Table 5: Prognostic factors for infectious complications: univariate analysis of 259 patients given rituximab prophylaxis

			Bacterial infection				Fungal infection				CMV disease			
Characteristics	Category	N	Odds ratio	95% CI	p-Value	p-Value (global association)	Odds ratio	95% CI	p-Value	p-Value (global association)	Odds ratio	95% CI	p-Value	p-Value (global association)
			Logistic regression analysis				Logistic regression analysis				Logistic regression analysis			
Local infusion	No	40	1.000	–	–	–	1.000	–	–	–	1.000	–	–	–
	Yes	218	1.449	0.671–3.128	0.345		0.830	0.173–3.993	0.816		2.945	1.373–6.319	0.006*	
	Unknown	1	–	–	–		–	–	–		–	–	–	
Splenectomy	No	90	1.000	–	–	–	1.000	–	–	–	1.000	–	–	–
	Yes	169	0.588	0.342–1.011	0.055		0.913	0.260–3.208	0.887		1.071	0.641–1.791	0.793	
Anti-lymphocyte antibodies	No	244	1.000	–	–	–	1.000	–	–	–	1.000	–	–	–
	Yes	15	2.010	0.703–5.747	0.193		1.650	0.197–13.82	0.644		1.049	0.369–2.982	0.929	
Prophylactic IVIG after transplantation	No	214	1.000	–	–	–	1.000	–	–	–	1.000	–	–	–
	Yes	45	1.792	0.925–3.471	0.084		1.922	0.489–7.559	0.350		1.626	0.851–3.106	0.141	
Timing of rituximab administration before transplantation	≤ 6 days	22	1.000	0.383–2.501	0.964	–	1.000	–	–	–	1.000	–	–	–
	>7 days	236	0.979	–	–		0.402	0.081–1.988	0.264		1.012	0.421–2.435	0.978	
	Unknown	1	–	–	–		–	–	–		–	–	–	
Number of doses of rituximab	1	225	1.000	–	–	0.513	1.000	–	–	0.010*	1.000	–	–	0.004*
	2	22	0.638	0.227–1.798	0.396		1.543	0.181–13.17	0.692		3.038	1.256–7.980	0.019*	
	3	12	1.549	0.475–5.050	0.468		10.288	2.278–46.47	0.002*		36.742	4.737–999.9	0.017*	
Dose of rituximab	Regular	162	1.000	–	–	–	1.000	–	–	–	1.000	–	–	–
	Small	66	1.742	0.948–3.203	0.074		0.122	0.000–0.984	0.152		0.455	0.249–0.832	0.011*	
	Unknown	31	–	–	–		–	–	–		–	–	–	
Dose and number of doses of rituximab	Regular × 1	134	1.000	–	–	0.283	1.000	–	–	0.040*	1.000	–	–	0.001*
	Regular × 2	16	0.679	0.182–2.526	0.563		2.243	0.220–12.32	0.412		14.802	3.517–137.3	0.003*	
	Regular × 3	12	2.101	0.625–7.058	0.230		8.542	1.756–37.86	0.006*		35.805	4.548–999.9	0.018*	
	Small × 1	60	1.828	0.955–3.501	0.069		0.192	0.001–1.734	0.270		0.780	0.412–1.451	0.440	
	Small × 2	6	1.471	0.258–8.390	0.664		2.108	0.015–23.08	0.657		0.110	0.000–0.964	0.167	
	Unknown	31	–	–	–		–	–	–		–	–	–	
Regimen	RS	30	1.000	–	–	0.266	1.000	–	–	0.685	1.000	–	–	0.034*
	R	10	2.611	0.574–11.71	0.221		3.105	0.232–41.87	0.366		2.609	0.574–11.71	0.221	
	RI	81	2.351	0.929–6.670	0.089		0.900	0.141–9.567	0.917		3.176	1.264–8.982	0.021*	
	RIS	137	1.566	0.642–4.318	0.357		0.980	0.195–9.654	0.983		4.053	1.688–11.07	0.004*	
	Unknown	1	–	–	–		–	–	–		–	–	–	

IVIG, intravenous immunoglobulin; R, only rituximab; regular dose, 500 mg/body or 375 mg/m^2; RI, rituximab and infusion; RIS, rituximab and infusion and splenectomy; RS, rituximab and splenectomy; small dose, 300 mg/body or less.
*$p < 0.05$.

