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 towards clinical transition following successful fundamental research and clinical trials. Immunotherapy by efficacious adjuvants is critical for increasing protective immune responses against infectious disease and cancer. STING and TLR9 agonists are interesting candidates for novel immunotherapies for the treatment of cancer. In this study, the antitumoral effects of ADU-S100 as a potent STING agonist and CpG ODN1826 as TLR9 agonist in single and combined forms in CT-26 colon adenocarcinoma model was 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, tumor, spleen and liver tissues of mice were isolated for histopathological analysis. Hematological analysis was performed on heart blood. Intratumoral injection of agonists induced significant

tumor suppression in all treatment groups with profound effect in combination group that received half concentration of single form. Also, histopathological analysis of tumor tissues showed the presence of apoptotic and inflammatory cells and increased number of lymphocytes in the blood samples of the treatment groups indicating the effective role of these agonists in clearing the tumor. Thus, such synergy of adjuvant may have an effective role in cancer immunotherapy and offer new perspectives on combination of agonists that trigger innate immune sensors during malignancy.

Abbreviation

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

NF-κB: Nuclear factor kappa B

TME: Tumor microenvironment

dsDNA: Double-stranded DNA

ER: Endoplasmic reticulum

IFN: Interferon

S.C: subcutaneous

HE: Hematoxylin-eosin

CBC: Complete blood count

cGAMP: Cyclic guanosine monophosphate–adenosine monophosphate

CDN: Cyclic dinucleotides

APC: Antigen presenting cell

Introduction

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

One of the key immunoadjuvants are 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 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 production of pro-inflammatory cytokine and antibody from B cells that has demonstrated encouraging outcomes by reducing tumor growth and increasing survival in clinical trials [6- 8]. Another important immunoadjuvant identified in recent years is STING which is stimulated with cytosolic dsDNA and induce the transcription of numerous innate immune gene [9].

STING as an adaptor protein localized predominantly on the ER membrane. In response to specific agonists, STING induce signaling pathway that led to the production of IFN-β as a critical pro-inflammatory cytokine [10]. This leads to activation of DCs and subsequent recruitment of cytotoxic T lymphocytes in TME. There are different agonists for inducing of

STING, among them 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 of antigen specific $CD8^+$ T cell and lasting immune-mediated tumor rejection [11]. Due to 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 considered the synergistic effect of ADU-S100 in combination with CpG ODN1826 in a mouse model of colon carcinoma. 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.

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 affect tumor cells by activating of STING signaling pathway in TME and priming of APC and $CD8^+$ T lymphocytes. Intratumoral injection of this molecules into various cancer models (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, utilized as an adjuvant for vaccines and has been successfully evaluated in a number of vaccination models [15]. This study evaluated whether combination of CpG-ODN 1826 and ADU-S100 will attenuate tumor growth more than single forms of agonists in 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 $^{(20\ \mu g)}$ was used to

confirm the synergistic effect of agonists. Tumor volumes and weights of mice were recorded every other day. As can be seen in table 1, a 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 mm^3.

In ADU-S100 $^{(40\ \mu g)}$ and combination groups, only one out of 7 treated mice did not show a decrease in tumor volume (Figure 1B and 1D). In CpG ODN $^{(40\ \mu g)}$ group, 5 mice showed a considerable decrease in tumor volume, but in the other two members of this group tumor volume reached to 358 and 624 mm^3 on 30^{th} day (Figure 1C). In ADU-S100 $^{(20\ \mu g)}$, the average tumor volume showed a smaller decrease compared to the double concentration of this agonist, but it was significant compared to the control (Figure 1A). These results indicated that the synergistic antitumor effects of ADU-S100 when combined with CpG ODN which 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 30^{th} day are represented in figure 2.

