injure compromised hepatocytes, which have acquired sensitivity to the Fas ligand and TNF-α, even if they are not infected with HCV (Fig. 3). TNF-α released from activated CTL can injure compromised non-infected cells without cell-to-cell interaction.[13] This phenomenon may contribute to inhibiting the spread of HCV infection, although there is a possibility that it may further aggravate the hepatitis.

Mechanisms of persistence of HCV infection

Escape from immune surveillance by amino acid mutations

HCV exists in patients as various quasispecies induced by low-fidelity replication by RNA polymerase that attempt to escape from immune surveillance of the host. A mutation in an immunogenic region results in ignorance of HCV-specific CTL and antibodies, and the induction of tolerance towards CTL.[14,15] In addition, it is thought that quasispecies of HCV acquire the ability to infect various kinds of cells and become tolerant against multiple drugs.

Escape from humoral immune responses

A virus-specific neutralizing antibody eliminates viruses in the body fluid, but not in the infected cells. It takes a long time to generate a neutralizing antibody in HCV infection. It is therefore thought that an HCV-specific neutralizing antibody response contributes to the prevention of secondary infection rather than first infection. Amino acid mutations within the hypervariable region 1 (HVR1) of the HCV E2 region are highly diverse, and this may contribute to the persistence of infection by facilitating escape from neutralizing antibodies. However, it was reported that in chimpanzees, both with and without anti-HVR1 antibodies, the major HVR1 sequences were unchanged for a long time, and that sequences of HVR1 variants converged finally in each chimpanzee.[16] The data show that anti-HVR1 antibodies are unlikely to induce variation in HVR1. Recently, it was reported that the humoral response continuously failed to neutralize viruses, and that during chronic infection, selective pressures favor the continuous generation of escape variants from humoral and cellular immunity.[17]

Escape from cellular immune responses

It is possible that mutations in epitopes of HLA class I or class II-restricted T cells contribute to viral persistence by interfering with the recognition of HCV-infected cells. An amino acid mutation that interfered with $CD8^+$ CTL recognition was found through cloning the infecting HCV. A mutation of one amino acid within the epitope of the NS3 region recognized by Th1 cells resulted in a shift in cytokine secretion patterns from Th1 to type 2 helper T (Th2) cells.[18] Th2 dominance may lead to decreased antiviral responses.

Mutations within CTL epitopes were found to emerge in three of six patients with acute hepatitis C. CD8 cells could recognize the parental epitopes, but not variant sequences, and the variant sequences could not induce a CD8 response efficiently *in vitro*.[19] It was suggested that immunodominant CD8 responses are influenced by inhibitory mechanisms occurring early post-infection. However, CTL responses are diverse in the early stage of HCV infection, and thus a single amino acid mutation would not be able to explain persistent infection. Escape mutations may therefore be a result rather than a cause of persistent infection.

Immune suppression induced by HCV infection

Anti-HCV antibodies do not appear until 2–4 months after infection, and even if they are produced, HCV infection will persist and hepatitis will progress in most cases. Various T-cell responses can be detected in the acute phase, yet they are dramatically reduced in the chronic phase of HCV infection. Although a large number of HCV-specific $CD8^+$ T cells infiltrate the liver, they are unable to eradicate HCV. Recently, many reports have suggested that HCV itself may actively suppress host immune responses.

Inhibition of NK cells

As the major HCV envelope protein E2 has been shown to bind to CD81 with high affinity, the CD81 molecule is thought to be one of the receptors for HCV infection. It has been reported that CD81 cross-linking via the immobilized HCV E2 protein inhibits non-specific cytotoxicity mediated by NK cells, as well as IFN-γ production by NK cells.[20] Thus HCV may directly suppress the function of NK cells. NK cell infiltration can be found in the liver of patients 1 week after starting IFN therapy. Further, cytotoxicity of NK cells is thought to be an indicator of the efficacy of IFN therapy. It is therefore apparent that NK cells, representing innate immunity, are involved in the eradication of HCV, while the direct suppression of NK cells may be implicated in HCV persistence.

The HCV core protein enhances major histocompatibility complex (MHC) class I molecules by increasing the expression of transporter associated with antigen processing 1 in a p53-dependent manner.[21] As a result, the enhancement of MHC class I expression contributes to HCV persistence by suppressing the cytotoxicity of NK cells.

The expression of CD94/NKG2A, which is an inhibitory receptor on NK cells, has been reported to be enhanced in patients with chronic hepatitis C.[22] HCV peptide aa35–44, which is known as a well-characterized HLA-A2-restricted T-cell epitope, stabilizes the expression of HLA-E, which is a ligand for CD94/NKG2A, and thereby inhibits NK cell-mediated lysis.[23] It has been also reported that another inhibitory receptor of NK cells, KIR2DL3, is implicated in HCV eradication.[24]

Inhibition of humoral immunity

Since peripheral lymphocytes of patients with HCV infection show a high expression of CD81,[25] it is possible that HCV infects these cells and may affect antibody production. B-cell response is thought to be impaired, since the titer of the neutralizing antibody is too low to prevent reinfection with HCV.

Inhibition of T lymphocytes

The function of HCV-specific CTL as effectors is clearly impaired in chronic HCV infection. The expression of the CD3 ζ chain,

which is crucial for T cells to function properly, has been reported to be reduced on the surface of peripheral lymphocytes in patients with chronic hepatitis C.[26] The CTL responses are functionally defective with impaired IFN-γ production, low perforin content, and decreased capacity for proliferation and cytotoxicity.[27]

The HCV core protein, which is released from HCV-infected cells and exists in peripheral blood, interacts with gC1qR on T cells. This results in the inhibition of T-cell activation, proliferation, and IFN-γ production by T cells.[28,29] The HCV NS4A/B protein blocks the expression of HLA class I molecules on the cell surface by the inhibition of endoplasmic reticulum-to-Golgi traffic.[30] This may be one of the reasons why CTL tend to ignore HCV-infected hepatocytes. These functional defects may lead to persistent infection with HCV.

In acute hepatitis C, the cytotoxic activity of $CCR7^{-}CD8^{+}$ memory T cells is impaired, but it has been reported that IL-2 pushes semi-effector CTL to complete their effector function.[31] Therefore, IL-2 deficiency during T-cell activation may be responsible for incomplete effector differentiation of the memory CTL shown in patients with acute HCV infection. In addition, it is supposed that circulating the HCV core protein suppresses IL-2 and IL-2 receptor α gene transcription by the inhibition of phosphorylation of extracellular signal-regulated kinase (ERK) and the mitogen-activated ERK kinase.[32] Furthermore, in exhausted CD8 cells, programmed cell death 1, the ligation of which inhibits T-cell effector function, has been reported to be upregulated in acute hepatitis C.[33]

In the chronic phase, HCV-specific $CD4^{+}$ T cells can be detected, but their antigen-specific proliferation is impaired. As described later, the production of IL-10 and transforming growth factor-β (TGF-β) are detected in an antigen-specific manner. It is thought that these phenomena lead to persistent HCV infection by suppressing the proliferation and activation of Th cells and CTL.

In the liver tissue, immune cells, such as sinusoidal endothelial cells and Kupffer cells, can present viral antigens, but exhibit few costimulatory molecules, such as CD80 and CD86. Thus they are not able to stimulate T cells effectively, and may induce immune tolerance.[34]

Inhibition of DC

DC play a crucial role in inducing immunity. In chronic hepatitis C, it has been reported that the ability of DC to stimulate allogenic T cells is impaired, and that HCV core and E1 proteins inhibit DC maturation.[35] A decrease in the number of plasmacytoid DC and impairment of IFN-α production by them have been reported, while the ability of myeloid DC to stimulate allogenic lymphocytes is reduced despite no reduction in overall frequency of these cells.[36] HCV core and NS3 proteins impair the function of DC, which recognize those HCV proteins through Toll-like receptor (TLR) 2.[36] The available evidence suggests that HCV directly inhibits cellular immune responses in the host. It has also been reported that DC activated by TLR ligands derived from bacteria or viruses have a reduced cross-presentation ability, and this plays an important role in inducing immune responses.[37] HCV may possess the same mechanism to evade immune responses. In fact, the expression of TLR2 on immature DC is reduced in patients with chronic hepatitis C compared with healthy donors, and DC stimulated through TLR2 impair T-cell proliferation.[38]

Another study in acute hepatitis C suggested that the frequency and IFN-α-producing capacity of peripheral blood plasmacytoid DC are apparently reduced and inversely correlated with the severity of liver inflammation. In the chronic state, the recovery of plasmacytoid DC function is incomplete; this could also be due to the chronic inflammation.[39]

