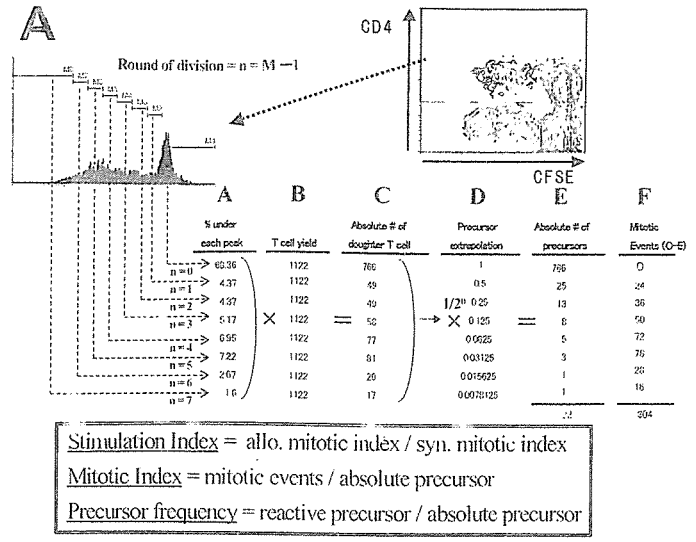
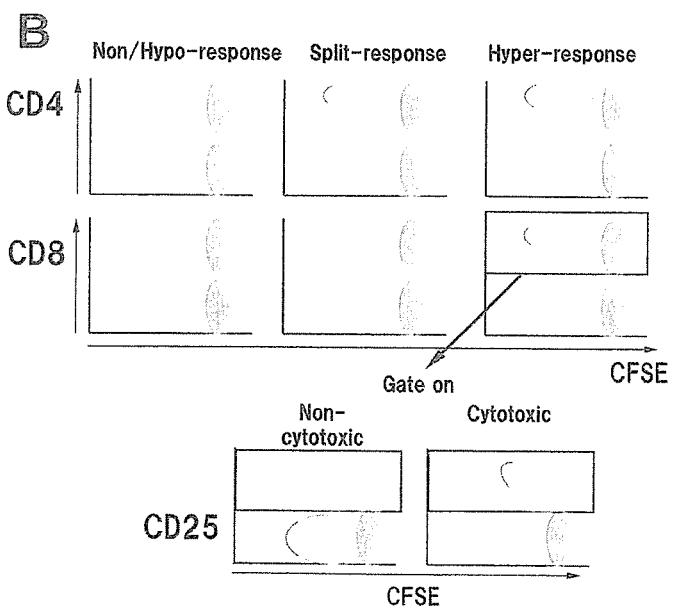
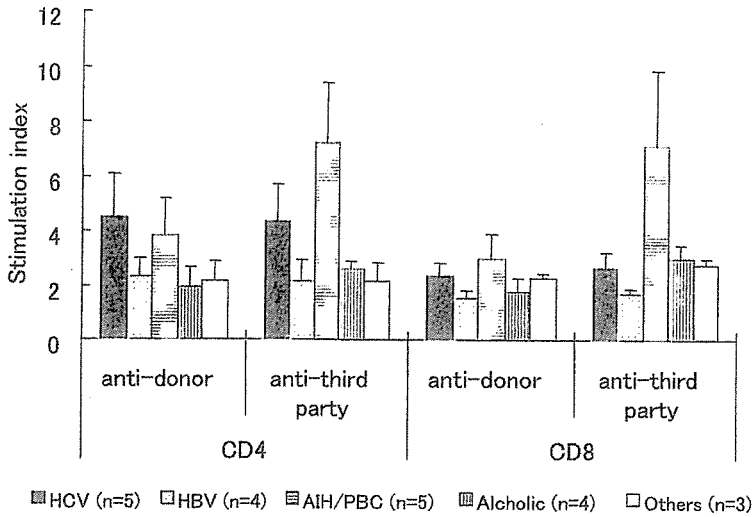


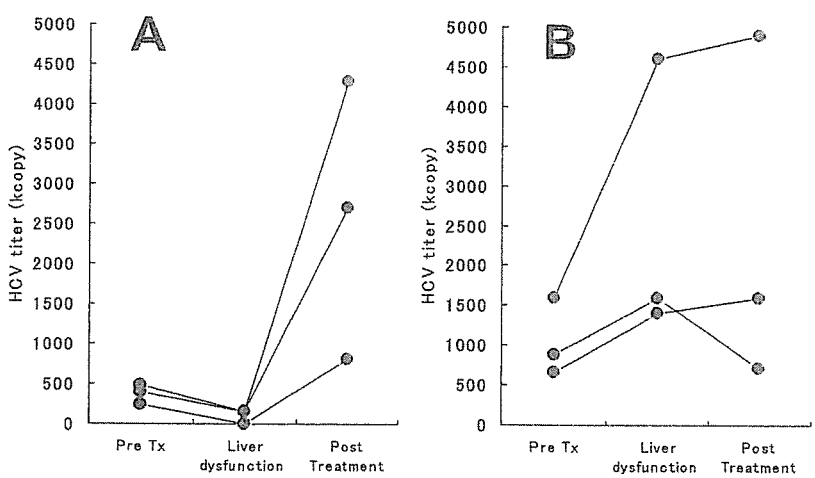


(A) ドナーの末梢血リンパ球を承諾の上採取して stimulator として使用した。また，健常ボランティアの末梢血リンパ球を third party stimulator として使用した。レシピエントの末梢血リンパ球を CFSE 染色し one-way MLR を施行した。MLR の解析には FCM を用い，FL-1 で CFSE の減衰による細胞分裂・増殖を，FL-2 と FL-4 で反応性 T 細胞のフェノタイプ（CD4，CD8，CD25）を評価し，FL-3 で PI 染色による生細胞抽出を行った。本法では，CD4 と CD8 T 細胞別に stimulation index（SI）の算出と CD 25 表出の定量評価が可能である。

(B) 横軸は CFSE，縦軸は CD4，あるいは CD8 の表出強度を示す。CD4，CD8 がともに分裂増殖を示さない場合，CFSE intensity の低下は認めず，アロ反応性に乏しい状態で，免疫抑制状態は適正あるいは過剰と判断する。次に，CD4 は分裂増殖するが，CD8 は反応していないとき，潜在的感作状態であることが示唆される。また，CD4，CD8 ともに分裂増殖を示すとき，現在進行中あるいは今後起こりうる拒絶反応を示唆する。同時に CD8T 細胞の CD25 の表出を解析すると，ドナーに対し特異的に反応しているものは，CD25 を表出し，非特異的に反応しているものは，CD25 の表出を認めない。われわれは，CD25（IL-2 R）抗体陽性 CD8T 細胞のみに細胞障害性を認めることを確認しているので，これが拒絶反応の特異的指標となりうると考えられる。

