stage,[12,13] it has become possible to repeatedly apply curative treatments.

To manage HCC patients surviving for a long term, preparing the treatment strategy for recurrent cancer becomes more important than that for initial treatment. This makes it important to predict the prognosis of recurrent patients. In other words, every time HCC is diagnosed, the prognostic value should be assessed, and then a treatment strategy should be decided. However, no attempts have been made to include prediction of the prognosis of recurrent HCC patients. The purpose of this article is to propose a new prognostic scoring system, which can be useful for deciding the treatment strategy not only for first-time diagnosed patients but also for recurrent HCC patients.

PATIENTS AND METHODS

Study Population

All (888) consecutive adult patients who were diagnosed as HCC and registered with the Division of Internal Medicine in the Osaka University Hospital between 1993 and 2003, were eligible for this study. Sixteen patients who could not be confirmed as having HCC were excluded. Three patients who underwent liver transplantation were also excluded. Eight patients who had local recurrences within 6 months were excluded because their admissions were not for the recurrent tumor but rather for the residual tumors caused by the insufficient ablation therapy. Thus, 861 patients composed the study population. The patient data were collected with both a survey of original medical records and access to the hospital information system. The patient data set was divided into 2 data sets for a split-sample validation procedure,[14] one set being retrospectively collected patients (n = 578) between September 1, 1993 and December 31, 2001, and the other being prospectively collected patients (n = 283) with the hospital database system between January 1, 2002 and December 31, 2003. The former was used as a training sample to construct a prognostic scoring system; the latter was used as a validation sample for the validation of the generated classification. HCC diagnosis was mainly established by the concomitant finding of 2 imaging techniques (n = 438), showing a nodule with arterial hypervascularization and portal hypovascularization, or by a positive imaging technique, showing hypervascularization associated with elevation of α-fetoprotein (AFP) or protein induced by vitamin K absence II (PIVKA-II) (n = 272). In addition, even if the above-mentioned features were not observed, target biopsy was performed when the findings of ultrasonography were consistent with HCC (n = 151). Details of the treatment modality showed that trans-catheter arterial chemoembolization alone or combined with percutaneous tumor ablation were mainly performed (n = 306 and 301, respectively). The number of patients treated with surgical resection, percutaneous tumor ablation alone, and best supportive care were 46, 188, and 20 respectively.

Statistical Methods

Overall survival was the only end point used in the analysis. It was defined as the time elapsed from the date of diagnosis and either the date of death related to liver disease or the date of the last follow-up information, with the final evaluation conducted on August 31, 2004. Patients lost before the last collection of follow-up information were censored at the time of their last visit. One hundred thirty-one of the 238 censored cases in the training sample were alive at the end of the period, whereas 22 patients had died from other diseases and 85 were lost to follow-up owing to change of residence (n = 21), introduction of a hospital near their residence (n = 50), and unknown reasons (n = 14). Two hundred and two of the 223 censored cases in the validation sample were alive, 3 patients died from other diseases, and 19 cases were loss to follow-up owing to the change of residence (n = 1), introduction of a hospital near their residence (n = 11), and unknown reasons (n = 7). Judging from the data at their last visit, all of the censored samples were considered to be independent of the future value of the hazard for the individual, in other words, they were noninformative censored cases. Figure 1 shows a schematic overview of investigated patients and dropouts for training and validation sample.

The following variables were used for the analysis: age and sex of the patient, date of HCC diagnosis, date of death or of last available information, viral status, the number of HCC recurrences, Child-Pugh score, the largest tumor size, tumor number, vascular invasion, AFP level, and PIVKA-II level. The cut-off levels of continuous variables were chosen on the basis of clinical meaning. For each variable, missing data were not used in the analysis if they accounted for less than 10% of the cases.

Univariate survival curves were estimated using the Kaplan-Meier method[15] and compared by means of the log-rank test.[16] The prognostic impact of the categories was assessed by means of the observed/expected ratio, as described previously.[6] Of the factors affecting patient survival in univariate analysis, baseline predictors were identified by the Akaike information criterion in a stepwise algorithm.[17] Next, a Cox proportional hazard

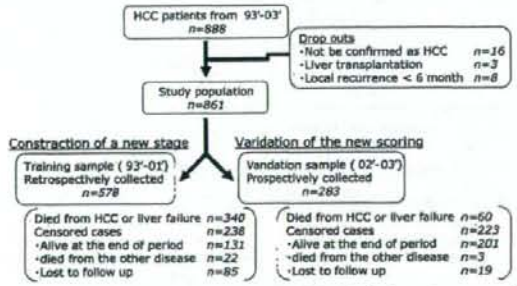

FIGURE 1. Schematic overview of included patients and dropouts for training and validation sample.

regression model was used for multivariate analyses.[18] Proportional hazard assumption was graphically assessed using plots of Log [− Log (survival time)]. Cases with missing values for one or more variables in the model were excluded from multivariate analysis. Treatment was not included in the model because the treatment choice was closely associated with the assessment of prognosis at the time of diagnosis.

Finally, the validity of the generated score was then assessed for the validation sample; a recent sample and a prospectively followed sample. The predictive accuracy of 3 models: this new score system, JIS score system, and CLIP score system was quantified by calculating the concordance index (C-index), which provides the area under the receiver operating characteristics (ROC) curve for the prediction of death at 3 years, as described previously.[19] A C-index of 0.5 indicates that outcomes are completely random, whereas a C-index of 1.0 indicates that the model is a perfect predictor.

All analyses were performed with R's software (R Foundation for Statistical Computing, Austria).[20] $P < 0.05$ was considered statistically significant in all analyses. The results were reported as a hazard ratio with 95% confidence intervals.

RESULTS

As of August 2004, 344 patients (59%) had died. The overall median survival time was 41 months (95% confidence interval, 36 to 46 mo); 1, 3, 5-year survival rates were 86%, 56%, and 35%, respectively. The baseline characteristics of the patients are given in Table 1. The first-time diagnosed HCC, shown as the number of HCC recurrences = 0 in Table 1, amounted to 295 cases, the first recurrence to 185, the second recurrence to 126, the third recurrence to 90, and more than the fourth recurrence to 165. Most cases were in the Child-Pugh A category. The baseline characteristics of the tumor are given in Table 2.

Nine variables were separately found to be associated with the outcome in univariate analysis of 11 variables (as shown in Table 3). Forward stepwise selection by Akaike information criterion was used to identify baseline predictors of 9 variables. Five variables were selected: the Child-Pugh score, the number of tumors, AFP, vascular invasion, and the number of HCC recurrences. To better reflect the treatment response, we combined 2 factors to create a single factor: we replaced "the number of tumors and vascular invasion" with "3 nodules less than 3 cm and none of vascular invasion, or not," called the tumor factor. This was done because the criterion "3 nodules less than 3 cm" reflects the possibility of complete response to ablation treatment[21] and was useful in the current clinical setting. We finally chose 4 factors for a new prognostic classification: the Child-Pugh score, tumor factor, AFP, and the number of HCC recurrences. These 4 covariates showed correlation with survival in the Cox regression analysis.

Each covariate selected by means of forward stepwise methods was divided into 2 categories to derive a simple scoring system. The cut-off levels were chosen where each estimated regression coefficient of the final Cox model was almost the same, that is, we made the relative prognostic weight of covariates the same, around 2 each (shown as in Table 4). A new scoring system was derived to assign scores (0/1) to each covariate of the final model as shown in Table 4. This classification was relatively easy to calculate by summing up each individual score of the 4 covariates. Five risk groups were constituted according to the score distribution. The survival curve of 578 patients calculated by the Kaplan-Meier method is shown in Figure 2A.

We assessed the new score system for 283 patients for the validation sample; prospectively obtained from 2002 to 2003 in Figure 2B. This result validated our scoring system and showed that it can be applied in today's clinical setting. This applicability to the present-day situation is very important, because diagnostic and

TABLE 1. Characteristics of Patients

Variables	Training Sample No. Patients	Validation Sample No. Patients
Median age, y (range)	64 (21-85)	67 (35-83)
Male (%)	425 (73.5)	192(67.8)
Cause of parenchymal disorder		
HBV/HCV/HB + HC	54/486/10	27/227/4
Alcoholic	8	10
Others	20	15
Child-Pugh score (unknown = 1)		
5-6 (A)/7-9 (B)/10-12 (C)	342/218/18	192/79/11
Number of HCC recurrence		
0/1/2/3/≥4	201/123/88/62/104	94/62/38/28/61

HBV indicates Hepatitis B virus; HCV, Hepatitis C virus.

TABLE 2. Characteristics of the Tumor

	Training Sample No. Patients	Validation Sample No. Patients
Number of tumor		
1/2/3/4/≥5	186/113/57/36/186	112/56/28/18/69
Largest size of tumor (cm)		
≤2.0/2.1-3.0/3.1-5.0/≥5.1	270/163/91/54	128/82/44/29
Vascular invasion		
Yes/no	534/44	266/17
Tumor factor [3 nodule less than 3 cm, vascular invasion (−)]		
Yes/no	285/293	159/124
AFP category (ng/mL)		
$\leq 10/10\text{-}10^2/10^2\text{-}10^3/>10^3$	137/230/129/82	65/108/70/40
PIVKA-II (mAU/mL) (unknown = 81)		
$\leq 10^2/10^2\text{-}10^3/10^3\text{-}10^4/>10^4$	327/118/64/27	110/58/20/14

TABLE 3. Univariate Analysis of Clinical Findings for Survival

Variables	No. Patients	O/E Ratio	*P*	DOF
Sex			0.00168	1
Male/female	425/153	1.11/0.73		
Age			0.00284	3
≤50/50-60/60-70/≥70	37/118/291/132	0.53/0.87/1.19/0.86		
Etiology			0.147	3
HCV/HBV/HB+HC/the others	486/54/10/28	1.03/0.9/1.38/0.54		
Number of HCC recurrence			< 0.0001	4
0/1/2/3/≥4	201/123/88/62/104	0.57/0.93/1.2/1.33/2.1		
Child-Pugh stage			< 0.0001	2
A/B/C	342/218/18	0.75/1.49/2.72		
Largest size of tumor (cm)			0.00467	3
≤2.0/2.1-3.0/3.1-5.0/≥5.1	270/163/91/54	0.86/1.06/1.16/1.65		
Number of tumor			< 0.0001	4
1/2/3/4/≥5	186/113/57/36/186	0.52/0.95/0.97/1.04/2.03		
Vascular invasion			< 0.0001	1
Yes/no	534/44	0.93/3.78		
Tumor factor [3 nodules less than 3 cm, vascular invasion (−)]			< 0.0001	1
Yes/no	285/293	0.67/1.53		
AFP (ng/mL)			< 0.0001	3
$\leq 10/10\text{-}10^2/10^2\text{-}10^3/>10^3$	137/230/129/82	0.56/0.95/1.2/2.19		
PIVKA-II (mAU/mL)			< 0.0001	3
$\leq 10^2/10^2\text{-}10^3/10^3\text{-}10^4/>10^4$	327/118/64/27	0.76/1.34/1.8/3.65		

DOF indicates degree of freedom; O/E ratio, observed/expected ratio; HBV, Hepatitis B virus; HCV, Hepatitis C virus.

therapeutic procedures for HCC have been improved over recent years.

