Table 2 Preventive effects of inoculation with DCs and the genetically modified MC38 cells on development of the parental MC38 tumor

Preventive inoculation	*Number of tumor-free mice*[a]	*P-value of tumor area*[b]
Splenocytes	1/17	<0.001
Splenocytes+MC38-Neo	3/17	0.004
Splenocytes+MC38-IFN-α	9/17	0.471
DC	1/17	<0.001
DC+MC38-Neo	3/17	0.008
DC+MC38-IFN-α	12/17	—

B6 mice were inoculated i.p. twice weekly (days −14 and −7) with 1×10^6 DCs or splenocytes with or without the genetically modified MC38 cells. At 7 days after the last injection (day 0), these mice received subsequent injection of 1×10^5 WT tumor cells. Results are reported as numbers of tumor-free mice on day 27 and as *P*-value of tumor area on day 27. Five or six mice per group were inoculated and the experiment was performed three times.
[a]Total numbers of tumor-free mice in three separate experiments.
[b]*P*-values of tumor area (mm^2) in DC+MC38-IFN-α group against that in each group in a representative experiment calculated with Wilcoxon's analysis.

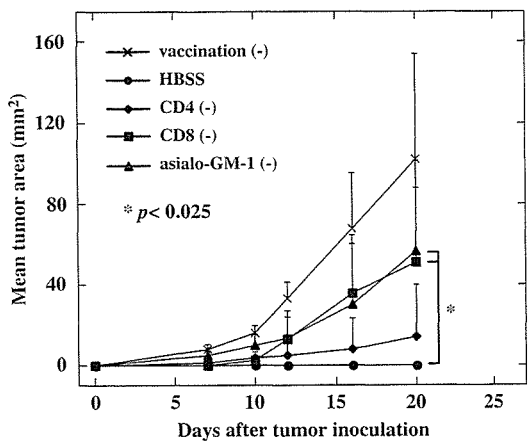

Figure 3 Antitumor effects of DCs and MC38-IFN-α cells in immune cell-depleted mice. $CD4^+$ T cells, $CD8^+$ T cells, or asialo-GM-1^+ cells were depleted by an antibody method as described in the Materials and methods section. After DCs and MC38-IFN-α cells were inoculated i.p. twice at a 7-day interval (days −14 and −7), we depleted $CD4^+$ T cells, $CD8^+$ T cells, or asialo-GM-1^+ cells (days −5, −4, and −3). At 1 week after the vaccination (day 0), the mice were injected s.c. with 1×10^5 MC38-WT cells in the right flank. Each experiment involved five mice per group. Tumor size was measured twice a week using vernier calipers. Results are reported as mean tumor area (mm^2)+s.e. The groups revealed in this figure are as follows: vaccination(−), without vaccination; HBSS, any immune cells were not depleted; CD4(−), $CD4^+$ T cells were depleted; CD8(−), $CD8^+$ T cells were depleted; and asialo-GM-1(−), asialo-GM-1^+ cells were depleted.

in Figure 4a, DCs+γ-irradiated MC38-IFN-α significantly suppressed the outgrowth of the tumors compared with splenocytes alone ($P = 0.037$). However, there was no difference between the DCs+γ-irradiated MC38-IFN-α-treated group and DCs+γ-irradiated MC38-Neo-treated group. Next, we used nonirradiated MC38 cells in anticipation of further therapeutic efficacy. DCs+non-

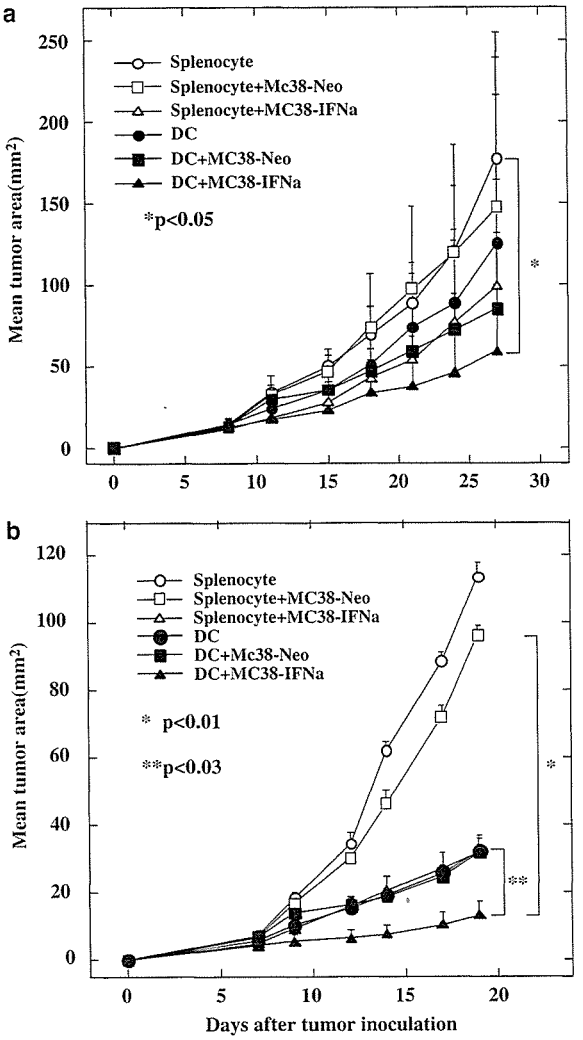

Figure 4 Therapeutic inoculation of DCs and MC38-IFN-α cells suppresses outgrowth of established parental MC38 tumors. Therapeutic inoculation of both DCs and MC38-IFN-α cells has synergistic antitumor effects on established parental MC38 tumor. B6 mice were injected s.c. in the right flank with 1×10^5 MC38-WT cells. At 7 and 14 days after WT inoculation, 1×10^6 DCs (or splenocytes) and (a) 1×10^5 γ-irradiated (100 Gy) genetically modified MC38 tumor cells or (b) the MC38 cells without γ-irradiation were inoculated around the established parental tumors. Each experiment involved six mice per group. Results are reported as mean tumor area (mm^2)+s.e. This experiment was performed two times with similar results.

irradiated MC38-Neo or splenocytes+nonirradiated MC38-IFN-α clearly suppressed the outgrowth of the established tumors compared with splenocytes alone as shown in Figure 4b. Moreover, DCs+nonirradiated MC38-IFN-α had further suppressive effects on the outgrowth of the established tumors compared with DCs+MC38-Neo or splenocytes+MC38-IFN-α ($P = 0.016$ or 0.024, respectively). Use of nonirradiated MC38-IFN-α cells had an advantage over irradiated MC38-IFN-α cells in therapy for the established parental tumors. These results suggest the presence of synergistic antitumor effects in the combination of DC-based immunotherapy and IFN-α gene therapy on established parental tumors.

Table 3 Immunohistologic analysis of established parental tumors of mice treated with DCs and genetically modified MC38 cells

(A) Treatment	*CD4⁺ cells*	*CD8⁺ cells*	*CD11c⁺ cells*
Splenocyte	5.2±1.1	3.8±0.6	6.2±2.6
Splenocytes+MC38-Neo	9.5±1.6	9.9±1.6	5.3±2.2
Splenocytes+MC38-IFN-α	7.2±1.3	13.4±2.4	13.0±4.3
DC	6.2±2.0	8.1±1.2	17.7±3.2
DC+MC38-Neo	12.3±1.6	19.5±3.9	16.0±2.6
DC+MC38-IFN-α	15.9±2.9	45.5±5.0	51.7±8.3

(B) Treatment	*Day 1*	*Day 3*
DC+MC38-Neo	1.8±0.8	Not detected
DC+MC38-IFN-α	1.8±0.8	Not detected

(A) B6 mice were injected (s.c.) in the right flank with 1×10^5 MC38-WT cells. On days 7 and 14, 1×10^6 DCs or splenocytes and 1×10^5 γ-irradiated (100 Gy) genetically modified MC38 cells were inoculated around the established parental tumors. Tumor tissues were harvested 3 days after the last inoculation (17 days after WT inoculation), and were exposed to anti-CD4, anti-CD8a, and anti-CD11c antibody. Immunoreactive cells were counted in 10 fields under a light microscopy (× 400) in a blinded manner. Results are reported as the mean number of positive cells±s.d. This experiment repeated twice with similar results.
(B) After labeling with the fluorescent dye, PKH26, DCs were inoculated around the established tumor. Tumor tissues were harvested 1 or 3 days after the DC inoculation, and numbers of the labeled DCs were counted in the tumor tissue under a fluorescence microscope. Each experiment involved two mice per group.

CD8⁺, CD11c⁺, and NK1.1⁺ cells markedly infiltrated the MC38-WT tumors of mice inoculated with DCs and MC38-IFN-α cells

We performed an immunohistochemical analysis of the established parental tumors of mice treated with DCs and MC38-IFN-α. Many mononuclear cells, especially CD8⁺ cells and CD11c⁺ cells, had infiltrated the tumors of mice that had received the combined therapy (Table 3A). The CD11c⁺ cells did not seem to be the injected DCs because we detected only a few PKH26-labeled cells infiltrating the tumor tissue 24 h after the inoculation (Table 3B). There was no difference in the number of PKH26-labeled DCs between the group of mice treated with DCs+MC38-Neo and the group treated with DC+MC38-IFN-α cells. In addition, more NK1.1⁺ cells were detected in the tumor-infiltrating mononuclear cells of mice treated with the combined therapy (Table 4). These results suggest that coinjection of DCs and MC38-IFN-α induces potent cellular immune responses, which includes recruitment of the host's own DCs.

Tumor-specific cytolysis was clearly detected when splenocytes of mice inoculated with both DCs and MC38-IFN-α cells were stimulated with MC38-IFN-α in vitro

To investigate the induction of tumor-specific immune responses in mice treated with DCs and MC38-IFN-α *in vivo*, we stimulated splenocytes of the mice with MC38-IFN-α *in vitro* because our previous study showed that MC38-IFN-α cells stimulate tumor-specific CTLs *in vitro* efficiently.[23] As shown in Figure 5, tumor-specific cytolysis was clearly detected, although splenocytes of naive mice did not display any tumor specificity after *in vitro* stimulation of MC38-IFN-α cells (data not shown). These results suggest that the combined use of DCs and MC38-IFN-α elicits potent tumor-specific cellular immune responses *in vivo*.

