a model induced by 90% hepatectomy and is considered to mimic the clinical status of human ALF fairly faithfully [14]. The rats lacked a functional liver and showed ischemic changes in the right lobe, resulting in regeneration failure of the remnant omental lobe, whose weight occupied about 8% of the total liver weight. This model has previously been used to demonstrate that FIHEP transplantation effectively prolongs the survival of rats suffering from ALF [15]. We reproduced similar results in the present study. Notably, CPHEPs, which had been prepared by multiplying FIHEPs 3 times, were as effective as FIHEPs in prolonging the survival of rats suffering from ALF. CPHEP transplantation improved all the liver functions tested in this study. In addition, the BrdU-labeling index of the hepatocytes in the remnant liver was comparable to that in the FIHEP group. Rats with CPHEPs gradually regained liver weight after ALF induction, as did those with FIHEPs. These results together indicate that both CPHEP and FIHEP could be a source for hepatocyte transplantation to promote regeneration of the remnant liver after ALF induction.

There have been two explanations for lethal hepatic failure after excessive hepatectomy: hepatectomy causes microcircular disturbances [20] or induces cytotoxic factors such as TNF-α, TGF-β1, and oxidative stress-related factors [21, 22]. In the present study, we did not find any evidence of microvascular disturbances on hematoxylin and eosin (H&E)-stained sections of the remnant lobe in the ALF-induced rats, but we did observe hypercytokinemia of cytokines such as IL-6 and TGF-β1. Apoptotic hepatocytes were frequently seen by TUNEL assay in the remnant liver lobe of the ALF-induced rats. CPHEP and FIHEP transplantation decreased the concentrations of IL-6 and TGF-β1 in sera, as well as the frequency of apoptotic hepatocytes. Therefore, it appears that both CPHEPs and FIHEPs prolonged the survival of ALF-induced rats by suppressing the hepatocytic apoptosis in the remnant liver.

In the present study, we demonstrated the presence of DPPIV$^+$ hepatocytes in the spleen at 24 h after ALF induction, which clearly indicated the engraftment of both transplanted CPHEPs and FIHEPs in the graft site. There were no significant differences in the frequency of DPPIV$^+$ hepatocytes between the FIHEP and CPHEP groups. However, the expression level of hepatocyte-specific mRNAs such as Alb, CYP2C7, and GS in the spleen of the CPHEP rats was considerably lower than that in the FIHEP rats. This might be explained by the fact that CPHEPs showed lower expression levels of these marker genes than FIHEPs at the time of transplantation; this was due to the fact that the CPHEP cells had been cultured for 11 d before transplantation, during which time the expression levels had decreased (Fig. 1B). Another explanation could be that the CPHEPs were more vulnerable than the FIHEPs, and that most of them became nonviable in the spleen after transplantation. We noticed the presence of many DPPIV$^+$ but Hoechst$^-$ cells in the middle of the CPHEP clusters, but not in the FIHEP clusters. These Hoechst$^-$ cells were considered to be nonviable.

It has previously been shown that homogenized hepatocytes were even effective as a treatment for liver failure [23], suggesting the effectiveness of nonviable hepatocytes. In the present study, we also showed that the survival rate of the rats in the DHEP group was better, to some extent, than that in the control CM group, although the rate was much lower than that of the CPHEP group. In light of these results, it is likely that transplanted CPHEPs contribute to the improvement of liver failure by substituting the function of the host liver. They may also provide some growth factors or enzymes to support the regeneration of the remnant liver. It remained to be elucidated whether the cryopreserved CPHEPs also display such beneficial effects. Hepatocytes are known to be very sensitive to freezing damage. Three distinct modes of cryopreservation-induced hepatocyte death have been identified, namely, physical cell rupture, necrosis, and apoptosis [24]. The susceptibility of hepatocytes to such freeze-thaw injury is attributed to the damage to mitochondria, including loss of mitochondrial membrane integrity, increase in membrane permeability, etc. The inhibition of mitochondria damage, for instance, by broad-spectrum caspase-inhibitor, would prevent cryopreservation-induced damage of propagated hepatocytes.

In conclusion, the transplantation of homologous CPHEPs has a remarkable therapeutic potential for ALF in rats. Since we have recently established a culture method that enables us to multiply human hepatocytes 50 to 100 times during 50 d of culture [25], CPHEPs might be a useful source of hepatocytes for transplantation to treat human patients with ALF.

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Selection Criteria for Hepatectomy in Patients with Hepatocellular Carcinoma Classified as Child-Pugh Class B

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Abstract
Background The appropriate surgical approach for hepatocellular carcinoma (HCC) patients of Child-Pugh class B is unclear. The aim of this study was to clarify the prognostic factors after hepatectomy in Child-Pugh class B patients and to delineate the selection criteria for hepatectomy.
Methods One hundred fifty patients of Child-Pugh class B who underwent hepatectomy were enrolled in this retrospective study (Hx group). Univariate and multivariate analyses were performed to identify prognostic factors. The prognosis was compared with that of 23 patients of Child-Pugh class B who underwent liver transplantation (LT group).
Results The overall survival rate of the Hx group was significantly worse than that of the LT group (5-year survival: 36.0 vs. 78.3%, $p = 0.001$). In multivariate analyses, diabetes mellitus ($p = 0.011$), preoperative total bilirubin level $\geq$1.5 mg/dl ($p = 0.038$), and Child-Pugh score of 8 or 9 ($p = 0.038$) were independent prognostic factors. Although the overall 5-year survival rate of patients with none of the three adverse prognostic factors was only 50.3%, that of patients with one or more adverse prognostic factors was only 27.2% ($p = 0.001$).
Conclusions Hepatectomy may be the optimal initial treatment for HCC patients classified as Child-Pugh class B and without any adverse prognostic factors.

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Introduction

The prognosis for hepatocellular carcinoma (HCC) patients with chronic liver disease depends not only on tumor factors and selected treatment modality but also on hepatic functional reserve [1]. The Child-Pugh classification was first reported to assess the severity of underlying cirrhosis in patients with bleeding esophageal varices [2]. This classification is simple, reliable, and widely used in deciding on HCC treatment.

Hepatectomy has been considered a reasonable treatment for patients with good liver function, such as those of Child-Pugh class A. It is now associated with reduced mortality [3, 4] and a 5-year overall survival rate of up to 70% [5–8]. Most HCC patients classified as Child-Pugh class A can undergo hepatectomy and have a good prognosis [9–12].

Liver transplantation (LT) based on Milan criteria (solitary liver nodule with a maximum diameter not exceeding 5 cm or 2 or 3 tumors with a diameter not exceeding 3 cm) has been shown to provide very good disease-free survival. Theoretically, LT has been considered the optimal treatment for HCC because it removes the underlying cirrhotic liver tissue that is at risk for the development of de novo HCC and restores normal hepatic function. LT is the only option for cirrhotic patients of Child-Pugh class C with HCC to achieve long-term survival. However, the limited availability of donor organs makes LT less available to individual patients [13–15]. Because of prolonged transplantation wait times, tumor progression may counteract the benefit of LT [6].

There are a few studies on the treatments for HCC patients classified as being of Child-Pugh class B [16–18]; however, the appropriate surgical approach for these patients is not decisive. The aims of this retrospective analysis were to clarify the prognostic factors after

hepatectomy in patients with HCC classified as Child-Pugh class B and to delineate the selection criteria of hepatectomy. This pragmatic attitude allows us to avoid unnecessary LT for patients who could survive long term with only hepatic resection and significantly spare their organs.

Patients and methods

Patients

Nine hundred nineteen patients with HCC underwent hepatectomy as initial treatment at our institute between 1986 and 2008. Among these patients, 150 patients with HCC classified as Child-Pugh class B who had been followed up for 1 or more years were enrolled in this retrospective study (Hx group). Their prognosis was compared with that of 23 patients with HCC who underwent living-donor liver transplantation (LDLT) for HCC of Child-Pugh class B at our institute between 1991 and December 2008 (LT group).

The clinicopathologic characteristics of the Hx group and the LT group are given in Table 1. Selection of hepatectomy type was made on the basis of liver function and tumor extent [5, 19]. Liver function was assessed by Child-Pugh classification [2] and indocyanine green retention rate at 15 min (ICGR15). If liver function would allow, anatomic resection [segmentectomy ($n = 15$), sectionectomy ($n = 7$), or hemihepatectomy ($n = 11$)] was performed. In patients with insufficient hepatic reserve, limited resection ($n = 117$) was performed. For example, right hemihepatectomy could be tolerated if ICGR15 was in the normal range. One third of the liver parenchyma could be resected in patients with ICGR15 of 10-19%, segmentectomy was possible with ICGR15 of 20-29%, and limited resection was possible with ICGR15 of 30% or more [19]. The hepatectomy procedures were the same as those described previously [5]. Eligibility for LDLT was determined by the following pretransplant criteria: technical unresectability of HCC or altered liver function (Child-Pugh class B or C), age <70 years, absence of metastatic lymph nodes or evidence of extrahepatic spreading, absence of macroscopic vascular invasion, no history of other malignant tumors for at least 5 years, and candidate for living donor. Selection of graft type was made on the basis of a remnant liver ratio greater than 30% and graft-to-recipient body weight ratio of greater than 0.7%. Postoperative follow-up included liver function tests, dosage of serum α-fetoprotein (AFP), hepatic ultrasound on a 3-month basis, and computed tomography scan every 6 months. Follow-ups were performed in outpatient clinics or by the patients' general practitioners, and data were updated until March 2010. Survival was computed from the date of the surgery.

Because LDLT was available throughout the study period, the policy was to consider LDLT for patients who presented with liver recurrence or hepatic deterioration after resection. Four patients underwent LDLT after hepatectomy for HCC of Child-Pugh class B.

Definitions

Liver cirrhosis was confirmed by histological examination of a resected specimen. Major hepatectomy was defined as the resection of three or more Couinaud segments. Curative

Table 1 Clinicopathologic characteristics of patients with hepatic resection (Hx group) or living-donor liver transplantation (LT group) for HCC as Child-Pugh class B

Features	Hx group ($N = 150$)	LT group ($N = 23$)	p value
Gender			
Male/Female	102/48	19/4	NS
Age	61.3 ± 8.6 (35–87)	55.0 ± 6.41 (38-65)	<0.001
Liver cirrhosis	120	23	0.039
Etiology of liver disease			
HBV/HCV/Other	18/111/15	5/16/2	NS
Total bilirubin (mg/dl)	1.0 ± 0.6 (0.2-5.3)	3.8 ± 6.9 (0.6-31.4)	<0.001
Maximum tumor diameter (mm)	41.3 ± 39.3 (5–300)	18.9 ± 8.4 (9–27)	<0.001
Number of tumors	2.2 ± 2.2 (1–20)	1.6 ± 2.2 (1–3)	NS
Milan criteria compatibility (Yes/No)	97/53	21/2	0.021
Histological grading			
Well/Moderately/Poorly/Unknown	34/86/24/6	3/18/2/0	NS
Microscopic vascular invasion	20	4/19	NS
T1, T2/T3, T4	67/83	20/3	<0.001
Extent of hepatic resection			
Minor hepatectomy/Major hepatectomy	139/11	–	
Curative/Noncurative	109/41	–	

HBV hepatitis B virus, *HCV* hepatitis C virus

hepatectomy was defined as removal of all recognizable tumors. In patients with multiple HCCs, the operation was defined as noncurative hepatectomy when some of the HCCs could not be resected. The operation was also defined as noncurative when some of the HCCs were resected and others were treated by intraoperative ablation therapies such as microwave coagulation therapy or radiofrequency ablation. All postoperative complications were reviewed for at least 30 days after surgery. The complications were graded according to the method described by Clavien et al. [20]. Complications of grade III or higher were categorized as morbid. Postoperative mortality was defined as any death that occurred within 30 days of surgery.