Finally, the prognostic ability of the new scoring system was compared with CLIP score system and the JIS score system. Kaplan-Meier survival curves were shown in Figs. 2C, D). In addition, the predictive accuracy of 3 models was quantified by calculating a C-index, which provides the area under the ROC curve (as shown in Fig. 3). CLIP stage and JIS scoring had a C-index of 7.05 and 6.93, respectively. This new scoring system had a C-index of 7.23. Our scoring system could discriminate the survival most precisely among them.

DISCUSSION

This article revealed that the number of HCC recurrences is a prognostic factor as well as the reserved liver function and the spreading of HCC, and we have proposed a new scoring system, comprised of 4 parameters: the number of HCC recurrences, the Child-Pugh score, the tumor factor of "3 nodules less than 3 cm and none of vascular invasion," and the AFP level. Each of these parameters has so far been reported to affect patient survival. The occurrence of HCC recurrence reflects disease progression.[3–5] The Child-Pugh score is a well-recognized prognostic variable and reflects reserved liver

TABLE 4. New Scoring System

Variables	Score 0	Score 1	RR
Number of HCC recurrence (n = 578)	0 or 1 (n = 324)	≥2 (n = 254)	2.26
Child-Pugh score (n = 578)	5–7 (n = 486)	≥8 (n = 92)	2.25
Tumor factor (n = 578)	Yes (n = 285)	No (n = 293)	1.90
AFP category (ng/ml) (n = 578)	≤1000 (n = 496)	≥1001 (n = 82)	2.08

RR indicates risk ratio of Score 1 compared with Score 0, assessed by multivariate analysis.

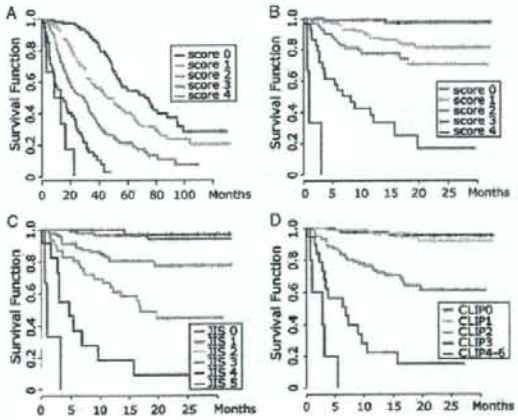

FIGURE 2. Kaplan-Meier–estimated survival curves. A, By our new scoring system in training samples. B, By our new scoring system in validation samples. C, By the CLIP score system in validation samples. D, By the JIS score system in validation samples.

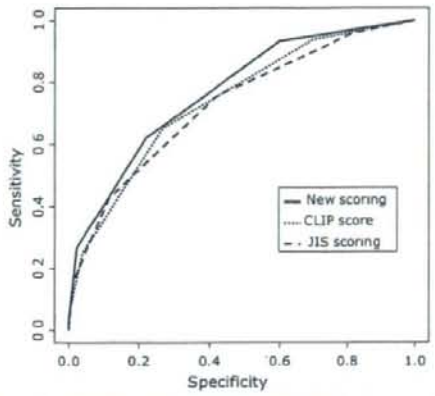

FIGURE 3. Discriminatory ability for the prediction of death at 3 years, evaluated by receiver operating characteristics curves of the new scoring, CLIP, and JIS staging systems.

function.[6,7] The criterion of 3 nodules less than 3 cm is related to the treatment response. Ablation therapy is highly effective for tumors smaller than 3 cm, achieving complete responses of around 80% to 100%.[22] The achievement of a complete and sustained response is an independent prognostic value.[23] AFP is also a well-recognized prognostic variable, and reflects the degree of cellular differentiation and the spreading of the tumor.[24] In the present study, these parameters were independent predictors of survival actually. Elevation of each parameter indicates the progression of HCC. As a result, this new scoring system reflects the spreading of HCC, the response to treatment, and the reserved liver function. In addition, our system is based on not pathologic but easily obtainable and reproducible clinical information. Therefore, this scoring system should be useful in many clinical settings.

A high possibility of recurrence is one of the major characteristics of HCC. Recurrences from either intrahepatic metastasis or de novo HCC exceed 50% at 3 years, even with hepatic resection as curative therapy.[3–5] The more the HCC recurs, the more the prognosis deteriorates because of treatment-induced liver damage and/or tumor progression. In clinical settings, it is very important to carefully follow HCC patients to detect recurrence as early as possible. More and more patients have been able to be frequently treated for recurrent HCC and prolong their survival. What is needed is a treatment strategy based on appropriate cancer staging systems for not only first-time diagnosed HCC but also for recurrent HCC. However, there has been no study reported on the prognosis of recurrent patients. Here, we first showed recurrence to be a prognostic factor with a Cox regression model, and furthermore developed a new scoring system to predict the prognosis of HCC patients including recurrent HCC patients.

What is the problem with applying the other staging systems for the recurrent cases? All of the following staging systems: the CLIP score system,[6] BCLC staging[7] and JIS scoring system[8] were derived from the analysis for first-time diagnosed HCC and were applied only at the initial treatment. Because hypothetical population is different between first-time HCC patients and all HCC patients, their baseline predictors for survival differ from the new scoring system. Indeed, the distributions of both the number of tumor and the largest size of HCC are significantly different between first-time HCC cases and all HCC patients in our cohort (data not shown). As a result, JIS system and CLIP score system may have poor stratification of survival. The goal of cancer staging is to separate patients into different groups on the basis of their predicted survival to help determine the most appropriate treatment modality. Therefore, it is unreasonable to apply their systems for recurrent HCC patients.

Although further evaluation is needed, this scoring system can be useful for conducting interventional trials. With the spread of routine screening and follow-up, the number of recurrent HCC patients can increase. More effective strategies to treat recurrent patients will be needed. In addition, a new modality of treatment will be necessary for HCC management, particularly for score 2 and 3 patients. Interventional trials may be needed to determine the most appropriate therapy for the patients in each group. This scoring system, because of good incorporation between prognosis estimation and potential treatment advances, may be useful for planning and evaluating interventional trials. It would allow us to follow a well-established treatment schedule and select the best treatment modality for each patient when managing long-term–surviving HCC patients.

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Impaired Cytokine Response in Myeloid Dendritic Cells in Chronic Hepatitis C Virus Infection Regardless of Enhanced Expression of Toll-Like Receptors and Retinoic Acid Inducible Gene-I

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Dendritic cells utilize various sets of Toll-like receptors (TLR) or cytosolic sensors to detect pathogens and evoke immune responses. In patients with hepatitis C virus (HCV) infection, a higher prevalence of various infectious diseases is reported; suggesting that innate immunity against pathogens is impaired. The aim of this study was to clarify whether the TLR and retinoic acid inducible gene-I (RIG-I) system in myeloid dendritic cells is preserved or not in chronic HCV infection. The expression of TLRs, RIG-I and its relatives were compared in myeloid dendritic cells between 39 patients and 52 healthy volunteers. The induction of type-I interferon (IFN) and inflammatory cytokines was examined in response to agonists for TLR2 (palmitoyl-3-cysteine-serine-lysine-4), TLR3/RIG-I (polyinosine–polycytidylic acid) or TLR4 (lipopolysaccharide). The relative expressions of TLR2, TLR4, RIG-I, and LGP2 from the patients were significantly higher than those from the volunteers, whereas TLR3 and MDA-5 expressions did not differ. In search for factors regulating TLR/RIG-I expression, it was shown that IFN-α, polyinosine–polycytidylic acid and lipopolysaccharide induced TLR3, TLR4 and RIG-I, but TNF-α, HCV core or HCV non-structural proteins did not. For the functional analyses, myeloid dendritic cells from the patients induced significantly less amounts of IFN-β, TNF-α and IL-12p70 in response to polyinosine–polycytidylic acid or lipopolysaccharide. It is noteworthy that the expression of TRIF and TRAF6, which are essential adaptor molecules transmitting TLR3 or TLR4-dependent signals, is reduced in the patients. Thus, innate cytokine responses in myeloid dendritic cells are impaired regardless of enhanced expressions of TLR2, TLR4, and RIG-I in HCV infection. ***J. Med. Virol. 80: 980–988, 2008.*** © 2008 Wiley-Liss, Inc.

KEY WORDS: chronic hepatitis C; myeloid dendritic cell; innate immunity; TLR3; RIG-I

INTRODUCTION

Hepatitis C virus (HCV) is a single-stranded RNA virus, which causes chronic liver disease in hosts. At primary HCV infection, approximately 80% of patients fail to eradicate HCV and eventually progress to a chronic infected state [Lauer and Walker, 2001]. It is very likely that escape mutation of the HCV genome and insufficient immune responses against HCV in hosts are involved in the persistence of infection, however, the precise mechanisms are still largely unknown. Type-I interferon (IFN) is a potent anti-viral agent that exerts its ability by suppressing viral replication or via modulating immune reactions. Gene expression analyses of HCV-infected livers obtained from chimpanzees revealed that type-I IFN and IFN-stimulated genes are highly induced even in the incubation phase [Bigger et al., 2004]. Nevertheless, HCV continues to replicate and remains at high titer levels, suggesting that HCV

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possesses some inhibitory mechanisms in IFN-inducible anti-viral responses.

