

The effect on portal hemodynamics was one of the major concerns in the RP-LDLT recipients. With partial liver graft LDLTs or split livers, the portal venous flow increases because of the reduced blood bed in the liver graft, resulting in the hyper-hemodynamics of the portal vein and possibly causing liver injury and impairment of the usual course of liver regeneration (known as small-for-size syndrome) (16). In our series, the portal venous flow was not elevated, compared to the average flow of well-functioning grafts, and the recovery of graft function did not differ from that of conventional right-lobe LDLT recipients.

The pressure of the left renal vein increases in subjects that undergo a reoportal anastomosis, possibly reducing the flow of the left renal vein. The function of the left kidney was slightly impaired in Case 1, but the findings were clinically negligible. The impairment of the left renal function may have started prior to the transplantation, since the venous pressure of the left renal vein was elevated because of the large splenorenal shunt in this particular case (17). In the remaining cases, left renal function was initially impaired slightly, but recovered fully by a year after the RP-LDLT, suggesting that the left renal dysfunction due to the alteration of drainage venous flow in this technique could be, if any, temporal and recovered.

Possible treatment alternatives for patients with large splenorenal shunts and insufficient portal venous flow, other than the reoportal anastomosis described here, include a portal vein thrombectomy or a thrombendovenectomy (18), an SMV-portal vein or collateral-portal vein anastomosis with or without an interposition vein graft, and a ligation of the splenorenal shunt with or without a splenectomy (19). These techniques are reportedly feasible in patients with portal vein thrombosis (1-4). However, the presence of large splenorenal shunts in the patients treated in these series was not noted. From the perspective of hemodynamic characteristics, simple anastomosis of the portal vein (without RP-LDLT), even if blood flow in the portal vein is present, may become inadequate and require a re-exploration to ligate the splenorenal shunt, increasing the risk of bleeding, infection, and mortality to the recipient, as described by Cescon et al. (8). Thus, an RP-LDLT may be the most appropriate treatment for patients with portal vein thrombus and a large splenorenal shunt, taking into account these advantages and disadvantages. Cavoportal hemitransposition is another option for these patients, but its morbidity and mortality cannot be ignored (20).

In conclusion, the present series of three patients suggests that hemodynamic changes in the portal venous system after the RP-LDLT were not significant and that the possible adverse effects of reoportal anastomosis, as discussed above, were clinically negligible, confirming the long-term effectiveness of the RP-LDLT. The RP-LDLT for spontaneous spleno-

renal shunt in end-stage liver disease patients appears to be a life-saving and safe technique and should be discussed as a treatment option for patients with splenorenal shunt.

REFERENCES

- Yerdel MA, Gunson B, Mirza D, et al. Portal vein thrombosis in adults undergoing liver transplantation: risk factors, screening, management, and outcome. *Transplantation* 2000; 69(9): 1873.
- Manzanet G, Sanjuan F, Orbis P, et al. Liver transplantation in patients with portal vein thrombosis. *Liver Transpl* 2001; 7(2): 125.
- Dumortier J, Czyglik O, Poncet G, et al. Eversion thrombectomy for portal vein thrombosis during liver transplantation. *Am J Transplant* 2002; 2(10): 934.
- Robles R, Fernandez JA, Hernandez Q, et al. Eversion thrombendovenectomy in organized portal vein thrombosis during liver transplantation. *Clin Transplant* 2004; 18(1): 79.
- Miyamoto A, Kato T, Dono K, et al. Living-related liver transplantation with reoportal anastomosis for a patient with large spontaneous splenorenal collateral. *Transplantation* 2003; 75(9): 1596.
- Mazzaferro V, Regalia E, Doci R, et al. Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. *N Engl J Med* 1996; 334(11): 693.
- Rousselot LM, Moreno AH, Panke WF. Studies on portal hypertension. IV. The clinical and physiopathologic significance of self-established (nonsurgical) portal systemic venous shunts. *Ann Surg* 1959; 150: 384.
- Cescon M, Sugawara Y, Kaneko J, et al. Restoration of portal vein flow by splenorenal shunt ligation and splenectomy after living-related liver transplantation. *Hepatogastroenterology* 2001; 48(41): 1453.
- Marubashi S, Sakon M, Nagano H, et al. Effect of portal hemodynamics on liver regeneration studied in a novel portohepatic shunt rat model. *Surgery* 2004; 136(5): 1028.
- de Jonge J, Zondervan PE, JN, JJ, et al. Importance of portal flow diversion in experimental auxiliary partial orthotopic liver transplantation. *Transplantation* 2000; 70(1): 44.
- Shaw Jr. BW, Iwatsuki S, Bron K, Starzl TE. Portal vein grafts in hepatic transplantation. *Surg Gynecol Obstet* 1985; 161(1): 66.
- Stieber AC, Zetti G, Todo S, et al. The spectrum of portal vein thrombosis in liver transplantation. *Ann Surg* 1991; 213(3): 199.
- Kato T, Levi DM, DeFaria W, et al. Liver transplantation with reoportal anastomosis after distal splenorenal shunt. *Arch Surg* 2000; 135(12): 1401.
- Wexler MJ, MacLean LD. Massive spontaneous portal-systemic shunting without varices. *Arch Surg* 1975; 110(8): 995.
- Rolffs J, Schmelzle R. Serious cerebral complications following unilateral ligation of the internal jugular vein. Review of the literature and a case report. *J Maxillofac Surg* 1977; 5(2): 118.
- Man K, Fan ST, Lo CM, et al. Graft injury in relation to graft size in right lobe live donor liver transplantation: a study of hepatic sinusoidal injury in correlation with portal hemodynamics and intragraft gene expression. *Ann Surg* 2003; 237(2): 256.
- Dash SC, Bhuyan UN, Dinda AK, et al. Increased incidence of glomerulonephritis following spleno-renal shunt surgery in non-cirrhotic portal fibrosis. *Kidney Int* 1997; 52(2): 482.
- Molmenti EP, Roodhouse TW, Molmenti H, et al. Thrombendovenectomy for organized portal vein thrombosis at the time of liver transplantation. *Ann Surg* 2002; 235(2): 292.
- Shapiro RS, Varma CV, Schwartz ME, Miller CM. Splenorenal shunt closure after liver transplantation: intraoperative Doppler assessment of portal hemodynamics. *Liver Transpl Surg* 1997; 3(6): 641.
- Gerunda GE, Merenda R, Neri D, et al. Cavoportal hemitransposition: a successful way to overcome the problem of total portosplenomesenteric thrombosis in liver transplantation. *Liver Transpl* 2002; 8(1): 72.