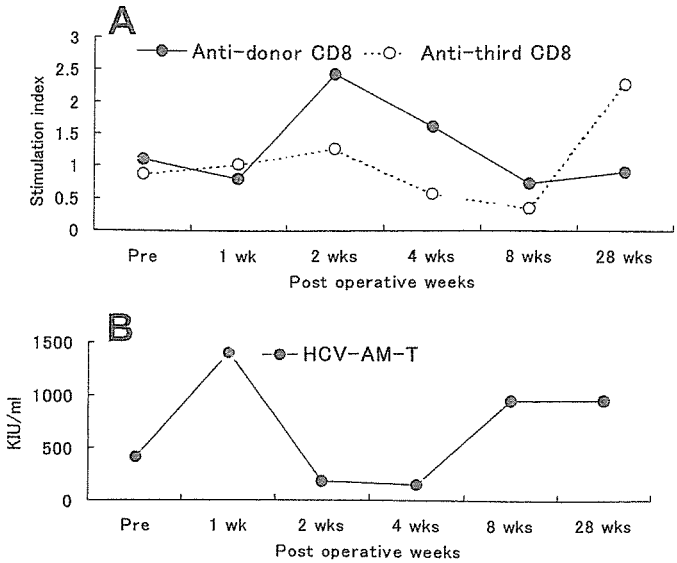


2004年8月～2006年2月の期間に術前CFSE-MLRを施行した患者21名を対象に検討した。CD4およびCD8T細胞別にドナーおよびサードパーティーに対するstimulation indexを原疾患別に比較検討した。
HCV: hepatitis C virus, HBV: hepatitis B virus, AIH: autoimmune hepatitis, PBC: primary biliary cirrhosis



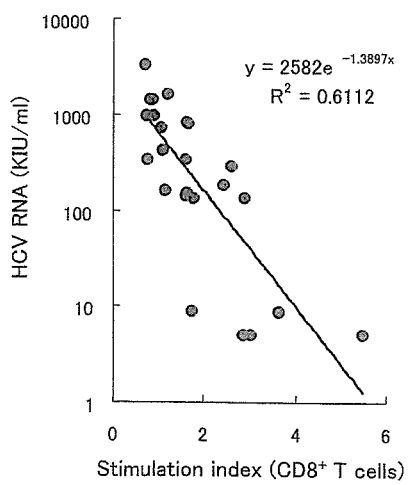
肝移植後1年以内に血液化学検査で肝機能異常を認め、急性拒絶反応を疑った症例は6例であった。
(A) CFSE-MLRで抗ドナー応答の亢進を認め急性拒絶反応と診断した3例の血清HCV RNA量。
(B) CFSE-MLRで抗ドナー応答の亢進を認めず急性拒絶反応が否定された3症例の血清HCV RNA量。

の関係は3例とも同様で，急性拒絶反応診断時にはHCVウイルス量が低下，拒絶治療後に急上昇していた（**図3**）。一方，抗ドナー応答の亢進を認めず急性拒絶反応を否定された3症例では，肝機能異常時にHCV RNA量が上昇していた。肝機能異常は遷延したが，その後のIFN療法により改善した。以上のように，HCV RNA量はアロ免疫応答と密接に関係し増減した。**図4**では，肝移植後抗ドナーCD8T細胞のstimu-



肝移植後 CFSE-MLR で定量化した抗ドナーおよび抗サードパーティー CD8T 細胞の stimulation index (A) と HCV RNA 量（B）の推移を比較した。

lation index と HCV RNA 量の推移を比較した。術後第 1 週では，導入期の強い免疫抑制のため抗ドナー応答は抑制されているが，第 2 週では亢進している。その後カルシニューリン抑制剤の血中濃度の安定に伴い抗ドナー応答は抑制された。HCV RNA 量はこの抗ドナー CD8T 細胞の stimulation index と鏡像を示して推移した。C 型肝炎患者に対する生体肝移植症例 12 例において術後 3 カ月以内（IFN 治療開始前）に施行した 31 回の CFSE-MLR から求めた抗ドナー CD8T 細胞 stimulation index と CFSE-MLR 施行時の HCV RNA 量を比較したところ，有意な逆相関が確認された（R^2= 0.61）（**図 5**）。この興味深い現象は，アロ免疫応答と抗 HCV 免疫応答との間にクロストークが存在する可能性と，あるいは T 細胞のアロ免疫応答の非特異的抑制が抗 HCV 応答も同等に抑制している可能性を示唆する。後者の場合，アロ免疫応答の特異的制御が可能であれば，抗 HCV 免疫応答を保持しつつ拒絶反応を防止しうるであろう。



C 型肝炎患者に対する生体肝移植症例 12 例において術後 3 カ月以内（IFN 治療開始前）に施行した 31 回の CFSE-MLR から求めた抗ドナー CD8T 細胞 stimulation index と CFSE-MLR 施行時の HCV RNA 量を比較した。

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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 histocompatobility 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 (×10⁵/ g)	HLA A	HLA B	HLA C	Relation	Age(y)/ Sex	Child Pugh	MELD	Original Disease	HCC	Liver Wt(g)	LMNC (×10⁵/ 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.

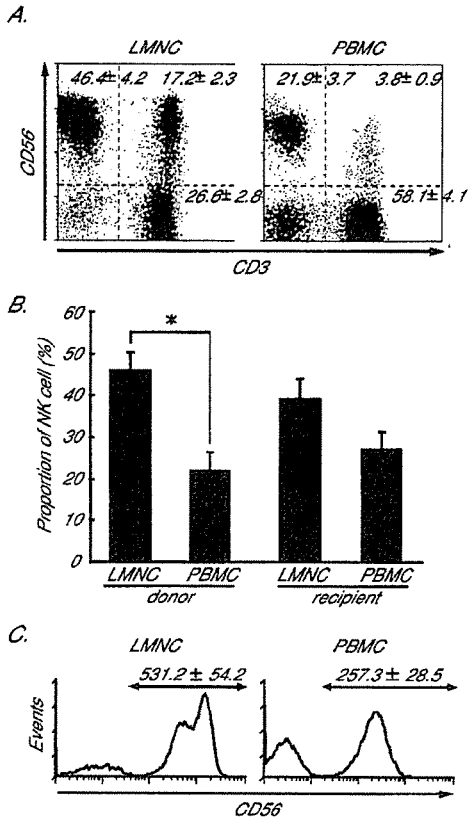

Fig. 1. Human liver mononuclear cell extracted from liver perfusates contain a large population of NK cells. (A) Flow cytometric (FCM) analysis of freshly isolated LMNCs obtained from liver perfusates and PBMCs from same donor after staining with MAbs against CD3 and CD56 were analyzed. Lymphocytes were 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-

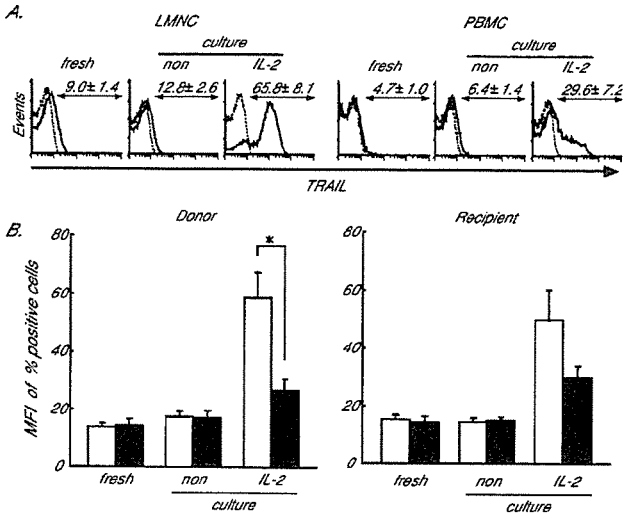

Fig. 2. Liver NK cells inductively express remarkable levels of TRAIL, but PB NK cells do not. Freshly isolated or cultivated with or without IL-2 LMNCs and PBMCs obtained from healthy donors and corresponding recipients were stained with CD3 and CD56 MAbs together with TRAIL MAb. (A) Histograms represent 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-

