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).

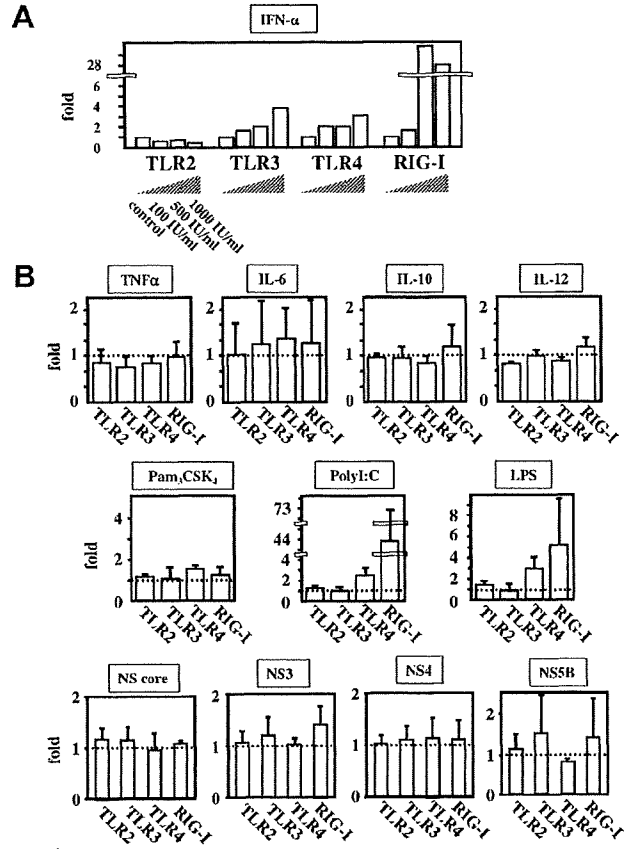

Fig. 3. IFN-α and polyI:C are inducers of TLR3, TLR4, or RIG-I in myeloid dendritic cells. **A**: Various doses. of IFN-α were added to myeloid dendritic cells obtained from healthy donors and their mRNA expressions of TLR2, TLR3, TLR4, and RIG-I were quantified by real-time RT-PCR as described in Materials 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

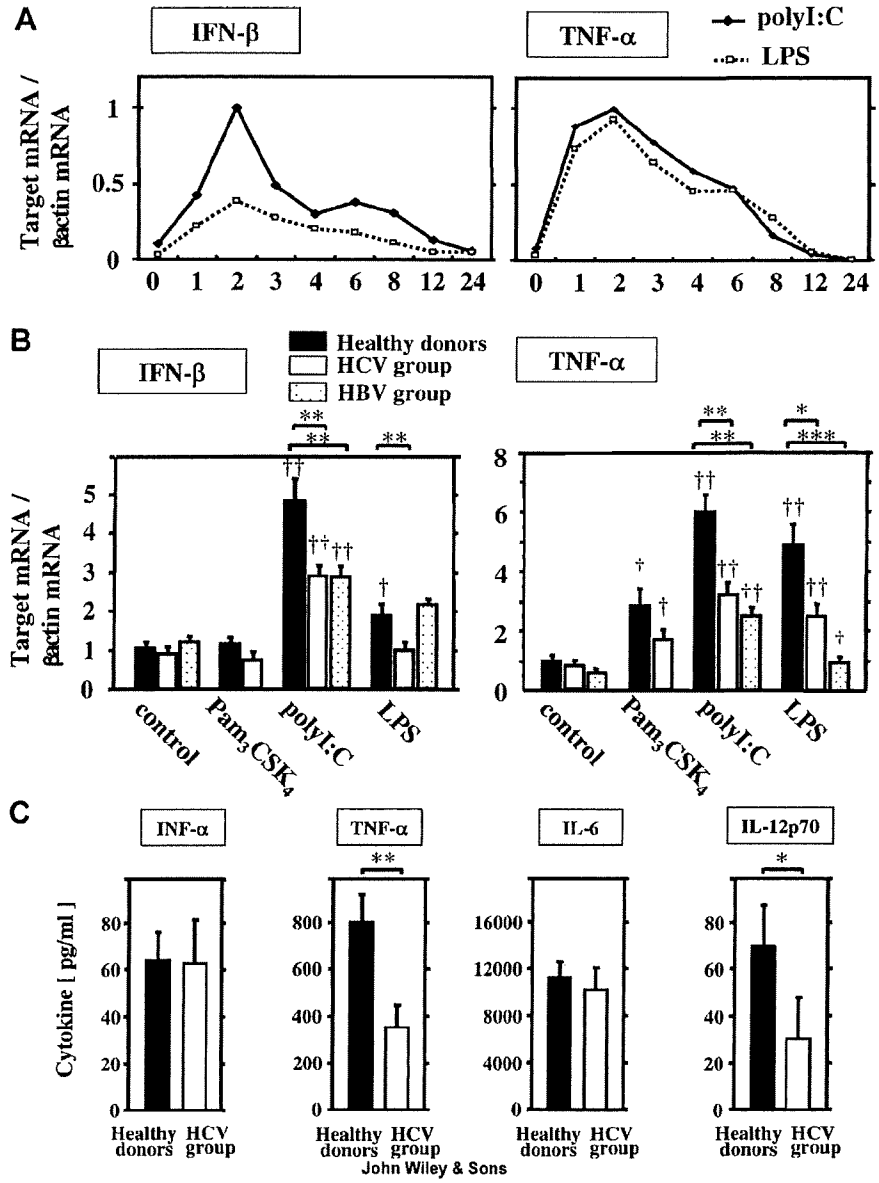

Fig. 4. Innate cytokine response is impaired in patient myeloid dendritic cells from HCV-infected patients. **A**: Kinetics of IFN-β and TNF-α in myeloid dendritic cells stimulated with polyI:C or LPS. The expressions of IFN-β and TNF-α in myeloid dendritic cells from healthy donors were quantified by 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

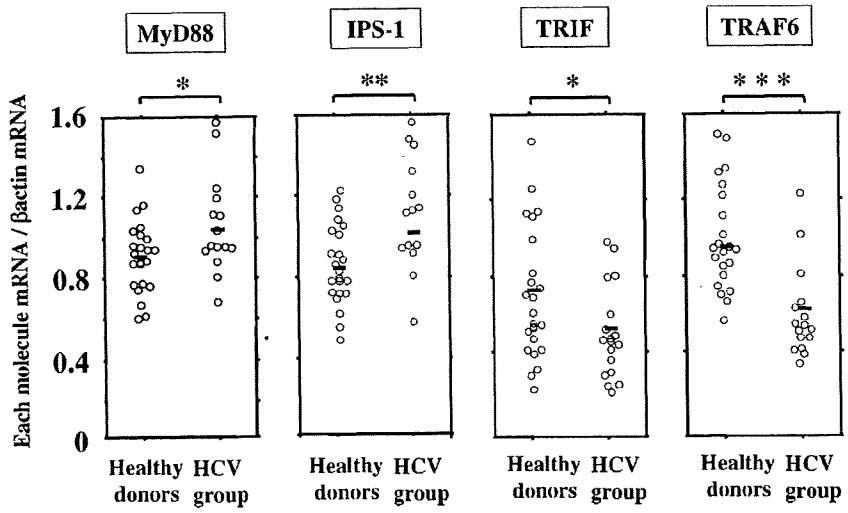

Fig. 5. Expressions of TRIF and TRAF6 are lower but those of MyD88, IPS-1 are higher in patient myeloid dendritic cells than those from healthy counterparts. Expressions of MyD88, IPS-1, TRIF TRAF6 were quantified by real-time RT-PCR as described in Materials and Methods Section. The results were expressed 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.

REFERENCES

Bigger CB, Guerra B, Brasky KM, Hubbard G, Beard MR, Luxon BA, Lemon SM, Lanford RE. 2004. Intrahepatic gene expression during chronic hepatitis C virus infection in chimpanzees. J Virol 78: 13779–13792.

Chang KM, Thimme R, Melpolder JJ, Oldach D, Pemberton J, Moorhead-Loudis J, McHutchison JG, Alter HJ, Chisari FV 2001. Differential CD4(+) and CD8(+) T-cell responsiveness in hepatitis C virus infection. Hepatology 33:267–276.

