HLA C	Relation	Age(y)/ Sex	Child Pugh	MELD	Original Disease	HCC	Liver Wt(g)	LMNC ($\times 10^5$/ g)	HLA A	HLA B	HLA C
1	27/F	Left	262	3.7	2,26	51,54	1,–	Offspring	59/F	B	13.4	HCV	-	800	4.4	11,26	54,–	1,–
2	29/F	Left	460	7.8	2,26	56,61	1,8	Offspring	56/F	C	13.6	HCV	-	776	2.7	26,–	61,–	8,–
3	21/M	Right	550	6.4	2,24	7,35	3,7	Offspring	49/M	C	18.9	HCV	+	718	0.4	2,24	13,35	3,–
4	24/M	Right	564	1.2	24,26	62,-	3,4	Offspring	60/M	B	10.4	HCV	+	846	0.6	24,–	60,62	3,4
5	20/M	Right	896	1.9	2,33	37,46	1,3	Offspring	54/M	B	13.3	HBV	+	665	0.2	31,33	37,61	3,4
6	20/M	Right	632	3.2	24,26	52,62	3,12	Offspring	47/M	A	10.6	HCV	+	1150	3.1	24,–	52,–	12,–
7	57/F	Right	678	9.2	2,24	52,54	1,11	Spouse	43/M	C	35.2	HBV	+	810	7.0	24,–	7,52	7,12
8	59/M	Left	398	4.2	11,31	39,61	7,8	Spouse	57/F	C	18.9	HCV	-	684	9.5	24,26	35,39	3,7
9	30/M	Right	550	3.7	26,33	44,62	3,14	Offspring	58/M	C	29.4	HBV	-	820	0.4	2,26	51,62	3,15
10	49/F	Right	660	7.4	2,24	7,61	7,8	Sibling	44/F	C	18.1	AIH	-	753	1.2	2,24	7,61	7,8
11	44/F	Right	576	4.0	26,-	35,62	3,–	Spouse	48/M	C	37.5	HCV	-	410	13.4	24,–	52,–	12,–
12	29/M	Right	900	4.4	2,24	51,54	1,14	Offspring	58/M	C	16.9	HCV	+	714	4.2	24,–	54,–	1,8
13	43/M	Left	350	10.6	24,–	7,46	1,7	Sibling	46/F	C	17.6	HBV	-	425	1.4	24,–	7,46	1,7
14	18/M	Right	630	3.2	2,31	54,61	1,3	Offspring	57/F	A	3.1	HBV	+	932	8.2	24,31	54,61	1,3

Abbreviations: F; female, M; male, Graft wt; graft weight, LMNC; liver mononuclear cell, HLA; human leukocyte antigens, MELD; Model for End-Stage Liver Disease, HCV; hepatitis C virus, HBV; hepatitis B virus, AIH; autoimmune hepatitis, HCC; hepatocellular carcinoma.

eral lymphatics.[13-15] The underlying reason for this anatomically biased distribution has not been fully elucidated. In addition, liver NK cells have been shown to mediate higher cytotoxic activity against tumor cells than spleen or peripheral blood (PB) NK cells in rodents.[13-16] However, such differences between liver and PB NK cells have not been extensively investigated in human because of the limited availability of appropriate human samples.

In the current study, we have determined phenotypical and functional properties of liver NK cells extracted from donor and recipient liver perfusates in clinical living donor liver transplantation (LDLT). Donor liver NK cells showed the most vigorous cytotoxicity against a hepatocellular carcinoma (HCC) cell line after *in vitro* IL-2 stimulation, compared with donor and recipient PB NK cells and recipient liver NK cells. IL-2 stimulation led to an increased expression of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) on liver NK cells, which has been shown to be critical for NK cell–mediated anti-tumor cell killing without affecting normal cells.[17-19] In addition, we have confirmed that HCC expressed the death-inducing TRAIL receptors (TRAIL-Rs), TRAIL-R1/death receptor (DR) 4, and TRAIL-R2/DR5 that contain cytoplasmic death domains and signal apoptosis.[20,21] These findings raise a novel concept to prevent recurrence of HCC after liver transplantation, in other words, adoptive transfer of IL-2–stimulated NK cells extracted from donor liver graft into 1-haplotype identical recipients.

Patients and Methods

Patients. Fourteen patients who underwent adult-to-adult LDLT at The Hiroshima University Hospital (Hiroshima, Japan) were involved in this study. The 14 patients with hepatic cirrhosis included 8 men and 6 women, ranging in age from 43 to 60 years [mean age (in years) ± SD, 52.6 ± 5.5]. Original diseases of the patients are shown in Table 1. Nine of the graft donors were offspring, two were siblings, and three were spouses, with ages ranging from 18 to 59 years (33.6 ± 12.0).