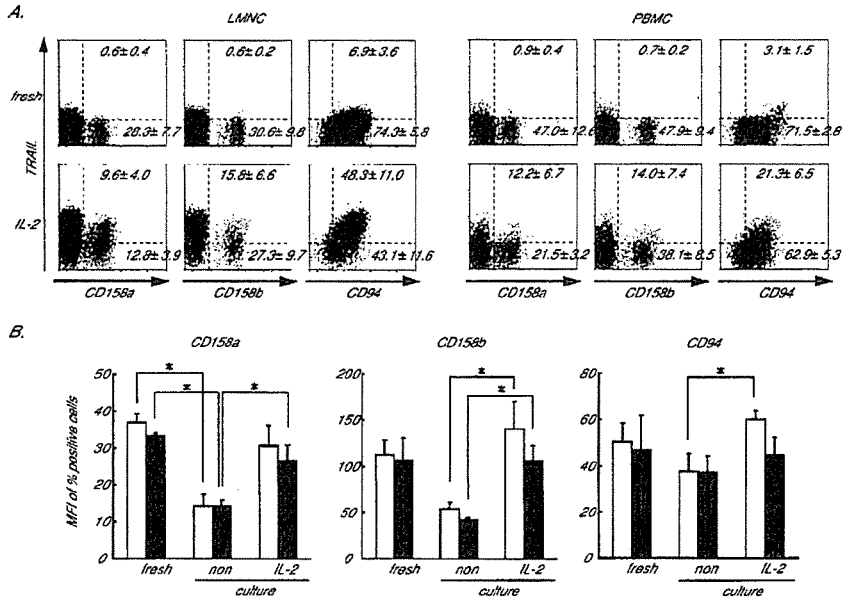

Fig. 3. IL-2 stimulation leads to maintenance of CD158a, CD158b, and CD94 expressions even on TRAIL expressing liver NK cells. Expression of various inhibitory receptors on the NK cells subsets among LMNCs or PBMCs freshly isolated or cultivated with or without IL-2 was analyzed. The LMNCs and PBMCs 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

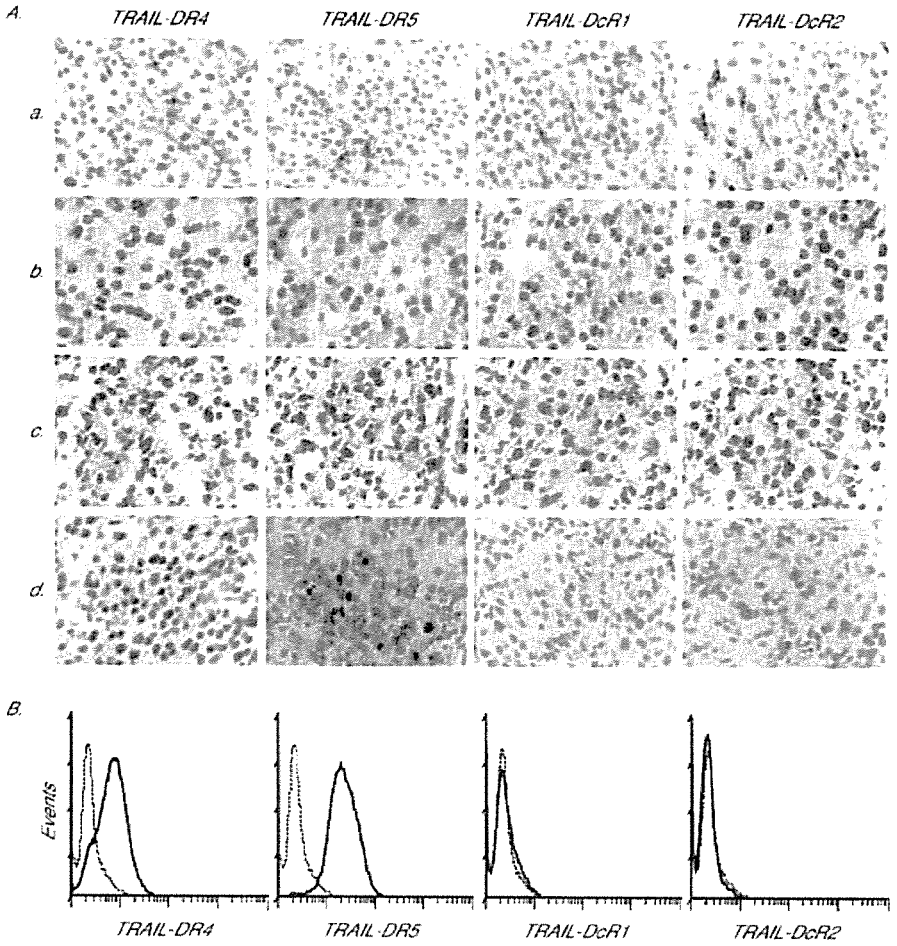

Fig. 4. Differential expression of TRAIL receptors in normal liver tissue and HCC tissue. (A) Immunohistochemical expression of TRAIL-DR4, -DR5, -DcR1, and -DcR2 in normal liver tissue (a), tumor site of well-differentiated HCCs (b), moderately differentiated HCCs (c), and poorly differentiated HCCs (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).

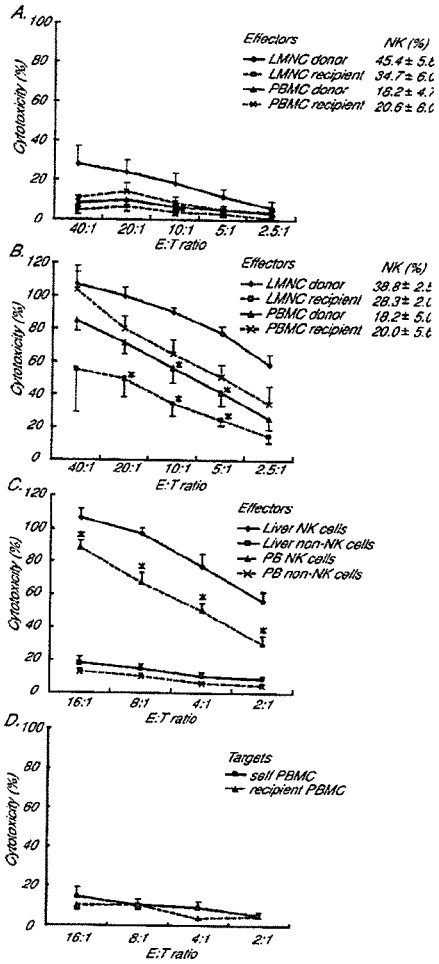

Fig. 5. IL-2-stimulated donor liver NK cells show vigorous cytotoxicity against HepG2. NK cytotoxic activities of indicated effectors against indicated target cells were analyzed by ^{51}Cr release assay. All data are represented as the mean ± SEM of assays, each set up in triplicate. Statistical analyses 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