Table 4 Infiltration of NK1.1⁺ cells in the established parental tumors of mice treated with DCs and genetically modified MC38 cells

Treatment	*NK1.1⁺ cells (%)*
HBSS	7.53
Splenocyte	3.86
Splenocytes+MC38-Neo	7.24
Splenocytes+MC38-IFN-α	11.54
DC	5.18
DC+MC38-Neo	4.67
DC+MC38-IFN-α	17.97

B6 mice were injected (s.c.) in the right flank with 1×10^5 MC38-WT cells. On days 7 and 14, 1×10^6 DCs or splenocytes and 1×10^5 genetically modified MC38 cells were inoculated around the established parental tumors. Tumor tissues were harvested 3 days after this third inoculation (17 days after WT inoculation). Tumor-infiltrating mononuclear cells were separated from tumor tissue, and NK1.1⁺ cell among these cells were stained and analyzed by flow cytometry.

Discussion

Several experimental therapies utilizing cytokine gene-transduced tumor cells have been performed. The local delivery of a high concentration of cytokine, which reduces systemic side effects, is implicated in the benefits of these therapies. IFN-α-expressing tumor cells have advantages in eliciting antitumor immune responses over IFN-α gene-transduced nontumor cells such as fibroblasts, because the expression of MHC molecules on which tumor-associated antigens would exist is upregulated,[29] and thus facilitate the recognition of tumor-associated antigens on MHC molecules of the transduced cells by tumor-specific T cells. However, for clinical use, patients' own tumor tissues are required to make genetically modified tumor cells. Effective transduction system is also needed to establish modified cells, which produce a large amount of the target cytokine.

DCs have been used widely for biologic therapy in cancer because of their physiologic roles in initiating and modulating the host's immune response. For therapeutic use, DCs are usually pulsed with tumor-associated antigen by incubating them with synthetic peptides corresponding to a known epitope, tumor lysate, or apoptotic tumor cells. A fusion technique to make hybrids of DCs and tumor cells is also performed for DC-based cancer therapy. In some trials, DC-based therapies resulted in better clinical courses compared with conventional therapies, such as chemotherapy and/or radiation therapy, without any severe side effects even in patients with advanced malignant tumors.

Induction of potent, long-lasting tumor-specific responses is crucial for preventing tumor enlargement and maintaining a tumor-free state. Both DC-based immunotherapy and IFN-α therapy have been widely evaluated, and antitumor effects induced by each

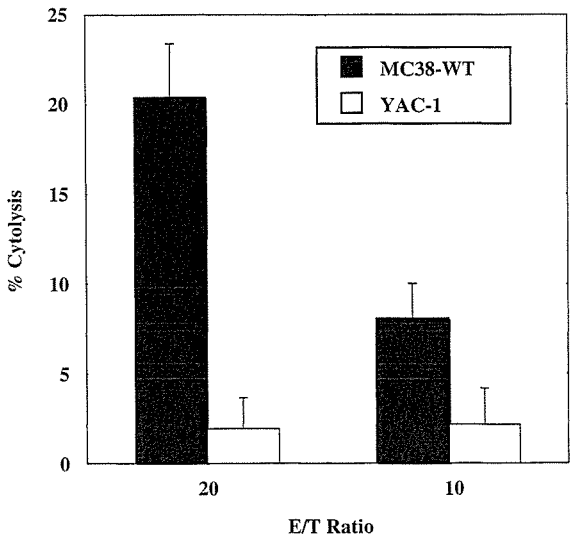

Figure 5 Tumor-specific cytolysis was clearly detected when splenocytes of mice inoculated with both DCs and MC38-IFN-α cells were stimulated with MC38-IFN-α *in vitro*. Tumor-specific CTLs were clearly induced from splenocytes of mice inoculated with both MC38-IFN-α cells and DCs. Mice, which received injection of MC38-WT on day 0 and subsequent therapeutic injection of DCs and MC38-IFN-α on day 7, were reinjected with 3×10^5 MC38-WT on day 50. After 2 weeks, splenocytes were incubated with MC38-IFN-α cells. After 7 days, responder cells were restimulated with irradiated MC38-IFN-α cells and irradiated syngeneic naive splenocytes in the presence of 50 IU/ml recombinant IL-2. Cytolytic assay against MC38 or YAC-1 cells was performed at indicated E:T ratios 7 days after the stimulation using the responder cells as effector cells. Results are reported as mean %cytotoxicity+s.d. This experiment was performed twice with similar results.

therapy, especially the induction of potent T helper1-type immune responses, have been reported. Recently, Okada *et al*.[31] reported that IFN-α-transfected DC showed marked antitumor effects against central nervous system tumors. Since IFN-α therapy is supposed to have additive or synergistic antitumor effects in cooperation with DC therapy, we investigated the efficacy of the combined therapy of bone marrow-derived DCs and IFN-α-expressing tumor cells in the present study.

When exogenous IFN-α was added to the culture of DCs and MC38-Neo cells, the proliferation of allogeneic splenocytes was significantly suppressed compared with the stimulation by DCs and MC38-IFN-α cells (Figure 1). In this experiment, we added 10 ng of IFN-α in the DC and MC38-Neo culture at the beginning of cultivation because the same number of MC38-IFN-α cells produces approximately total 10 ng of IFN-α for 48 h. Since it has been reported that IFN-α has suppressive effects on cell proliferation, 10 ng of IFN-α might be too much to observe proliferation of the allogeneic splenocytes. We observed less antiproliferative effects of IFN-α at the concentration of 1 ng. Furthermore, we could not observe DC maturation by coincubation with MC38-IFN-α cells. Thus, we hypothesize that continuous supply of small amount of IFN-α might be effective on the proliferation of allogeneic splenocytes. We are planning to use IFN-α-expressing fibroblasts instead of MC38-IFN-α cells to evaluate this hypothesis.

DCs coinjected with MC38-IFN-α cells prevented the development of parental tumors effectively in mouse model experiments. Therapeutic injection of DCs with MC38-IFN-α cells also suppressed the outgrowth of established parental tumors. Although IFN-α has a suppressive effect on cell growth, the proliferation of MC38-IFN-α cells was almost the same as that of the parental tumor cells. Cell numbers (survival) and the proportion of apoptotic cells among MC38-IFN-α cells after γ-irradiation were almost the same as those of MC38-WT or MC38-Neo cells. Thus, IFN-α gene transduction did not affect the growth, viability, or sensitivity to γ-irradiation of the MC38 cells *in vitro* in this model. Furthermore, our previous data suggest that an anti-angiogenic effect is not induced by inoculation of IFN-α-expressing MC38.[29] Therefore, antitumor effects induced by coinjection of DCs and IFN-α-expressing tumor cells in this study are thought to be due to the host's immune responses. These interpretations are supported by the immunohistologic analysis, experiments in mice depleted the individual immune cells, and investigation of cytolytic activity using splenocytes of mice treated with the combined therapy. These experiments showed that $CD8^+$ cells and asialo-$GM\text{-}1^+$ cells contributed to the antitumor effects induced by DC and IFN-α therapy, and host's DCs also seem to be involved in the effects.

In the present study, we inoculated therapeutically DCs admixed with MC38 cells, but not DCs preincubated with MC38 cells *in vitro* before injection, because a simple procedure is better for clinical use. In addition, since we lost many DCs after *in vitro* cultivation, and we could not recover enough DCs to use for the therapy. We thought it better to avoid loosing DCs, as it is often difficult to obtain many DCs from patients with advanced malignant tumors.

In general, as a live tumor vaccine may lead to the establishment of new tumors and metastases, it may be difficult to use in a clinical study. However, we tried to perform therapeutic inoculation of live MC38-IFN-α cells because almost all mice inoculated with these cells were free of tumors in our previous study.[29] We demonstrated the preferable effects of nonirradiated to γ-irradiated MC38-IFN-α cells on established tumors. The difference of antitumor effects between γ-irradiated and nonirradiated MC38-IFN-α cells might be due to the amount of IFN-α secreted by these genetically modified tumor cells after injection. Now, we are trying to establish new MC38 cell lines, which produce less IFN-α to compare the antitumor effects of the MC38-IFN-α used in this study.

To promote immune responses *in vivo*, DCs capture antigens at peripheral tissues, and then migrate to lymph nodes where T cells are activated and stimulated by mature DCs presenting tumor-associated antigens. In a previous study, some DCs migrated from the tumor site to draining lymph nodes within 24 h after inoculation.[32] The expression of CC chemokine receptor-7 on the DCs may be upregulated by coincubation with apoptotic tumor cells as reported previously,[33] facilitating the migration to lymph nodes. As we observed that few DCs infiltrated the tumor tissue 24 h after the inoculation, the inoculated DCs would have migrated into draining lymph nodes. However, we could find only a few tiny swollen lymph nodes in mice treated with coinjection of DCs and genetically modified tumor cells in the present study, and could not prove the migration

and maturation of the injected DCs in lymph nodes. Additional studies are required for further understanding of the transfer of DCs in this system.

Although the combined use of DCs and IFN-α-expressing tumor cells reduced parental tumor growth significantly, we did not observe any eradication of established parental tumors using this therapy. More effective modifications of the therapy, such as the administration of immunoadjuvants, or other cytokines may be necessary before clinical use for patients with immunesuppression. A new therapy to use DCs, irradiated MC38-WT cells and IFN-α-expressing fibroblasts is under investigation in our laboratory. From our results, immunotherapy by combined use of DCs and IFN-α may be a candidate for clinical cancer therapy, although further investigation is needed to augment the effectiveness.

Materials and methods

Mice

Female C57BL/6 (B6) and BALB/c mice 6 to 8 weeks old were purchased from Sankyo Lab Service (Tokyo, Japan) for use in experiments at ages from 8 to 12 weeks. Mice were maintained in an animal care facility at Showa University. This study has been approved by the Ethical Committee for Animal Experiments of Showa University.

Cell lines, culture medium, and reagents

The MC38 murine colorectal adenocarcinoma cell line (B6 mouse origin) and the YAC-1 lymphoma cell line were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES buffer, 1 mM minimum essential medium sodium pyruvate, and 0.1 mM minimum essential medium nonessential amino acids (complete medium, 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).