Concerning the relationship between DC and NK cells, it has been suggested that DC inhibit NK cell activation in HCV infection. DC activate NK cells by the expression of major histocompatibility complex class I-related chain A and B (MICA/B) on their surface after stimulation with IFN-α. However, in HCV-infected patients, the MICA/B expression is reduced. Impaired IL-15 production is one of the mechanisms of insufficient MICA/B expression on DC in response to type I IFN.[40]

HCV is thought to infect DC through the binding of the HCV E2 protein to DC-specific intercellular adhesion molcure-3 grabbing non-integrin on their cell surface.[41,42] Since the soluble E2 protein is also able to bind to DC, HCV may directly suppress DC function. Furthermore, it has been suggested that HCV core, NS3, NS5A, and NS5B proteins may induce apoptosis of mature DC.[43]

In an HCV-infected chimpanzee model, it has been shown that impairment of the above maturation and allostimulatory function are not necessary prerequisites, but rather consequences of chronic HCV infection.[44] Furthermore, there are some reports that neither plasmacytoid DC nor monocyte-derived DC are impaired in HCV-infected patients.[45] Human DC expressing HCV core and NS3 proteins showed normal phenotypic function, cytokine production, and normal T-cell stimulation capabilities.[46] It has also been reported that there are lower frequencies of myeloid DC and plasmacytoid DC in chronic hepatitis C patients than in healthy individuals. However, despite the decreased circulating myeloid DC, no phenotypic or functional defects were identified.[47] Although lower IFN-α production was responsible for the decreased numbers of plasmacytoid DC, these DC from HCV-infected patients produced almost the same level of IFN-α as DC from healthy individuals. Further investigations are needed to clarify DC function in chronic hepatitis C, the reason for its impairment, and the nature (if any) of functional defects.

Other effects of HCV proteins on immunity

It has been reported that IFN-induced signal transduction through the Jak–signal transducers and activators of transcription pathway is impaired in transgenic mice that express HCV proteins in their liver cells.[48] The HCV core protein induces apoptosis in Jurkat cells and immune cells via the Fas system.[49,50] The HCV core protein also drives liver injury by increasing Fas-mediated apoptosis and liver infiltration of peripheral T cells.[51] However, it has been also reported that although HCV-transgenic mice have an apparently normal T-cell response, their hepatocytes cannot eliminate an adenoviral infection, and that the defect in adenoviral clearance is responsible for resistance of the hepatocytes to apoptosis induced by Fas/APO1/CD95 death receptor stimulation.[52] *In vitro* data have also shown that the HCV core protein binds to TNF receptors. It has also been reported that the expression of the HCV core protein inhibits TNF-α-mediated apoptosis by the sustained upregulation of cellular Fas-associated via death domain-like IL-1β-converting enzyme-like inhibitory protein.[53] These data

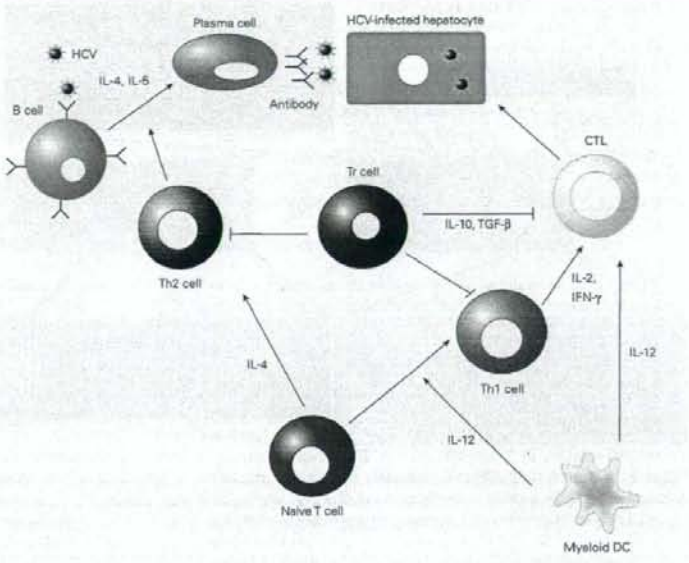

Figure 4 Induction of immune cells and inhibition of regulatory T (Tr) cells. IL-12 produced by myeloid dendritic cells (DC) differentiates T-helper (Th) cells towards type 1 helper (Th1) cells and activates CTL. Type 2 helper (Th2) cells stimulate humoral immune responses. Tr cells inhibit both cellular and humoral immune responses against hepatitis C virus (HCV). IL, interleukin; TGF, transforming growth factor.

emphasize that HCV can regulate apoptosis by direct effects of its own proteins, thereby escaping from host immune responses.

Involvement of regulatory T cells in persistent infection with HCV

Recently, the focus has been on regulatory T (Tr) cells, which secrete IL-10 and TGF-β, as potential regulators of immune responses.[54] In chronic hepatitis C, the frequency of $CD4^+$ $CD25^+$ T cells (considered Tr cells) is high, and these cells suppress T-cell function directly.[55] These cells can reduce HCV-specific cellular immune responses, thereby leading to persistent hepatitis (Fig. 4).[56] HCV core-specific Tr cells are induced from the peripheral blood of patients with chronic hepatitis C, and IL-10 produced by those cells is involved in the persistence of HCV infection.[57] Forkhead/winged helix transcription factor 3 Tr cells and IL-10 producing HCV-specific $CCR7^-$ $CD8^+$ Tr cells infiltrate the liver of patients with chronic HCV infection; IL-10 is identified as a soluble inhibitory factor mediating immune suppression.[58,59] These cells may play a crucial role in regulating intrahepatic T-cell responses. In addition, peripheral blood mononuclear cells from normal individuals secrete IL-10 in response to NS3 and NS4, suggesting that cells of the innate immune system, in addition to T cells, produce IL-10 in HCV-infected patients.[60,61]

While these data suggest the involvement of Tr cells in HCV-persistent infection, the involvement of Tr cells in the pathogenesis of chronic hepatitis C still remains to be clarified.

Other mechanisms of HCV persistence

The HCV NS5A protein binds to the IFN-induced, double-stranded, RNA-activated protein kinase, protein kinase R (PKR), which is a critical component of the cellular antiviral and antiproliferative responses, and inhibits PKR function.[62] This mechanism may also play a critical role in HCV persistence. The IFN sensitivity-determining region is a 40 amino-acid sequence located in the NS5A protein of hepatitis C virus genotype 1b. Mutations in this region abrogate the interaction of NS5A with PKR, and may result in increasing sensitivity of HCV to antiviral therapy.[63] HCV NS3/4A serine protease blocks the phosphorylation of interferon regulatory factor-3, which is a key cellular antiviral signaling molecule.[64] The NS3/4A serine proteases target Cardif, a caspase activation and recruitment-domain containing adaptor protein that interacts with retinoic acid-inducible gene I, and the interaction with Cardif leads to the inhibition of antiviral effects.[65] Immune suppression by HCV proteins is summarized in Figure 5.

Relevance of immune responses for HCV infection therapy

IFN-α is the mainstay for HCV antiviral treatment. Low doses of human IFN-α augment the cellular immune response by three to four-fold, whereas a further increase in IFN dosage suppresses the CTL response significantly.[66] A Th1 response is dominant in patients with sustained viral response to IFN-α-based therapies. Ribavirin, which is used in combination with IFN-α, has an antiviral mechanism that drives the Th2 response towards a Th1 response.[67] It was reported that intrahepatic and peripheral blood HCV-specific CTL activity could be detected in patients with a sustained response to IFN therapy more frequently than in patients who relapsed or had no treatment response.[68] The authors of that report stated that: "rather than the combination therapy acting to enhance the CTL response to achieve viral clearance, detectable CTL prior to treatment increases the likelihood of the host responding to the direct antiviral activity of IFN-α and ribavirin".