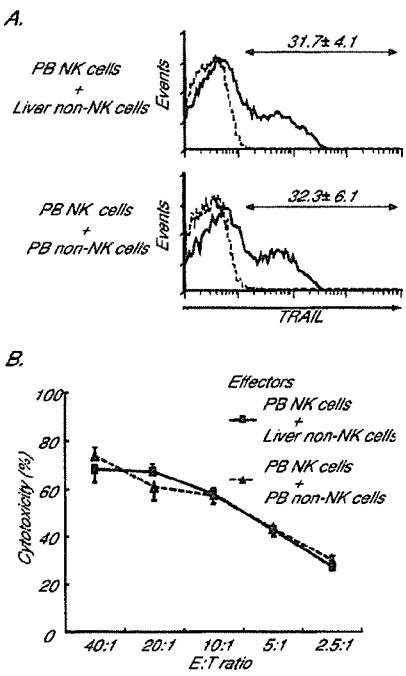

Fig. 6. Liver non-NK cells are not responsible for the increased anti-tumor activity of NK cells. Before IL-2 stimulation, NK and non-NK cells were isolated from donor LMNCs and same donor PBMCs. Then, NK cells from PBMCs were cultured with either liver non-NK or peripheral blood (PB) non-NK cells at 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.

that the recurrence rate should be lower than for recipients who wait for an organ from a deceased donor.[37] When liver transplantation is performed as a therapy for HCC, recurrent HCC is nevertheless one of the most fatal complications. The management for the prevention of organ rejection requires the use of postoperative immunosuppressive therapy; however, immunosuppressants increase the incidence of recurrence or metastasis of cancer and induce cancer progression. The immunosuppressive regimen currently used after liver transplantation, consisting of tacrolimus/cyclosporine and methylprednisolone, reduces adaptive components of cellular immunity (predominantly T cell–mediated immune responses), while maintaining innate components of cellular immunity.[3,38,39] Because immune surveillance against tumors is mediated by both innate and adoptive components of cellular immunity, augmentation of NK cell responses to tumors, which have been thought to play a central role in innate immunity against tumors, might be a promising immunotherapy approach. Several therapeutic cytokines including IL-2 and IFNs primarily act through NK cells, and many studies have shown that activation of NK cell differentiation and function leads to a more efficient elimination of tumor growth[5-9]; however, the systemic administration of those cytokines is likely associated with an acceleration of alloimmune responses leading to liver allograft rejection. Hence, the adoptive transfer of cytokine-modulated NK cells might be a reasonable approach in preventing recurrence of HCC after liver transplantation, while minimizing effects on alloimmune responses.

NK cells in the blood stream are ready to kill any cell.[1,2] Healthy cells are spared by their MHC class I molecules, which bind to corresponding inhibitory receptors on NK cells so that only cells with altered or lacking MHC class I molecules, a mechanism to escape recognition by MHC-restricted $CD8^+$ T lymphocytes, are killed because of missing inhibitory mechanisms.[32,33,40,41] The cytotoxic activities of NK cells are controlled by a variety of receptors including CD94/NKG2 and KIRs (CD158a/CD158b), which bind to respective MHC class I molecules on target cells. NK cells can discriminate not only between different class I molecules but also between certain allotypes that differ in single amino acid substitutions at positions 77 and 80 in the 1 domain of the 2 HLA-C groups.[42,43] This process can contribute to detection of transformed cells because downregulation of selected allotypes is an event often occurring in the progression of some tumor types. The group of inhibitory receptors is reported to have higher affinity of ligand binding than the activating group, and thus the inhibitory receptors may play a more dominant role in regulating the cytotoxic activities of NK cells.[44] HCC cell lines, such as HepG2 and HuH-7, have been reported to lose or decrease the expression of HLA-B and -C alleles on the cell surface.[23,45] The only requirement for NK cell receptor repertoire development appears to be that every NK cell express at least one inhibitory receptor specific for autologous HLA class I, thereby ensuring tolerance against healthy cells sharing 1-haplotype MHC molecules.[46,47]

In the current study, we have determined functional properties of liver NK cells extracted from donor and recipient liver perfusates in clinical LDLT. Liver NK cells have never been used for the adoptive transfer to mount anti-tumor activity, because of the limited availability of liver NK cells in a clinical setting. In liver transplantation, *ex vivo* perfusion of the liver through the portal vein should inevitably be done for flushing blood from the liver graft before implantation to avoid intragraft coagulation. We have demonstrated that liver perfusates, which usually are thrown away, contain large amounts of NK cells. Donor liver NK cells showed the most vigorous cytotoxicity against a HCC cell line after *in vitro* IL-2 stimulation, when compared with donor and recipient PB NK cells and recipient liver NK cells. The higher anti-tumor activity of liver NK cells than PB NK cells has been well demonstrated in mice, although mechanisms underlying this fact remain unclear.[15,16] The consistent results were observed in healthy donors, but not in recipients with cirrhosis. The cytotoxicity of PB NK cells against HepG2 per se did not differ between donors and recipients, but that of liver NK cells from livers with cirrhosis were significantly impaired, regardless of the presence or absence of IL-2 stimulation.

Previously published data redefine NK cells as potent constitutive immune effectors, which are able to use not only the perforin-mediated secretory/necrotic mechanism to kill rare leukemia cell targets, but also a powerful TNF family ligand-mediated nonsecretory apoptotic mechanism to destroy most solid tumor cell targets.[34] TRAIL is highly expressed on most NK cells after stimulation with IL-2, IFNs, or IL-15 in mice.[9,19,48] Neutralization of TRAIL additively enhanced liver metastasis in perforin-deficient mice but not in IFN-γ–deficient mice.[9] These findings clearly place perforin and TRAIL as the 2 key cytotoxic effector pathways used by NK cells. From the current study in human, we now appreciate that freshly isolated liver NK cells barely express a detectable level of TRAIL on their surface, but a remarkable level of TRAIL expression can be induced, preferentially on liver NK cells, by stimulation with IL-2. Taken together with the finding that poorly differentiated HCCs highly express the death-inducing TRAIL receptors (DR 4 and DR5), which contain cytoplasmic death domains and signal ap-

optosis, as well as HepG2, adoptive transfer of IL-2–stimulated NK cells extracted from donor liver grafts are likely to mount an anti-tumor response without causing toxicity against recipient intact tissue, at least in 1-haplotype identical combination. If this approach were to be used in clinical applications, it should be taken into account that T cells contaminants in donor LMNCs might cause graft-versus-host responses after adoptive transfer to the corresponding recipient. Hence, we confirmed that *in vitro* treatment with muromonab-CD3 (Janssen-Cilag, The Netherlands) leads to complete ligation of MAbs on $CD3^+$ T and $CD3^+CD56^+$ NKT cells in IL-2–stimulated LMNCs (data not shown). Such MAbs-binding cells should be opsonized *in vivo* after adoptive transplantation. Furthermore, this treatment did not attenuate cytotoxic activity of IL-2–stimulated donor LMNCs against HepG2 (data not shown).

The mean yield of NK cells from donor liver perfusates was $0.5 \pm 0.1 \times 10^6$ cells/g liver-weight in this study. Because graft weight and graft-to-recipient body weight ratio ranged from 262 to 900 g (mean weight, 579 ± 132 g) and from 0.51% to 1.42% (mean ratio, 0.97% ± 0.17%), respectively, a maximum of 100 to 250×10^6 NK cells can be used for the adoptive transfer even without further treatment to promote their proliferation. The optimal dose of liver NK cells needs to be elucidated to bring this approach closer to clinical application.

In conclusion, liver NK cells inductively express TRAIL. IL-2–stimulated donor liver NK cells have the vigorous cytotoxicity against HCC.

