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Therapeutic Effects of ADU-S100 as STING Agonist and CpG ODN1826 as TLR9 Agonist in CT-26 Model of Colon Carcinoma

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ABSTRACT

Cancer immunotherapy emerged as a novel therapeutic approach to destroy tumor cells, and it has grown toward clinical transition following successful fundamental research and clinical trials. Immunotherapy by efficacious adjuvants is critical for increasing protective immune responses against infectious diseases and cancers. STING and TLR9 agonists are interesting candidates for novel immunotherapies of cancers. In this study, the antitumoral effects of ADU-S100, as a potent STING agonist, and CpG ODN1826, as a TLR9 agonist, in single and combined forms in CT-26 colon adenocarcinoma model were evaluated. This model was induced in female BALB/c mice which were divided into five groups treated with PBS, ADU-S100 (20 and 40 µg), CpG ODN (40 µg), and ADU-S100 (20 µg)+CpG ODN (20 µg). The tumor volumes and weights of mice were measured every other day. On the 30th day, the tumor, spleen, and liver tissues of mice were isolated for histopathological assessment. Hematological analysis was performed on heart blood. Intratumoral injection of agonists induced significant tumor suppression in all treatment groups with profound effect in the combination group that received half concentration of single form. Moreover, the histopathological analysis of tumor tissues showed the presence of apoptotic and inflammatory cells and increased the number of lymphocytes in the blood samples of the treatment groups indicating the effective role of these agonists in clearing the tumor. Therefore, a such synergy of adjuvants may have an effective role in cancer immunotherapy and offer new perspectives on the combination of agonists that trigger innate immune sensors during malignancy.

Keywords

STING agonist, TLR9 agonist, Synergistic effect, Immunotherapy, Colon carcinoma model

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Abbreviations

STING: Stimulator of interferon genes
TLR9: Toll-like receptor 9
CpG ODN: CpG oligodeoxynucleotides
IRF7: Interferon regulatory factor 7
MYD88: Myeloid differentiation primary response 88
DCs: Dendritic cells

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Introduction

There are different treatment methods for cancers, including radiotherapy, chemotherapy, and surgery. However, each procedure has limitations that affect the diagnosis and survival of patients [1]. In recent years, immunotherapy, as a promising treatment approach that triggers the immune system against a broad category of cancers, has had principal efficacy on some patients with metastatic tumors [2]. Recent investigations opened a novel chapter as immunoadjuvants for cancer immunotherapy by the initial activation of innate and subsequent adaptive immune responses [3].

One of the key immunoadjuvants is TLR agonists that bind to the receptors on the endosomal compartments of mainly immune cells and lead to inflammatory reactions and adaptive immune responses. One of the important agonists of TLR9 is CpG ODN as a synthetic TLR9 ligand that can activate the signaling pathway through MyD88 and IRF7 to produce type I interferons and through NF-κB signaling pathway to stimulate immune cells to induce pro-inflammatory cytokines production [4]. CpG ODNs as a potent cancer vaccine adjuvant can increase the proliferation of cytotoxic CD8+ T cells against tumor antigens and trigger Th1-type immune responses that stimulate antigen-specific adaptive immunity [4, 5]. CpG-ODN 1826, as a class B CpG specific for murine TLR9, induces the production of pro-inflammatory cytokine and antibodies from B cells and has demonstrated encouraging outcomes by reducing tumor growth and improving survival in clinical trials [6-8]. Another important immunoadjuvant identified in recent years is STING which is stimulated with cytosolic dsDNA and induces the transcription of numerous innate immune genes [9].

STING is an adaptor protein localized predominantly on the ER membrane. In response to specific agonists, STING induces a signaling pathway that leads to the production of IFN-β as a critical pro-inflammatory cytokine [10]. Consequently, DCs are activated, and cytotoxic T lymphocytes are recruited in TME. There are different agonists for inducing STING, among which MLRRS2 CDA (also called MIW815 or ADU-S100) is known as a powerful vaccine adjuvant. It induces TME activation in a variety of tumor types by priming the antigen-specific CD8+ T cell and lasting immune-mediated tumor rejection [11]. Due to the improved features of ADU-S100 compared to other STING agonists, the therapeutic effects of this agonist in combination with different immunomodulatory agents have been evaluated [12].

