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ORIGINAL ARTICLE

Dendritic cell therapy with interferon-α synergistically suppresses outgrowth of established tumors in a murine colorectal cancer model

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Both dendritic cell (DC)-based immunotherapy and interferon (IFN)-α therapy have been proved to have potent long-lasting antitumor effects. In anticipation of synergistic antitumor effects, we performed combination therapy with DCs and IFN-α gene-transduced murine colorectal cancer MC38 cells (MC38-IFN-α). DCs incubated with MC38-IFN-α, but not neomycin-resistance gene-transduced MC38 cells (MC38-Neo), effectively enhanced proliferation of allogeneic splenocytes in vitro. *In 12 of 17 mice, DCs in combination with MC38-IFN-α prevented the development of a parental tumor, while DCs and MC38-Neo did in only three of 17 mice (P = 0.008). In a therapeutic model of an established parental tumor, inoculation of DCs and MC38-IFN-α suppressed the growth of the established parental tumors significantly compared with the administration of DCs with MC38-Neo or naive splenocytes with MC38-IFN-α (P = 0.016 and 0.024, respectively). Analyses of immunohistochemistry and tumor-infiltrating mononuclear cells showed that CD8+, CD11c+, and NK1.1+ cells markedly infiltrated the established tumors of mice treated with DCs and MC38-IFN-α. From the results of observation of parental tumor outgrowth in immune cell-depleted mice, CD8+ cells, and asialo-GM-1+ cells were thought to contribute to the antitumor effects induced by the combination therapy. Furthermore, MC38-specific cytolysis was detected when splenocytes of mice inoculated with DCs and MC38-IFN-α cells were stimulated with MC38-IFN-α cells* in vitro. *Since DC-based immunotherapy in combination with IFN-α-expressing tumor cells induces potent antitumor cellular immune responses, it should be considered for clinical application.*
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Keywords: *dendritic cell; maturation; interferon-α; immunotherapy*

Introduction

Although therapies for malignant tumors including operation, chemotherapy, and radiation therapy have developed remarkably, the complete conquest of malignant tumors has not been achieved yet. In addition, in many cases, patients suffer from side effects of these therapies such as fatigue, anorexia, pyrexia, infection, and suppression of bone marrow. Thus, new anticancer therapies that have more efficacy and fewer side effects are required. The cellular immune response is considered not to work functionally and sufficiently in patients with advanced malignant tumors, and it has been reported that some tumors escape immune surveillance by several mechanisms.[1] To overcome immune suppression or immune escape in patients with advanced malignant tumors, many biologic therapies that aim at inducing potent antitumor immune responses have been tried.

Dendritic cells (DCs) are potent antigen-presenting cells that can elicit primary and secondary immune responses to foreign antigens.[2,3] Immature DCs express low levels of major histocompatibility complex (MHC) class I, class II, and costimulatory molecules (CD80, 86) that play important roles in T cell stimulation.[4] When immature DCs capture and take up antigens, DCs downregulate the function of antigen acquisition, but upregulate MHC and costimulatory molecules. As DCs mature, they will express higher levels of MHC and costimulatory molecules and present antigens to antigen-specific T cells and induce immune responses.[4–8] Since DCs are playing a crucial role in controlling immunity, the use of DCs may be ideal for cancer therapy.[4] Recently, various DC-based therapies have been tried to elicit antitumor responses,[6,8–15] and DCs have been used for therapy in patients with some malignant tumors such as malignant melanoma, lymphoma, renal cell carcinoma, pancreatic, and gastric cancer.[16]

Interferon (IFN)-α has been used to treat patients with not only viral infections such as hepatitis B and C but also some malignant tumors such as melanoma, renal cell carcinoma, and leukemia. The effects of type I IFN include antiviral function, enhancement of IFN-α/β production,[17,18] and inhibition of cell growth and angiogenesis.[19] In addition, IFN-α plays a crucial role in the immune system. IFN-α upregulates the expression of MHC class I on the cell surface and enhances the proliferation of Th1-lymphocytes.[20] Previous studies emphasized the importance of IFN-α for the generation of cytotoxic T lymphocytes (CTLs) in specific antitumor

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immune responses.[21,22] In addition, we reported previously that IFN-α transduction of poorly immunogenic tumor cells reduces tumorigenicity and leads to a long-lasting tumor immunity,[23] and that IFN-α-expressing tumor cells promote the survival of tumor-specific CTLs by preventing apoptosis.[24]

IFN-α has been reported to induce the maturation of DCs. Santini *et al*.[25] demonstrated that IFN-α upregulated the expression of costimulatory molecules (CD80, 86) and MHC class II (HLA-DR) and induced CD83 expression, which is considered to be a marker of mature and activated human DCs. Then, they showed that DCs gained a greater capability to stimulate the proliferation of allogeneic lymphocytes in the presence of IFN-α.[25] Others have also reported the effects of IFN-α on enhancement of DC maturation.[26,27] However, it has also 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.[28]