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Induction of endotoxin tolerance inhibits alloimmune responses

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Abstract

It was recently reported that the induction of endotoxin tolerance (ET), which is defined as a reduced response to a lipopolysaccharide (LPS) challenge following the first LPS encounter, inhibits major histocompatibility complex (MHC)-restricted antigen presentation. This raises the question whether alloimmune responses can be inhibited by inducing ET in transplant donors. C57BL/6 mice were treated with a low dose of LPS prior to a challenge with a high dose of LPS to induce ET. Hearts from endotoxin-tolerized C57BL/6 mice were transplanted to BALB/c mice. The survival of the endotoxin-tolerized heart allografts was significantly prolonged. By using irradiated splenocytes from C57BL/6 mice and allogeneic splenocytes from BALB/c mice, a mixed lymphocyte reaction (MLR) assay was performed. The MLR assay used CFSE, and revealed that the splenocytes from the endotoxin-tolerized mice failed to induce the proliferation of allogeneic $CD4^+$ and $CD8^+$ T cells. Cytokine analyses of the supernatant of the MLR culture using endotoxin-tolerized stimulators revealed a distinct shift in the Th 1/Th 2 balance toward the Th 2-type response. The induction of ET increased the proportion of myeloid-related dendritic cells (DCs) expressing molecules necessary for antigen presentation, which favor the development of a Th 2 response; however, it reduced the proportion of lymphoid-related DCs expressing those molecules, which favor the development of the Th 1 response. Although the relevance of these findings with regard to the prolonged survival of the endotoxin-tolerized heart allografts remains to be elucidated, this is the first study to demonstrate that the induction of ET in donor animals inhibits alloimmune responses.

Keywords: Endotoxin tolerance; Mixed lymphocyte reaction; Alloimmune response; Transplantation

1. Introduction

Lipopolysaccharide (LPS), endotoxin, can stimulate the release of a number of proinflammatory mediators by macrophages and monocytes [1,2]. Although LPS is a potent cellular activator, repeated LPS challenge may desensitize the cells to subsequent LPS stimulation. This phenomenon known as endotoxin tolerance (ET) has been demonstrated *in vivo* by reduced LPS lethality in rodents that were pretreated with a low dose of LPS [3,4] and *in vitro* by the reduced capacity of isolated macrophages or monocytes to release proinflammatory cytokines after repeated LPS stimulation [5,6]. ET is considered to be an adaptive response that evolved as a protective mechanism to curb the infected host's continuous response to invading bacterial organisms, and thereby reduced the auto-toxic effect precipitated by the overproduction of inflammatory mediators [7,8].

In addition to the altered capacity of monocytic cells to produce soluble immunomodulators during ET, the impaired capacity of endotoxin-tolerized monocytes to stimulate various T cell responses has been recently demonstrated [7]. It has been demonstrated that *in vitro* priming of peripheral blood mononuclear cells using LPS caused marked down-regulation of major histocompatibility complex (MHC) class II and costimulatory molecules. A similar phenomenon has been observed in dendritic cells (DCs), which are the most potent antigen presenting cells (APCs), during experimental ET [9].

Abbreviations: ET, endotoxin tolerance; LPS, lipopolysaccharide; MHC, major histocompatibility complex; CFSE, carboxyfluorescein diacetate succinimidyl ester; MLR, mixed lymphocyte reaction; SI, stimulation index; FCM, flow cytometry; APC, antigen presenting cell; DC, dendritic cell.

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Although LPS has been used to induce resistance against subsequent identical insults, it has also been demonstrated that LPS induces cross-tolerance against other types of insults. For instance, LPS-derived protection has been demonstrated against ischemia/reperfusion injury in the kidney and liver [10,11]. This observation indicates the possibility of a novel approach–using synthetic LPS analogs–to prevent ischemia/reperfusion injury to cadaveric organ grafts that are used for transplantation [12,13]. For this concept to be clinically applicable, the possible interference of the LPS preparation in subsequent alloimmune responses following transplantation needs to be elucidated. Considering the phenotypical alterations in the endotoxin-tolerized APCs described above, it might be possible that inducing ET in transplant donors is beneficial, even in terms of preventing an allograft rejection reaction of organs. In the present study, we investigated whether ET induced transplant donors can inhibit alloimmune responses by using a murine system.

2. Objective

ET has been demonstrated *in vivo* by reduced LPS lethality in rodents pretreated with a low dose of LPS and *in vitro* by the reduced capacity of isolated macrophages or monocytes to release proinflammatory cytokines after repeated LPS stimulation. Although LPS has been used to induce resistance against subsequent identical insults, it has also been demonstrated that LPS induces cross-tolerance against other insults. It was recently reported that ET induction inhibits MHC-restricted antigen presentation. This raises the question whether alloimmune responses can be inhibited by ET induction in transplant donors. This study was conducted to investigate whether ET induction inhibits alloimmune responses *in vivo* and *in vitro*.

3. Materials and methods

3.1. Animals

Female C57BL/6J (B6) (H-2^b) mice and BALB/c (H-2^d) mice were purchased from Clea Japan, Inc. (Osaka, Japan). All animals were maintained in a specific pathogen-free microisolator environment and were used at 10 weeks of age. Animal experiments were conducted according to the guidelines of the National Institutes of Health (NIH publication no. 8623, revised 1985).

3.2. Mice treatment protocol

In order to induce ET, B6 mice were pretreated as follows: protocol 1, intravenous injection of low doses of *Escherichia coli* LPS (serotype 0111:B4; Sigma, Saint Louis, MO) (20 μg/mouse) three times on days −6, −3 and 0; protocol 2, intravenous injection of low doses of LPS twice on days −6 and −3 and of a high dose of LPS (200 μg/mouse) on day 0. B6 mice that were injected PBS (Nissui, Tokyo, Japan) on days −6, −3 and 0 served as controls.

3.3. Flow cytometric (FCM) analysis

To quantify the expression of DC surface molecules, splenocytes were stained with the following monoclonal antibodies (mAbs) after erythrocyte lysis with ammonium chloride/potassium solution: FITC-conjugated anti-I-A/I-E mAb (2G9), anti-CD40 mAb (HM40-3), anti-CD80 mAb (16-10A1), anti-CD86 mAb (GL1) (BD PharMingen, San Diego, CA), anti-CD11b mAb (M1/70) (eBioscience, San Diego, CA), PE-conjugated anti-CD11b mAb (M1/70) (BD PharMingen), anti-Fas ligand (FasL) mAb (MFL3) (eBioscience) and biotin-conjugated anti-CD11c mAb (HL-3) (BD PharMingen). The biotinylated mAbs were visualized with allophycocyanin (APC)–streptavidin (BD PharMingen). For all analyses, non-specific FcγR binding of labeled Abs was blocked by CD16/32 (2.4G2) (BD PharMingen). Dead cells were excluded from the analysis by light-scatter and propidium iodide staining. All analyses were performed using a FACSCaliber® cytometer (Becton Dickinson, Mountain View, CA).