Doyle SE, O'Connell R, Vaidya SA, Chow EK, Yee K, Cheng G. 2003. Toll-like receptor 3 mediates a more potent antiviral response than Toll-like receptor 4. J Immunol 170:3565–3571.

El-Serag HB, Anand B, Richardson P, Rabeneck L. 2003. Association between hepatitis C infection and other infectious diseases: A case for targeted screening? Am J Gastroenterol 98:167–174.

Fitzgerald KA, McWhirter SM, Faia KL, Rowe DC, Latz E, Golenbock DT, Coyle AJ, Liao SM, Maniatis T. 2003. IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. Nat Immunol 4:491–496.

Foy E, Li K, Sumpter R Jr, Loo YM, Johnson CL, Wang C, Fish PM, Yoneyama M, Fujita T, Lemon SM, Gale M Jr. 2005. Control of antiviral defenses through hepatitis C virus disruption of retinoic acid-inducible gene-I signaling. Proc Natl Acad Sci USA 102:2986–2991.

Hornung V, Ellegast J, Kim S, Brzozka K, Jung A, Kato H, Poeck H, Akira S, Conzelmann KK, Schlee M, Endres S, Hartmann G. 2006. 5'-Triphosphate RNA is the ligand for RIG-I. Science 314:994–997.

Iwasaki A, Medzhitov R. 2004. Toll-like receptor control of the adaptive immune responses. Nat Immunol 5:987–995.

Kaimori A, Kanto T, Kwang Limn C, Komoda Y, Oki C, Inoue M, Miyatake H, Itose I, Sakakibara M, Yakushijin T, Takehara T, Matsuura Y, Hayashi N. 2004. Pseudotype hepatitis C virus enters immature myeloid dendritic cells through the interaction with lectin. Virology 324:74–83.

Kanto T, Inoue M, Miyatake H, Sato A, Sakakibara M, Yakushijin T, Oki C, Itose I, Hiramatsu N, Takehara T, Kasahara A, Hayashi N.

2004. Reduced numbers and impaired ability of myeloid and plasmacytoid dendritic cells to polarize T helper cells in chronic hepatitis C virus infection. J Infect Dis 190:1919–1926.

Lauer GM, Walker BD. 2001. Hepatitis C virus infection. N Engl J Med 345:41–52.

Li K, Foy E, Ferreon JC, Nakamura M, Ferreon AC, Ikeda M, Ray SC, Gale M Jr, Lemon SM. 2005. Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. Proc Natl Acad Sci USA 102:2992–2997.

Machida K, Cheng KT, Sung VM, Levine AM, Foung S, Lai MM. 2006. Hepatitis C virus induces toll-like receptor 4 expression, leading to enhanced production of beta interferon and interleukin-6. J Virol 80:866–874.

Noborg U, Gusdal A, Pisa EK, Hedrum A, Lindh M. 1999. Automated quantitative analysis of hepatitis B virus DNA by using the Cobas Amplicor HBV monitor test. J Clin Microbiol 37:2793–2797.

Pawlotsky JM, Bouvier-Alias M, Hezode C, Darthuy F, Remire J, Dhumeaux D. 2000. Standardization of hepatitis C virus RNA quantification. Hepatology 32:654–659.

Riordan SM, Skinner NA, Kurtovic J, Locarnini S, McIver CJ, Williams R, Visvanathan K. 2006. Toll-like receptor expression in chronic hepatitis C: Correlation with pro-inflammatory cytokine levels and liver injury. Inflamm Res 55:279–285.

Rodrigue-Gervais IG, Jouan L, Beaule G, Sauve D, Bruneau J, Willems B, Sekaly RP, Lamarre D. 2007. Poly(I:C) and lipopolysaccharide innate sensing functions of circulating human myeloid dendritic cells are affected in vivo in hepatitis C virus-infected patients. J Virol 81:5537–5546.

Saito T, Hirai R, Loo YM, Owen D, Johnson CL, Sinha SC, Akira S, Fujita T, Gale M Jr. 2007. Regulation of innate antiviral defenses through a shared repressor domain in RIG-I and L GP2. Proc Natl Acad Sci USA 104:582–587.

Spanakis NE, Garinis GA, Alexopoulos EC, Patrinos GP, Menounos PG, Sklavounou A, Manolis EN, Gorgoulis VG, Valis D. 2002. Cytokine serum levels in patients with chronic HCV infection. J Clin Lab Anal 16:40–46.

Sumpter R Jr, Loo YM, Foy E, Li K, Yoneyama M, Fujita T, Lemon SM, Gale M Jr. 2005. Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. J Virol 79:2689–2699.

Szabo G, Dolganiuc A. 2005. Subversion of plasmacytoid and myeloid dendritic cell functions in chronic HCV infection. Immunobiology 210:237–247.

Tavakoli S, Schwerin W, Rohwer A, Hoffmann S, Weyer S, Weth R, Meisel H, Diepolder H, Geissler M, Galle PR, Lohr HF, Bocher WO. 2004. Phenotype and function of monocyte derived dendritic cells in chronic hepatitis B virus infection. J Gen Virol 85:2829–2836.

van der Molen RG, Sprengers D, Binda RS, de Jong EC, Niesters HG, Kusters JG, Kwekkeboom J, Janssen HL. 2004. Functional impairment of myeloid and plasmacytoid dendritic cells of patients with chronic hepatitis B. Hepatology 40:738–746.

Wedemeyer H, He XS, Nascimbeni M, Davis AR, Greenberg HB, Hoofnagle JH, Liang TJ, Alter H, Rehermann B. 2002. Impaired effector function of hepatitis C virus-specific CD8+ T cells in chronic hepatitis C virus infection. J Immunol 169:3447–3458.

Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M, Taira K, Akira S, Fujita T. 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat Immunol 5:730–737.

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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

Shinjiro Yamaguchi and Tomohide Tatsumi contributed equally to this work.

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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 $P < 0.05$.

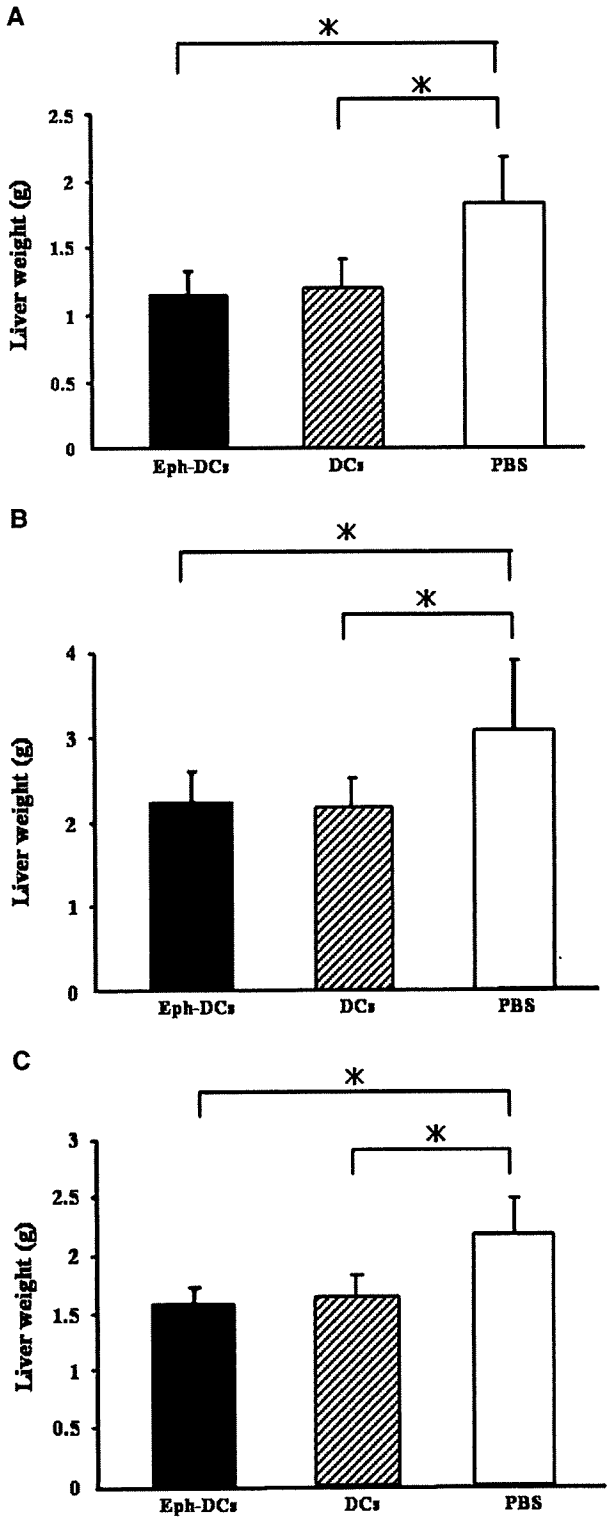
Results

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-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