Retroviral transduction and genetically modified tumor cell lines

Tumor cells were transduced using retroviral vectors according to standard protocols[34] and selected for antibiotic resistance in culture medium containing 0.5 mg/ml. G418 (Sigma, St Louis, MO, USA). The MC38 cell line genetically modified to produce IFN-α (MC38-IFN-α) was established as described previously.[23] Expression of murine 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). MC38 cells expressing the neomycin-resistance gene following retroviral transduction with MFG-Neo (MC38-Neo) were used as control cells.[23]

Characterization of γ-irradiated tumor cells

γ-Irradiation (100 Gy) was performed with Gammacell 3000 Elan (Nordion International Inc., Kanata, Canada). After irradiation, MC38-IFN-α cells (5×10^5 cells) were incubated in CM in six-well plates, and were enumerated every day in duplicate. Cell numbers in each well were determined microscopically. To detect the apoptosis of tumor cells induced by γ-irradiation, TUNEL was performed using an APO-DIRECT™ Kit (Pharmingen, San Diego, CA, USA). To determine the changes of IFN-α production with γ-irradiation, the culture supernatant of 1×10^5 irradiated tumor cells was examined using the IFN-α ELISA 48 h after γ-irradiation.

Phenotypic change of DCs and cytokine production by DCs after coincubation with MC38-IFN-α

To evaluate DC maturation induced by coincubation with MC38-IFN-α, expression of surface molecules on DCs were examined using fluorscein isothiocyanate (FITC)-conjugated anti-I-A^b CD80, and CD86 monoclonal antibody (Pharmingen). Furthermore, cytokine production by DCs was detected by ELISA using a commercially available kit according to the manufacturer's instructions (mouse IFN-α ELISA obtained from PBL Biomedical Laboratories, and Endogen mouse IL-1β ELISA, mouse iL-12p70 ELISA, and mouse TNF-α ELISA obtained from Pierce Biotechnology Inc., Rockford, IL, USA).

Preparation and purification of DCs

DCs were generated as reported previously with some modification.[35] In brief, bone marrow cells were obtained from the femurs and tibias of B6 mice. After treatment to lyse erythrocytes, the bone marrow cells were depleted of B- and T-lymphocytes with anti-B220 (RA3-3A1/6.1, TIB-146; American Type Culture Collection (ATCC), Manassas, VA, USA), anti-CD4 (GK1.5, TIB207; ATCC), and anti-CD8 antibodies (2.43, TIB210; ATCC) for 1 h on ice, followed by a 30-min incubation with rabbit compliment (obtained from Cedarlane, Hornby, Canada). The recovered cells were put in six-well plates in 4 ml of CM at a concentration of 1×10^6 cells/ml and incubated overnight in 5% CO_2 in air at 37°C. After overnight incubation, nonadherent cells were harvested, and put into flasks in CM containing 50 ng/ml each of murine GM-CSF and IL-4 (both were obtained from Pepro Tech EC, London, UK) at a concentration of 1.5×10^5 cells/ml. After 7 days, nonadherent cells were harvested, and were centrifuged at 200 *g* for 10 min. The cells were washed with Hank's balanced salt solution (HBSS; Life Technologies) supplemented with 0.5% BSA, and then were incubated with anti-CD11c (N418)-magnetic beads and passed through a positive selection column (Type MS+) in a magnetic field (MACS; Miltenyi biotec, Bergisch Gladbach, Germany). After rinsing, the column was removed from the magnetic field, and $CD11c^+$ cells were eluted from the column and washed with HBSS before use. The cells were 80–90% CD11c positive after selection with the MACS system.

Proliferation of allogeneic splenocytes after stimulation with DCs and genetically modified MC38 cells

Purified DCs (2×10^6) were incubated with γ-irradiated (100 Gy) MC38-Neo or MC38-IFN-α cells (2×10^5) in CM (2 ml). In some wells, exogenous IFN-α was added to the culture of DCs and MC38-Neo cells at a concentration of 10 ng/ml (corresponding to the amount of IFN-α secreted by 2×10^5 γ-irradiated MC38-IFN-α cells for 48 h). After incubation for 2 days, the cells were washed twice with HBSS, and were γ-irradiated (30 Gy) for use as stimulator cells. Then, allogeneic splenocytes (5×10^5)

obtained from BALB/c mice were incubated with the stimulator cells (5×10^4) in a 96-well plate for 3 days. To investigate the ability to stimulate allogeneic splenocytes of the DCs incubated with the genetically modified MC38 cells, a cell proliferation assay was performed with the MTT Proliferation Assay kit (ATCC) according to the manufacturer's directions.

Preventive effects of inoculation with DCs and genetically modified MC38 cells on development of parental tumors in vivo

B6 mice were injected intraperitoneally (i.p.) with 1×10^6 DCs (or splenocytes) and 1×10^5 γ-irradiated (100 Gy) genetically modified MC38 cells twice at a 7-day interval (days −14 and −7). At 1 week after the final vaccination (day 0), the mice were injected s.c. with 1×10^5 MC38-WT cells in the right flank. Each experiment involved five or six mice per group. Tumor size was measured twice a week using vernier calipers. Mice with ulcerated tumors or tumors larger than 20 mm in diameter were killed.

Antitumor effects of DCs and MC38-IFN-α cells in immune cell-depleted mice

To determine the role of the immune system in the reduction of *in vivo* tumor growth in the establishment model, $CD4^+$ T cells, $CD8^+$ T cells, or asialo-GM-1^+ cells were depleted by an antibody method as we performed previously.[29] Culture medium from hybridomas producing the following antibodies was used at appropriate dilutions/concentrations: anti-CD4 (GK1.5, TIB207; ATCC) and anti-CD8 (2.43, TIB210; ATCC). For depletion of asialo-GM-1^+ cells, anti-asialo-GM-1 was obtained from WAKO (Osaka, Japan). All antibody doses and treatment regimens were determined in preliminary studies using the same lots of antibody employed for the experiments. Treatment was confirmed to delete completely the desired cell population for the entire duration of the study, as determined by flow cytometric analysis (data not shown). After DCs and MC38-IFN-α cells were inoculated i.p. twice at a 7-day interval (days −14 and −7), we depleted $CD4^+$ T cells, $CD8^+$ T cells, or asialo-GM-1^+ cells (days −5, −4, and −3). At 1 week after the vaccination (day 0), the mice were injected s.c. with 1×10^5 MC38-WT cells in the right flank. Each experiment involved five mice per group. Tumor size was measured twice a week using vernier calipers.

Therapeutic effects of DCs and genetically modified MC38 cells on established parental tumors in vivo

To evaluate therapeutic effects of DCs and the modified MC38 cells on established wild-type tumors, we measured the size of established MC38-WT tumors in mice before and after treatment with DCs and the modified MC38 cells as described previously.[23] B6 mice were injected s.c. with 1×10^5 MC38-WT cells in the right flank. At 7 and 14 days after the WT inoculation, 1×10^6 DCs (or naive splenocytes) and 1×10^5 genetically modified MC38 cells with or without γ-irradiation (100 Gy) were inoculated s.c. around the established parental tumors, which had reached 9–25 mm^2 in size. Each experiment involved six mice per group. Tumor size was measured twice a week using vernier calipers. Mice with ulcerated tumors or tumors larger than 20 mm in diameter were killed.

Immunohistologic analysis

B6 mice were injected s.c. in the right flank with 1×10^5 MC38-WT cells. On days 7 and 14, 1×10^6 DCs or splenocytes and 1×10^5 genetically modified MC38 cells were inoculated around the established parental tumors. Tumor tissues were harvested 3 days after the last inoculation (17 days after WT inoculation), and were immediately embedded in optimal clotting temperature (OCT) compound (Tissue Tek, Elkhart, IN, USA) and frozen. Serial 5-μm sections were exposed to anti-CD4, CD8a, and CD11c antibody (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 a light microscopy (×400) in a blinded manner. To observe the localization of the injected DCs in the established tumors of mice that received the combined therapy, DCs were labeled with a fluorescent dye using a PKH26 red fluorescent cell linker kit (obtained from Sigma-Aldrich Japan, Tokyo, Japan). After labeling, DCs were inoculated around the established tumor. Tumor tissues were harvested 1 day or 3 days after the DC inoculation, and the labeled DCs in the tumor tissue were enumerated under a fluorescence microscope. Each experiment involved two mice per group.

Analysis of NK1.1$^+$ cell infiltration in established parental tumors

B6 mice were injected (s.c.) in the right flank with 1×10^5 MC38-WT cells. On days 7 and 14, 1×10^6 DCs or splenocytes and 1×10^5 genetically modified MC38 cells were inoculated around the established parental tumors. Tumor tissues were harvested 3 days after this third inoculation (17 days after WT inoculation). Tumor-infiltrating mononuclear cells were separated from tumor tissue as reported previously.[29] The cells were washed three times with CM. Flow cytometric analyses were performed using FACS calibur (Becton Dickinson) to analyze the phenotype of tumor-infiltrating mononuclear cells of mice treated with DCs and genetically modified tumor cells. Monoclonal antibodies used in this analysis were FITC-conjugated anti-NK1.1 antibody (Becton Dickinson).

Induction of MC38-specific CTLs from splenocytes of mice immunized with DCs and MC38-IFN-α cells by stimulation in vitro

Tumor-free mice, which received injections of 1×10^6 DCs and 1×10^5 MC38-IFN-α on days 0 and 7, and a subsequent injection of MC38-WT (1×10^5 cells) on day 14, were reinjected with 3×10^5 MC38-WT on day 50. After 2 weeks, splenocytes were harvested and, 2×10^6 splenocytes were incubated with γ-irradiated MC38-IFN-α cells (100 Gy, 2×10^5 cells/ml) in 24-well plates. After 7 days, responder cells (1×10^6 cells/ml) were restimulated with irradiated MC38-IFN-α cells (100 Gy, 1×10^5 cells/ml) and irradiated syngeneic naive splenocytes (30 Gy, 1×10^6 cells/ml) in the presence of 50 IU/ml of recombinant mouse IL-2 (Becton Dickinson). Cytolytic assays were performed 7 days after the last stimulation using the responder cells as effector cells.