As for the mechanisms of HCV persistence, the alteration or impairment of various immune cells has been reported, such as T cells, NK cells and dendritic cells [Chang et al., 2001; Wedemeyer et al., 2002; Kanto et al., 2004; Szabo and Dolganiuc, 2005]. In clear contrast with the human immunodeficiency virus, HCV does not lead to generalized immune suppression in infected hosts. Large-scale epidemiological study on US veterans revealed that the prevalence of various infectious diseases was significantly higher in HCV-positive individuals than in HCV-negative ones, including viral, bacterial, and parasite diseases [El-Serag et al., 2003]. These observations suggest that HCV infection raises the susceptibility to pathogens, not profoundly but significantly, in infected patients. However, the underlying mechanisms in the increased prevalence of infection are yet to be determined.

Toll-like receptors (TLR) are expressed in epithelial cells or antigen presenting cells and act as sensors of bacterial or viral infection. These cells utilize specific TLR for the recognition of pathogen-associated molecular patterns and eventually induce type I IFN or inflammatory cytokines. In addition to the TLR system, the existence of cytoplasmic receptors for dsRNA has been reported as virus sensors, which are retinoic acid inducible protein I (RIG-I) and melanoma differentiation associated gene 5 (MDA-5) [Yoneyama et al., 2004]. Since dsRNA is a replicative intermediate of RNA virus, RIG-I and MDA-5 induce IFN-β in response to virus infection independently of TLR3. It is thus plausible that a disabled TLR/RIG-I system may be involved in the increased susceptibility to pathogens or the mechanisms of persistent virus infection [Sumpter et al., 2005]. In human hepatoma cells harboring HCV replicons, it has been shown that HCV NS3/4A protease impedes TLR3-dependent or RIG-I-dependent IFN-β induction by means of the cleavage of relevant adaptor molecules, such as TIR domain-containing adapter inducing IFN-β (TRIF) or interferon-β promoter stimulator-1 (IPS-1), respectively [Foy et al., 2005; Li et al., 2005]. However, it is not clear whether similar inhibitory machinery of HCV operates or not in immune cells, such as dendritic cells.

Dendritic cells are immune sentinels that play a central role against pathogens in inducing innate as well as adaptive immune responses. Dendritic cells consist of myeloid and plasmacytoid subsets that play distinct roles in the regulation of immune responses. Dendritic cells utilize various sets of TLR or RIG-I/MDA-5 to sense virus infection. After the recognition, dendritic cells begin to mature and gain the ability to produce type-I IFN and inflammatory cytokines. It has been reported that blood dendritic cells expresses distinct profiles of TLRs; human myeloid dendritic cells express TLR2, -3, -4, -5, -6, -7, and -8, while plasmacytoid dendritic cells express TLR7, -8 and -9 [Iwasaki and Medzhitov, 2004]. Numerical and/or functional impairment of blood dendritic cells in acute or chronic HCV infection has been reported by several investigators including us [Kanto et al., 2004; Szabo and Dolganiuc, 2005]. One of the plausible mechanisms leading to dendritic cells impairment may be direct HCV infection to blood dendritic cells or their precursors. In support for this, it was shown that myeloid dendritic cells are susceptible to HCV infection, judging from the results of an inoculation study with pseudo-HCV particles or detection of negative strand HCV-RNA [Kaimori et al., 2004]. According to another report, myeloid dendritic cells displayed impaired expression of IL-12 and TNF-α in response to poly-inosine–polycytidylic acid (polyI:C) and lipopolysaccharide (LPS) in patients with a large amount of cell-associated HCV [Rodrigue-Gervais et al., 2007], suggesting a possible link between direct HCV infection to myeloid dendritic cells and an impaired innate response.

Taking these reports into consideration, the current study focused on myeloid dendritic cells in order to clarify the roles of the TLR/RIG-I system in HCV infection, by comparing the expression of TLR, RIG-I, and MDA-5 and the induction of cytokines in response to specific agonists for these virus sensors. The study demonstrated that myeloid dendritic cells from HCV-infected patients induces a significantly lesser amount of cytokines in spite of enhanced expressions of TLR2, TLR4, and RIG-I. These findings imply that alteration of the TLR/RIG-I system is instrumental in impairment of innate immunity in HCV infection, where myeloid dendritic cells play a key role as immune sentinels against pathogens.

MATERIALS AND METHODS

Subjects

Thirty-nine patients (male/female: 22/17, mean age: 53.4 ± 10.3 years old, mean serum ALT levels: 93.9 ± 51.0 IU/L, HCV serotype 1/serotype 2: 39/0) with chronic hepatitis C (HCV group) followed at Osaka University Hospital (Osaka, Japan) were enrolled in the present study. All of them were confirmed to be positive for both serum anti-HCV antibody and HCV RNA (mean HCV RNA quantity assayed by Cobas Amplicor HCV monitor v 2.0, Roche Diagnostics, Tokyo, Japan; [Pawlotsky et al., 2000]: 1,637 ± 402 KIU/ml) but were negative for other viral infections, including hepatitis B virus (HBV) and human immunodeficiency virus (HIV). The presence of other liver diseases, such as alcoholic, metabolic or autoimmune hepatitis, was ruled out. Thirteen patients with chronic HBV infection determined by serum HBsAg-positive and ALT abnormality (male/female: 6/7, HBeAg+/HBeAg−: 7/6, mean age: 45.9 ± 14.4 years old, mean serum ALT levels: 95.2 ± 145 IU/L, mean HBV-DNA levels assayed by Cobas Amplicor HBV monitor Roche Diagnostics; [Noborg et al., 1999]: 6.1 ± 1.7 $\log_{10}$ copies/ml) were also enrolled as disease controls (HBV group). The study protocol was approved by the ethical committee of Osaka University Graduate School of Medicine. At enrolment, written informed consent was obtained from each patient. The

controls were 52 healthy volunteers or blood donors (healthy donors group) at the Osaka Red Cross Blood Center (Osaka, Japan), who were confirmed to be negative for HCV, HBV, and HIV. The background data of the blood donors were not accessible due to the confidentiality regulations of the blood center, but their serum ALT levels were confirmed to be within the normal range.

Reagents

Palmitoyl-3-cysteine-serine-lysine-4 (Pam_3CSK_4) was purchased from InvivoGen (San Diego, CA). Polyinosine–polycytidylic acid (polyI:C) and lipopolysaccharide (LPS) from *Escherichia coli* were purchased from Sigma (St. Louis, MO). Recombinant human IL-6, IL-10, and IL-12 were purchased from InvivoGen. Recombinant TNF-α was purchased from Genzyme (Framingham, MA). Recombinant HCV structural or non-structural (NS) proteins expressed by *E. coli* were purchased from Virogen (Watertown, MA). They were HCV core (amino acid positions, from 2 to 192), NS3 (from 1,450 to 1,643), and NS4 (from 1,658 to 1,863), respectively. HCV NS5B protein (from 2,421 to 2,965) was kindly provided by Japan Tobacco Corp. (Tokyo, Japan). Natural human interferon-α was purchased from Otsuka Pharmaceutical Co. (Tokyo, Japan).

Isolation of Myeloid Dendritic Cells

Peripheral blood mononuclear cells were isolated from heparinized venous blood by centrifugation on Ficoll-Hypaque cushion as described previously [Kanto et al., 2004]. Myeloid dendritic cells were magnetically isolated using a BDCA-1 Isolation Kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. The purity of myeloid dendritic cells (Lineage-negative, HLA-DR^-, $CD11c^+$, and $CD123^{dim+}$ cells) was more than 95% as assessed by FACS (data not shown). Short-term culture of myeloid dendritic cells was performed in cytokine-free Isocove's modified Dulbecco's medium (GIBCO Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum, 100 μg/mL streptomycin, 100 U/mL penicillin, 2 mmol/L L-glutamine, 5 mmol/L HEPES, and 5 mmol/L non-essential amino acid at 37°C in 5% CO_2.

To clarify the factors influencing the expressions of TLR or RIG-I in myeloid dendritic cells, fresh myeloid dendritic cells obtained from uninfected controls were incubated for 2 hr in the presence or absence of various cytokines, agonists for TLR/RIG-I or recombinant HCV proteins. After the incubation, they were subjected to RT-PCR analyses for the comparison.

In order to compare the function of TLR/RIG-I-mediated responses in myeloid dendritic cells between the groups, myeloid dendritic cells were incubated with various agonists for 2 hr and subjected them to cytokine analysis by RT-PCR. Alternatively, myeloid dendritic cells were cultured in the presence or absence of 25 μg/ml of polyI:C for 24 hr and collected supernatants for subsequent cytokine analyses.

Flowcytometric Analysis

The phenotypes of myeloid dendritic cells were analyzed using FACS Calibur and CellQuest software (BD Biosciences, San Jose, CA). For the staining, myeloid dendritic cells were incubated with specific antibodies for 15 min at room temperature in phosphate buffered saline (PBS) containing 2% of bovine serum albumin and 0.1% of sodium azide. The following FITC-, PE-, or APC-conjugated anti-human monoclonal antibodies were used: CD11c (clone, B-ly6), HLA-DR (L243), CD80 (L307.4), CD86 (IT2.2), CD40 (5C3), and CD83 (HB15e). All were purchased from BD Biosciences.

Real-Time Quantitative PCR

Total RNA was extracted from more than 10^6 myeloid dendritic cells using RNeasy Mini kit (Qiagen, Hilden, Germany), which was subsequently reverse transcribed in 20 μl volume using SuperScript III First-Strand Synthesis System (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. Random hexamers were added as primers. The mRNA levels were evaluated using ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). For the quantification of TLR2, TLR3, TLR4, RIG-I, MDA-5, LGP2, myeloid differentiation factor 88 (MyD88), IPS-1, TRIF, TNF receptor associated factor 6 (TRAF6), TNF-α and IFN-β, ready-to-use assays (Taqman Gene Expression Assays, Applied Biosystems) were utilized, according to the manufacturer's instructions. All of the reagents used for PCR were purchased from Applied Biosystems. All of the reactions were performed in duplicate. The thermal cycling conditions for all genes were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. A calibrator sample from healthy volunteers was identified. The expressions of molecule were expressed as the relative values to the calibrator samples. To standardize the amount of total RNA added to each reaction mixture, β-actin mRNA from each sample was quantified as a control of internal RNA and corrected all values with this.