3.4. Mixed lymphocyte reaction (MLR) assay

The splenocytes used as responder cells were obtained from BALB/c mice and labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR), and those used as stimulator cells were prepared from B6 mice, each mouse was exposed to 30 Gy radiation. Due to CFSE's property of being equally distributed to daughter cells upon division, this dye can be used to study lymphocyte division kinetics and differentiation. CFSE labeling was performed as described previously [14]. After culturing the cells for the MLR assay, the harvested cells were stained with PE-conjugated anti-CD4 mAb (RM4-5) or anti-CD8 mAb (53-6.7) (BD PharMingen). The proliferation of the CFSE-labeled lymphocytes after the MLR assay was detected in a multi-parameter FCM setting (FACSCaliber® cytometer).

Stimulation index (SI) as alloreactivity of responder $CD4^+$ and $CD8^+$ T cells were quantified by their CFSE fluorescence intensities modified as reported previously [14]. In brief, $CD4^+$ and $CD8^+$ T cells were selected by gating on CFSE fluorescence histograms and were analyzed for CFSE fluorescence. Theoretically, the CFSE fluorescence intensity of cells that have divided once is half that of non-divided cells. According to this theory, the number of divisions of alloreactive T cells can be mathematically determined by the logarithmic CFSE intensities on the basis of the peak at the extreme right (the peak of undivided cells). 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 resulting from each division and the mitotic events occurring in each $CD4^+$ and $CD8^+$ T cell subset were calculated. Using these values, the mitotic indexes (MI) were calculated by dividing the total number of mitotic events by the total number of precursors.

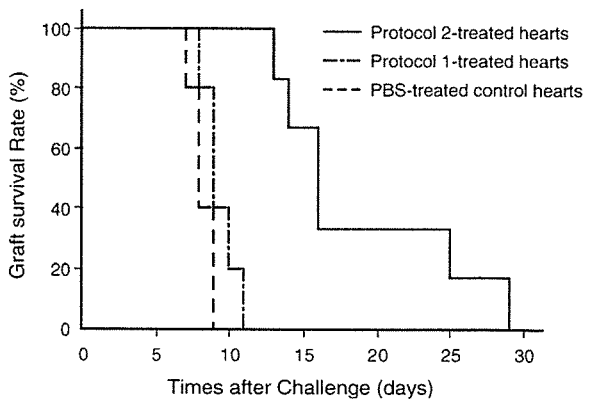


Fig. 1. The survival of endotoxin-tolerized heart allografts was significantly prolonged as compared with that of untreated heart allografts. B6 mice were pretreated as follows: protocol 1, intravenous injection with low doses of LPS (20 μg) three times on days −6, −3 and 0; protocol 2, intravenous injection with low doses of LPS twice on days −6 and −3 and with a high dose of LPS (200 μg) on day 0. B6 mice that were injected with PBS on days −6, −3 and 0 served as controls. The hearts were harvested for heterotopic transplantation into BALB/c mice 3 days after the final administration of LPS. The survival curves of the heart allografts are shown—heart grafts from B6 mice treated with protocol 2 ($n=6$) and heart grafts from B6 mice treated with protocol 1 ($n=5$). PBS-treated control B6 mouse heart grafts ($n=5$). Graft survival curves were calculated by the Kaplan–Meier method, with differences between groups compared by the log-rank test. $p<0.01$ by log-rank test.

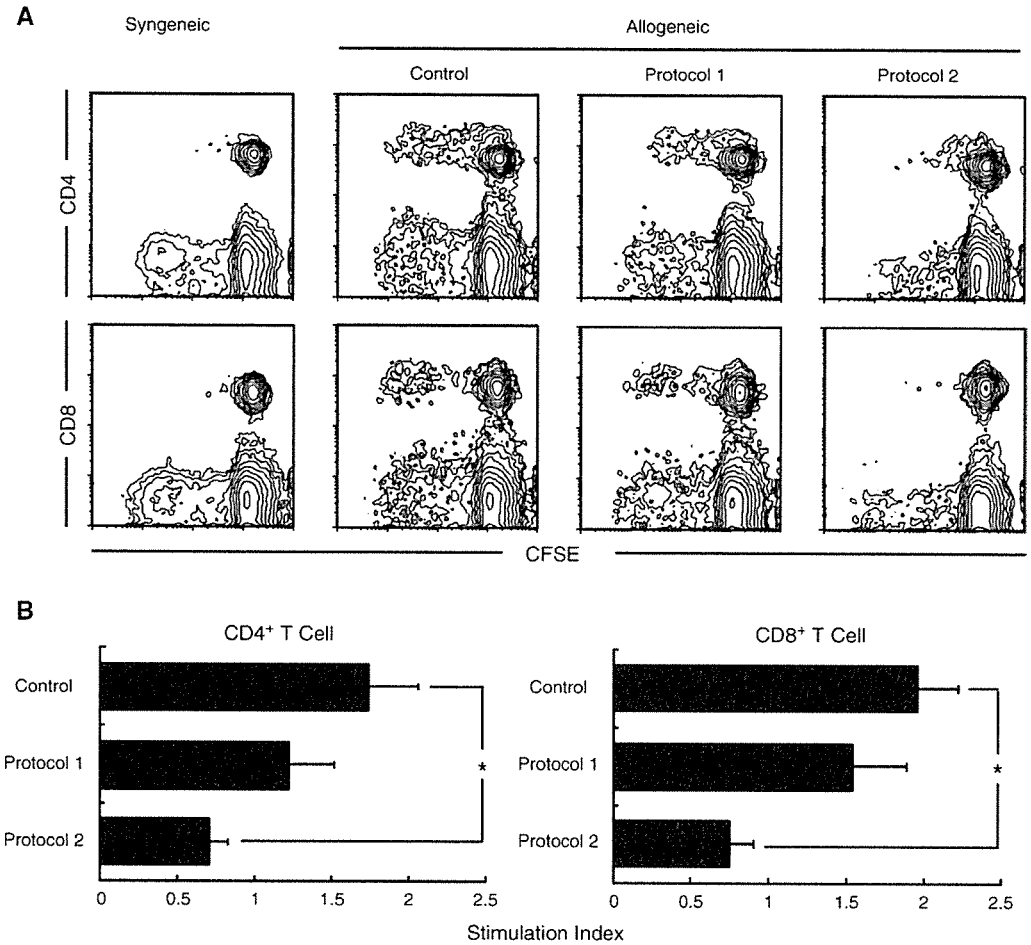


Fig. 2. Induction of endotoxin tolerance in stimulator mice reduced alloimmune responses. B6 mice were pretreated with the following protocol to induce ET: protocol 1, intravenous injection with low doses of LPS (20 μg) three times on days −6, −3 and 0; protocol 2, intravenous injection with low doses of LPS twice on days −6 and −3 and with a high dose of LPS (200 μg) on day 0. B6 mice that were injected PBS on days −6, −3 and 0 served as controls. The mice were killed 3 days after the final administration of LPS. Ether-irradiated splenocytes obtained from the control or LPS-treated B6 mice were used as stimulator cells, and splenocytes from CFSE-labeled naive BALB/c mice were used as responder cells. For 5 days, CFSE-labeled responder cells (4×10^6) were cultured with 4×10^6 irradiated stimulator cells in the dark. Following the MLR assay, harvested lymphocytes were stained with PE-conjugated anti-CD4 or CD8 mAbs. Subsequently, T cell proliferation (division) was visualized as serial halving of the fluorescence intensity of CFSE by FCM analysis. (A) Representative FCM results of CFSE-labeled $CD4^+$ and $CD8^+$ T cell division. (B) The bar graph depicts the stimulation index (SI) of $CD4^+$ and $CD8^+$ T cells with direct allospecificity in the MLR assay for each protocol. The data represents mean (±S.E.M.) of five mice per group. Statistical analyses were performed using ANOVA (*$p<0.05$).