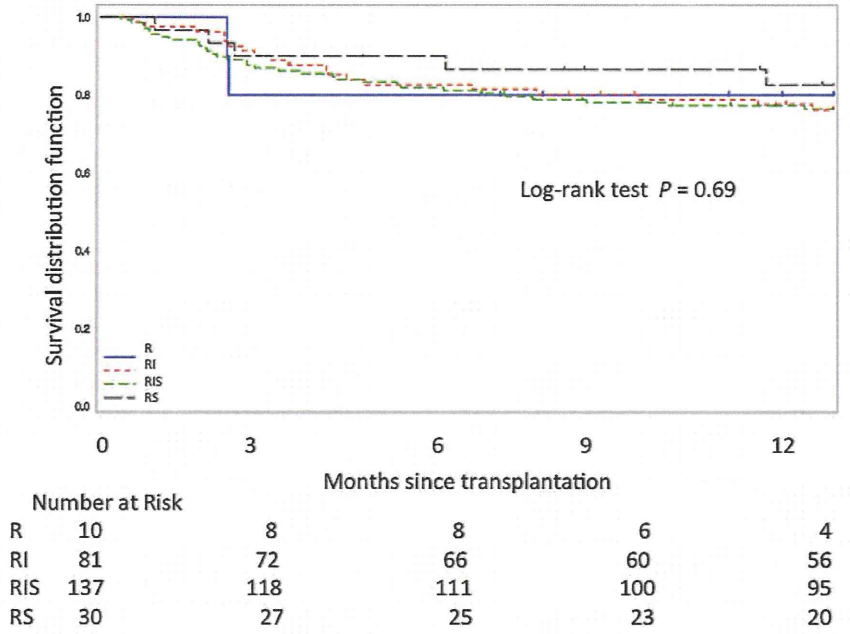

Figure 5: One-year survival of patients in the rituximab group. R, rituximab without splenectomy or local infusion (n = 10); RI, rituximab with infusion but without splenectomy (n = 81); RIS, rituximab with both infusion and splenectomy (n = 137); RS, rituximab with splenectomy but without infusion (n = 30). There were no significant differences among regimens with additional desensitization in patients with rituximab prophylaxis.

is performed, the greater the potential for an increase in DSA titer. However, we observed no significant relationship between the number of plasmapheresis procedures and clinical outcomes (Table 1).

IVIG is also a standard procedure, especially for human leukocyte antigen-related DSA in kidney transplantation, and the IVIG dose often ranged from 0.1 to 2 g/kg (18,19). In liver transplantation, Ikegami et al (4) reported a small series with desensitization by rituximab and IVIG (0.8 g/kg), and their cases were included here. We found no significant effect of IVIG on overall survival or AMR in the entire adult cohort (Table 1) and no additional effects in the rituximab group (Table 5). We analyzed the AMR incidence in each regimen with IVIG versus without IVIG (Figure 6). The AMR incidence was reduced from 26% to 9% in the local infusion and splenectomy (IS; no rituximab) regimen when IVIG was added, but this difference was not significant (p = 0.19). Among regimens with rituximab (R, RI, RIS and RS), the incidences were similar between with IVIG and without IVIG. IVIG is not approved in Japan and is not covered by insurance. IVIG costs 1.5–2.0 million yen per injection, whereas 500 mg of rituximab costs 0.3 million yen. A prospective study is required to elucidate the effects of IVIG in patients after rituximab prophylaxis.

The incidence of adverse effects of rituximab was 1.6% (4/258), and all patients recovered and underwent LDLT. Rituximab prophylaxis could be tolerated by patients with end-stage liver diseases. The incidences of bacterial

Table 6: Comparison of antibody titers between patients with and without AMR under rituximab prophylaxis

		AMR+			AMR−			
		N	Median	Mean ± SD	N	Median	Mean ± SD	p-Value
IgM	Peak before transplantation	15	64	158 ± 255	211	64	147 ± 199	0.881
	At transplantation	16	4	7 ± 8	213	4	16 ± 48	0.700
	Peak posttransplantation	16	64	593 ± 1091	223	8	49 ± 181	<0.001*
IgG	Peak before transplantation	14	128	408 ± 584	215	64	319 ± 771	0.221
	At transplantation	13	16	27 ± 35	210	8	34 ± 96	0.265
	Peak posttransplantation	13	256	1002 ± 2196	212	16	68 ± 187	<0.001*

AMR, antibody-mediated rejection. p-values are derived from Wilcoxon sum-rank test.
*$p < 0.05$ for AMR+ versus AMR−.

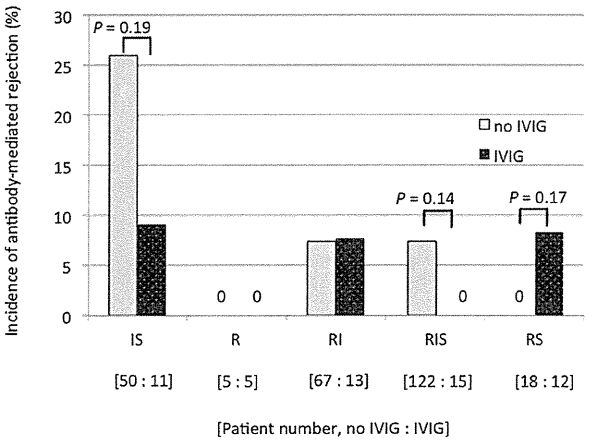

Figure 6: Comparison of the incidences of antibody-mediated rejection (AMR) with and without intravenous immunoglobulin (IVIG) in each regimen. IS, local infusion with splenectomy without rituximab; R, rituximab without splenectomy or local infusion; RI, rituximab with infusion but without splenectomy; RIS, rituximab with both infusion and splenectomy; RS, rituximab with splenectomy but without infusion. There were no significant differences in the incidence of AMR.

infections and CMV disease after transplantation were similar between the nonrituximab and rituximab groups, but the incidence of fungal infection was significantly lower in the rituximab group. Although data for the amount of steroid and trough levels of calcineurine inhibitors were not collected here, the total amount of conventional immunosuppressant might be reduced in light of the expected beneficial effects of rituximab. Lower amounts of conventional immunosuppressants might be a reason for the lower fungal infections.

