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Original Article

Disulfiram combined with chemoimmunotherapy potentiates pancreatic cancer treatment efficacy through the activation of cGAS-STING signaling pathway via suppressing PARP1 expression

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Abstract: Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related mortality globally with limited effective treatment options. Although the combination of immunotherapy and chemotherapy has been attempted in clinical trials to treat PDAC, the results are not promising. Therefore, in this study, we explored the application of a novel combination strategy with disulfiram (DSF) to enhance the treatment efficacy of PDAC as well as its underlying molecular mechanism. We compared the antitumor effects between single agents and the combination therapy by using mouse allograft tumor model and found DSF combined with chemoimmunotherapy significantly suppressed the growth of subcutaneous PDAC allograft tumor in mice and prolonged the survival of mice. To further investigate the alterations in the immune microenvironment of tumors from different treatment groups, we employed flow cytometry and RNA-seq analysis to examine the composition of tumor-infiltrating immune cells as well as the expression level of a variety of cytokines. Our results revealed that the proportion of CD8 T cells was notably elevated and that multiple cytokines were upregulated in the combination therapy group. Furthermore, qRT-PCR results indicated that DSF could upregulate the mRNA levels of IFNα and IFNβ, which could be reversed by STING pathway inhibitor. Mechanistically, we found that DSF activated STING signaling pathway through Poly (ADP-ribose) polymerases (PARP1) inhibition. Taken together, our findings highlight the potential clinical application of this novel combination strategy using DSF and chemoimmunotherapy in the treatment of patients with PDAC.

Keywords: Pancreatic ductal adenocarcinoma, disulfiram, chemotherapy, immunotherapy, PD1 inhibitor, PARP1, cGAS-STING signaling pathway

Introduction

PDAC is the fourth leading cause of cancer-related mortality globally and is predicated to be the second leading cause of cancer-related death in the next decade [1]. Although the treatment of pancreatic cancer has been improved in recent years, the 5-year survival rate still remains at 9% [2], due to the limited therapeutic options and the development of chemotherapy resistance [3]. Despite immunotherapy using immune checkpoint inhibitors (ICIs) has shown encouraging efficacy in multiple cancers [4], it is mostly ineffective in patients with PDAC [5]. Additionally, the therapeutic efficacy of ICIs combined with chemo-

therapies to treat PDAC was not satisfactory [6]. Therefore, it is imperative to explore more efficient therapeutic strategies for the treatment of PDAC and to improve the prognosis of patients with PDAC.

Disulfiram (DSF), an inhibitor of PARP1 that has been employed to treat alcohol aversion for more than 6 decades, has been reported to have anticancer effect in a broad spectrum of malignancies [7]. One Study has shown that DSF can interact with nuclear protein localization protein 4 (NPL4) and induce its aggregation to contribute to the vital p97-NPL4-ubiquitin fusion degradation 1 (UFD1) pathway, thereby leading to cell death [8]. Another study reports that DSF/Cu^{2+} overcomes cytarabine and bortezomib resistance in vitro in cell lines derived from Down-syndrome-associated acute myeloid leukemia patients [9]. In our previous study, we also indicated that a combination therapy of DSF/Cu^{2+} with anti-PD-1 antibody showed much better antitumor efficacy than monotherapy in treating hepatocellular carcinoma (HCC) [10].

Stimulator of interferon gene (STING), an intracellular receptor in the endoplasmic reticulum, can promote the expression of cytokines including type I interferon (IFN) through activating the cyclic guanosine monophosphate adenosine monophosphate (cGAMP) synthase (cGAS)-STING signaling pathway [11]. In 2013, Chen et al. has demonstrated that cGAS is a direct cytoplasmic DNA transducer [12]. During chemotherapy, self-DNA is released and can be detected by cGAS, which subsequently generates the second messenger cGAMP. cGAMP binds to and activates STING, resulting in the recruitment and the activation of Tank-binding kinase I (TBK1). TBK1 then phosphorylates the transcription factor interferon regulatory factor 3 (IRF3), leading to its nuclear translocation as well as the IRF3-dependent activation of IFN signaling [13, 14]. Notably, STING agonists and ICIs combination therapy have shown a potent effect in improving antitumor immunity [15, 16].

Here, we found that DSF combined with chemoimmunotherapy could significantly suppress the growth of allograft tumor in mice compared with monotherapy. Specifically, this novel therapy enhanced the antitumor immunity through upregulating IFNs to promote CD8 cytotoxic T cells infiltration in tumor. Mechanistically, DSF inhibited the activation of PARP1 triggered by chemotherapy and thus upregulated STING signaling pathway to activate antitumor immunity. These findings may explain the low therapeutic efficacy of the combination of chemotherapy and immunotherapy in treating PDAC and provide a novel therapeutic strategy.