Isolation of Liver and Peripheral Blood Lymphocytes. Donor hepatectomy and the recipient transplantation procedure were performed as described previously.[22] In brief, the right or left lobe was harvested from the donor. After hepatectomy, *ex vivo* perfusion of the liver graft was performed through the portal vein. The initial perfusate consisted of saline solution (500 mL) followed by University of Wisconsin solution (1,000 mL). For the recipient, the implantation was performed after total hepatectomy. *Ex vivo* perfusion of the removed recipient liver was also performed through the portal vein by using the same perfusates. Liver mononuclear cells (LMNCs) were obtained from those perfusate effluents from healthy donor liver grafts and recipient livers with cirrhosis as follows. The effluents were condensed by centrifuging and LMNCs were isolated by gradient centrifugation with Separate-L (Muto Pure Chemicals Co., Ltd, Tokyo, Japan). PBMCs were also isolated by gradient centrifugation with Separate-L from 40 mL heparinized peripheral

blood from donors and recipients. LMNCs and PBMCs were suspended in RPMI 1640 medium that was supplemented with 10% heat-inactivated fetal calf serum (Sanko Chemical Co., Ltd., Tokyo, Japan), 25 mmol/L HEPES Buffer (Gibco, Grand Island, NY), 50 μmol/L 2 mercaptoethanol (Katayama Chemical Co., Osaka, Japan), 50 U/mL penicillin, and 50 μg/mL streptomycin (Gibco) (from hereon we refer to this medium as 10% RPMI). Ethical approval for this study was obtained from the Ethics Committee at The Hiroshima University Hospital.

Flow Cytometric Analyses. All flow cytometric (FCM) analyses were performed on a FACS Calibur dual-laser cytometer (BD Biosciences, Mountain View, CA). For phenotyping of NK cells, LMNCs and PBMCs were stained with fluorescein isothiocyanate–conjugated anti-CD3 (BD Pharmingen, San Diego, CA), phycoerythrin-conjugated anti-CD56 (B159) (BD Pharmingen), and biotin-conjugated anti-TRAIL monoclonal antibodies (MAbs) (RIK-2) (e Bioscience, Oxford, U.K.). For analyzing inhibitory receptors on NK cells, LMNCs and PBMCs were stained with peridin chlorophyll protein–conjugated anti-CD3 (SP34-2) (BD Pharmingen), phycoerythrin-conjugated anti-CD56, biotin-conjugated anti-TRAIL and fluorescein isothiocyanate–conjugated anti-CD-158a (HP-3E4) (BD Pharmingen), anti-CD-158b (CH-L) (BD Pharmingen), or anti-CD94 MAbs (HP-3D9) (BD Pharmingen). For analyzing TRAIL receptors on the HCC cell line, HepG2 cells 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). All the biotinylated MAbs were visualized with allophycocyanin-streptavidin (BD Pharmingen). Dead cells were excluded from the analysis by light-scatter and propidium iodide staining.

Cell Culture. LMNCs and PBMCs were cultured with or without human recombinant IL-2 (100 U/mL) (Takeda, Tokyo, Japan) in 10% RPMI at 37°C in a 5 % CO_2 incubator. After 4 days in culture, cells were harvested for further analyses.

Cytotoxicity Assay. HepG2 cells established from HCC tissue from a hepatitis B virus– and hepatitis C virus–negative HCC patient (HLA; human leukocyte antigens: −A02,24, −B35,51 −CO,4) were purchased from The Japanese Cancer Research Resources Bank and were maintained in 10% RPMI.[23] HepG2 were labeled with 100 μCi Na2 (^{51}Cr) O4 for 60 minutes at 37°C in 5% CO_2 in 10% RPMI, washed 3 times with medium, and then subjected to the cytotoxicity assay. The labeled HepG2 cells were adjusted to 1×10^6 cells in 10 mL volumes (1×10^4/well) and were incubated in a total volume of 200 μL with effector cells in 10% RPMI in round-bottomed 96-well microtiter plates (Nunclon; Inter Med, Denmark). LMNCs or PBMCs from healthy donors or recipients with cirrhosis were used as effectors at effector-target (E:T) ratios of 2.5:1 to 40:1. When indicated, LMNCs and PBMCs were cultured *in vitro* with IL-2 for 4 days before using as effectors. As a control, the target cells were incubated either in culture medium alone to determine spontaneous release, or in a mixture of 2% Nonidet P-40 (Nacalai Tesque, Inc., Kyoto, Japan) to define the maximum ^{51}Cr release. The plates were centrifuged at 1,000 rpm for 3 minutes to pack the cell layer at the end of the reaction, after which the cell-free supernatants were carefully harvested, and its radioactivity was measured with a gamma counter. The percentage of specific ^{51}Cr release was calculated by the following formula: % cytotoxicity = [(cpm of experimental release − cpm of spontaneous release)]/[(cpm of maximum release − cpm of spontaneous release)] × 100. The spontaneous release was less than 20% of the maximum release. All assays were performed in triplicate.

