Original

Effects of Interleukin-4-Transduced Tumor Cell Vaccines and Blockade of Programmed Cell Death 1 on the Growth of Established Tumors

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Abstract : Interleukin (IL)-4 exhibits strong antitumor effects and IL-4 gene therapy has been used clinically in the treatment of some types of cancer. In the present study, we evaluated the efficacy of IL-4-transduced tumor cell vaccines in combination with blockade of programmed cell death 1 (PD-1) and investigated the mechanisms underlying the antitumor effects of this therapy. A poorly immunogenic murine colorectal cancer cell line (i.e. MC38) was transduced to overexpress IL-4. In a therapeutic model, MC38-IL4 cells and anti-PD-1 antagonistic antibodies (Ab) were inoculated into parental tumor-bearing mice. Immunohistochemical analyses and tumor-specific lysis were also performed. Additive antitumor effects were observed when mice were treated with IL-4 in combination with an anti-PD-1 Ab. Immunohistochemical analysis of the therapeutic model showed marked infiltration of $CD4^+$ and $CD8^+$ cells into established MC38 tumors of mice treated with anti-PD-1 Ab. Significant tumor-specific cytolysis was detected when the splenocytes of mice treated with both IL-4 and anti-PD-1 Ab were used as effector cells. These results suggest that blockade of the interaction between PD-1 and programmed death ligand 1 (PD-L1) enhances the antitumor immune responses induced by IL-4. Thus, IL-4 gene-transduced tumor cell vaccines in combination with PD-1 blockade may be considered as possible candidates for clinical trials of new cancer vaccines.

Key words : Interleukin-4, Programmed cell death 1, Immunotherapy, Tumor-based vaccine, Colorectal cancer

Introduction

Cellular immune responses are thought to be impaired in patients with advanced malignant tumors, and tumors are thought to escape immune surveillance by several mechanisms[1,2]. To overcome immune suppression in patients or the immune escape of malignant

tumors, novel approaches to induce a strong cellular immune response are needed. Several studies in murine models have reported that the cytokine gene transduction of tumor cells induces potent antitumor immune responses without systemic adverse effects[3,4]. Subcutaneous injection of the transduced cells can induce local inflammation at the injection site via the accumulation of inflammatory cells, such as activated natural killer (NK) cells, macrophages, dendritic cells (DCs), and T lymphocytes[3,5,6]. Based on these observations, the development of vaccinations for clinical use using gene-transduced tumor cells as a vehicle to deliver cytokines is an attractive proposition.

Interleukin (IL)-4, a type 2 response inducer, plays a major role in both B and T cell development in the immune system[7,8]. In addition, it causes a class switch of B cells, upregulates major histocompatibility complex (MHC) Class II and adhesion molecules, and prevents T cell apoptosis. With respect to its antitumor effects, IL-4 has been shown to have a direct inhibitory effect on tumor cell growth *in vivo* and *in vitro*, as well as antiangiogenic effects[9,10]. It has been reported that IL-4 activates endothelia in the tumor microenvironment, which results in increased infiltration of immune cells[11]. Other reports suggest that eosinophils and neutrophils are responsible for the antitumor effects of IL-4[12,13]. A recent study has demonstrated that local IL-4 delivery at the site of vaccination activates local DCs, which play a critical role in the initiation, promotion, and regulation of host immune responses and promote T helper (Th) 1 cell responses[4]. In that study, IL-4 appeared to support DC maturation and to enhance IL-12p70 secretion from DCs. On the basis of these findings supporting the therapeutic effects of IL-4 against tumors, it has been used clinically in the treatment of tumors.

Programmed death 1 (PD-1) was first described as a member of the B7 family of costimulatory molecules that modulate T cell antigen-specific receptor signaling and control T cell activation, inactivation, and survival[14]. Recently, PD-1 was identified as a marker of exhausted T cells in chronic infectious disease[15,16]. Of the various mechanisms underlying tumor-induced immunosuppression by which tumors escape immune surveillance, numerous studies have suggested a role for an interaction between PD-1 and programmed death ligand 1 (PD-L1) in inhibiting the effector functions of antigen-specific CD8$^+$ T cells[17,18]. PD-1 is expressed on tumor-infiltrating CD8$^+$ T cells in tumors or on antigen-specific CD8$^+$ T cells in hosts with tumors, and the function of these PD-1$^+$ T cells is impaired[19,20]. PD-L1 has been shown to be expressed at high levels in several different types of cancer[21], and there is a strong correlation between high PD-L1 expression in tumors and a poor prognosis[22]. Recent studies have shown that blockade of PD-1−PD-L1 signaling restores functional T cell responses in several cancers, subsequently improving clinical outcome[23,24].