The SI of allogeneic combinations was calculated by dividing the MI of an allogeneic combination by that of the control syngeneic combination.

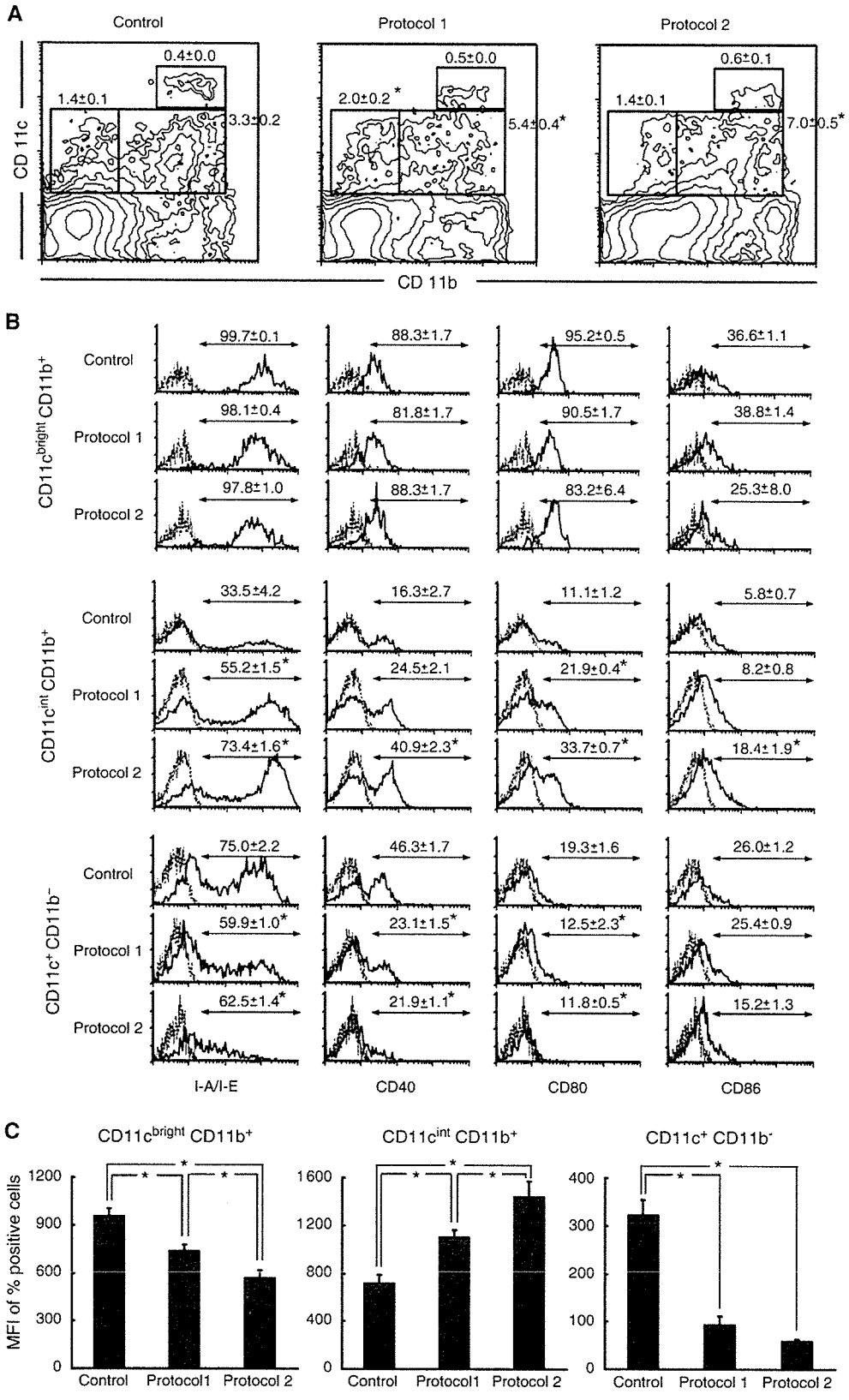
3.5. Cytokine production in MLR culture supernatants

The concentration of Th 1 and Th 2 cytokines in the MLR culture supernatants were measured by FCM using a BD Mouse Th 1/Th 2 Cytokine Cytometric Bead Array (CBA) Kit® (BD PharMingen) according to the manufacturer's instructions.

3.6. Heterotopic heart transplantation

Cervical heterotopic heart transplantation was performed using the cuff technique modified from a previously described method [15]. Briefly, the right

Fig. 3. Dendritic cells from LPS-treated mice with significant alteration in the expression of MHC class II and costimulatory molecules. The splenocytes from control or LPS-treated B6 mice were stained with anti-CD11b-PE and anti-CD11c Bio+APC-streptavidin together with various mAbs-FITC (anti-I-A/I-E, CD40 and CD80 or CD86). (A) Representative contour plots obtained by flow cytometric analysis are shown. Splenic DCs could be separated into three different subsets by a combination of CD11c and CD11b staining, i.e., $CD11c^{bright}$ $CD11b^+$, $CD11c^{int}$ $CD11b^+$ and $CD11c^+$ CD11b DCs. Percentages given (mean±S.E.M. from five independent experiments) are for the total splenocytes. Statistical analyses were performed using ANOVA (*$p<0.05$ vs. control). (B) Evaluation of the different DC subsets for their expression of MHC class II and costimulatory molecules. Dotted lines represent negative control staining with isotype-matched mAbs. FCM profiles shown are representative of five independent experiments. The numbers (mean±S.E.M.) indicate the percentages of cells in each group that were positive for I-A/I-E, CD40, CD80 and CD86 expression ($n=5$ each). Statistical analyses were performed using ANOVA (*$p<0.05$ vs. control). (C) Surface expression of I-A/I-E on the different DC subsets was analyzed by FCM. The numbers indicate the mean fluorescence intensity (MFI) of cells in each group that were staining positively for I-A/I-E on $CD11c^{bright}$ $CD11b^+$, $CD11c^{int}$ $CD11b^+$ and $CD11c^+$ CD11b DCs. The data represents mean±S.E.M. ($n=5$). Statistical analyses were performed using ANOVA (*$p<0.05$).


A
Control
Protocol 1
Protocol 2
CD 11c
CD 11b
0.4±0.0
1.4±0.1
3.3±0.2
0.5±0.0
2.0±0.2*
5.4±0.4*
0.6±0.1
1.4±0.1
7.0±0.5*
B
CD11c^bright CD11b+
CD11c^int CD11b+
CD11c+ CD11b-
Control
Protocol 1
Protocol 2
99.7±0.1
88.3±1.7
95.2±0.5
36.6±1.1
98.1±0.4
81.8±1.7
90.5±1.7
38.8±1.4
97.8±1.0
88.3±1.7
83.2±6.4
25.3±8.0
33.5±4.2
16.3±2.7
11.1±1.2
5.8±0.7
55.2±1.5*
24.5±2.1
21.9±0.4*
8.2±0.8
73.4±1.6*
40.9±2.3*
33.7±0.7*
18.4±1.9*
75.0±2.2
46.3±1.7
19.3±1.6
26.0±1.2
59.9±1.0*
23.1±1.5*
12.5±2.3*
25.4±0.9
62.5±1.4*
21.9±1.1*
11.8±0.5*
15.2±1.3
I-A/I-E
CD40
CD80
CD86
C
CD11c^bright CD11b+
CD11c^int CD11b+
CD11c+ CD11b-
MFI of % positive cells
1200
900
600
300
0
1600
1200
800
400
400
300
200
100
Control
Protocol1
Protocol 2
Protocol 1

external jugular vein and the right common carotid artery in the recipients were dissected, mobilized and fixed to the appropriate cuffs. The cuffs were composed of polyethylene tubes (2.5F; Portex Co. Ltd., London, United Kingdom). The aorta and the main pulmonary artery of the harvested donor heart were drawn over the end of the common carotid artery and the external jugular vein for anastomoses. The grafts were monitored by daily inspection and palpation. Rejection was determined by the cessation of beating of the graft and was confirmed by histology.