Materials and methods

The antibodies listed below were used in Western blotting, immunohistochemical and flow cytometry analyses: anti-CD8 (ab22378; Abcam; 560776; BD Biosciences), anti-CD11b (557395; BD Biosciences), anti-NK1.1 (557391; BD Biosciences), anti-CD19 (553785; BD Biosciences), and anti-CD4 (550954; BD Biosciences; code ab183685, Abcam), anti-CD45 (code #70257, Cell Signaling Technology), anti-Foxp3 (code #12653, Cell Signaling Technology), anti-γH2AX antibody (code #9718, Cell Signaling Technology), anti-Phospho-TBK1 antibody (code #5483, Cell Signaling Technology), anti-TBK1 antibody (code #3504, Cell Signaling Technology), anti-GAPDH antibody (code #5174, Cell Signaling Technology), anti-cGAS antibody (code #9718, Cell Signaling Technology), anti-PARP1 antibody (code #9532, Cell Signaling Technology) and anti-PAR antibody (code #83732, Cell Signaling Technology). The STING agonist, diABZI STING agonist-1 was purchased from MCE (HY-112921B).

Western blot analysis

Western blot analysis was performed as described previously [10, 17]. Protein was extracted by RIPA buffer (Beyotime, Shanghai, China) containing protease and inhibitor mixes (Thermo Fisher Scientific, New York, USA) on ice. BCA Protein Assay kit (Thermo Scientific, Waltham, MA, USA) was employed to evaluate protein level. After being incubated with the second antibody for 2 h at room temperature, band intensity quantitation for western blotting was performed using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Immunohistochemistry and immunofluorescence

Paraffin-embedded subcutaneous tumor tissues were cut into 5 μm sections. Sections were deparaffined through immersing into

xylene and a series of concentration gradients of ethanol. Next, antigen retrieval was performed through keeping sections in microwave-heated antigen unmasking solution (EDTA, pH 8.0) for 30 mins. After incubation in goat serum for 30 mins, tissued sections were incubated by anti-CD8 antibodies (code ab-217344, Abcam) or anti-CD4 antibodies (code ab183685, Abcam) overnight at 4°C. The next day, goat anti-mouse or anti-rabbit secondary antibody was used to incubate sections for 1 h at room temperature and DAB Detection Kit (Maxim) was adopted for immunostaining for 3 mins. The proportion of positive areas were scored semi-quantitatively by 3 pathologists who were blind to the clinical parameters.

Cell culture

All human PDAC cell lines PANC-1, BXPC-3 and mouse PDAC cell line PAN02 were purchased from the Shanghai Institute of Cell Biology in China and Cell Bank of Type Culture Collection of Chinese Academy of Sciences. The cells were cultured in Dulbecco's modified Eagle's Medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal calf serum (FBS, Gibico) at 37°C in a humidified incubator containing 5% CO_2.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated by using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Equal amounts of RNA were reversely transcribed into Complementary DNA (cDNA) using the PrimeScript RT Reagent Kit (TaKaRa Shiga, Japan) and qRT-PCR was performed using SYBR Premix Ex Taq (TaKaRa), according to the manufacturer's instructions.

Cell transfection

Short hirepin RNAs (shRNAs) targeting human cGAS were synthesized by Genomeditech (Shanghai, China). Lipofectamine 3000 (Invitrogen) reagents were employed to transiently knock-down the target genes, according to the manufacturer's instructions. Two days after transfection, the cells were used for the indicated experiments. The knock-down of target genes was validated by western blot analysis.

Animal experiments

Male C57BL/6 mice (5-6 weeks old) were subcutaneously injected with PAN02 cells (10^6 cells/mouse) to generate allograft tumor and then were randomly divided into 8 groups (8 mice per group). The tumor growth was monitored, and when the tumor volume reached to 108-171.5 mm^3, the mice in different groups were treated as follows: Group 1: rat IgG (control; Bio X Cell) was injected intraperitoneally (i.p.) every 3 days; Group 2: anti-PD-1 antibody (250 μg, RMP1-14; Bio X Cell, West Lebanon, NH, USA) was injected via i.p. every 3 days; Group 3: gemcitabine (60 mg/kg) was administered via i.p. every 4 days; Group 4: mice were treated with anti-PD-1 antibody and gemcitabine; Group 5: DSF (50 mg/kg) was orally administered once a day; Group 6: mice were treated with a combination of DSF and gemcitabine; Group 7: mice were treated with a combination of DSF and anti-PD-1 antibody; Group 8: mice were treated with a combination of DSF plus gemcitabine and anti-PD-1 antibody.

The tumor size was measured every 3 days by caliper, and the tumor volume was calculated using the formula: V = length × $width^2/2$. Mice were sacrificed at day 30 after the inoculation of tumor cells, and the tumors were dissected and weighed. This study was approved by the Shanghai Medical Experimental Animal Care Committee, and all animal handlings were performed according to the National Institutes of Health "Guide for the Care and Use of Laboratory Animals".