In the present study, a preliminary investigation of the effects of combination therapy prior to clinical studies, the antitumor effects of a combination of an IL-4-transfected tumor cell vaccine and PD-1 blockade were evaluated in a poorly immunogenic murine colorectal cancer system. When parental tumor-bearing mice were injected with IL-4-overexpressing

tumor cells and an anti-PD-1-blocking antibody (Ab), the outgrowth of the established parental tumor was significantly suppressed. Furthermore, to explore the mechanisms underlying the antitumor effects of the IL-4 plus anti-PD-1 combination, tumors were stained immunohistologically and we tried to induce tumor-specific T lymphocytes.

Materials and Methods

Mice

Female 6-week-old C57BL/6 (B6) mice were purchased from Sankyo Lab Service (Tokyo, Japan) for use in experiments when they were 8−12 weeks of age. Mice were maintained in an animal care facility at Showa University. The present study was approved by the Ethics Committee for Animal Experiments of Showa University (permission no. 2011-1111).

Cell lines, culture medium, and reagents

The MC38 murine colorectal adenocarcinoma cell line (B6 mouse origin) and yeast artificial chromosome-1 (YAC-1) lymphoma cells 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 (MEM) sodium pyruvate, and 0.1 mM MEM 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). The YAC-1 cells were used as target cells to assess non-specific killing in cytolytic assays.

The MC38 cell line was genetically modified to produce murine IL-4 (MC38-IL4), as described previously[25]. Expression of IL-4 was confirmed by ELISA using a commercially available kit (mouse IL-4 ELISA ; PBL Biomedical Laboratories, New Brunswick, NJ, USA) according to the manufacturer's instructions. MC38 cells expressing the neomycin-resistance gene following retroviral transduction with MFG-Neo (MC38-Neo) were used as control cells[26]. γ-Irradiation (100 Gy for tumor cells) was performed using a Gammacell 3000 Elan (Nordion International, Kanata, Canada). As reported previously, 1×10^5 MC38-IL4 cells produce approximately 360 ± 108 ng IL-4/48 h, whereas wild-type MC38 (MC38-WT) cells do not produce any IL-4[25]. IL-4 gene transduction does not affect the growth of tumor cells *in vitro* or the survival of γ-irradiated tumor cells[25].

Therapeutic models

To evaluate the potential to treat established tumors, we measured the size of established wild-type (WT) tumors in mice treated with genetically modified MC38 cells and antibody, as described previously[26]. Briefly, B6 mice were injected with 1×10^5 MC38-WT cells subcutaneously (s.c.) into the right flank. Then, 7, 10 and 14 days after WT inoculation, mice were treated with an injection of 1×10^5 genetically modified MC38 or 200 μg anti-

PD-1 antagonistic Ab （BioLegend, San Diego, CA, USA）, alone or in combination, into the contralateral （left） flank. Each experiment involved six mice per group. Tumor size was measured twice a week using vernier calipers. Experiments with the therapeutic model were performed three times.

Immunohistologic analysis

B6 mice were injected with 1×10^5 MC38-WT cells s.c. in the right flank. On Days 7, 10, and 14, mice were injected with 1×10^5 MC38-IL-4 and / or 200 μg anti-PD-1 antagonistic Ab into the contralateral （left） flank. Tumor tissues were harvested 4 days after the last inoculation （18 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 and anti-CD8a Abs （Nippon Becton Dickinson, Tokyo, Japan） and anti-PDL1 Ab （BioLegend）. Rat IgG2a （Nippon Becton Dickinson） was used as a control Ab. Immunostaining was completed using a Vectastain ABC kit （Vector, Burlingame, CA, USA）. Immunoreactive cells were counted in 10 fields using light microscopy at ×400 magnification in a blinded manner. Each experiment involved two mice per group.