Enzyme-Linked Immunosorbent Assay and Cytokine Beads Assay

The quantity of IFN-α in culture supernatants was evaluated using Human Interferon Alpha ELISA kit (PBL Biomedical Laboratories, New Brunswick, NJ) according to the manufacturer's instructions. The concentration of TNF-α, IL-6, and IL-12p70 in the supernatants was assayed by the use of BD cytokine beads assay (CBA) Flex Sets (BD Biosciences) and analyzed by FACS Calibur according to the manufacture's instructions. The detection limits of IFN-α, TNF-α, IL-6, and IL-12p70 are 10–5,000 pg/ml, respectively.

Statistical Analysis

The Mann–Whitney *U*-test was performed to evaluate differences among the groups using StatView

5.0 software (SAS Institute, Cary, NC). A P-value of <0.05 was considered to be statistically significant.

RESULTS

Expressions of TLR2, TLR4, and RIG-I Were Higher in Myeloid Dendritic Cells From Chronic Hepatitis C Patients

With respect to the phenotypes of fresh myeloid dendritic cells, the expressions of maturation markers such as CD40, CD80, CD83, and CD86 were relatively low and were not different between the HCV group and healthy donor group (Fig. 1). The similar results were obtained from HBV group (data not shown). These results show that myeloid dendritic cells from all groups are equally immature phenotypes.

First, the expressions of TLR2, TLR3, and TLR4 in myeloid dendritic cells were examined. The relative amounts of TLR2 and TLR4 in the HCV group were higher than those in healthy donors or the HBV group (Fig. 2). In contrast, the TLR3 expression was not different among the groups (Fig. 2). In comparison between HBV and healthy donor groups, there was no difference in the expressions of these TLRs in myeloid dendritic cells (Fig. 2).

The expression of cytoplasmic receptors for dsRNA in myeloid dendritic cells was also compared. The RIG-I and LGP2 expression in the HCV or the HBV group was significantly higher than those from healthy donors, whereas MDA-5 did not differ among the groups (Fig. 2). No correlation was found among the expressions of any TLR and dsRNA receptors (data not shown).

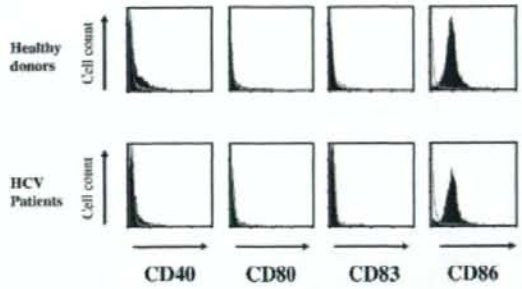

Fig. 1. Fresh myeloid dendritic cells are immature regardless of HCV infection. Myeloid dendritic cells were obtained from HCV-infective patients or healthy donors and their expressions of CD40, CD80, CD83, and CD86 were analyzed by flow cytometry. The shaded histograms are the results with specific Abs, while the open ones are those with isotype Abs. Representative results from five HCV-infected patients and five controls are shown.

IFN-α or PolyI:C Enhanced RIG-I Expression in Myeloid Dendritic Cells

To clarify the factors influencing TLR2, 3, 4, or RIG-I expression in myeloid dendritic cells, it was examined

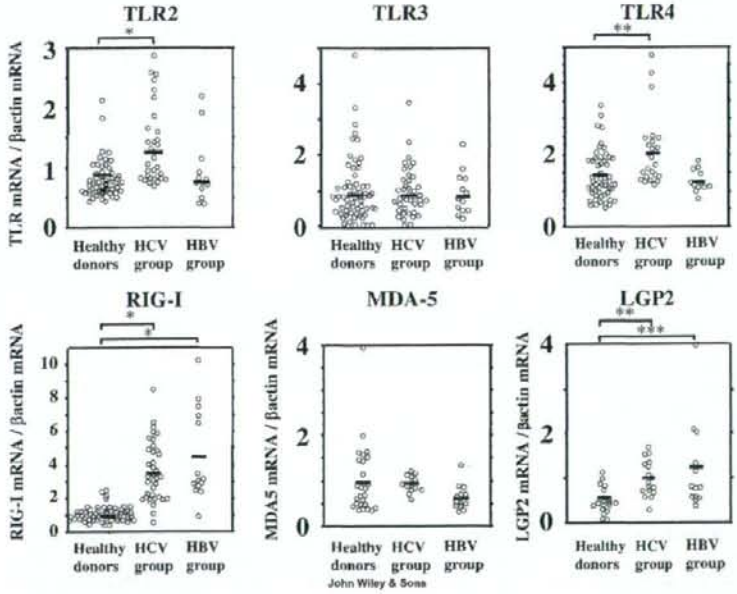

Fig. 2. Expressions of TLR2, TLR4, RIG-I, and LGP2 in patient myeloid dendritic cells from HCV-infected patients are higher than those from healthy donors, while TLR3 and MDA-5 are comparable. Expressions of TLR2, TLR3, TLR4, RIG-I, MDA-5, and LGP2 in myeloid dendritic cells were quantified by real-time RT-PCR as described in Materials and Methods Section. Horizontal bars represent the median. The statistical difference was evaluated by the Mann–Whitney U-test. $^{*}P < 0.0001$, $^{**}P < 0.0005$, $^{***}P < 0.005$.

whether they correlated with clinical parameters, such as age, serum ALT, HCV-RNA, and HBV-DNA titers. No correlation was found between any of these markers and TLR2, TLR3, TLR4, or RIG-I expressions (data not shown). Therefore, the degree of expression of these sensors is not involved in the control of virus replication or liver inflammation. Their expressions in myeloid dendritic cells cultured with and without various reagents were compared. The ratio of the quantity was determined between samples with and without treatments and their positive induction was defined as more than 2.0. The kinetics of agonist-induced TLR2, TLR3, TLR4, or RIG-I expression were preliminarily examined in myeloid dendritic cells recovered from volunteers or patients. It was found that they showed a peak at 2 hr after the stimulation, which were the same either they were HCV-infected or not (data not shown). Thus, in the following experiments, cells were obtained at this point and subsequently analyzed transcripts of target genes.

In the present study, IFN-α significantly enhanced RIG-I expression in myeloid dendritic cells (Fig. 3A). A similar effect of IFN-α was observed in TLR3 and TLR4 expression, although at much lesser degrees than those of RIG-I. In chronic hepatitis C patients, serum levels of IL-6, TNF-α, or IL-10 have been reported to be higher than those in uninfected individuals, suggesting their roles in the pathogenesis of HCV infection [Spanakis et al., 2002]. However, the addition of these cytokines or IL-12 to myeloid dendritic cell did not influence TLR or RIG-I expression (Fig. 3B). As for TLR agonists, polyI:C or LPS significantly enhanced RIG-I expression, but only slightly enhanced TLR4 (Fig. 3B). TLR2 agonist Pam_3CSK_4 did not influence the levels of TLR and RIG-I (Fig. 3B). None of the HCV proteins had a positive impact on TLR2, TLR3, TLR4, and RIG-I expressions (Fig. 3B).

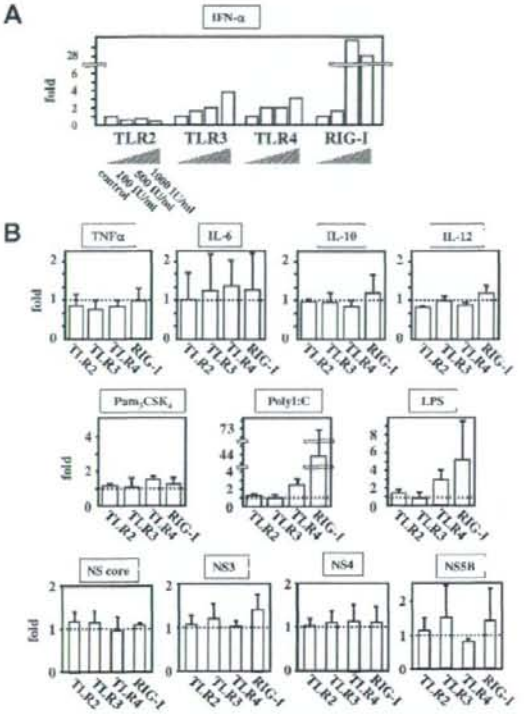

Fig. 3. IFN-α and polyI:C are inducers of TLR3, TLR4, or RIG-I in myeloid dendritic cells. **A**: Various doses of IFN-α were added to myeloid dendritic cells obtained from healthy donors and their mRNA expressions of TLR2, TLR3, TLR4, and RIG-I were quantified by real-time RT-PCR as described in Materials and Methods Section. Bars represent the mean fold increase of relevant transcripts to those of each control. Representative results from three donors are shown. **B**: Changes of TLR2, TLR3, TLR4, and RIG-I expression in myeloid dendritic cells were examined by the addition of various cytokines, TLR agonists or recombinant HCV proteins as described in Materials and Methods Section. The fold increase was determined by the ratio of each transcript of samples with reagents to those without and expressed as the mean – SEM. The concentration of reagents were 10 ng/ml of TNF-α or IL-6, 20 ng/ml of IL-10, 200 pg/ml of IL-12, 100 ng/ml of Pam3CSK4, 25 μg/ml of polyI:C, 100 ng/ml of LPS and 2.5 μg/ml each of HCV core, NS3, NS4, and NS5B. Representative results from five donors are shown.

Induction of IFN-β, TNF-α, and IL-12 p70 With TLR Agonists Is Impaired in Myeloid Dendritic Cells From Chronic Hepatitis C Patients

First, IFN-β and TNF-α expression were examined in myeloid dendritic cells as representatives in response to specific agonists. Since the expression of these genes in myeloid dendritic cell showed a peak at 2 hr after the stimulation either they were from donors or patients (Fig. 4A), samples were collected at this point. In myeloid dendritic cells stimulated with polyI:C, IFN-β was significantly induced in the HCV, the HBV, and healthy donor groups (Fig. 4B). However, their expression from HCV or HBV-infected patients was significantly lower than that from healthy donors (Fig. 4B). Agonists for TLR3 or TLR4 significantly stimulated myeloid dendritic cells to induce TNF-α regardless of HCV or HBV infection. As the same IFN-β, TNF-α induction in myeloid dendritic cells stimulated with polyI:C or LPS was lower in the HCV or the HBV group (Fig. 4B). Therefore, in myeloid dendritic cells from hepatitis C patients, in spite of higher expression of TLR2, TLR4, and RIG-I, their levels of agonist-induced IFN-β and TNF-α were less than those in healthy donors.