In this study, we investigated the synergistic effect of ADU-S100 in combination with CpG ODN1826 in a mouse model of colon carcinoma. Furthermore, we evaluated the ability of single and combination forms of agonists on tumor growth and survival rate in a CT-26 colon carcinoma model. Our results suggest that combined ADU-S100 and CpG ODN1826 have a significant antitumor impact, making them a promising immunotherapeutic agent for the treatment of colon cancer.

Abbreviations-Cont'd

NF-κB: Nuclear factor kappa B
TME: Tumor microenvironment
dsDNA: Double-stranded DNA
ER: Endoplasmic reticulum
IFN: Interferon
SC: subcutaneous
HE: Hematoxylin-eosin
CBC: Complete blood count
cGAMP: Cyclic guanosine monophosphate–adenosine monophosphate
CDN: Cyclic dinucleotides
APC: Antigen-presenting cell

Results

Anti-tumoral activity of CpG ODN 1826 and ADU-S100 in single and combined forms against established CT-26 colon adenocarcinoma

ADU-S100 is a new STING agonist that affects tumor cells by activating the STING signaling pathway in TME and priming APC and CD8+ T lymphocytes. Intratumoral injection of these molecules into various cancer models, including CT26 colon cancer, B16 melanoma, and 4T1 breast cancer models, showed stronger antitumor outcomes [11, 13, 14]. On the other hand, CpG ODN 1826, as a well-known B-type CpG, has been utilized as an adjuvant for vaccines and successfully evaluated in a number of vaccination models [15]. This study investigated whether the combination of CpG-ODN 1826 and ADU-S100 attenuates tumor growth more than the single forms of agonists in the CT-26 cancer model.

In order to induce mice with tumors, CT-26 cell suspensions were subcutaneously implanted into the right flank of animals and treated with 20 μg of CpG ODN 1826 and ADU-S100 together or twice the concentration of single agonists (40 μg). ADU-S100 (20 μg) was used to confirm the synergistic effect of agonists. The volume and weight of tumors in the mice were recorded every other day. As can be seen in Table 1, significant tumor regression was observed in all treatment groups compared to the control group ($p < 0.0001$). The highest suppression of tumor growth was observed with the synergy of two agonists from 1952 to 32 mm3.

In ADU-S100 (40 μg) and combination groups, only one out of seven treated mice did not show a

decrease in tumor volume (Figure 1B and 1D). In CpG ODN (40 µg) group, five mice showed a considerable shrinkage in tumor volume, but in the other two members of this group, tumor volume reached 358 and 624 mm3 on the 30th day (Figure 1C). Although in ADU-S100 (20 µg), the average tumor volume showed a smaller decrease compared to the double concentration of this agonist, the change was significant compared to the control (Figure 1A). These results indicated that the synergistic antitumor effects of ADU-S100 when combined with CpG ODN is approximately equal to those of ADU-S100 when administrated at higher doses (twice the concentration). Images of CT26 tumor-bearing mice and treated mice of all groups on the 30th day are represented in Figure 2.

At the end of the experiment, mean tumor weight was considerably lower in the combination group (0.08 g) and other treatment groups than in the control (2.01g) ($p < 0.05$) (Figure 3A). The curve of spleen weight is shown in Figure 3B and spleen weight was positively correlated with tumor weight. This differ-