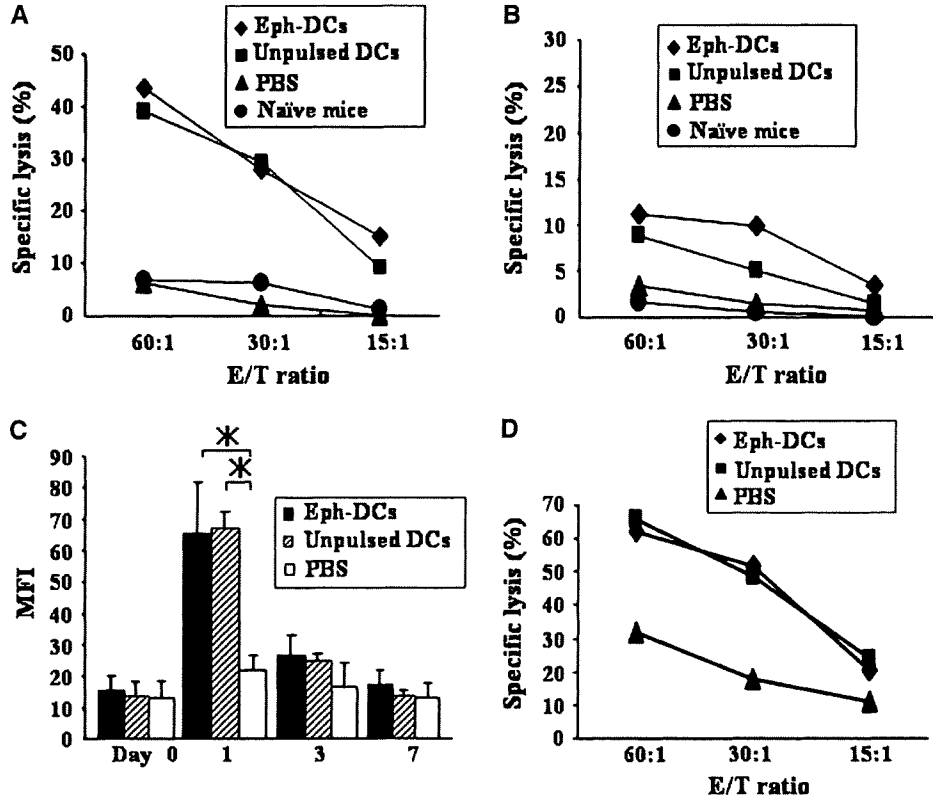


Fig. 2 Liver NK cells are activated by DC-based vaccination. LMNCs (**a**) or splenocytes (**b**) were isolated from the various treatment groups (Eph-DCs, unpulsed DCs or PBS) of mice or naïve mice 1 day after tumor inoculation, and subjected to 4-h ^{51}Cr release assays against YAC-1 targets at the 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

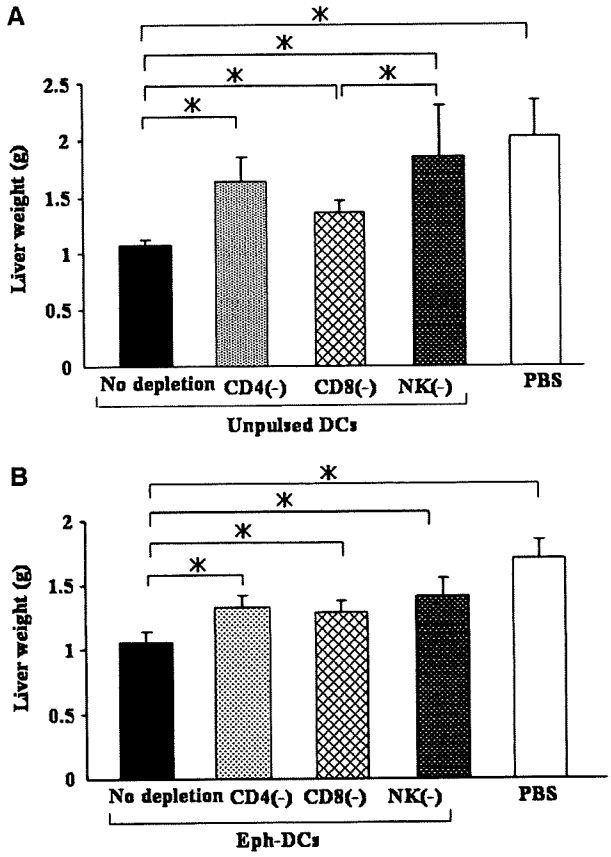


Fig. 3 DC-based vaccine efficacy is dependent upon T cells and NK cells. Ab-mediated in vivo depletion of CD4+, CD8+ T cells and NK cells were performed (as described in "Materials and methods"), with the depleted mice then receiving unpulsed DC (**a**) or Eph-DC vaccines (**b**) (on day-7 and 0) and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

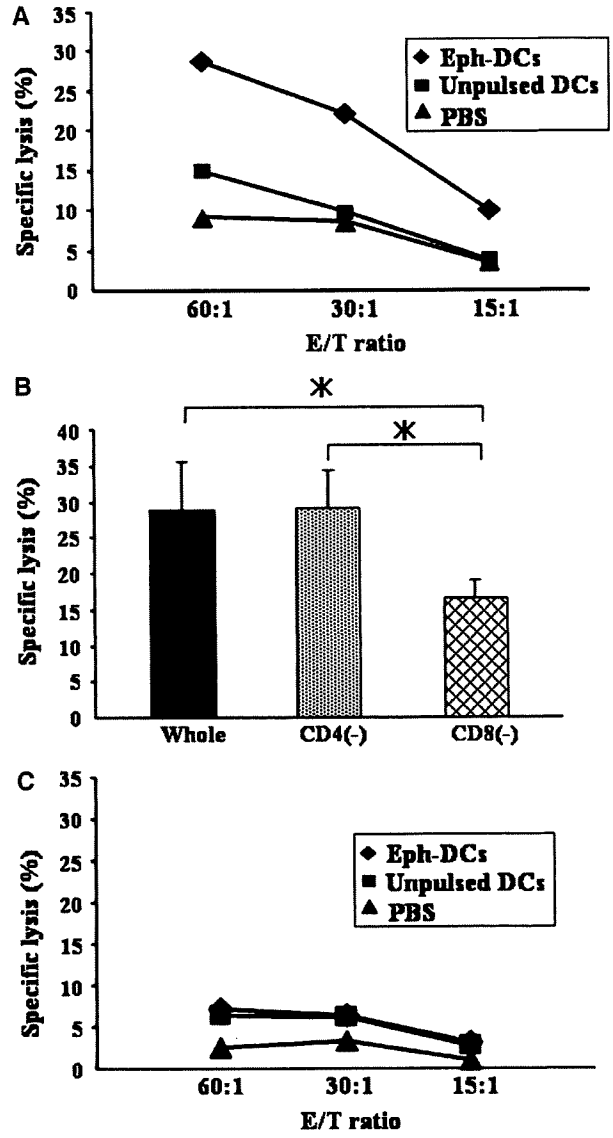


Fig. 4 DC-based vaccines induce anti-tumor T cells. Splenocytes were harvested from tumor-bearing mice 14 days after final treatment with Eph-DCs, unpulsed DCs or PBS. Splenocytes were stimulated in vitro with MMC-treated MC38 cells in the presence of low-dose recombinant human IL-2. After 5 days of 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