Statistical analysis

Parametric analyses were performed using Student's t test, and nonparametric analyses were performed using the Mann-Whitney U test. The overall survival rate was calculated using the Kaplan-Meier method. Univariate analysis of factors thought to influence overall survival was carried out using the log-rank test. The Cox proportional hazards model was used in the multivariate analysis of the factors that were determined to be significant for overall survival by univariate analysis. A difference was considered significant if $p < 0.05$. Statistical analyses were performed using SPSS statistical software version 16 (SPSS, Chicago, IL, USA).

Results

Patient gender, age, etiology, number of liver tumors, maximum tumor diameter, Milan criteria compatibility, histological grading of liver tumors, microvascular invasion, extent of hepatic resection, and surgery curability are listed in Table 1. Median follow-up periods of the Hx group and the LT group were 34.2 (range = 0.8–165) months and 40.9 (0.7–100.9) months, respectively.

In the Hx group, one patient died of ruptured esophageal varices within 1 month after surgery (operative mortality rate: 0.7%). On the other hand, one patient died of sepsis after LDLT (operative mortality rate: 4.3%). The morbidity rate of the Hx group was 12.7% (19/150).

A total of 116 patients died after hepatectomy for HCC. The causes of death were cancer recurrence in 72 patients and liver failure or bleeding from the gastrointestinal tract in 44 patients. Overall survival rates at 1, 3, and 5 years after hepatectomy were 81.9, 52.9, and 36.0%, respectively. On the other hand, overall survival rates at 1, 3, and 5 years after LT were 87.0, 78.3, and 78.3%, respectively. Outcomes of the Hx group were significantly worse than those of the LT group ($p = 0.001$) (Fig. 1). A total of 115 patients (76.7%) developed recurrence after hepatectomy for HCC. Disease-free survival rates at 1, 3, and 5 years after hepatectomy were 74.5, 49.7, and 20.4%, respectively. On the other hand, no patients experienced recurrence of HCC in the LT group.

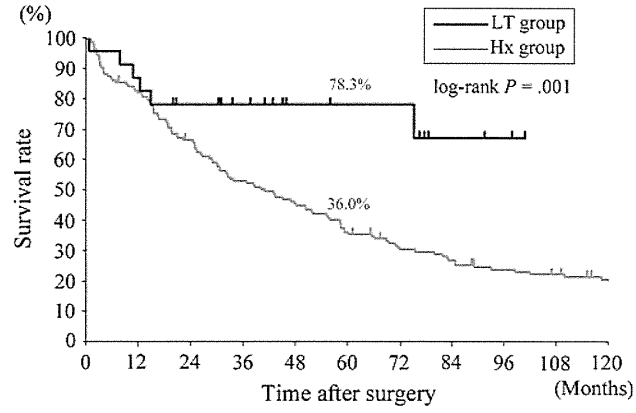

Fig. 1 Kaplan-Meier curves for the overall survival rate after hepatectomy (Hx group; $N = 150$) or living-donor liver transplantation (LT group; $N = 23$) for HCC classified as Child-Pugh class B. The overall survival rates of the Hx group (81.9, 52.9, and 36.0% at 1, 3, and 5 years) were significantly lower than those of the LT group (87.0, 78.3, and 78.3% at 1, 3, and 5 years) ($p = 0.001$)

In the univariate analysis, 7 of 29 variables were found to be significant prognostic factors for overall survival. Presence of liver cirrhosis ($p = 0.004$), presence of diabetes mellitus ($p = 0.001$), Child Pugh score of 8 or 9 ($p = 0.010$), preoperative total bilirubin (TBIL) level $\geq$1.5 mg/dl ($p = 0.028$), operative blood loss $\geq$1000 g ($p = 0.015$), transfusion of fresh frozen plasma (FFP) ($p = 0.004$), and noncurative operation ($p = 0.008$) were significant adverse prognostic factors for overall survival (Table 2). Other prognostic factors related to liver tumors were not identified as being statistically significant. In multivariate analyses, liver cirrhosis ($p = 0.005$), preoperative TBIL level $\geq$1.5 mg/dl ($p = 0.030$), and Child Pugh score of 8 or 9 ($p = 0.024$) were independent variables related to poor prognosis (Table 3). Presence of liver cirrhosis cannot be clearly demonstrated until resected specimens have been examined microscopically by a pathologist, i.e., it is difficult to preoperatively predict the presence of this factor. In multivariate analysis excluding the factor of liver cirrhosis, diabetes mellitus ($p = 0.011$), a preoperative TBIL level $\geq$1.5 mg/dl ($p = 0.038$), and Child Pugh score of 8 or 9 ($p = 0.038$) were independent variables related to poor prognosis (Table 4).

Overall survival rates at 1, 3, and 5 years of 58 patients in the Hx group with none of the three adverse prognostic factors (score 0) were 89.7, 66.4, and 50.3%, respectively. The survival rate of the LT group tended to be higher than that of the Hx group with none of the three adverse

Table 2 Univariate analysis of prognostic factors after hepatectomy for HCC classified as Child-Pugh class B

Variable	*N*	3-year survival rate (%)	5-year survival rate (%)	*p* value
All patients	150	52.9	36.0	
Age				
≥70 years	19	67.7	45.1	0.235
<70 years	131	50.8	34.7	
Gender				
Male	102	52.4	35.3	0.319
Female	48	54.2	37.5	
HBs antigen				
Positive	29	43.2	32.4	0.895
Negative	121	55.2	36.8	
Anti-HCV antibody				
Positive	111	54.8	34.7	0.323
Negative	39	47.7	39.8	
Liver cirrhosis				
Yes	120	50.0	31.7	0.004
No	30	65.1	54.3	
Esophageal varices				
Yes	7	57.1	47.9	0.401
No	143	52.7	35.6	
Ascites				
Yes	56	48.2	30.4	0.267
No	94	55.8	39.4	
Diabetes mellitus				
Yes	66	40.9	24.2	0.001
No	84	62.5	45.3	
Child-Pugh score				
7 point	108	58.8	41.7	0.010
8 or 9 point	42	38.1	21.4	
Platelet count (/mm^3)				
<100,000	89	53.9	36.0	0.945
≥100,000	61	51.5	36.1	
Prothrombin time (%)				
<70	102	55.7	36.8	0.668
≥70	48	47.2	34.3	
Total bilirubin (mg/dl)				
<1.5	120	57.9	40.0	0.028
≥1.5	30	30.0	20.0	
AST (IU/L)				
≥50	86	50.7	33.0	0.337
<50	64	56.0	40.0	
ALT (IU/L)				
≥50	71	54.9	38.0	0.822
<50	79	51.3	34.2	
Albumin (g/dl)				
<3.5	120	50.5	34.5	0.774
≥3.5	30	62.6	41.7	
Total cholesterol (mg/dl)				
<150	93	49.0	34.8	0.263
≥150	57	59.3	37.7	
ICG-R15 (%)				
≥20	109	49.5	33.0	0.068
<20	41	62.3	49.3	
AFP (ng/ml)				
≥400	38	42.1	23.7	0.063
<400	110	57.1	40.5	
PIVKA-II (mAU/ml)				
≥400	13	58.0	58.0	0.210
<400	118	54.2	37.3	
Number of tumors				
Single	74	60.6	44.1	0.089
Multiple	76	45.5	28.1	
Tumor size (mm)				
<30	82	46.2	33.9	0.385
≥30	68	47.7	35.4	
Milan criteria compatibility				
Yes	97	54.6	35.4	0.653
No	53	50.0	38.0	
Microscopic vascular invasion				
Positive	20	27.8	27.8	0.064
Negative	130	56.8	37.3	
Histological grading				
Well	39	71.8	48.7	0.210
Moderately/Poorly	111	47.1	31.4	
Extent of hepatic resection				
Major hepatectomy	11	63.6	53.0	0.175
Minor hepatectomy	139	52.4	34.9	
Intraoperative blood loss (g)				
≥1000	29	32.7	21.8	0.015
<1000	121	57.7	39.3	
Blood transfusion				
Performed	26	44.7	28.4	0.312
Not performed	124	54.7	37.5	
FFP transfusion				
Performed	61	43.6	23.5	0.004
Not performed	89	59.3	44.5	
Curability				
Noncurative	41	35.2	22.7	0.008
Curative	109	59.5	40.9	

HBs antigen hepatitis B surface antigen, *HCV* hepatitis C virus, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *ICG-R15* indocyanine green retention rate at 15 min, *AFP* α-fetoprotein, *PIVKA-II* protein induced by vitamin K absence II

All data were analyzed by the log rank test

Table 3 Multivariate analysis of prognostic factors after hepatectomy for HCC classified as Child-Pugh class B

Variable	Hazard ratio	Confidence interval (95%)	*p* value
Liver cirrhosis	2.247	1.271–3.968	0.005
Total bilirubin (mg/dl) ≥1.5	1.675	1.065–2.667	0.030
Child-Pugh score 8 or 9 point	1.597	1.065–2.392	0.024

Multivariate analysis of factors shown to be significant in the univariate analysis was performed by logistic regression

Table 4 Multivariate analysis of prognostic factors excluding cirrhosis after hepatectomy for HCC classified as Child-Pugh class B

Variable	Hazard ratio	Confidence interval (95%)	*p* value
Diabetes mellitus	1.637	1.117–2.392	0.011
Total bilirubin (mg/dl) ≥1.5	1.616	1.065–2.545	0.038
Child-Pugh score 8 or 9 point	1.534	1.025–2.294	0.038

Multivariate analysis of factors shown to be significant in the univariate analysis was performed by logistic regression

prognostic factors (score 0, $n = 58$), but there was no statistically significant difference between the two groups ($p = 0.127$). On the other hand, overall survival rates at 1, 3, and 5 years of 92 hepatectomized patients with one or more of the three adverse prognostic factors (score ≥1) were 77.2, 44.6, and 27.2%, respectively, significantly lower than those of the LT group ($p = 0.002$) (Fig. 2).