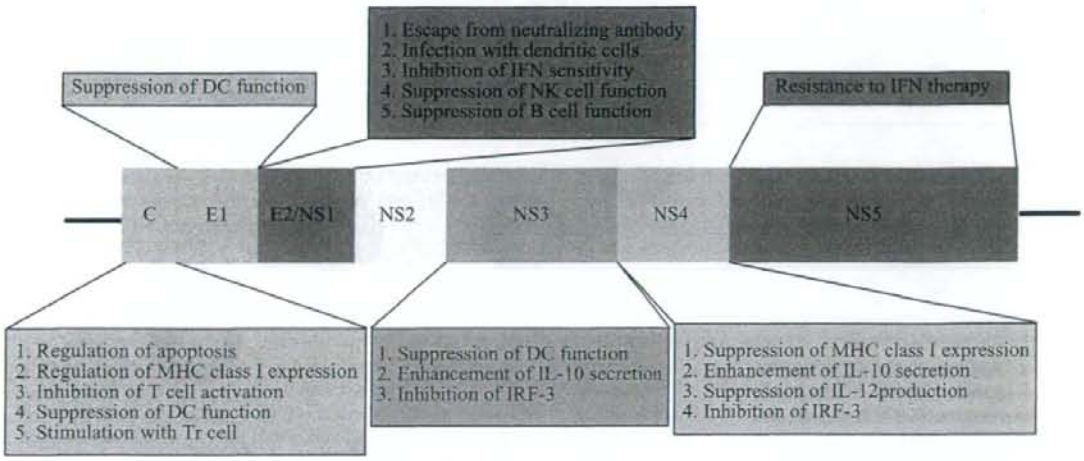

Figure 5 Immune suppression by hepatitis C virus (HCV) proteins. Suppressive effects of HCV proteins on host immune responses have been reported. Suppressive effects of HCV proteins at every HCV region are summarized. DC, dendritic cells; IFN, interferon; IL, interleukin; IRF, interferon regulatory factor; MHC, major histocompatibility complex; NK, natural killer; Tr cells, regulatory T cells.

In a mouse model, the adoptive transfer of HCV NS3 protein-pulsed DC matured with an oligodeoxynucleotide containing cytidine-phosphate-guanosine motifs *ex vivo* effectively promoted potent HCV-specific protective immune responses.[69] Thus DC-based therapy may be a candidate for immune therapy for chronic HCV infection in the future.

Conclusions and future directions

Immune responses against HCV have been investigated by analyses of clinical samples and animal models. It has been clarified that cellular immune responses play an important role in the pathogenesis of hepatitis. The mechanisms of liver damage and inhibition of immune responses have also been investigated. The appropriate control of immune responses would contribute to the eradication of HCV and improvement of hepatitis, but there are still many issues to be clarified. Therapies capable of complete HCV eradication could be developed by detailed exploration of immunological mechanisms as well as the virology of HCV.

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Dendritic cells stimulated with cytidine-phosphate-guanosine oligodeoxynucleotides and interferon-α-expressing tumor cells effectively reduce outgrowth of established tumors *in vivo*

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Dendritic cells (DC) are potent antigen-presenting cells that elicit immune responses to foreign antigens. We have previously demonstrated the synergistic effects of cytidine-phosphate-guanosine (CpG) oligodeoxynucleotides (ODN) and interferon (IFN)-α on DC maturation *in vitro*. In the present study, the antitumor effects of DC preincubated with IFN-α gene-overexpressing murine colorectal cancer MC38 cells (MC38-IFN-α) and CpG ODN were evaluated in a poorly immunogenic murine cancer system. When we injected DC preincubated with MC38-IFNα and CpG ODN subcutaneously to mice bearing MC38 wild-type tumors, the outgrowth of the established parental tumors was suppressed significantly compared with that following administration of DC with MC38-IFN-α ($P = 0.008$). All mice injected with DC preincubated with MC38-IFN-α and CpG ODN rejected a subsequent parental tumor challenge. Immunohistochemical and flow cytometric analyses showed that CD4$^+$, CD8$^+$, and NK1.1$^+$ cells markedly infiltrated the established tumors of mice treated with DC preincubated with MC38-IFN-α and CpG ODN. From the results in immune cell-depleted mice, CD4$^+$ and asialo-GM-1$^+$ cells seemed to contribute to the antitumor effects induced by the combination DC therapy. Furthermore, non-specific cytolysis was detected when splenocytes of mice inoculated with DC preincubated with MC38-IFNα and CpG ODN were used as effector cells. Using an interleukin (IL)-12-neutralizing antibody it was suggested that IL-12 stimulates natural killer cells and contributes in part to the antitumor effects induced by DC incubated with CpG ODN and IFN-α. As DC-based immunotherapy with CpG ODN and IFN-α-expressing tumor cells induces a potent antitumor immune response, it should be considered for clinical application. (*Cancer Sci* 2008; 99: 1663–1669)

Dendritic cells are known as professional APC, characterized by their potent ability to activate and stimulate naive T lymphocytes *in vivo*. It has been hypothesized that the colocalization of DC and tumor cells may generate APC capable of stimulating tumor-reactive T cells *in vivo* because the histological infiltration of DC into primary tumors is associated with prolonged patient survival and reduces the incidence of metastatic disease in patients with bladder, lung, esophageal, gastric, and nasopharyngeal carcinoma.[1–5] Therefore, DC are thought to be attractive adjuvant agents for cancer therapy and a number of clinical studies, as well as immunotherapy using DC in murine models, have been carried out.

In the immature state, DC are able to capture and process antigens. Then, as they mature, they express surface molecules, including CD40, CD80, and CD86, that appear to play crucial roles in costimulating the activation and expansion of antigen-specific CD8$^+$ CTL, and they migrate to draining lymph nodes where they encounter and prime CD4$^+$ and CD8$^+$ T cells. Concomitantly, their capacity to acquire antigens is decreased. Therefore, to establish DC-based clinical therapy, the changing phenotype should be characterized in detail.

Microbial molecules such as lipopolysaccharides, bacteria-derived RNA, and DNA are recognized by host cells through the TLR family, which belongs to the family of pattern-recognition receptors[6] and stimulates immune responses. It has been reported that synthetic oligodeoxynucleotides (ODN) containing the unmethylated CpG motif promote Th1-type immune responses.[7] CpG ODN bind to TLR9 and are then endocytosed by DC. The signal transduction cascade in such DC ultimately leads to their activation with subsequent maturation into professional APC,[8] which have enhanced expression of costimulatory molecules such as CD80 and CD86 and produce high amounts of cytokines such as IL-12 and TNF-α. Because matured DC may improve the effects of therapy on established tumors, many investigations have been carried out in murine models as well as human clinical studies. CpG-based immunotherapy has demonstrated enhanced antitumor responses in murine models[9–12] and, indeed, CpG ODN has been applied to clinical cancer therapy for melanoma patients.[13]

Interferon-α has many biological effects, including enhancement of IFN-α/β production,[14,15] antiviral function, inhibition of cell growth, and angiogenesis.[16] IFN-α upregulates the expression of MHC class I on the cell surface and enhances the proliferation of Th1,[17] and is important for the generation of CTL in specific antitumor immune responses.[18,19] In addition, we reported previously that IFN-α-expressing tumor cells promote the survival of tumor-specific CTL by preventing apoptosis.[20] Based on these immunomodulating effects of IFN-α, it has been used to treat patients with tumors such as melanoma, renal cell carcinoma, and leukemia.

It has been reported that DC maturation, such as upregulation of costimulatory molecules (CD80, CD86), MHC class II, and CD83 expression on human DC, follows stimulation with IFN-α,[21] and in the presence of IFN-α it has been reported that DC show a greater capability to stimulate the proliferation of allogenic lymphocytes.[21] Furthermore, we reported that IFN-α gene therapy

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Abbreviations: APC, antigen-presenting cell; B6, C57BL/6; CM, complete medium; CpG, cytidine-phosphate-guanosine; CTL, cytotoxic T lymphocyte; DC, dendritic cell; DC + a, DC preincubated with MC38-IFN-α; DC + a + C, DC preincubated with MC38-IFN-α and CpG ODN-1826; DC + WT, DC preincubated with MC38-WT; DC + WT + C, DC preincubated with MC38-WT and CpG ODN-1826; ELISA, enzyme-linked immunosorbent assay; E : T, effector to target ratio; FITC, fluorescein isothiocyanate; IFN, interferon; IL, interleukin; MC38-IFN-α, IFN-α gene-overexpressing murine colorectal cancer MC38; MC38-WT, MC38 wild type; MHC, major histocompatibility complex; NK, natural killer; OCT, optimal clotting temperature; ODN, oligodeoxynucleotide; Th1, type I helper T cell; TLR, toll-like receptor; TNF, tumor necrosis factor; WT, wild type; YAC, yeast artificial chromosome.

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in combination with DC-based immunotherapy reduces the outgrowth of established tumors in a poorly immunogenic tumor model.[22] In that model, we demonstrated that CD4+, CD8+, and NK cells are involved in the antitumor effects induced by DC and IFN-α therapy. However, tumor regression was not observed in the parental tumor-bearing mice treated with DC and IFN-α, and it was considered that more effective treatment was required for clinical trials.