In our previous studies, we demonstrated that IFN-α gene therapy in combination with CD80 transduction reduces tumorigenicity and the growth of established tumors in poorly immunogenic tumor models,[23] and that IFN-α has additive effects on suppressing tumor growth in cooperation with interleukin (IL)-12.[29] Costimulatory molecules such as CD80 are highly expressed on the surface of DCs, and IL-12 is produced mainly by DCs. Therefore, the combined use of DC-based immunotherapy with IFN-α gene therapy is considered reasonable. Recently, Tsugawa *et al*.[30] reported that combined use of DC with adenoviral vector encoding IFN-α elicits antitumor response in a murine intracranial gliomas model. In this study, as a preliminary investigation of the combined therapy, we investigated the effects of bone marrow-derived DCs and IFN-α-expressing colorectal cancer cells on the proliferation of allogeneic splenocytes. Then, we examined whether DCs and IFN-α-expressing tumor cells display synergistic effects on the induction of antitumor immunity in a therapeutic model to evaluate the possibility of applying this combined therapy to clinical trial.

Results

IFN-α gene transduction does not affect growth of tumor cells in vitro

Each of the tumor cells (wild type (WT), neomycin-resistance gene-transduced MC38 (MC38-Neo)-, murine IFN-α-overexpressing MC38 cells (MC38-IFN-α)) with or without γ-irradiation were seeded at 5×10^5 cells/well in six-well plates, and enumerated every day in duplicate to compare the growth *in vitro* of the genetically modified MC38 cells. The growth rates did not differ significantly between MC38-WT, MC38-Neo, and MC38-IFN-α cells within 72 h incubation (data not shown). Cell counts of each tumor cell were decreased to approximately one-third 48 h after 100 Gy γ-irradiation.

When nonirradiated tumor cells were incubated with DCs, cell growth of each tumor cell line was almost the same with that of MC38-WT without DCs (data not shown). DCs did not affect the cell growth of tumor cell lines.

With the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling assay (TUNEL), we found approximately 50% of the γ-irradiated (100 Gy) MC38 cells to be apoptotic 48 h after irradiation (data not shown). The proportion of apoptotic cells after γ-irradiation did not differ between the genetically modified MC38 cell lines.

Coincubation with MC38-IFN-α cells does not enhance cytokine production by DCs and phenotypic maturation of DCs

The production of murine IFN-α by modified tumor cells or DCs was confirmed by enzyme-linked immunosorbent assay (ELISA). Nonirradiated MC38-IFN-α cells produced large amounts of IFN-α as shown in Table 1. On the other hand, 100 Gy γ-irradiated MC38-IFN-α cells produced about half as much IFN-α as nonirradiated MC38-IFN-α. There was no difference in IFN-α production between the irradiated MC38-IFN-α cells alone and DCs coincubated with irradiated MC38-IFN-α. IFN-α was not detected in supernatants of MC38-WT cells, MC38-Neo cells, DCs, splenocytes, and DCs with MC38-Neo cells.

IL-1β, IL-12, and TNF-α production by DCs was also confirmed by ELISA. Enhancement of these cytokine production was not observed when DCs were coincubated with parental or genetically modified tumor cells (data not shown). Expression of CD80, CD86, and I-A^b molecules on DCs was compared by flow cytometry. Coincubation with genetically modified tumor cells did not enhance the expression of those molecules (data not shown). From these data, coincubation with parental or genetically modified tumor cells does not enhance the cytokine production by DCs as well as the phenotypic maturation of DCs in this system.

Proliferation of allogeneic splenocytes is markedly enhanced by coincubation with MC38-IFN-α cells

To investigate proliferative effects of DCs and MC38-IFN-α cells on allogeneic splenocytes, we performed cell proliferation assays. As shown in Figure 1, stimulation by both DCs and MC38-IFN-α cells markedly enhanced the proliferation of allogeneic splenocytes compared with stimulation by DCs alone or by DCs and MC38-Neo ($P = 0.007$ or 0.020, respectively). When exogenous IFN-α was added to the culture of DCs and MC38-Neo cells, the proliferation of allogeneic splenocytes was

Table 1 Production of IFN-α by genetically modified MC38 tumor cells and DCs

Cell	*IFN-α production (ng/48 h)*
DC	Not detected
MC38-Neo	Not detected
MC38-IFN-α	20.8±0.5
MC38-IFN-α (irradiated)	10.8±0.3
DC+MC38-IFN-α (irradiated)	11.0±1.1
Splenocyte+MC38-IFN-α (irradiated)	7.2±0.6

A total of 1×10^6 DCs or splenocytes were incubated with or without 1×10^5 γ-irradiated (100 Gy) MC38-IFN-α cells in 5 ml of CM in six-well plates. After 48 h incubation, concentration of IFN-α in the culture supernatant was confirmed by ELISA according to the manufacture's protocol.