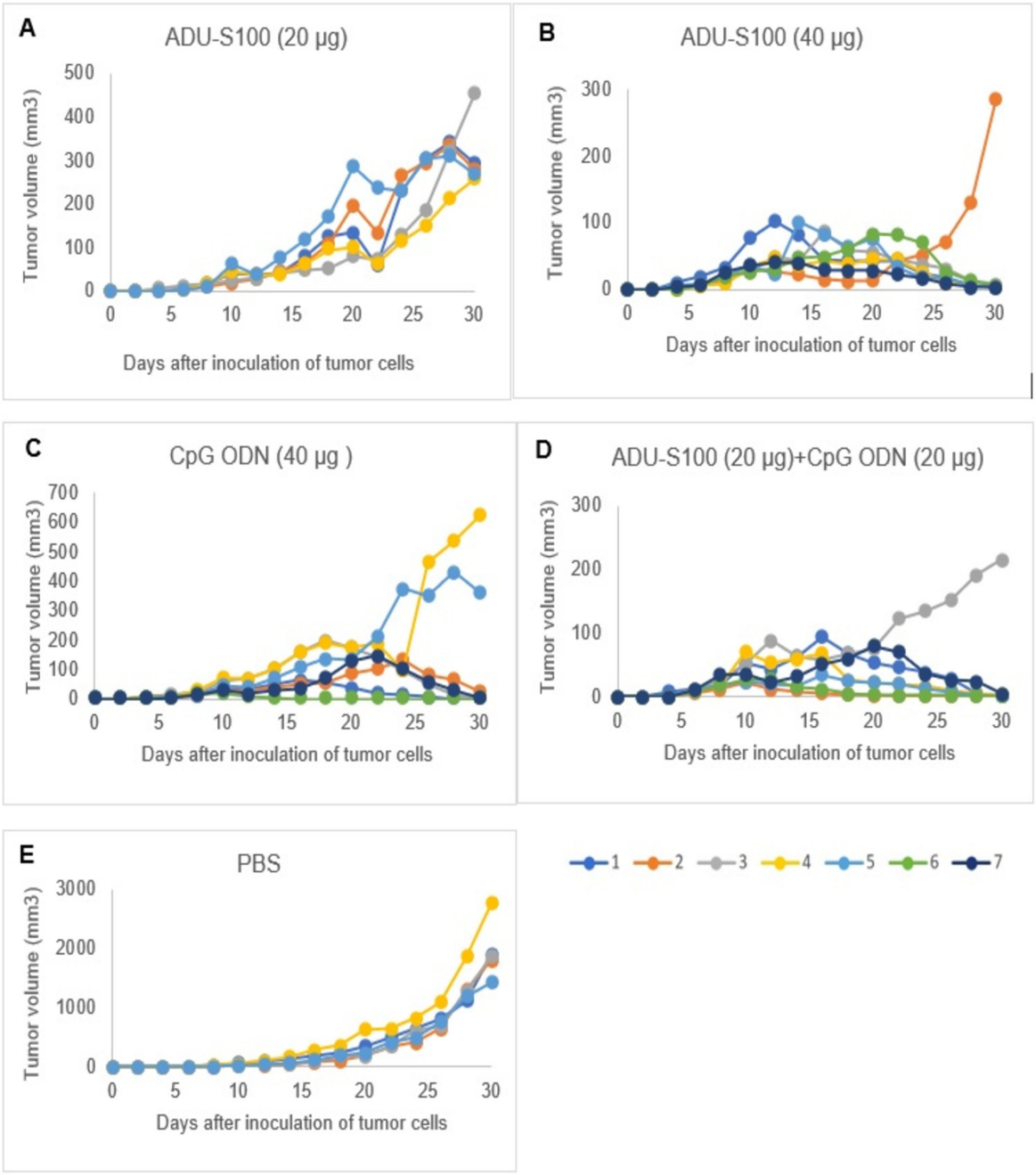

Figure 1.
Intratumoral (IT) administration of STING and TLR9 agonist reduces tumor volume in CT-26 adenocarcinoma model. (A) Mice were injected with 3×105 CT-26 cells (in 100 µl of PBS) SC on day 0. On days 10 and 16, mice were given IT injections of PBS, ADU-S100 (20 and 40 µg), CpG ODN (40 µg), and ADU-S100 (20 µg) + CpG ODN (20 µg) (n=7). tumor volumes were monitored every other day for 30 days.