Cytolytic assays

Tumor-stimulated effector cells were assessed for cytolytic activity against MC38-WT and YAC-1 cells in triplicate in 4-h ^{51}Cr-release assays. Target cells (1×10^6 cells/ml) were labeled with 100 μCi 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 indicated effector to target ratios (E:T) were plated in 200 μl of CM in each well of the 96-well round-bottomed plates. ^{51}Cr-release was measured after a 4-h incubation at 37°C. Percent lysis was determined using the formula: (release in assay−spontaneous release) × 100/(maximum release−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.

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.

Abbreviations

DC, dendritic cell; IFN, interferon; MC38-IFN-α, IFN-α-overexpressing MC38; MC38-Neo, neomycin-resistance gene-transduced MC38; MC38-WT, MC38 wild type; MHC, major histocompatibility complex; CTL, cytotoxic T-lymphocytes; IL, interleukin; B6, C57BL/6; CM, complete medium; ELISA, enzyme-linked immunosorbent assay; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling assay; ATCC, American Type Culture Collection; HBSS, Hank's balanced salt solution; FITC, fluorescein isothiocyanate; OCT, optimal clotting temperature; E:T, effector to target ratio.

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Cytidine-phosphate-guanosine Oligodeoxynucleotides and Interferon-alpha-expressing Tumor Cells Effectively Induce Dendritic Cell Maturation *In Vitro*

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Abstract. *Background: Dendritic cells (DCs) play an important role in immune response and cytidine-phosphate-guanosine (CpG) oligodeoxynucleotides (ODN) as well as interferon (IFN)-α have been proven to induce DC maturation. In this study, the synergistic effects of CpG-ODN and IFN-α on DC maturation were evaluated. Materials and Methods: Surface molecules on DCs and the stimulatory responses of DCs to allogeneic splenocytes were analyzed after cultivation with CpG-ODN and IFN-α-overexpressing murine colorectal cancer MC38 cells (MC38-IFNα). Results: Co-incubation with CpG-ODN and MC38-IFNα, but not wild-type MC38 cells (MC38-WT), effectively up-regulated co-stimulatory molecules on the DCs. CpG, in combination with IFN-α, stimulated IL-1β and TNF-α production by DCs effectively. When DCs pre-incubated with CpG-ODN and MC38-IFNα were co-incubated with allogeneic splenocytes in vitro, the proliferation of these splenocytes was significantly enhanced compared with that of splenocytes incubated with CpG-ODN and MC38-WT cells (p=0.041). Conclusion: Since CpG-ODN and IFN-α have synergistic effects on DC maturation, they may induce potent antitumor immune responses and combination therapy should be considered for clinical application.*

Dendritic cells (DCs) play crucial roles in eliciting primary and secondary immune responses to foreign antigens as antigen-presenting cells (1, 2) and their characteristics change upon maturation. Immature DCs express low levels of major histocompatibility complex (MHC) class I, class II and co-stimulatory molecules (CD80, 86) (3). When immunogenic antigens are captured by immature DCs, these DCs down-regulate the functions of antigen acquisition such as phagocytosis, but up-regulate MHC and co-stimulatory molecules to induce immune responses (3-7). Thus, mature DCs may be preferable for tumor immunotherapy. We previously reported that apoptotic human colorectal cancer cells induce DC maturation functionally and phenotypically (8). Recently, various DC-based therapies against malignant tumors have been tested for antitumor responses (5, 7, 9-14).

Microbial molecules, such as lipopolysaccharides or bacteria-derived DNA, are recognized by the host immune cells through the family of toll-like receptors (TLR) (15) and stimulate immune responses. It has been reported that DNA vaccines and synthetic oligodeoxynucleotides (ODN) containing an unmethylated cytidine-phosphate-guanosine (CpG) motif promote Th1-type immune responses (16). CpG stimulates DCs *via* TLR9 and enhances DC maturation (17), which may improve the therapeutic effects on established tumors. In a murine model, CpG-based immunotherapy enhanced the antitumor responses (18-21). Therefore, CpG-ODN may be applied to clinical cancer therapy as an immune adjuvant.

Interferon (IFN)-α has many biological effects, including an antiviral function, enhancement of IFN-α/β production (22, 23), inhibition of cell growth and angiogenesis (24). Based on its immunomodulating effects, IFN-α has been used to treat patients with some malignant tumors such as melanoma, renal cell carcinoma and leukemia. IFN-α up-regulates the expression of MHC class I on the cell surface and enhances the proliferation of Th1 lymphocytes (25) and is important for the generation of cytotoxic T lymphocytes (CTLs) in specific antitumor immune responses (26, 27). In addition, we reported, previously, that IFN-α-expressing tumor cells promote the survival of tumor-specific CTLs by preventing apoptosis (28).

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Concerning the relationship between DCs and IFN-α, it has been reported that DC maturation including the up-regulation of co-stimulatory molecules (CD80, 86), MHC class II and CD83 expression in human DCs is observed by stimulation with IFN-α (29). A greater capability of DCs to stimulate the proliferation of allogeneic lymphocytes has also been reported in the presence of IFN-α (29). In a previous study, we also demonstrated that IFN-α gene therapy, in combination with DC-based immunotherapy, reduced the growth of established tumors in a poorly immunogenic tumor model (30).

As a preliminary investigation of the combined therapy before clinical use, the synergistic effects of CpG-ODN and IFN-α on the phenotypical and functional maturation of DCs, using both murine bone marrow-derived DCs and a poorly immunogenic colorectal cancer cell line genetically modified to overexpress murine IFN-α *in vitro,* were investigated in this study.

Materials and Methods

Mice. Female C57BL/6 (B6) and BALB/c 6-week-old mice were purchased from Sankyo Lab Service (Tokyo, Japan) for use in the experiments. The mice were maintained in an animal care facility at Showa University, Japan. This study had been approved by the Ethical Committee for Animal Experiments of Showa University.

Cell lines, culture medium and reagents. The MC38, murine poorly immunogenic colorectal adenocarcinoma cell line (B6 mouse origin) was maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES buffer, 1 mM minimum essential medium, sodium pyruvate and 0.1 mM minimum essential medium with non-essential amino acids (complete medium; 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).

Establishment of IFN-α-overexpressing tumor cell line. The MC38 cell line, genetically modified to produce murine IFN-α (MC38-IFNα), was established as described previously (31). The expression of IFN-α was confirmed by enzyme-linked immunosorbent assay (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 DCs) was performed with a Gammacell 3000 Elan (Nordion International Inc., Kanata, Canada). In our previous observation, $1x10^5$ cells of MC38-IFNα produced approximately 20.8±0.5 ng/48 h, and IFN-α gene transduction did not affect the growth of tumor cells *in vitro* or the survival of γ-irradiated tumor cells (30).

Preparation and purification of bone marrow-derived DCs. DCs were generated as reported previously (30). The bone marrow cells from the femurs and tibias of B6 mice were incubated overnight after depletion of the B and T cells. Following incubation, the non-adherent cells were put into flasks in CM containing 50 ng/ml each of murine GM-CSF and IL-4 (both obtained from Pepro Tech EC, London, UK). Seven days later, the non-adherent cells were harvested and the DCs were collected by the MACS system (Miltenyi biotec, Bergisch Gladbach, Germany). The cells were 80-90% CD11c-positive after selection by the MACS system.

Synthesis of CpG-ODNs. CpG-ODN-1826 has been reported to have maturation effects on DCs (32). Both CpG-ODN-1826, 5'-TCC ATG ACG TTC CTC ACG TT-3' and ODN-1911 serve as control; 5'-TCC AGG ACT TTC CTC AGG TT-3' (non-CpG) were synthesized by Sigma-Aldrich Japan (Tokyo, Japan).

Phenotypic changes of DCs after co-incubation with CpG-ODN and the IFN-α-overexpressing tumor cell line. To assess the effects of CpG-ODN on DC maturation, purified DCs were incubated with CpG-ODN-1826 at the indicated concentrations (1, 6, 10 μg/ml), non-CpG-ODN-1911 (6 μg/ml) or lipopolysaccharide (LPS) (10 μg/ml) in CM. To elucidate the additive effects of CpG in combination with IFN-α, DCs were co-cultured with γ-irradiated (100 Gy) MC38-WT or MC38-IFNα cells at a DC to tumor ratio of 10, with CpG-ODN in CM. After a 48-h incubation, the DCs were harvested and washed with HBSS 3 times. The flow cytometric analyses were then performed using FACScan (Becton Dickinson) to observe the phenotypic change of DCs pulsed with the irradiated tumor cells. The monoclonal antibodies used in this analysis were fluorecein isothiocyanate (FITC)-conjugated anti-MHC class I ($H\text{-}2K^b$), class II ($I\text{-}A^b$), CD80 and CD86 (Nippon Becton Dickinson, Tokyo, Japan). The results were shown as mean fluorescence ratios, which were calculated by the following formula: mean fluorescence ratios = mean fluorescence intensity of sample / mean fluorescence intensity of DCs alone (without CpG-ODN or IFN-α).

Cytokine production of DCs after co-incubation with CpG-ODN and the IFN-α-overexpressing tumor cell line. To determine the changes of cytokine production after incubation, the culture supernatant of $1x10^6$ DCs and/or $1x10^5$ tumor cells with or without 6 μg CpG-ODN was examined, after 48-h incubation, by ELISA using a commercially available kit, according to the manufacturers' instructions (mouse IFN-α ELISA obtained from PBL Biomedical Laboratories, and Endogen mouse IL-1β ELISA. mouse IL-12p70 ELISA and mouse TNF-α ELISA obtained from Pierce Biotechnology Inc, Rockford, IL, USA).

Proliferative effects of DCs co-incubated with CpG-ODN and genetically modified MC38 cells on allogeneic splenocytes. The DCs ($2x10^6$) were incubated with γ-irradiated (100 Gy) MC38-WT or MC38-IFNα cells ($2x10^5$) in CM. In some wells, CpG-ODN-1826 (6 μg/ml) was added to the culture of DCs and/or MC38-WT cells. After incubation for 2 days, the cells were washed twice with HBSS, and were γ-irradiated (30 Gy) for use as stimulator cells. Allogeneic splenocytes ($5x10^5$), as responder cells, obtained from BALB/c mice were subsequently incubated with the stimulator cells ($5x10^4$) in a 96-well plate for 3 days. To investigate the ability to stimulate allogeneic splenocytes of the DCs incubated with CpG-ODN-1826 and the MC38-IFNα cells, a cell proliferation assay was performed with the MTT Proliferation Assay kit (ATCC), according to the manufacturer's directions.