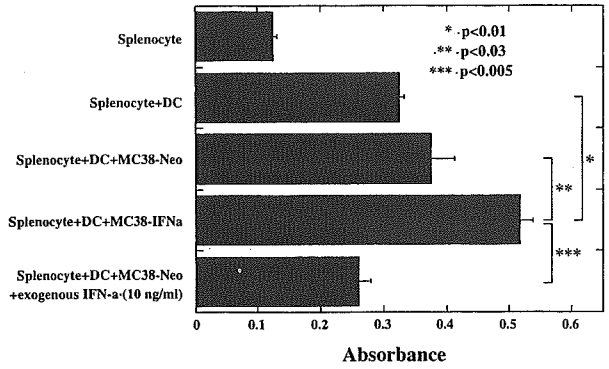

Figure 1 Proliferation of allogeneic splenocytes is markedly enhanced by coincubation with MC38-IFN-α cells. Proliferation of allogeneic splenocytes is markedly enhanced by coincubation with MC38-IFN-α cells. Purified DCs were incubated with γ-irradiated (100 Gy) MC38-Neo or MC38-IFN-α cells at a DC to tumor ratio of 10 for 2 days. The cells were γ-irradiated (30 Gy) for the purpose of using as stimulator cells. In some wells, exogenous murine IFN-α was added at a concentration of 10 ng/ml. After allogeneic splenocytes (5×10^5) were incubated with the stimulator cells (5×10^4) for 3 days, cell proliferation assay was performed. This experiment was performed twice with similar results.

significantly suppressed compared with the stimulation by DCs and MC38-IFN-α cells ($P = 0.005$). The results suggested that MC38-IFN-α cells and DCs stimulate allogeneic splenocytes more effectively than control gene-transduced MC38 cells, and that continuous secretion of IFN-α may be responsible for the proliferative effect on allogeneic splenocytes in this system.

Inoculation of DCs and MC38-IFN-α cells prevents development of parental MC38 tumors in vivo

We investigated the preventive effects of DCs and MC38-IFN-α cells on the development of parental MC38 tumors. At 1 week after the second intraperitoneal inoculation of DCs (or splenocytes) and the modified MC38 cells, MC38-WT cells were inoculated subcutaneously (s.c.). 12 of 17 mice inoculated with DCs+MC38-IFN-α cells did not develop parental tumors, although all mice injected with only DCs or splenocytes had growing parental tumors on day 28 as shown in Figure 2 and Table 2. All these 12 tumor-free mice rejected the subsequent parental MC38 cell challenge. DCs+MC38-Neo and splenocytes+MC38-IFN-α cells also had preventive effects on the development of parental tumors compared with splenocytes alone, although the preventive effects were less than those of DCs+MC38-IFN-α.

$CD8^+$ cells and asialo-GM-1^+ cells contribute to the antitumor effects induced by DCs and IFN-α-expressing tumor cells

We depleted immune cells using anti-CD4, anti-CD8, and anti-asialo-GM-1 antibodies to explore the mechanism of the antitumor effects induced by DCs and IFN-α-expressing tumor cells. We depleted these immune cells *in vivo* after inoculation with DCs and IFN-α-expressing tumor cells. Then, MC38-WT cells were injected and the WT tumor development was measured. When $CD8^+$ cells or asialo-GM-1^+ cells were depleted, we observed obvious growing WT tumors in those mice, whereas

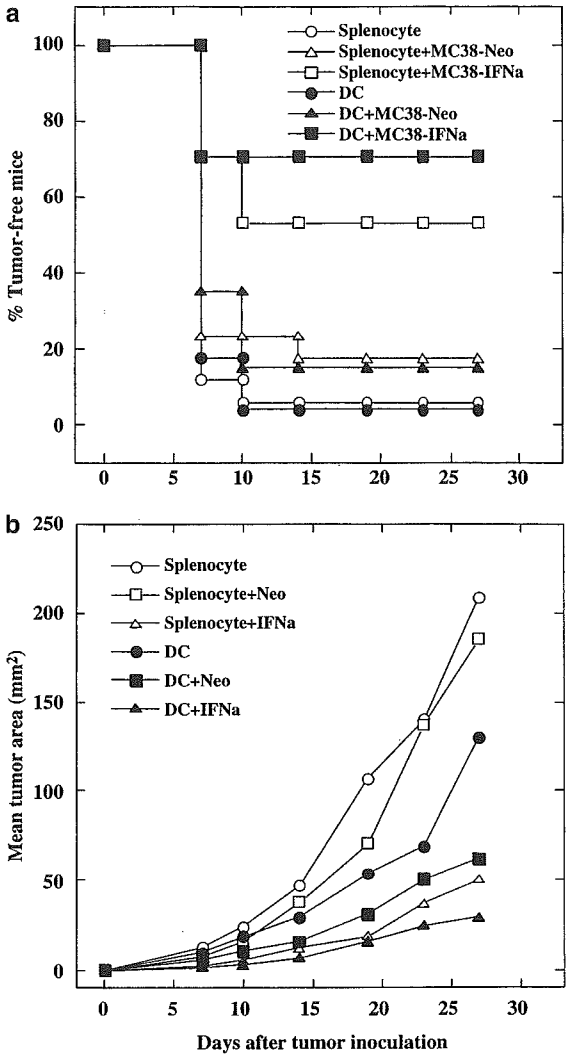

Figure 2 Inoculation of DCs and MC38-IFN-α cells prevents development of parental MC38 tumors *in vivo*. Inoculation of both MC38-IFN-α cells and DCs prevents development of parental MC38 tumor. Mice were injected i.p. with 1×10^6 DCs or splenocytes and 1×10^5 the genetically modified tumor cells twice 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. Results are reported as (**a**) percent of tumor-free mice and (**b**) mean tumor area (mm^2). This experiment was performed three times with similar results.