In this study, half the patients were given 500 mg/body, a quarter were given 300 mg/body and a quarter were given 375 mg/m^2 (corresponding to 430–762 mg/body; median, 600 mg/body). One reason for dose reduction could be concern about potential adverse effects in patients with end-stage liver diseases. In kidney transplantation, Shirakawa et al (20) reported a successful trial to reduce rituximab from 500 to 200 mg/body. Here, there was a tendency toward a higher incidence of AMR in patients treated with $\leq$300 mg/body compared with 500 mg/body or 375 mg/m^2; however, three patients treated with 130 mg/body or 200 mg/body belonged to the same center, and one of them died from severe AMR. More evidence is needed before we can recommend reducing the rituximab dose below 300 mg/body in liver transplantation.

Multiple administrations of rituximab are standard in the treatment of B cell lymphoma. However, because the amount of targeted B cells is expected to be much smaller in transplant patients, a single dose is usually applied. A single dose is standard in kidney transplantation. Here, there were patients with two administrations in six centers and with three administrations in three centers, but the majority of these patients underwent transplantations in 2010 or earlier. All three centers changed their policy to one dose in 2012 on the basis of our data. The current study clearly demonstrates that multiple doses provide no significant benefit in terms of AMR incidence or survival, whereas they increase the incidences of fungal and CMV infections.

The Kyoto group recommended early administration of rituximab to deplete B cells, although the incidence of clinical AMR did not increase significantly in patients with late administration (2). Here, the timing of rituximab administration had no significant effect on AMR incidence on patient survival. Furthermore, 6 of 22 patients with FHF were given rituximab within 6 days before transplantation and survived without AMR. Hence, administration of rituximab immediately before transplantation is a promising therapeutic strategy.

The titers decrease after desensitization before transplantation and increase or do not change immediately after transplantation, and they usually decrease thereafter when patients survive (1). Hence, the optimum cut-off values vary among time points, between IgM and IgG. In rituximab-treated patients, peak IgG and IgM DSA titers posttransplantation were significantly greater in those with AMR, and the AMR incidence was significantly higher in patients with peak titers posttransplantation above optimum cut-off values calculated from ROC curves (i.e. IgM, $\geq$64; IgG, $\geq$128). Theoretically, it is an option to treat patients preemptively by using other desensitization methods such as IVIG and plasmapheresis when antibody titers are above the cut-off values; however, the decision is still difficult.

This study had limitations. It was an uncontrolled retrospective observational study with many confounders, some of which may have been nonrandom and unaccounted for, and thus despite the use of appropriate multivariate statistics unknown bias was possible. Because of the extent of co-linearity between rituximab and era, estimates of regression coefficients still might be unstable, although we tried to adjust era effects as much as possible. Prospective studies are required to examine the causality of the relationships found.

In conclusion, outcomes in adult ABO-I LDLT have significantly improved in the latest era coincident with the introduction of rituximab.

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Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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INVITED COMMENTARY

Is living donor liver transplantation really equivalent to deceased donor liver transplantation?*

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*Invited commentary on "Living donor versus deceased donor liver transplantation: A surgeon-matched comparison of recipient morbidity and outcomes", by Reichman *et al.*

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Living donor liver transplantation (LDLT) has developed as an alternative to deceased donor liver transplantation (DDLT) in order to overcome the critical shortage of deceased organ donations. Particularly in regions with low deceased donation rates, like Asian, LDLT for end stage liver disease significantly reduces the risk of death or drop off the wait list without compromising post-transplant survival. A preference for LDLT to DDLT may depend on the original disease representing the indication for liver transplantation (LT). LDLT offers a timely alternative to DDLT for patients with hepatocellular carcinoma (HCC). However, the higher recurrence rate of HCC after LDLT and the indication criteria remain controversial. One of the recent quantitative meta-analyses revealed the comparable patient survival rates and no significant differences in the recurrence rates between LDLT and DDLT recipients [1]. Another meta-analysis provided evidence of lower disease-free survival (DFS) after LDLT compared with DDLT for HCC [2]. Hence, LDLT likely represents an acceptable option that does not compromise patient survival or increase HCC recurrence in comparison with DDLT at this moment.