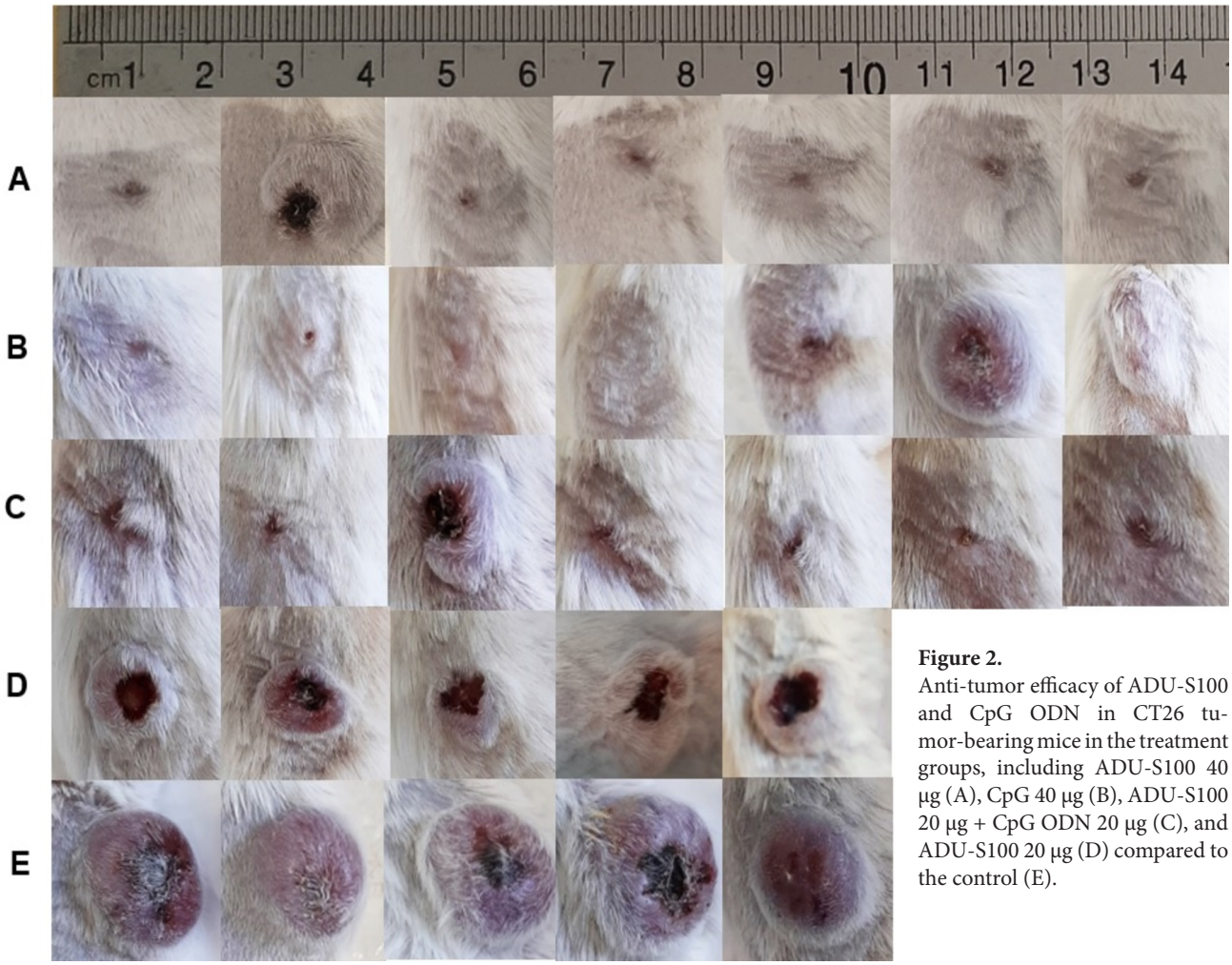

Figure 2.
Anti-tumor efficacy of ADU-S100 and CpG ODN in CT26 tumor-bearing mice in the treatment groups, including ADU-S100 40 µg (A), CpG 40 µg (B), ADU-S100 20 µg + CpG ODN 20 µg (C), and ADU-S100 20 µg (D) compared to the control (E).