we detected no tumor in mice not depleted of any immune cells ($P = 0.015$ or 0.023, respectively, Figure 3). On the other hand, in $CD4^+$ cell-depleted mice, the antitumor effects induced by DCs and IFN-α-expressing tumor cells were diminished marginally. Thus, DCs and IFN-α therapy seemed to stimulate $CD8^+$ cells and asialo-GM-1^+ cells mainly *in vivo*.

Therapeutic inoculation of DCs and MC38-IFN-α cells suppresses outgrowth of established parental MC38 tumors

We evaluated the therapeutic effects of DCs and MC38-IFN-α on established parental MC38 tumors. As shown

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.

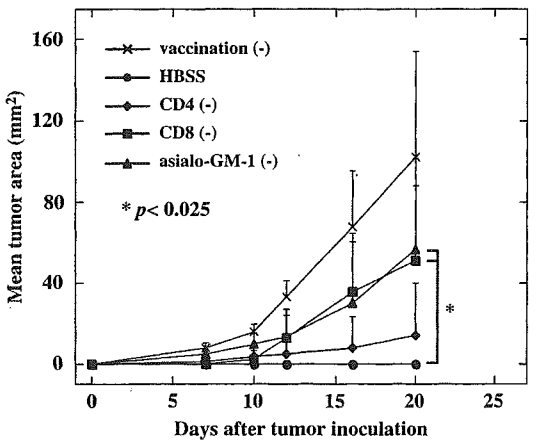

Figure 3 Antitumor effects of DCs and MC38-IFN-α cells in immune cell-depleted mice. $CD4^+$ T cells, $CD8^+$ T cells, or asialo-$GM\text{-}1^+$ cells were depleted by an antibody method as described in the Materials and methods section. After DCs and MC38-IFN-α cells were inoculated i.p. twice at a 7-day interval (days −14 and −7), we depleted $CD4^+$ T cells, $CD8^+$ T cells, or asialo-$GM\text{-}1^+$ 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-$GM\text{-}1^+$ 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-

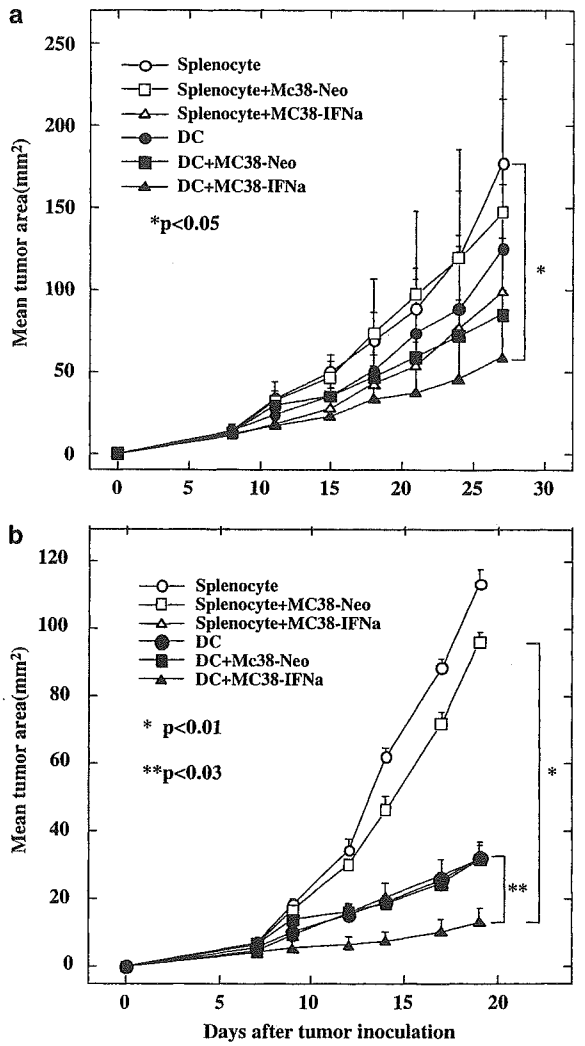

Figure 4 Therapeutic inoculation of DCs and MC38-IFN-α cells suppresses outgrowth of established parental MC38 tumors. Therapeutic inoculation of both DCs and MC38-IFN-α cells has synergistic antitumor effects on established parental MC38 tumor. B6 mice were injected s.c. in the right flank with 1×10^5 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

