Interleukin-4 and CpG oligonucleotide therapy suppresses the outgrowth of tumors by activating tumor-specific Th1-type immune responses

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Received November 14, 2011; Accepted January 9, 2012

DOI: 10.3892/or.2012.1723

Abstract. Because IL-4 and CpG oligodeoxynucleotides (CpG-ODNs) are immune stimulants, we evaluated the antitumor effects of IL-4 gene therapy and CpG-ODN treatment in a poorly immunogenic murine cancer model. We used a murine colorectal cancer MC38 cell line overexpressing the IL-4 gene (MC38-IL4). Incubation with MC38-IL4 and CpG-ODN enhanced bone marrow-derived dendritic cell (DC) maturation in vitro. In addition, interferon (IFN)-γ production was significantly increased in naïve splenocytes after they were coincubated with MC38-IL4 and CpG-ODN. When mice bearing MC38 wild-type tumors were inoculated subcutaneously with MC38-IL4 cells and CpG-ODN, the outgrowth of established parental tumors was significantly suppressed compared to those in the MC38-IL4-treated group (IL-4 vs. IL-4 + CpG-ODN, P=0.015). A marked infiltration of CD8⁺ cells in the established parental tumors of mice treated with MC38-IL4 and CpG-ODN was confirmed by immunohistochemical analyses (MC38-IL4, 2.8±1.9 cells/field vs. MC38-IL4 + CpG-ODN, 20.7±15.3 cells/field, P=0.027). Significant tumor-specific cytolysis was detected when splenocytes of MC38-IL4 + CpG-ODN-treated mice were stimulated by γ-irradiated MC38-IL4 cells and CpG-ODN twice weekly in vitro and used as effector cells in a chromium-release assay (32.2±3.5% for MC38 cells vs. 3.2±1.1% for YAC-1 cells; at an effector to target ratio of 40). These results suggest that IL-4 and CpG-ODN treatment promotes potent Th1-type antitumor immune responses. Therefore, the combination of IL-4 gene therapy and CpG-ODN treatment for cancer should be evaluated in clinical trials.

Introduction

Several original treatments of cancer have been developed in the last decade. Many anticancer drugs, including molecule-targeted drugs, have been applied clinically. Although some of these drugs have dramatically changed the prognoses of a number of diseases, several harmful side effects were observed. Because cancer immunotherapy is thought to induce few adverse events compared to other therapies, such as chemotherapy and radiation, the establishment of novel treatments that elicit potent immune responses against cancer has been anticipated. Indeed, many clinical trials of such treatments have been conducted. However, most of them have shown only minimal effects, and immunotherapy has not yet been applied as a conventional clinical treatment for cancer. Moreover, patients with advanced cancer exhibit impaired immune responses. Therefore, new strategies are needed to induce more potent antitumor immunities.

We have previously reported the antitumor effects and mechanisms of cell-based immunotherapies that were combined with treatments with cytokines, such as interferon (IFN)-α (1-3), IL-4 (4) and IL-12 (1). The administration of each cytokine gene-transduced tumor cells appears to induce potent cellular immune responses. However, the antitumor immune response mechanisms that were induced by these cytokine therapies differ from each other. In particular, we reported that IL-4 treatment resulted in unique and interesting biological effects.

IL-4, which is a type-2 response inducer (5,6), plays a major role in both B-cell and T-cell development in the immune system (7). In addition, it causes a class switch of B cells, upregulates MHC class II and adhesion molecules, and prevents the apoptosis of T cells (8). With respect to the antitumor effects, IL-4 has a direct inhibitory effect on tumor cell growth in vivo and in vitro (9,10) and an antiangiogenic effect (11). It has been reported that IL-4 activates endothelia in the tumor microenvironment, which results in an increased infiltration of immune cells (12). Other reports have suggested...
that eosinophils and neutrophils are responsible for the anti-
tumor effects induced by IL-4 (13-15). In addition, a recent
study demonstrated that local IL-4 delivery at the site of
vaccination activates local dendritic cells (DCs), which play a
critical role in the initiation, promotion, and regulation of host
immune responses and promote type-I T-helper (Th1) cell
responses (16). In that investigation, IL-4 appeared to support
DC maturation and enhance IL-12p70 secretion from DCs.
Because these findings support the therapeutic effects of IL-4
on tumors, IL-4 has been applied in the clinical treatment of
tumors (17,18).