In general, LT is not the accepted treatment for patients with HCC beyond the Milan criteria. Thus, we compared 21 patients classified as Child-Pugh class B who underwent LT for HCC and who met the Milan criteria to 48 patients

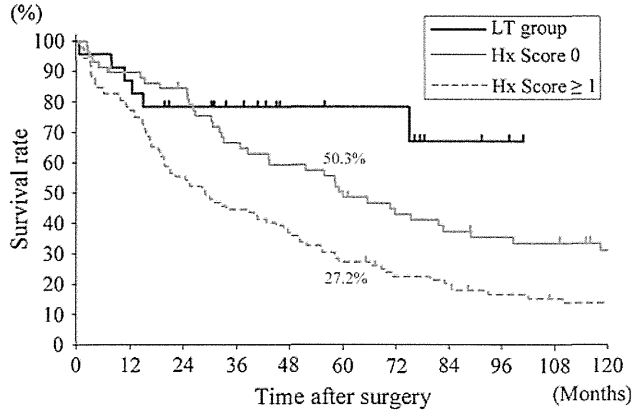

Fig. 2 Patients who underwent hepatectomy for HCC classified as Child-Pugh class B (Hx group; $N = 150$) were divided into two groups by the number of risk factors (score = 0, $N = 58$; score ≥ 1, $N = 92$). Overall survival rates of those groups were compared with those of patients who underwent living-donor liver transplantation (LT group; $N = 23$). Overall survival rates of patients in the score 0 group (89.7, 66.4, and 50.3% at 1, 3, and 5 years) were similar to those of the LT group (87.0, 78.3, and 78.3% at 1, 3, and 5 years) ($p = 0.127$). On the other hand, the corresponding survival rates of the score ≥ 1 group (77.2, 44.6, and 27.2% at 1, 3, and 5 years) were significantly lower than those of the LT group ($p = 0.002$)

with one or more of the three adverse prognostic factors who underwent curative hepatectomy for HCC and met the Milan criteria. Overall survival rates at 1, 3, and 5 years of patients who underwent LT were significantly higher than those of patients who underwent hepatectomy ($p = 0.017$); the 3- and 5-year overall survival rates were 75.9 and 75.9%, respectively, in the LT group and 54.2 and 33.3%, respectively, in the Hx group. On the other hand, the overall survival rates of 32 patients who underwent curative hepatectomy for HCC with none of the three adverse prognostic factors within Milan criteria tended to be lower than those of patients who underwent LT within Milan criteria, but there was no statistically significant difference between the two groups (90.6, 70.9, and 47.2% at 1, 3, and 5 years) ($p = 0.213$) (Fig. 3).

In addition, we divided 58 hepatectomized patients with none of the three adverse prognostic factors (score 0) into two groups, those with and without liver cirrhosis, which were the independent prognostic factors demonstrated after surgery. Overall survival rates at 1, 3, and 5 years of 15 patients without liver cirrhosis were 100, 85.1, and 77.4%, respectively. On the other hand, those of 43 patients with liver cirrhosis were 86.0, 60.5, and 41.9%, respectively. Although the overall survival rate of Hx patients without liver cirrhosis was similar to that of the LT group ($p = 0.867$), outcomes of Hx patients with liver cirrhosis were significantly worse than those of the LT group ($p = 0.041$) (Fig. 4).

Discussion

There is a limit to the extent of resectable liver volume in patients classified as Child-Pugh class B because they do not have sufficient hepatic functional reserve to tolerate a

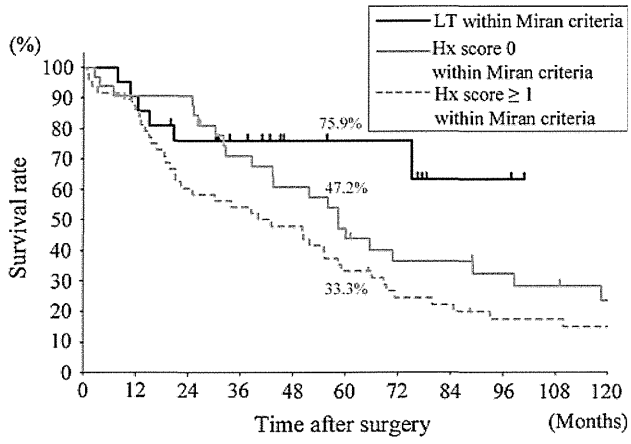

Fig. 3 Within the Milan criteria, patients who underwent curative hepatectomy for HCC classified as Child-Pugh class B with one or more of the three adverse prognostic factors (Hx score ≥ 1 within Milan criteria; $N = 48$) were compared to patients who underwent liver transplantation (LT within Milan criteria; $N = 21$). The overall survival rates of patients in the Hx score ≥ 1 within the Milan criteria group (85.4, 54.2, and 33.3% at 1, 3, and 5 years) were significantly lower than those of patients in the LT within the Milan criteria group (90.5, 75.9, and 75.9% at 1, 3, and 5 years) ($p = 0.017$). The overall survival rates of patients who underwent curative hepatectomy for HCC with none of the three adverse prognostic factors within the Milan criteria group (Hx score 0 within Milan criteria; $N = 32$) tended to be lower than those of patients in the LT within the Milan criteria group, but there was no statistically significant difference between the two groups (90.6, 70.9, and 47.2% at 1, 3, and 5 years) ($p = 0.213$)

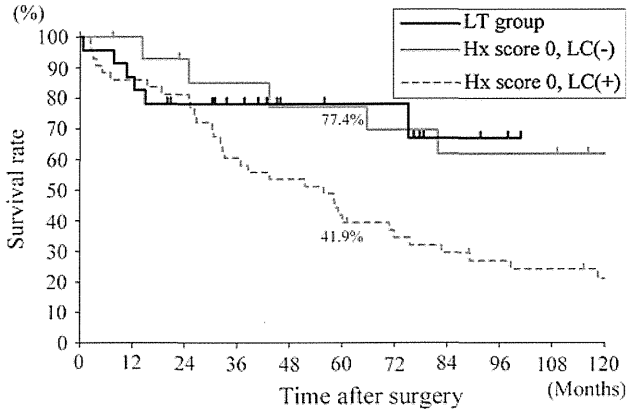

Fig. 4 Fifty-eight patients who underwent hepatectomy for HCC classified as Child-Pugh class B (Hx group) with none of the three adverse prognostic factors (score 0) were divided into two groups: with or without liver cirrhosis [LC(+) and LC(−), respectively]. The overall survival rates at 1, 3, and 5 years of the LC(−) group ($N = 15$) were 100, 85.1, and 77.4%, respectively, and those of the LC(+) group ($N = 43$) were 86.0, 60.5, and 41.9%, respectively. Although the overall survival rate of the LC(−) group was similar to that of the LT group ($p = 0.867$), that of the LC(+) group was significantly lower than that of the LT group ($p = 0.041$)

major hepatectomy. As a result, a minor hepatectomy was selected most often in the present study. Furthermore, hepatectomy leaves the diseased liver in place and is understandably associated with high rates of intrahepatic recurrence. Local recurrence and de novo HCC are the main types of recurrence. Local recurrence may be the result of inadequate R1 resection or of secondary progression of microscopic vascular invasion, mainly through subsegmental portal branches. De novo HCC is another major cause of post-hepatectomy recurrence. Either way, hepatectomy for patients with HCC classified as Child-Pugh class B has the disadvantage of tumor recurrence. It has been shown that the recurrence rate grows with time and reaches 80% or more at 5 years [21].

Few studies have compared the outcomes of patients classified as Child-Pugh class B who underwent hepatectomy for HCC with those of patients who underwent LT. To clarify which subgroup has a good prognosis after hepatectomy, we investigated the adverse prognostic factors and compared the prognoses of patients who underwent hepatectomy with those who underwent LT.

We identified the presence of liver cirrhosis, preoperative TBIL level ≥1.5 mg/dl, and Child-Pugh score of 8 or 9 as the independent variables related to poor prognosis. Excluding the factor that is difficult to predict preoperatively (i.e., presence of liver cirrhosis), diabetes mellitus ($p = 0.011$), preoperative total bilirubin level ≥1.5 mg/dl ($p = 0.038$), and Child-Pugh score of 8 or 9 ($p = 0.038$) were independent prognostic factors. Two of these three prognostic factors are the variables related to liver function. Liver function can be a variable in patients classified as Child-Pugh class B; therefore, our institute uses the Child-Pugh score of 8 or 9 as one of the standards for choosing LT. This score is a simple measure of hepatic functional reserve. In this study, patients with Child-Pugh scores of 8 or 9 actually had significantly poor prognoses.

In this analysis, diabetes mellitus is also one of the independent prognostic factors. Diabetes has been suggested as a potential risk factor for HCC in some published studies [22–26], but the mechanism for this remains unclear. One of the suggested mechanisms is that insulin resistance leads to a state of hyperinsulinemia, which, via interaction with the insulin receptor, promotes increased phosphorylation and activation of the downstream serine/threonine kinase and extracellular signal-regulated kinase pathways [27]. The other involves insulin-like growth factor, which, while acting through separate binding proteins and receptors, has the same downstream intracellular mediators as the insulin receptor pathway [28]. On the one hand, diabetes is a risk factor for nonalcoholic fatty liver disease or nonalcoholic steatohepatitis [24], which can lead to liver fibrosis, cirrhosis, and subsequent HCC. More research is necessary to clarify whether diabetes itself directly predisposes patients to HCC.

Ueno et al. [18] found serum albumin level, presence of esophageal varices, number of tumors, tumor diameter, and

AFP as negative prognostic factors in HCC patients of Child-Pugh class B or C. With respect to the prognostic factors for survival, our results differed from those reported by Ueno et al. [18]. The difference might be due to patients' characteristics, including transcatheter arterial chemoembolization and local ablation. Our previous reports have shown that adverse prognostic factors for overall survival in cirrhotic patients with HCC classified as Child-Pugh class B were the presence of ascites, elevated TBIL, reduced choline esterase, elevated AFP, microvascular invasion, and noncurative hepatectomy [17]. These adverse prognostic factors include the variables related to tumor factors. The difference between our current study and the previous study may be due to the fact that the previous study was restricted to cirrhotic patients with Child-Pugh class B, whereas the present study included noncirrhotic patients. Next, in our study the overall survival of patients classified as Child-Pugh B was significantly longer in the LT group than those in the Hx group. However, we identified a subgroup in which HCC patients of Child-Pugh class B had relatively good prognoses (1-, 3-, and 5-year survival rates of 89.7, 66.4, and 50.3%, respectively) after Hx, which was compatible with the LT results. Hepatectomy may be acceptable even in Child-Pugh class B patients because proper selection of candidates for resection may result in better outcomes. The subgroup comprises patients with none of the three adverse prognostic factors described above. This new knowledge is very important in the era of donor organ scarcity. On the other hand, the 5-year survival rate after hepatectomy in patients with one or more adverse prognostic factors was 27.2%. Accordingly, such patients should be evaluated for LT before hepatectomy is performed.