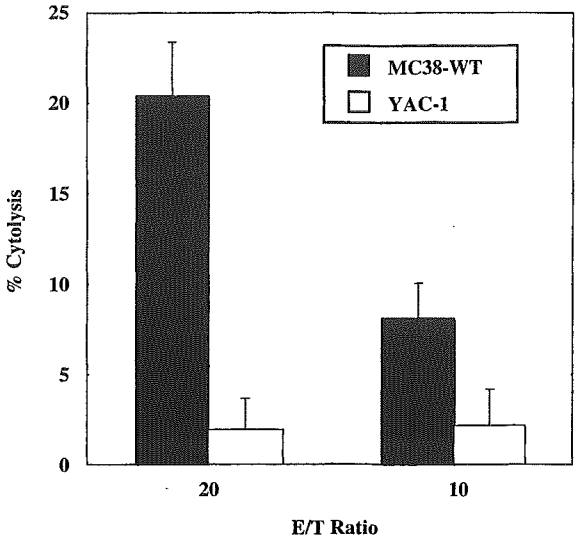

Figure 5 Tumor-specific cytolysis was clearly detected when splenocytes of mice inoculated with both DCs and MC38-IFN-α cells were stimulated with MC38-IFN-α *in vitro*. Tumor-specific CTLs were clearly induced from splenocytes of mice inoculated with both MC38-IFN-α cells and DCs. Mice, which received 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-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.

Acknowledgements

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C型肝炎の発症機序とHCV持続感染

Immunopathogenesis of hepatitis C and mechanism of HCV persistence

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永井書店

肝疾患の病態と対策

特集

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肝臓の臨床最前線

Key words 細胞障害性T細胞 ヘルパーT細胞 調節性T細胞 ナチュラルキラー細胞 サイトカイン

一般に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障害するというよりも，患者自身の細胞障害性T細胞(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されている．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以前から報告されているが，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．

HCV感染の持続化の機序については，HCVに対して生体の免疫機構に欠陥があることや，HCV自身が免疫逃避を起こす蛋白を産生していることなどが報告されている．また，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．

本稿では，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き述べる．

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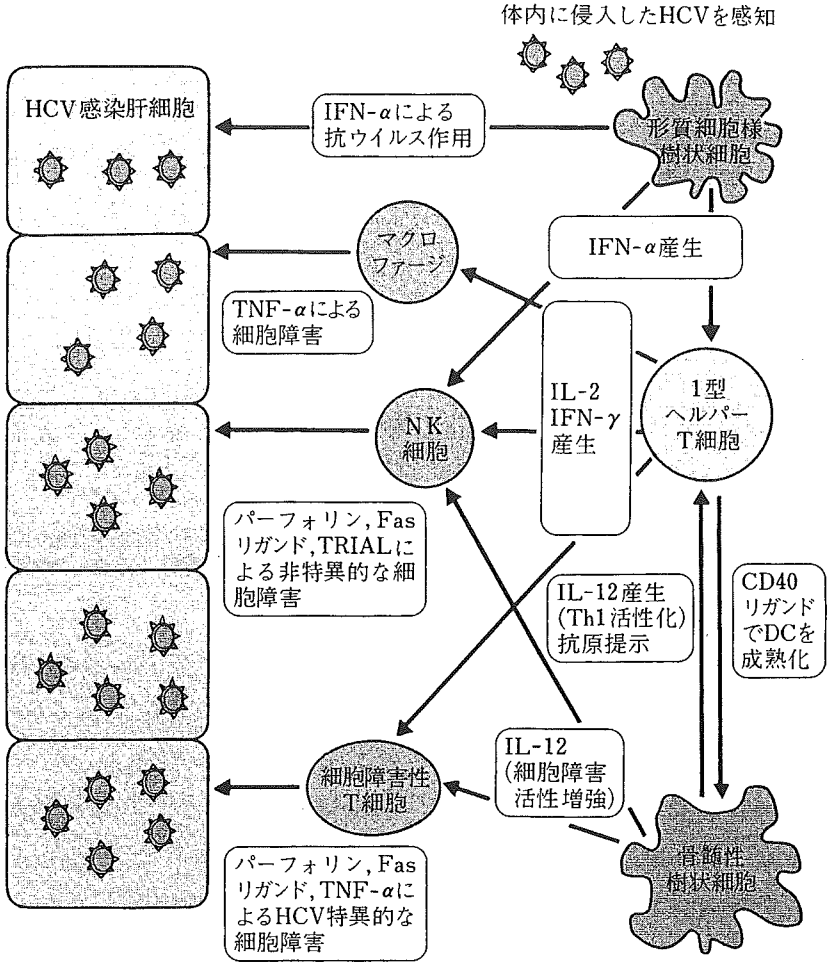

図1 HCV 感染における生体の細胞性免疫応答

Ⅰ. HCV 感染に対する免疫応答

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(interferon ; 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，ナチュラルキラー(natural killer ; 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．肝細胞が障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持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．