The potent immunomodulator CpG is present at the
expected frequency of about 1 in 16 bases in bacterial DNA,
but it is hardly observed in vertebrate DNA. It has been
reported that DNA vaccines and synthetic oligodeoxynucleo-
tides (ODN) containing an unmethylated CpG motif promote
Th1-type immune responses. CpG stimulates DCs through the
toll-like receptor 9 and enhances DC maturation, which may
improve therapeutic effects on established tumors. Recently,
we reported the synergistic effects of the combination of
CpG-ODN and IFN-α on DC maturation (19). Furthermore,
DC-based therapy with CpG-ODN and IFN-α exhibited
potent antitumor effects by inducing tumor-specific cytotoxic
T-lymphocyte (CTL) responses (3).

Although we have previously demonstrated that IL-4
promotes a Th1-type antitumor immune response, evidence of
the induction of a more potent immune response that dimin-
ishes established tumors in vivo is required before justification
of a clinical trial. In the present study, we anticipated that the
combination of IL-4 gene therapy and CpG-ODN would have
additive effects on tumors. Thus, we investigated the anti-
tumor effects of the combination therapy and the underlying
mechanisms of these effects in order to determine whether the
combined therapy would be appropriated in a clinical trial.

Materials and methods

Mice. Female C57BL/6 (B6) mice that were 6-8 weeks old
were purchased from Sankyo Lab Service (Tokyo, Japan) for
use in experiments when they were 8-12 weeks old. Mice were
maintained in an animal care facility at Showa University.
This study was approved by the Ethics Committee for Animal
Experiments of Showa University (permission #2011-1111).

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 that was supplemented with 10% heat-inactivated
fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin,
and 100 μg/ml streptomycin (complete medium; CM) in a
humidified incubator with 5% CO₂ in air at 37°C. All cell
culture reagents were purchased from Life Technologies Corp.
(Gaithersburg, MD). CpG-ODN-1826 and non-CpG-ODN-1911
(used as control ODN) were synthesized by Sigma-Aldrich
Corp., St. Louis, MO. We have established a IL-4-overexpressing MC38 cell line (MC38-IL4), which produces a large amount of IL-4 (359.9±108.2 ng/10⁶ cells/48 h)
(4). A neomycin-resistant gene-transduced MC38 (MC38-Neo)
cell line was used as a control (21). The growth rate of the
MC38-IL4 cells did not differ from those of MC38-wild-type
(WT) or MC38-Neo cells in vitro.

Phenotypic changes of DCs after coincubation with IL-4-
overexpressing tumor cells and CpG-ODN. In order to observe
the immunogenic effects of IL-4 and CpG-ODN on DC matu-
ration, flow cytometry was performed using FACScalibur
(Nippon Becton-Dickinson Co., Ltd., Tokyo, Japan). Bone
marrow-derived DCs were generated, as previously reported
(2). We incubated the DCs (5x10⁶-6x10⁶ cells/flask) with either MC38-IL4 or MC38-Neo cells (1x10⁶ cells) that were
100 Gy-irradiated in combination with 6 μg/ml of either
CpG-ODN-1826 or non-CpG-ODN-1911 in vitro. After a 24-h
incubation, cells were harvested and stained with fluorescein
isothiocyanate (FITC)-conjugated monoclonal antibodies.
The monoclonal antibodies used in this assay were FITC-
conjugated anti-H-2Kb, I-Aβ, CD80, and CD86 antibodies
(obtained from Nippon Becton-Dickinson). The results were
shown as ratios of the mean fluorescent intensity (MFI) of
the DCs that were incubated with each monoclonal antibody
to the MFI of DCs incubated with the FITC-conjugated control
IgG antibody.