3.7. Statistical analyses

Data are presented as mean±S.E.M. Statistical differences of the results among the experimental groups were calculated using one-way ANOVA. A value of $p<0.05$ was accepted as statistically significant.

4. Results

4.1. Induction of endotoxin tolerance by pretreatment with low doses of LPS

Prior to the challenge with a high dose of LPS, the mice were treated with low doses of LPS twice, in order to induce ET. The survival rate at 72 h after challenging with a high dose of LPS showed a dramatically improved in mice by pretreatment with low doses of LPS (100%, $n=6$). In contrast, the survival curve of the control mice that were challenged with a high dose of LPS without pretreatment had a steep slope (survival rate at 72 h=0%, $n=6$). All the endotoxin-toleraized mice were not immobile; they exhibited shivering and diarrhea even after the challenge with a high dose of LPS. However, the high dose LPS challenge caused septic syndrome in the control mice.

4.2. The time course of the endotoxin-tolerized B6 mouse heart rejection was significantly slower than that of the untreated B6 mouse heart rejection in BALB/c mice

In order to determine whether the induction of ET in donor animals prior to harvesting organ grafts would have a prolonged effect on the graft survival in allogeneic recipients, the hearts of B6 mice that were treated with either only low doses of LPS (protocol 1) or low doses of LPS followed by a high dose of LPS (protocol 2), and then transplanted into BALB/c mice. The control hearts from PBS-treated B6 mice were also transplanted into BALB/c mice. In the absence of any immunosuppressive therapy, the median survival time of the hearts from B6 mice treated with protocols 1 and 2 were 9 and 16 days, respectively, whereas that of the control hearts transplanted into in BALB/c mice was 8 days (Fig. 1). Thus, the survival of the heart allografts was significantly prolonged if the organ was obtained from endotoxin-tolerized mice that were prepared by challenging the mice with a low dose LPS followed by a high dose LPS.

4.3. Splenocytes from B6 mice that had been rendered endotoxin tolerant had lowered allogeneic T cell stimulation ability

In order to evaluate the capacity of splenocytes from endotoxin-tolerized mice to stimulate allogeneic T cells, the MLR assay was performed using irradiated splenocytes from either PBS-injected control or LPS-treated B6 mice as stimulators and CFSE-labeled splenocytes from BALB/c mice as responders. When the splenocytes from the control mice were used as stimulators, a remarkable proliferation of allogeneic $CD4^+$ and $CD8^+$ T cells was observed. In contrast, such a proliferation of allogeneic $CD4^+$ and $CD8^+$ T cells was suppressed when splenocytes from LPS-treated mice were used as stimulators (Fig. 2A). The SI of allogeneic $CD4^+$ and $CD8^+$ T cells in response to the splenocyte stimulators from LPS-treated mice was significantly lower than those in response to the splenocyte stimulators from control mice (Fig. 2B). Protocol 2 induced more profound suppressive effects on allostimulation than protocol 1. Thus, treating a transplant donor with a low dose of LPS followed by challenge with a high dose of LPS inhibits the capacity of the donor's APCs to stimulate allogeneic T cells.

4.4. DCs from the endotoxin-tolerized mice significantly altered the expression of molecules necessary for antigen presentation

The impaired stimulation of allogeneic T cell proliferation in the splenocyte stimulators from endotoxin-tolerized mice might be due to their incompetence in presenting alloantigens. To address this possibility, the phenotypical alterations in DCs, which are the predominant APCs among splenocytes, were analyzed after the LPS treatments. The method of staining with a combination of CD11c and CD11b was used to separate the myeloid-related and lymphoid-related DCs in the splenocytes (Fig. 3A). $CD11c^+$ $CD11b^+$ is the phenotype of myeloid-related DCs, residing in the marginal zones of the spleen [16,17]. In our study, the $CD11c^+$ $CD11b^+$ subset was further divided into $CD11c^{int}$ $CD11b^+$ and $CD11c^{bright}$ $CD11b^+$ subsets; this probably reflects the difference in maturation. Cells from the control mice belonging to the $CD11c^{bright}$ $CD11b^+$ subset expressed all the molecules necessary for effective T cell stimulation (MHC class II, CD40, CD80 and CD86), thus indicating their maturity (Fig. 3B). LPS treatments–using protocols 1 or 2–did not affect the percentage of $CD11c^{bright}$ $CD11b^+$ DCs; instead it reduced the expression level of MHC class II molecules on this subset (Fig. 3C). The $CD11c^{int}$ $CD11b^+$ subset in the spleen of control mice comprised class $II^{-/dim}$ $CD40^-$ $CD80^-$ immature DCs and class II^{bright} $CD40^+$ $CD80^+$ mature DCs. LPS treatments resulted in the expansion of the $CD11c^{int}$ $CD11b^+$ subset and increased the proportion of mature DCs in this subset (Fig. 3B). In addition, the expression level of MHC class II molecules on mature DCs in the $CD11c^{int}$ $CD11b^+$ subset of the splenocytes from LPS-treated mice was significantly higher than that of the splenocytes from the control mice (Fig. 3C). $CD11c^+$ $CD11b^-$ is the phenotype of lymphoid-related DCs, which reside in the T cell zones [16,17]. Further, the $CD11c^+$ $CD11b^-$ subset comprised class II^{dim} $CD40^{dim}$ immature DCs and class II^{bright} $CD40^+$ mature DCs. In contrast to the $CD11b^+$ myeloid-related DC subset, the LPS treatments resulted in a decreased proportion of mature DCs in the $CD11b^-$ lymphoid-related DC subset, i.e., reduction of class II^{bright} $CD40^+$ DCs. (Fig. 3B). In addition, the expression level of MHC class II molecules on mature DCs in the $CD11c^+$ $CD11b^-$ subset of the splenocytes from LPS-treated mice was significantly lower than that of the splenocytes from the control mice (Fig. 3C). Such LPS-induced modulations on the lymphoid-related DCs by protocol 2 were more notable than those induced by protocol 1. Thus, the induction of ET increased the proportion of myeloid-related DCs expressing molecules necessary for antigen presentation, but reduced the proportion of lymphoid-related DCs expressing these molecules.

4.5. DCs from the endotoxin-tolerized mice showed up-regulation of FasL expression on their cell surface

It has been demonstrated that the expression of FasL molecules are induced in well-developed mature DCs; Fas–FasL interactions usually lead to the apoptosis-induced cell death of antigen-primed T cells [18]. This may be a regulatory mechanism to curb the excessive adaptive