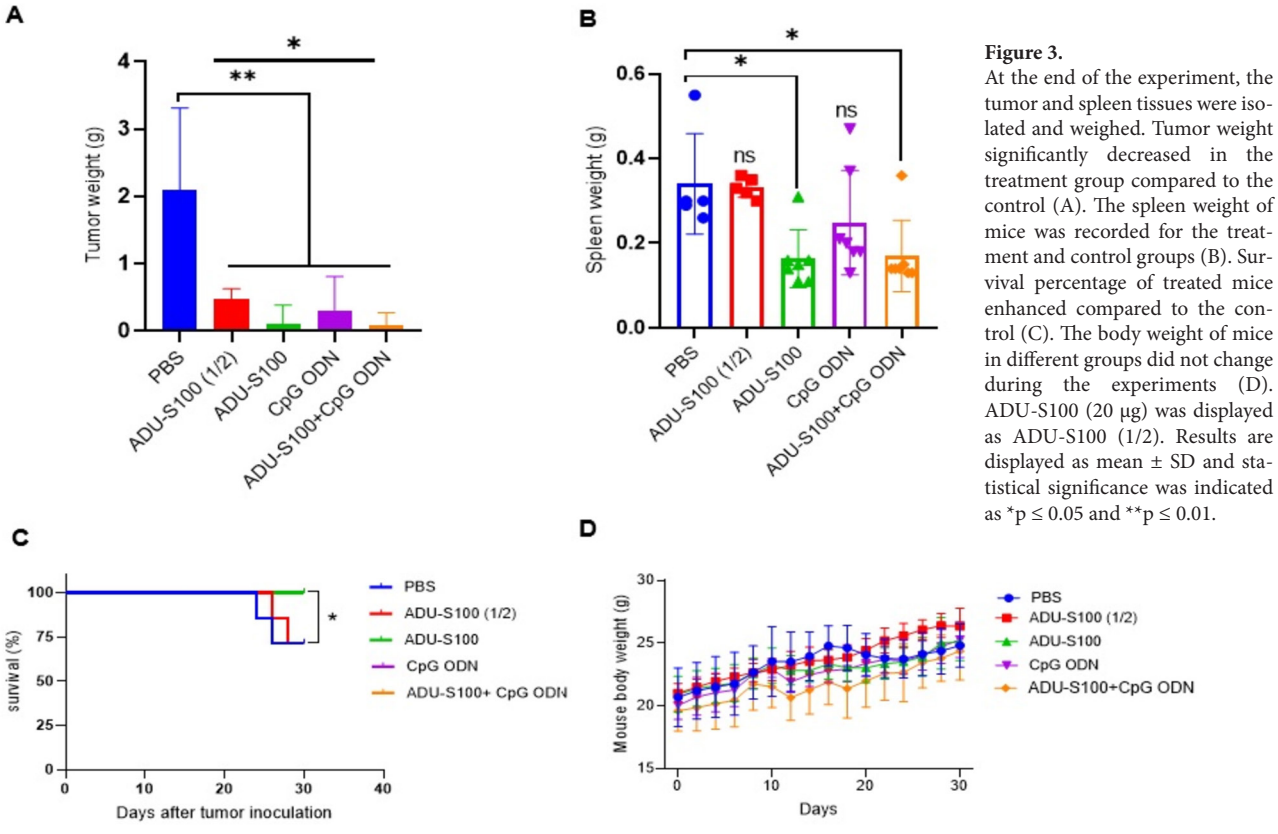

Figure 3.
At the end of the experiment, the tumor and spleen tissues were isolated and weighed. Tumor weight significantly decreased in the treatment group compared to the control (A). The spleen weight of mice was recorded for the treatment and control groups (B). Survival percentage of treated mice enhanced compared to the control (C). The body weight of mice in different groups did not change during the experiments (D). ADU-S100 (20 µg) was displayed as ADU-S100 (1/2). Results are displayed as mean ± SD and statistical significance was indicated as *p ≤ 0.05 and **p ≤ 0.01.

ence was significant in the ADU-S100 (40 µg) and combined groups which had the smallest tumor volume at the end of the experiment. During the study, the mice survival rate increased from 71.4% in the control group and ADU-S100 (20 µg) to 100% in other treatment groups (Figure 3C). Furthermore, the body weight of animals was also monitored during the experiment and no significant weight change was observed compared to the control group (Figure 3D).

Histological examination of the tumor, spleen, and liver tissues

Microscopic examination of the tumor tissues showed round, polygonal, spindle-like, polymorph neoplastic cells of varied sizes, containing prominent one or more nuclei, within a delicate stroma. The control group displayed a large number of tumor cells undergoing mitosis (Figure 4A). In addition, some criteria, such as necrosis and hemorrhage as signs of malignancy, were detected in the control group. Compared to the control group, increased numbers of apoptotic cells were observed in the treatment groups (Figure 4 B-E). Histological analysis of the spleen and liver tissues was performed to assess the abnormal