特集「ウイルス肝炎の肝移植」

C型肝炎肝移植患者の T細胞性免疫応答の特性

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はじめに

C型肝炎ウイルス (HCV) 性肝硬変は肝移植の最も頻度の高い適応疾患のひとつであるが, 移植後C型肝炎の再発が高率に起こり, また肝炎の進行も移植患者以外と比較すると急速であることが分かっている^{1,2)}。HCV陰性患者では術後急性拒絶反応は予後と関連しないのに対し, HCV陽性の移植患者における急性拒絶反応は予後不良因子とされている。HCV肝炎合併例では免疫抑制療法がウイルスの増勢を助長するためと理解されている³⁾。従って, 急性拒絶反応とC型肝炎再発, あるいはその他の原因とを的確に鑑別し, 必要最低限の免疫抑制療法を行なうため, 個々の患者の免疫状態を把握することが重要となる。信頼性のある免疫監視法の確立が求められるゆえんである。われわれは, carboxyfluorescein diacetate succinimidyl ester (CFSE) 細胞質染色とマルチパラメーターフローサイトメトリーを応用した mixed lymphocyte reaction assay (以後, CFSE-MLR と略す) を臨床導入している^{4,5)}。CFSE色素は細胞傷害性がなく細胞内蛋白を染色し, 細胞分裂回数に比例して色素が半減化する性質を有し, 反応性リンパ球の表面マーカーと同時に precursor frequency, mitotic index や stimulation index の定量化が可能である (図1)^{6,7)}。CFSE-MLRによりHCV罹患肝移植患者のT細胞性免疫応答の特性を解析したので報告する。

HCV肝硬変患者のT細胞アロ免疫応答

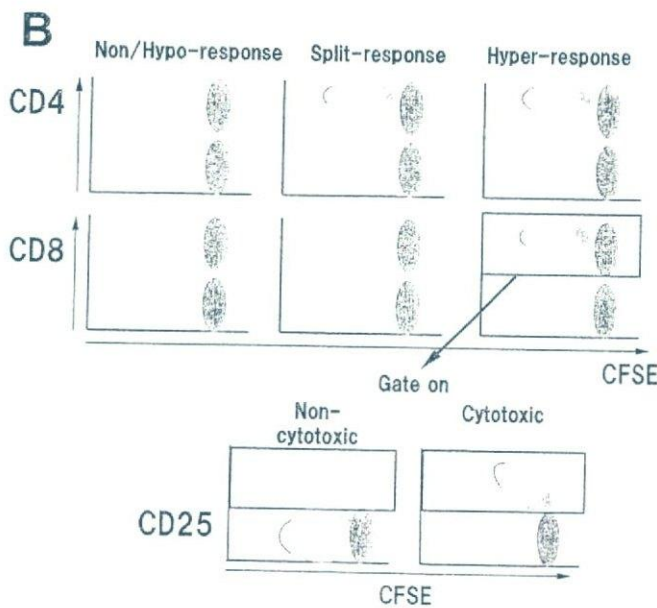
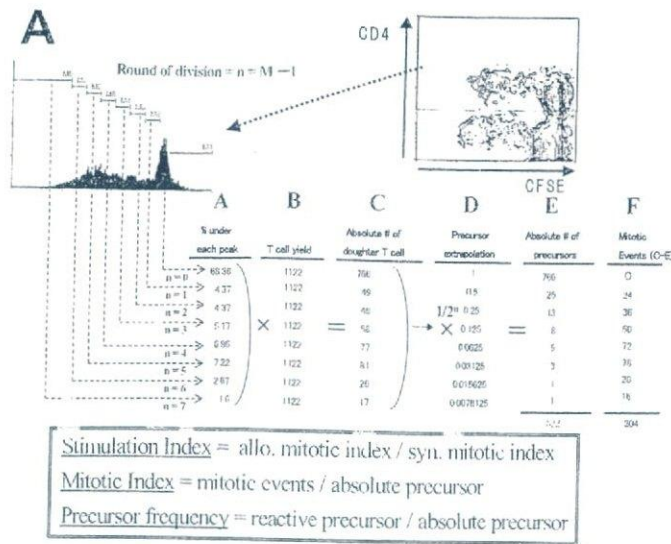
HCV患者では健常人に比べ樹状細胞の抗原提示能

の低下が報告されている^{8,9)}。樹状細胞への直接的なHCV感染が起因する可能性やHCV感染が間接的に樹状細胞の成熟を抑制する可能性が指摘されているが, HCV患者におけるT細胞応答能の低下にかかわる機序はいまだ明らかではない。HCV肝硬変患者の抗原提示能の低下は, 肝移植後に間接認識経路で惹起されるT細胞応答が他疾患による肝移植後に比べて弱い可能性を示唆する。しかし, 肝移植後にグラフト由来の健常抗原提示細胞から直接認識経路で惹起されるT細胞応答について解析された報告は認められない。これを解析する目的でわれわれは, 2004年8月~2006年2月の間に術前の末梢血リンパ球を用いたCFSE-MLRを施行した患者21名を対象に, その結果を解析した。T細胞のstimulation indexを原疾患別に比較したところ, ドナー由来の抗原提示細胞を認識したT細胞は, B型肝炎やアルコール性肝硬変などの他疾患で肝移植を要した患者と同等のアロ応答を示した (図2)。従ってHCV肝移植患者の拒絶予防には, 他疾患による肝移植患者と同等の免疫抑制療法が必要であるものと考えられた。

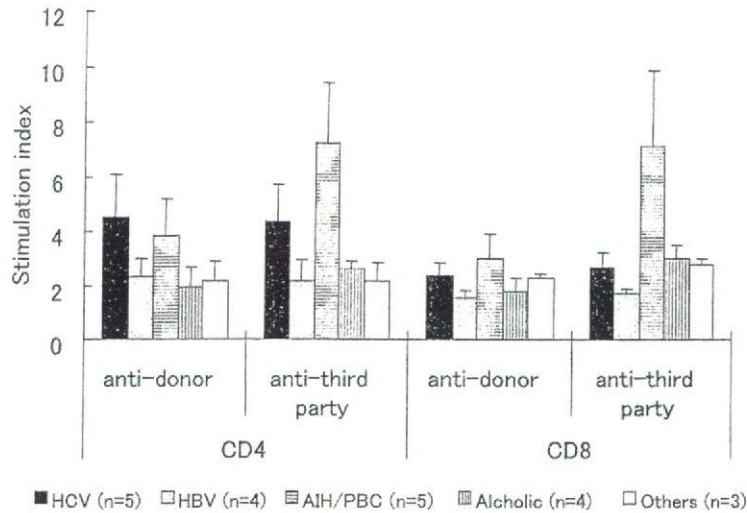
肝移植後のアロ応答と

HCVウイルス量の関係

当科においてCFSE-MLRで免疫監視を行ったC型肝炎患者に対する生体肝移植症例14例のうち, 術後1年以内に血液生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。このうち, CFSE-MLRによって抗ドナー応答の亢進を認め急性拒絶反応と診断し拒絶治療を要したのは3例であった。HCVウイルス量と急性拒絶反応および拒絶治療