Induction of tumor-specific cytotoxic T lymphocytes

Mice initially were inoculated with 1×10^5 MC38-IL4 tumor cells and / or 200 μg anti-PD-1 Ab on Days 0, 7, and 14. Subsequently, on Day 28, MC38-immune mice were challenged with 3×10^5 MC38-WT cells. Splenocytes （3×10^6 cells / mL） were harvested from these mice on Day 42 and were then stimulated *in vitro* with irradiated （100 Gy） MC38-WT tumor cells （3×10^5 cells / mL）. Seven days later, responder cells （1×10^6 cells / mL） were restimulated with irradiated MC38-WT tumor cells （100 Gy ; 1×10^5 cells / mL） and syngeneic DCs （2×10^5 cells / mL） in the presence of 50 IU / mL recombinant mouse IL-2 （R&D Systems, Minneapolis, MN, USA）. DCs were generated from bone marrow cells of B6 mice using murine granulocyte–macrophage colony stimulating factor （GM-CSF ; 10 ng / mL） and IL-4 （10 ng / mL）, obtained from Pepro Tech EC （London, UK）, as reported previously[27]. 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 a 4-h ^{51}Cr-release assay. Target cells （1×10^6 cells / mL） were labeled with 3.7 MBq Na$_2$ ^{51}CrO$_4$ （Amersham Pharmacia Biotech, Tokyo, Japan） for 1 h at 37℃. Labeled cells were washed and resuspended in CM. Target cells （5×10^3） and various numbers of effector cells at specific effector-to-target ratios （E:T） were plated in 200 μL CM in each well of 96-well round-bottomed plates. ^{51}Cr release was measured after

4 h incubation at 37℃.   Percent lysis was determined using the formula (release in assay −spontaneous release) / (maximum release−spontaneous release) ×100.   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 ＜15% of maximum release.

Statistical analyses

The comparative growth of established tumors and T cell responses were assessed using Student's *t*-tests for two samples with unequal variance.   Differences between groups were considered significant when $P < 0.05$.

Results

Therapeutic inoculations with IL-4-transduced MC38 tumor cells in combination with anti-PD-1 Ab suppress the in vivo growth of established MC38 tumors

We examined the therapeutic effects of MC38-IL4 and anti-PD-1 Ab.   Mice bearing established WT MC38 tumors were treated with injections of MC38-IL4 and anti-PD-1 Ab, either alone or in combination.   Starting 7 days after inoculation of MC38-WT, mice were injected every 3 days with the IL-4 cells and / or the Ab in the opposite flank.   The mean area of established tumors in mice treated with MC38-IL4 and MC38-IL4 plus the anti-PD-1 Ab was significantly less than that of tumors in the control group (203.2±31.9, 177.8± 23.9 and 309.5±27.6 mm^2, respectively, on Day 28 ; $P = 0.041$ and $P = 0.008$, respectively ; Fig. 1).   Although there was a tendency for combination therapy with MC38-IL4 plus anti-PD-1 Ab to suppress the growth of established tumors somewhat more effectively than MC38-IL4 therapy alone, the difference failed to reach statistical significance ($P = 0.289$).

Combined MC38-IL4 plus anti-PD-1 Ab treatment induces CD4$^+$ and CD8$^+$ cell infiltration into established WT tumors

To analyze the antitumor mechanisms induced by combined MC38-IL4 plus anti-PD-1 Ab therapy, we performed immunohistochemical staining using WT tumor tissues of treated mice.   Marked infiltration of both CD4$^+$ and CD8$^+$ cells was observed following the addition of anti-PD-1 Ab compared with the control group and the group treated with MC38-IL4 alone (Table 1 ; Fig. 2).   These results suggest that the antitumor effect of MC38-IL4 plus anti-PD-1 Ab combination therapy is dependent on both CD4$^+$ and CD8$^+$ cells.