At the end of the experiment, mean tumor weight was considerably lower in combination group (0.08 g) and other treatment groups with respect to control (2.01g) (p<0.05) (Figure 3A). The curve of spleen weight is shown in figure 3B and displayed spleen weight was positively correlated with tumor weight. This difference was significant in ADU-S100 $^{(40\ \mu g)}$ and combined groups which had the smallest tumor volume at the end of the experiment. During the study, mice survival rate was obviously increased from 71.4% in control group and ADU-S100 $^{(20\ \mu g)}$ 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 abnormal effects of the agonists on the reticuloendothelial tissues. No obvious histological changes were detected in the spleen tissue in all treatment groups. The liver microstructure of the control group showed 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 ADU-S100 $^{(40\,\mu g)}$, CpG ODN $^{(40\,\mu g)}$ and ADU-S100 $^{(20\,\mu g)}$ + CpG ODN $^{(20\,\mu g)}$ groups (p<0.05) indicating the recruitment of immune cells to the TME (Table 2). Other hematological factors measured in this experiment were not significantly different between the control and treatment groups.

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 the stimulation of apoptosis and anti-proliferative responses acting directly on tumor cells leading to tumor suppression [17]. Due to the role of STING pathway in generation of spontaneous immune responses against tumors, STING agonists were discovered for direct pharmacologic stimulation of this signaling pathway [18-20]. Among STING agonists, 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 that capable for rejecting distant metastases, improved survival of mouse and induced direct apoptosis of cancer cells [13, 21- 23]. On the other hand, CpG ODNs have been considered widely as new choice for vaccine adjuvants because of their powerful capacity to increase 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 CT-26 colon cancer model. Our results showed that ADU-S100 at the 40μg dose suppressed the CT-26 tumor growth more effective than 20 μg dosage. Also, combination of ADU-S100 and CpG ODN1826 showed the smallest tumor volume (Figure 1 and 2). As a result, the intratumoral injection of this combination in half concentration effectively reduced tumor growth in the CT-26 model (Fig. 1A). Also, the survival rate of treated mice (100%) was more than control group (71.4%). Corrales *et al*., showed that intratumoral injection of ADU-S100 in CT26 colon carcinoma model, displayed T cell memory and durable tumor regression. When the tumor cell line was injected to mice again, the animals were completely resistant to re-challenge. This study had similar results in 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 adenocarcinoma, reduced the antitumor effects of cGAMP as a potent STING agonist. cGAMP displayed remarkable antitumor activity against CT-26 adenocarcinoma (2569 mm^3 to 967 after treatment). This agonist increased survival rates of mice from 40% up to 90% for 20 days. Also, cGAMP induced apoptosis of tumor cells and increased the expression levels of critical cytokines such as IFN-β and IFN-γ in the mouse serum [20]. Furthermore, intratumoral injection of cGAMP by induction of $CD8^+$ T cell responses delayed 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 decreased radiotherapy resistance [25]. Furthermore, synergistic effects of K3CpG and cGAMP in melanoma, lymphoma and panceratic models displayed significant tumor suppression compared to single form of agonists [26, 27]. Cai *et al*., investigated the synergism effects of CpG ODN, CGAMP and anti-OX40 in TC1 and B16 models. This combination induced tumor regression and cytokines production through activation of 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 IFN-γ secreting cells number [29]. In HE staining assays, cell apoptosis was observed in the tumor tissues upon ADU-S100 and CpG ODN treatment with respect to the control group that maybe 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 increased in all treatment groups (except ADU-S100$^{(20\ \mu g)}$) especially in combination group and this lymphocytosis probably played a crucial role in tumor suppression.

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

Materials and Methods

Animals and cells

Six- to eight-week-old female BALB/c mouse were purchased from Royan Institute (Tehran, Iran). All *in vivo* studies were performed according to 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 to 25ºC and 12L:12D lighting cycle. Mouse colon cancer cell line, CT26, was bought from research institute of biotechnology (Mashhad, Iran) and 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 5% CO2 atmosphere.

Tumor model

To set up the animal tumor model, CT26 cells (100 µl PBS containing 3 $\times 10^5$ cells for each mouse) were injected subcutaneously into the right flank of mouse (day 0). Tumor bearing mice were randomly assigned to five groups (n=7): (a) Control group received 20µl 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 10^{th} and 16^{th} days post tumor inoculation.