To compare more precisely the cytokine response in myeloid dendritic cell between HCV-infected patients and donors, the levels of IFN-α, TNF-α, IL-6, and IL-12 p70 in supernatants were examined. Since the induction of IFN-β and TNF-α in myeloid dendritic cell was profound in the presence of polyI:C, samples were collected from myeloid dendritic cells stimulated with polyI:C. The levels of IFN-α and IL-6 were not different between the groups (Fig. 4C). In contrast, the amounts of TNF-α and IL-12 p70 from patients group were significantly lower than those from the donor group (Fig. 4C). These results suggest that some inhibitory

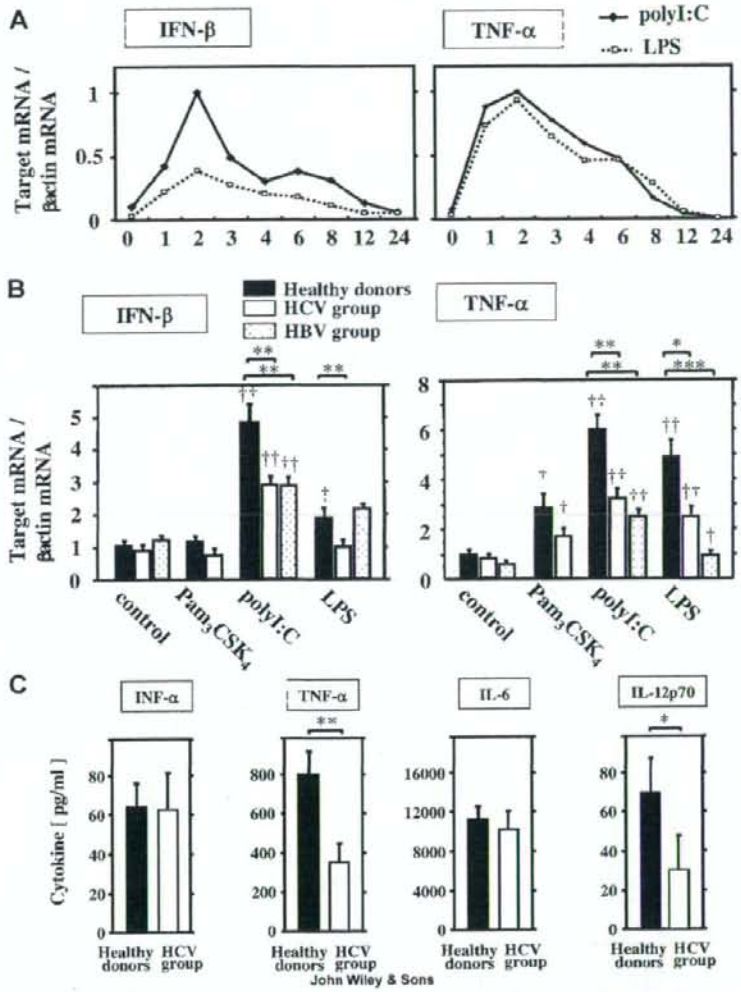

Fig. 4. Innate cytokine response is impaired in patient myeloid dendritic cells from HCV-infected patients. **A**: Kinetics of IFN-β and TNF-α in myeloid dendritic cells stimulated with polyI:C or LPS. The expressions of IFN-β and TNF-α in myeloid dendritic cells from healthy donors were quantified by real-time RT-PCR as described in Materials and Methods Section. At several time points before and after the stimulation of myeloid dendritic cell with 25 μg/ml of poly I:C or 100 ng/ml of LPS, the samples were subjected to RT-PCR analyses. The results are expressed as the ratio of IFN-β or TNF-α transcripts to that of β-actin. Representative results from three healthy donors are shown. **B**: Expressions of IFN-β and TNF-α in myeloid dendritic cells stimulated with various TLR agonists were quantified by real-time RT-PCR as described in Materials and Methods Section. Two hours after the stimulation of myeloid dendritic cells with Pam_3CSK_4, polyI:C or LPS, the samples were subjected to RT-PCR analyses. The results were expressed as the ratio of IFN-β or TNF-α transcripts to that of β-actin. The concentrations of agonists were 100 ng/ml of Pam_3CSK_4, 25 μg/ml of polyI:C and 100 ng/ml of LPS. The bars represent mean + SEM. $^{\dagger}P < 0.05$ vs. control, $^{\dagger\dagger}P < 0.01$ versus control, $^{*}P < 0.05$ versus healthy donors, $^{**}P < 0.01$ versus healthy donors, $^{***}P < 0.001$ versus healthy donors. Representative results from 14 HCV-infected patients, 13 HBV-infected patients and 25 controls are shown. Statistical differences were evaluated by the Mann–Whitney *U*-test. **C**: Myeloid dendritic cells in both groups were stimulated with polyI:C for 24 hr. The supernatants were collected and the levels of IFN-α, TNF-α, IL-6, and IL-12p70 were examined by ELISA or cytokine beads assay as described in Materials and Methods Section. The bars represent mean + SEM. Statistical differences were evaluated by the Mann–Whitney *U*-test. Representative results from 11 HCV-infected patients and 17 controls are shown. $^{*}P < 0.05$, $^{**}P < 0.01$.

mechanisms exist downstream of TLR or RIG-I in myeloid dendritic cells from the HCV-infected patients.

Expressions of TRIF and TRAF6 Were Lower in Myeloid Dendritic Cells From the HCV-Infected Patients

In order to seek the inhibitory mechanisms of TLR or RIG-I signaling in myeloid dendritic cells, the expressions of adapter molecules, MyD88, IPS-1, TRIF, or TRAF6 were compared between the HCV and donor groups. The expressions of MyD88 and IPS-1 were higher in myeloid dendritic cells from the HCV group (Fig. 5). By contrast, the levels of TRIF and TRAF6 in myeloid dendritic cells from HCV-infected patients were significantly lower than in those from healthy counterparts (Fig. 5).

DISCUSSION

The present study demonstrated that myeloid dendritic cells from HCV-infected patients express higher levels of TLR2, TLR4, and RIG-I than those from healthy subjects. Regardless of such enhanced expression, specific agonists stimulated patient myeloid dendritic cells to induce lesser degrees of IFN-β/TNF-α/IL-12 than those from the healthy counterparts. Two conclusions were reached from the current study findings: HCV enhances expression of some TLR and RIG-I in myeloid dendritic cells, but HCV impedes TLR or RIG-I-mediated cytokine responses in them. Since dendritic cells play a role as immune sentinels, such impaired cytokine response in myeloid dendritic cell may be one of the mechanisms in enhanced susceptibility to various pathogens in HCV-infected individuals as reported elsewhere [El-Serag et al., 2003].

It has been reported that TLRs are expressed in epithelial cells and immune cells, and RIG-I is ubiquitously expressed in various cells [Yoneyama et al., 2004]. However, it remains obscure how their expressions are regulated. It is generally accepted that TLR3 and RIG-I are inducible by type-I IFN [Doyle et al., 2003; Yoneyama et al., 2004]. The current study confirmed this phenomenon also in myeloid dendritic cells, since IFN-α up-regulated TLR3, TLR4, and RIG-I expression in a dose-dependent manner. Gene expression analyses revealed that HCV infection induces type-I IFN and IFN-stimulated genes in HCV-infected liver from chimpanzees or humans [Bigger et al., 2004]. One of the triggers leading to IFN production is the presence of double-strand RNA in infected tissues, which is a replicative intermediate of HCV. The current study also showed that polyI:C is a prominent inducer of RIG-I and TLR4. Since polyI:C is a synthetic mimic of double-strand RNA, its positive impact suggests that HCV replication in myeloid dendritic cells and/or subsequent IFN production may be involved in RIG-I or TLR4 induction.

Several investigators have reported that TLR2, TLR3, or TLR4 expression is enhanced in monocytes or B cells obtained from chronic hepatitis C patients, both of which are known to be susceptible to HCV [Machida et al., 2006; Riordan et al., 2006]. Regardless of the difference in cell types, the present study offers support for the enhanced TLR2 and TLR4 expression in HCV infection described by these reports. As for the mechanisms, TNF-α or HCV NS5A has been reported to be involved in TLR2 or TLR4 up-regulation [Machida et al., 2006]. However, in this study, addition of recombinant TNF-α or the HCV proteins failed to induce any TLR or RIG-I in

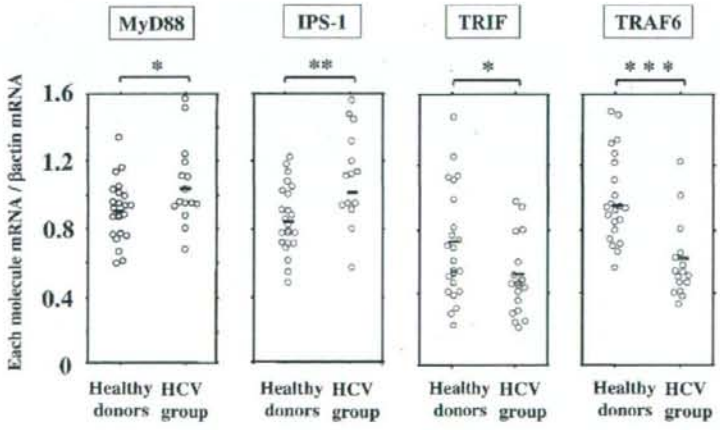

Fig. 5. Expressions of TRIF and TRAF6 are lower but those of MyD88, IPS-1 are higher in patient myeloid dendritic cells than those from healthy counterparts. Expressions of MyD88, IPS-1, TRIF TRAF6 were quantified by real-time RT-PCR as described in Materials and Methods Section. The results were expressed as the ratio of each transcript to those of β-actin. Horizontal bars represent the median. Statistical differences were evaluated by the Mann–Whitney *U*-test. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$.

myeloid dendritic cells. Therefore, enhanced expressions of TLR2, TLR4, and RIG-I in myeloid dendritic cells may be due to, not completely but in some part, the existence of HCV in cells or the exposure to endogenous IFN-α. To check this, it may be necessary to conduct studies with inoculation of HCV particles or transduction of the viral genome in myeloid dendritic cells.