We previously demonstrated that coincubation with CpG ODN and IFN-α-overexpressing tumor cells, but not WT cells, effectively upregulated costimulatory molecules on the murine bone marrow-derived DC, and that CpG in combination with IFN-α effectively stimulated production by DC of cytokines such as IL-1β and TNF-α.[23] When DC that had been preincubated with CpG ODN and IFNα-overexpressing tumor cells were coincubated with allogenic splenocytes *in vitro*, proliferation of the splenocytes was enhanced significantly compared with those incubated with CpG ODN and WT cells. Because CpG ODN and IFN-α have synergistic effects on DC maturation, they are expected to induce potent antitumor immune responses, and should be considered for clinical application in combination therapy.

In the present study, a preliminary investigation of the combined therapy prior to clinical studies, the antitumor effects of DC preincubated with CpG and IFN-α-overexpressing murine colorectal cancer cells were evaluated in a poorly immunogenic murine cancer system. We showed that when parental tumor-bearing mice were injected with DC preincubated with CpG and IFN-α-overexpressing tumor cells outgrowth of the established parental tumor was suppressed significantly, and CD4+, CD8+, and NK1.1+ cells markedly infiltrated the established tumors of mice treated with DC preincubated with CpG and IFN-α-overexpressing tumor cells. Combined therapy with DC showed a profound preventive antitumor effect on the parental tumor. Furthermore, we confirmed that IL-12 production contributes in part to the antitumor effects induced by DC preincubated with CpG and IFN-α-overexpressing tumor cells.

Materials and Methods

Mice. Female 6-week-old B6 mice were purchased from Sankyo Laboratory Service (Tokyo, Japan) for use in experiments from 8 to 12 weeks of age. Mice were maintained in an animal care facility at Showa University. The present study has been approved by the Ethical Committee for Animal Experiments of Showa University.

Cell lines, culture medium, and reagents. The MC38 poorly immunogenic murine colorectal adenocarcinoma cell line (B6 mouse origin) was maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mmol/L L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, 10 mmol/L HEPES buffer, 1 mmol/L Minimum Essential Medium sodium pyruvate, and 0.1 mmol/L Minimum Essential Medium non-essential amino acids (CM) in a humidified incubator with 5% CO_2 in air at 37°C. All cell culture reagents were purchased from Life Technologies (Gaithersburg, MD, USA).

The MC38 cell line was genetically modified to produce murine IFN-α (MC38-IFNα) as described previously.[24] Expression of IFN-α was confirmed by ELISA using a commercially available kit according to the manufacturer's instructions (mouse IFN-α ELISA; PBL Biomedical Laboratories, New Brunswick, NJ, USA). Gamma-irradiation (100 Gy for tumor cells and 30 Gy for DC) was carried out with Gammacell 3000 Elan (Nordion International, Kanata, Canada). As reported previously, 1×10^5 cells of MC38-IFNα produce approximately 20.8 ± 0.5 ng/48 h of IFN-α, and wild type MC38 (MC38-WT) cells do not produce any IFN-α. IFN-α gene transduction does not affect the growth of tumor cells *in vitro* or survival of γ-irradiated tumor cells.[22]

Cytidine-phosphate-guanosine (CpG) ODN-1826 has been reported to have maturation effects on DC.[25] CpG ODN-1826 (5′-TCC ATG ACG TTC CTC ACG TT-3′) and ODN-1911 (served as the control, 5′-TCC AGG ACT TTC CTC AGG TT-3′, non-CpG) were synthesized by Sigma-Aldrich Japan (Tokyo, Japan). In our previous study, we confirmed that ODN-1911 does not affect DC maturation[23] or antitumor activity (data not shown).

Preparation of DC and incubation with CpG ODN and tumor cells. DC were generated from bone marrow cells of B6 mice using murine granulocyte macrophage-colony stimulating factor (GM-CSF) (10 ng/mL) and IL-4 (10 ng/mL), obtained from Pepro Tech EC (London, UK) as reported previously.[22] DC were preincubated with MC38-WT (DC + WT), MC38-IFNα (DC + a), CpG ODN-1826 and MC38-WT (DC + WT + C), or CpG ODN-1826 and MC38-IFNα (DC + a + C). CpG ODN-1826 was added at a concentration of 6 μg/mL, and all tumor cells were γ-irradiated (100 Gy) before incubation with DC at a DC to tumor ratio of 10.[23]

Therapeutic effects of DC and genetically modified MC38 cells on established parental tumors *in vivo*. To evaluate the therapeutic effects of DC incubated with CpG ODN and IFN-α-overexpressing MC38 cells on established parental tumors, we measured the size of established MC38-WT tumors in mice before and after treatment with these DC, as described previously.[24] B6 mice were injected subcutaneously with 1×10^5 MC38-WT cells in the right flank. Seven, 10, and 14 days after the WT-inoculation, 1×10^6 DC prepared as above (DC + WT, DC + a, DC + C, DC + WT + C, and DC + a + C) were inoculated subcutaneously around the established parental tumors, which had reached 9–25 mm^2 in size. Tumor size was measured twice a week using vernier calipers. Each experiment involved six mice per group. Mice with ulcerated tumors or tumors larger than 20 mm in diameter were killed.

Preventive effects of inoculation with DC with CpG ODN and MC38-IFN-α cells on the development of parental tumors *in vivo* after immune-cell depletion. To determine the role of the immune system in reduction of *in vivo* tumor growth in the preventive model, CD4+ T cells, CD8+ T cells, or asialo-GM-1+ cells were depleted by an antibody method as described previously.[26] Culture medium from hybridomas producing the following antibodies was used at appropriate concentrations: anti-CD4 (GK1.5, TIB207; American Type Culture Collection (ATCC) Manassas, VA, USA) and anti-CD8 (2.43, TIB210; ATCC). For depletion of asialo-GM-1+ cells, antiasialo-GM-1 antibody was obtained from Wako (Osaka, Japan). All antibody doses and treatment regimens were determined in preliminary studies using the same antibodies used for the experiments. Treatment was confirmed by flow cytometric analysis to completely delete the desired cell population for the entire duration of the study (data not shown). After DC with MC38-IFNα cells (DC + a) or DC with MC38-IFNα cells and CpG ODN-1826 (DC + a + C) were inoculated twice intraperitoneally at 7-day intervals (days −14 and −7), we depleted CD4+ T cells, CD8+ T cells, or asialo-GM-1+ cells (days −5, −4, and −3). Otherwise, rat IgG (obtained from Wako) was inoculated as a control on the same days. In another experiment, depletion of both CD4+ and asialo-GM-1+ cells was carried out to evaluate additional suppressive effects on antitumor immunity. One week after the vaccination (day 0), these mice were injected subcutaneously with 1×10^5 MC38-WT cells in the right flank. Tumor size was measured twice a week using vernier calipers. On day 35, tumor-free mice were reinjected subcutaneously with 3×10^5 MC38-WT cells, then tumor establishment was observed. Each experiment involved five or six mice per group. Mice with ulcerated tumors or tumors larger than 20 mm in diameter were killed.

Immunohistological analysis. B6 mice were injected subcutaneously in the right flank with 1×10^5 MC38-WT cells. On days 7, 10, and 14, 1×10^6 DC prepared as above (DC + WT, DC + a,

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DC + CC, DC + WT + C, DC + a +C) were inoculated around the established parental tumors. Tumor tissues were harvested 4 days after the last inoculation (18 days after WT inoculation), and were embedded immediately in optimal clotting temperature compound (Tissue Tek, Elkhart, IN) and frozen. Serial 5-μm sections were exposed to anti-CD4, CD8a, and CD11c antibodies (Nippon Becton Dickinson, Tokyo, Japan). Rat IgG2a (Nippon Becton Dickinson) was used as a control antibody. Immunostaining was completed with a Vectastain ABC kit (Vector, Burlingame, CA, USA). Immunoreactive cells were counted in 10 fields using light microscopy (×400) in a blinded fashion. Each experiment involved two mice per group.