The tumor volume (V) was estimated every other day with a digital caliper and calculated according to the equation $V= (L \times W^2)/2$, where W is the small diameter and L is the large diameter of tumor [11, 31, 32]. The animals were sacrificed on day 30 and 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 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 were stained with hematoxylin and eosin (HE). The sections were surveyed by light microscope (Labomed, Labo America Inc, USA) for the presence of histopathological lesions in the all groups.

Hematological parameters

At the end of the study, whole blood was collected from mouse heart in a tube containing anticoagulant. Hematological parameters including white blood cell (WBC), red blood cell (RBC), hematocrit (Hct), hemoglobin (Hbg), mean cellular volume (MCV), mean cellular hemoglobin (MCH), mean cellular hemoglobin concentration (MCHC), platelet count (PLT), red blood cell distribution width (RDW), Procalcitonin (PCT), mean platelet volume (MPV), Platelet Distribution Width (PDW), Neutrophil, Lymphocyte, Monocyte and total protein were counted 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). 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 $*p \leq 0.05$.

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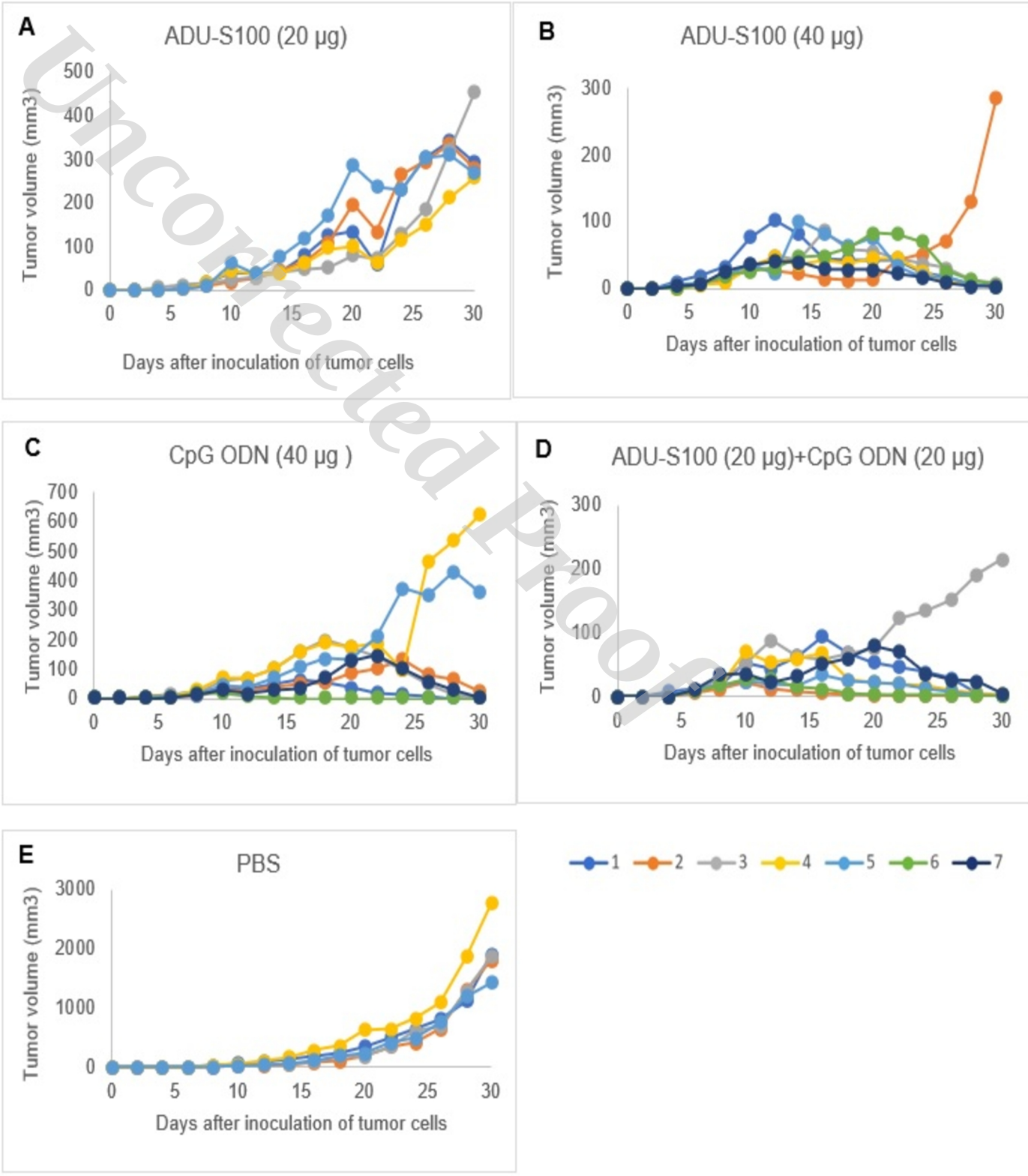
Figure legends