IFN-γ and IL-10 production in naïve splenocytes cocoinoculated
with IL-4-overexpressing tumor cells and CpG-ODN in vitro.
In order to assess the effects of IL-4 and CpG-ODN on immune
balance, we performed an enzyme-linked immunosorbent
assay (ELISA) of the representative Th1-type cytokine, IFN-γ
and the representative Th2-type cytokine, IL-10. Splenocytes
(1x10⁶ cells/ml) from naive mice were stimulated with either
MC38-IL4 or MC38-Neo cells (1x10⁵ cells/ml) either alone or
in combination with 6 μg/ml of CpG-ODN-1826 or non-CpG-
ODN-1911 in vitro. After a 24-h incubation, we measured the
centrations of IFN-γ and IL-10 in the supernatant of
each culture with ELISA using a commercially available kit,
according to the manufacturer’s instructions (mouse IFN-γ
ELISA and mouse IL-10 ELISA, Thermo Fisher Scientific,
Inc., Rockford, IL). This experiment was repeated twice.

Combination therapy of IL-4-overexpressing tumors with
CpG-ODN in therapeutic models. In order to evaluate the in vivo
therapeutic effects of the combination of IL-4-
overexpressing tumor cells and CpG-ODN on established
tumors, we measured the size of established MC38-WT
tumors in mice before and after treatment, as previously
described (21). In brief, B6 mice were first injected s.c. with
3x10⁵ MC38-WT cells in the right flank. Seven, 9, and 11 days
after the WT inoculation, 3x10⁵ MC38-Neo or MC38-IL4 cells
were inoculated s.c. with 30 μg/mouse of either CpG-ODN-
1826 or non-CpG-ODN-1911 around the established parental
tumors, which had reached 4-20 mm in size. Otherwise,
tumor-bearing B6 mice (5x10⁵ MC38-WT cells/mouse) were
inoculated 5x10⁵ MC38-Neo or MC38-IL4 cells were inocu-
sated s.c. with either CpG-ODN-1826 or ODN-1911 in the same
manner. Tumor size was measured twice a week using vernier calipers. Each experiment involved 4–6 mice per group. Mice with ulcerated tumors or tumors >20 mm in diameter were sacrificed. Experiments with the therapeutic model were performed twice.

**Immunohistological analysis.** For leukocyte detection in tumor tissues, B6 mice were injected s.c. with $3 \times 10^5$ MC38-Neo or -IL4 cells with or without 30 µg/mouse of CpG-ODN-1826 in the area surrounding the established wild-type tumor 7 days after inoculation with $3 \times 10^5$ MC38-WT cells. Tumor tissues, which were harvested 3 days after inoculation with the genetically modified tumor. Serial 5-µm sections were exposed to anti-Gr-1, anti-CD11c, anti-CD4, and anti-CD8a antibodies (Nippon Becton-Dickinson). Rat IgG2a was used as a control antibody. Immunostaining was completed with a Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA). Immunoreactive cells were counted in 6 fields in a light microscope (magnification, x400) in a blinded fashion.

**Induction of tumor-specific CTL.** We assessed the tumor-specific cytolytic activity of immune mice. Mice were initially immunized with MC38-IL4 cells in combination with 30 µg of either CpG-ODN-1826 or non-CpG-ODN-1911 in vitro. Seven groups were compared: i) DC only, ii) DC + non-CpG, iii) DC + IL4, iv) DC + non-CpG + MC38-Neo, v) DC + non-CpG + MC38-IL4, vi) DC + CpG + MC38-Neo, and vii) DC + CpG + MC38-IL4. After a 24-h incubation, cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-H-2Kb, I-A^b, CD80, and CD86 monoclonal antibodies. The results are shown as ratios of mean fluorescent intensity (MFI) of the incubated DCs to the MFI of FITC-conjugated control IgG of each group.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Coincubation with interleukin (IL)-4 and CpG-oligodeoxynucleotides (ODN) induces DC maturation in vitro. We incubated bone marrow-derived DCs with irradiated IL-4-overexpressing or control gene (Neo)-transduced MC38 cells either alone or in combination with CpG-ODN-1826 or non-CpG-ODN-1911 in vitro. Seven groups were compared: i) DC only, ii) DC + non-CpG, iii) DC + IL4, iv) DC + non-CpG + MC38-Neo, v) DC + non-CpG + MC38-IL4, vi) DC + CpG + MC38-Neo, and vii) DC + CpG + MC38-IL4. After a 24-h incubation, cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-H-2Kb, I-A^b, CD80, and CD86 monoclonal antibodies. The results are shown as ratios of mean fluorescent intensity (MFI) of the incubated DCs to the MFI of FITC-conjugated control IgG of each group.