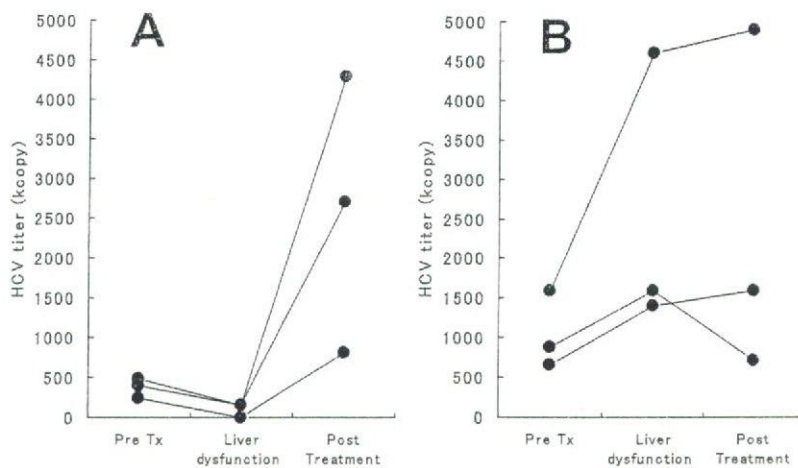


- (A) ドナーの末梢血リンパ球を承諾の上採取して stimulator として使用した。また、健常ボランティアの末梢血リンパ球を third party stimulator として使用した。レシピエントの末梢血リンパ球を CFSE 染色し one-way MLR を施行した。MLR の解析には FCM を用い、FL-1 で CFSE の減衰による細胞分裂・増殖を、FL-2 と FL-4 で反応性 T 細胞のフェノタイプ (CD4, CD8, CD25) を評価し、FL-3 で PI 染色による生細胞抽出を行った。本法では、CD4 と CD8 T 細胞別に stimulation index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



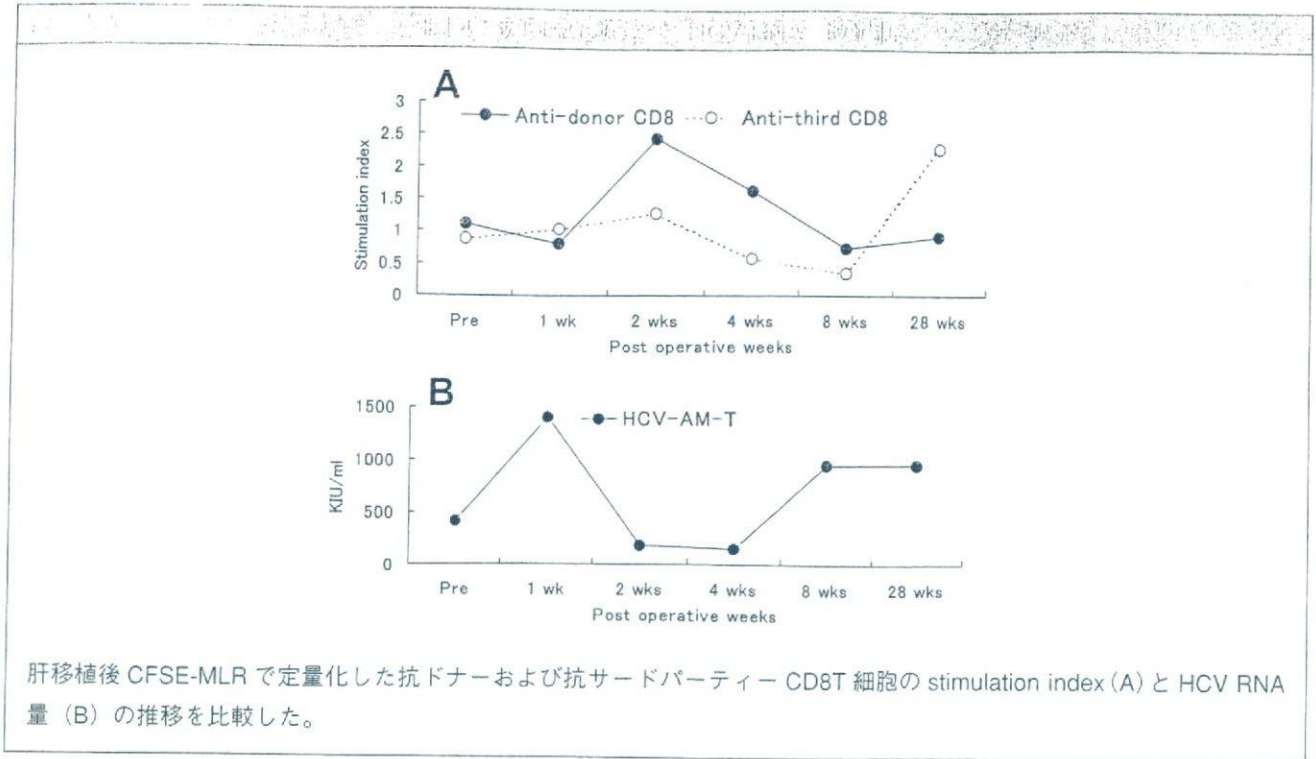
肝移植後1年以内に血液化学検査で肝機能異常を認め、急性拒絶反応を疑った症例は6例であった。