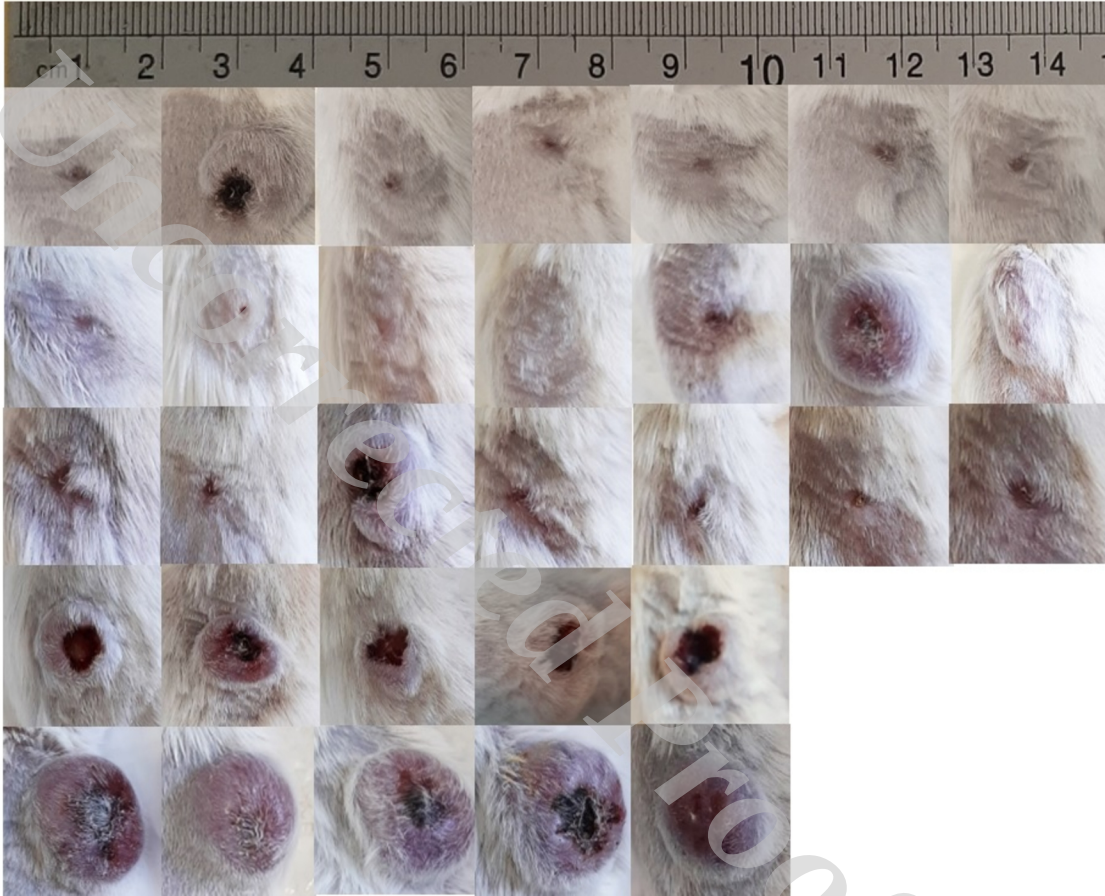
Figure 1: Intratumoral (IT) administration of STING and TLR9 agonist reduces tumor volume in CT-26 adenocarcinoma model. (A) Mice were injected with 3 $\times 10^5$ CT-26 cells (in 100 µl of PBS) S.C on day 0. On days 10 and 16, mice were given IT injections of PBS, ADU-S100 (20 and 40 µg), CpG ODN (40 µg), 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 treatment groups including ADU-S100 40 µg (A), CpG 40 µg (B), ADU-S100 20 µg + CpG ODN 20 µg (C), ADU-S100 20 µg (D) compared to control (E).