Marked tumor-specific cytolysis is detected when splenocytes from mice treated with both MC38-IL4 and anti-PD-1 Ab are used as effector cells

Mice were initially inoculated with MC38-IL4 and / or anti-PD-1 Ab on Days 0, 7, and 14.   Subsequently, these mice where challenged with MC38-WT cells on Day 28.   Splenocytes were harvested from the treated mice on Day 35 and were stimulated weekly twice *in vitro*
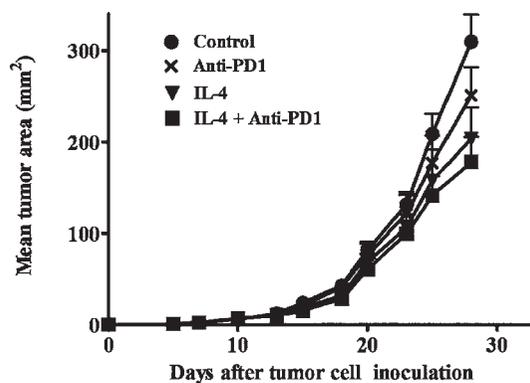
Fig. 1. Therapeutic inoculation of IL-4-transduced MC38 tumor cells in combination with anti-PD-1 Ab sup-
presses the *in vivo* growth of established MC38 tumors. Mice were inoculated with MC38-WT cells in
the flank on Day 0. On Day 7, mice were treated three times with injections of MC38-IL4 and anti-
PD-1 Ab, alone or in combination, into the contralateral flank every 3rd day starting 14 days after
wild-type tumor injection. Six mice were studied in each group. Results show mean±SEM tumor area
（mm^2）, with significance at 95% confidence limits indicated. This experiment was performed twice with
similar results.

Table 1. Immunohistologic analysis of CD4$^+$ and CD8$^+$ T
cell infiltration in the established wild-type tumors
treated with MC38-IL and / or anti-PD1 antagonistic
antibody.

Treatment	Number of positive cells	
	CD4$^+$ cells	CD8$^+$ cells
Control	2.4±2.3	11.4±5.3
Anti-PD1	61.4±38.7	68.2±38.7
IL-4	2.4±1.8	12.8±7.9
IL-4＋Anti-PD1	58.0±17.6	60.0±22.2

Immunoreactive cells were counted in 5 microscopic fields
（×400）without-knowledge of the experimental group.
Results are reported as mean number of positive cells±S.D.

with MC38-IL4 cells. Cytolytic assays against MC38 or YAC-1 cells, which are sensitive for
NK cells, were performed 7 days after the second stimulation. High specificity for MC38
was observed when mice were treated with MC38-IL4 alone （17.9±3.5% for MC38 vs 9.5
±2.9% for YAC-1 ; E:T＝20, $P＝0.032$; Fig. 3）or in combination with anti-PD-1-Ab （27.4
±4.6% for MC38 vs 16.9±3.9% for YAC-1 ; E:T＝20 ; $P＝0.039$）. When splenocytes from
mice treated with the anti-PD-1 Ab alone were used as effector cells, comparable cytolysis
was detected for both MC38 and YAC-1 cells as target cells （18.6±5.9% for MC38 vs 18.2
±2.7% for YAC-1 ; E:T＝20 ; $P＝0.231$）. Significant cytotoxicity was observed against
MC38 cells in the group treated with the combination of IL-4 plus anti-PD-1 Ab compared
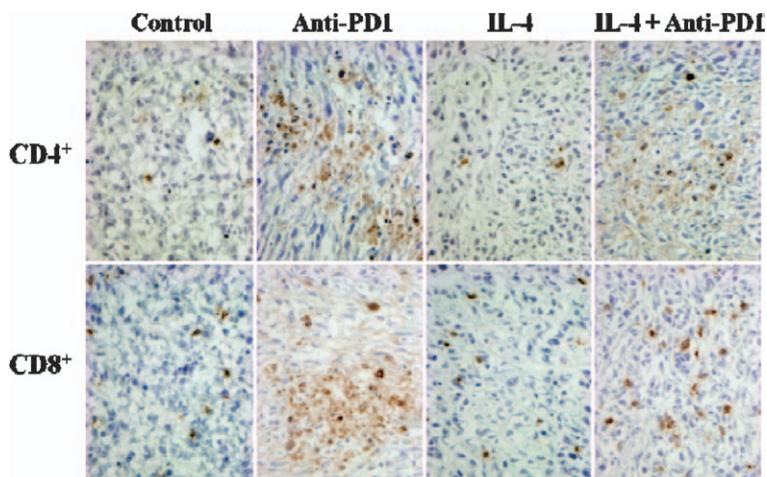with IL-4 alone （$P＝0.046$）. These data suggest that the anti-PD-1 Ab has minimal ability