Analysis of the role of IL-12 and NK1.1⁺ cell infiltration in established parental tumors of mice treated with DC preincubated with CpG ODN and MC38-IFN-α. To explore the antitumor mechanism of DC preincubated with CpG ODN and MC38-IFN-α, we neutralized IL-12 using monoclonal antibody (C17.8; Nippon Becton Dickinson), and assessed the NK-cell infiltration of the tumors. B6 mice were injected subcutaneously in the right flank with 2×10^5 MC38-WT cells. On days 7, 10, and 14, 2×10^6 DC incubated with MC38-IFN-α and CpG ODN (DC + a + C) supplemented with 100 μg neutralizing rat antimouse IL-12 antibody[27] or control rat IgG (Nippon Becton Dickinson) were inoculated intraperitoneally. Tumor size was measured twice a week using vernier calipers. Each experiment involved five mice per group. Mice with ulcerated tumors or tumors larger than 20 mm in diameter were killed. Tumor tissues were harvested 4 days after the third inoculation (18 days after WT inoculation). Tumor-infiltrating mononuclear cells were separated from tumor tissue as described previously.[26] The cells were washed three times with CM. Flow cytometric analyses were carried out using FACScalibur (Nippon Becton Dickinson) to investigate the phenotype of the tumor-infiltrating mononuclear cells. The monoclonal antibodies used in this analysis were fluorescein isothiocyanate-conjugated anti-CD4, CD8, and NK1.1 antibodies (Nippon Becton Dickinson).

Evaluation of cytolytic activity of splenocytes from mice immunized with DC preincubated with CpG ODN and MC38-IFN-α cells by stimulation *in vitro*. Mice received injections of 1×10^6 DC preincubated with MC38-IFN-α (DC + a) or with MC38-IFN-α and CpG ODN (DC + a + C) on days 0 and 7. One week later, splenocytes were harvested and 2×10^6 splenocytes were incubated in the presence of 100 IU/mL recombinant mouse IL-2 (Nippon Becton Dickinson). Seven days later, cytolytic assays were carried out using the splenocytes as effector cells to assess non-specific cytolytic activity. For evaluation of cytolytic activity against MC38-WT cells, splenocytes from the immunized mice were stimulated three times weekly *in vitro* with 30 Gy γ-irradiated DC + a or DC + a + C, as described previously.[22] One week later, the stimulated cells were harvested and used as effector cells.

Cytolytic assays. Tumor-stimulated effector cells were assessed for cytolytic activity against MC38-WT and YAC-1 cells in triplicate in a 4-h ^{51}Cr-release assay. Target cells (1×10^6 cells/mL) were labeled with 3.7×10^6 Bq of $Na_2{}^{51}CrO_4$ (Amersham Pharmacia Biotech, Tokyo, Japan) for 1 h at 37°C. Labeled cells were washed and resuspended. Target cells (5×10^3) and various numbers of effector cells at the indicated E : T were plated in 200 μL CM in each well of the 96-well round-bottom plates. ^{51}Cr release was measured after a 4-h incubation at 37°C. Percentage lysis was determined using the formula:

$$(\text{release in assay} - \text{spontaneous release}) \times 100/(\text{maximum release} - \text{spontaneous release}).$$

Maximum release was determined by lysis of labeled target cells with 1% Triton X-100. Spontaneous release was measured by incubating target cells in the absence of effector cells, and was less than 15% of maximum release.

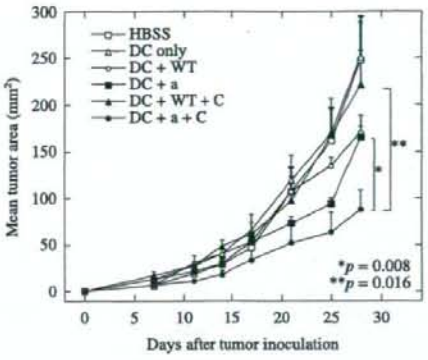


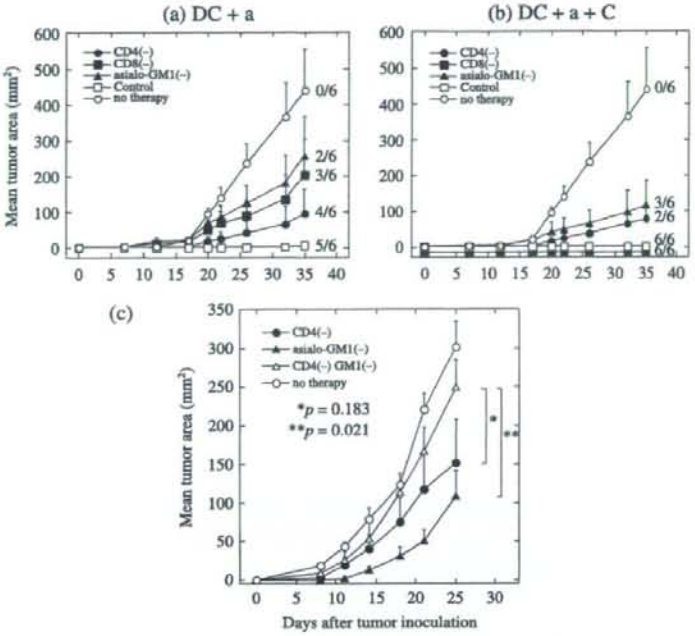
Fig. 1. Dendritic cells (DC) incubated with interferon (IFN)-α gene-overexpressing murine colorectal cancer MC38 (MC38-IFN-α) and cytidine-phosphate-guanosine (CpG) effectively suppressed established wild-type (WT) tumors. B6 mice were injected subcutaneously with 1×10^5 MC38-WT cells in the right flank. Seven, 10, and 14 days after the WT inoculation, 1×10^6 DC incubated with or without tumor cells and/or CpG oligodeoxynucleotides (ODN) were inoculated around the established parental tumors, which had reached 9–25 mm² in size. Tumor size was measured twice a week using vernier calipers. Each experiment involved six mice per group. The experiment was repeated twice and a representative result is shown. Results are shown as mean tumor area + SEM. DC + WT, DC incubated with MC38-WT; DC + a, DC incubated with MC38-IFNα; DC + WT + C, DC incubated with MC38-WT and CpG ODN; DC + a + C, DC incubated with MC38-IFN-α and CpG ODN; HBSS, Hank's balanced salt solutions.

Statistical analyses. Significance was assessed with Student's *t*-test or Wilcoxon's analysis. Differences between groups were considered significant when the *P*-value was lower than 0.05.

Results

Dendritic cells incubated with CpG ODN and IFN-α-overexpressing tumor cells effectively suppressed outgrowth of the established WT tumors. In our previous study, we demonstrated that DC maturation was effectively enhanced by coincubation with CpG ODN and IFN-α-overexpressing tumor cells *in vitro*.[23] To assess the antitumor effects of these DC *in vivo*, DC incubated with CpG ODN and MC38-IFN-α were injected into mice with established WT tumors. As shown in Figure 1, outgrowth of the established tumors was suppressed significantly by treatment with DC preincubated with CpG ODN and MC38-IFN-α (DC + a + C) compared with treatment with DC preincubated with MC38-IFN-α (DC + a) (DC + a + C vs DC + a, $P = 0.008$). In addition, we did not observe any therapeutic benefits of DC preincubated with MC38-WT and CpG ODN (DC + WT + C) in suppressing the outgrowth of parental tumors (Hank's balanced salt solutions (HBSS) vs DC + WT + C, $P = 0.663$; DC + a + C vs DC + WT + C, $P = 0.016$). Furthermore, of the 12 mice treated with DC + a + C, we observed regression of the parental tumor size in two mice.

CD4⁺ and asialo-GM-1⁺ cells were involved in the antitumor effects induced by DC incubated with CpG ODN and IFN-α-overexpressing tumor cells. DC in combination with IFN-α-overexpressing tumor cells have been proven to effectively suppress outgrowth of established WT tumors.[22] To clarify the different antitumor mechanisms in DC preincubated with MC38-IFN-α (DC + a) and DC preincubated with MC38-IFN-α and CpG (DC + a + C), we injected MC38-WT and observed tumor growth after treatment and depletion of immune cells. In mice treated with DC + a, when CD4⁺, CD8⁺, or asialo-GM-1⁺ cells were depleted by monoclonal antibodies, obvious tumor growth was observed in some



(d)

Therapy	CD4(–)	CD8(–)	Asialo-GM1(–)	Control	No therapy
DC + a	2/4	0/3	2/2	4/5	0/0
DC + a + C	2/2	0/6	3/3	6/6	0/0

Fig. 2. CD4⁺ and asialo-GM-1⁺ cells are involved in the antitumor effects induced by dendritic cells (DC) incubated with cytidine-phosphate-guanosine (CpG) oligonucleotides (ODN) and interferon (IFN)-α-overexpressing tumor cells. After (a) DC with IFN-α gene-overexpressing murine colorectal cancer MC38 (MC38-IFN-α) cells (DC + a) or (b) DC with MC38-IFN-α cells and CpG ODN-1826 (DC + a + C) were inoculated intraperitoneally twice at 7-day intervals (days –14 and –7), CD4⁺ T cells, CD8⁺ T cells, or asialo-GM-1⁺ cells were depleted on days –5, –4, and –3. Otherwise, rat IgG was inoculated as a control on the same days. (c) In another experiment, depletion of both CD4⁺ cells and asialo-GM-1⁺ cells was carried out to evaluate the additional suppressive effects on antitumor immunity. One week after the vaccination (day 0), these mice were injected with 1×10^5 MC38-WT cells in the right flank. Tumor size was measured twice a week using vernier calipers. The number of tumor-free mice on day 35 is also shown. (d) Numbers of tumor-free mice treated with DC + a or DC + a + C after the second parental tumor cell challenge (on day 70). On day 35, tumor-free mice were reinjected with 3×10^5 MC38-WT cells, and then tumor establishment was observed, and the numbers of tumor-free mice on day 70 are shown in the panel. Each experiment involved five or six mice per group.