In comparison of the results between the HCV and the HBV groups, the expressions of TLR2 and TLR4 in the HBV group were comparable with those from healthy donor group, suggesting that the induction of TLR2 and TLR4 in myeloid dendritic cells is unique in HCV infection. In contrast, the levels of RIG-I and LGP2 were comparable between the HCV and the HBV groups, both of which were higher than those from healthy donors. These results raise the possibility that, regardless of the difference of hepatitis virus, similar mechanisms may be involved in the induction of RIG-I and LGP2 in myeloid dendritic cells.In cells bearing HCV replicons, it has been reported that HCV NS3/4A inhibits TLR3 or RIG-I-mediated IFN-β induction by the cleavage of relevant adaptor molecules TRIF or IPS-1, respectively [Foy et al., 2005; Li et al., 2005]. In the present study, in myeloid dendritic cells from the HCV group, polyI:C-stimulated IFN-β, TNF-α, and IL-12 p70 induction is impaired. As for the adaptor molecules in TLR-dependent signals, TRIF and TRAF6 expression was lower in HCV-infected patients than those in healthy donors. Since it has been proven that the cleavage of TRIF hampers TLR3-mediated IFN production [Fitzgerald et al., 2003], the current study implies that lower expression of TRIF is involved in the inhibition of TLR3 or TLR4-mediated signals in myeloid dendritic cells. Of particular interest is the possibility that such reduction of TRIF and TRAF6 in myeloid dendritic cells is caused by the cleavage by NS3/4A, as shown in hepatoma cells [Foy et al., 2005; Li et al., 2005]. If this does occur, the inhibitor of NS3/4A serine protease may be able to restore TLR-dependent innate responses in myeloid dendritic cells, in addition to its potent suppressive ability of HCV replication. Machida et al. reported that enhanced expression of TLR4 in HCV-infected B cells is related to the TLR4-dependent up-regulation of IFN-β and IL-6, suggesting that TLR4-dependent signals are not impaired in B cells [Machida et al., 2006]. Further study is necessary to reveal whether HCV does actually influence innate immunity according to differences in blood cell types. In the current study, polyI:C or LPS-stimulated myeloid dendritic cells from HBV-infected patients induced lesser degree of IFN-β or TNF-α, respectively. Several investigators reported that the function of blood dendritic cells in HBV-infected patients were impaired [Tavakoli et al., 2004; van der Molen et al., 2004]. It is yet to be determined whether HBV infects to myeloid dendritic cells or not. The current study raises the possibility that distinct mechanisms are involved in the impairment of TLR or RIG-I pathway according to the difference of virus. Further study depending on expression as well as functional assay of virus recognition system in HBV infection is needed to clarify these important issues.

In contrast with RIG-I and LGP2, MDA-5 expression in myeloid dendritic cells from HCV-infected patients was comparable with that from healthy donors, suggesting that these cytosolic RNA sensors are regulated independently. Recently, it has been reported that RIG-I is expected to be involved in the detection of Flaviviridae, which HCV belong to, but MDA-5 is not [Hornung et al., 2006]. Active involvement of RIG-I in HCV infection has been reported, demonstrating that RIG-I, but not MDA-5, efficiently binds to secondary structured HCV RNA to confer induction of IFN-β [Saito et al., 2007]. In this study, although the polyI:C-stimulated cytokine response in patient myeloid dendritic cells was impeded, IPS-1 expression was higher than that in myeloid dendritic cells from the healthy donor group, suggesting a lesser possibility of IPS-1 as a cleavage target of HCV in myeloid dendritic cells. Alternatively, higher expression of LGP2 may contribute to the inhibitory machinery against RIG-I-mediated responses in myeloid dendritic cells, as reported elsewhere [Saito et al., 2007].

In summary, in myeloid dendritic cells from HCV-infected patients, innate cytokine responses were impaired regardless of the enhanced expressions of TLR2, TLR4, and RIG-I. These findings provide insights into the roles of the TLR/RIG-I system in the pathogenesis of HCV infection and their potentials as therapeutic targets for immune modulation.

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ORIGINAL ARTICLE

Dendritic cell-based vaccines suppress metastatic liver tumor via activation of local innate and acquired immunity

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Abstract
Background Dendritic cell (DC)-based vaccines have been applied clinically in the setting of cancer, but tumor-associated antigens (TAAs) have not yet been enough identified in various cancers. In this study, we investigated whether preventive vaccination with unpulsed DCs or peptide-pulsed DCs could offer anti-tumor effects against MC38 or BL6 liver tumors.
Methods Mice were subcutaneously (s.c.) immunized with unpulsed DCs or the recently defined TAA EphA2 derived peptide-pulsed dendritic cells (Eph-DCs) to treat EphA2-positive MC38 and EphA2-negative BL6 liver tumors. Liver mononuclear cells (LMNCs) from treated mice were subjected to ^{51}Cr release assays against YAC-1 target cells. In some experiments, mice were injected with anti-CD8, anti-CD4 or anti-asialo GM1 antibody to deplete each lymphocyte subsets.
Results Immunization with unpulsed DCs displayed comparable efficacy against both MC38 and BL6 liver tumors when compared with Eph-DCs. Both DC-based vaccines significantly augmented the cytotoxicity of LMNCs against YAC-1 cells. In vivo antibody depletion studies revealed that NK cells, as well as, CD4+ and CD8+ T cells play critical roles in the anti-tumor efficacy associated with either DC-based modality.
Tumor-specific cytotoxic T lymphocyte (CTL) activity was generally higher if mice had received Eph-DCs versus unpulsed DCs. Importantly, the mice that had been protected from MC38 liver tumor by either unpulsed DCs or Eph-DCs became resistant to s.c. MC38 rechallenge, but not to BL6 rechallenge.
Conclusions These results demonstrate that unpulsed DC vaccines might serve as an effective therapy for treating *metastatic liver tumor*, for which TAA has not yet been identified.

Keywords Dendritic cells · Innate immunity · Liver tumor · Cancer immunotherapy

Abbreviations

DC	Dendritic cell
Eph-DCs	EphA2 derived peptide-pulsed dendritic cells
CTLs	Cytotoxic T cell lymphocytes
s.c.	Subcutaneously
SCID	Severe combined immuno-deficiency
BM	Bone marrow
GM-CSF	Granulocyte/macrophage-colony stimulating factor
PBS	Phosphate-buffered saline
LMNC	Liver mononuclear cell
TAA	Tumor-associated antigen

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Introduction

Dendritic cell (DC)-based vaccines are attractive cancer modalities since DCs are competent to coordinately induce both tumor antigen-specific cytotoxic T lymphocytes (CTLs) and helper T cells [1–3]. In this regard, DCs pulsed with TAA derived peptides have proven clinically effective in eliciting protective and therapeutic anti-tumor immunity in the setting of a broad range of cancer types [3]. Recent studies have also suggested that DCs may effectively activate elements of innate immunity (NK cells [4–9] and NKT cells [10–12]) via IL-12 secretion and direct cellular interaction. The liver is an enriched source of innate immune cells such as NK cells and NKT cells compared with other organs, supporting the specialized role of this organ in the immune system [13–15]. Indeed, it has been shown that liver-associated innate immune cells play a critical role in the first-line defense against metastatic liver tumors [16, 17]. However, despite numerous reports supporting the efficacy of DC-based vaccines in murine s.c. tumor models [18–20], the efficacy of this approach in liver tumor models remains under developed. Given the possibility that DC-based vaccines may efficiently activate NK cells, NKT cells and specific T cells in the liver, they could offer a preferred immunotherapy for liver cancer.

The liver is the most common site of distal metastasis for tumors developing in distal organs, and physiologic status of this organ correlates with survival in patients with advanced disease, even if primary tumor site are resected curatively [21, 22]. Recently, adjuvant chemotherapies have been reported to yield significant improvement in disease (including liver metastasis) free interval and overall patient survival, however, dose-limiting toxicities were often observed and liver metastasis could not be completely prevented [23–25]. In contrast, several peptide-pulsed DC vaccines have been shown to be clinically capable of stimulating tumor-specific T cells in patients with tumor liver metastasis [26–28], suggesting that such treatments may represent a new strategy option in the setting of metastatic liver cancer. In this context, several tumor-associated antigen (TAA) peptides have been identified in various types of cancer that often metastasize to liver [29–31], however, this approach remains encumbered by the necessity to restrict patient accrual to those individuals harboring specific HLA types.

We previously demonstrated that the recently defined TAA EphA2 derived peptide-pulsed DC (Eph-DC) vaccine prevented the subcutaneous tumor growth in mice, but unpulsed DC vaccine did not [32]. In the present study, we examined the anti-tumor protection of Eph-DC vaccines in the liver tumor model, which is under the unique immunological environment. Unexpectedly, we observed that preventive vaccination with not only Eph-DCs but also unpulsed DCs provide anti-tumor protection as a result of the activation of both innate immune cells and specific T cells. This suggests that cultured autologous DC (alone or pulsed with TAA peptides) may represent an effective modality for patients with tumors localized to their livers.

Materials and methods

Mice

Female C57BL/6 mice and severe combined immuno-deficiency (SCID) mice were purchased from Clea Japan, Inc. (Tokyo, Japan) and were used at 6–8 weeks of age. They were housed under conditions of controlled temperature and light with free access to food and water at the Institute of Experimental Animal Science, Osaka University Graduate School of Medicine. All animals received humane care and our study protocol complied with the institution's guideline.

Cell lines and culture

MC38, a mouse colon carcinoma cell derived from C57BL6/J mice, was generously provided by Dr. Kazumasa Hiroishi (Showa University School of Medicine, Tokyo, Japan). BL6, a melanoma cell line, and YAC-1, a sensitive cell line to NK cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). These cell lines were maintained in Complete Medium [RPMI medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin] at 37°C in 5% CO_2.

Generation of dendritic cells in vitro from bone marrow (BM) and DC-based peptide vaccines

The procedure used in this study was described previously [32]. Briefly, BM-DCs were separated by magnetic cell sorting using CD11c Micro Beads (Miltenyi Biotec) and typically represented >90% of the harvested population of the cells based on morphology and expression of the CD40, CD80, CD86 and MHC class II (data not shown). The H-2 K^b-binding $mEphA2_{682-689}$ epitope (VVSKYKPM) was kindly provided by Dr. Walter Storkus (University of Pittsburgh Cancer Institute). BM-DCs were incubated with the $mEph_{682-689}$ peptide at a concentration of 10 μg/ml/10^6 DC/ml CM for 2 h at 37°C. The cells were harvested and washed three times with phosphate-buffered saline (PBS) before use [20].