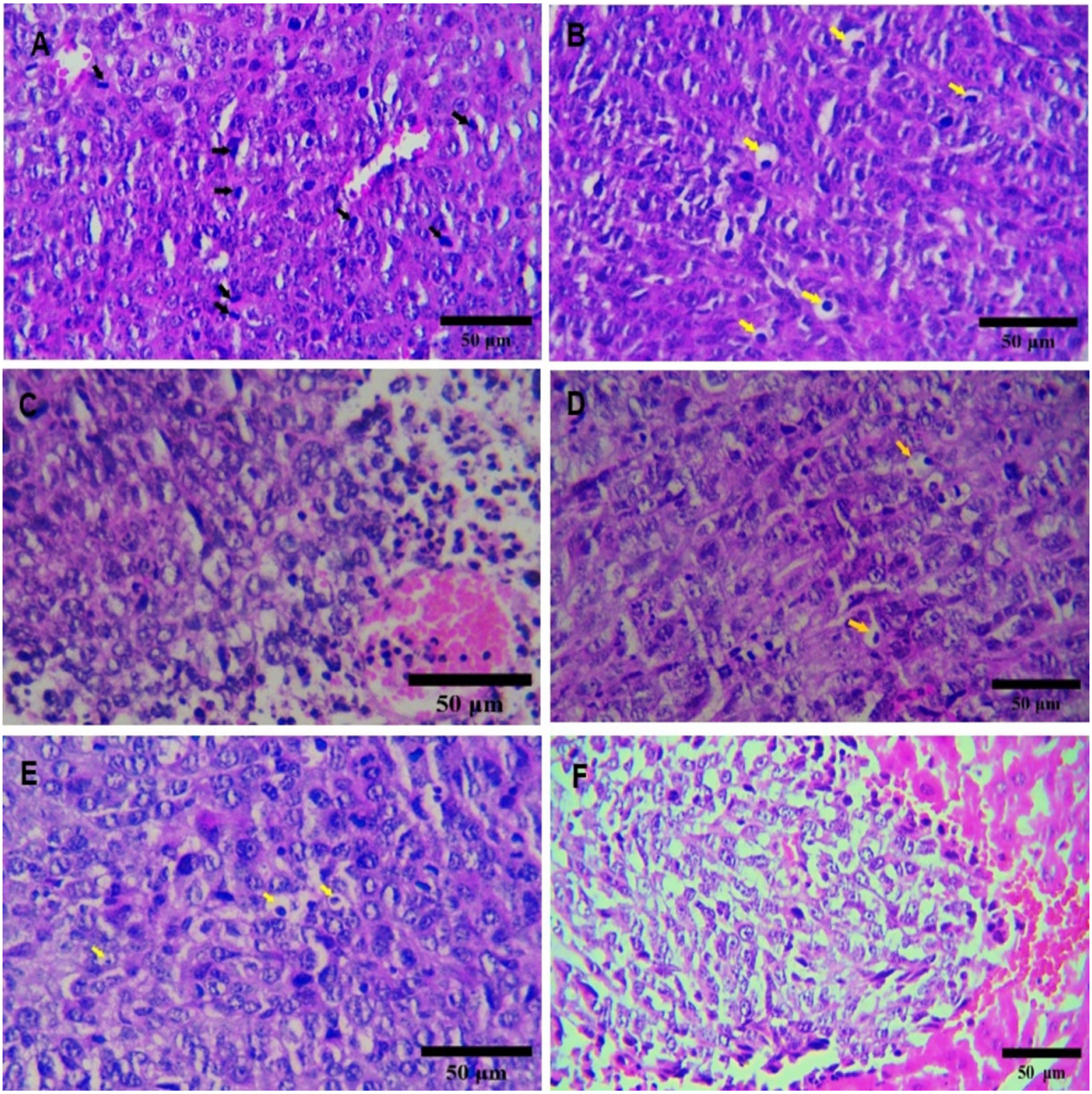

Figure 4.
Hematoxylin-Eosin staining of tumor tissues in the control (A), ADU-S100 20 µg (B), ADU-S100 40 µg (C), CpG 40 µg (D), and ADU-S100 20 µg + CpG ODN 20 µg (E). Liver metastasis in the control group (F). Cells undergoing mitosis in the control group are displayed with black arrows (A). Typical features of apoptosis (B-E, yellow arrows) are shown in the treatment groups.

effects of the agonists on the reticuloendothelial tissues. No obvious histological changes were detected in the spleen tissue in none of the treatment groups. The liver microstructure of the control group revealed tumor metastasis, while the treatment groups were normal (Figure 4F).

Hematological analysis of whole blood

CBC experiments showed that the number of lymphocytes increased significantly in the ADU-S100 (40 µg), CpG ODN (40 µg), and ADU-S100 (20 µg) + CpG ODN (20 µg) groups ($p < 0.05$) indicating the recruitment of immune cells to the TME (Table 2). Other hematological factors measured in this research were not significantly different between the control and treatment groups.

Table 1.
The average tumor volume (mm^3) in control and all treatment groups on 30th day

Group	Average tumor volume (mm^3) ± SEM	*p*- value
Control	1952±194	
ADU-S100 (20 µg)	310±32	<0.0001
ADU-S100 (40 µg)	44.8±36	<0.0001
CpG ODN (40 µg)	144±87	<0.0001
ADU-S100 (20 µg) + CpG ODN (20 µg)	32±28	<0.0001

Mean in each group is significantly different compared to control group ($p < 0.0001$).
SEM: Standard error of means. CpG ODN: CpG oligodeoxynucleotides.