Cytolytic assays. Cytolytic assays were performed as previously described (22). Tumor-stimulated effector cells were assessed for cytolytic activity against MC38-WT and YAC-1 cells, which are sensitive to non-specific killing, in triplicate in 4-h $^{51}$Cr-release assays. The percentage of lysis was determined using the following formula: (release in assay - spontaneous release) x 100/(maximum release - spontaneous release). Maximum release was determined by the lysis of labeled target cells with 1% Triton X-100. Spontaneous release, which was measured by incubating target cells in the absence of effector cells, was <10% of maximum release.

**Statistical analyses.** Significance was assessed by Student's t-tests. Differences between groups were considered significant when the P-value was <0.05.

**Results**

**Maturation of DCs was induced by coinoculation with IL-4-overexpressing tumor cells and CpG-ODN.** We investigated the effects of IL-4 and CpG-ODN on DC maturation by flow cytometry. While the coinoculation of DCs with MC38-IL4
enhanced the expression of MHC class I, class II, and costimulatory molecules, only minimal phenotypic changes in DCs were detected after coincubation with CpG-ODN. When we compared DCs that were incubated with IL-4-overexpressing or control gene-transduced MC38 cells either alone or in combination with CpG-ODN-1826 or ODN-1911 in vitro. After a 24-h incubation, we measured interferon (IFN)-γ and IL-10 concentrations in the culture supernatant by enzyme-linked immunosorbent assay (ELISA).

The combination of IL-4-overexpressing MC38 cells and CpG-ODN promotes the production of Th1-type cytokines by naïve splenocytes in vitro. Since the combination of IL-4 and CpG-ODN enhanced DC maturation, we observed their effects on naïve splenocytes. ELISAs confirmed that naïve splenocytes produced high amounts of IFN-γ after a 24-h coincubation with MC38-IL4 and CpG-ODN-1826 (Fig. 2A), whereas IFN-γ production could not be detected after coincubation with either IL-4 or CpG-ODN alone. While IL-10 production was clearly detected when naïve splenocytes were incubated with CpG-ODN (Fig. 2B), coincubation with MC38-IL4 suppressed the production of IL-10. These results suggest that the combination of IL-4 and CpG-ODN promoted Th1-type immune responses.

Therapeutic inoculation of IL-4-overexpressing MC38 cells and CpG-ODN reduces the outgrowth of established tumors in vivo. Next, we observed whether CpG-ODN had additive antitumor effects on the effects of IL-4 on established tumors in vivo. We treated tumor-bearing mice with MC38-IL4 and CpG-ODN-1826 and compared the parental tumor size. As shown in Fig. 3A, the combination therapy of MC38-IL4 (3x10^5 cells/mouse) and CpG-ODN (30 µg/mouse) significantly reduced the outgrowth of the parental tumors (IL-4 vs. IL-4 + CpG, P=0.048) whereas treatment with MC38-Neo + CpG and MC38-IL4 + non-CpG did not show any antitumor effects. Furthermore, the combination of increased cell number of MC38-IL4 (5x10^5 cells/mouse)
and CpG-ODN (30 µg/mouse) was more effective against the established tumors (Fig. 3B; IL-4 vs. IL-4 + CpG, *P* = 0.015) while treatment with MC38-IL4 (5x10^5 cells/mouse) only also revealed antitumor activity. Therefore, the combination therapy of IL-4 and CpG-ODN has an additive antitumor effect *in vivo*.