Next, even without adverse prognostic factors, the overall survival rate of Hx patients with liver cirrhosis was significantly worse than that of patients who underwent LT. If the presence of histological liver cirrhosis was demonstrated after hepatic resection, salvage LT was considered after HCC recurrence.

In this decade, the strategy of liver resection as a bridge to transplantation for HCC, so-called "salvage" LT, was suggested when patients had one or two nodules, good hepatic function, and preoperative and intraoperative absence of macroscopic vascular invasion and extrahepatic tumors [29–32]. In the study by Adam et al. [30], outcomes of salvage LT were poor, largely because of the high operative mortality compared with that of primary LT. By contrast, Belghiti et al. [29], Del Gaudio et al. [31], and Cherqui et al. [32] found no increased mortality and morbidity after salvage LT compared with primary LT, as well as identical long-term survival. Salvage LT may become the strong option after hepatectomy for HCC in patients of Child-Pugh class B using our new criteria because they have enough liver function after hepatectomy to undergo secondary LT.

In summary, diabetes mellitus, preoperative TBIL level $\geq$1.5 mg/dl, and Child-Pugh score of 8 or 9 were shown to be adverse prognostic factors in hepatectomized HCC patients of Child-Pugh class B. Hepatectomy can be indicated as the initial treatment for HCC patients of Child-Pugh class B with none of the three adverse prognostic factors, whereas LT should be considered the initial treatment for patients of Child-Pugh class B with one or more adverse prognostic factors.

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Possibility of Adoptive Immunotherapy With Peripheral Blood-derived $CD3^-CD56^+$ and $CD3^+CD56^+$ Cells for Inducing Antihepatocellular Carcinoma and Antihepatitis C Virus Activity

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Summary: We recently showed that interleukin (IL)-2-stimulated $CD56^+$ cells derived from the liver exert vigorous cytotoxicity against hepatocellular carcinoma (HCC) by their binding to the tumor necrosis factor-related apoptosis-inducing ligand expressed on natural killer cells and the corresponding death receptors, and exhibit inhibitory effects on hepatitis C virus (HCV) replication by production of a high level of interferon-γ. These findings prompted us to develop a technique to increase the number of such innate components of cellular immunity from peripheral blood mononuclear cells (PBMCs) so that, they can be easily applied for immunotherapy clinically. We expanded $CD3^-CD56^+$ and $CD3^+CD56^+$ cells ex vivo from PBMCs of human volunteers by using media containing IL-2 and anti-CD3 monoclonal antibody. Among the various culture media used, autoserum supplemented X-VIVO 15 most efficiently supported PBMCs expansion and maintained the viability of the expanded cells (approximately 60-fold expansion after 28-d culture). Cultivation of PBMCs in this medium resulted in the highest proportion of $CD3^-CD56^+$ cells among the propagated lymphocytes (approximately 40% after 28-d culture). An experiment using genomic HCV replicon-containing hepatic cells showed that the $CD3^-CD56^+$ cell-enriched expansion strongly inhibited HCV replication when compared with freshly isolated PBMCs. The additional anti-CD3 monoclonal antibody pulse stimulation induced anti-HCV activity even in the $CD3^+CD56^+$ cells among the propagated PBMCs. Further, cytotoxic assay showed that the expansion of $CD3^-CD56^+$ and $CD3^+CD56^+$ cells resulted in vigorous cytotoxicity against HCC. In conclusion, $CD56^+$ cells obtained from the PBMCs show anti-HCV activity in addition to anti-HCC activity.

Key Words: adoptive immunity, cytotoxicity, hepatocellular carcinoma, hepatitis C virus, peripheral blood mononuclear cells

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Liver failure and hepatocellular carcinoma (HCC) because of chronic hepatitis C infection are the most common indications for liver transplantation (LT). The incidence of both the conditions has been projected to increase further. Recurrent hepatitis C virus (HCV) infection of allografts is universal, occurs immediately after LT, and is associated with accelerated progression to liver cirrhosis, graft loss, and death.[1,2] The infection is thought to reflect suppression of the host-effector immune responses that usually control the HCV replication.[3,4] Further, this immunosuppressive condition is considered to increase the incidence of HCC recurrence after LT.

We recently proposed a novel strategy of adjuvant immunotherapy for preventing HCC recurrence after LT: in this immunotherapy, transplant recipients are intravenously injected with lymphokine-activated killer (LAK) cells including activated $CD3^-CD56^+$ and $CD3^+CD56^+$ cells derived from the liver allografts. Considering that the immunosuppressive regimen currently used after LT reduces the components of adaptive immunity, but effectively maintains the innate components of cellular immunity,[5–7] augmentation of $CD3^-CD56^+$ and $CD3^+CD56^+$ cells that are thought to play a pivotal role in innate immunity, may be a promising immunotherapeutic approach.[8] We confirmed that $CD56^+$ cells derived from the liver allografts treated with interleukin (IL)-2 and anti-CD3 monoclonal antibody (mAb) express a significantly high level of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which is a critical molecule for tumor cell killing; further, these cells showed high cytotoxicity against HCC, with no such effect on normal cells.[8] After obtaining approval from the ethical committee of our institute, we successfully applied adoptive immunotherapy with liver lymphocytes treated with IL-2 and anti-CD3 mAb to patients with HCC having liver cirrhosis in a phase I trial.[9] Although the long-term benefits of this approach with regard to the control of HCC recurrence after LT remain to be elucidated, the trial provided a unique opportunity to study whether the administration of liver lymphocytes treated with IL-2 and anti-CD3 mAb can also induce an anti-HCV response in transplant recipients with HCV infection. During the first month after LT, the HCV ribonucleic acid (RNA) titers in the sera of the patients who underwent immunotherapy were markedly low compared with those in the sera of the patients who did not undergo immunotherapy; however, such anti-HCV responses were transient. This observation prompted us to develop a technique to increase the number of innate components of cellular immunity from peripheral blood mononuclear cells (PBMCs) so that they can be easily applied for immunotherapy in the clinical setting.

In this study, we attempted to establish a procedure for the ex vivo expansion of $CD3^-CD56^+$ and $CD3^+CD56^+$ cells obtained from PBMCs of healthy volunteers.

MATERIALS AND METHODS

Isolation and Cell Culture

PBMCs obtained from healthy volunteers were isolated by gradient centrifugation with Separate-L (Muto Pure Chemicals Co. Ltd, Tokyo, Japan), and suspended in either X-VIVO 15 medium (Lonza, Walkersville, Inc., MD) or RPMI 1640 medium (Gibco Brl, Grand Island, NY). X-VIVO 15 was supplemented with a 50 mmol/L HEPES buffer (Gibco Brl) and used with or without the addition of heat-inactivated 5% autoserum. RPMI 1640 was supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sanko Chemical Co. Ltd., Tokyo, Japan), 25 mmol/L HEPES buffer (Gibco Brl), 50 mol/L 2 mercaptoethanol (Katayama Chemical Co., Osaka, Japan), 50 U/mL penicillin, and 50 g/mL streptomycin (Gibco Brl). Human recombinant IL-2 (1000 Japanese reference units/mL; Takeda Pharmaceutical, Tokyo, Japan) and 100 ng/mL of anti-CD3 mAb OKT3 (Janssen-Kyowa Co. Ltd., Tokyo, Japan) were added to all culture media, which were maintained at 37°C in a 5% CO_2 incubator during the indicated period. The medium was replaced every 7 days, and cell concentrations were adjusted to 1×10^6 cells/mL. The cell viability was assessed by using the trypan blue dye exclusion test.

Flow Cytometry

Flow cytometric analyses were carried out by using an FACSCalibur dual-laser cytometer (BD Biosciences, San Jose, CA). The following mAbs were used for surface staining of the lymphocytes: flourescein isothiocyanate (FITC)-conjugated anti-CD3 mAb (clone HIT3a; BD Biosciences), phycoerythrin (PE)-conjugated anti-CD56 mAb (clone B159; BD Biosciences), and biotinylated anti-TRAIL (biotin-conjugated anti-TRAIL) mAb (clone RIK-2; eBioscience, Inc., San Diego, CA). The biotinylated mAb was visualized by using allophycocyanin (APC)-streptavidin (BD Biosciences, San Diego, CA). Dead cells identified by light scattering and propidium iodide staining were excluded from the analysis.

Interferon (IFN)-γ production in the lymphocytes was measured by a combination of cell surface and cytoplasmic mAb staining according to the manufacturer instruction. In brief, 4 hours after treatment with leukocyte activation cocktail (BD GolgiPlug, BD Biosciences), the lymphocytes were stained with surface markers anti-CD3-FITC and anti-CD56-APC (BD Biosciences). After washing, the cells were fixed and permeabilized with Cytofix/Cytoperm solution (BD Biosciences), and washed with 10% Perm/Wash Buffer (BD Biosciences). Subsequently, aliquots were stained with either a mAb against intracellular cytokine anti-IFN-γ-PE or isotype-matched control (BD Biosciences).

Cytotoxicity Assay

HepG2 cells, a hepatoma cell line, were purchased from the Japanese Cancer Research Resources Bank and maintained in 10% RPMI.[10] The cells were labeled with 100 μCi Na_2 (^{51}Cr) O_4 for 60 minutes at 37°C in 5% CO_2 in Dulbecco modified eagle medium (DMEM: Gibco Brl) supplemented with 10% FBS, and then subjected to a cytotoxic assay. The labeled HepG2 cells were adjusted to 1×10^4 cells in 100 μL volumes and were incubated for 4 hours in a total volume of 200 μL with effector cells in 10% DMEM in U-bottomed 96-well tissue culture plates (Falcon; Becton Dickinson, Franklin Lakes, NJ). PBMCs from healthy volunteers were used as effectors at effector-target (E:T) ratios of 5 : 1 to 40 : 1. As a control, the target cells were incubated in either culture medium alone, to determine spontaneous release, or a mixture of 2% Nonidet P-40 (Nacalai Tesque, Inc., Kyoto, Japan), to define the maximum ^{51}Cr release. The plates were centrifuged at 1000 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 their radioactivity was measured with a gamma counter. The percentage of specific ^{51}Cr release was calculated by the following formula:

$$\% \text{ cytotoxicity} = \frac{[(\text{cpm of experimental release} - \text{cpm of spontaneous release})]}{[(\text{cpm of maximum release} - \text{cpm of spontaneous release})]} \times 100.$$

The spontaneous release was < 20% of the maximum release. When indicated, the cytotoxic assay was performed in the presence of 10 μg/mL of anti-TRAIL mAb (N2B2) or, 10 μg/mL of anti-Fas ligand (FasL) mAb (MFL3) (both from BD Biosciences), or 50 nmol/L of Concanamycin A (Wako Pure Chemicals, Osaka, Japan), which inhibited perforin-mediated cytotoxicity. All the assays were performed in triplicate.