Isolation of NK Cells. LMNCs and PBMCs were separated into a $CD3^-CD56^+$ NK cell fraction and a non-NK cell fraction (T cells, NKT cells, B cells, and monocytes/macrophages) by magnetic cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany) using the human NK cell isolation Kit II (Miltenyi Biotec) according to the manufacturer's instructions. The purity of isolated fractions was assessed by FCM, and only preparations whose purities were greater than 95% were used for functional studies. Using a similar method as described, NK and non-NK cells isolated from IL-2–stimulated donor LMNCs and PBMCs were subjected to the cytotoxicity assay against HepG2 cells. When indicated, the isolated NK cells were pre-incubated for 30 minutes at 4°C with neutralizing Abs against TRAIL (purified anti-TRAIL MAbs; eBioscience) (final concentration 20 μg/mL) or with isotype-matched non-reactive Abs before the cytotoxicity assay. In addition, a similar cytotoxicity assay with those donor NK cells was performed against autologous and allogenic lymphoblasts, which had been prepared from PBMCs of donors and corresponding recipients by cultivating with 5 μg/mL PHA (Sigma, Poole, UK) for 4 days.

Immunohistochemistry. Surgically resected liver specimens were obtained from patient with HCC who had undergone potentially curative tumor resection at The Hiroshima University Hospital. All HCC tissues were pathologically confirmed. Normal liver samples obtained from patients with metastatic liver tumors were used as control. Informed consent was obtained from all

patients. To localize TRAIL receptors *in situ* in the liver, immunohistochemistry was performed on frozen tissue sections of normal liver tissues and carcinoma cases as described previously.[24] For protein detection by immunohistochemistry, 4-μm frozen sections from representative tumor tissue of at least 1 cm^2 were fixed in acetone for 15 minutes. Sections were incubated for 60 minutes with the following MAbs: biotin-conjugated anti-TRAIL-DR4, anti-TRAIL-DR5, anti-TRAIL-DcR1, and anti-TRAIL-DcR2 at appropriate concentrations in phosphate-buffered saline. Binding sites of primary antibodies were visualized using the Dako EnVision kit (Dako, Copenhagen, Denmark) according to the manufacturer's instructions. Finally, sections were faintly counterstained with Harris' hematoxylin and mounted with glycerol gelatin. Negative controls were performed by omission of the primary antibody.

Statistical Analyses. Data are presented as mean ± SEM. The statistical differences of the results were analyzed by ANOVA analysis with the Scheffe F test using the Stat View program. A P value of .05 or less was accepted as statistically significant.

Results

LMNCs Contained a Large Population of NK Cells. The liver contains significant numbers of resident mononuclear cells (MCs) in human. These cells include a large number of T cells, B cells, NK cells, and NKT cells, many of which differ phenotypically and functionally from circulating lymphocytes.[25,26] Characterization of liver NK cells requires the isolation of viable LMNCs that can be analyzed by FCM and in functional assays. The techniques used to isolate LMNCs usually involve mechanical and enzymatic dissociation of liver tissue.[25-27] However, the difficulties in getting liver samples and processing liver biopsy have been obstacles to such studies. In the current study, instead of dispersal of liver tissue, *ex vivo* perfusion of the liver through the portal vein, which was inevitably done to flush blood from the liver graft before implantation in LDLT, provided LMNCs by extraction from liver perfusates. In our preliminary experiments, the proportions of $CD3^-CD56^+$ NK and $CD3^+CD56^+$ NKT cells in LMNCs extracted from liver perfusates (38.1% ± 4.5 % and 14.0% ± 3.0 %, respectively) were almost identical to those in LMNCs collected by method using enzymatic dissociation (33.7% ± 1.3 % and 14.8% ± 0.7 %, respectively, n = 3). These data were consistent with data in previous reports that used the enzymatic dissociation method.[25,26] Although this nondestructive method might allow contamination with circulating MCs to some extent, the possibility of enzyme-induced alteration/disruption of specific epitopes could be disregarded.