Table 2.
The number of lymphocytes (%) of blood samples in control and all treatment groups on 30th day

Group	Number of lymphocytes (%) ± SEM	*p*- value
Control	13.5 ± 3	
ADU-S100 (20 µg)	21 ± 5	0.17
ADU-S100 (40 µg)	42 ± 7	<0.05
CpG ODN (40 µg)	44 ± 3	<0.05
ADU-S100 (20 µg) + CpG ODN (20 µg)	50 ± 6	<0.05

Mean in three treatment groups is significantly different compared to control group ($p < 0.05$).
SEM: Standard error of means. CpG ODN: CpG oligodeoxynucleotides.

Discussion

In the TME, immune cells and tumor cells can be affected by STING and TLRs activation. In addition, T cells, endothelial cells, fibroblasts, and APCs as the main cells in this pathway, can lead to type I IFN production with STING stimulation [11, 16]. Type I IFN signaling is important for antitumor immune responses through stimulating apoptosis and anti-proliferative responses acting directly on tumor cells leading to tumor suppression [17]. Due to the role of the STING pathway in the generation of spontaneous immune responses against tumors, STING agonists were discovered for direct pharmacologic stimulation of this signaling pathway [18-20]. Among STING agonists, the intratumoral administration of CDNs, such as ADU-S100, into different types of cancer inhibited tumor growth, generated lasting and systemic antigen-specific T cell immunity capable of rejecting distant metastases, improved survival of mice, and induced direct apoptosis of cancer cells [13, 21- 23]. On the other hand, CpG ODNs have been considered widely as a new choice for vaccine adjuvants because of their powerful capacity to enhance vaccine immunogenicity against cancer [3, 24]. In this research, we examined the antitumor effects of single and combined forms of ADU-S100 and CpG ODN1826 in the CT-26 colon cancer model. Our results showed that ADU-S100 at 40 µg dose suppressed the CT-26 tumor growth more effectively than at 20 µg dosage. Moreover, the combination of ADU-S100 and CpG ODN1826 resulted in the smallest tumor volume (Figures 1 and 2). As a result, the intratumoral injection of this combination in half concentration effectively reduced tumor growth in the CT-26 model (Figure 1A). In addition, the survival rate of treated mice (100%) was more than the control group (71.4%). Corrales et al. showed that the intratumoral injection of ADU-S100 in the CT26 colon carcinoma model caused T cell memory and durable tumor regression. When the tumor cell line was injected into mice again, the animals were completely resistant to re-challenge. This study had similar results in the 4T1 breast cancer model [11]. Other STING agonists also showed effective therapeutic results in the colon carcinoma model.

STING deficiency in mouse models bearing colon 26 adenocarcinomas reduced the antitumor effects of cGAMP as a potent STING agonist. The cGAMP displayed remarkable antitumor activity against CT-26 adenocarcinoma (2569 mm3 to 967 post-treatment). This agonist increased the survival rates of mice from 40% up to 90% in 20 days. Furthermore, cGAMP in-

duced the apoptosis of tumor cells and raised the expression levels of critical cytokines, such as IFN-β and IFN-γ, in the mouse serum [20]. The intratumoral injection of cGAMP by inducing CD8+ T cell responses delayed the growth of injected tumors and controlled the growth of distant tumors [21]. Deng et al. explained that STING pathway signaling was effective in type I IFNs induction and promotion of innate and adaptive immune responses upon radiation in MC-38 tumor models. The antitumor effects of radiation were significantly dependent on STING signaling as shown by impairing this antitumor effect in STING-deficient mice. Moreover, cGAMP and radiation synergistically increased the antitumor responses and reduced radiotherapy resistance [25]. The synergistic effects of K3CpG and cGAMP in melanoma, lymphoma, and pancreatic models displayed significant tumor suppression compared to single forms of agonists [26, 27]. Cai et al. investigated the synergism of CpG ODN, CGAMP, and anti-OX40 in TC1 and B16 models. This combination induced tumor regression and cytokines production by activating innate and adaptive immunity [28]. Formulation of recombinant protein HPV with 2'-3'cGAMP CDN and CpG-C ODN induced remarkable tumor suppression in TC-1 harboring mice as a cervical cancer model. This combination caused lymphocyte proliferation and increased the number of IFN-γ secreting cells [29].