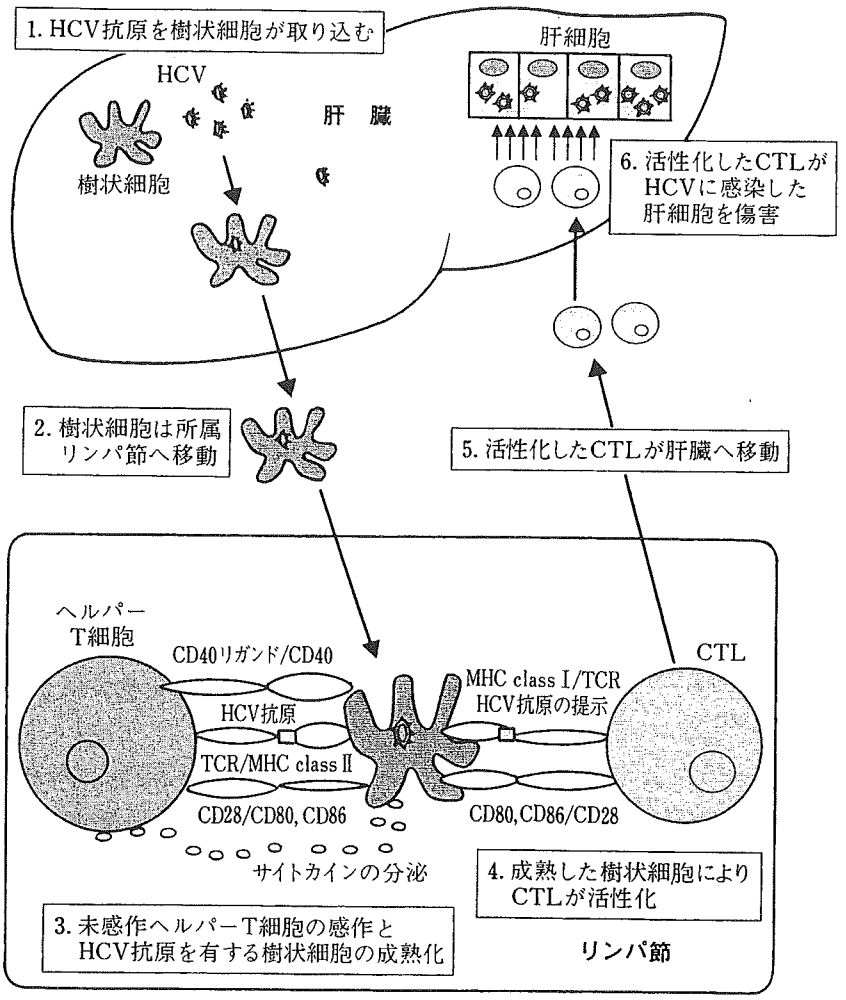

図2 HCV 特異的細胞障害性 T 細胞の誘導
CTL；細胞障害性 T 細胞，TCR；T 細胞レセプター，MHC；major histocompatibility complex

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，CD40リガンドを表出し，さらにサイトカインを分泌することで樹状細胞をさらに成熟化，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 Th1細胞に分化，誘導し，その後 Th1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，初めて感作される．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(図2)．

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．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[1]．このように，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される．しかし，HCV 感染では生体での免疫応答が一般に弱いため，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[2]．C 型肝炎治癒症例はTh1優位であるといわれているが，近年，C 型慢性肝炎の治療に用いられているリバビリン(商品名レベトール®)は，患者の免疫応答をTh2からTh1優位に変化させることが，抗ウイルス効果の一つの機序と考えられている．

Ⅱ．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 は表面のT 細胞受容体により，ウイルス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から11個のアミノ酸よりなるウイルス抗原ペプチドを認識し，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．さらに，活性化したCTL はFas リガンドや tumor necrosis factor (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持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障害すると考えられる(**図3**)[3]．

Ⅲ．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

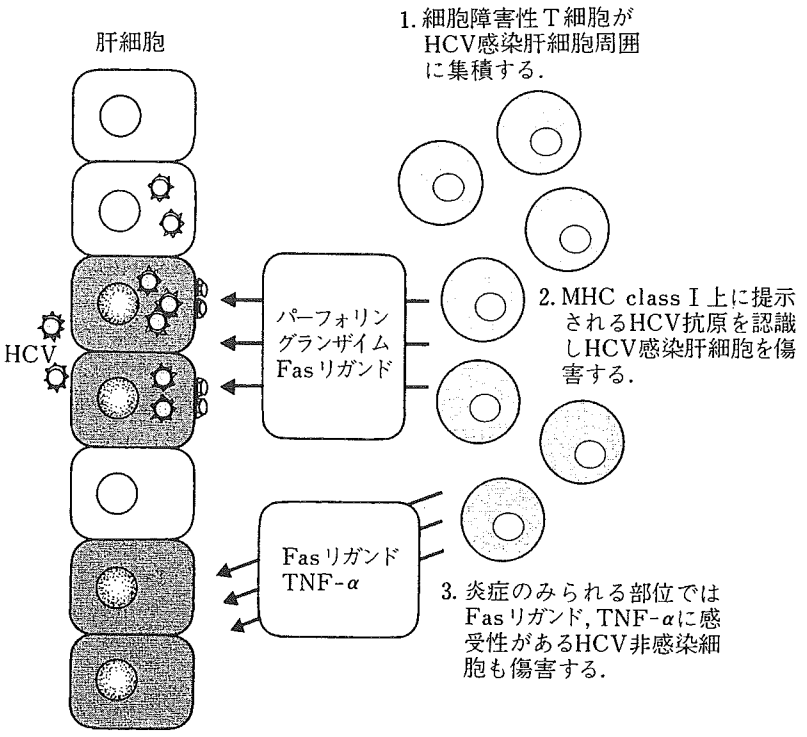

図3 HCV特異的細胞障害性T細胞による肝細胞傷害

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領域の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持続感染に寄与しているとされている．しかし一方，チンパンジーでのHCV感染実験では，HVR1に変異はみられずに持続感染に発展しているとの報告もあり，E2領域の変異が感染の持続化に大きな役割を果たしているかを疑問視する意見もある．