Coculture With HCV Replicon Cells

The Huh7/Rep-Feo cell line (HCV replicon cells) was kindly gifted by Dr N. Sakamoto (Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan). An HCV subgenomic replicon plasmid, pRep-Feo, was derived from pRep-Neo (originally pHCVIbneo-delS).[11] pRep-Feo carries a fusion gene comprising firefly luciferase (*Fluc*) and neomycin phosphotransferase, as described elsewhere.[12,13] After culture in the presence of G418 (Wako Pure Chemical Industries), Huh7/Rep-Feo cell lines stably expressing the replicons were established. For the coculture experiments, transwell tissue culture plates (pore size, 1 μm; Costar, Cambridge, MA) were used. HCV replicon cells (10^5 cells) were incubated in the lower compartment with different numbers of lymphocytes in the upper compartment. DMEM supplemented with 10% FBS was used in this assay. The HCV replicon cells in the lower compartments were collected 48 hours after coculture for luciferase assay. When indicated, anti-CD3 mAb pulse (1 μg/mL) was added 24 hours before harvesting. The luciferase activities were measured with a luminometer (Lumat LB940; Berthold Technologies, Germany) using the Bright-Glo Luciferase Assay System (Promega). When indicated, the assays were performed in the presence of 100 μg/mL anti-human IFN-γ mAb and 100 μg/mL isotype-matched control mAb (R&D Systems, Minneapolis, MN). All the assays were performed in duplicate.

ELISA Assay

Supernatants of propagated PBMCs were collected after 28 days of culture with or without additional stimulation by anti-CD3 mAb pulse (1 day before harvest). IFN-γ levels in the cell culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) using Quantikine kit (R&D systems, Minneapolis, MN), according to the manufacturer instructions. Absorbance was measured at 492 nm using a microplate reader MTP-300 (CORONA Electric, Ibaraki, Japan).

Statistical Analysis

The results were statistically analyzed by using either unpaired or paired Student *t* test or the linear regression analysis when appropriate. *P*-values < 0.05 were considered to be statistically significant.

RESULTS

Ex Vivo Expansion of $CD3^-CD56^+$ and $CD3^+CD56^+$ Cells From PBMCs

Although RPMI 1640 medium supplemented with FBS is widely used to propagate PBMCs in the experimental setting, several studies have shown that X-VIVO 15 medium has been used under serum-free conditions to support the growth of human monocytes, macrophages, lymphocytes, and natural killer (NK) cells.[14–16] X-VIVO 15 contains pharmaceutical grade human albumin, recombinant human insulin, and pasteurized human transferrin; hence, it seems to be favorable in terms of clinical applicability. We compared the capacity of RPMI 1640 and X-VIVO 15 to support the growth of $CD3^-CD56^+$ and $CD3^+CD56^+$ cells from human PBMCs in the presence of IL-2 and anti-CD3 mAb, which are crucial for the expansion of LAK cells.[17–21] PBMCs obtained from 10 human volunteers expanded after 28 days of culture by about 22-fold (range, 8–55) in RPMI 1640 containing FBS, 38-fold (range, 10–93) in serum free X-VIVO 15, and 62-fold (range 32–97) in X-VIVO 15 containing autoserum (Fig. 1A). Autoserum supplemented X-VIVO 15 most efficiently supported cell expansion and also maintained the viability of the expanded cells (Figs. 1A, B).

Further, the cultivation in X-VIVO 15 containing 5% autoserum constantly resulted in the highest proportion of $CD3^-CD56^+$ cells among the propagated lymphocytes (Fig. 1C). Hence, X-VIVO 15 containing autoserum was used for the subsequent experiments. After being cultured for 28 days in the presence of IL-2 and anti-CD3 mAb, propagated PBMCs exclusively consisted of $CD3^-CD56^+$ and $CD3^+CD56^+$ cells (Fig. 2). The proportion of those 2 cell fractions in the propagated PBMCs varied among individuals. The average proportion of $CD3^-CD56^+$ and $CD3^+CD56^+$ cells was 41.5% (range, 4.6%–97.1%) and 51.1% (range, 0.8%–93.7%), respectively.

Vigorous Cytotoxicity of Propagated PBMCs Against HCC

Cytotoxic assays using freshly isolated and propagated PBMCs as the effectors and the HepG2 cell line as target were performed. As shown in Figure 3, the freshly isolated PBMCs barely mediated potent cytotoxicity against HepG2 cells. Although the ratio of $CD3^-CD56^+$ and $CD3^+CD56^+$ cells highly varied among the propagated PBMCs of each individual after 28 days of culture, all propagated PBMCs uniformly exhibited vigorous cytotoxicity against HCC, regardless of the ratio of these cell types, while those propagated PBMCs showed no cytotoxicity against self-lymphoblasts (data not shown).

Anti-HCC Activity of the Propagated PBMCs By TRAIL

The propagated $CD3^-CD56^+$ and $CD3^+CD56^+$ cells expressed TRAIL on their surface (Fig. 4). To determine the contribution of TRAIL to the anti-HCC activity of the propagated PBMCs, the effect of a neutralizing anti-TRAIL mAb was examined in a cytotoxicity assay using the propagated PBMCs (cultivated for 28 d) as the effector and HepG2 cells as the target. The cytotoxicity of propagated PBMCs against hepatoma cells was partially inhibited by the anti-TRAIL mAb alone and more profoundly inhibited by the combination of anti-TRAIL and anti-FasL mAbs together with Concanamycin A. However, cytotoxicity was detected even in the presence of all those blockers, suggesting that other unknown effectors, in addition to TRAIL, FasL, and perforin, are also involved in hepatoma cell cytotoxicity induced by propagated PBMCs (Fig. 5). These findings indicated that TRAIL expressed on the propagated PBMCs was involved in anti-HCC activity.

Anti-HCV Activity of the Propagated PBMCs

We next analyzed the anti-HCV activity of the propagated PBMCs. Propagated PBMCs strongly inhibited luciferase reporter activity compared with freshly isolated PBMCs (Fig. 6A). The additional anti-CD3 mAb pulse administered 1 day before culturing with HCV replicon-containing cells increased the anti-HCV activity of propagated PBMCs. To identify the cell type expressing the anti-HCV activity, we analyzed the relationship between anti-HCV activity and the ratio of $CD3^-CD56^+$ cells in propagated PBMCs (Fig. 6B). Notably, propagated PBMCs containing more $CD3^-CD56^+$ cells (and, thus, fewer $CD3^+CD56^+$ cells) showed lower luciferase reporter activity, indicating that the anti-HCV activity of $CD3^-CD56^+$ cells was more vigorous than that of $CD3^+CD56^+$ cells. However, this trend was not observed when anti-CD3 mAb pulse was added to the culture medium 1 day before the anti-HCV assay; rather, all propagated PBMCs strongly inhibited luciferase reporter activity regardless of the $CD3^-CD56^+/CD3^+CD56^+$ cell ratio. This indicates that the additional anti-CD3 mAb pulse stimulation increased anti-HCV activity of the $CD3^+CD56^+$ cells to levels exhibited by $CD3^-CD56^+$ cells.

We earlier reported that IFN-γ secreted from liver lymphocytes activated by IL-2 and anti-CD3 mAb is responsible for the anti-HCV activity of these cells.[9] Propagated PBMCs also actively produced IFN-γ (Fig. 7A), which likely played a pivotal role in the anti-HCV activity of these cells. IFN-γ expression was significantly higher in $CD3^-CD56^+$ cells than in $CD3^+CD56^+$ cells, consistently with the anti-HCV activity levels of these cells (Fig. 7B). Even after being cultured for 28 days in the presence of IL-2 and anti-CD3 mAb, a considerable number of $CD3^+CD56^+$ cells could be detected by Flow Cytometry, indicating that CD3 molecules not coated with anti-CD3 mAb were present on the surface of propagated PBMCs. The additional anti-CD3 mAb pulse stimulation 1 day before coculturing propagated PBMCs with HCV replicon-containing cells significantly promoted IFN-γ secretion from $CD3^+CD56^+$ cells (Fig. 8). In addition, blocking IFN-γ with a mAb abrogated the anti-HCV activity of propagated PBMCs cocultured with HCV replicon-containing cells (Fig. 9). Thus, IFN-γ secreted by propagated PBMCs can inhibit HCV virion production probably by suppressing viral RNA and protein synthesis.

DISCUSSION

LAK cells for immunotherapy are conventionally generated after expansion in the presence of IL-2 for a relatively short culture period. The heterogeneous LAK cell population consists of nonmajor histocompatibility complex-restricted $CD3^-CD56^+$ and $CD3^+CD56^+$ cell