Animal experiments

C57BL/6 mice or SCID mice were immunized s.c. in the flank with 1×10^6 Eph-DCs or unpulsed DCs in a total

volume of 100 µl of PBS twice a week. On day 0, at the time of the second injection with Eph-DCs or unpulsed DCs, 2×10^6 MC38 cells (EphA2-positive) or 5×10^5 BL6 (EphA2-negative) tumor cells were inoculated intrahepatically. Mice were sacrificed 14 days after tumor inoculation and liver weight was measured. Data are reported as the average liver weight ± SD.

Cytolytic assays

Liver mononuclear cells (LMNCs) were isolated from the liver 1 day after tumor inoculation, and subjected to 4-h ^{51}Cr release assays against NK-sensitive YAC-1 target cells. In some experiments, whole splenocytes were harvested 14 days after tumor inoculation, with T cells stimulated in vitro using MC38 cells pre-treated with Mitomycin C (Kyowa Hakko, Tokyo, Japan) in the presence of 30 IU/ml murine IL-2 (Strathmann Biotech, Hannover, Germany) for 5 days. Lymphocytes (bulk, CD4-depleted, or CD8-depleted) were then harvested and analyzed for their ability to kill MC38 tumor cells in 4-h ^{51}Cr-release assays.

Flow cytometric analysis

Liver mononuclear cells were isolated from the liver prior to the first vaccination and on days 1, 3, 7 after tumor inoculation as previously described [16]. The phenotype of LMNCs from mice treated with Eph-DCs, unpulsed DCs or PBS was examined by flow cytometric analysis. NK and NKT cells in the liver were identified separately by using PE anti-mouse NK1.1 monoclonal antibody and FITC anti-mouse TCR (BD Pharmingen, San Diego, CA, USA). Furthermore, NK activation was examined by using FITC anti-mouse CD69, the early activation marker (BD Pharmingen). Analysis was performed using a FACScan (Becton Dickinson, Mountain View, CA) with the resulting data analyzed using the CELLQuest program (Becton Dickinson). NK cells were identified as NK1.1+/TCR-lymphocytes and NKT cells as NK1.1+/TCR+ lymphocytes. Activated NK1.1+ cells were identified as NK1.1+/CD69+ lymphocytes.

In vivo depletion experiments

The procedure used in this study was described previously [32]. The efficiency of specific subset depletions (CD4+, CD8+ T cell or NK cell) was comfirmed by flow cytometric analysis. In all cases, 99% of the targeted cell subset was specifically depleted (data not shown).

Tumor rechallenge

C57BL/6 mice were immunized s.c. with 1×10^6 Eph-DCs or unpulsed DCs twice a week and then challenged intrahepatically with 2×10^6 MC38 cells, at the time of the second Eph-DC or unpulsed DC immunization. On day 14 after tumor inoculation, 2×10^5 MC38 or 5×10^4 BL6 cells were injected s.c. in the flank. As a control, 2×10^5 MC38 or 5×10^4 BL6 cells were injected s.c. into naïve C57BL/6 mice. Tumor size was assessed on a weekly basis and recorded in mm^2 by determining the product of the largest perpendicular diameters measured by vernier calipers. Data are reported as the average tumor area ± SD.

Statistical analyses. Statistical differences between the groups was determined by applying a Student's t test with Welch correction or one-way ANOVA after each group had been tested with equal variance and Fisher's exact probability test. Statistical significance was defined as $P < 0.05$.

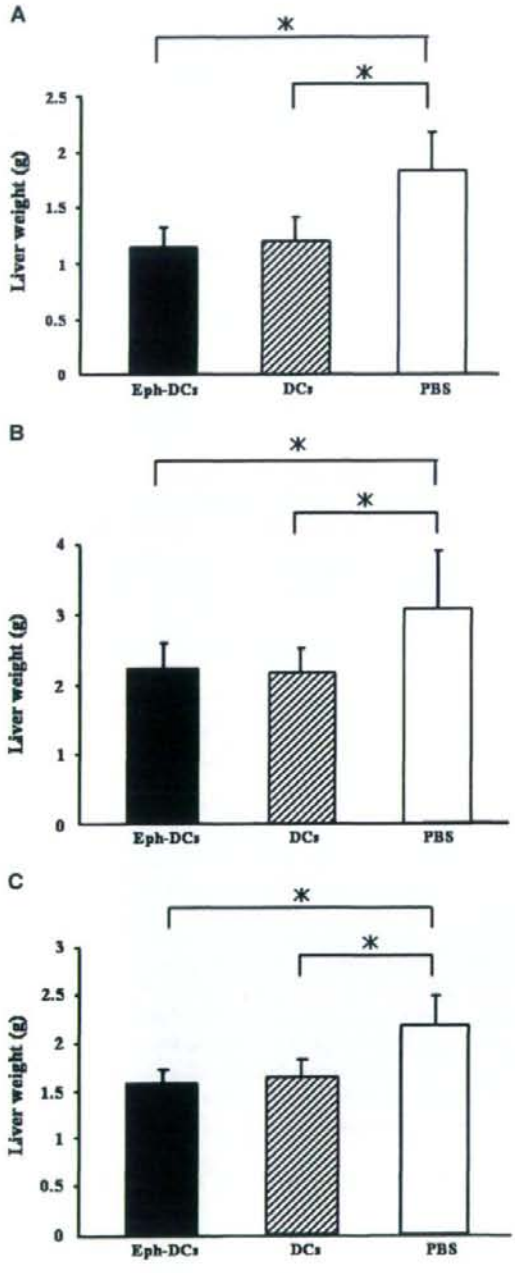
Results

Immunization with Eph-DCs or unpulsed DCs prevents progression of liver tumors in vivo

We examined whether immunization with Eph-DCs or unpulsed DCs would promote protective anti-tumor effects against the EphA2-positive MC38 or EphA2-negative BL6 liver tumors. MC38 liver tumor growth in mice immunized with either Eph-DCs or unpulsed DCs was significantly inhibited when compared to mice treated with PBS. Immunization with unpulsed DCs provided an equitable degree of anti-MC38 protection to that observed for immunization using Eph-DCs (Fig. 1a). BL6 tumor growth was also significantly inhibited by Eph-DCs or unpulsed DCs, to a comparable degree (Fig. 1b). These results suggest that immunization with DCs (whether pulsed with peptide or not) successfully inhibits the growth of two distinct $H\text{-}2^b$ tumors established in the liver. Moreover, MC38 liver tumor growth in SCID mice (T cell, B cell and NKT cell deficient mice) immunized with DCs (either Eph-DCs or unpulsed DCs) was also significantly inhibited when compared to PBS treated mice, with no significant difference between the Eph-DC and the unpulsed DC groups (Fig. 1c). These results suggest that hepatic NK cells play an important role in regulating tumor growth in the liver after being activated by DC-based vaccination.

Liver NK cells are activated by DC vaccination

We examined whether LMNCs isolated from the liver 1 day after tumor inoculation displayed increased cytolytic activity against YAC-1 target cells in vitro. LMNCs harvested from mice treated with DCs (±peptide) were better killers of YAC1 cells than control LMNCs from PBS-treated or naïve mice (Fig. 2a). In contrast, splenocytes harvested from these same animals displayed only weak



◀ **Fig. 1** Anti-tumor effects with DC-based vaccination against liver and lung tumor. C57BL/6 mice were immunized on day-7 and 0 with 1×10^6 Eph-DCs, unpulsed DCs or PBS. On day 0, 2×10^6 MC38 cells (**a**) or 5×10^5 BL6 cells (**b**) were injected intrahepatically. Mice were sacrificed and liver weight was then determined 14 days after tumor inoculation, $^*P < 0.05$; $N = 10$/group. *Each data point* represents the mean liver weight ± SD. **c** SCID mice that lack T cells, B cells and NKT cells were immunized with 1×10^6 Eph-DCs, unpulsed DCs or PBS on day-7 and 0. On day 0, 2×10^6 MC38 cells were injected intrahepatically. Mice were sacrificed and liver weight was then determined 14 days after tumor inoculation, $^*P < 0.05$, $N = 8$/group. *Each data point* represents the mean liver weight ± SD

anti-YAC1 killing capacity (Fig. 2b). These results suggest the preferential activation of liver versus splenic NK effector cells by DC-based vaccination. We next examined the activation status (expression of CD69) of NK1.1+ cells by flow cytometry after DC-based vaccination. The frequency of hepatic NK1.1+ cells in mice immunized with Eph-DCs or unpulsed DCs were equal to those noted for mice treated with PBS alone (data not shown). The CD69 expression level on NK1.1+ cells in mice treated with either form of DC-based vaccine was significantly stronger than that of mice treated with PBS on day 1 after tumor inoculation, with this level of expression decreasing gradually on days 3, 7 after tumor inoculation (Fig. 2c). These results suggested that hepatic NK1.1+ cells were efficiently activated by DC vaccination versus PBS treatment. NK cells isolated from mice treated with Eph-DCs or unpulsed DCs displayed stronger cytolytic activity against MC38 targets when compared with PBS treatment, and there is no difference in anti-tumor killing by liver NK cells between Eph-DC group and unpulsed DC group (Fig. 2d).

Depletion of CD4+ T cells, CD8+ T cells or NK cells impairs the anti-tumor effects of immunization with DCs

To prove whether the therapeutic benefit associated with DC vaccination in the MC38 liver tumor model was dependent on CD4+, CD8+ T cells or NK cells in vivo, we performed selective cell subset depletion studies. The anti-tumor efficacy of DC-based immunization was significantly reduced in CD4+, CD8+ T cell or NK cell-depleted mice (Fig. 3). Notably, the liver weights of NK cell-depleted mice were significantly heavier than those of CD8+ T cell-depleted mice if the animals received unpulsed DC injections (Fig. 3a), while this was not observed for mice injected with Eph-DCs (Fig. 3b). These results suggest that not only NK cells, but also CD4+ T cells and CD8+ T cells are required for optimal anti-tumor effects associated with either DC vaccines, but that NK cells may play a greater role than CD8+ T cells in regulating tumor growth in mice receiving unpulsed DCs.