Table 1. Immunohistochemical analyses of tumor-infiltrating immune cells after treatment with dendritic cell (DC), interferon (IFN)-α gene-overexpressing murine colorectal cancer MC38 (MC38-IFN-α), and cytidine-phosphate-guanosine (CpG)

Treatment	CD4⁺ cells	CD8⁺ cells	CD11c⁺ cells
DC only	4.2 ± 1.6	2.4 ± 1.1	6.0 ± 2.4
DC + WT	9.8 ± 3.5	0.6 ± 0.9	4.6 ± 3.4
DC + a	62.6 ± 31.9	44.2 ± 13.3	14.2 ± 11.1
DC + C	4.4 ± 2.9	0.0 ± 0.0	0.4 ± 0.55
DC + WT + C	6.4 ± 3.8	21.4 ± 4.1	0.0 ± 0.0
DC + a + C	94.6 ± 59.6	98.8 ± 79.6	44.8 ± 37.5
HBSS (control)	10.0 ± 7.1	3.6 ± 3.1	7.0 ± 14.6

Results are shown as mean counts ± SD of each cell in 10 fields using light microscopy (×400) in a blinded fashion. Each experiment involved two mice per group. DC + WT, DC incubated with MC38-WT; DC + a, DC incubated with MC38-IFNα; DC + C, DC incubated with CpG ODN; DC + WT + C, DC incubated with MC38-WT and CpG ODN; DC + a + C, DC incubated wit MC38-IFNα and CpG ODN. HBSS, Hank's balanced salt solutions.

mice of each group (Fig. 2a). In contrast, we did not detect any tumors in mice treated with DC + a + C when CD8⁺ cells were depleted (Fig. 2b). In other words, CD4⁺ and asialo-GM-1⁺ cells may be involved in the antitumor effects induced by DC + a + C, whereas CD8⁺ cells did not seem to be critical for the effects.

When both anti-CD4 antibody and anti-asialo GM-1 antibody were injected (Fig. 2c), the antitumor immunity was reduced significantly compared with administration with only anti-asialo GM⁻¹ antibody ($P = 0.021$). The mean parental tumor size of mice injected with anti-CD4 antibody was smaller than that of mice injected with both antibodies, although it did not reach a significant difference ($P = 0.183$). Thus, an additional suppressive effect of both antibodies on antitumor immune responses induced by the combined DC therapy was observed.

To evaluate the prolonged antitumor activity induced by the combined therapy, tumor-free mice were reinjected subcutaneously with 3×10^5 MC38-WT cells, and then tumor establishment was observed. As shown in Figure 2d, all six mice treated with DC + a + C rejected the subsequent parental tumor cell challenge, as did four of five mice treated with DC + a. In addition, all six mice treated with DC + a + C following injection with anti-CD8 antibody rejected the first parental tumor cell challenge, but obvious tumors were observed in all of these mice after rechallenge of the parental tumor cells. In three of six mice that were vaccinated with DC + a + C following depletion of asialo-GM-1⁺ cells, no obvious tumors were observed, and all three tumor-free mice rejected a subsequent challenge of the parental tumor cells. These results suggest that CD8⁺ cells are concerned with maintaining long-lasting antitumor immunity.

CD4⁺, CD8⁺, and asialo-GM-1⁺ cells markedly infiltrated the established parental tumors of mice treated with DC preincubated with CpG ODN and IFN-α-overexpressing tumor cells. To analyze the antitumor mechanisms induced by DC preincubated with MC38-IFN-α and CpG (DC + a + C), we carried out immunohistochemical staining using WT tumor tissue of mice treated with the DC therapy. The results showed that CD4⁺, CD8⁺, and CD11c⁺ cells clearly infiltrated the WT tumors of mice treated with DC + a (Table 1). Furthermore, extreme infiltration of these

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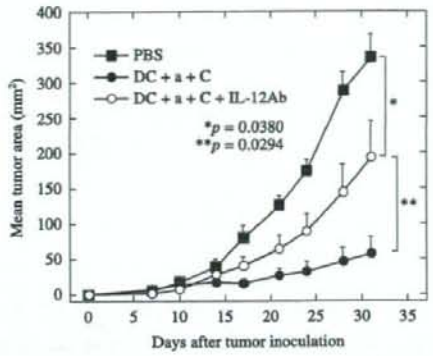

Fig. 3. Decreased antitumor effects of mice treated with dendritic cells (DC) incubated with cytidine-phosphate-guanosine (CpG) oligonucleotides (ODN) and interferon (IFN)-α-overexpressing tumor cells after neutralization of interleukin (IL)-12. B6 mice were injected in the right flank with 2×10^5 MC38-WT cells. On days 7, 10, and 14, 2×10^6 DC incubated with MC38-IFN-α and CpG ODN together with 100 μg IL-12-neutralizing antibody or control IgG were inoculated intraperitoneally. Tumor size was then measured twice a week using vernier calipers. Each experiment involved five mice per group. PBS, phosphate-buffered saline.

Table 2. Comparison of tumor-infiltrating immune cells after dendritic cell (DC) therapy with interferon (IFN)-α gene-overexpressing murine colorectal cancer MC38 (MC38-IFN-α), and cytidine-phosphate-guanosine (CpG) with or without anti-interleukin (IL)-12-neutralizing antibody

Treatment	$CD4^+$ cells	$CD8^+$ cells	$NK1.1^+$cells
Control IgG	38.02 ± 2.04	33.02 ± 6.73	19.90 ± 1.15
IL-12 antibody	38.91 ± 0.38	20.33 ± 0.12	11.23 ± 2.74

B6 mice were injected subcutaneously in the right flank with 2×10^5 MC38-WT cells. On days 7, 10, and 14, 1×10^6 DC incubated with MC38-IFN-α and CpG ODN, with 100 μg IL-12-neutralizing antibody or with control IgG were inoculated. Tumor tissues were harvested 4 days after this third inoculation (18 days after wild-type inoculation). Tumor-infiltrating mononuclear cells were separated from tumor tissue. Flow cytometric analyses were carried out using fluorescein isothiocyanate-conjugated anti-CD4, -CD8, or -NK1.1 antibodies to investigate the phenotype of tumor-infiltrating mononuclear cells. The results are shown as mean percentage positive cells ± SD.

cells was observed in the parental tumors of mice treated with DC + a + C (Table 1). These results suggest that the antitumor immune response would be activated effectively by DC preincubated with CpG and IFN-α-overexpressing tumor cells.

Decreased antitumor effects of mice treated with DC incubated with CpG ODN and IFN-α-overexpressing tumor cells after neutralization of IL-12. As shown previously, IL-12 production by DC was enhanced markedly by the addition of CpG ODN.[23] We carried out ELISA and detected 2.99 ± 0.15 ng IL-12 production by 1×10^6 mature DC incubated with CpG and IFN-α-overexpressing tumor cells. We evaluated the relationship between IL-12 and the antitumor effects of the DC therapy using neutralizing antibody against mouse IL-12. As shown in Figure 3, the outgrowth of WT tumors increased significantly in mice treated with DC + a + C together with injected IL-12 neutralizing antibody ($P = 0.029$) compared with control antibody, whereas the tumor size was still clearly smaller than the parental tumor of mice with no treatment (phosphate-buffered saline (PBS); $P = 0.038$). Because the antitumor effects of DC incubated with MC38-IFN-α and CpG ODN were partially abrogated by neutralization of IL-12, this cytokine contributes in part to the effects.