2. 細胞性免疫からの逃避

HLA class Ⅰあるいはclass Ⅱ拘束性T細胞のエピトープの変異は，HCV感染肝細胞の排除を妨げることにより感染の持続化に寄与する可能性がある．これまでの研究では，感染HCVのクローニングにより，$CD8^+$ CTLの認識を妨げるアミノ酸変異が認められている．また，HLA-DRB1拘束性でNS3由来のペプチドを認識するTh1タイプのT細胞に対し，このエピトープ内の1つのアミノ酸に変異が起こると，分泌するサイトカインがTh1タイプからTh2タイプに変化したことが報告された[4]．

一方，HCV感染に対する初期のCTL応答は多様であり，一つのエピトープの変異のみで持続化は説明できないとの指摘もある．エスケープミュータントは持続感染の原因というより，持続感染の結果を見ている可能性も否定できない．

Ⅳ. HCV感染による免疫細胞の機能抑制

抗HCV抗体は感染後2～4ヵ月間もの間出現せず，さらに抗体が現れてもHCV感染は持続し肝炎は進行する．また，細胞性免疫においても，急性期には多様なT細胞応答が認められるが，慢性化するとその応答は劇的に減弱してしまう．肝内には多数のHCV特異的$CD8^+$ T細胞が存在してはいるものの，HCVを排除できない．さらに，C型慢性肝炎患者にはB型肝炎ウイルスや細菌感染などの合併も多く，以前から生体での免疫力の低下が想定されてきた．近年，HCV自体が能

表1 HCV持続感染におけるHCV蛋白の作用

免疫細胞	作用	参考文献
NK細胞	CD81と結合しNK細胞の機能を抑制	Tseng CT, J Exp Med 195：43, 2002
T細胞	アミノ酸変異により分泌するサイトカインがTh1からTh2に変化	Wang JH, Hum Immunol 64：662, 2003
	C型肝炎患者の末梢リンパ球でCD3ζ鎖の発現が低下	Maki A, Hepatol Res 27：272, 2003
	C型肝炎患者の肝浸潤リンパ球でT細胞レセプターδ鎖の発現が低下	Leroy V, Hepatology 38：829, 2003
	IL-2の欠乏によりCTLのエフェクター機能が低下	Francavilla V, Eur J Immunol 34：427, 2004
B細胞	B細胞にもCD81分子が発現	Zuckerman E, J Virol 77：10432, 2003
樹状細胞	アロT細胞刺激能の低下	Bain C, Gastroenterology 120：512, 2001
	形質細胞様樹状細胞におけるIFN-α産生能の低下	Goutagny N, J Infect Dis 189：1646, 2004
	HCV E1蛋白が樹状細胞の成熟化を抑制	Sarobe P, J Virol 77：10862, 2003
	HCV CoreとNS3蛋白が樹状細胞の分化とアロ刺激能を抑制	Dolganiuc A, J Immunol 170：561, 2003
	MIC A／B発現の抑制	Jinushi M, J Immunol 171：5423, 2003
	C型肝炎患者の樹状細胞にHCVが増殖	Goutagny N, J Infect Dis 187：1951, 2003
	チンパンジーのHCV感染実験では樹状細胞の機能低下は認められない	Larsson M, J Virol 78：6151, 2004
調節性T細胞	$CD4^+$ $CD25^+$細胞が肝炎の持続化に関与	Sugimoto K, Hepatology 38：1437, 2003
	C型肝炎患者の末梢血からHCV Core蛋白特異的調節性T細胞を分離	MacDonald AJ, J Infect Dis 185：720, 2002
	C型肝炎患者の肝内に調節性T細胞が存在し，CTLの機能を抑制	Accapezzato D, J Clin Invest 113；963, 2004
	NS4蛋白は健常者の末梢単核球からIL-10の産生を促進	Brady MT, Eur J Immunol 33：3448, 2003
その他	Jak-STAT系の抑制	Blindenbacher A, Gastroenterology 124：1465, 2003
	Fasを介したアポトーシスを抑制	Moorman JP, Virology 312：320, 2003
	MHC class Ⅰ分子の発現抑制	Konan KV, J Virol 77：7843, 2003
	MHC class Ⅰ分子の発現増強	Herzer K, J Virol 77：8299, 2003