Induction of specific CTLs against MC38 cells after immunization with DCs

We next examined whether either Eph-DC or unpulsed DC immunization induced specific splenocyte (harvested 14 days after tumor inoculation) cytolytic activity against

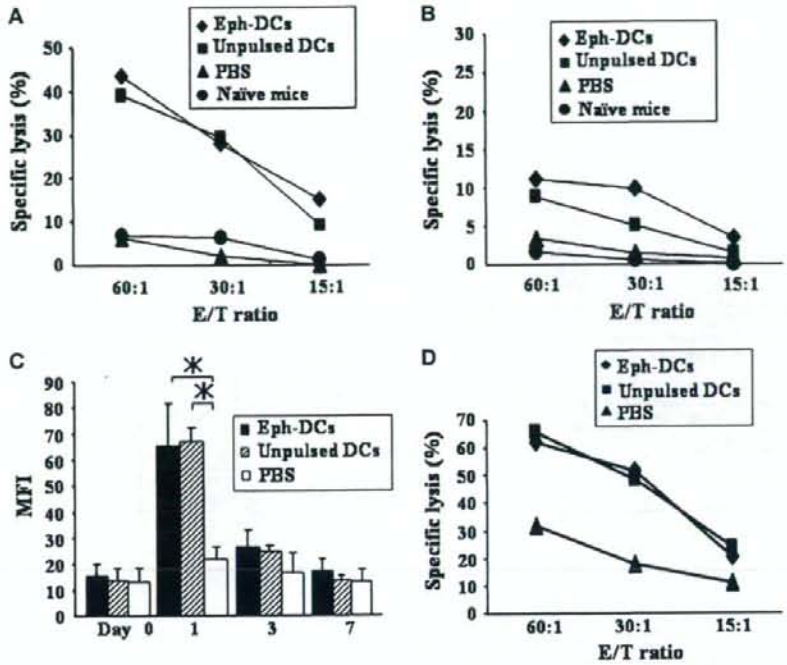


Fig. 2 Liver NK cells are activated by DC-based vaccination. LMNCs (a) or splenocytes (b) were isolated from the various treatment groups (Eph-DCs, unpulsed DCs or PBS) of mice or naïve mice 1 day after tumor inoculation, and subjected to 4-h ^{51}Cr release assays against YAC-1 targets at the indicated E:T ratios. Similar results were obtained in three experiments. c LMNCs were harvested before the first vaccination and on days 1, 3, 7 after tumor inoculation, and the phenotype of LMNCs from mice treated with Eph-DCs, unpulsed DCs or PBS was examined by flow cytometric analysis. Activated NK1.1 + cells were identified as NK1.1 + /CD69 + lymphocytes. *MFI* mean fluorescence intensity, $N = 3$/group. d LMNCs were isolated from the various treatment groups of mice 1 day after tumor inoculation, and liver NK cells were isolated from LMNCs by magnetic cell sorting using DX-5 MicroBeads (Miltenyi Biotec) and then subjected to 4-h ^{51}Cr release assays against MC38 target cells

MC38 or BL6 cells. Splenocytes isolated from mice treated with unpulsed DCs displayed stronger cytolytic activity against MC38 targets when compared with PBS treatment. Furthermore, splenocytes harvested from mice treated with Eph-DCs displayed stronger anti-MC38 cytolytic activity than unpulsed DC or PBS group (Fig. 4a), with this activity mediated by CD8+ T cells, but not CD4+ T cells (Fig. 4b). Cytolytic activity was not observed against EphA2-negative cells (i.e. BL6; Fig. 4c). These results suggest that either format of DC immunization induces MC38-specific CTLs in vivo, with somewhat greater levels of response observed in the case of peptide-specific vaccination.

Immunity to tumor rechallenge

We next determined whether DC vaccines that slow liver tumor progression protected mice against a consequent s.c. rechallenge with that same tumor. C57BL/6 mice were s.c. immunized with Eph-DCs or unpulsed DCs and MC38 liver tumors implanted. On day 14 post-intrahepaic tumor inoculation, 2×10^5 MC38 or 5×10^4 BL6 cells were injected s.c. into the flank of these mice and tumor growth monitored. We observed that s.c. MC38 (EphA2-positive) was inhibited by prior vaccination with Eph-DCs > unpulsed DCs versus naïve mice (Fig. 5a), and that the growth of BL6 (EphA2-negative) was not inhibited in any cohort analyzed versus control (Fig. 5b). These results suggested that immunization with either format of DCs elicited some degree of systemic anti-tumor effects against the EphA2-positive tumor, but that the specific immunization (Eph-DCs) was superior to unpulsed DCs in generating protective effects against EphA2-positive tumor outside the liver.

Discussion

DCs pulsed with TAA derived peptides (Peptide-DCs) have proven effective in eliciting protective and therapeutic anti-tumor immunity in patients against a diverse range of

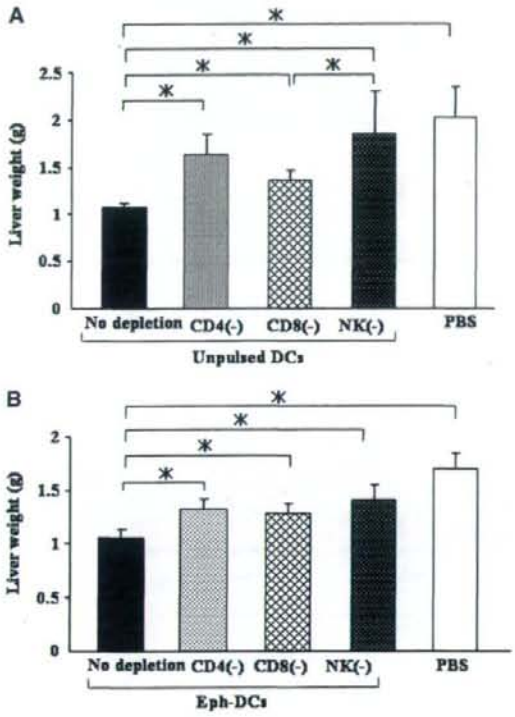


Fig. 3 DC-based vaccine efficacy is dependent upon T cells and NK cells. Ab-mediated in vivo depletion of CD4+, CD8+ T cells and NK cells were performed (as described in "Materials and methods"), with the depleted mice then receiving unpulsed DC (**a**) or Eph-DC vaccines (**b**) (on day-7 and 0) and intrahepatic 2×10^6 MC38 cell injection (day 0), $^*P < 0.05$, $N = 8$/group. *Each data point* represents the mean liver weight ± SD

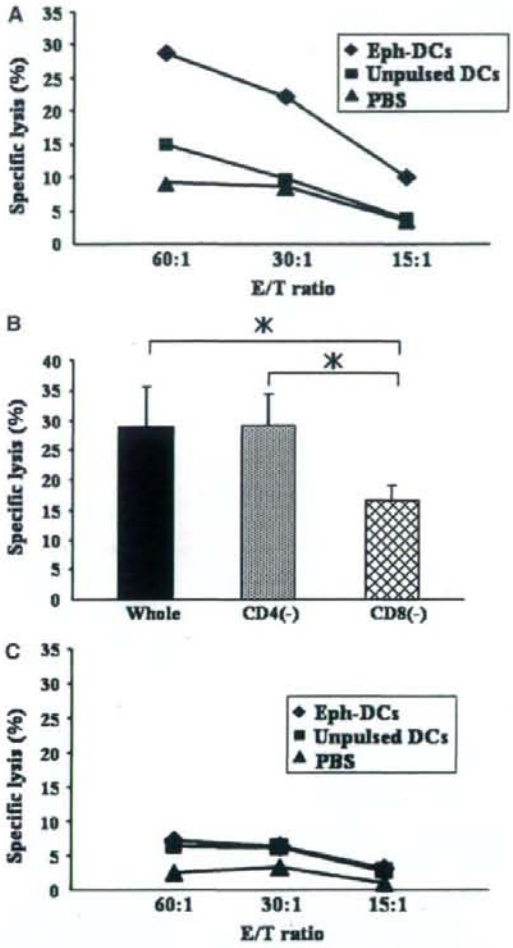


Fig. 4 DC-based vaccines induce anti-tumor T cells. Splenocytes were harvested from tumor-bearing mice 14 days after final treatment with Eph-DCs, unpulsed DCs or PBS. Splenocytes were stimulated in vitro with MMC-treated MC38 cells in the presence of low-dose recombinant human IL-2. After 5 days of culture, the cytolytic activity of the expanded T cells was evaluated using 4-h ^{51}Cr release assays against MC38 (**a**) or irrelevant BL6 (**c**) tumor target cells at the indicated E:T ratios. **b** Before performance of 4-h ^{51}Cr release cytolytic assays, CD4+ or CD8+ T cells were depleted from whole splenocytes of Eph-DC treated mice using specific MicroBeads. Similar results were obtained in three independent experiments

cancers [3]. However, at present, peptide-DC vaccines have not been comprehensively evaluated in clinical trials for treatment of metastatic liver cancers [25–27]. The liver uniquely contains an abundance of not only T cells, but also NK cells and NKT cells when compared with other organs [13–15]. Recently, DCs have been implicated as playing an important role in the activation of NK and NKT cells in both mice and humans [4–12, 33, 34], suggesting that DC-based therapies would be poised to activate an array of innate immune effector cells in the liver and might mediate clinical benefit within that organ. In this study, we demonstrated that administration of DCs prior to tumor implantation successfully promoted protective anti-tumor immunity against two distinct tumors in the liver (with little requirement for antigen-loading of DCs). Moreover, either Eph-DCs or unpulsed DCs have also proven effective in eliciting equally protective anti-tumor immunity against BL6 metastasis models in the lung that contains relatively high frequencies of innate immune effector cells (unpublished data).

In vitro cytotoxicity assays performed against the YAC-1 target cell line revealed that hepatic (and to a lesser extent splenic) NK cells were activated and mediated stronger killing function as a result of mice being treated with DCs (unpulsed or EphA2 peptide-pulsed) vaccination versus PBS controls. Consistent with this functional finding, hepatic NK 1.1+ cells (probably NK and NKT cells) in DC