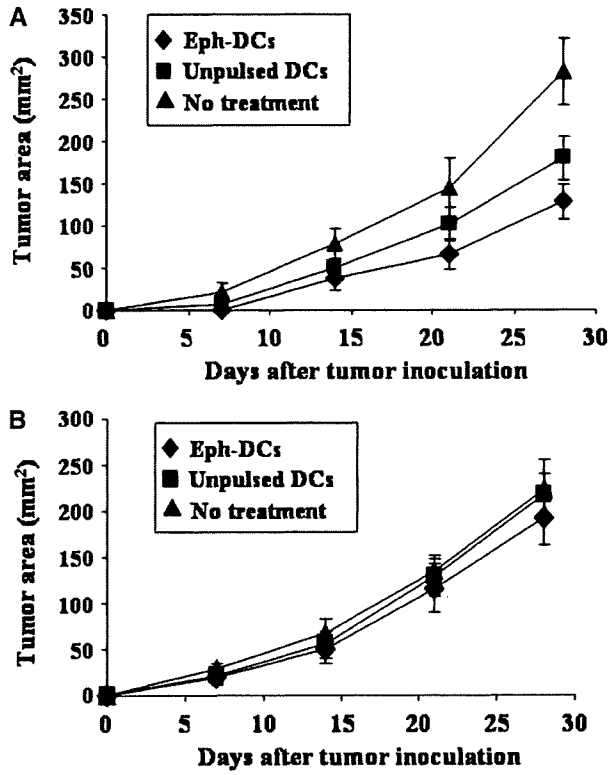


Fig. 5 DC vaccinated mice are partially protected against subcutaneous rechallenge with MC38 (EphA2-positive) but not BL6 (EphA2-negative) tumors. MC38-bearing mice that were successfully treated with DC-based vaccines were rechallenged s.c. in the flank with MC38 (**a**) or BL6 (**b**) tumor cells 14 days after the final DC injection. As controls, naïve mice were injected s.c. with MC38 or BL6 tumor cells. MC38 tumor growth in mice immunized with either format of DCs was significantly inhibited when compared to naive mice ($P < 0.05$ on day 28). In addition, tumor growth in the mice immunized with Eph- DCs was inhibited to a greater extent; i.e. $P < 0.05$ on day 28 versus unpulsed DCs, $P < 0.05$ on days 14, 21, 28 versus naive mice, $N = 8$/group. **b** Growth of the EphA2-negative BL6 tumor was not inhibited regardless of the treatment received, $N = 8$/group

treated mice also expressed elevated levels of the activation marker CD69 when compared to control treated animals.

Overall, these results suggest organ (i.e. Liver) focused anti-tumor "clinical" benefit derived from DC administration and that in conjunction with our results obtained in SCID models (lacking B cells, T cells, NKT cells) and in vitro cytotoxicity assays of liver NK cells performed against MC38 target cells, NK cells are major mediators of hepatic protection against tumor progression.

Recent studies have also suggested that DCs may effectively activate elements of innate immunity (NK cells [4–9] and NKT cells [10–12]) via direct cellular interaction and IL-12 secretion. In this study, the injected green fluorescence protein (GFP) mice-derived BM-DCs did not migrate to the liver following s.c. injection.in the flow cytometric analysis and IL-12 production in the serum harvested 1 day after either DC vaccination and tumor inoculation was not detected using IL-12 ELISA kits (unpublished data). Therefore, we would speculate that the injected DCs effectively enhance NK cell activity in the liver by some unknown humoral factors (cytokines except for IL-12, etc) or by secondary activated immune cells after DC immunization in vivo.

However, it is also clear that based on our in vivo lymphocyte depletion studies, CD4+ T cells and CD8+ T cells also contribute to suppression of tumor growth in the liver after DC vaccination. We would therefore speculate that hepatic NK cells may be a crucial early mediators of anti-tumor activity, with MC38 liver tumor-derived antigens then taken up by dedicated professional antigen-presenting cells in the liver that consequently cross-prime MC38 tumor-specific CD4+ or CD8+ T cells. In animals pre-vaccinated with Eph-DCs, the initial wave of tumor killing by NK cells may be boostered by EphA2-specific CD8+ T cells in the MC38 (EphA2-positive) model. This added degree of protection appeared to be dependent solely upon the presence of the loaded antigenic peptide on DCs, since we observed no other differences in Eph-DCs versus unpulsed DCs with regard to DC expression of a broad range of markers/parameters: CD40, CD80, CD86, MHC class II and IL12 production (S. Yamaguchi, unpublished data).

Data from our tumor rechallenge experiments indicate that unpulsed DC vaccines are not only effective in limiting MC38 liver tumor progression, but also offer protection against reintroduction of MC38 tumor at an extrahepatic site. However, Eph-DC immunizations were more effective in this endpoint, consistent with their superior capacity to promote tumor (i.e. EphA2)-specific CTLs. In contrast, in all cases when animals were rechallenged with the unrelated, EphA2-negative BL6 tumor, lesions grew progressively, suggesting that MC38-specific immunity was generated after liver tumor treatment. These results demonstrated that both unpulsed DC and peptide-pulsed DC vaccines generated systemic tumor-specific anti-tumor immunity against *metastatic liver tumor*, and that peptide-pulsed DC vaccination offered more optimal preventive treatment for extrahepatic tumor recurrence than unpulsed DC vaccines.

In spite of recent progress and early success reported for adjuvant chemotherapy trials in the prevention of liver metastasis, there remains a great need for developing novel and effective treatment modalities for this indication. In the current study, we have demonstrated that unpulsed DC vaccines, that are competent to activate both innate and acquired immunity within the liver, may represent a novel treatment option for metastatic liver tumors which expressed unknown TAAs. In cases where more disseminated disease is also present (or likely to occur) in non-liver

sites, the greatest degree of clinical efficacy may be expected of Peptide-DC vaccines, such as Eph-DCs.