(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-



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

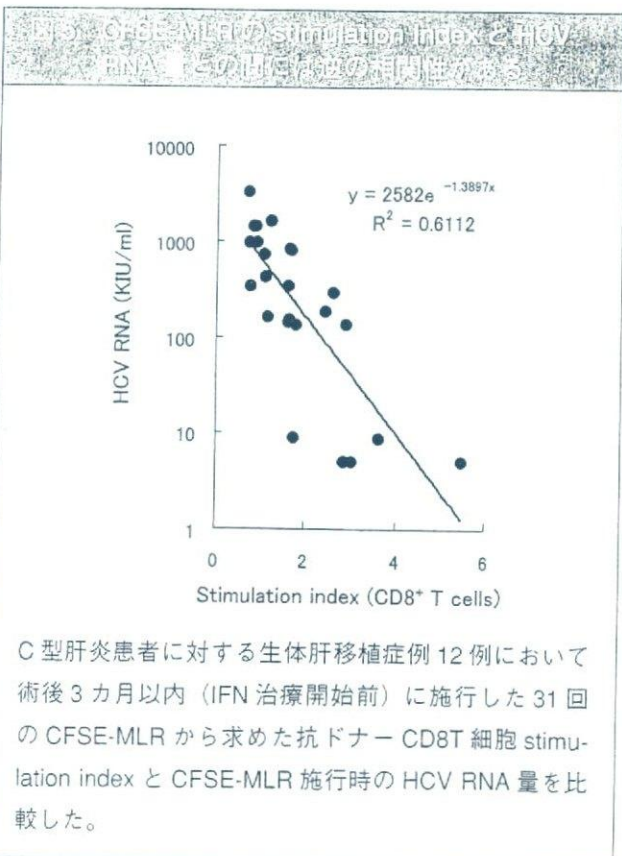
の後カルシニューリン抑制剤の血中濃度の安定に伴い抗ドナー応答は抑制された。HCV RNA 量はこの抗ドナー CD8T 細胞の stimulation index と鏡像を示して推移した。C 型肝炎患者に対する生体肝移植症例 12 例において術後 3 カ月以内 (IFN 治療開始前) に施行した 31 回の CFSE-MLR から求めた抗ドナー CD8T 細胞 stimulation index と CFSE-MLR 施行時の HCV RNA 量を比較したところ、有意な逆相関が確認された ($R^2 = 0.61$) (図 5)。この興味深い現象は、アロ免疫応答と抗 HCV 免疫応答との間にクロ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。

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文 献

- 1) Sheiner PA, Schwartz ME, Mor E, *et al.* Severe or multiple rejection episodes are associated with early recurrence of hepatitis C after orthotopic liver transplantation. *Hepatology* 1995; 21: 30-34.



- 2) Rosen HR, Shackleton CR, Higa L, *et al.* Use of OKT 3 is associated with early and severe recurrence of hepatitis C after liver transplantation. *Am J Gastroenterol* 1997; 92: 1453-1457.
- 3) Charlton M, Seaberg E. Impact of immunosuppression and acute rejection on recurrence of hepatitis C: results of the National Institute of Diabetes and Digestive and Kidney Diseases Liver Transplantation Database. *Liver Transpl Surg* 1999; 5(4 Suppl 1): S107-S114.
- 4) Hara H, Ohdan H, Tashiro H, *et al.* Differential diagnosis between graft-versus-host disease and hemophagocytic syndrome after living-related liver transplantation by mixed lymphocyte reaction assay. *J Invest Surg* 2004; 17: 197-202.
- 5) Tanaka Y, Ohdan H, Onoe T, *et al.* Low incidence of acute rejection after living-donor liver transplantation: immunologic analyses by mixed lymphocyte reaction using a carboxyfluorescein diacetate succinimidyl ester labeling technique. *Transplantation* 2005; 79: 1262-1267.
- 6) Wells AD, Gudmundsdottir H, Turka LA. Following the fate of individual T cells throughout activation and clonal expansion. Signals from T cell receptor and CD 28 differentially regulate the induction and duration of a proliferative response. *J Clin Invest* 1997; 100: 3173-3183.
- 7) Tanaka Y, Ohdan H, Onoe T, *et al.* Multiparameter flow cytometric approach for simultaneous evaluation of proliferation and cytokine-secreting activity in T cells responding to allo-stimulation. *Immunol Invest* 2004; 33: 309-324.
- 8) Kanto T, Hayashi N, Takehara T, *et al.* Impaired allostimulatory capacity of peripheral blood dendritic cells recovered from hepatitis C virus-infected individuals. *J Immunol* 1999; 162: 5584-5591.
- 9) Bain C, Fatmi A, Zoulim F, *et al.* Impaired allostimulatory function of dendritic cells in chronic hepatitis C infection. *Gastroenterology* 2001; 120: 512-524.

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 ($90.5\% \pm 2.2\%$ at E:T = 10:1), compared with donor and recipient PB NK cells and recipient liver NK cells ($64.8\% \pm 8.2\%$, $56.1\% \pm 8.9\%$, and $34.6\% \pm 7.5\%$, 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; PBM, 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; MA, monoclonal antibody; HLA, human leukocyte antigen; E:T, effector:target; MC, mononuclear cell; KIR, killer cell immunoglobulin-like receptors; MHC, major histocompatibility complex.

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

Case No.	Donor								Recipient									
	Age(y)/Sex	Graft Liver	Graft Wt(g)	LMNC ($\times 10^5/g$)	HLA			Relation	Age(y)/Sex	Child Pugh	MELD	Original Disease	HCC	Liver Wt(g)	LMNC ($\times 10^5/g$)	HLA		
					A	B	C									A	B	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) \pm 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 μ mol/L 2 mercaptoethanol (Katayama Chemical Co., Osaka, Japan), 50 U/mL penicillin, and 50 μ g/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% CO₂ 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 μ Ci Na² (⁵¹Cr) O₄ for 60 minutes at 37°C in 5% CO₂ in 10% RPMI, washed 3 times with medium, and then subjected to the cytotoxicity assay. The labeled HepG2 cells were adjusted to 1 \times 10⁶ cells in 10 mL

volumes (1 \times 10⁴/well) and were incubated in a total volume of 200 μ L 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 ⁵¹Cr 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 ⁵¹Cr release was calculated by the following formula: % cytotoxicity = [(cpm of experimental release - cpm of spontaneous release)]/[(cpm of maximum release - cpm of spontaneous release)] \times 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 μ g/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 μ g/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- μ m frozen sections from representative tumor tissue of at least 1 cm² 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 \pm 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% \pm 4.5% and 14.0% \pm 3.0%, respectively) were almost identical to those in LMNCs collected by method using enzymatic dissociation (33.7% \pm 1.3% and 14.8% \pm 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 ex-

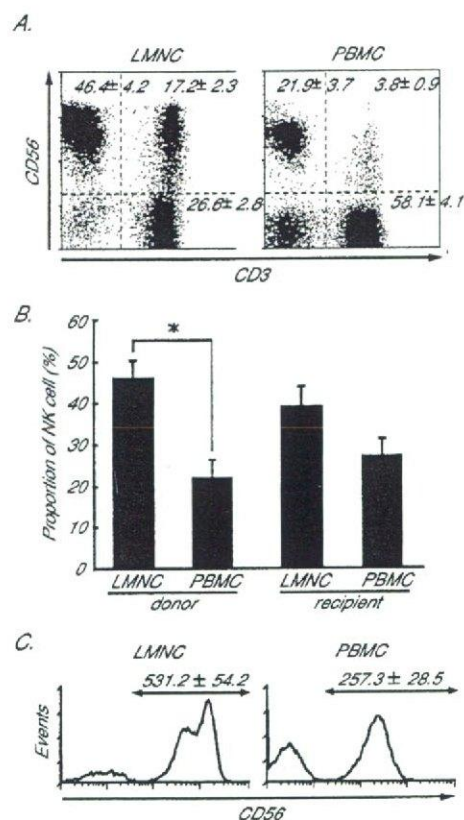


Fig. 1. Human liver mononuclear cell extracted from liver perfusates contain a large population of NK cells. (A) Flow cytometric (FCM) analysis of freshly isolated LMNCs obtained from liver perfusates and PBMCs from same donor after staining with MAbs against CD3 and CD56 were analyzed. Lymphocytes were 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 \pm SEM, *n* = 14 each). (B) The numbers represent the means \pm 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 \pm 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.

tent, the possibility of enzyme-induced alteration/disruption of specific epitopes could be disregarded.