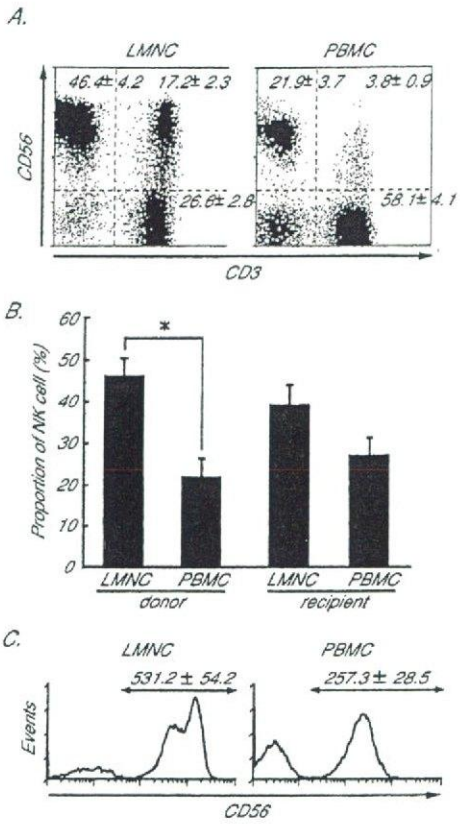

Fig. 1. Human liver mononuclear cell extracted from liver perfusates contain a large population of NK cells. (A) Flow cytometric (FCM) analysis of freshly isolated LMNCs obtained from liver perfusates and PBMCs from same donor after staining with MAbs against CD3 and CD56 were analyzed. Lymphocytes were gated by forward scatter and side scatter. FCM profiles shown are representative of 14 independent experiments. Percentages of $CD3^+CD56^-$ (T), $CD3^-CD56^+$ (NK) and $CD3^+CD56^+$ (NKT) cells are indicated at each quadrant (mean ± SEM, n = 14 each). (B) The numbers represent the means ± SEM of the $CD3^-CD56^+$ NK cell populations in total LMNCs or PBMCs obtained from 14 adult healthy donors and 14 corresponding recipients with liver cirrhosis. Statistical analyses were performed using ANOVA (*$P < .05$). (C) Expression of CD56 on electronically gated $CD3^-$ cells were analyzed by FCM. Histogram profiles shown are representative of 14 independent experiments. The data shown were obtained from donor LMNCs and PBMCs (similar results were obtained from recipient LMNCs and PBMCs). The numbers indicate the mean ± SEM of the mean fluorescence intensity (MFI) of CD56 expression on $CD3^-$ LMNCs and PBMCs cells (n = 14 each). NK, natural killer; LMNC, liver mononuclear cell; PBMC, peripheral blood mononuclear cell; MAb, monoclonal antibody; FCM, flow cytometric.

The number of LMNCs extracted from donor normal livers and recipient livers with cirrhosis were 0.5 ± 0.1 and 0.4 ± 0.1 × 10^6 cells/g, respectively, and were not statistically different (Table 1). FCM profiles of LMNCs and PBMCs from donor are shown in Fig. 1A. Proportions of $CD3^-CD56^+$ NK and $CD3^+CD56^+$ NKT cells in LMNCs were significantly higher than in PBMCs from same donors (Fig. 1B). Such a difference in NK cell pro-

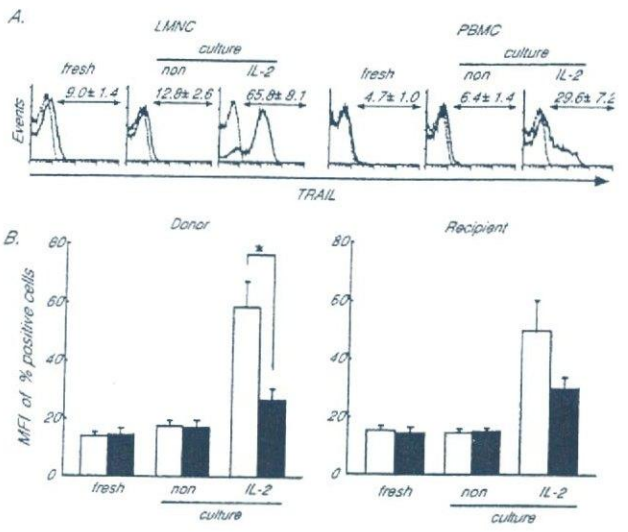

Fig. 2. Liver NK cells inductively express remarkable levels of TRAIL, but PB NK cells do not. Freshly isolated or cultivated with or without IL-2 LMNCs and PBMCs obtained from healthy donors and corresponding recipients were stained with CD3 and CD56 MAbs together with TRAIL MAb. (A) Histograms represent the log fluorescence intensities obtained on staining for TRAIL after gating on the $CD3^{-}CD56^{+}$ NK cells subsets obtained from healthy donors. Dotted lines represent negative control staining with isotype-matched MAbs. The numbers (mean ± SEM) indicate the percentages of cells in each group that were positive for TRAIL expression (n = 7 each). Histogram profiles shown are representative of 7 independent experiments. (B) The numbers indicate the mean fluorescence intensity (MFI) of cells in each group that were staining positively for TRAIL on freshly isolated, or cultivated with/without IL-2 NK cells (LMNC; open column, PBMC; closed column). The data represent mean ± SEM (n = 7). Statistical analyses were performed using ANOVA ($*P < 0.05$). NK, natural killer; TRAIL, TNF-related apoptosis-inducing ligand; LMNC, liver mononuclear cell; PBMC, peripheral blood mononuclear cell; MAb, monoclonal antibody.

portion between LMNCs and PBMCs was not conspicuous in recipients with cirrhosis, because of a relatively reduced proportion of NK cells in LMNCs from livers with cirrhosis. In LMNCs from donors and recipients, the $CD3^{-}CD56^{high}$ NK cell subpopulation, which is known to produce preferentially large amounts of cytokines,[28,29] was easily detectable, whereas it was undetectable in PBMCs (Fig. 1C).