Figure 3: At the end of experiment tumor and spleen tissues were isolated and weighed. Tumor weight significantly decreased in treatment group compared to control (A). spleen weight of mice was recorded among treatment groups and control (B). Survival percent of treated mice enhanced compared to 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 by $*p \leq 0.05$ and $**p \leq 0.01$.

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

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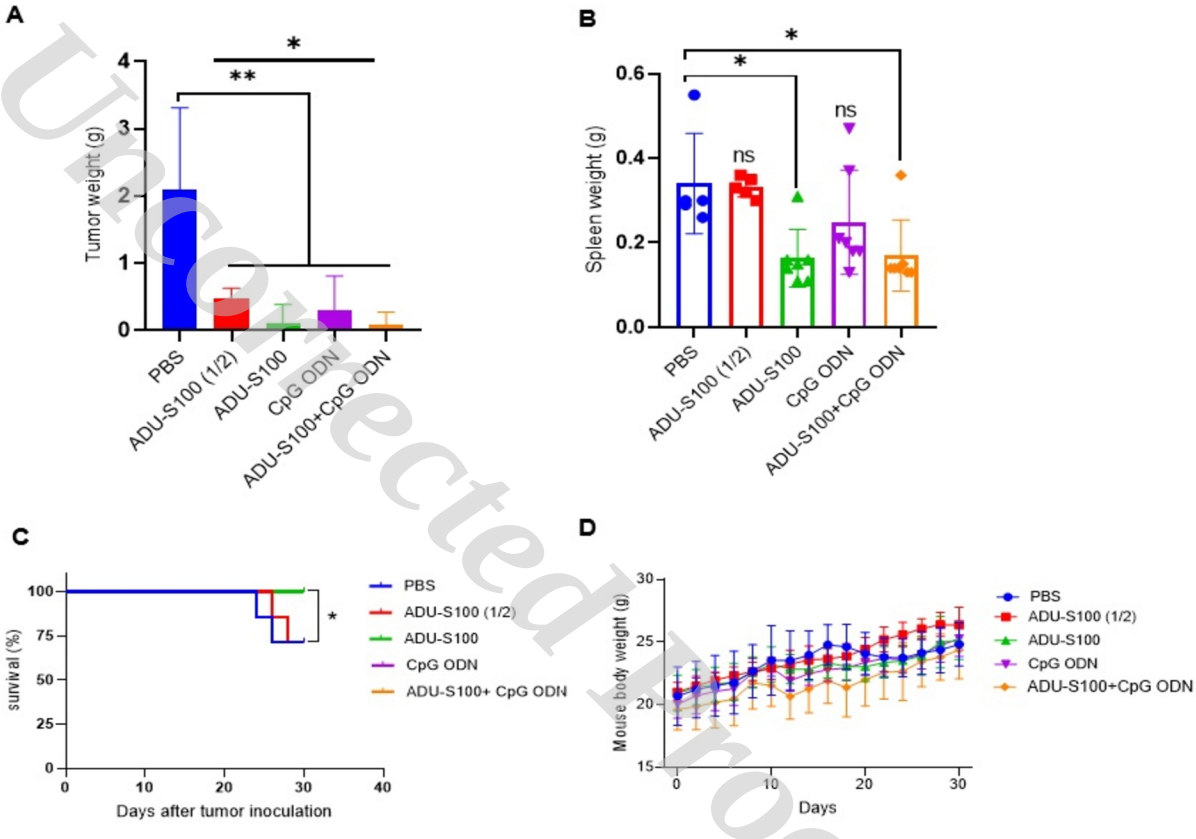