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References

1. Steinman RM (1991) The dendritic cell system and its role in immunogenicity. Annu Rev Immunol 9:271–296
2. Hart DN (1997) Dendritic cells: unique leukocyte populations which control the primary immune response. Blood 90:3245–3287
3. Smith CM, Wilson NS, Waithman J, Villadangos JA, Carbone FR, Heath WR, Belz GT (2004) Cognate CD4(+) T cell licensing of dendritic cells in CD8(+) T cell immunity. Nat Immunol 5:1143–1148
4. O'Neill DW, Adams S, Bhardwaj N (2004) Manipulating dendritic cell biology for the active immunotherapy of cancer. Blood 104:2235–2246
5. Fernandez NC, Lozier A, Flament C, Ricciardi-Castagnoli P, Bellet D, Suter M, Perricaudet M, Tursz T, Maraskovsky E, Zitvogel L (1999) Dendritic cells directly trigger NK cell functions: cross-talk relevant in innate anti-tumor immune responses in vivo. Nat Med 5:405–411
6. Gerosa F, Baldani-Guerra B, Nisii C, Marchesini V, Carra G, Trinchieri G (2002) Reciprocal activating interaction between natural killer cells and dendritic cells. J Exp Med 195:327–333
7. Miller G, Lahrs S, Dematteo RP (2003) Overexpression of interleukin-12 enables dendritic cells to activate NK cells and confer systemic antitumor immunity. FASEB J 17:728–730
8. Piccioli D, Sbrana S, Melandri E, Valiante NM (2002) Contact-dependent stimulation and inhibition of dendritic cells by natural killer cells. J Exp Med 195:335–341
9. Jinushi M, Takehara T, Kanto T, Tatsumi T, Groh V, Spies T, Miyagi T, Suzuki T, Sasaki Y, Hayashi N (2003) Critical role of MHC class I-related chain A and B expression on IFNα-stimulated dendritic cells in NK cell activation: Impairment in chronic hepatitis C virus infection. J Immunol 170:1249–1256
10. Kawano T, Cui J, Koezuka Y, Toura Y, Kaneko Y, Motoki K, Ueno H, Nakagawa R, Sato H, Kondo E, Koseki H, Taniguchi M (1997) CD1d-restricted and TCR-mediated activation of Vα14NKT cells by glycosylceramids. Science 278:1626–1629
11. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Sato H, Kondo E, Harada M, Koseki H, Tanaka Y, Taniguchi M (1998) Natural killer-like nonspecific tumor cell lysis mediated by specific ligand-activated Vα14NKT cells. Proc Natl Acad Sci USA 95:5690–5693
12. Kitamura H, Iwakabe K, Yahata T, Nishimura S, Ohta A, Ohmi Y, Sato M, Takeda K, Okumura K, Van Kaer L, Kawano T, Taniguchi M, Nishimura T (1999) The natural killer T (NKT) cell-ligand-Galactosylceramide demonstrates immunopotentiating effects by inducing interleukin (IL)-12 production by dendritic cells and IL-12 receptor expression on NKT cells. J Exp Med 189:1121–1128
13. Wiltrout RH (2000) Regulation and antimetastatic functions of liver-associated natural killer cells. Immunol Rev 174:63–76
14. George AP, Catherine AP (2005) Liver immunobiology. Toxicol Pathol 33:52–62
15. Doherty DG, O'Farrelly C (2000) Innate and adaptive lymphoid cells in human liver. Immunol Rev 174:5–20
16. Miyagi T, Takehara T, Tatsumi T, Kanto T, Suzuki T, Jinushi M, Sugimoto Y, Sasaki Y, Hori M, Hayashi N (2003) CD1d-mediated stimulation of natural killer T cells selectively activates hepatic natural killer T cells to eliminate experimentally disseminated hepatoma cells in murine liver. Int J Cancer 106:81–89
17. Nakagawa R, Motoki K, Ueno H, Iijima R, Nakamura H, Kobayashi E, Shimosaka A, Koezuka Y (1998) Treatment of hepatic metastasis of the colon 26 adenocarcinoma with a Galactosylceramide, KRN7000. Cancer Res 58:1202–1207
18. Mayordome JI, Zorina T, Storkus WJ, Zitvogel L, Celluzzi C, Falo LD, Melief CJ, Ildstad ST, Kast WM, Deleo AB et al (1995) Bone marrow-derived dendritic cells pulsed with synthetic tumor peptide elicits protective and therapeutic antitumor immunity. Nat Med 12:1297–1302
19. Paglia P, Chiodoni C, Rodolfo M, Colombo MP (1996) Murine dendritic cells loaded in vitro with soluble protein prime cytotoxic T lymphocytes against tumor antigen in vivo. J Exp Med 183:317–322
20. Mayordomo JI, Loftus DJ, Sakamoto H, De Cesare CM, Appasamy PM, Lotze MT, Storkus WJ, Appella E, DeLeo AB (1996) Therapy of murine tumors with p53 wild-type and mutant sequence peptide-based vaccines. J Exp Med 183:1357–1365
21. Olson RM, Perencevich NP, Malcolm AW, Chaffey JT, Wilson RE (1980) Patterns of recurrence following curative resection of adenocarcinoma of the colon and rectum. Cancer 45:2969–2974
22. Malcolm AW, Perencevich NP, Olson RM, Hanley JA, Chaffey JT, Wilson RE (1981) Analysis of recurrence patterns following curative resection for carcinoma of thecolon and rectum. Surg Gynecol Obstet 152:131–136
23. Ota M, Shimada H, Masui H, Tanaka K, Yamaguchi S, Ichikawa Y, Togo S, Ike H, Oki S (2004) Adjuvant hepatic arterial infusion chemotherapy after curative resection for Dukes colorectal cancer: a pilot study. Hepatogastroenterology 51:124–127
24. Arkenau HT, Rettig K, Porschen R (2005) Adjuvant chemotherapy in curative resected colon carcinoma: 5-fluorouracil/leucovorin versus high-dose 5- fluorouracil 24-h infusion versus high-dose 5-fluorouracil 24-h infusion. Int J Colorectal Dis 20:258–261
25. Hayashibe A, Kameyama M, Shinbo M, Makimoto S (2007) Clinical results on intra-arterial adjuvant chemotherapy for prevention of liver metastasis following curative resection of pancreatic cancer. Ann Surg Oncol 14:190–194
26. Liu KJ, Wang CC, Chen LT, Cheng AL, Lin DT, Wu YC, Yu WL, Hung YM, Yang HY, Juang SH, Whang-Peng J (2004) Generation of carcinoembryonic antigen (CEA)- specific T-cell responses in HLA-A*0201 and HLA-A*2402 late-stage colorectal cancer patients after vaccination with dendritic cells loaded with CEA peptides. Clin Cancer Res 10:2645–2651
27. Sadanaga N, Nagashima H, Mashino K, Tahara K, Yamaguchi H, Ohta M, Fujie T, Tanaka F, Inoue H, Takesako K, Akiyoshi T, Mori M (2001) Dendritic cell vaccination with MAGE peptide is a novel therapeutic approach for gastrointestinal carcinomas. Clin Cancer Res 7:2277–2284
28. Timmerman JM, Levy R (1999) Dendritic cell vaccines for cancer immunotherapy. Annu Rev Med 50:507–529
29. Nevellino L, Castelli C, Parmiani G (2005) A listing of human tumor antigens recognized by T cells: March 2004 update. Cancer Immunol Immunother 54:187–207
30. Simone M, Carlo RR, Mario L, Donato N (2004) Colorectal cancer vaccines: principles, results, and perspectives. Gastroenterology 27:1821–1837
31. Oka Y, Tsuboi A, Taguchi T, Osaki T, Kyo T, Nakajima H, Elisseeva OA, Oji Y, Kawakami M, Ikegame K, Hosen N, Yoshihara S, Wu Y, Fujiki F, Murakami M, Masuda T, Nishida S, Shirakata T, Nakatsuka S, Sasaki A, Udaka K, Dohy H, Aozasa K, Noguchi S, Kawase I, Sugiyama H (2004) Induction of WT1 (Wilms' tumor gene)-specific cytotoxic T lymphocytes by WT1 peptide vaccine

and the resultant cancer regression. Pro Natl Acad Sci USA 138:13885–13890
32. Yamaguchi S, Tatsumi T, Takehara T, Sakamori R, Uemura A, Mizushima T, Ohkawa K, Hayashi N (2007) Immunotherapy of murine colon cancer using receptor tyrosine kinase EphA2-derived peptide-pulsed dendritic cell vaccines. Cancer 110(7):1469–1477
33. Felazzo G, Munz C (2004) NK cell compartments and their activation by dendritic cells. J Immunol 172:1333–1339
34. Nieda M, Okai M, Tazbirkova A, Lin H, Yamamura A, Ide K, Abraham R, Juji T, Macfarlane DJ, Nicol AJ (2004) Therapeutic activation of Vα24 + Vβ11 + NKT cells in human subjects results in highly coordinates secondary activation of acquired and innate immunity. Blood 103:383–389

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Case Report

Early emergence of entecavir-resistant hepatitis B virus in a patient with hepatitis B virus/human immunodeficiency virus coinfection

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The efficacy of entecavir for patients with hepatitis B virus/human immunodeficiency virus coinfection has not been fully elucidated. Here we examined a patient coinfected with both viruses in whom entecavir-resistant hepatitis B virus appeared. The 60-year-old Japanese male with the coinfection received antiretroviral therapy including lamivudine. The therapy initially suppressed replication of both viruses, followed by reactivation of the hepatitis B virus alone by 2 years of therapy. He subsequently received entecavir therapy in addition to the antiretroviral regimen. After entecavir administration, the hepatitis B virus DNA level was slightly reduced, but then increased after 6 months of entecavir therapy. In the sequencing analysis of hepatitis B virus, no drug resistance-associated amino acid substitutions were observed in the reverse transcriptase (rt) domain before antiretroviral therapy. The lamivudine-resistant amino acid substitutions at rt173, rt180 and rt204 were detected before entecavir administration, and further the entecavir-resistant rt202 substitution was observed after 6 months of entecavir therapy. The full-length hepatitis B sequences showed that the viral strain derived from the patient belonged to genotype H. In summary, this report describes a patient with hepatitis B virus/human immunodeficiency virus coinfection who received entecavir therapy in addition to an antiretroviral regimen and showed the early emergence of entecavir-resistance hepatitis B virus. In entecavir therapy for patients infected with both viruses, great care should be taken with respect to the emergence of entecavir-resistant hepatitis B virus, especially in patients with pre-existing lamivudine-resistant virus.