In HE staining assays, cell apoptosis was observed in the tumor tissues upon ADU-S100 and CpG ODN treatment concerning the control group which may be displayed that the therapeutic effect of agonists is because of stimulation of tumor cells apoptosis. Circulating Lymphocyte-mediated immune response against tumor cells is critically important in tumor suppression [30]. Hematology experiments displayed that the number of lymphocytes rose in all treatment groups except ADU-S100 (20 μg), especially in the combination group and this lymphocytosis probably played a crucial role in tumor suppression.

In conclusion, our study suggests that the combination of ADU-S100 and CpG ODN with a reduced concentration is a potent adjuvant in tumor regression and antitumor immunity and emphasizes the potency of such combination adjuvants as a potential cancer immunotherapy approach.

Materials & Methods

Animals and cells

Six- to eight-week-old female BALB/c mice were purchased from Royan Institute (Tehran, Iran). All in vivo studies were performed according to the animal experimental guidelines approved by the Institutional Animal Care and Use Committee at Ferdowsi University of Mashhad. The animals were kept in standard cages at a temperature of 20°C-25°C and a 12L:12D lighting cycle. Mouse colon cancer cell line, CT26, was bought from the research institute of biotechnology (Mashhad, Iran), cultivated in RPMI 16-40 medium (Gibco, Grand Island, USA), and supplemented with heat-inactivated fetal bovine serum (10% v/v) and penicillin-streptomycin (1% v/v) (Gibco, Grand Island, USA). The cells were incubated at 37°C with a 5% CO2 atmosphere.

Tumor model

To set up the animal tumor model, CT26 cells (100 μl PBS containing 3105 cells for each mouse) were injected SC into the right flank of the animals (day 0). Tumor-bearing mice were randomly assigned to five groups (n=7): (a) Control group received 20 μl PBS; (b, c) tumor groups treated with ADU-S100 disodium salt (Med Chem Express, New Jersey, NJ, USA) at various dosages of 20 or 40 μg; (d) tumor group treated with 40 μg of CpG ODN (InvivoGen, San Diego, USA), and combination group (e) received 20 μg ADU-S100 and 20 μg CpG ODN simultaneously. The agonists were injected intratumorally on the 10th and 16th days after tumor inoculation.

The tumor volume (V) was estimated every other day with a digital caliper and calculated according to the equation V=(L×W2)/2, where W is the small diameter and L represents the large diameter of the tumor [11, 31, 32]. The animals were sacrificed on day 30 and the body weight of each mouse was measured. The spleen, liver, and tumor tissues were isolated for histological analysis.

Histopathological evaluation

The tumor, spleen, and liver tissues were fixed in 10% buffered neutral formalin for 24 h. The tissues were then dehydrated by enhancing the concentrations of ethanol solution (Merck, Munich, Germany), embedded in paraffin wax, and cut by microtome. The 5-μm sections were mounted on the slides and stained with HE. The sections were surveyed by light microscope (Labomed, Labo America Inc, USA) for the presence of histopathological lesions in all groups.

Hematological parameters

At the end of the study, whole blood was collected from mouse hearts in a tube containing an anticoagulant. Hematological parameters, including white blood cell, red blood cell, hematocrit, hemoglobin, mean cellular volume, mean cellular hemoglobin, mean cellular hemoglobin concentration, platelet count, red blood cell distribution width, plateletcrit, mean platelet volume, platelet distribution width, neutrophil, lymphocyte, monocyte, and total protein were assessed by a cell counter (Nihon Kohden, Nima Pouyesh Teb, Iran)

Statistical analysis

All data were analyzed using GraphPad Prism software version 9.0 (La Jolla, San Diego, California). The Mann–Whitney test was used for the statistical analyses. Results are displayed as mean ± SD or mean ± SEM. The significant differences between groups are shown as **** $p \leq 0.0001$, *** $p \leq 0.001$, ** $p \leq 0.01$, and * $p \leq 0.05$.

Authors' Contributions

S.H. performed the experiments, analyzed data and wrote the main draft of the manuscript., S.A. analyzed the histopathological experiments data., Z.M.F. assisted in in vivo experiments, M.G.S. and H.F assisted in data analysis.,

A.H. conceived the idea, designed experiments, provided reagents, analyzed data, edited and revised the manuscript and supervised the whole study.

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Competing Interests

The authors declare that they have no conflict of interest according to the work presented in this report.

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