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

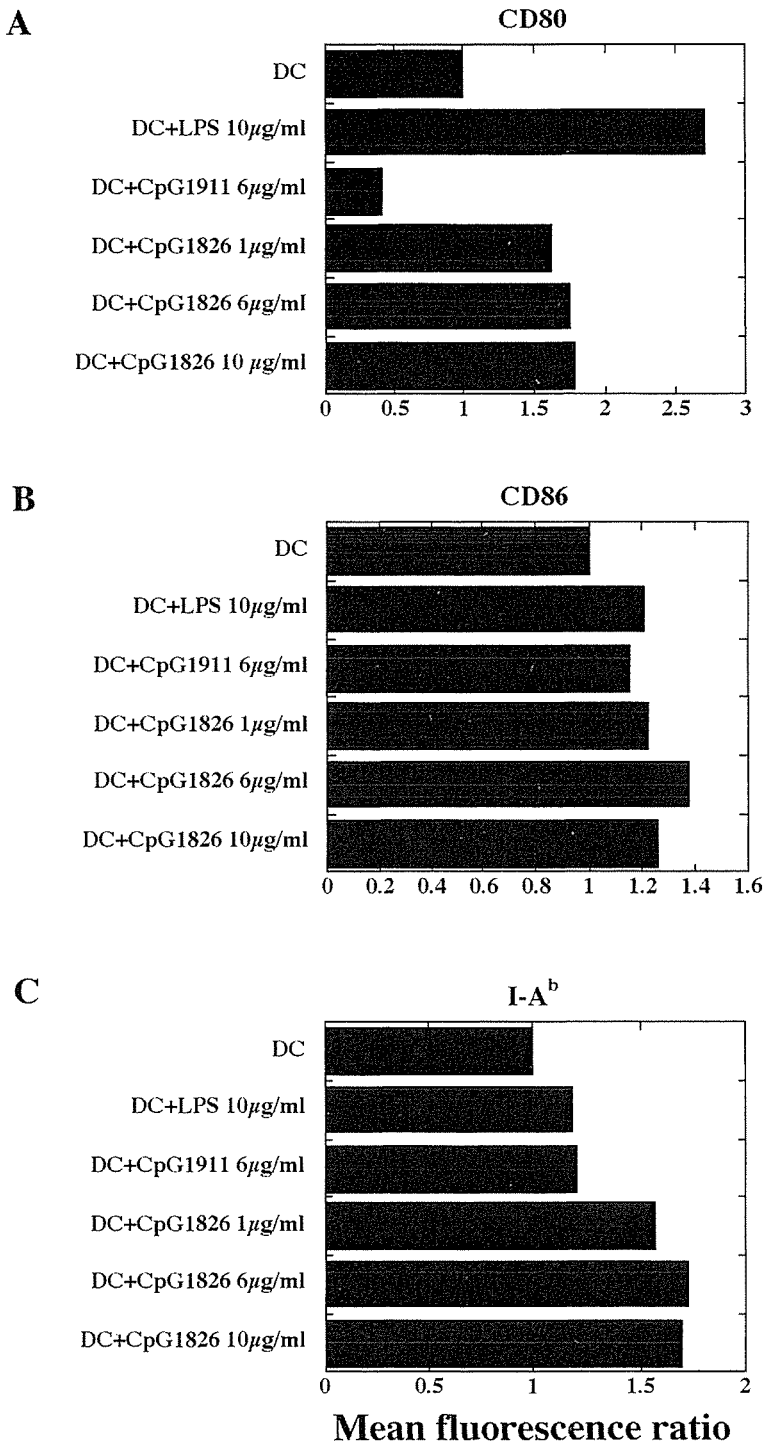

Figure 1. *CpG-ODN-1826 induced DC maturation effectively at the concentration of 6 μg/ml. Purified DCs were incubated with CpG-ODN-1826 at the indicated concentrations (1, 6, 10 μg/ml), non-CpG-ODN-1911 (6 μg/ml) or lipopolysaccharide (LPS) (10 μg/ml) in CM. After 48-h incubation, (A) CD80, (B) CD86 and (C) anti-MHC class II (I-A^b) expressions were analyzed by flow cytometry. The results are shown as mean fluorescence ratios, which were calculated by the following formula: mean fluorescence ratios = mean fluorescence intensity of sample / mean fluorescence intensity of DCs alone (without CpG-ODN nor IFN-α). This experiment was performed twice with similar results.*

Results

CpG-ODN-1826 effectively phenotypically enhanced DC maturation. As a preliminary study to assess the specific effects of CpG-ODN-1826 on DC maturation, the DCs were incubated with CpG-ODN-1826 or non-CpG-ODN-1911. As shown in Figure 1, the expression of the MHC class II and co-stimulatory molecules on DCs was enhanced after 48-h incubation with CpG-ODN-1826. In particular, CpG-ODN-1826 markedly enhanced I-A^b expression on DCs even when compared with LPS 10 μg/ml. CpG-ODN-1826 effectively enhanced phenotypic DC maturation. On the other hand, non-CpG-ODN-1911 did not affect DC maturation after incubation. Since the expression levels of these molecules on DCs were almost the same between 6 μg/ml and 10 μg/ml, 6 μg/ml was considered adequate to induce DC maturation.

CpG-ODN enhances IL-12 p70 production by DCs and the production of IL-1β and TNF-α by DCs and IFN-α-overexpressing tumor cells. The cytokine production by DCs and/or IFN-α-expressing tumor cells was evaluated in response to stimulation with CpG-ODN. In our previous study, MC38-IFNα did not enhance IFN-α production by DCs (30). As shown in Table I, CpG-ODN did not enhance the IFN-α production by DCs incubated with MC38-IFNα. IL-12 production by DCs was markedly enhanced by the addition of CpG-ODN. IL-1β and TNF-α could be detected only in the supernatant of DCs incubated with MC38-IFNα and CpG. These findings suggest that CpG-ODN enhances IL-12 production by DCs and CpG, in combination with IFN-α, effectively stimulates IL-1β and TNF-α production by DCs.

Expression of MHC class II, CD80 and CD86 molecules on the surface of DCs is up-regulated after co-incubation with IFN-α-expressing MC38 cells and CpG-ODN-1826. The phenotypic changes of DCs after co-incubation with IFN-α-expressing MC38 cells and CpG-ODN-1826 were investigated. As shown in Figure 2, the expression of MHC class II and co-stimulatory molecules on the surface of DCs co-incubated with γ-irradiated MC38-IFNα and CpG-ODN-1826 was clearly up-regulated compared with that of DCs co-incubated with MC38-IFNα or with MC38-WT and CpG-ODN. The expression of H-$2K^b$ on DCs was clearly up-regulated by MC38-IFNα. Exogenous IFN-α also enhanced the H-$2K^b$, but not the CD80 or CD86 expressions of DCs (data not shown). In our previous study, MHC and co-stimulatory molecules on DCs incubated with MC38-WT and exogenous IFN-α were not up-regulated compared with those on DCs incubated with MC38-IFNα cells (30). These data suggest that co-incubation with γ-irradiated IFN-α-expressing tumor cells and CpG-ODN-1826 enhances the expression of MHC class II and co-stimulatory molecules on DCs.

Table I. *Production of IFN-α, IL-1β, IL-12p70 and TNF-α by DCs with or without MC38-IFNα (-WT) tumor cells and/or CpG-ODN-1826.*

Cells	IFN-α	IL-1β	IL-12	TNF-α
DC	ND	ND	0.33±0.16	ND
DC + MC38-WT	ND	ND	0.25±0.14	ND
DC + MC38-IFNα	3.55±0.13	ND	0.25±0.20	ND
DC + CpG	ND	ND	3.41±0.24	ND
DC + MC38-WT + CpG	ND	ND	2.13±0.26	ND
DC + MC38-IFNα + CpG	2.18±0.08	1.55±0.78	2.39±0.12	0.25±0.13

(ng/48h)

ND; not detected
DCs ($8x10^6$) and/or tumor cells ($8x10^5$), with or without 6 μg/ml CpG-ODN, were examined after 48-h incubation by ELISA using a commercially available kit according to the manufacturer's instructions. The results are reported as mean (ng/48h) ± S.D.

Proliferation of allogeneic splenocytes is markedly enhanced by DC co-incubation with IFN-α-expressing MC38 cells and CpG-ODN-1826. To investigate the functional changes of DCs after co-incubation with IFN-α-expressing MC38 cells and CpG-ODN-1826, cell proliferation assays using allogeneic splenocytes were performed. As shown in Figure 3, the stimulation by DCs co-incubated with both MC38-IFNα cells and CpG-ODN-1826 markedly enhanced the proliferation of allogeneic splenocytes compared with stimulation by DCs alone, those co-incubated with CpG-ODN, MC38-WT or those co-incubated with MC38-WT and CpG-ODN ($p<0.001$, $p=0.003$, $p<0.001$ and $p=0.041$, respectively). Although statistical significance was not reached, the allogeneic splenocytes proliferated effectively by DCs co-incubated with MC38-IFNα and CpG-ODN-1826 compared with by those co-incubated with MC38-IFNα ($p=0.115$). When exogenous IFN-α was added to the culture of DCs and MC38-WT cells, the proliferation of the allogeneic splenocytes was significantly suppressed compared with the stimulation by DCs with MC38-IFNα cells (30). These results suggest that IFN-α-expressing MC38 cells and CpG-ODN-1826 enhance DC maturation more effectively than IFN-α alone.