Early data suggested that patients with Hepatitis C virus (HCV) that received a LDLT had worse outcomes, including increased rates of cholestatic HCV than did recipients of DDLT [3,4]. This is currently thought to be because of an increased rate of biliary complications or other problems seen during the learning curve of early LDLT experience. More recent data demonstrated that there is no difference in recurrent HCV between recipients of DDLT and LDLT [5,6]. The latest meta-analysis demonstrated that LDLT was equivalent to DDLT in terms of long-term patient or graft survival, HCV recurrence, and acute rejection with a potential lower short-term graft survival [7].

There are limited convincing data comparing outcomes of LDLT and DDLT for autoimmune hepatitis (AIH) and cholestatic liver diseases. It has been previously reported that the overall survival outcomes of LDLT were similar to DDLT in patients with AIH and primary biliary cirrhosis

[8]. In contrast, patients with primary sclerosing cholangitis undergoing LDLT, especially with biologically related donors, are thought to have a higher risk to develop recurrent disease compared with the DDLT setting, probably because of sharing antigens targeted by autoimmunity between recipients and the related donors [9]. Further prospective studies at transplant centers performing both LDLT and DDLT might be needed to confirm these issues.

Regardless of such original disease, LDLT offers several advantages over DDLT, which include the reduction in waiting time mortality, the reduction in cold ischemic time (CIT) and the feasibility of various preoperative interventions, such as nutritional treatment for both the donor and recipient [10]. However, it remains unclear whether those advantages offset disadvantages peculiar to LDLT, such as the smaller graft volume than DDLT and the highly technical procedure, which may be associated with higher complication rates. This seems to be caused by a fact that direct comparison of the results between LDLT and DDLT inevitably involves various biases in nature.

Reichman *et al.* [11] have performed a retrospective matched-cohort study to compare postoperative complication rate and patient survival in the two groups of patients submitted to LDLT and to DDLT. Six clinical variables for recipients: age, Meld, date of transplant, gender, primary diagnosis, and recipient surgeon were matched in each group (n = 145 in each group). They found that the overall complication rate was similar between two groups. In further detail, biliary complications were higher in LDLT although the complications that occurred in the DDLT were strongly associated with graft loss. Graft and patient survival outcomes for LDLT versus DDLT were similar. From those findings, they concluded that LDLT offers an excellent alternative to DDLT in areas of deceased donor organ shortages. This study defined surgical complications that are more frequent in LDLT, i.e., biliary complications (34% and 17% in LDLT and DDLT cohorts, respectively). Despite a higher rate of complications among LDLT recipients, complications leading to death were not significantly higher in LDLT in the experienced center. These findings, in concert with the current common consent that the incidence of complications, even biliary complications, can decline with center experience to levels comparable with DDLT [12], underscore the impact of the learning curve on this highly technical procedure. Potential recipients need to hear about both the rates of complications after LDLT and DDLT, and this study with control for recipient variables will help to define those rates. As pointed out by the authors, this study left control for donor variables out of consideration, despite a well known fact that donor age/gender and donor-recipient human leukocyte antigen matching correlate with either the incidence of certain complications or the severity of original disease recurrence. Nevertheless, this case control comparison of the outcome of LDLT and DDLT convincingly reported that these procedures had different complication profiles but the overall outcomes were similar with expert management, suggesting that the biological advantage in LDLT could compensate for a higher rate of surgical complications caused by greater technical complexity.

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ELSEVIER

Comparative Analysis of T-Cell Depletion Method for Clinical Immunotherapy—Anti–Hepatitis C Effects of Natural Killer Cells Via Interferon-γ Production

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ABSTRACT

Liver transplantation (LT) is a life-saving treatment for liver cirrhosis patients with hepatocellular carcinoma (HCC). However, 10%–20% HCC recurrence rate after LT is due to the immunosuppression inducing tumor growth. We recently reported a novel immunotherapy with donor liver natural killer (NK) cells to prevent HCC and hepatitis C virus (HCV) recurrence after LT. In this cell processing procedure, Muromonab-CD3 (Orthoclone OKT3, an anti-CD3 antibody) was added to the culture medium to deplete $CD3^+$ T cells to prevent graft-versus-host disease. However, the manufacture of OKT3 was discontinued in 2010, when other treatments with similar efficacy and fewer side effects became available. In this study, we examined alternative reagents for T-cell depletion-MACS GMP CD3 pure (GMP CD3), antithymocyte globulin, and alemtuzumab-for NK cell immunotherapy in the allogeneic setting. We observed that GMP CD3 showed exactly the same effects on liver mononuclear cells as OKT3, including activation of NK cells and depletion of T cells. Interestingly, binding of T-cell depletion antibodies to NK cells led to an anti-HCV effect via interferon-γ production. These results with the use of in vitro culture systems suggested that antibodies which produce T-cell depletion affected NK cell function.

Liver failure and hepatocellular carcinoma (HCC) caused by chronic hepatitis C virus (HCV) infection are the most common indications for liver transplantation (LT). The incidences of both conditions have been projected to increase further. On the one hand, the rate of HCC recurrence after LT is 10%–20%.[1,2] On the other hand, recurrent HCV infection in the allograft, which is universal, occurs immediately after LT and is associated with accelerated progression to liver cirrhosis, graft loss, and death.[3,4] These recurrences remains the most serious issue with LT. The use of postoperative immunosuppressants poses an additional risk for recurrences and hinders the use of chemotherapeutic or interferon (IFN) agents.[5,6] However, no definitive treatment or prevention for HCC recurrence after LT is known.