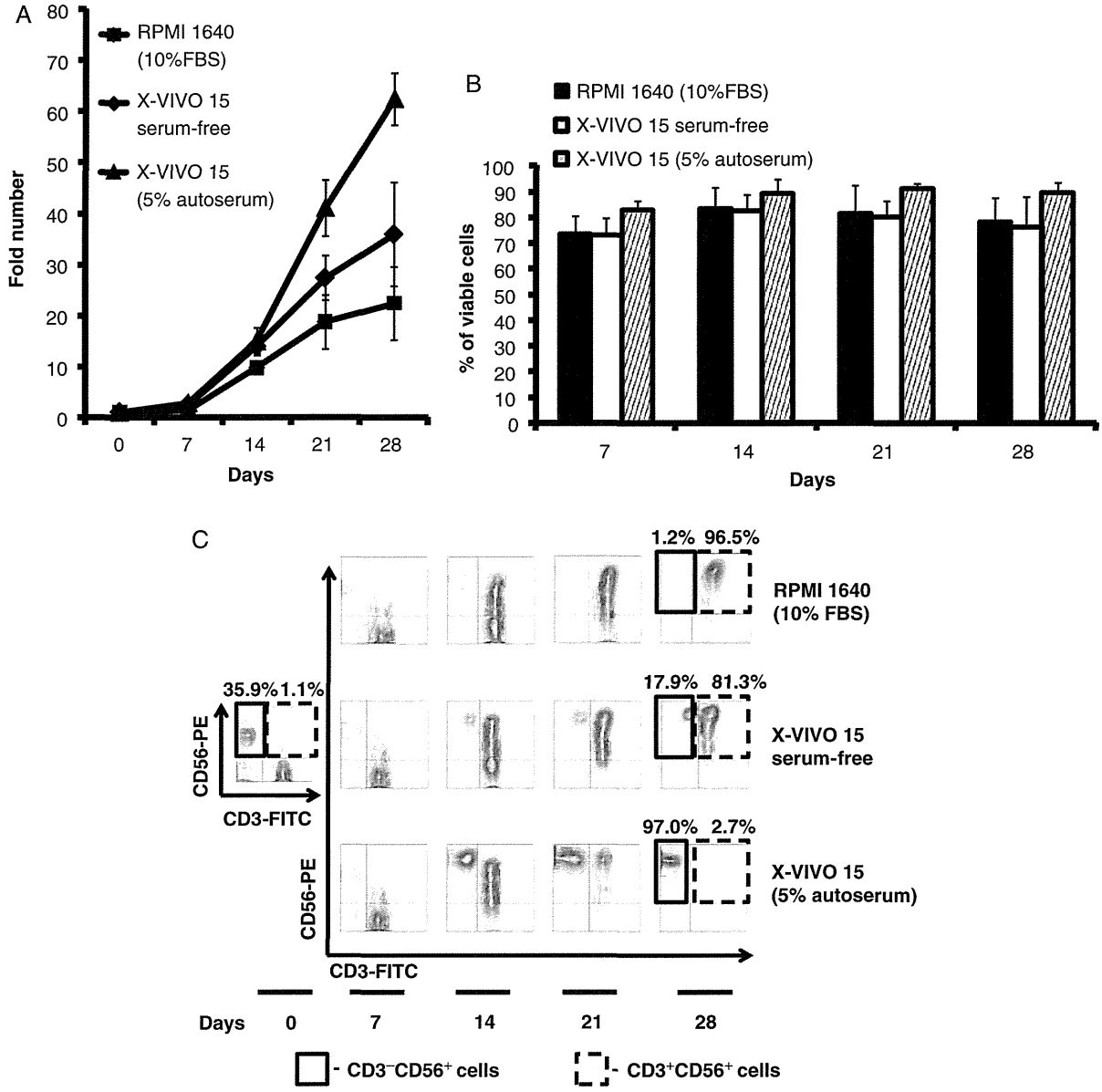

FIGURE 1. A, Comparative study for evaluating the capacity of various medium to propagate PBMCs. PBMCs obtained from healthy volunteers (n = 10) were cultured in X-VIVO 15 supplemented with/without 5% autoserum and RPMI 1640 supplemented with 10% FBS. All cultures were performed in the presence of interleukin-2 (IL-2; 1000 JRU/mL) and anti-CD3 monoclonal antibody (100 ng/mL) during 28 days. B, Viability of the PBMCs propagated in the various media. At 7-day culture, cell viability was assessed by using trypan blue dye exclusion test. The data represent the mean ± SEM (n = 10 individuals). C, Phenotypic characteristics of the PBMCs in various mediums. The representative flow cytometric profiles of the propagated PBMCs from a certain volunteer are shown. The PBMCs were stained with monoclonal antibodies against CD3 and CD56, and analyzed by flow cytometry on day 0 and during culture (on days 7, 14, 21, and 28). FBS indicates fetal bovine serum; PBMCs, peripheral blood mononuclear cells.

subsets, both of which contribute to the cytolytic property of LAK cells.[22] The unique $CD3^+CD56^+$ cells are generally referred to as NK-like T cells, because, similar to NK cells, they do not require prior specific sensitization to induce recognition of target cells. Addition of anti-CD3 mAb at the initiation of culture, prolongation of culture duration, and addition of various stimuli at the end of culture are improved methodologies to culture LAK cells and reportedly result in better expansion over the original described method.[23] Such expanded LAK cells have clinically shown modest efficacy against metastatic renal cell carcinoma and melanoma.[24] In this study, X-VIVO 15 medium containing autoserum efficiently supported expansion of $CD3^-CD56^+$ and $CD3^+CD56^+$ cells. The $CD3^+CD56^+$ cells in propagated PBMCs did not express invariant T-cell receptors, but express conventional T-cell receptors-α/β in the preliminary experiments (data not shown), indicating that these cells are different from the invariant natural killer T cells. Further studies are required to define the phenotypic and functional properties of

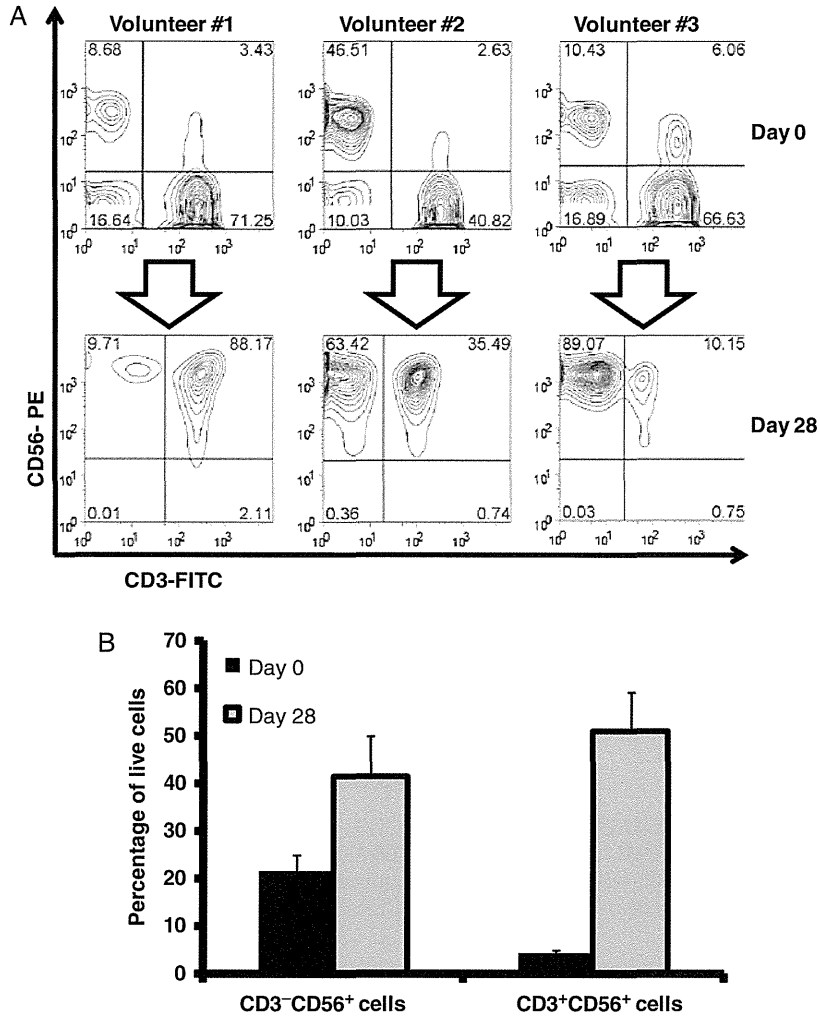

FIGURE 2. The proportion of $CD3^-CD56^+$ and $CD3^+CD56^+$ cells among the propagated PBMCs varied among individuals. A, Representative flow cytometric profiles of the propagated PBMCs from 3 different individuals are shown. The PBMCs were cultured in X-VIVO 15 medium supplemented with 5% autoserum in the presence of interleukin-2 and anti-CD3 monoclonal antibody. The harvested cells were stained with monoclonal antibodies against CD3 and CD56, and analyzed on day 0 and 28. B, The proportion of $CD3^-CD56^+$ and $CD3^+CD56^+$ cells among the propagated PBMCs before (day 0) and after (day 28) culture. The data represent mean ± SEM (n = 15 individuals). PBMCs indicates peripheral blood mononuclear cells.

$CD3^-CD56^+$ and $CD3^+CD56^+$ cells among the propagated PBMCs.

X-VIVO 15 medium is more efficient in proliferation $CD56^+$ cells than RPMI medium, particularly $CD3^-CD56^+$ cells. To address the possible mechanism underlying this phenomenon, we analyzed the amino acid and cytokine content of X-VIVO 15 and RPMI mediums. X-VIVO 15 contains higher concentrations of branched chain amino acids (valine, leucine, and isoleucine) than RPMI (data not shown). In addition, X-VIVO 15 contains higher concentrations of intercellular adhesion molecule 1 (ICAM)-1. As $CD56^+$ cells express the ICAM-1 ligand lymphocyte function-associated antigen 1 (LFA-1), ICAM-1 and LFA-1 binding might promote NK cell activation.

We recently showed that $CD3^-CD56^+$ and $CD3^+CD56^+$ cells can be extracted from the liver allograft perfusate during transplant surgery, and short culture with IL-2 and anti-CD3 mAb induces the anti-HCV activity and the anti-HCC activity of the NK and NK-like T cells.[9] Short-term (3 days) stimulation with IL-2 significantly upregulates the expression of TRAIL on liver NK cells, but this effect is barely observed on NK cells from PBMCs. Molecular cloning of TRAIL-receptors elucidated that TRAIL binds to at least 4 receptors: 2 are death-inducing receptors (TRAIL-R1/DR4 and TRAIL-R2/DR5), containing cytoplasmic death domains and mediate signal apoptosis; the other 2 are death-inhibitory receptors (TRAIL-R3/DcR1 and TRAIL-R4/DcR2), lacking a functional death domain and do not mediate apoptosis. However, all have similar affinities and the latter pair may act as decoys.[25,26]

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: this process is mediated primarily by death receptor-ligand interactions.[27] We have found that normal hepatocytes express TRAIL-DR4 and TRAIL-DR5 together with TRAIL-DcR1 and TRAIL-DcR2, but moderately or poorly differentiated HCCs highly express TRAIL-DR4 and

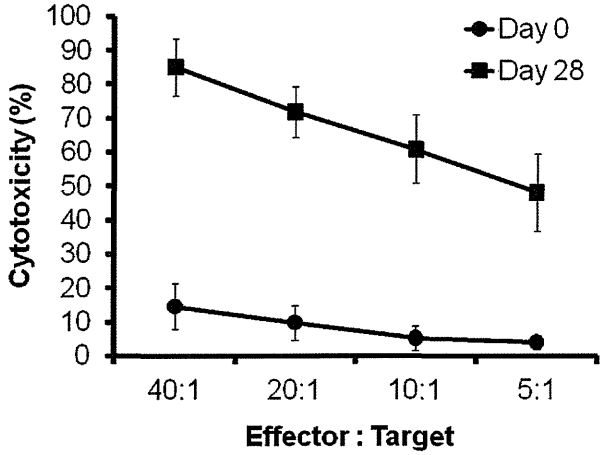

FIGURE 3. The propagated PBMCs showed vigorous cytotoxicity against hepatoma cells. Cytotoxic activities of freshly isolated PBMCs (day 0) and propagated PBMCs after 28 days culture (day 28) against HepG2 cells (a hepatoma cell line) were analyzed by ^{51}Cr release assay. The data represent the mean ± SEM of the percentage of target lysis at effector-target (E:T) ratios of 40:1, 20:1, 10:1, and 5:1 (n = 5 for the freshly isolated PBMCs and n = 10 for the propagated PBMCs). There was statistically significant difference in cytotoxicity against the hepatoma cells between the propagated PBMCs and the freshly isolated PBMCs ($P < 0.05$). The data of each person was determined as average of ^{51}Cr titer in triplicate wells. PBMCs indicates peripheral blood mononuclear cells.