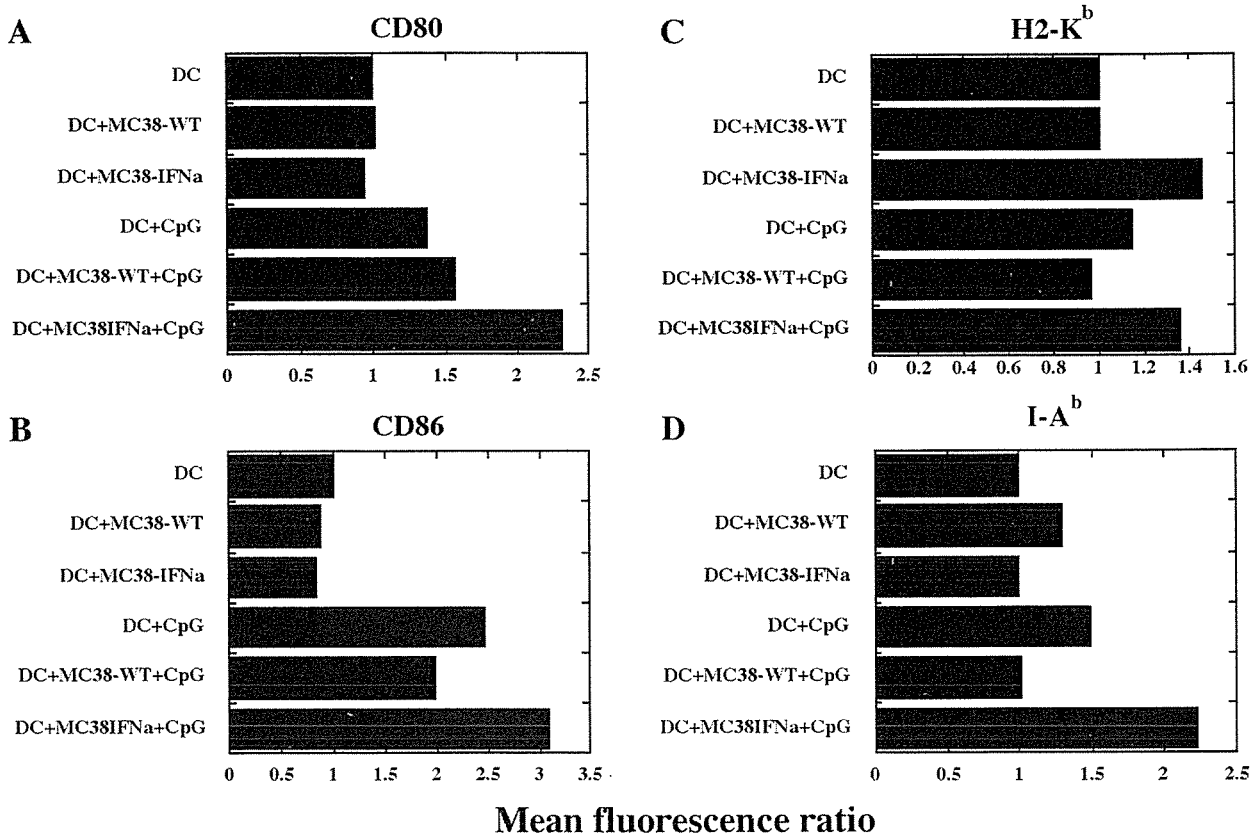

Figure 2. *CpG-ODN-1826 and irradiated IFN-α-expressing tumor cells markedly enhanced DC maturation. Purified DCs were co-cultured with γ-irradiated (100 Gy) MC38-WT or MC38-IFNα cells at a DC to tumor ratio of 10, with or without CpG-ODN-1826 (6 μg/ml) in CM. After 48-h incubation, (A) CD80, (B) CD86, (C) anti-MHC class I (H-2k^b and (D) anti-MHC class II (I-A^b) expressions were analyzed by flow cytometry. The results are shown as mean fluorescence ratios. This experiment was performed 3 times with similar results.*

Discussion

DCs have been used for clinical therapy for patients with advanced tumors. It remains to be clarified which type of DCs is suitable for cancer therapy, mature or immature. Mature DCs express more surface molecules, including MHC class I and class II and co-stimulatory molecules, compared with immature DCs. On the other hand, the ability of phagocytosis and migration diminishes as they mature. Further investigation is needed to elucidate which is better for anticancer treatment.

It has been demonstrated that IFN-α induces DC maturation. IFN-α has also been reported to up-regulate the expression of co-stimulatory molecules (CD80, 86) and MHC class II (HLA-DR) and to induce CD83 expression, which is considered to be a marker of mature and activated human DCs (29). It has also been shown that DCs gain a greater capability to stimulate the proliferation of allogeneic lymphocytes in the presence of IFN-α (29). Other groups have reported the enhancing effects of IFN-α on DC maturation (33, 34). However, it has been reported that IFN-α fails to induce DC maturation and that the presence of IFN-α prior to or during the differentiation of DCs from the monocyte precursors alters their response to maturation stimuli in the human system (35).

We previously reported that DCs incubated with MC38-IFNα cells had proliferative effects on allogeneic splenocytes, whereas exogenous IFN-α did not show such effects (30). We confirmed that high levels of IFN-α have antiproliferative effects on allogeneic splenocytes. Thus, it was thought that continuous IFN-α provide might be preferable for cell proliferation. Furthermore, DCs and IFN-α-expressing tumor cells elicited potent antitumor immune responses and suppressed the outgrowth of established parental tumors. Since we expected to induce more potent antitumor effects, we performed the present experiments with DCs, IFN-α-expressing tumor cells and CpG-ODN.

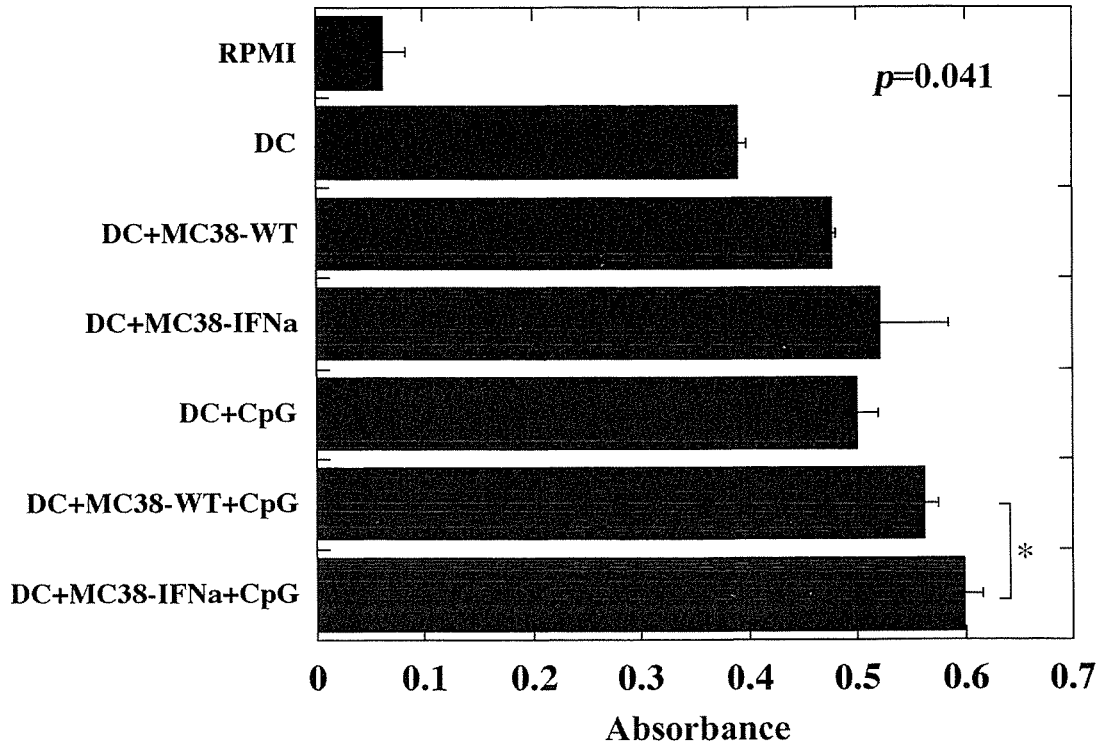

Figure 3. *Proliferation of allogeneic splenocytes is markedly enhanced by DCs co-incubated with IFN-α-expressing MC38 cells and CpG-ODN. Purified DCs were incubated with γ-irradiated (100 Gy) MC38-WT or MC38-IFNα cells at a DC to tumor ratio of 10 and with or without 6 μg/ml CpG-ODN-1826 for 48 h. These cells were γ-irradiated (30 Gy) to be used as stimulator cells. After the allogeneic splenocytes ($5x10^5$) had been incubated with the stimulator cells ($5x10^4$) for 3 days, the cell proliferation assay was performed. This experiment was performed twice with similar results.*

In humans, the expression of TLR9 is restricted to B cells and to a certain subset of DCs, the plasmacytoid DCs (36). It has been reported that conventional CpG DNAs, phosphorothioate-modified oligodeoxynucleotides called CpG-B, induced splenic B cell proliferation, DC maturation and cytokine production from a variety of immune cells (37, 38). TLR9 is expressed on human plasmacytoid DCs, but not on human myeloid DCs. However, it has been reported that TLR9 is expressed by all murine DC subsets (39) and that CpG ODNs can stimulate not only splenic, but also bone marrow-derived myeloid DCs through TLR9 (40). Our results are consistent with these previous reports that CpG-ODN enhances murine bone marrow-derived DC maturation. However, in humans, CpG-ODN may not affect DC maturation as effectively as it affects murine myeloid DC maturation, because human myeloid DCs do not have TLR9. Thus, for clinical applications, further modification may be required.

In a previous study (32), 6 μg/ml of CpG-ODN-1826 were used for DC maturation. We compared 6 μg/ml with 1 μg/ml and 10 μg/ml of CpG-ODN-1826. The results confirmed that 6 μg/ml of CpG-ODN-1826 was suitable for DC maturation and this concentration was employed in the present study. A concentration of IL-12 p70, a bioactive form of IL-12, approximately 10 times higher was detected when the DCs were incubated with 6 μg/ml of CpG-ODN-1826. On the other hand, no enhancement of IFN-α production was observed in the present study because myeloid DCs were used instead of plasmacytoid DCs, which secrete a high amount of IFN-α upon simulation by CpG-ODN (41). IL-1β and TNF-α were detected in the culture supernatant only when the DCs were incubated with MC38-IFNα and CpG-ODN. Thus, we believe that MC38-IFNα and CpG-ODN have synergistic effects on cytokine production by DCs.