Natural killer (NK) cells are innate immune lymphocytes that are identified by their expression of the CD56 surface antigen and the absence of CD3 markers.[7,8] NK cells can directly kill targets through the release of granzymes, which are granules containing perforin and serine proteases, and/or by surface-expressed ligands that engage and activate death receptors expressed on target cells. Unlike T cells, NK cells do not require the presence of a specific antigen to kill cancer cells, modified cells, or invading infectious microbes. NK cells are abundant in the liver, in

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contrast to their relatively small distribution in peripheral lymph and lymphatic organs in rodents[9–11] and humans.[12,13] In addition, hepatic NK cells in humans have been shown to mediate cytotoxic activity against HCC[12] and to display anti-HCV effects[14] compared with their peripheral blood counterparts. We have successfully applied adoptive immunotherapy with liver NK cells to LT recipients with HCC in Japan and the United States.[14–16] In this regimen, LT recipients are injected intravenously with interleukin (IL) 2–activated NK cells derived from the donor liver allograft. After treatment with IL-2 and OKT3 (Orthoclone OKT3, an anti-CD3 monoclonal antibody [mAb]; Ortho Biotech, Raritan, NJ), liver NK cells expressed significantly elevated levels of the tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), a crucial molecule for killing of tumor cells. Furthermore, these cells showed great cytotoxicity against HCC without any effect on normal cells.[12]

OKT3, a potent immunosuppressant, has been shown to reverse renal allograft rejection episodes.[17,18] It has also been widely used for immunotherapy, as well as to expand cytotoxic T cells[19] and enhance the activity of lymphokine-activated killer (LAK) cells,[20–25] and prevent graft-versus-host disease (GVHD).[26–29] In the latter setting, administration of OKT3-coated T cells in vivo opsonizes for the reticuloendothelial system to subsequently trap or lyses cells.[30–32] This method has been used for clinical NK therapy in Japan, achieving protection against GVHD.[14] However, because of its numerous side effects, the availability of better-tolerated alternatives, and its declining use, OKT3 has been recently removed from the market. Therefore, alternative reagents need to be evaluated for this immunotherapy. In the present study, we evaluated the effect of alternative reagents-GMP CD3 (MACS GMP CD3 pure; Miltenyi Biotec, Bergisch Gladbach, Germany), antithymocyte globulin (Thymoglobulin; Genzyme, Cambridge, MA), and alemtuzumab (Campath; Genzyme) using culture systems with NK and T cells for subsequent application in clinical trials.

MATERIALS AND METHODS

Isolation of Liver Mononuclear Cells

Liver mononuclear cells (LMNCs) from liver perfusates were isolated by gradient centrifugation with Ficoll-Hypaque (GE Healthcare, Pittsburgh, PA) before suspension in X-Vivo 15 medium (Lonza, Walkersville, MD) supplemented with 100 μg/mL gentamicin (APP Pharmaceuticals, Schaumburg, IL), 10% human AB serum (Valley Biomedical, Winchester, VA), and 10 U/mL sodium heparin (APP Pharmaceuticals), as previously described.[16] Our Institutional Review Board (IRB) approved this study.

Cell Culture

LMNCs were cultured with 1,000 U/mL human recombinant IL-2 (Proleukin; Novartis, Emeryville, CA) in culture medium at 37°C in an atmosphere supplemented with 5% CO_2. LMNCs were exposed to a OKT3 (1 μg/mL), GMP CD3 (1 μg/mL), antithymocyte globulin (100 μg/mL), or alemtuzumab (100 μg/mL) at 1 day before cell harvest. After 4 days of culture, cells were subjected to further analyses.

Flow Cytometry

All flow cytometry (FCM) analyses were performed on an LSR II Flow Cytometer (BD Biosciences, San Jose, CA). The following mAbs were used for surface staining of the lymphocytes: fluorescein isothiocyanate–conjugated anti-CD3 (HIT3a; BD Pharmingen, San Diego, CA) or anti-CD56 (B159; BD Pharmingen); phycoerythrin (PE)-conjugated anti-TRAIL (RIK-2; BD Pharmingen), anti-NKp44 (P44-8.1; BD Pharmingen), or anti-CD158b (CH-L; BD Pharmingen); allophycocyanin (APC)–conjugated anti-CD56 (B159; BD Pharmingen), anti-CD25 (M-A251; BD Pharmingen), or anti-NKG2A (Z199; Beckman Coulter, Fullerton, CA); APC-eFluor780–conjugated anti-CD3 (UCHT1; eBioscience, San Diego, CA); PE-Cy7–conjugated anti-CD69 (FN50; Biolegend, San Diego, CA), or anti-NKG2D (1D11; Biolegend); eFluor 605NC–conjugated anti-CD16 (eBioCB16; eBioscience); Alexa Fluor 647–conjugated anti-NKp30 (P30-15; Biolegend); peridinin chlorophyll protein complex (PerCP)-Cy5.5–conjugated anti-CD158a (HP-MA4; eBioscience); and biotin-conjugated anti-CD122 (Mik-b3; BD Pharmingen), anti-NKp46 (9E2; Biolegend), or CD132 (TuGh4; BD Pharmingen). The biotinylated mAbs were visualized with the use of PerCP-Cy5.5-streptavidin (eBioscience) or PE-Cy7-streptavidin (Biolegend). Dead cells were excluded by light scatter and 4′,6-diamidino-2-phenylindole staining (DAPI; Invitrogen, Carlsbad, CA). FCM analyses were performed with Flowjo software (Tree Star, Ashland, OR).