The number of LMNCs extracted from donor normal livers and recipient livers with cirrhosis were 0.5 ± 0.1 and $0.4 \pm 0.1 \times 10^6$ 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-

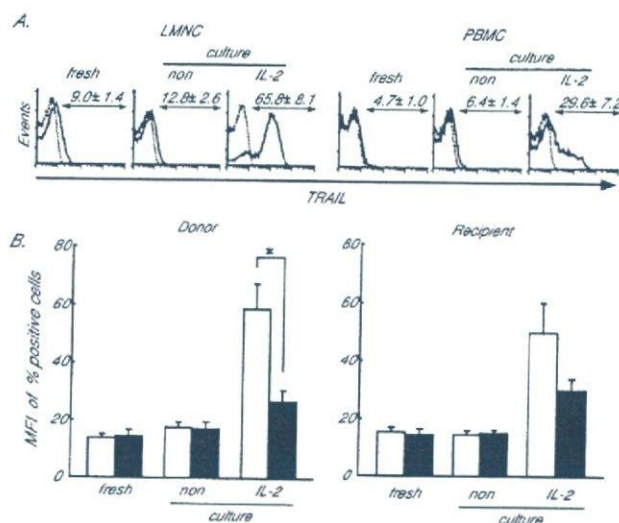


Fig. 2. Liver NK cells inductively express remarkable levels of TRAIL, but PB NK cells do not. Freshly isolated or cultivated with or without IL-2 LMNCs and PBMCs obtained from healthy donors and corresponding recipients were stained with CD3 and CD56 MAb together with TRAIL MAb. (A) Histograms represent the log fluorescence intensities obtained on staining for TRAIL after gating on the CD3⁺CD56⁺ NK cell subsets obtained from healthy donors. Dotted lines represent negative control staining with isotype-matched MAbs. The numbers (mean \pm 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 \pm 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-

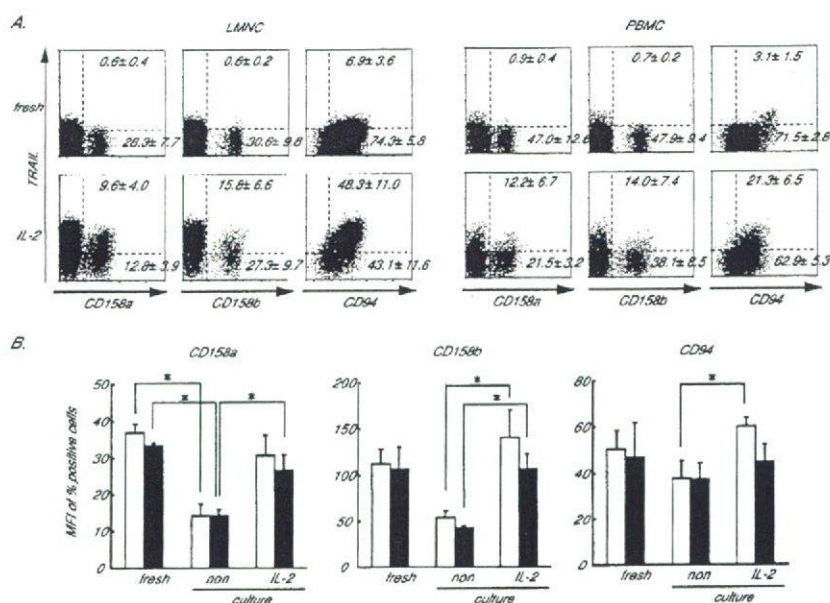


Fig. 3. IL-2 stimulation leads to maintenance of CD158a, CD158b, and CD94 expressions even on TRAIL expressing liver NK cells. Expression of various inhibitory receptors on the NK cells subsets among LMNCs or PBMCs freshly isolated or cultivated with or without IL-2 was analyzed. The LMNCs and PBMCs obtained from healthy donors were stained with CD3, CD56, and TRAIL MAb together with CD-158a, CD-158b, or CD94 MAb. (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

