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.

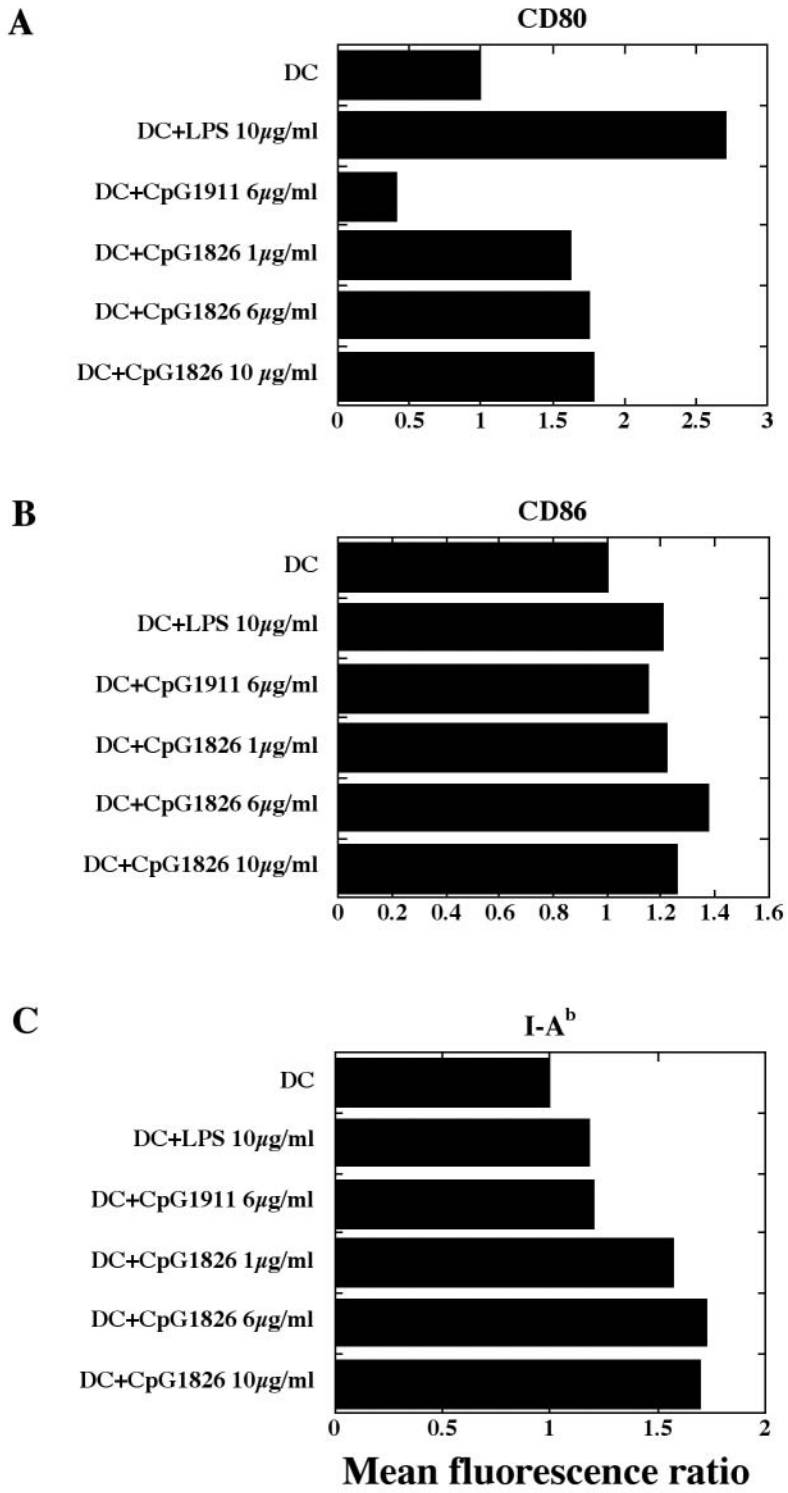

Figure 1. *CpG-ODN-1826 induced DC maturation effectively at the concentration of 6 μg/ml. Purified DCs were incubated with CpG-ODN-1826 at the indicated concentrations (1, 6, 10 μg/ml), non-CpG-ODN-1911 (6 μg/ml) or lipopolysaccharide (LPS) (10 μg/ml) in CM. After 48-h incubation, (A) CD80, (B) CD86 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.