Cytotoxic Assay

The cytotoxicity assay was performed by FCM as previously described.[16] Briefly, target cells labeled with 0.1 μmol/L carboxyfluorescein diacetate succinimidyl ester Cell Tracer Kit (Invitrogen) for 5 minutes at 37°C in 5% CO_2 were washed twice in phosphate-buffered saline solution, resuspended in complete medium, and counted with the use of trypan blue staining. The effector and target cells were coincubated at various ratios for 1 hour at 37°C in 5% CO_2. As a control, target cells or effector cells were incubated alone in complete medium to measure spontaneous cell death after DAPI was added to each tube. The data were analyzed with the use of Flowjo software. Cytotoxic activity was calculated as a percentage with the following formula: % cytotoxicity = [(% experimental $DAPI^+$ dead targets) − (% spontaneous $DAPI^+$ dead targets)]/[(100 − (% spontaneous $DAPI^+$ dead targets)] × 100.

ELISA

IFN-γ production of LMNCs during the culture was measured by enzyme-linked immunosorbent assay (ELISA) (Biolegend). Supernates collected after the incubation were stored at −80°C until further use. IFN-γ ELISA was performed according to the manufacturer's instructions.

Coculture with HCV Replicon Cells

The Huh7/Rep-Feo cell line (HCV replicon cells) was kindly provided by Dr N Sakamoto (Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan). The HCV subgenomic replicon plasmid, pRep-Feo, was derived from pRep-Neo (originally pHCVIbneo-delS).[33] pRep-Feo carries a fusion gene comprising firefly luciferase and neomycin phosphotransferase, as described elsewhere.[34,35] After culture in the pres-

ence of G418 (Invitrogen), Huh7/Rep-Feo cell lines showed stable expression of the replicons. We used transwell tissue culture plates (pore size 1 μm; Costar, Cambridge, MA) for coculture experiments. HCV replicon cells (10^5 cells) were incubated in the lower compartment with various numbers of lymphocytes in the upper compartment. The HCV replicon cells in the lower compartments were collected at 48 hours after the coculture for luciferase assays in duplicate with the use of a luminometer (TriStar LB 941; Berthold Technologies, Oak Ridge, TN) with the Bright-Glo Luciferase Assay System (Promega, Madison, WI).

Statistical Analysis

Data are presented as mean ± SEM. The statistical difference between results were analyzed by Student *t* test (2 tailed), using the Statistical Package for the Social Sciences (SPSS) software version 19 for Windows (IBM Corp, Armonk, NY). *P* values of ≤.05 were considered to be statistically significant.

RESULTS

Effect on the Surface Phenotype of LMNCs

In 5 LMNC preparations, the addition of OKT3 GMP CD3 to IL-2–stimulated LMNCs decreased $CD3^+CD56^-$ T cells to 0.2% ± 0.1% and 0.2% ± 0.1%, respectively, from the IL-2–only control value of 28.1% ± 12.3%. In contrast, $CD3^+CD56^-$ T cells were retained among LMNCs with the addition of antithymocyte globulin or alemtuzumab: 3.3% ± 2.0% and 17.2% ± 7.3%, respectively. The proportion of $CD3^-CD56^+$ NK cells increased by ~10% in all groups (Fig 1A).

Addition of OKT3 or GMP CD3 to IL-2–stimulated LMNCs maintained both activation and inhibitory markers on NK cells. Interestingly, the expressions of TRAIL, CD25 (IL-2αR), and CD132 (IL-2γR) were increased in the antithymocyte globulin group. Furthermore, both antithymocyte globulin and alemtuzumab completely blocked the expression of CD16 on NK cells (Fig 1B).