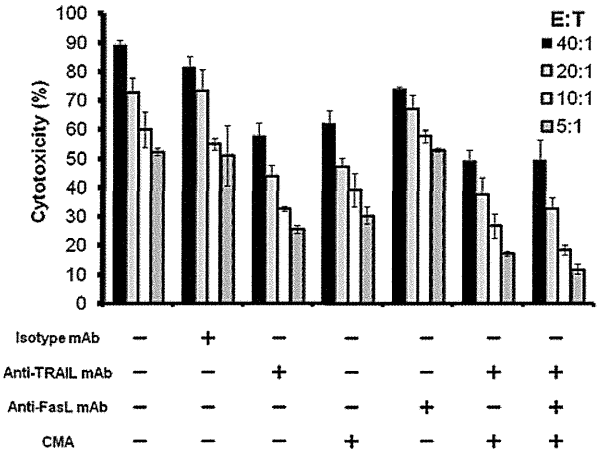

FIGURE 5. The cytotoxicity of the propagated PBMCs against hepatoma cells was partially inhibited by an anti-TRAIL mAb alone and more profoundly inhibited by the combination of the anti-TRAIL mAb and anti-FasL mAb together with CMA. The propagated PBMCs were used as effector cells in assays of cytotoxicity against HepG2 cells (hepatoma cell line) at effector-target (E:T) ratios of (40:1, 20:1, 10:1, and 5:1) in the presence or absence of anti-TRAIL (N2B2) mAb (10 μg/mL), anti-FasL (MFL3) mAb (10 μg/mL), and/or CMA (50 nmol/L). The results shown are the mean ± SD of values from triplicate samples and are representative of 3 similar experiments. CMA indicates Concanamycin A; FasL, Fas ligand; mAb, monoclonal antibody; PBMCs, peripheral blood mononuclear cells; TRAIL, tumor necrosis factor-related apoptosis inducing ligand.

TRAIL-DR5 but did not express TRAIL-DcR1 and TRAIL-DcR2, indicating a susceptibility to TRAIL-expressing NK cell-mediated activity toward HCC.[8] We now showed that long-term (28 d) cultivation in the presence of IL-2 and anti-CD3 mAb significantly upregulated the expression of TRAIL even on $CD56^+$ cells derived from PBMCs and induces significant cytotoxicity of LAK cells including the $CD56^+$ subsets against HCC (Figs. 3, 4). The contribution of

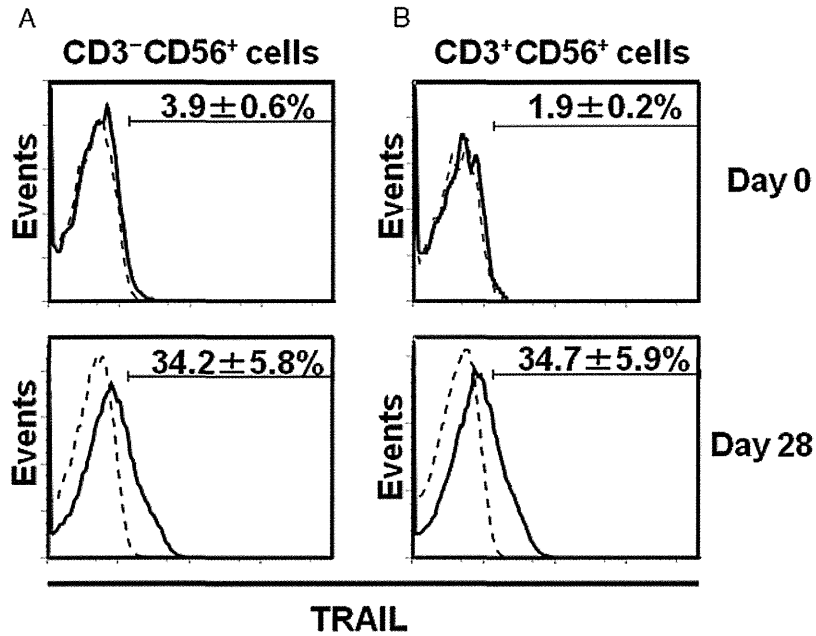

FIGURE 4. The propagated $CD3^-CD56^+$ and $CD3^+CD56^+$ cells expressed the TRAIL on their surface. The histograms represent the log fluorescence intensities obtained on staining for TRAIL after gating on the $CD3^-CD56^+$ (A) and the $CD3^+CD56^+$ (B) cells among freshly isolated PBMCs (day 0) and propagated PBMCs after 28-day culture (day 28). The dotted lines represent negative control staining with isotype-matched monoclonal antibodies. The numbers indicate the percentages of cells positive for TRAIL expression (mean ± SEM, n = 10 individuals). PBMCs indicates peripheral blood mononuclear cells; TRAIL, tumor necrosis factor-related apoptosis inducing ligand.

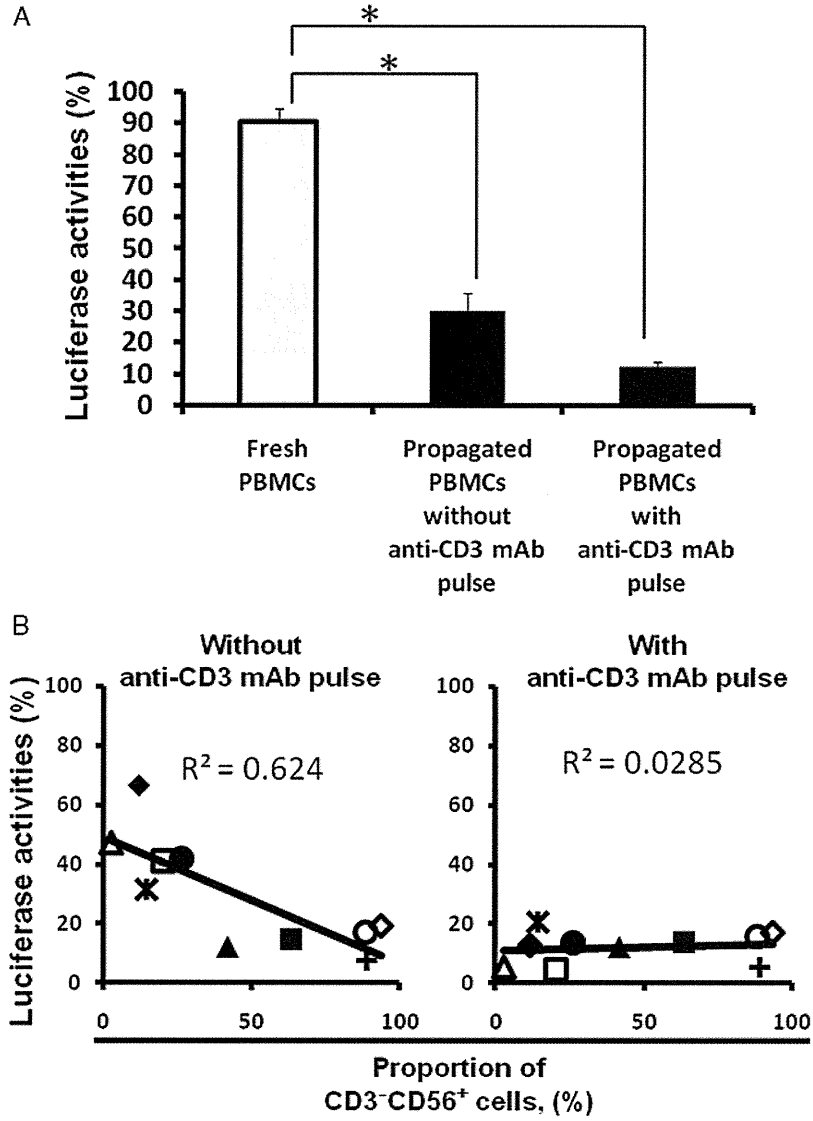

FIGURE 6. Propagated PBMCs exhibit marked anti-HCV activity. A, Propagated PBMCs cultured for 28 days in the presence of interleukin-2 and anti-CD3 mAb were incubated with HCV replicon-containing hepatic cells for 48 hours in transwell tissue culture plates (effector:target ratio = 10:1). One day before coculturing PBMCs with HCV replicon-containing cells, anti-CD3 mAb pulse (1 μg/mL) was added or not added to the culture of propagated PBMCs. Bar graphs indicate luciferase activity of HCV replicon-containing cells in the presence of effectors normalized to luciferase activity in the absence of effectors (% of luciferase activity; 5 individuals were used for freshly isolated PBMCs, and 10 individuals for propagated PBMCs). The difference in anti-HCV effect between the propagated PBMCs and the freshly isolated PBMCs was statistically significant (*$P < 0.05$). B, Propagated PBMCs containing more $CD3^-CD56^+$ cells showed lower luciferase reporter activity without the anti-CD3 mAb pulse. All of the propagated PBMCs strongly inhibited luciferase reporter activity, regardless of the $CD3^-CD56^+/CD3^+CD56^+$ cell ratio after the anti-CD3 mAb pulse. Data were obtained from 10 volunteers. Each symbol indicates data from 1 individual. HCV indicates hepatitis C virus; mAb, monoclonal antibody; PBMCs, peripheral blood mononuclear cells.

TRAIL molecules to the cytotoxicity of NK cells against HCC was proved by the effect of a neutralizing anti-TRAIL mAb (Fig. 5).

In addition to anti-neoplastic effects, adoptive immunotherapy with LAK cells may lead to viral clearance. In fact, a reduction in hepatitis B virus (HBV) load has been described in patients undergoing treatment with LAK cells.[28] LAK cells might suppress HBV replication through the secretion of IFN-γ and tumor necrosis factor-α. Despite such an attractive approach, this therapy has never been applied to suppress HCV replication. In general, in the early phase of viral infection, the first line of host defense may be effective in removing the virus; however, recent reports have indicated that HCV effectively escapes the innate immune system comprising NK and natural killer T cells, resulting in persistent infection.[29,30] It has been also reported that cross-linking of CD81 on NK cells by the major envelope protein of HCV, HCV-E2, blocks NK cell activation, IFN-γ production, cytotoxic granule release, and proliferation.[29] Engagement of CD81 on NK cells blocks tyrosine phosphorylation through a mechanism that is distinct from the negative signaling pathways associated