Decreased tumor-infiltrating $CD8^+$ and $NK1.1^+$ cells in the established parental tumors of mice treated with DC preincubated with CpG ODN and IFN-α-overexpressing tumor cells after neutralization of IL-12. We analyzed tumor-infiltrating NK cells of mice treated with the DC therapy because we had found previously that DC in combination with IFN-α-overexpressing tumor cells recruits many NK cells in tumors.[22] As shown in Table 2, we detected a high level of $CD8^+$ and $NK1.1^+$ cells in the parental tumors of mice treated with DC preincubated with MC38-IFN-α and CpG ODN. When anti-IL-12 neutralizing antibody was injected into mice treated with these DC, the levels decreased. However, the amount of intrahepatic $CD4^+$ cells in mice injected with anti-IL-12 neutralizing antibody was almost the same as that of mice injected with control antibody. These data suggest that IL-12 produced by the injected mature DC stimulates $CD8^+$ and NK cells *in vivo* and contributes to the antitumor effect of the combined DC therapy.

Cytolytic activity of splenocytes obtained from mice treated with DC incubated with CpG ODN and IFN-α-overexpressing tumor cells. To evaluate the cytolytic activity of mice treated with the DC therapy *in vivo*, splenocytes obtained from mice treated with DC incubated with MC38-IFN-α and CpG ODN were examined with a 4-h ^{51}Cr-release assay against YAC-1 cells, which are known to be sensitive to NK cells. As shown in Figure 4a, meaningful

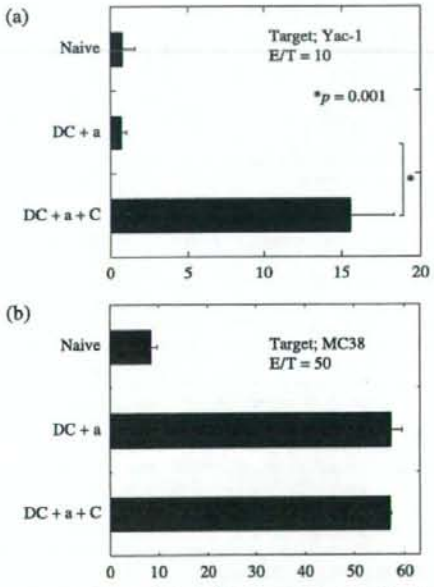

Fig. 4. Non-specific cytolytic activity was detected clearly when splenocytes obtained from mice treated with dendritic cells (DC) incubated with cytidine-phosphate-guanosine (CpG) oligonucleotides (ODN) and interferon (IFN)-α-overexpressing tumor cells were used as effector cells. Mice received injections of 1×10^6 DC preincubated with IFN-α gene-overexpressing murine colorectal cancer MC38 (MC38-IFN-α) (DC + a) or with MC38-IFN-α and CpG ODN (DC + a + C) on days 0 and 7. (a) To assess non-specific cytolytic activity, splenocytes were harvested and 2×10^6 splenocytes were incubated in the presence of 100 IU/mL recombinant mouse interleukin (IL)-2. Seven days later, a ^{51}Cr-release assay was carried out using the splenocytes as effector cells. The results are shown as mean percentage cytolysis against YAC-1 cells + SD. (b) For evaluation of cytolytic activity against MC38-WT cells, splenocytes from the immunized mice were stimulated three times weekly *in vitro* with 30 Gy γ-irradiated DC + a or DC + a + C. One week later, the stimulated cells were harvested and used as effector cells. The results are shown as mean percentage cytolysis against MC38-WT cells + SD.

cytolysis against YAC-1 cells was detected only with splenocytes from mice treated with DC preincubated when MC38-IFN-α and CpG ODN (% cytolysis = 15.5 ± 2.9, E : T = 10), and not with DC preincubated with MC38-IFN-α (% cytolysis = 0.7 ± 0.4, E : T = 10), were used as effector cells ($P = 0.001$). This result suggests that NK cells are activated *in vivo* by DC incubated with IFN-α-overexpressing tumor cells and CpG ODN.

We induced MC38-specific CTL using splenocytes of tumor-free mice treated with DC + a or DC + a + C by following repetitive *in vitro* stimulation with DC + a or DC + a + C, respectively, as reported previously.[22] Although we detected cytolytic activity against MC38 cells, there was no difference in tumor-specific cytotoxicity against MC38-WT cells between DC + a-treated mice (% cytolysis = 57.5 ± 2.1, E : T = 50) and DC + a + C-treated mice (% cytolysis = 57.1 ± 0.4, E : T = 50) (Fig. 4b). When splenocytes from mice treated with DC + a or DC + a + C twice weekly, but without any rechallenge of WT tumor cells *in vivo*, were stimulated with DC + a or DC + a + C *in vitro*, the cytotoxic activity against MC38 was 1.651% or 4.875%, respectively (E : T = 40). We could not detect any differences of CTL cytotoxicity against MC38-WT cells between them at any time points.

Discussion

We have demonstrated that DC preincubated with CpG and IFN-α-overexpressing tumor cells significantly suppress the outgrowth of an established parental tumor, and that the therapy recruits $CD4^+$ cells, $CD8^+$ cells, DC, and $NK1.1^+$ cells in the established tumors. The combined DC-based therapy showed a profound preventive antitumor effect on the parental tumor. IL-12 and NK cells contributed to the antitumor effects induced by DC preincubated with CpG and IFN-α-overexpressing tumor cells.

Dendritic cell therapy has been tried for patients with advanced tumors such as melanoma and renal cancer. However, the outcomes have been unsatisfactory; for example, the DC used in the clinical trials are not uniform, and it remains to be clarified which type of DC, mature or immature, is suitable for clinical cancer therapy. Mature DC would induce and activate antitumor immune cells more effectively than immature DC, but their phagocytosis and migration abilities diminish as they mature. DC that do not perceive an activating environment do not mature, and they induce tolerance rather than immunity.[28] As we revealed that mature DC stimulated by IFN-α-overexpressing tumor cells have potent antitumor activity *in vivo*,[22] we tried to induce more mature DC by CpG ODN, which is thought to stimulate DC through TLR9. Moreover, our previous study demonstrated that IFN-α-overexpressing tumor cells in combination with CpG ODN markedly induce DC maturation phenotypically as well as functionally, such as cytokine production and proliferative effects on allogenic lymphocytes.[23] Therefore, in anticipation of further antitumor effects of the DC therapy, we evaluated the therapeutic inoculation of more mature DC incubated with IFN-α-overexpressing tumor cells and CpG ODN.

In our previous study, IFN-α-overexpressing tumor cells, but not the parental tumor cells with exogenous IFN-α, promoted a tumor-specific immune response *in vivo*.[20,22] This suggested that a continuous source of IFN-α would be crucial for the induction and maintenance of immune responses. Therefore, IFN-α gene-transduced cells were used in our studies although establishment of the transduced cells, which produce a high amount of IFN-α, would be difficult in the clinical setting. We are now planning to use allogenic IFN-α-overexpressing tumor cell lines to treat hepatocellular carcinoma-bearing mice and evaluate the antitumor effects.

No tumors were observed in mice that were vaccinated with DC, CpG ODN, and MC38-IFN-α cells, and then depleted of $CD8^+$ cells following parental tumor cells, suggesting that $CD8^+$ cells do not contribute to the antitumor immune responses induced by the DC therapy with IFN-α-overexpressing tumor cells and CpG ODN. However, other results did not support this hypothesis. Cytolytic activity against MC38-WT cells was detected clearly in mice treated with DC, CpG ODN, and MC38-IFNα cells. All $CD8^+$ cell-depleted mice had the parental tumor after subsequent inoculation of MC38-WT. In addition, we did not detect any obvious tumors in three of six mice that had been vaccinated with DC, CpG ODN, and MC38-IFNα cells, and following depletion of asialo-GM-1^+ cells, and all three of these mice rejected a subsequent challenge of parental tumor cells. In these mice, a long-lasting antitumor immune response would be induced, and effector cells, which diminish tumor cells directly, would be $CD8^+$ cells. All $CD8^+$ cell-depleted mice were tumor-free because of potent activation of NK cells induced by the combined DC therapy, as NK cells exterminate the parental tumor cells without $CD8^+$ cells. In the previous study, we demonstrated the potent activation of tumor-specific $CD8^+$ cells as well as non-specific NK cells *in vivo* by DC with IFN-α therapy.[22] In addition, it was reported that peptide-coated DC generated memory $CD8^+$ T cells within 4–6 days, and that coinjection of CpG ODN with those DC prevented the rapid generation of memory T cells in an IFN-γ-dependent manner.[29] Thus, NK cells may mainly contribute to the antitumor effects induced by the DC-based therapy with CpG ODN in the early stage. From these phenomena, we speculate that potent activity of NK cells would reject the parental tumor cells in the $CD8^+$ cell-depleted mice, whereas stimulated $CD8^+$ cells could eliminate the tumor cells in some asialo-GM-1^+ cell-depleted mice.