Cytotoxic Capacity

Cytotoxicity assays were performed with the use of freshly isolated cultured LMNCs as effectors and K562 cells as targets. Fig 2 shows freshly isolated LMNCs barely mediated cell death, whereas IL-2–stimulated LMNCs produced significant cytotoxicity. Although the ratios of $CD3^-CD56^+$ to $CD3^+CD56^+$ cells varied after treatment with various

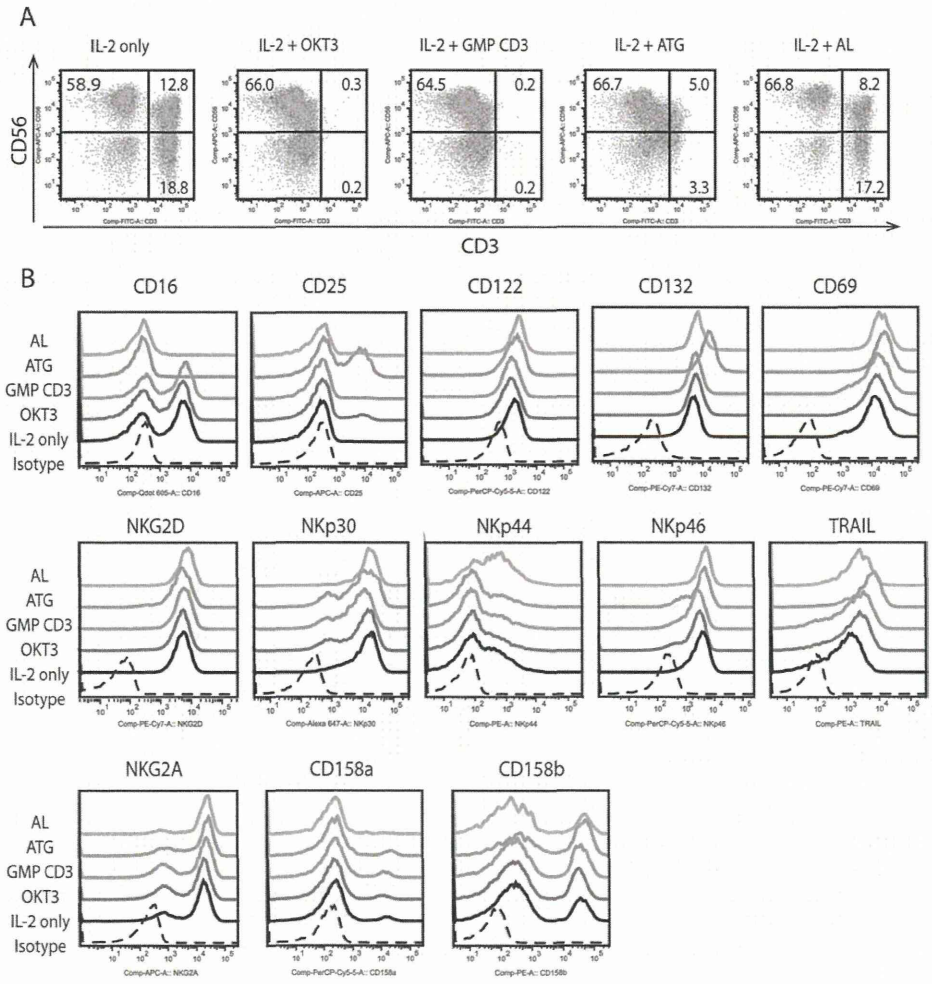

Fig 1. Effect of the T-cell depletion antibodies on the phenotypic characteristics of liver mononuclear cells (LMNCs). LMNCs obtained from cadaveric donors were stimulated with IL-2 (1000 U/mL) for 4 days. Anti-CD3 mAb (OKT3; 1 μg/mL), MACS GMP CD3 pure (GMP CD3; 1 μg/mL), antithymocyte globulin (ATG; 100 μg/mL), or alemtuzumab (AL; 100 μg/mL) was added to the culture medium 1 day before cell harvesting. (A) The LMNCs were stained with monoclonal antibodies against CD3 and CD56. The numbers indicate the mean percentages of the population. (B) Histograms show the logarithmic fluorescence intensities obtained on staining for each surface marker after gating on the $CD3^-CD56^+$ NK cells. Dotted lines indicate negative control samples with isotype-matched mAbs. The flow cytometry dot plot and histogram profiles represent 5 independent experiments. TRAIL, tumor necrosis factor–related apoptosis-inducing ligand.