Key words: coinfection, drug-resistant hepatitis B virus, entecavir, hepatitis B virus, human immunodeficiency virus, lamivudine

INTRODUCTION

CHRONIC CARRIERS OF hepatitis B virus (HBV) number more than 350 million worldwide.[1] Chronic HBV infection is seen in approximately 10% of human immunodeficiency virus (HIV)-infected patients,[2] and coinfection with HBV and HIV is a serious health problem due to the shared mode of transmission. Since the prognosis of HIV-infected patients can be dramatically improved by highly active antiretroviral therapy (HAART), one of the major causes of mortality in HIV-infected patients is chronic liver disease due to HBV infection.[3]

Lamivudine (LAM, also abbreviated to 3TC), one of the antiretroviral drugs, has also been used for the reduction of HBV replication and improvement of HBV-related liver diseases.[4,5] However, the anti-HBV effect of LAM is hampered by the emergence of LAM-resistant mutant virus in cases of HBV monoinfection and HBV/

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HIV coinfection.[6,7] The LAM-resistant HBV strain is based on point mutation occurring within the reverse transcriptase (rt) domain of the polymerase gene. A methionine-to-valine/isoleucine amino acid substitution at rt204 (rtM204V/I) is known to confer LAM resistance.[8,9] A leucine-to-methionine substitution at rt180 (rtL180M) and a valine-to-leucine substitution at rt173 (rtV173L) have also been shown to appear in association with LAM resistance.[8,10,11] The emergence rate of LAM-resistant virus in patients coinfected with HBV and HIV has been reported to be approximately 50% after 2 years of therapy.[9]

Recently, entecavir (ETV) has been reported to be superior to LAM for the suppression of viral replication and disease activity in patients with HBV monoinfection who had not received previous treatment with other anti-HBV drugs (naïve patients).[12,13] ETV has also been shown to be effective in HBV-infected patients who had been treated with LAM and showed LAM resistance.[14] It has been demonstrated that ETV resistance occurs based with amino acid substitution(s) at rt184, rt202 and/or rt250, together with the LAM-resistant rtM204V/I and rtL180M substitutions.[15] The emergence rate of ETV-resistant virus after 3 years of therapy has been reported to be less than 1% in naïve patients and 15% in LAM-resistant patients with chronic HBV monoinfection.[16] However, the anti-HBV efficacy of ETV for HBV/HIV coinfection has not been fully clarified.

In this study, we examined a patient with concomitant HBV/HIV infection who underwent HAART including LAM, and showed the appearance of LAM-resistant HBV. Subsequent ETV administration did not lead to an adequate reduction of the HBV replicative level, followed by the early emergence of the ETV-resistant virus. We investigated the serial change in the drug resistance-associated mutation status within the rt domain of the HBV polymerase gene, as well as full-length nucleotide sequences of the ETV-resistant HBV strain derived from the patient.

CASE REPORT

Patient and serum sampling

A 60-YEAR-OLD JAPANESE heterosexual male first visited to the National Hospital Organization Osaka National Hospital in December 2001 due to a positive result from an HIV antibody (anti-HIV) test in voluntary HIV screening. From his anamnestic record, he had been admitted with type B acute hepatitis to another hospital 3 years earlier. Anti-HIV had been negative at that time. On his first visit, the anti-HIV positivity was confirmed by Western blot analysis. Antibodies to HIV-1 proteins, gp160, gp110/120, p68, p52, gp41, p40 and p34 were positive. As for antibodies to HIV-2 proteins, only an antibody to p68 was positive. According to these, he was judged to be infected with HIV-1. The HIV-RNA level was $10^{4.3}$ copies/mL, and the CD4+ T cell counts were 275/mm^3 (normal range, >300/mm^3). He tested positive for hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg), and negative for antibody to HBsAg (anti-HBs) and antibody to HBeAg (anti-HBe). The HBV-DNA level was $>10^{7.6}$ copies/mL, and the alanine aminotransferase (ALT) level was 106 IU/L. The patient was free of HIV-related symptoms and had no opportunistic infectious diseases. HAART with LAM (300 mg/day), zidovudine (AZT) (600 mg/day) and efavirenz (EFV) (600 mg/day) was started in April 2002. AZT and EFV were then substituted for sanilvudine (d4T) (60 mg/day) and avacavir (ABC) (600 mg/day) in July 2002 because of anemia and dizziness. By July 2002, HIV-RNA decreased to below the detection limit ($<10^{1.7}$ copies/mL), whereas the CD4+ T cell counts tended to rise up to >500/mm^3. In August 2006, fosamprenavir (FPV) (2400 mg/day) was commenced in place of d4T due to peripheral nerve palsy. Suppression of HIV-RNA below the detection limit continued at the end of follow-up, irrespective of repeated alterations in the therapeutic regimen of HAART. As for HBV status, HBV-DNA declined to $10^{3.9}$ copies/mL in April 2003 but increased again to $>10^{7.6}$ copies/mL in May 2005. To control HBV replication, ETV (0.5 mg/day) was added in October 2006. After the ETV administration, HBV-DNA slightly decreased from $>10^{7.6}$ to $10^{6.2}$ copies/mL in January 2007 but rose to $10^{7.2}$ copies/mL 3 months later. ALT remained abnormal and HBeAg continued to be positive throughout the follow-up period. The clinical course of the patient is summarized in Figure 1a.

For the nucleotide sequencing of HBV-DNA, the serum samples were obtained in December 2001 (before HAART), August 2006 (before ETV administration), and April 2007 (after 6 months of ETV therapy). These serum sampling points were designated as P1, P2 and P3 (see Fig. 1a). Serum samples were stored at −80°C until use. Informed consent was obtained from the patient.

Virus markers and nucleotide sequencing

HBsAg, anti-HBs, HBeAg, anti-HBe and anti-HIV were tested by chemiluminescent immunoassay. A

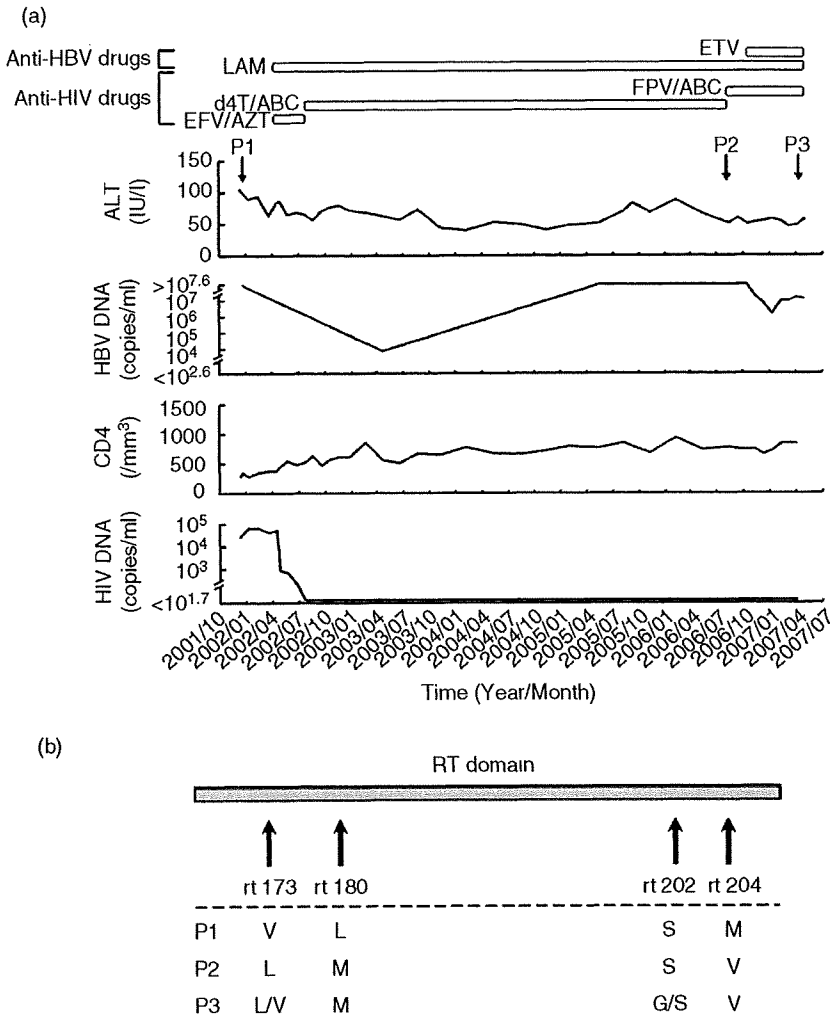

Figure 1 (a) Patient clinical course and serum sampling points. P1, P2 and P3 are the points at which serum samples were obtained. P1 was taken in December 2001 (before HAART), P2 in August 2006 (before ETV administration) and P3 in April 2007 (after 6 months of ETV therapy). ABC, avacavir; ALT, alanine aminotransferase; AZT, zidovudine; d4T, sanilvudine; EFV, efavirenz; ETV, entecavir; FPV, fosamprenavir; HBV, hepatitis B virus; HIV, human immunodeficiency virus; LAM, lamivudine. (b) Serial change in the status of drug resistance-associated amino acid substitutions.