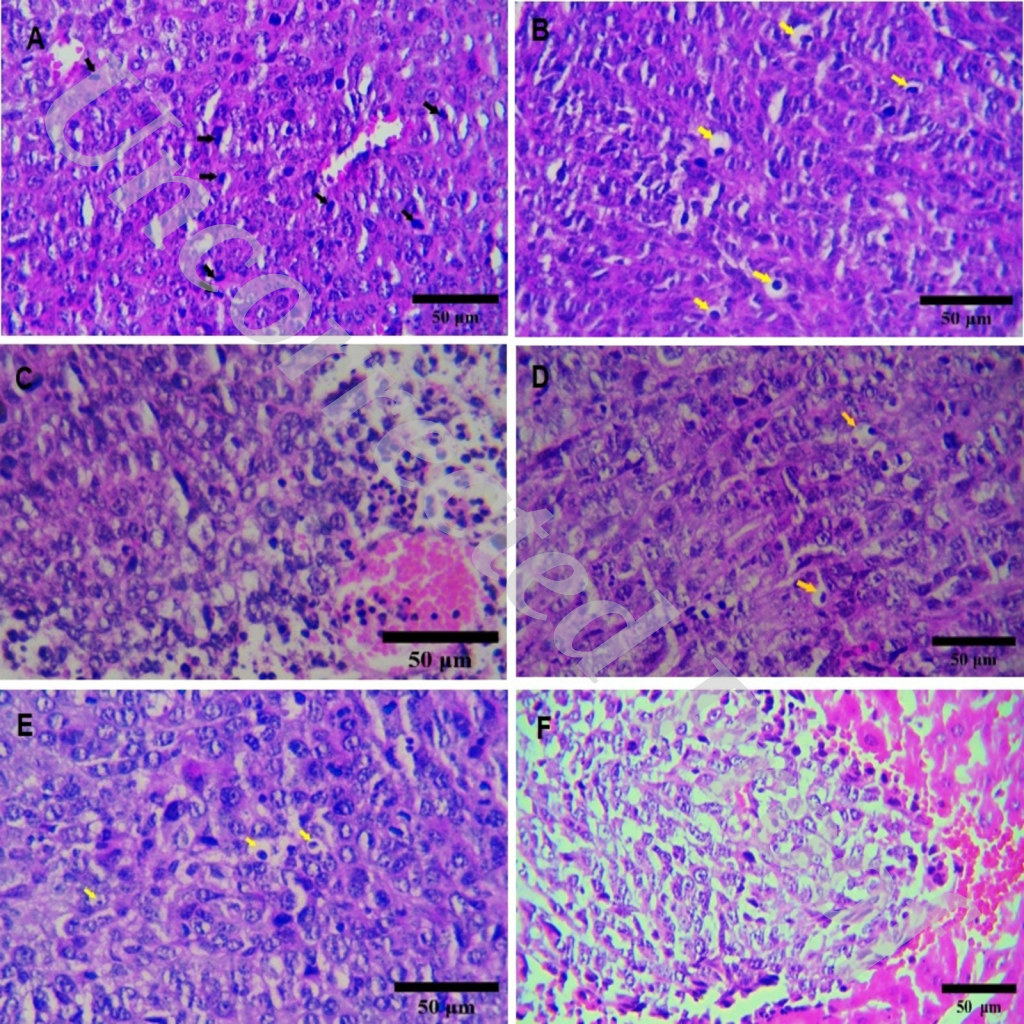
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Table 1.
The average tumor volume (mm^3) in control and all treatment groups on 30th day

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.