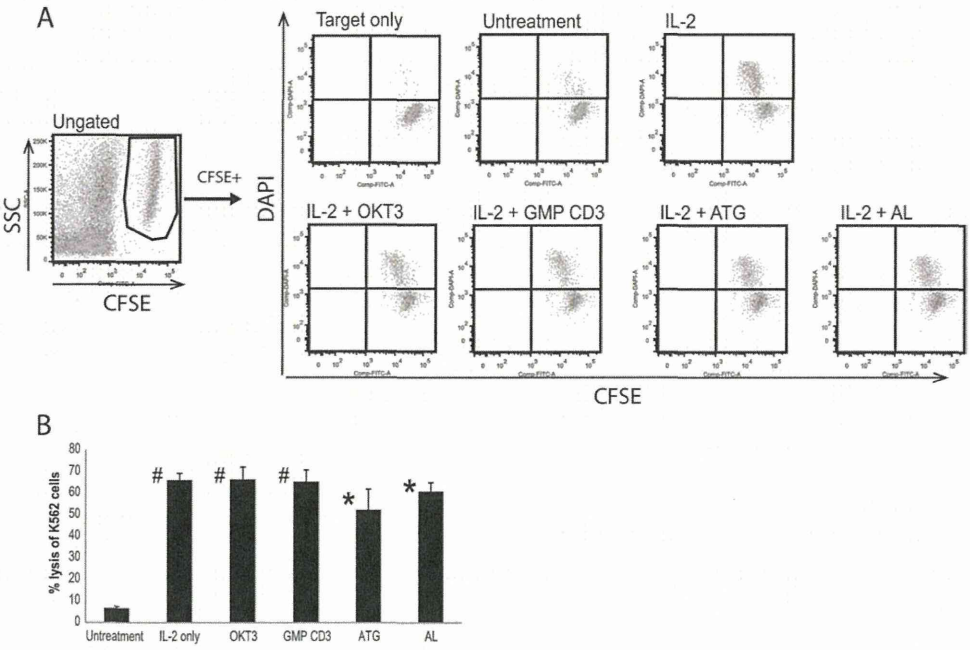

Fig 2. Antitumor effect of the T-cell depletion antibodies on IL-2–stimulated liver mononuclear cells (LMNCs). The NK cell cytotoxic activities of untreated cells and IL-2–stimulated LMNCs treated with various reagents were analyzed by a flow cytometry (FCM)–based cytotoxic assay. (A) Gate is set on cells to discriminate $CFSE^+$ targets from LMNCs. Gate is set on target to obtain the number of live and dead K562 cells. The FCM dot plot profiles represent 5 independent experiments. (B) The data represent the mean ± SEM of the percentage of target lysis at effector-to-target (E:T) ratios of 10:1 (5 LMNCs; $^{\#}P < .01$; $^{*}P < .05$ vs untreated group, *t* test).

T-cell depletion reagents for 4 days in culture, all cultured LMNCs exhibited vigorous cytotoxicity against K562. LMNCs treated with antithymocyte globulin showed slightly decreased cytotoxicity compared with the other groups, but the difference was not significant. This tendency was similar to that reported in an earlier study.[36] The cultured LMNCs did not show cytotoxicity against self-lymphoblasts (data not shown).

Anti-HCV Activity

IL-2–cultured LMNCs inhibited 40% luciferase reporter activity compared with freshly isolated LMNCs (Fig 3A). As we have reported before, the anti-HCV effect of IL-2–activated LMNCs was strongly enhanced by OKT3 treatment.[14] GMP CD3 treatment showed ~80% decreased HCV replication, which was almost the same effect as that caused by OKT3. Surprisingly, antithymocyte globulin and alemtuzumab treatment also elicited robust anti-HCV effects on LMNCs. We previously reported that IFN-γ secreted from LMNCs activated by IL-2 and OKT3 was responsible for the anti-HCV activity of these cells.[14] Cultured LMNCs also actively produced large amounts of IFN-γ (Fig 3B), which probably played a pivotal role in their anti-HCV activity.

DISCUSSION

In this study, we discovered GMP CD3 to be an alternative reagent to OKT3 for immunotherapy using liver NK cells.

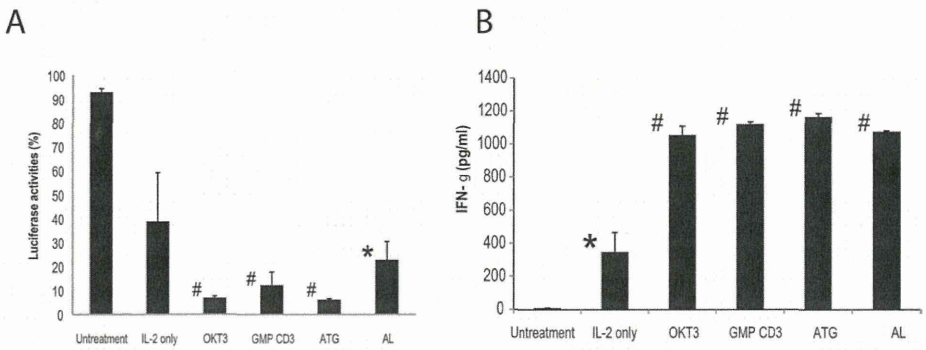

Fig 3. Anti–hepatitis C virus (HCV) effect of the T-cell depletion antibodies on IL-2–stimulated liver mononuclear cells (LMNCs). The LMNCs cultured for 4 days in the presence of IL-2 and various reagents were incubated with HCV replicon–containing cells for 48 hours in transwell tissue culture plates (effector-to-target ratio, 10:1). (A) Luciferase activity of HCV replicon–containing cells in the presence of effectors, normalized to luciferase activity in the absence of effectors. The difference in anti-HCV effect between the reagent-treated LMNCs and the freshly isolated LMNCs was statistically significant (5 LMNCs; $^{\#}P < .01$; $^{*}P < .05$ vs untreated group, *t* test). (B) IFN-γ production during the culture, as measured by ELISA [mean ± SEM (5 samples; $^{\#}P < .01$; $^{*}P < .05$ vs untreated group, *t* test)].