Liver NK Cells Inductively Expressed TRAIL. TRAIL is a type II transmembrane protein that belongs to the TNF family, which preferentially induces apoptotic cell death in a wide variety of tumor cells but not in most normal cells.[17-19] We and others have previously reported that a subpopulation of NK cells in adult mouse liver, unlike other tissues, constitutively express TRAIL, and these liver NK cells were partially responsible for the natural anti-tumor function against TRAIL-sensitive tumor cells.[9,16,17] As shown in Fig. 2, freshly isolated liver NK cells from normal liver and liver with cirrhosis barely expressed TRAIL, whereas freshly isolated PB NK cells completely lacked TRAIL-expression. *In vitro* stimulation with IL-2 significantly up-regulated the expression of TRAIL on liver NK cells (the expression of TRAIL on liver NK cells from donor normal livers was somewhat higher than that on liver NK cells from recipient livers with cirrhosis). Conversely, even after IL-2 stimulation, PB NK cells expressed little TRAIL. Thus, liver NK cells inductively expressed remarkable levels of TRAIL, but PB NK cells did not.

We have recently demonstrated that most murine TRAIL-expressing liver NK cells lack expression of Ly-49 inhibitory receptors, which recognize self-MHC class I.[16] To address whether the same is true in humans, we have analyzed inhibitory receptors on human liver NK cells. Inhibitory receptors on human NK cells can be subdivided into 2 groups: killer cell immunoglobulin-like receptors (KIRs) (belonging to the immunoglobulin superfamily) including CD158a and CD158b; and C-type lectin-like receptors, CD94/NKG2. KIRs are major histocompatibility class (MHC) class I–restricted molecules that recognize HLA-A, -B, -C, and -G molecules, whereas CD94 recognizes the nonclassical MHC class Ib molecule HLA-E. CD94 is expressed essentially on all NK cells, and uses HLA-E expression as a sensor for the overall MHC class I level of a cells.[30,31] In contrast, individual KIR family members express on certain NK cell subsets, exhibit finer specificity for HLA class I allotypes, and can distinguish between groups of HLA-A, -B, and -C allotypes. Ligation of such KIRs/CD94 to HLA class I molecules on self cells results in inhibition of NK cytotoxic activity, as originally predicted by the "missing-self" hypothesis.[32,33] This regulation ensures that cells expressing none, altered, or reduced MHC-I molecules, such as malignant or virus-infected cells, can be killed by NK cells. All freshly isolated liver NK cells expressed CD94 and subpopulations of those cells expressed CD158a/CD158b (Fig. 3A). Cultivation of liver NK cells with no stimulants resulted in reduced expressions of CD158a, CD158b and CD94 (Fig. 3B). However, IL-2 stimulation led to the maintenance of those expressions even on TRAIL expressing liver NK cells (Fig. 3A-B). Thus, as opposed to TRAIL-expressing NK cells in mice, those cells in humans equip a compensatory mechanism to protect the self-MHC class I–expressing cells from NK cell-mediated cell killing.

HCCs Express the Death-Inducing TRAIL-DR4 and -DR5. The susceptibility to TRAIL-induced apoptosis may be related to the expression levels of multiple receptors on target cells. Recent molecular cloning of the TRAIL-receptors elucidated that TRAIL binds to at least four receptors, two of these death-inducing receptors (TRAIL-R1/DR4 and TRAIL-R2/DR5) contain cyto-