Treatment of IL-4-overexpressing MC38 cells with the combination of CpG-ODN induces the infiltration of CD8-positive cells in established wild-type tumors. We analyzed the mechanisms of the antitumor effects that were induced by the inoculation of MC38-IL4 cells with CpG-ODN-1826. As shown in Fig. 4, WT tumors of mice treated with MC38-IL4 showed significant infiltration with Gr-1^+^ cells, as previously reported (4). More CD4^+^ cells infiltrated tumors in mice treated with MC38-IL4 alone, as well as with MC38-IL4 and CpG-ODN, compared with those in mice treated with MC38-Neo (*P* = 0.039 and *P* = 0.026, respectively). While only a few CD8^+^ cells could be detected in the tumors of mice treated with MC38-IL4 alone, a marked infiltration of CD8^+^ cells was observed in the tumors of mice treated with MC38-IL4 and CpG-ODN (Fig. 4; MC38-IL4 alone: 2.8±1.9 cells/field vs. MC38-IL4 + CpG-ODN, 20.7±15.3 cells/field, *P* = 0.027). CD11c^+^ cells were rarely seen in any of the groups. These results suggest that the initial antitumor effects induced by MC38-IL4 and CpG-ODN may be dependent on CD8^+^ and CD4^+^ cells.

Potent tumor-specific cytology is detected when splenocytes of MC38-immune mice are stimulated by the combination of IL-4-overexpressing tumor cells and CpG-ODN. Because the combination of IL-4 and CpG-ODN seemed to induce potent Th1-type immune responses, we tried to detect the generation of tumor-specific CTL in mice immunized with IL-4 and CpG-ODN. As shown in Fig. 5, effector cells that were stimu-
lated with IL-4 and CpG-ODN revealed marked cytolytis that was specific for MC38-WT cells (32.2±3.5% for MC38 vs. 3.2±1.1% for YAC-1; at effector to target ratio, E:T=40). Although tumor-specific cytolytis was also detected when the splenocytes of immunized mice that had been inoculated with MC38-IL4 cells and non-CpG-ODN-1911 in vivo were stimulated with MC38-IL4 cells in vitro, the specific lysis was lower compared to that of the splenocytes of mice treated with IL-4 and CpG-ODN (15.8±2.1% for MC38 vs. 0.5±0.3% for YAC-1; E:T=40, P=0.002 when compared with IL4 + CpG for lysis of MC38). Naïve splenocytes stimulated with IL4 + CpG-ODN did not show any MC38-specific killing (Fig. 5). The results suggested that immunization with IL-4 and CpG-ODN effectively induce tumor-specific immune response in vivo.

Discussion

In this study, we observed that the combination of IL-4 and CpG-ODN enhanced the expression of MHC and costimulatory molecules on the surface of DCs. In addition, the combination promoted IFN-γ production and suppressed IL-10 production in naïve splenocytes in vitro, which strongly suggested that the combination of IL-4 and CpG-ODN contributed to T-cell differentiation towards a Th1-type immune response. The combined therapy of IL-4 and CpG effectively suppressed the outgrowth of parental tumors in vivo compared to IL-4 monotherapy. The results of both the immunohistochemical and the tumor-specific cytolytis analyses suggested that Th1-type immune responses were strongly induced in mice treated with IL-4 and CpG-ODN.