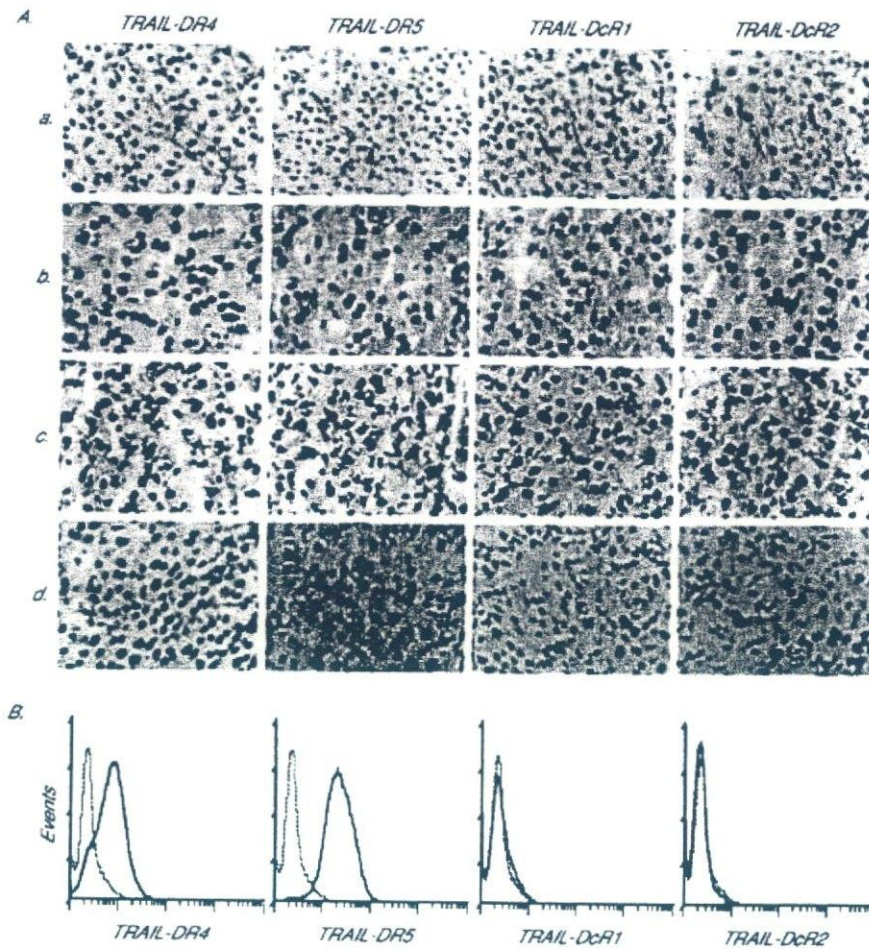


Fig. 4. Differential expression of TRAIL receptors in normal liver tissue and HCC tissue. (A) Immunohistochemical expression of TRAIL-DR4, -DR5, -DcR1, and -DcR2 in normal liver tissue (a), tumor site of well-differentiated HCCs (b), moderately differentiated HCCs (c), and poorly differentiated HCCs (d). Magnification $\times 400$. 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% \pm 8.2%, 56.1% \pm 8.9%, and 34.6% \pm 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% \pm 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).

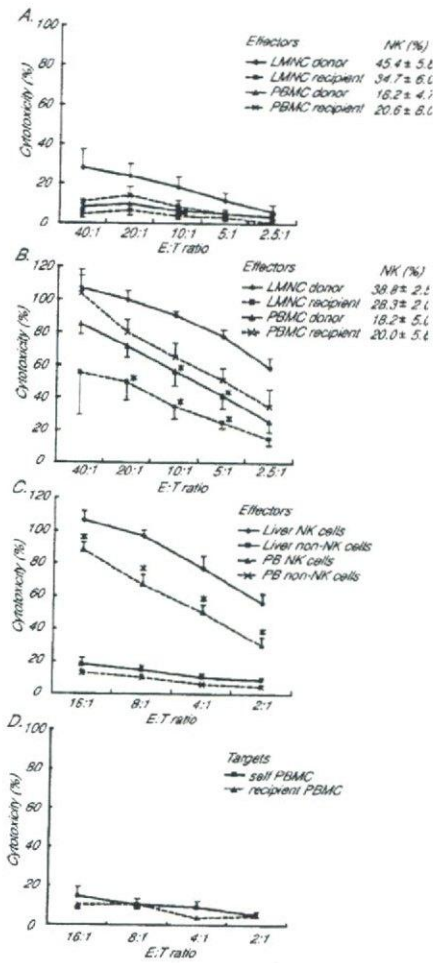


Fig. 5. IL-2-stimulated donor liver NK cells show vigorous cytotoxicity against HepG2. NK cytotoxic activities of indicated effectors against indicated target cells were analyzed by ⁵¹Cr release assay. All data are represented as the mean ± SEM of assays, each set up in triplicate. Statistical analyses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

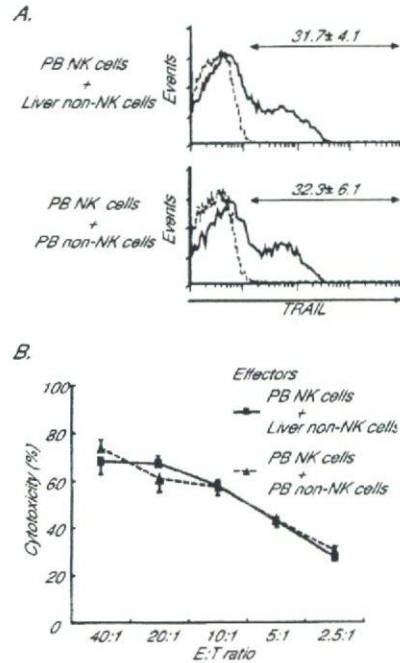


Fig. 6. Liver non-NK cells are not responsible for the increased anti-tumor activity of NK cells. Before IL-2 stimulation, NK and non-NK cells were isolated from donor LMNCs and same donor PBMCs. Then, NK cells from PBMCs were cultured with either liver non-NK or peripheral blood (PB) non-NK cells at 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 growth⁵⁻⁹; 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, $0.97\% \pm 0.17\%$), 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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References