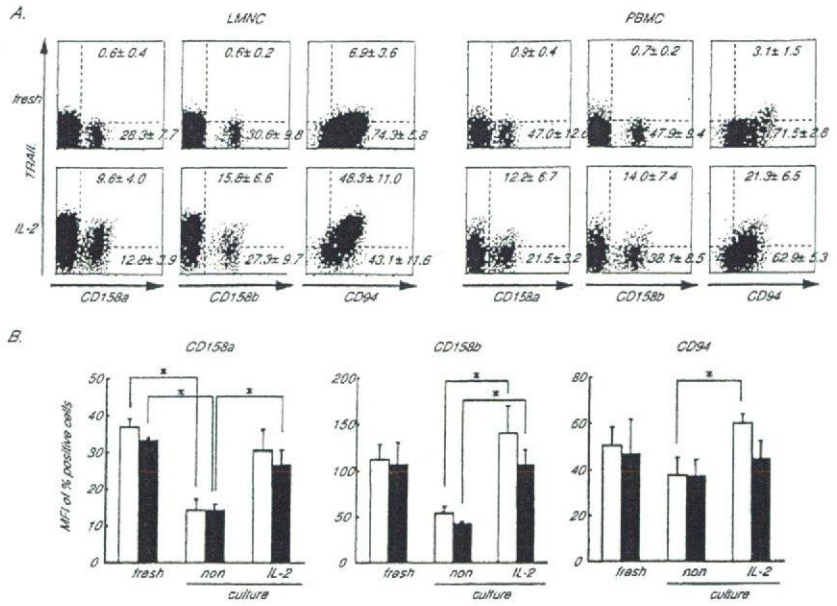

Fig. 3. IL-2 stimulation leads to maintenance of CD158a, CD158b, and CD94 expressions even on TRAIL expressing liver NK cells. Expression of various inhibitory receptors on the NK cells subsets among LMNCs or PBMCs freshly isolated or cultivated with or without IL-2 was analyzed. The LMNCs and PBMCs obtained from healthy donors were stained with CD3, CD56, and TRAIL MAbs together with CD-158a, CD-158b, or CD94 MAbs. (A) Expression of CD158a, CD158b, CD94, and TRAIL on electronically gated $CD3^-CD56^+$ NK cells were analyzed by FCM. Representative dot plots from freshly isolated NK cells (top panel) or IL-2–stimulated NK cells (second panel) obtained by FCM analysis. Percentages of $TRAIL^+$ NK and $TRAIL^-$ NK cells expressing killing inhibitory receptors are shown in the right upper and lower quadrants, respectively (mean ± SEM, n = 4 each). The results shown are representative of 4 independent experiments. (B) The percentages of cells staining positively for CD158a, CD158b, and CD94 on $CD3^-CD56^+$ NK cells are shown (LMNC; open column, PBMC; closed column). Data represent mean ± SEM of 4 cases per group. Statistical analyses were performed using ANOVA (*$P < .05$). IL-2, interleukin-2; NK, natural killer; TRAIL, TNF-related apoptosis-inducing ligand; LMNC, liver mononuclear cell; PBMC, peripheral blood mononuclear cell; MAb, monoclonal antibody; FCM, flow cytometric.

plasmic death domains and signal apoptosis, whereas two other death-inhibitory receptors (TRAIL-R3/DcR1 and TRAIL-R4/DcR2) lack a functional death domain and do not mediate apoptosis, all have similar affinities, and the latter may act as decoys.[20,21] Many cancer cell lines preferentially express TRAIL-DR4 and -DR5, suggesting differential regulation of the death and decoy receptors.[24] The preferential expression of these decoy receptors in normal tissue suggests that TRAIL may be useful as an anti-cancer agent that induces apoptosis in cancer cells while sparing normal cells. Recent studies have demonstrated that NK cells can destroy many solid tissue-derived malignant cells, such as melanoma, breast cancer, lung cancer, gastric cancer, colon cancer, renal cancer, and ovarian cancer cell lines, and that this process is mediated primarily by death receptor/ligand interactions.[34] We investigated the expression patterns of TRAIL-DR and -DcR on or in both normal liver tissues and HCC samples. As shown in Fig. 4A, the endothelial cells in normal liver tissues expressed TRAIL-DR4 and -DR5 together with TRAIL-DcR1 and -DcR2, but hepatocytes did not. Well-differentiated HCCs weakly expressed TRAIL-DR4, -DR5, -DcR1, and -DcR2. Moderately differentiated HCCs showed a higher expression of TRAIL-DR4 and -DR5 than well-differentiated HCCs but little TRAIL-DcR1 and DcR2. In particularly, poorly differentiated HCCs expressed remarkable levels of TRAIL-DR4 and -DR5 but did not express TRAIL-DcR1 and -DcR2, suggesting a susceptibility to TRAIL-expressing NK cell–mediated cell killing. To address this possibility, HepG2, an HCC cell line, could be used as target cells for natural killing activity of LMNCs, because HepG2 expressed high TRAIL-DR4 and -DR5 but no TRAIL-DcR1 and -DcR2, most resembling poorly differentiated HCCs (Fig. 4B).

IL-2–Stimulated Donor Liver NK Cells Showed the Vigorous Cytotoxicity Against HepG2. NK cell cytotoxicity assays using LMNCs and PBMCs isolated from same donors and recipients with liver cirrhosis as effectors and HepG2 as targets were performed. As shown in Fig. 5A, the freshly isolated donor LMNCs were able to mediate potent cytotoxicity against HepG2, whereas the recipient LMNCs and both donor and recipient PBMCs were not able to mediate cytotoxicity without stimulation. After IL-2 stimulation, the donor LMNCs showed the most vigorous natural cytotoxicity against HepG2 (90.5% ± 2.2% at E:T = 10:1), when compared with the donor and recipient PBMCs and the recipient LMNCs