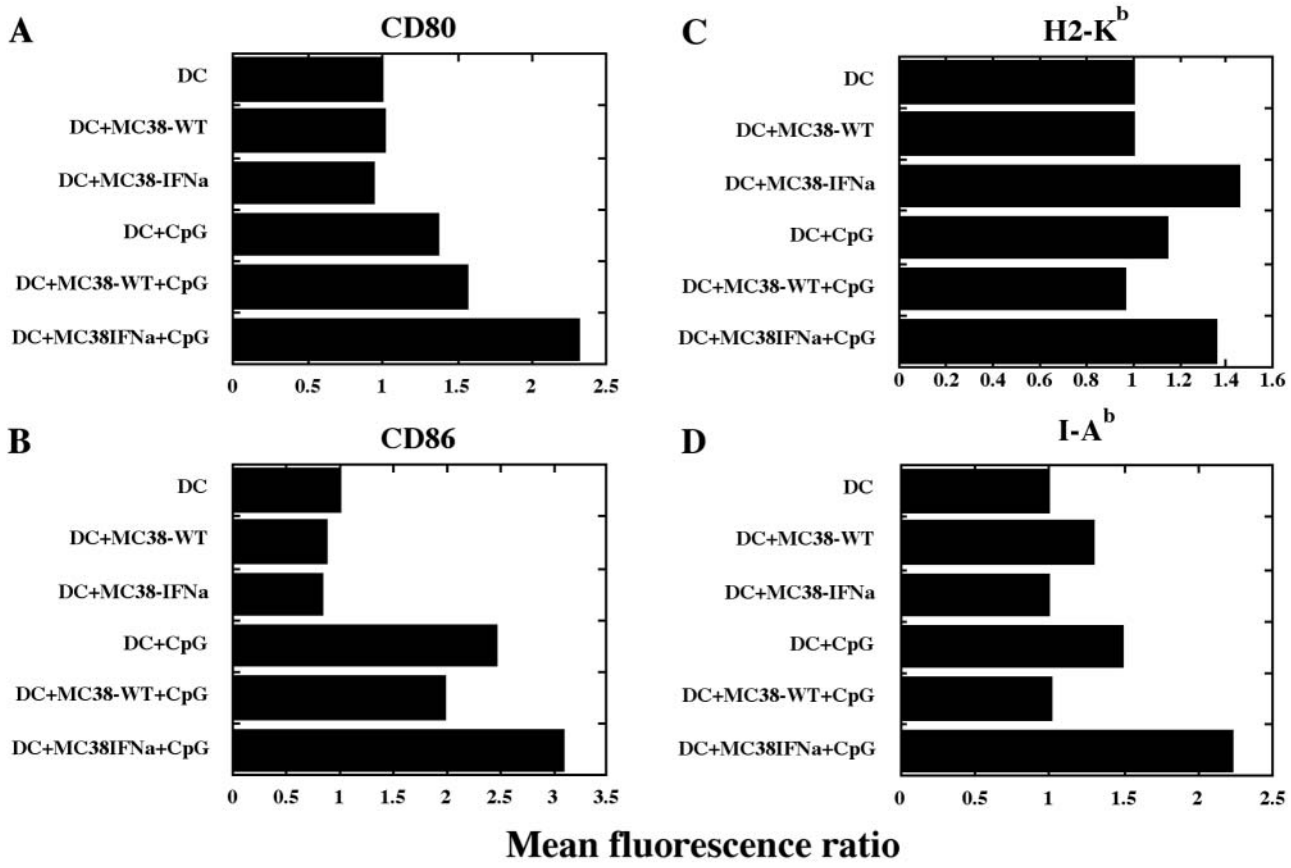

Figure 2. *CpG-ODN-1826 and irradiated IFN-α-expressing tumor cells markedly enhanced DC maturation. Purified DCs were co-cultured with γ-irradiated (100 Gy) MC38-WT or MC38-IFNα cells at a DC to tumor ratio of 10, with or without CpG-ODN-1826 (6 μg/ml) in CM. After 48-h incubation, (A) CD80, (B) CD86, (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.

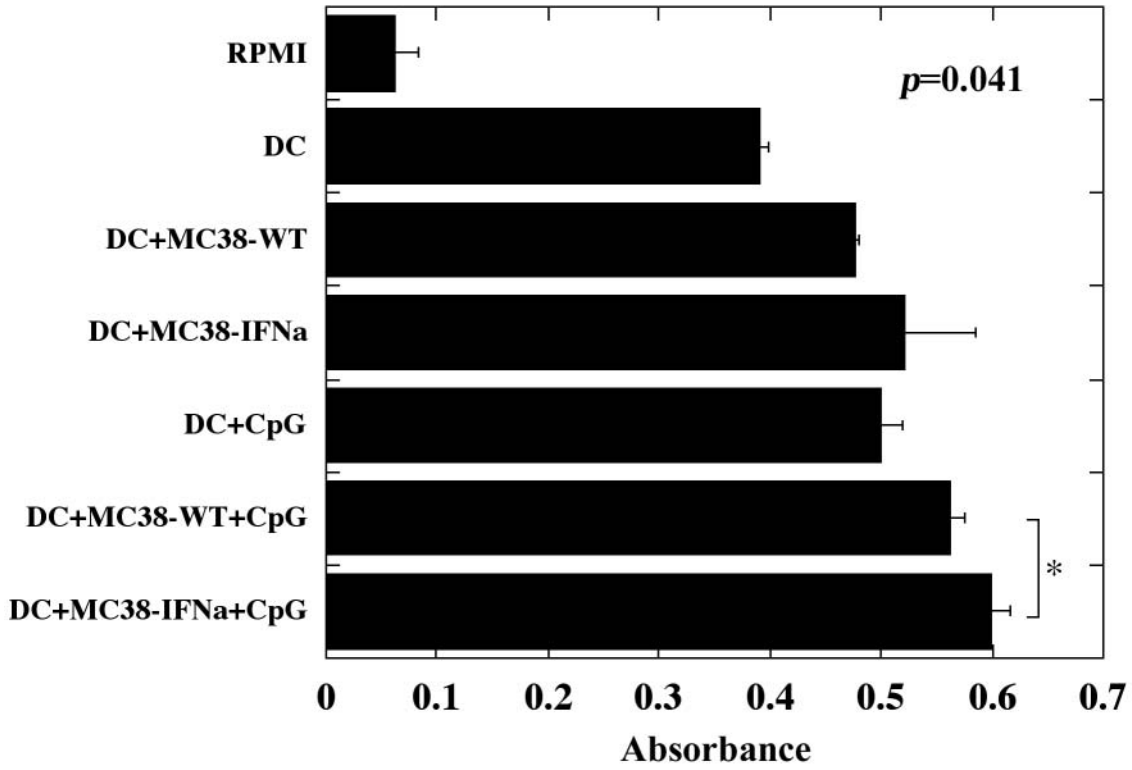

Figure 3. *Proliferation of allogeneic splenocytes is markedly enhanced by DCs co-incubated with IFN-α-expressing MC38 cells and CpG-ODN. Purified DCs were incubated with γ-irradiated (100 Gy) MC38-WT or MC38-IFNα cells at a DC to tumor ratio of 10 and with or without 6 μg/ml CpG-ODN-1826 for 48 h. These 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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