動的に生体の免疫機構を抑制している可能性が考えられており，それを示唆する報告が多数なされるようになった．HCV感染による免疫細胞の機能抑制を表1に示す．

1. 自然免疫に対する抑制

HCVのE2タンパクは細胞表面上のCD81と高い親和性を持ち，CD81はHCVが細胞感染する際の受容体となりうると考えられている．HCVのE2タンパクはNK細胞上に発現するCD81と結合し，直接NK細胞の機能を低下させる作用があることが報告された[5]．NK細胞はIFN治療一週間後には肝内への浸潤が観察されるが，治療有効群と治療不応群との間でNK細胞の細胞障害活性は異なり，IFN治療の有効性を予知する指標となるとされている．このことからも，NK細胞を中心とした自然免疫系もHCV排除には重要な役割を果たすと考えられるが，HCVが直接NK細胞の活性を抑制することは感染の持続化に対し大きな意味を持つ．また，HCVコア蛋白はp53依存性にtransporter associated with antigen processing 1(TAP1)の発現を増強することで，MHC class Ⅰ発現を増強するとも報告されている．この中で筆者らはMHC class Ⅰの発現増強は，NK細胞のHCV感染肝細胞に対する細胞障害活性を低下させ感染の持続化に寄与すると推論している．

2. 液性免疫に対する抑制

C型肝炎患者の末梢リンパ球は，CD81分子が

強発現しており，HCVが感染しやすい状態になっていることが考えられ，感染を介して抗体産生などに影響を及ぼしている可能性がある．また，他の感染実験でも，中和抗体の抗体価は低く再感染を防止することはできなかったため，B細胞応答もHCVにより抑制されていることが想定されている．

3. T細胞に対する抑制

HCV特異的CTLのエフェクターとしての機能は明らかに低下している．C型慢性肝炎患者の末梢リンパ球においてはCD3ζ鎖の発現が，また肝浸潤リンパ球においてはT細胞レセプターδ鎖やCD56の発現が低下していることが報告されている．また，HBV特異的CTLに比しHCV特異的CTLは明らかにパーフォリンの発現量が少なく，これは機能低下を示す一つの例とされているが，このような免疫細胞自体の機能低下もC型肝炎の持続化に関与することが想定されている．また，C型急性肝炎時には$CCR7^{-}$ $CD8^{+}$ T細胞(メモリー・エフェクター細胞)は細胞障害活性が低下しているが，これにIL-2を加えると完全にエフェクター機能を有する細胞になることから，T細胞が活性化する際のIL-2の欠乏がCTLの機能低下主な原因であるとの報告もある[6]．循環しているコア蛋白がIL-2産生のシグナル伝達の抑制に関与していることも想定されている．

C型慢性肝炎患者において，HCV特異的$CD4^{+}$ T細胞の存在は認められるものの抗原特異的な増殖能は抑制され，さらに抗原刺激に特異的なIL-10やTGF-β産生も有意に認められており，HCV持続感染の一因となりうる．

また，HCV感染肝細胞より遊離し末梢血中に存在するHCVコア蛋白は，T細胞のgC1qRと結合することで，T細胞の増殖や活性，IFN-γ産生能を阻害することが報告された．HCVコア蛋白は血中にナノグラムの単位で存在しており，gC1qRと結合するには十分量と考えられるが，肝組織内ではさらに高濃度のコア蛋白が存在していると想定され，肝浸潤リンパ球に少なからず影響を与えていると推測される．

HCV NS4A/B蛋白は，細胞内で小胞体からゴルジ体への輸送を妨げることにより，major histocompatibility complex (MHC) class Ⅰ分子の細胞上への発現を抑制することが報告された[7]．これにより，HCV特異的$CD8^{+}$ T細胞がHCV感染肝細胞を認識しにくくなり，HCVの感染持続化に繋がることも考えられる．

さらに，肝臓には類洞内皮細胞やKupffer細胞といった免疫に関与する細胞が存在するが，それらは成熟した樹状細胞とは異なり，ウイルス抗原は提示するもののCD80やCD86といった共刺激分子に乏しいためT細胞を十分に刺激できないばかりか，かえって免疫寛容を誘導してしまうことも考えられている[8]．

4. 樹状細胞に対する抑制作用

樹状細胞は免疫を誘導するうえで，重要な役割を担っている．C型慢性肝炎患者においては，樹状細胞のallogeneicのT細胞を刺激する能力が低下していることや，形質細胞様樹状細胞の減少によりインターフェロンα産生が低下していること，さらに，HCVコアとE1蛋白は樹状細胞の成熟化を抑制することでT細胞刺激能を減弱させていたことがこれまでに指摘されている．また，Jinushiらは，IFN-α刺激後に樹状細胞は，その表面にMHC class Ⅰ-related chain A and B (MICA/B)を発現させNK細胞を活性化することに着目し，C型慢性肝炎患者においてNK細胞が有効に活性化できないのは，type Ⅰ IFNによるIL-15産生能が低下しており，MICA/B発現の増強が抑制されていることが原因であると報告している[9]．

HCVはE2蛋白が樹状細胞上に発現するDC-SIGNに結合することより樹状細胞にも感染することや，soluble E2蛋白も樹状細胞と結合が可能であることも報告され，それらにより樹状細胞は機能低下に陥る可能性も考えられている．