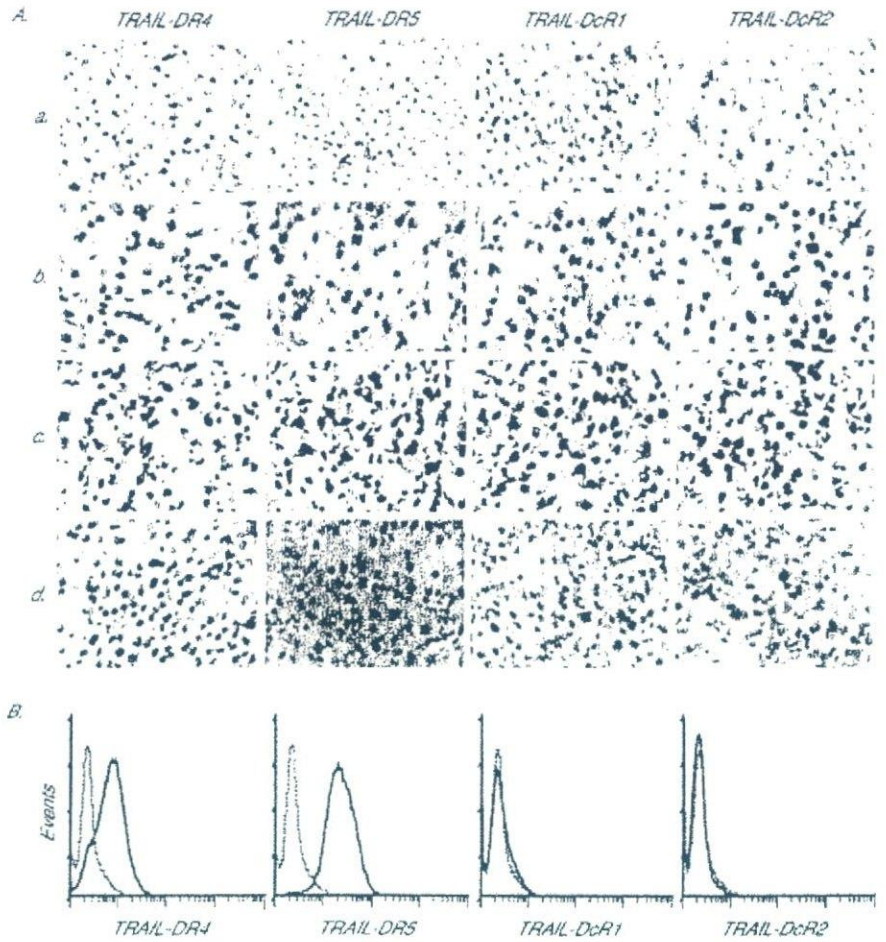

Fig. 4. Differential expression of TRAIL receptors in normal liver tissue and HCC tissue. (A) Immunohistochemical expression of TRAIL-DR4, -DR5, -DcR1, and -DcR2 in normal liver tissue (a), tumor site of well-differentiated HCCs (b), moderately differentiated HCCs (c), and poorly differentiated HCCs (d). Magnification ×400. Immunopathological findings shown are representative of 3 individual samples in each categorized HCCs. (B) Surface expression of TRAIL receptors on the surface of HepG2 was analyzed by FCM. Dotted lines represent negative control staining with isotype-matched MAbs. HepG2 expressed high TRAIL-DR4 and -DR5 but no TRAIL-DcR1 and DcR2, resembling poorly differentiated HCCs. TRAIL, TNF-related apoptosis-inducing ligand; HCC, hepatocellular carcinoma; FCM, flow cytometric; MAb, monoclonal antibody; TNF, tumor necrosis factor.

(64.8% ± 8.2%, 56.1% ± 8.9%, and 34.6% ± 7.5%, respectively) (Fig. 5B). By magnetic sorting, NK and non-NK cells were isolated from donor LMNCs and PBMCs and resulting populations were then analyzed for cytotoxicity against HepG2. As expected, the non-NK cell fraction did not mediate cytotoxicity. The higher cytotoxicity of NK cells isolated from LMNCs was observed than that of NK cells from PBMCs (Fig. 5C). Addition of neutralizing anti-TRAIL MAb partially reduced the cytotoxicity of IL-2–stimulated donor NK cells toward HepG2 cells (24.2% ± 9.1% reduction at E:T = 8:1, data not shown), indicating that TRAIL-mediated NK cell cytotoxicity was involved. Despite strong cytotoxicity of IL-2–stimulated donor liver NK cells, the cytotoxicities of those cells toward 1-haplotype identical allogeneic recipient- and autologous donor-derived lymphoblasts were negligible, indicating their capacity to distinguish tumor cells from normal cells (Fig. 5D).

To determine whether liver non-NK cells are responsible for the increased anti-tumor activity of liver NK cells, we performed additional experiments mixing PB NK and non-NK cells isolated from either LMNCs or PBMCs. Before IL-2 stimulation, NK and non-NK cells were isolated from donor LMNCs and same donor PBMCs. Then, for subsequent cytotoxic assays, PB NK cells were cultured with either liver non-NK or PB non-NK cells at the same ratio in the presence of IL-2. Even when PB NK cells were cultured with liver non-NK cells in the presence of IL-2, PB NK cells expressed little TRAIL (Fig. 6A). No difference in cytotoxicity against HepG2 was observed between a mixture of PB non-NK and PB NK cells and one of liver non-NK and PB NK cells (Fig. 6B).