Fig. 2. There was marked infiltration of CD4$^+$ and CD8$^+$ cells into established wild-type tumors in mice inoculated with MC38-IL4 cells in combination with anti-PD-1 Ab. B6 mice were injected s.c. three times with MC38-IL4 cells and anti-PD-1 Ab, either alone or in combination, in the contralateral flank to that containing the established wild-type tumor. Tumor tissues were harvested 4 days after therapeutic inoculation and were stained with anti-CD4 and anti-CD8 Abs. Immunoreactive cells were observed using light microscopy（×400）.
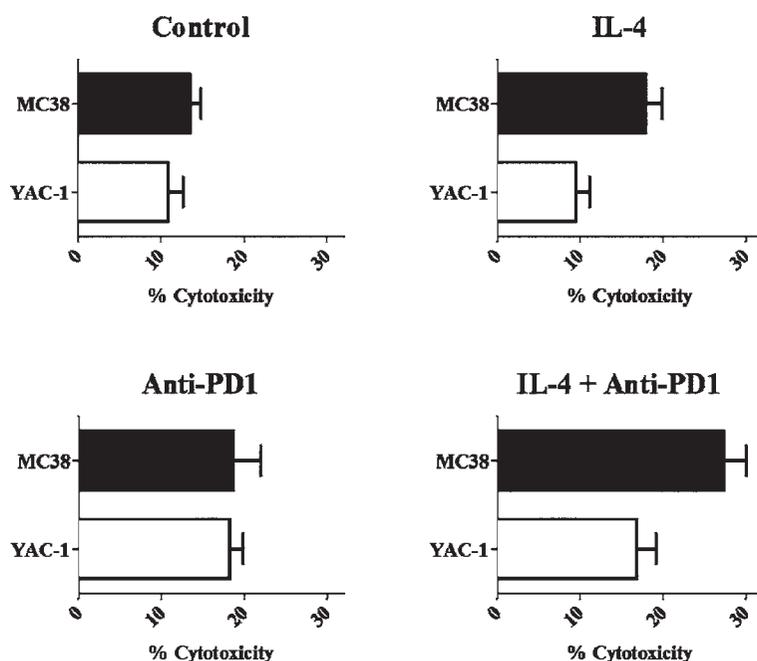

Fig. 3. Treatment with IL-4 in combination with anti-PD-1 Ab induced potent tumor-specific cytolysis. Mice were initially inoculated with MC38-IL4 cells and / or anti-PD-1 Ab on Days 0, 7 and 14. Subsequently, MC38-immune mice were challenged with MC38-WT cells on Day 28. Splenocytes were harvested from these mice on Day 42 and then stimulated *in vitro* with MC38-IL4 cells and syngeneic DCs twice weekly. A cytolytic assay against MC38, MCA205, or YAC-1 cells was performed 7 days after the last stimulation. Results show mean±SD percentage cytotoxicity. This experiment was performed twice with similar results. The cytolytic assay against MC38 was performed using an effector-to-target（E:T）ratio of 20.

to induce tumor-specific cytotoxicity, but does enhance the strong specific response induced by IL-4.

Discussion

IL-4, a representative Th2-type cytokine, is produced by Th2-type cells. The Th2-type response is thought to suppress the generation of Th1 cells [28], which are usually involved in antitumor immunity. Although IL-4 is generally regarded as an inducer of Th2-type responses, recent studies clearly demonstrate that IL-4 has pleiotropic effects on immune cells of multiple lineages [29] and that it plays an important role as an inducer of Th1-type immunity [30]. In particular, IL-4 supports DC maturation and promotes enhanced IL-12p70 secretion from DCs [4]. IL-4 gene therapy for cancer using genetically engineered tumor cells has been shown to induce potent protective and therapeutic antitumor immunity in animal models [29]. IL-4 induces tumor-specific cellular immune responses that contribute to long-lasting immunity against parental tumors, and we have previously reported that IL-4 appears to promote a Th1-type antitumor immune response that is enhanced in cooperation with interferon（IFN）-α [25].