- Trinchieri G. Biology of natural killer cells. *Adv Immunol* 1989;47:187-376.
- Yokoyama WM. Natural killer cells. In: Paul EW, ed. *Fundamental Immunology*. 4th ed. Philadelphia: Lippincott-Raven, 1999:575-603.
- Miller JS, Soignier Y, Panoskaltis-Mortari A, McNearney SA, Yun GH, Fautsch SK, et al. Successful adoptive transfer and *in vivo* expansion of human haploidentical NK cells in patients with cancer. *Blood* 2005;105:3051-3057.
- Klingemann HG, Martinson J. *Ex vivo* expansion of natural killer cells for clinical applications. *Cytotherapy* 2004;6:15-22.
- Rosenberg SA, Lotze MT, Muul LM, Chang AE, Avis FP, Leitman S, et al. A progress report on the treatment of 157 patients with advanced cancer using lymphokine-activated killer cells and interleukin-2 or high-dose interleukin-2 alone. *N Engl J Med* 1987;316:889-897.
- Brunda MJ, Luistro L, Warrior RR, Wright RB, Hubbard BR, Murphy M, et al. Antitumor and antimetastatic activity of interleukin 12 against murine tumors. *J Exp Med* 1993;178:1223-1230.
- Takeda K, Hayakawa Y, Smyth MJ, Kayagaki N, Yamaguchi N, Kakuta S, et al. Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. *Nat Med* 2001;7:94-100.
- Kennedy MK, Glaccum M, Brown SN, Butz EA, Viney JL, Embers M, et al. Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. *J Exp Med* 2000;191:771-780.
- Smyth MJ, Cretney E, Takeda K, Wilttrout RH, Sedger LM, Kayagaki N, et al. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) contributes to interferon gamma-dependent natural killer cell protection from tumor metastasis. *J Exp Med* 2001;193:661-670.
- Talmadge JE, Phillips H, Schindler J, Tribble H, Pennington R. Systematic preclinical study on the therapeutic properties of recombinant human interleukin 2 for the treatment of metastatic disease. *Cancer Res* 1987;47:5725-5732.
- Leung W, Iyengar R, Turner V, Lang P, Bader P, Conn P, et al. Determinants of antileukemia effects of allogeneic NK cells. *J Immunol* 2004;172:644-650.
- Meller B, Frohn C, Brand JM, Lauer I, Schelper LF, von Hof K, et al. Monitoring of a new approach of immunotherapy with allogeneic (111)In-labelled NK cells in patients with renal cell carcinoma. *Eur J Nucl Med Mol Imaging* 2004;31:403-407.
- Takahashi M, Ogasawara K, Takeda K, Hashimoto W, Sakihara H, Kumagai K, et al. LPS induces NK1.1 + alpha beta T cells with potent cytotoxicity in the liver of mice via production of IL-12 from Kupffer cells. *J Immunol* 1996;156:2436-2442.
- Crispe IN, Mehal WZ. Strange brew: T cells in the liver. *Immunol Today* 1996;17:522-525.
- Seki S, Habu Y, Kawamura T, Takeda K, Dobashi H, Ohkawa T, et al. The liver as a crucial organ in the first line of host defense: the roles of Kupffer cells, natural killer (NK) cells and NK1.1 Ag+ T cells in T helper 1 immune responses. *Immunol Rev* 2000;174:35-46.
- Ochi M, Ohdan H, Mitsuta H, Onoe T, Tokita D, Hara H, et al. Liver NK cells expressing TRAIL are toxic against self hepatocytes in mice. *HEPATOLOGY* 2004;39:1321-1331.
- Walczak H, Miller RE, Ariail K, Gliniak B, Griffith TS, Kubin M, et al. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand *in vivo*. *Nat Med* 1999;5:157-163.
- Wiley SR, Schooley K, Smolak PJ, Din WS, Huang CP, Nicholl JK, et al. Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity* 1995;3:673-682.
- Pitti RM, Marsters SA, Ruppert S, Donahue CJ, Moore A, Ashkenazi A. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. *J Biol Chem* 1996;271:12687-12690.
- Pan G, O'Rourke K, Chinnaiyan AM, Gentz R, Ebner R, Ni J, et al. The receptor for the cytotoxic ligand TRAIL. *Science* 1997;276:1111-1113.
- Sheridan JP, Marsters SA, Pitti RM, Gurney A, Skubatch M, Baldwin D, et al. Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. *Science* 1997;277:818-821.
- Ohdan H, Mizunuma K, Tashiro H, Tokita D, Hara H, Onoe T, et al. Intraoperative near-infrared spectroscopy for evaluating hepatic venous outflow in living-donor right lobe liver. *Transplantation* 2003;76:791-797.
- Kurokohchi K, Carrington M, Mann DL, Simonis TB, Alexander-Miller MA, Feinstone SM, et al. Expression of HLA class I molecules and the transporter associated with antigen processing in hepatocellular carcinoma. *HEPATOLOGY* 1996;23:1181-1188.
- Strater J, Hinz U, Walczak H, Mechttersheimer G, Koretz K, Herfarth C, et al. Expression of TRAIL and TRAIL receptors in colon carcinoma: TRAIL-R1 is an independent prognostic parameter. *Clin Cancer Res* 2002;8:3734-3740.
- Kawarabayashi N, Seki S, Hatsuse K, Ohkawa T, Koike Y, Aihara T, et al. Decrease of CD56(+)T cells and natural killer cells in cirrhotic livers with hepatitis C may be involved in their susceptibility to hepatocellular carcinoma. *HEPATOLOGY* 2000;32:962-969.
- Doherty DG, Norris S, Madrigal-Estebas L, McEntee G, Traynor O, Hegarty JE, et al. The human liver contains multiple populations of NK

- cells, T cells, and CD3+CD56+ natural T cells with distinct cytotoxic activities and Th1, Th2, and Th0 cytokine secretion patterns. *J Immunol* 1999;163:2314-2321.
27. Yuen MF, Norris S. Expression of inhibitory receptors in natural killer (CD3(-)CD56(+)) cells and CD3(+)CD56(+) cells in the peripheral blood lymphocytes and tumor infiltrating lymphocytes in patients with primary hepatocellular carcinoma. *Clin Immunol* 2001;101:264-269.
 28. Cooper MA, Fehniger TA, Turner SC, Chen KS, Ghaheri BA, Ghayur T, et al. Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. *Blood* 2001;97:3146-3151.
 29. Jacobs R, Stoll M, Stratmann G, Leo R, Link H, Schmidt RE. CD16-CD56+ natural killer cells after bone marrow transplantation. *Blood* 1992;79:3239-3244.
 30. Braud VM, Allan DS, O'Callaghan CA, Soderstrom K, D'Andrea A, Ogg GS, et al. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature* 1998;391:795-799.
 31. Borrego F, Ulbrecht M, Weiss EH, Coligan JE, Brooks AG. Recognition of human histocompatibility leukocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NKG2 confers protection from natural killer cell-mediated lysis. *J Exp Med* 1998;187:813-818.
 32. Moretta A, Vitale M, Sivori S, Bottino C, Morelli L, Augugliaro R, et al. Human natural killer cell receptors for HLA-class I molecules: evidence that the Kp43 (CD94) molecule functions as receptor for HLA-B alleles. *J Exp Med* 1994;180:545-555.
 33. Karre K, Ljunggren HG, Piontek G, Kiessling R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature* 1986;319:675-678.
 34. Vujanovic NL, Nagashima S, Herberman RB, Whiteside TL. Nonsecretory apoptotic killing by human NK cells. *J Immunol* 1996;157:1117-1126.
 35. Wiesner RH, Freeman RB, Mulligan DC. Liver transplantation for hepatocellular cancer: the impact of the MELD allocation policy. *Gastroenterology* 2004;127:S261-267.
 36. Kulik L, Abecassis M. Living donor liver transplantation for hepatocellular carcinoma. *Gastroenterology* 2004;127:S277-282.
 37. Axelrod D, Koffron A, Kulik L, Al-Saden P, Mulcahy M, Baker T, et al. Living donor liver transplant for malignancy. *Transplantation* 2005;79:363-366.
 38. Hirata M, Kita Y, Saito S, Nishimura M, Ito M, Mizuta K, et al. Increase in natural killer cell activity following living-related liver transplantation. *Transpl Int* 1998;11(Suppl. 1):S185-S188.
 39. Harada N, Shimada M, Okano S, Suehiro T, Soejima Y, Tomita Y, et al. IL-12 gene therapy is an effective therapeutic strategy for hepatocellular carcinoma in immunosuppressed mice. *J Immunol* 2004;173:6635-6644.
 40. Alcami A, Koszinowski UH. Viral mechanisms of immune evasion. *Immunol Today* 2000;21:447-455.
 41. Falk CS, Mach M, Schendel DJ, Weiss EH, Hilgert I, Hahn G. NK cell activity during human cytomegalovirus infection is dominated by US2-11-mediated HLA class I down-regulation. *J Immunol* 2002;169:3257-3266.
 42. Colonna M, Moretta A, Vely F, Vivier E. A high-resolution view of NK-cell receptors: structure and function. *Immunol Today* 2000;21:428-431.
 43. Winter CC, Gumperz JE, Parham P, Long EO, Wagtmann N. Direct binding and functional transfer of NK cell inhibitory receptors reveal novel patterns of HLA-C allotype recognition. *J Immunol* 1998;161:571-577.
 44. Lopez-Botet M, Bellon T. Natural killer cell activation and inhibition by receptors for MHC class I. *Curr Opin Immunol* 1999;11:301-307.
 45. Matsui M, Machida S, Tomiyama H, Takiguchi M, Akatsuka T. Introduction of tapasin gene restores surface expression of HLA class I molecules, but not antigen presentation of an HIV envelope peptide in a hepatoma cell line. *Biochem Biophys Res Commun* 2001;285:508-517.
 46. Shilling HG, McQueen KL, Cheng NW, Shizuru JA, Negrin RS, Parham P. Reconstitution of NK cell receptor repertoire following HLA-matched hematopoietic cell transplantation. *Blood* 2003;101:3730-3740.
 47. Valiante NM, Uhrberg M, Shilling HG, Lienert-Weidenbach K, Arnett KL, D'Andrea A, et al. Functionally and structurally distinct NK cell receptor repertoires in the peripheral blood of two human donors. *Immunity* 1997;7:739-751.
 48. Kayagaki N, Yamaguchi N, Nakayama M, Takeda K, Akiba H, Tsutsui H, et al. Expression and function of TNF-related apoptosis-inducing ligand on murine activated NK cells. *J Immunol* 1999;163:1906-1913.