confirmatory anti-HIV-1/2 testing was carried out by Western blot analysis. Serum HBV-DNA was detected by means of a PCR assay (Amplicor HB monitor; Roche Diagnostics, Basel, Switzerland) with a lower detection limit of $10^{2.6}$ (=400) copies/mL. Plasma HIV-RNA was quantified by a PCR assay (Amplicor HIV-1 monitor; Roche) whose lower detection limit was $10^{1.7}$ (=50) copies/mL.

The nucleotide sequences of HBV-DNA were determined by a method based on nested PCR and direct sequencing, as described elsewhere.[17] In this study, primers BF5-2 (5′-TCC TCA GGC CAT GCA GTG GA-3′, nt 3201-20) and BR8 (5′-TTG CGT CAG CAA ACA CTT GG-3′, nt 1195-76) were also used. Nucleotide sequences of the entire rt domain in the polymerase gene were examined in HBV strains derived from the P1 and P2 serum samples (GenBank accession nos. AB353765 and AB353766), whereas the full-length HBV-DNA was determined in the strain derived from the P3 serum sample (GenBank accession no. AB353764). The full-length HBV strain obtained in this study (designated as HBDI03), the seven representative HBV strains of genotypes A–G and the eight previously isolated HBV strains of genotype H were aligned, and the phylogenetic tree was constructed. These analyses were done at the homepage of the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp).

Results of sequencing analysis of HBV

The serial change in the nucleotide sequences in the rt domain of the HBV polymerase gene was first examined

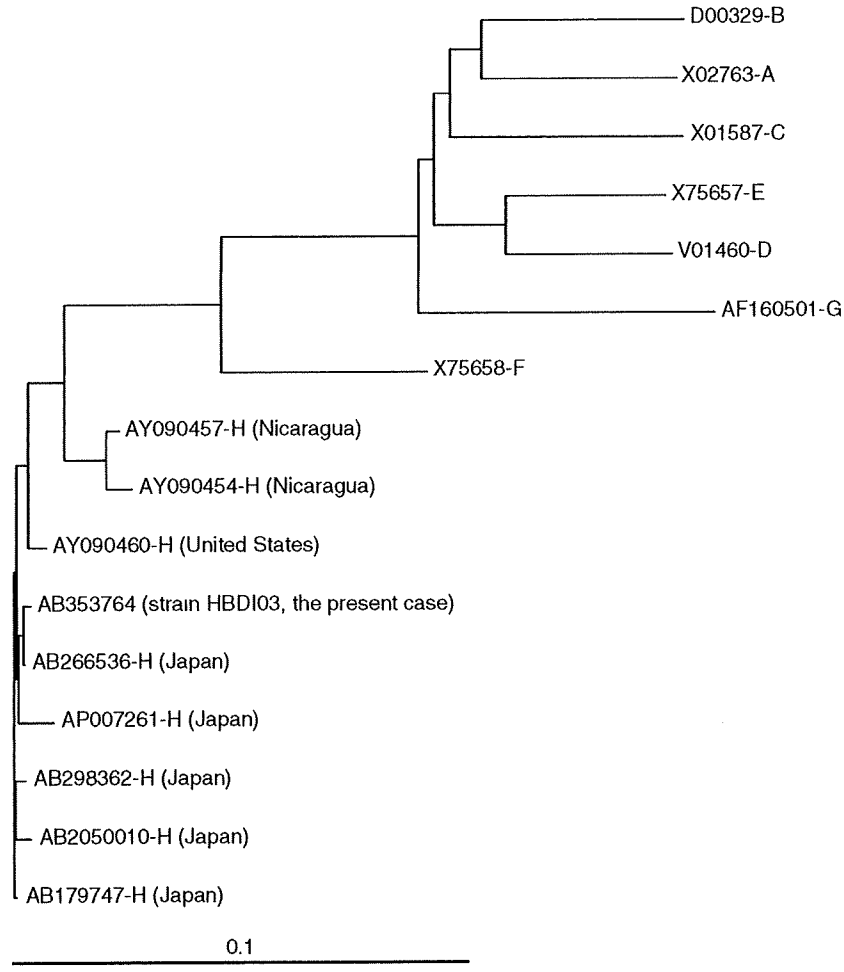

Figure 2 Phylogenetic tree analysis including the HBV strain HBDI03 obtained in this study, the seven representative HBV strains of genotypes A–G, and the eight previously isolated HBV strains of genotype H.

using serum samples obtained at P1–P3 (Fig. 1b). At point P1, no drug resistance-associated mutations were found in the rt domain, but three LAM resistance-associated substitutions, rtM204V, rtL180M and rtV173L, emerged at point P2. A serine-to-glycine substitution at rt202 (rtS202G), which has been shown to be one of the ETV resistance-associated substitutions,[15] was further observed at point P3, although rtS202G and rtV173L substitutions occurred incompletely. No other amino acid substitutions were seen in the rt domain of the HBV polymerase gene from point P1 to P3. Thus, in the patient with HBV/HIV coinfection, the emergence of the drug resistance-associated amino acid substitutions revealed a close relationship with the poor anti-HBV efficacy of LAM and ETV.

Next, the full-length nucleotide sequences of HBV were determined from the P3 serum sample of the patient with HBV/HIV coinfection showing ETV resistance. The full-length HBV strain HBDI03 comprised a total of 3215 nucleotide lengths. The phylogenetic tree was depicted using the HBV strain HBDI03, the seven representative HBV strains of genotypes A–G and the eight previously identified genotype H HBV strains. As shown in Figure 2, the HBV strain HBDI03 obtained in this study was classified as genotype H. When the nucleotide sequences of the strain HBDI03 were compared with the eight reported genotype H HBV strains, the strain HBDI03 showed a 97.2–99.8% identity with these strains. The unique amino acid substitutions in the strain HBDI03 were further investigated in comparison with these eight genotype H HBV strains. As shown in Table 1, four drug resistance-associated substitutions within the rt domain were observed, as described above. The two amino acid substitutions in the S gene were also caused by the same mutations of the drug resistance-associated rtV173L and rtM204V

Table 1 The unique amino acid substitutions in strain HBDI03 in comparison with eight previously isolated genotype H hepatitis B virus strains

Amino acid position	Consensus residue of genotype H	Residue unique to strain HBDI03
Polymerase		
519 (rt173)	V	L/V
526 (rt180)	L	M
548 (rt202)	S	G/S
550 (rt204)	M	V
Surface		
164	E	D/E
195	I	M
X		
32	W	G

Consensus residues of genotype H were from the eight reported hepatitis B virus (HBV) strains (GenBank accession nos. AY090454, AY090457, AY090460, AP007261, AB179747, AB205010, AB266536 and AB298362).

changes. As for the remaining one amino acid substitution in the X gene, the substituted glycine residue observed in the HBDI03 strain was a common one in the representative HBV strains of genotypes A–G at the corresponding codon position. Taken together, the HBDI03 strain did not appear to have any distinctive features other than the presence of the drug-associated amino acid substitutions.