IL-4, which is a representative Th2-type cytokine, is produced by Th2-type cells. The Th2-type response is thought to suppress the generation of Th1 cells (23), which are usually involved in antitumor immunity. However, it has been reported that IL-4 shows multiple suppressive effects on tumors, and our previous investigation showed that IL-4 gene transduction in MC38 cells did not affect in vitro tumor growth, while tumors were not seen in most mice injected with IL-4-overexpressing MC38 cells. This observation implies that IL-4 does not injure MC38 cells directly but reduces their tumorigenicity by inducing host immune responses. Our previous study and other investigations have suggested that IL-4 recruits and activates granulocytes in the microenvironment of parental tumors in order to attack and kill the tumor cells during the primary response. However, how IL-4 recruits or stimulates granulocytes in the tumor microenvironment remains unclear (4,24). Thus, IL-4 induces tumor-specific cellular immune responses, which contribute to long-lasting immunity against the parental tumors.

For further improvement of the antitumor effects of IL-4-based immune therapy, we performed combination therapy, which consisted of IL-4 with CpG-ODN, and this combination has been reported as a potent inducer of immune responses. As previously reported, treatment with CpG-ODN alone did not reduce tumor outgrowth (3). Although CpG-ODN is thought to promote the induction of Th1-type cytokines, CpG-ODN promoted IL-10 production by naïve splenocytes in our system. Reports show that CpG induces Th2-type cytokines in certain conditions support our observation (25,26). MC38-IL4 did not promote IL-10 production, whereas exogenous IL-4 might. We speculate that irradiated tumor cells modified the immune circumstances in the culture. In any case, we suggest that the balance of Th1/Th2 cytokine production is critical to the induction of potent immune responses in the early stages of tumor development and that the great suppression of outgrowth of the parental tumors that was seen in this study occurred because the combination of IL-4 and CpG enhanced IFN-γ production and suppressed IL-10 production. Further investigation is required in order to clarify the mechanisms by which IL-4 reduces the IL-10 production that is induced by CpG.

However, opposite effects of IL-4 on tumors have also been reported. IL-4 itself may affect tumor growth, especially on those that have abundant IL-4 receptors. The levels of expression of the IL-4 receptor correlated with the tumorigenic potential in a murine model (27). Several tumors appeared to be resistant to apoptosis, which is induced by chemotherapeutic agents or CD95 ligation, in an IL-4-dependent manner. IL-4 seemed to modify the immunological functions of effector cells. The cultivation of naïve CD8+ T cells in the presence of IL-4 resulted in poor cytolytic function in the cells by reducing the levels of perforin and granzymes (27,28). Whether an immune response is predominantly Th1 or Th2 may depend on a number of other factors, such as mouse strain, kind of tumor, amount of tumor antigen, and the amount of cytokine (29,30).

It has been reported that the combination of CpG, IL-4, and CD40 ligand effectively induce CTLs by enhancing the expression of immunogenic molecules on B-cell precursor acute lymphoblastic leukemia cells (31). Although MHC class I molecules were slightly upregulated by IL-4 transduction in MC38 cells, changes in other molecules, such as costimulatory and MHC class II molecules, could not be detected in our system (unpublished data). It was thought that IL-4 and CpG-ODN resulted in antitumor effects by inducing Th1-type immune responses, especially that of tumor-specific CTLs, rather than by modulating the immunogenic molecules in the tumor cells. The mechanism by which cytokines and immunomodulators exhibit antitumor effects may also depend on the models used in the experiments.

In order to perform tumor-based gene therapy, there are a number of issues, such as dose determination, site of therapeutic injection, and therapeutic interval, that need to be determined, but the most critical issue is the establishment of the patient's cytokine gene-transduced tumor cells. The development of a new technique that transduces a targeted gene to a patient's cells may be required.

Finally, we found in this study that combination therapy with IL-4 and CpG-ODN had potent antitumor effects on established tumors through the induction of potent Th1-type immune responses in the hosts. Thus, this combination therapy may be a candidate for clinical cancer therapy, but further investigation is needed before clinical trials.

Acknowledgements

This study was supported in part by a grant from the Ministry of Health, Labor, and Welfare of Japan (K. Hiroishi); a Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Culture, Sports, Science and Technology of
Japan (K. Hiroishi); a grant for the High-Technology Research Center Project from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (M. Imawari); and the Research Program of Intractable Disease provided by the Ministry of Health, Labor and Welfare of Japan (K. Hiroishi).

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