To further improve the antitumor effects of IL-4-based immune therapy, we focused on PD-1, which has been identified as a marker of exhausted T cells [15, 16]. Because blockade of PD-1 signaling has been shown to improve clinical outcome and restore functional T cell responses in cancer [23, 24], we hypothesized that PD-1 blockade has the potential to enhance the Th1-type antitumor response elicited by IL-4 gene-transduced tumor-based vaccination therapy. In the present study, we investigated the antitumor effects, as well as the underlying mechanisms, of combined treatment with IL-4-transduced tumor cell vaccine therapy and PD-1 blockade.

First we examined the therapeutic effect of IL-4-transduced tumor cells and the anti-PD-1 Ab in an established tumor model. Following treatment of tumor-bearing mice with MC38-IL4 and anti-PD-1 Ab, significant suppression of the outgrowth of established tumors was observed in the combination treatment group. Combination treatment with IL-4 plus anti-PD-1 Ab tended to suppress the growth of established tumors somewhat more effectively than IL-4 alone, although the differences failed to reach statistical significance. We performed this experiment three times with similar results. Thus, additive antitumor effects were observed using a combination of IL-4 plus anti-PD-1 Ab to treat established tumors.

Immunohistologic analyses of established tumors in mice treated with anti-PD-1 Ab revealed marked infiltration of both CD4$^+$ and CD8$^+$ cells compared with the control and MC38-IL4-treated groups. These data are in accord with the results of a previous study that reported that blockade of PD-1 signaling pathways reversed T cell exhaustion and restored antitumor immunity [24]. Interestingly, in the present study the proliferation rate of splenocytes did not differ significantly between the control and anti-PD-1 Ab-treated groups in the setting of an *in vitro* culture（data not shown）. It is likely that this apparent

discrepancy between *in vitro* and *in vivo* findings is due to the condition and microenvironment of the local tumor because it was confirmed by immunohistochemical staining that established MC38-WT tumors express abundant PD-L1 molecules (data not shown). These observations suggest that blockade of PD-1 will avoid lymphocyte apoptosis, and will maintain the survival and infiltration of tumor-specific T cells in the local tumor environment, which contains abundant PD-L1 molecules.

Although PD-1 blockade recruits lymphocytes to local tumor sites, the therapeutic effect of anti-PD-1 Ab treatment alone did not have marked effects. To investigate this finding further, we tried to induce tumor-specific cytotoxic T lymphocytes (CTLs) from the splenocytes of IL-4- or anti-PD-1-treated mice and performed cytolytic assays. A high specificity for MC38 cells was observed following IL-4 treatment, whereas only non-specific cytolysis was detected following treatment with the anti-PD-1 Ab. Furthermore, specific cytolysis against MC38 cells was more potent following combined IL-4 plus anti-PD-1 Ab treatment compared with IL-4 alone. These observations suggest that IL-4 elicits tumor-specific CTLs, whereas blockade of PD-1 mainly maintains the specific response induced by IL-4. This finding further supports the rationale behind the use of combination therapy because both IL-4 and blockade of PD-1 have antitumor effects, but these effects are mediated by different mechanisms.

Before tumor-based gene therapy can be performed, there are a number of issues that need to be considered, including dose determination, the site of therapeutic injection, and the therapeutic interval. However, the most critical issue remains the establishment of cytokine gene-transduced tumor cells for individual patients. Thus, new techniques may need to be developed to allow the transduction of targeted genes to a patient's cells.

Overall, the findings of the present study suggest that combination therapy with IL-4 and PD-1 blockade has potent antitumor effects on established tumors via the induction of potent Th1-type immune responses in the host. This form of combination therapy may be a candidate for ultimate application in the clinical treatment of cancer, although further investigations are required before clinical trials can be started.

Acknowledgements

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