DISCUSSION

RECENTLY, ETV HAS been widely accepted as an effective drug for the treatment of HBV monoinfection because of its stronger inhibitory effect on HBV replication and lower emergence rate of drug-resistant mutant virus compared to LAM.[12–14] ETV-resistant HBV has been demonstrated to be established by amino acid substitution(s) at rt184, rt202 and/or rt250, in addition to the LAM-resistant rtM204V/I and rtL180M substitutions.[15] The emergence rate of ETV-resistant virus has been reported to be higher in LAM-resistant patients than in naïve patients.[16] There has so far been little evidence concerning the anti-HBV efficacy of ETV for patients with HBV/HIV coinfection. In particular, LAM-resistant HBV has been shown to emerge frequently in patients with HBV/HIV coinfection who received LAM therapy as a component of HAART.[7] The therapeutic efficacy of ETV on LAM-resistant HBV should be assessed in patients with HBV/HIV coinfection. In this study, we examined a patient with HBV/HIV coinfection who had LAM-resistant HBV induced by HAART including LAM, and underwent subsequent ETV therapy. The patient showed a rather weak suppressive effect of ETV on HBV replication, followed by the emergence of ETV-resistant HBV in the early phase of therapy.

In the sequence analysis of the HBV genome, no drug-resistant HBV mutations were detected before HAART, but continuous LAM administration induced the LAM-resistant mutant HBV with rtM204V, rtL180M and rtV173L amino acid substitutions. Subsequent ETV therapy resulted in the emergence of an ETV-resistant virus possessing the rtS202G substitution in addition to the three LAM resistance-associated substitutions after no more than 6 months of ETV therapy, although the rtS202G and rtV173L substitutions were incomplete. In LAM-resistant patients with HBV monoinfection, the emergence rate of the ETV-resistant mutation has been reported to be merely 15% after 3 years of therapy.[16] In comparison with this, ETV-resistant HBV appeared in an extremely early phase of therapy in our patient with HBV/HIV coinfection. According to this, ETV resistance is speculated to be established earlier in patients with HBV/HIV coinfection than in those with HBV monoinfection, although concomitant HIV infection has not thus far been suggested to result in a higher incidence of the drug-resistant HBV strain in the treatment with other anti-HBV drugs in chronic HBV infection. The latent immune deficiency caused by HIV infection might prevent HBV eradication through a host immune response, resulting in poor anti-HBV efficacy of ETV. Alternatively, simultaneous usage of multiple antiretroviral drugs might in some way contribute to the emergence of ETV-resistant HBV

Very recently, it has been shown that ETV possesses modest anti-HIV activity both *in vitro* and *in vivo* and can induce the drug-resistant mutant HIV strain in patients with HBV/HIV coinfection.[18] This suggests that ETV may not be appropriate for the treatment of patients with HBV/HIV coinfection in whom HAART is not needed. On the other hand, ETV is considered to be beneficial for patients with HBV/HIV coinfection undergoing a stable continuation of HAART. In particular, the therapeutic efficacy of ETV may be more promising in patients without LAM-resistant HBV than in those with it. Although the present case of the patient under discussion, who already displayed LAM-resistant HBV due to the preceding HAART, did not support the usefulness of ETV therapy because of the early emergence of ETV-resistant HBV, further studies with a large number of

patients should be completed to assess the antiviral efficacy and deliberate clinical application of ETV therapy for HBV/HIV coinfection.

Both adefovir dipivoxil (ADV) and tenofovir disoproxil fumarate (TDF) have recently been shown to effectively inhibit HBV replication in patients with HBV/HIV coinfection, irrespective of LAM resistance.[19,20] ADV exerts only anti-HBV activity and is available for patients with HBV/HIV coinfection who have no need for HAART or who are receiving a stable HAART regimen. In contrast, TDF can be used as a component of HAART because of its valuable antiviral activity against both HBV and HIV. Accordingly, ADV and TDF are currently useful drugs for patients with HBV/HIV coinfection and may be subsequent therapeutic options for the patient reported in this study.

Our patient was found to be infected with HBV of genotype H, a globally rare genotype. To date, the full-length sequences of eight genotype H HBV strains have been reported from the USA, Nicaragua and Japan (see Fig. 2). Of them, one strain has been obtained from a Japanese patient with chronic HBV monoinfection who underwent ETV therapy as a naïve patient and showed ETV resistance later.[21] The relevance of the genotype frequency to the therapeutic efficacy of ETV should be studied extensively in HBV-infected patients treated with ETV.

In Japan, genotypes B and C are prevalent in chronic HBV carriers who acquire the infection mainly through the mother-to-child transmission route. In contrast, the foreign HBV strains other than genotypes B and C have been shown to be involved in a considerable proportion of patients with acute HBV infection.[22] Infection of such foreign types of HBV possibly occurs through sexual contacts in Japan. In our patient with HBV/HIV coinfection who had genotype H HBV of foreign origin, it is speculated that acute HBV infection occurring 3 years before his first visit led to the transition to chronicity. The time of HIV infection cannot be defined due to the lack of HIV-RNA testing during the period of acute HBV infection. The possibility of simultaneous infection with HBV and HIV cannot be excluded, despite the negative result of anti-HIV at that time, because the test may have taken place during the immunological window period of HIV infection.

In summary, we have introduced a patient with HBV/HIV coinfection who underwent ETV therapy in addition to the HAART regimen and showed ETV resistance in the early phase of therapy. Our finding suggests that, in ETV therapy for patients with HBV/HIV infection, great care should be taken against the emergence of ETV-resistant HBV, especially in patients with pre-existing LAM-resistant HBV.

REFERENCES

1 Lee WM. Hepatitis B virus infection. *N Engl J Med* 1997; 337: 1733–45.
2 Konopnicki D, Mocroft A, de Wit S *et al.* EuroSIDA Group. Hepatitis B and HIV: prevalence, AIDS progression, response to highly active antiretroviral therapy and increased mortality in the EuroSIDA cohort. *AIDS* 2005; 19: 593–601.
3 Thio CL, Seaberg EC, Skolasky R Jr *et al.* Multicenter AIDS Cohort Study. HIV-1, hepatitis B virus, and risk of liver-related mortality in the Multicenter Cohort Study (MACS). *Lancet* 2002; 360: 1921–6.
4 Lai CL, Chien RN, Leung NW *et al.* A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. *N Engl J Med* 1998; 339: 61–8.
5 Benhamou Y, Katlama C, Lunel F *et al.* Effects of lamivudine on replication of hepatitis B virus in HIV-infected men. *Ann Intern Med* 1996; 125: 705–12.
6 Lai CL, Dienstag J, Schiff E *et al.* Prevalence and clinical correlates of YMDD variants during lamivudine therapy for patients with chronic hepatitis B. *Clin Infect Dis* 2003; 36: 687–96.
7 Benhamou Y, Bochet M, Thibault V *et al.* Long-term incidence of hepatitis B virus resistance to lamivudine in human immunodeficiency virus-infected patients. *Hepatology* 1999; 30: 1302–6.
8 Allen MI, Deslauriers M, Andrews CW *et al.* Identification and characterization of mutations in hepatitis B virus resistant to lamivudine. Lamivudine Clinical Investigation Group. *Hepatology* 1998; 27: 1670–7.
9 Ono-Nita SK, Kato N, Shiratori Y *et al.* Susceptibility of lamivudine-resistant hepatitis B virus to other reverse transcriptase inhibitors. *J Clin Invest* 1999; 103: 1635–40.
10 Ono SK, Kato N, Shiratori Y *et al.* The polymerase L528M mutation cooperates with nucleotide binding-site mutations, increasing hepatitis B virus replication and drug resistance. *J Clin Invest* 2001; 107: 449–55.
11 Delaney WE, Yang H, Westland CE *et al.* The hepatitis B virus polymerase mutation rtV173L is selected during lamivudine therapy and enhances viral replication in vitro. *J Virol* 2003; 77: 11833–41.
12 Chang TT, Gish RG, de Man R *et al.* BEHoLD AI463022 Study Group. A comparison of entecavir and lamivudine for HBeAg-positive chronic hepatitis B. *N Engl J Med* 2006; 354: 1001–10.
13 Lai CL, Shouval D, Lok AS *et al.* BEHoLD AI463027 Study Group. Entecavir versus lamivudine for patients with HBeAg-negative chronic hepatitis B. *N Engl J Med* 2006; 354: 1011–20.
14 Sherman M, Yurdaydin C, Sollano J *et al.* AI463026 BEHoLD Study Group. Entecavir for treatment of