The results of an IL-12 neutralization model showed that IL-12 plays a partial role in the antitumor activity induced by DC therapy with IFN-α-overexpressing tumor cells and CpG ODN. IL-12 was identified as a NK cell stimulatory factor, and was characterized as a heterodimeric cytokine with multiple biological effects on T and NK cells.[30,31] IL-12 is produced mainly by APC such as macrophages and DC following activation.[32] In the present study, not only the injected matured DC but also host DC stimulated with the therapy would produce IL-12, and then IL-12 might activate NK cells as well as $CD4^+$ and $CD8^+$ T cells. We observed partial inhibition of the antitumor effects induced by DC with IFN-α and CpG ODN by neutralization of IL-12. Further antitumor mechanisms other than IL-12 should exist in the combined DC therapy, although it is possible that the dose of IL-12 neutralizing antibody was insufficient because a high amount of IL-12 might be produced by the host immune cell stimulated with the treatment.

It has been reported that conventional CpG DNA, phosphorothioate-modified ODN called CpG-B, induce splenic B cell proliferation, DC maturation, and cytokine production from a variety of immune cells.[33,34] TLR9 is expressed essentially on human plasmacytoid DC, but not on human myeloid DC. However, it has been reported that TLR9 is expressed by all subsets of murine DC[35] and that CpG ODN can stimulate not only splenic but also bone marrow-derived myeloid DC through TLR9.[36] Our results support these previous observations that CpG ODN enhances murine bone marrow-derived DC maturation. However, as myeloid DC do not express TLR9 in humans, CpG ODN would not affect DC maturation effectively and might not induce antitumor effects as observed in this murine model. Therefore, further modifications such as OK-432, which is a potent stimulator to myeloid DC[37] and has indeed been used in clinical cancer therapy, are required for clinical application.

Here, we have demonstrated that DC preincubated with IFN-α-overexpressing tumor cells and CpG ODN have therapeutic benefits in suppressing parental tumor growth compared with DC preincubated with the parental tumor cells and CpG ODN, and with DC preincubated with IFN-α-overexpressing tumor cells. Thus, CpG ODN and IFN-α-overexpressing tumor cells have additive effects on DC-based immune therapy for tumors. To the

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best of our knowledge, this is the first demonstration of additive antitumor effects of IFN-α and CpG ODN on DC-based therapy *in vivo*. Our findings suggest that DC-based immunotherapy in combination with CpG ODN and IFN-α gene therapy has potential for inducing potent immune responses and that it would be applicable for clinical antitumor therapy, although further investigations are required.

Acknowledgments

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A New Prognostic System for Hepatocellular Carcinoma Including Recurrent Cases

A Study of 861 Patients in a Single Institution

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Objective: To manage hepatocellular carcinoma (HCC) patients surviving for a long term, the treatment strategy for recurrent cancer is as important as that for the initial treatment. However, no prognostic scoring system has been available for patients with HCC recurrence. The purpose of this study was to develop a new staging system for deciding the treatment strategy not only for first-time diagnosed patients but also for recurrent patients.

Methods: A total of 861 cases diagnosed at our single institution from 1993 to 2003 were included. Overall survival was the only end point. The Cox model was used for multivariate analyses.

Results: As of August 2004, 344 cases (59%) had died. Overall median survival time was 41 months. For multivariate Cox regression analysis, independent predictive factors of survival were the number of recurrences, the Child-Pugh score, 3 nodules less than 3 cm and none of vascular invasion, and the α-fetoprotein level. A simple scoring system was thus developed, assigning scores (0/1) to the 4 covariates of the final model. Compared with the other scoring systems, the new scoring system has a greater discriminant ability.

Conclusions: We concluded that our scoring system can serve as a new prognostic system that reflects the spread of HCC, treatment response, and liver function. It should be very useful as the only method which can be applied for patients with recurrence.

Key Words: hepatocellular carcinoma, recurrence, staging system, predictive factor, cox regression analysis

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Recently, various nonsurgical treatment modalities for hepatocellular carcinoma (HCC) have been developed and surgical techniques have been also improved.[1,2] However, HCC with cirrhosis remains one of the diseases that is extremely difficult to manage, because survival in HCC is not predominantly based on the biology of the tumor, but also on the underlying hepatic function. Actually, we need consider 2 distinctive features in planning the HCC treatment from other cancers. First, even if HCC can be completely treated, the residual cirrhotic liver displays a high risk of recurrence, including new primary cancers.[3–5] Second, most options for the treatment of HCC lead to a decrease in the reserved hepatic function. In other words, they take the risk of future liver failure in return for HCC treatment. Taken together, the complexity of these factors makes HCC management difficult.

The prognosis of HCC patients is highly variable and hard to predict, which makes it difficult to effectively treat patients or to design good clinical trials. To provide guides for assessing disease severity and making therapeutic decisions, several staging or prognostic scoring systems for HCC have been proposed: the Cancer of the Liver Italian Program (CLIP) score,[6] BCLC staging,[7] and Japan Integrated Staging (JIS) scoring system,[8] which were produced on the basis of prognostic values. These staging systems can be used for assessing the prognosis of HCC patients as well as the efficiency of therapeutic modalities. Although these systems may be useful for predicting the prognosis of HCC patients at the time of the initial treatment,[9–11] there is considerable doubt about whether these systems are suitable for cases of recurrent cancer, because they cannot distinguish HCC diagnosed for the first time from recurrent HCC. In clinical practice, recurrent HCC patients are encountered 2.5 times more frequently in our institution than first-time HCC patients. Because the development of screening and follow-up programs and the improvement of radiologic techniques have facilitated the recognition of HCC at an earlier

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

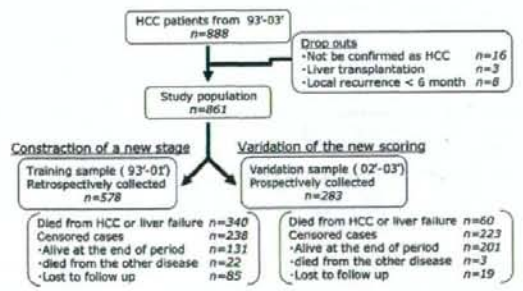

FIGURE 1. Schematic overview of included patients and dropouts for training and validation sample.

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.

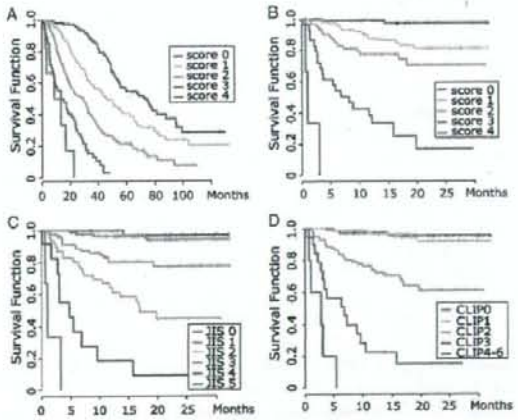

FIGURE 2. Kaplan-Meier–estimated survival curves. A, By our new scoring system in training samples. B, By our new scoring system in validation samples. C, By the CLIP score system in validation samples. D, By the JIS score system in validation samples.

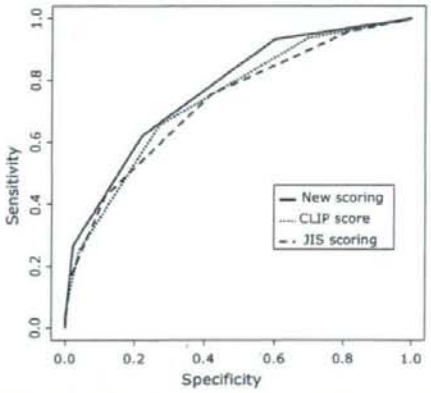

FIGURE 3. Discriminatory ability for the prediction of death at 3 years, evaluated by receiver operating characteristics curves of the new scoring, CLIP, and JIS staging systems.

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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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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