We previously reported that the addition of exogenous IFN-α had only minimal effects on the phenotypic maturation of DCs (30). The up-regulation of co-stimulatory molecules was clearly observed when CpG-ODN was added to the DCs. Further up-regulation of those molecules as well as MHC class II molecules on DCs was seen when CpG-ODN and MC38-IFNα cells were added to the culture. From the results of cytokine production and the analyses of surface molecules, CpG-ODNs in combination with

IFN-α-expressing tumor cells effectively enhanced DC maturation *in vitro*, thereby elucidating the additive effects of CpG-ODN on the proliferation of allogeneic splenocytes induced by DC and MC38-IFNα cells.

To our knowledge, this is the first report of the additive effects of IFN-α and CpG-ODN on DC maturation. We are currently exploring the antitumor effects of DCs, MC38-IFNα cells and CpG-ODNs *in vivo* using a murine model. The present findings suggest that DC-based immunotherapy in combination with CpG-ODN and IFN-α gene therapy has the potential to induce potent immune responses in clinical antitumor therapy. Further investigations are needed.

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C型肝炎ウイルス感染における免疫応答

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はじめに

1989年に，米国Chiron社によってはじめてC型肝炎ウイルス(hepatitis C virus ： HCV)がクローニングされる以前[1]，非A非B型肝炎の大部分はウイルスによるものと考えられていたが，その病因を探るべくさまざまな試みが行われていた。当時われわれは，非A非B型肝炎患者の末梢リンパ球から，非A非B型肝炎に罹患した患者の肝細胞のみを特異的に認識し傷害する細胞障害性T細胞(cytotoxic T lymphocyte ： CTL)クローンを樹立した[2]。HCV発見により，それまで非A非B型肝炎といわれてきた肝炎のうち，その多くがHCVによるC型肝炎であると報告されたが，それ以降ウイルス性肝炎に対する免疫応答の研究は飛躍的に進められ，肝炎ウイルスによる肝細胞障害は，ウイルス自身が直接肝細胞を傷害するのではなく，患者自身のナチュラルキラー(natural killer ： NK)細胞やCTLなどの免疫細胞がウイルス感染肝細胞を排除するために肝細胞を破壊する，という免疫応答の結果生じると考えられるに至った。

この肝炎ウイルス由来のペプチドを特異的に認識するCTLは，ウイルス肝炎患者の肝臓内や末梢血中に少なからず存在していることが報告されているが，これら肝炎ウイルス特異的CTLはウイルス感染を終息させようとする生体防御にかかわる一方で，肝細胞を破壊して肝炎の慢性化や重症化にも関連していると考えられる。

感染したHCVが，約70％という高い割合で持続肝感染に至る原因として，HCVの増殖能の高さが考えられる。HCV感染初期にはインターフェロン(interferon ： IFN)-αが誘導されるが，獲得免疫が十分に誘導される前に，HCVは瞬く間に増殖してしまう。さらに，HCV感染の持続

化の機序については，ウイルス側の因子として，HCV自身が免疫逃避を起こす蛋白を産生していることが報告されている。生体側の因子としては，HCVに対して免疫機構に欠陥があることが考えられている。これまでの経験から，小児や若年者ではHCV排除率が高く，逆に移植後の免疫抑制状態やアルコール大量摂取などにより免疫機構に異常がみられる状態では，排除率は低下することがこれまでに知られてきており，患者の免疫応答の強さがウイルス性肝炎の転帰を大きく左右すると考えられる。したがって，ウイルス肝炎に対する生体の免疫応答を観察し，ウイルス肝炎の発症機序やウイルスの生体免疫応答からの逃避機構を詳細に検討することは，ウイルスの排除や肝炎の終息を目的とした治療法の確立，さらにはウイルス感染の予防法の開発に大きな意味をもつ。

これまでに免疫学的に解明，報告されているHCVに対する生体の免疫応答を中心に，C型肝炎における肝障害の発症機序や，HCVの生体免疫からの逃避機構などについて述べる。

HCV感染に対する免疫応答と肝炎の発症機序

1．非特異的免疫応答

HCV感染後，まず生体内では，他のウイルス感染と共通する非特異的応答が生じる(図1)。HCV感染初期は，HCVに感染した肝細胞や，Toll様受容体(toll-like receptor：TLR)を介して感染を認知した形質細胞様樹状細胞(plasmacytoid dendritic cell)などから産生されるインターフェロンIFN-α/β(I型IFN)によりウイルスの増殖抑制が試みられる。I型IFNは2′-5′オリゴアデニル酸合成酵素などを誘導しHCVの増殖を抑制するほか，樹状細胞など抗原提示細胞においてヒト白血球抗原(human leukocyte antigen：HLA) class I分子の表出を増強させる作用や，NK細胞，CTLなどの免疫細胞を活性化させる作用などの免疫応答増強作用を有する。IFN-αにより活性化したNK細胞は，HCVに感染した肝細胞を認識し障害を起こす。肝細胞が傷害を受けることにより刺激を受けた骨髄系樹状細胞(myeloid dendritic cell)は，NK細胞や，NK細胞とT細胞の両者の性質をもち肝臓に多く存在するNKT細胞を活性化し，それらの細胞はIFN-γを多量に分泌する。さらに，IFN-γはマクロファージの活性化を増強し，局所の炎症反応を増強する。

2．HCV特異的細胞性免疫応答

HCV感染において，上記の非特異的免疫応答により肝炎ウイルスが十分に生体から排除できない場合，特異的免疫応答が誘導され，さらなるウイルスの排除が試みられる。

図2に，HCV特異的細胞免疫が誘導される過程を示す。1型ヘルパーT細胞(type I helper T：Th1)は，ウイルス特異的CTLやNK細胞などの細胞性免疫の誘導や活性化に重要な役割を担う。骨髄系樹状細胞は，肝内で死滅したウイルス感染肝細胞などからHCV抗原を取り込むと所属リンパ節に遊走する。リンパ節で表面に共刺激分子などの発現を増強させて成熟した樹状細胞となり，T細胞を活性化する。樹状細胞は，表面のHLA class II分子上に提示されたHCV抗原を認識する未感作ヘルパーT細胞(helper T：Th)を刺激し活性化させる。それにより活性化したTh細胞は，CD 40リガンドを表出し，さらに腫瘍壊死因子(tumor necrosis factor：TNF)-αなどのサイトカインを分泌することで樹状細胞をさらに成熟，活性化させる。主に骨髄系樹状細胞が産生するインターロイキン(interleukin：IL)-12は，感作されたTh細胞をTh 1細胞に分化・誘導し，その後Th 1細胞はIL-2やIFN-γを産生してCTLやNK細胞を刺激し，活性化や増殖を促す。それらにより未感作CTLは樹状細胞が提示するHCV抗原を認識し，はじめて感作される。感作し活性化されたHCV特異的CTLはリンパ

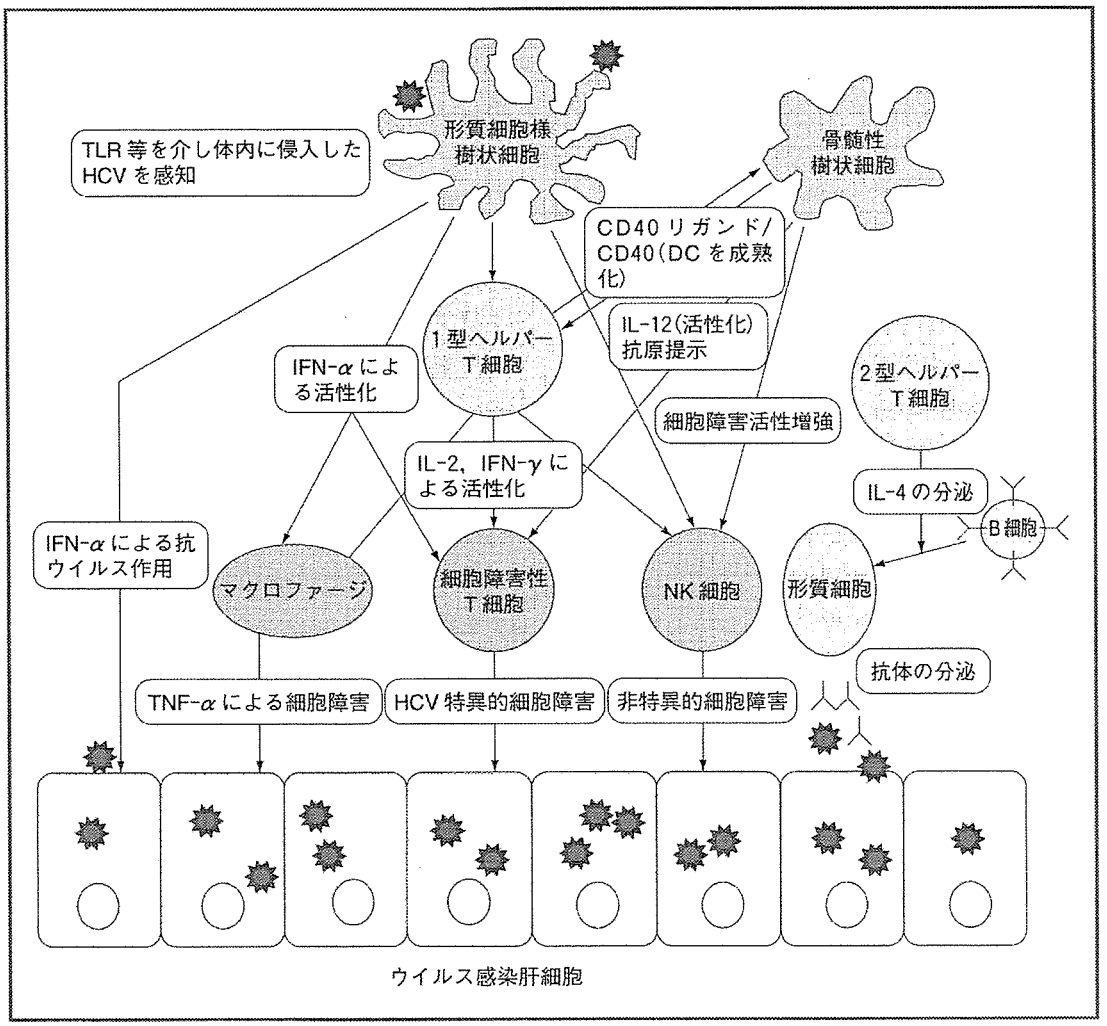

図1　HCV 感染における生体の免疫応答

節を離れ末梢に到達して，HCV に感染した細胞の表面にある HLA class I 分子上に提示された HCV 抗原を認識し，感染細胞の細胞死を誘導することによりウイルスを排除する。