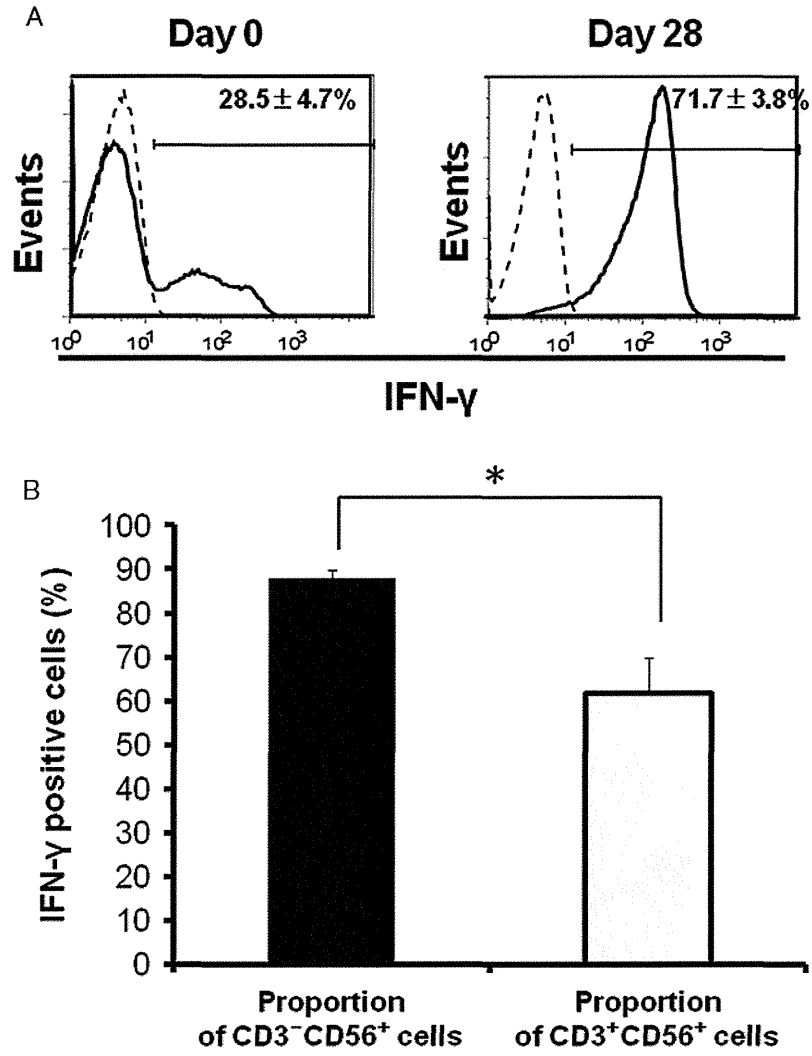

FIGURE 7. A, IFN-γ production by propagated PBMCs was evaluated on days 0 and 28 by a combination of cell-surface and cytoplasmic mAb staining and subsequent flow cytometry analysis. Histograms represent log fluorescence intensities obtained by staining whole propagated PBMCs for IFN-γ. Dotted lines represent negative control staining with isotype-matched mAb. Numbers indicate the percentage of cells positive for IFN-γ expression (mean ± SEM, n = 5 individuals). B, IFN-γ expression is higher in $CD3^-CD56^+$ cells than in $CD3^+CD56^+$ cells propagated for 28 days in the presence of interleukin-2 and anti-CD3 mAb (*$P < 0.05$). The data were obtained after gating by staining $CD3^-CD56^+$ and $CD3^+CD56^+$ cells for IFN-γ after 28 days of culture. Data represent mean ± SEM (n = 5 individuals). IFN indicates Interferon; mAb, monoclonal antibody; PBMCs, peripheral blood mononuclear cells.

with NK cell inhibitory receptors for major histocompatibility complex class I molecules.[30] These findings prove that HCV-E2-mediated inhibition of NK cells is an efficient HCV evasion strategy, which involves targeting the early antiviral activities of NK cells and allowing the virus to establish itself as a chronic infection. We earlier explored whether CD81 cross-linking-induced inhibitory effects occur even in IL-2-stimulated NK cells. CD81 cross-linking by a mAb specific for CD81 inhibited antitumor cytotoxicity and anti-HCV activity mediated by resting NK cells, but this manipulation did not alter both these activities of IL-2-stimulated NK cells.[9] This indicated that exposure to IL-2 before CD81 cross-linking abrogates subsequent inhibitory signals in NK cells and encourages us to study the possibility of adoptive immunotherapy with LAK cells to inhibit HCV replication. We have recently shown that $CD3^-CD56^+$ and $CD3^+CD56^+$ cells derived from liver resident lymphocytes show anti-HCV activity after short-term culture with IL-2 and anti-CD3 mAb through the secretion of IFN-γ.[9] Similarly, long-term cultivation in the presence of IL-2 and anti-CD3 mAb promotes the inhibitory effects of LAK cells from PBMCs on HCV replication. Although the role of NK cells in controlling HCV infection and replication has not been completely elucidated, a recent report has indicated that NK cells are not directly cytolytic towards HCV replicon-containing hepatic cells, but rather release IFN-γ, suppressing HCV RNA expression.[31] The role of IFN-γ in the expression of NK cell-mediated anti-HCV activity has been supported by the observation that NK cell-conditioned media have higher levels of signal transducer and activator of transcription 1, a nuclear factor essential in IFN-γ-mediated antiviral pathways. It has also been reported that hepatocytes cultured in NK cell-conditioned media express higher levels of IFN-α/β, IFN regulatory factor 3, and IFN

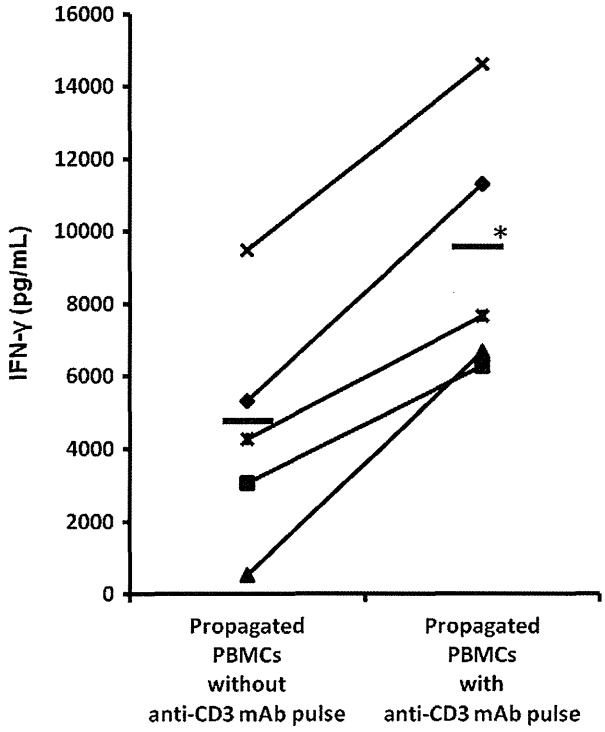

FIGURE 8. IFN-γ concentrations in culture supernatant increased with the addition of an anti-CD3 mAb pulse. PBMCs were propagated for 28 days in X-VIVO 15 medium supplemented with 5% autoserum in the presence of interleukin-2 and anti-CD3 mAb. One day before harvest, anti-CD3 mAb pulse (1 μg/mL) was added to the culture. IFN-γ was detected by enzyme-linked immunosorbent assay. Each symbol indicates IFN-γ culture supernatant concentration from each volunteer (n = 5 individuals). Horizontal line indicates the mean. IFN-γ expression was significantly different between the supernatants of nonstimulated and stimulated cells (*$P < 0.05$). IFN indicates Interferon; mAb, monoclonal antibody.

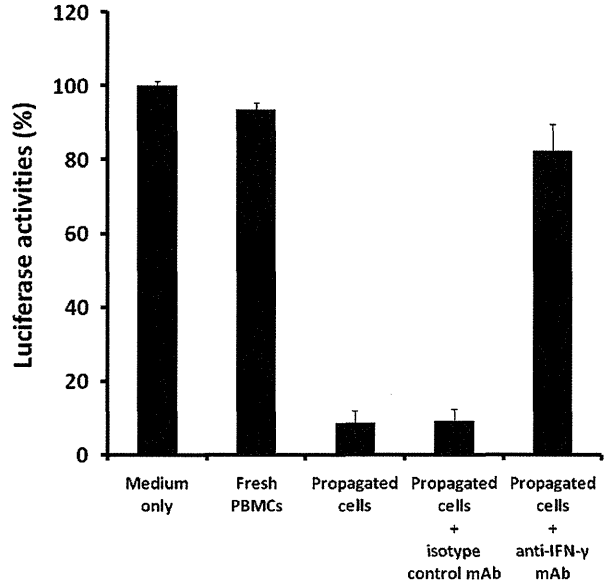

FIGURE 9. Blocking of IFN-γ with mAb in coculture with HCV replicon-containing hepatic cells abrogated the anti-HCV activity of the propagated PBMCs. HCV replicon-containing hepatic cells incubated with the control medium alone served as control. When HCV replicon-containing hepatic cells were incubated with freshly isolated PBMCs in transwell tissue culture plates (effector: target ratio = 10 : 1), the luciferase activities (indicating HCV replication) were not altered. However, when HCV replicon-containing hepatic cells were incubated with the PBMCs propagated with interleukin-2 and anti-CD3 mAb and additional anti-CD3 mAb pulse stimulation, the luciferase activities were significantly reduced. The propagated PBMCs were used as effector cells in assays of cytotoxicity against HCV replicon-containing hepatic cells in the presence or absence of anti-IFN-γ mAb (100 μg/mL) and isotype-matched control mAb (100 μg/mL). Blocking of IFN-γ with mAb elucidated the significant role played by IFN-γ in producing the anti-HCV effect. The bar graphs indicate the rate of luciferase activity of HCV replicon cells in the presence of effectors to that in the absence of effectors (% of luciferase activity) in each group. The data represent the mean ± SEM of 3 similar experiments. HCV indicates hepatitis C virus; IFN, Interferon; mAb, monoclonal antibody; PBMCs, peripheral blood mononuclear cells.

regulatory factor 7, confirming that NK cells suppress HCV infection of and replication in human hepatocytes via IFN.[32] Similar to recent studies, this study showed that propagated PBMCs consisting of $CD56^+$ cells vigorously produce IFN-γ, thus suppressing HCV replication (Figs. 7, 8). However, we have confirmed that IFN-α/β production by propagated $CD56^+$ cells was undetectable (data not shown).

Regarding preventing replication of other viruses, earlier studies have shown that IFNs are a group of inducible cytokines with a central role in innate antiviral immune responses because they establish an intracellular antiviral state that prevents viral replication.[33] Mice lacking either IFN-γ or its functional receptor are more susceptible to both viral and bacterial infections, indicating that IFN-γ plays an important role in antiviral and antibacterial responses.[34,35] It is possible that these propagated PBMCs can prevent the replication of other viruses through an IFN-γ-dependent mechanism. Further studies are required to address this possibility.

We have observed earlier that IL-2-stimulated NK cells were negligibly cytotoxic towards allogeneic and autologous lymphoblasts with 1 shared haplotype despite being strongly cytotoxic to HCC cells.[8] Therefore, we propose immunotherapy with propagated PBMCs of liver allografts derived from healthy donors with 1 shared haplotype or from the recipient patients themselves, to mount anti-HCC and anti-HCV responses in HCV-infected LT recipients. Living donor LTs are frequently performed between parent and child who share 1 haplotype of major histocompatibility complex. In this study, we confirmed that $CD56^+$ cells from healthy donors expand well, indicating that our approach is realistic. However, it remains to be elucidated whether similar cells can be obtained from recipient patients for adoptive immunotherapy.

In conclusion, $CD3^-CD56^+$ and $CD3^+CD56^+$ cells obtained from the PBMCs show anti-HCV activity in addition to anti-HCC activity and would be used for adoptive immunotherapy in clinical setting.

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