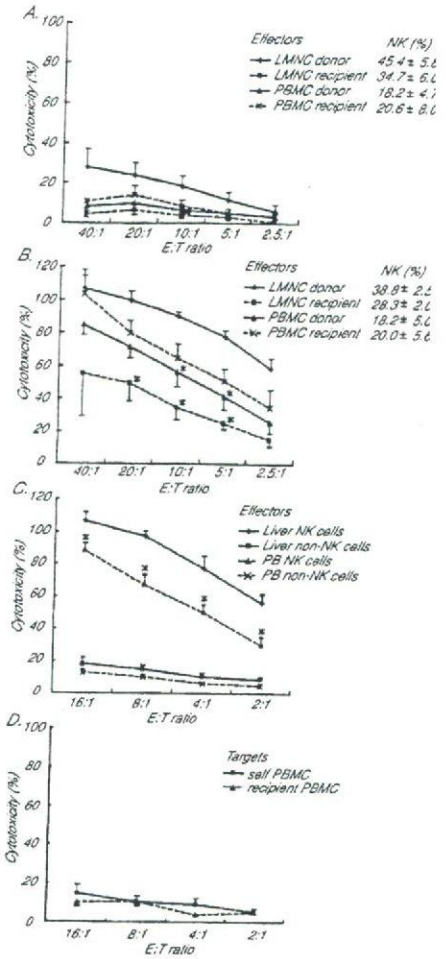

Fig. 5. IL-2-stimulated donor liver NK cells show vigorous cytotoxicity against HepG2. NK cytotoxic activities of indicated effectors against indicated target cells were analyzed by ^{51}Cr release assay. All data are represented as the mean ± SEM of assays, each set up in triplicate. Statistical analyses were performed using ANOVA (*$P < .05$). (A) NK cytotoxic activities of freshly isolated LMNCs and PBMCs obtained from liver transplant donors and recipients against HepG2 target cells (n = 4 each). Percentages of $CD3^-CD56^+$ NK cells in LMNCs or PBMCs obtained from 4 adult healthy donors and 4 corresponding recipients with cirrhosis are shown in the upper right corner (mean ± SEM). (B) NK cytotoxic activities of IL-2-stimulated LMNCs and PBMCs obtained from liver transplant donors and recipients against HepG2 target cells. LMNCs and PBMCs were cultivated for 4 days in the presence of IL-2 before the cytotoxicity assay (n = 4 each). Percentages of $CD3^-CD56^+$ NK cells in LMNCs or PBMCs obtained from 4 adult healthy donors and 4 corresponding recipients with cirrhosis are shown in the upper right corner (mean ± SEM). (C) NK cytotoxic activities of NK and non-NK cells isolated from IL-2-stimulated donor LMNCs and PBMCs against HepG2 target cells (n = 4 and 5, respectively). (D) NK cytotoxic activities of NK cells isolated from IL-2-stimulated donor LMNCs against autologous and allogeneic lymphoblasts, which had been prepared from PBMCs of donors and corresponding recipients by cultivating with PHA for 4 days (n = 4). IL-2, interleukin-2; NK, natural killer; LMNC, liver mononuclear cell; PBMC, peripheral blood mononuclear cell.

These findings are consistent with a model whereby donor LMNCs have greater NK activity because NK cells from LMNCs are more functional on a per cell basis than those from PBMCs, and that liver non-NK cells are not responsible for such altered function of liver NK cells in our experimental system.

Discussion

The role of liver transplantation in patients with HCC has evolved over the past 2 decades, and transplantation has become one of the few curative treatment modalities for patients with unresectable HCC.[35,36] LDLT has become an acceptable therapy for patients with HCC in response to the pervasive shortage of deceased donor livers. In addition, waiting time for HCC patients to receive a deceased donor has decreased significantly, and the number of patients dropping out from the waiting list has decreased because of a decrease of advanced-stage disease. As a result, this may decrease the progression of disease so

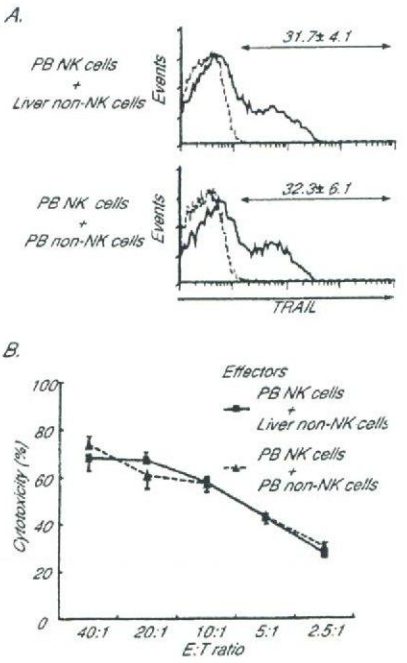

Fig. 6. Liver non-NK cells are not responsible for the increased anti-tumor activity of NK cells. Before IL-2 stimulation, NK and non-NK cells were isolated from donor LMNCs and same donor PBMCs. Then, NK cells from PBMCs were cultured with either liver non-NK or peripheral blood (PB) non-NK cells at the same ratio (3: 7) in the presence of IL-2 for subsequent NK cytotoxic assays. (A) Histograms represent the log fluorescence intensities obtained on staining for TRAIL after gating on the $CD3^-CD56^+$ NK cells subsets obtained from each group. Dotted lines represent negative control staining with isotype-matched MAbs. The numbers indicate the percentages of cells in each group that were positive for TRAIL expression (mean ± SEM, n = 3 each). Histogram profiles shown are representative of 3 independent experiments. (B) NK cytotoxic activities of IL-2-stimulated PB NK cells cultured with either liver non-NK or PB non-NK cells against HepG2 target cells. Data are represented as the mean ± SD of triplicate samples and similar results were obtained in 3 independent experiments. IL-2, interleukin-2; NK, natural killer; LMNC, liver mononuclear cell; PBMC, peripheral blood mononuclear cell; TRAIL, TNF-related apoptosis-inducing ligand.