HCV 感染で CTL 応答がウイルスの増殖を抑制していることが示唆されており，初感染時におけるウイルス排除には，ウイルス特異的 CTL の存在が重要であると報告されている。われわれは HLA B 44 に拘束性の CTL エピトープを報告して[3)4)]以来，合成ペプチドや ELISPOT 法などを用いて，HCV の CTL エピトープをこれまでに 10 数個発見し報告してきた[5)6)]。これらのエピトープのアミノ酸配列に相当する合成ペプチドで末梢リンパ球を刺激したところ，3 年以下の感染後比較的早期の慢性肝炎患者では HCV 特異的 CTL 応答が検出されやすいのに対し，10 年以上経過した例では CTL 応答がほとんどみられなかった。一方で，HCV を排除した症例では，排除後 35 年という長期間にわたり HCV に対する $CD4^+$ や $CD8^+$ T 細胞の応答が認められることも報告されている[7)]。肝炎ウイルス感染時に適度な細胞性免疫応答が生じた場合には，ウイルスは完全に排除

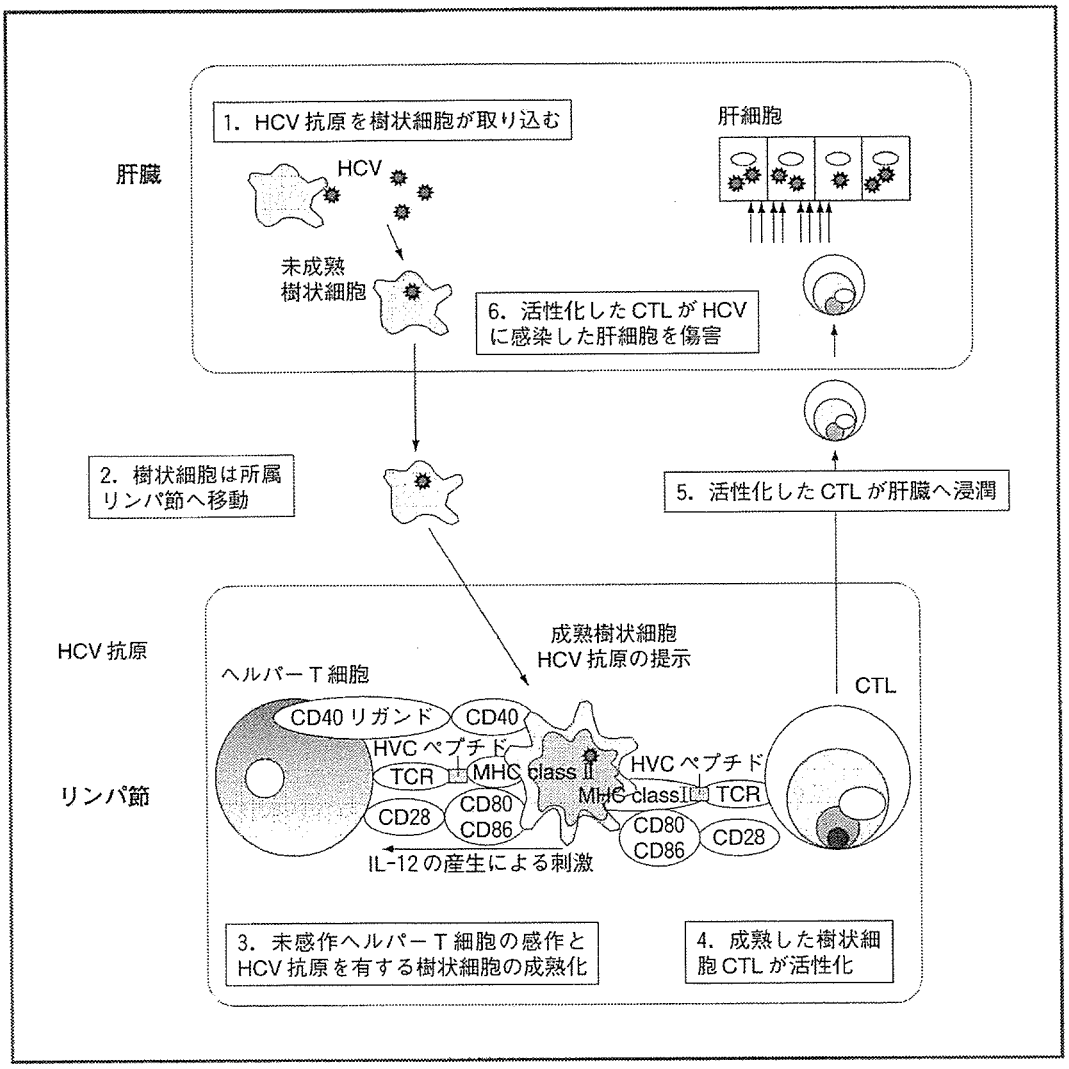

図 2　HCV 特異的細胞障害性 T 細胞の誘導

CTL：細胞障害性 T 細胞，TCR：T 細胞レセプター，MHC：major histocompatibility complex

される。しかし，HCV 感染では生体での免疫応答が一般的に弱いため，HCV による急性肝炎では肝障害の程度が軽く，感染が持続化しやすいと考えられている。それに対して，非常に強い免疫応答が誘導されると，劇症肝炎などの重症な肝障害を引き起こす可能性がある。このように，HCV 量と HCV 特異的 CTL 活性のバランスによりさまざまな程度の肝障害が起こりうると考えられる。

また，C 型慢性肝炎で活性化したウイルス特異的 CTL は肝臓に集積しており，ウイルスの増殖抑制と肝障害に関与していることが報告されている。また，HCV 量の少ない C 型慢性肝炎患者では末梢血中にも HCV 特異的 CTL が検出され，多量のウイルスが T 細胞を抑制あるいは消費しているものと考えられている[8]。HCV 特異的 CTL 応答のみられた進行した C 型慢性肝炎患者では，有意に血清 ALT 値が高値を示していたが，他の臨床像や病理組織像とは関連が少なかったことも報告された[9]。

さらに強い肝炎をともなった患者では，肝炎が軽度である場合には認められないHCV NS3領域の1073-1081のアミノ酸に対する強いCTL応答がみられたが，この部分はインフルエンザウイルスのノイラミニダーゼ(neuraminidase)の配列と交叉反応性を有していた[10]。こうしたCTLの交叉反応性が，C型肝炎の発症や肝炎の炎症の強さに影響することも考えられる。また，この部分のアミノ酸変異は，HCVにとってCTLの認識から逃れることができる一方，HCVの生存や複製に必要なプロテアーゼ活性が失われる可能性も高く，この免疫原性の高いエピトープ領域にアミノ酸変異のあるHCV株が出現しにくい1つの理由になりうる[11]。

3．CTLのHCV感染肝細胞に対する障害機序

成熟した樹状細胞により刺激を受け活性化したウイルス特異的CTLは，類洞から肝組織内に浸潤し，HCV感染細胞の表面に存在するHLA class I分子とその上に提示されている8～11個のアミノ酸よりなるHCV抗原ペプチドをT細胞受容体により認識して，細胞表面に孔を形成するパーフォリンや，細胞をアポトーシスに陥らせる蛋白分解酵素グランザイムを，標的であるウイルス感染細胞に放出して細胞死を引き起こす(図3)。さらに，活性化したCTLはFasリガンドやTNF-αの発現も増強し標的細胞の障害に携わる。パーフォリンはほとんどすべての細胞に細胞障害活性を示すが，FasリガンドやTNF-αはそれらの受容体をもち感受性がある細胞のみに効力を発揮する。正常の肝細胞はFasリガンドやTNF-αに対して抵抗性を示すが，一方，肝炎患者の肝組織中の炎症が強い部位では肝細胞のFas抗原やTNF受容体の発現が増強しており，Fasリガンドや TNF-αに対しての感受性も高まっていることが考えられる。Fas系やTNF-αを介した細胞障害活性はパーフォリンと比較すると細胞障害効率は低いものの，活性化したCTLはこれらの系を介して感受性が高まったウイルス非感染細胞をも傷害すると考えられる(図3)[12]。われわれの実験系から，TNF-αはHCV感染細胞に隣接していない比較的離れた部位にある非感染細胞をも傷害しうることも確認された。これは，ウイルスの拡散を防止する一方で，肝炎の悪化を来す可能性も考えられる。

HCV感染持続化の機序

1．HCVのアミノ酸変異による生体免疫機構からの逃避

HCVは，自らのアミノ酸に変異を起こさせやすいRNAポリメラーゼの作用や高い複製能により，生体内にさまざまなタイプのHCVが存在するというクアシスピーシスを形成し，宿主の免疫監視機構からの逃避を試みている。抗体結合部やT細胞が認識する抗原エピトープのアミノ酸を変異させることにより，抗体がウイルス自体に結合できなくなること，HCV特異的CTLが感染細胞を認識できないようにすること，CTLにトレランスを誘導すること，あるいは未感作CTLを感作させにくくすること[13][14]などにより，生体の免疫応答から逃れていることが想定されている。さらに，クアシスピーシスは多様な細胞への感染を可能にすることや薬剤耐性の獲得にも影響を及ぼすと考えられる。

1)液性免疫からの逃避

一般に，ウイルス特異的中和抗体は体液中のウイルスの排除に作用するが，HCVの初感染から十分な中和抗体が産生されるまでには時間がかかることから，HCV中和抗体はウイルス初感染におけるウイルス排除というより，二次感染の予防に関与していると考えられている。HCV E2領域内の超可変領域(hypervariable region 1：HVR1)のアミノ酸変異はきわめて多様であり，生体で産生される中和抗体による認識から逃れて