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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.

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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.

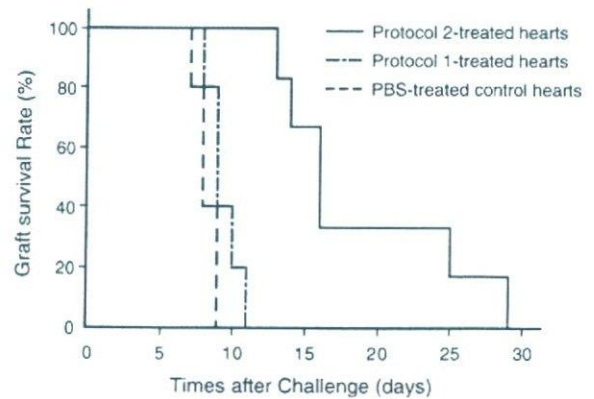


Fig. 1. The survival of endotoxin-tolerized heart allografts was significantly prolonged as compared with that of untreated heart allografts. B6 mice were pretreated as follows: protocol 1, intravenous injection with low doses of LPS (20 µg) three times on days -6, -3 and 0; protocol 2, intravenous injection 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.

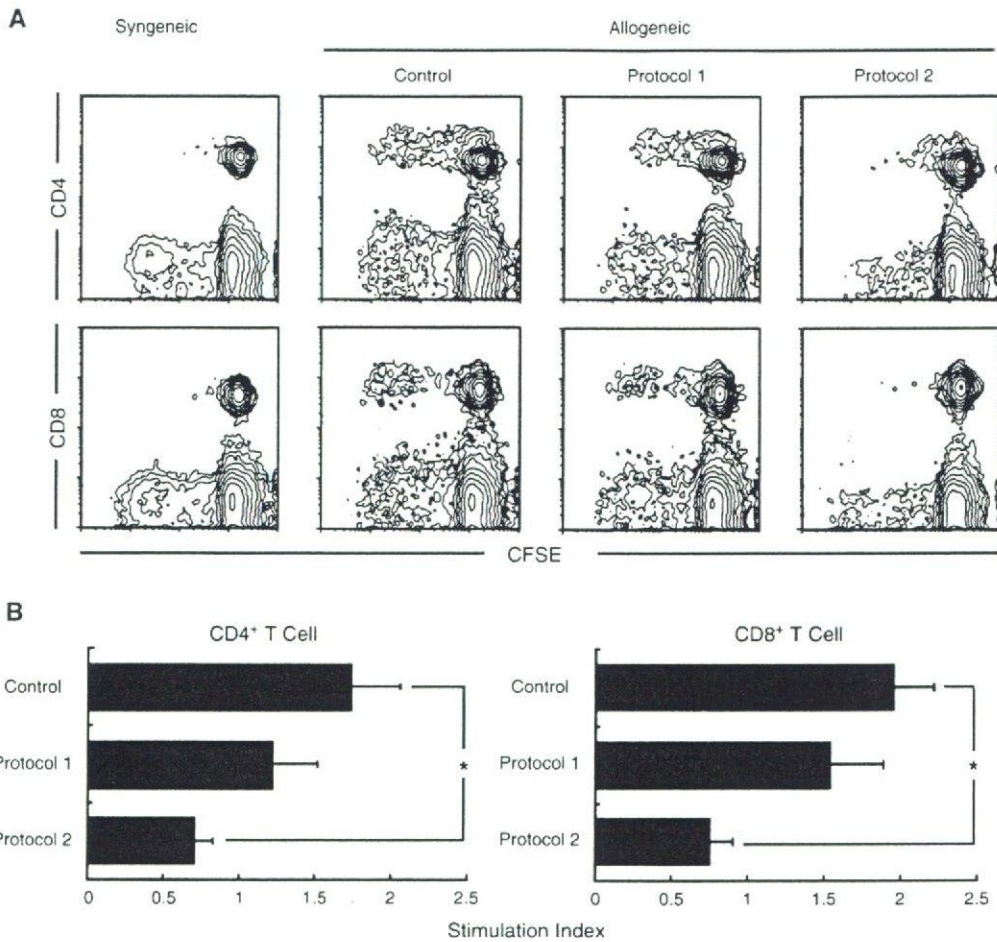


Fig. 2. Induction of endotoxin tolerance in stimulator mice reduced alloimmune responses. B6 mice were pretreated with the following protocol to induce ET: protocol 1, intravenous injection with low doses of LPS (20 µg) three times on days –6, –3 and 0; protocol 2, intravenous injection with low doses 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 (\pm 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 \pm 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 \pm 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 \pm S.E.M. ($n = 5$). Statistical analyses were performed using ANOVA ($*p < 0.05$).