Statistical analysis

All results were shown as mean ± SD from at least three independent experiments. All statistical analyses including unpaired Student's t-tests, one- and two-way ANOVA tests were performed through the GraphPad Prism software (v8.2.0). $P < 0.05$ was considered statistically significant.

Results

DSF in combination with anti-PD1 therapy and chemotherapy demonstrated the best antitumor efficacy

To determine the treatment efficacy of DSF in combination with immunotherapy (anti-PD1 antibody) and chemotherapy (gemcitabine), we used subcutaneous PAN02 tumor model to compare the effect of single drug and drug combination on tumor growth. We found that anti-PD1 therapy showed minimal effect on

tumor growth, although chemotherapy demonstrated a positive tumor growth-suppressing effect. However, the combination of immunotherapy and chemotherapy didn't improve the treatment efficacy compared with chemotherapy treatment alone (**Figure 1A-C**). Consistent with the effect on tumor growth, there was no significant difference on the proportion of tumor-infiltrating immune cells in these tumors (**Figure 1D**). In contrast, when we assessed the impact of DSF on the antitumor function of immunotherapy and chemotherapy, the results were promising. Specifically, when we compared the monotherapy alone with different combinations among DSF, anti-PD1 therapy, and chemotherapy, we observed that the combination of these three drugs had the best effect on suppressing tumor growth (**Figure 1E-G**), suggesting that DSF in combination with anti-PD1 therapy and chemotherapy might be a promising therapeutic strategy in treating PDAC.

DSF in combination with anti-PD1 therapy and chemotherapy remodeled the tumor immune microenvironment

To understand the mechanisms mediating the function of DSF combined with anti-PD1 therapy and chemotherapy in inhibiting the growth of PDAC tumors, we examined the molecular and cellular changes by RNA-seq and flow cytometry in the treated or untreated subcutaneous tumors. Intriguingly, compared with other groups, the proportion of CD4 T cells and CD8 T cells was markedly upregulated in the combination treatment group (**Figure 2A** and Supplementary Figure 1A). Furthermore, we evaluated weather DSF could enhance the cytotoxic effect of chemotherapy by using two human pancreatic cell lines (PANC-1, BXPC-3 cells), and the results were negative (**Figure 2B**). However, RNA-seq analysis indicated a series of cytokines including IFNα, IFNγ, TNFα, CCL5, CXCL9 and CXCL10 were markedly upregulated (**Figure 2C**). It is known that these cytokines can activate and attract CD8 T cells for anti-tumor function [18, 19]. To further verify this result, we performed qRT-PCR to detect the expression of these cytokines and obtained the same results (**Figure 2D**). As we know, when the cGAS-STING pathway in tumor cells is activated, cytokines such as type I IFN are induced, contributing to tumor cell apoptosis or death [20]. Hence, we speculated that cGAS-STING signaling pathway might be activated by the combined treatment to upregulate the expression of cytokines. While our results showed that STING pathway agonist significantly promoted the expression of IFNα and IFNβ in PANC-1 (**Figure 2E** and **2F**), both 5 μM and 10 μM DSF alone could not upregulate the expression of IFNα and IFNβ, suggesting that DSF might enhance STING signaling pathway through the combination effect with immunochemotherapy. Taken together, these results indicated that DSF in combination with anti-PD1 therapy and chemotherapy could remodel the tumor immune microenvironment through upregulating the expression of cytokines to activate CD8 T cells.

DSF activated antitumor immune response via the cGAS-STING pathway

It is well recognized that chemotherapy can cause the formation of cytosolic dsDNA and micronuclei, which subsequently activates of the cGAS-STING signaling pathway as well as the inflammatory responses in patients with malignancies [21, 22]. To further investigate whether DSF in combination with immunochemotherapy activates cGAS-STING signaling pathway, we examined the expression levels of γH2AX (a marker for DNA double strand breaks) and p-TBK1 by western blot in PDAC cell lines treated by DSF and chemotherapy drug. Our results showed that the combined DSF and chemotherapy drug treatment could notable upregulate γH2AX level (**Figure 3A**). Additionally, we found that DSF combined with chemotherapy was able to induce the formation of cytosolic dsDNA and to upregulate the S172 phosphorylation of TBK1 (**Figure 3B** and **3C**). Conversely, knockdown of GAS reversed the upregulated expression of IFNα and IFNβ induced by STING agonist or the combined DSF and chemotherapy treatment (**Figure 3D-F**). Consistently, GAS knockdown significantly inhibited the S172 phosphorylation of TBK1, and the combined DSF and chemotherapy treatment showed minimal effect on the S172 phosphorylation of TBK1 (**Figure 3G**). Collectively, these results suggested that DSF and chemotherapy could effectively activate cGAS-STING pathway in PDAC cell lines and that the S172 phosphorylation of cGAS was indispensable for this activation.

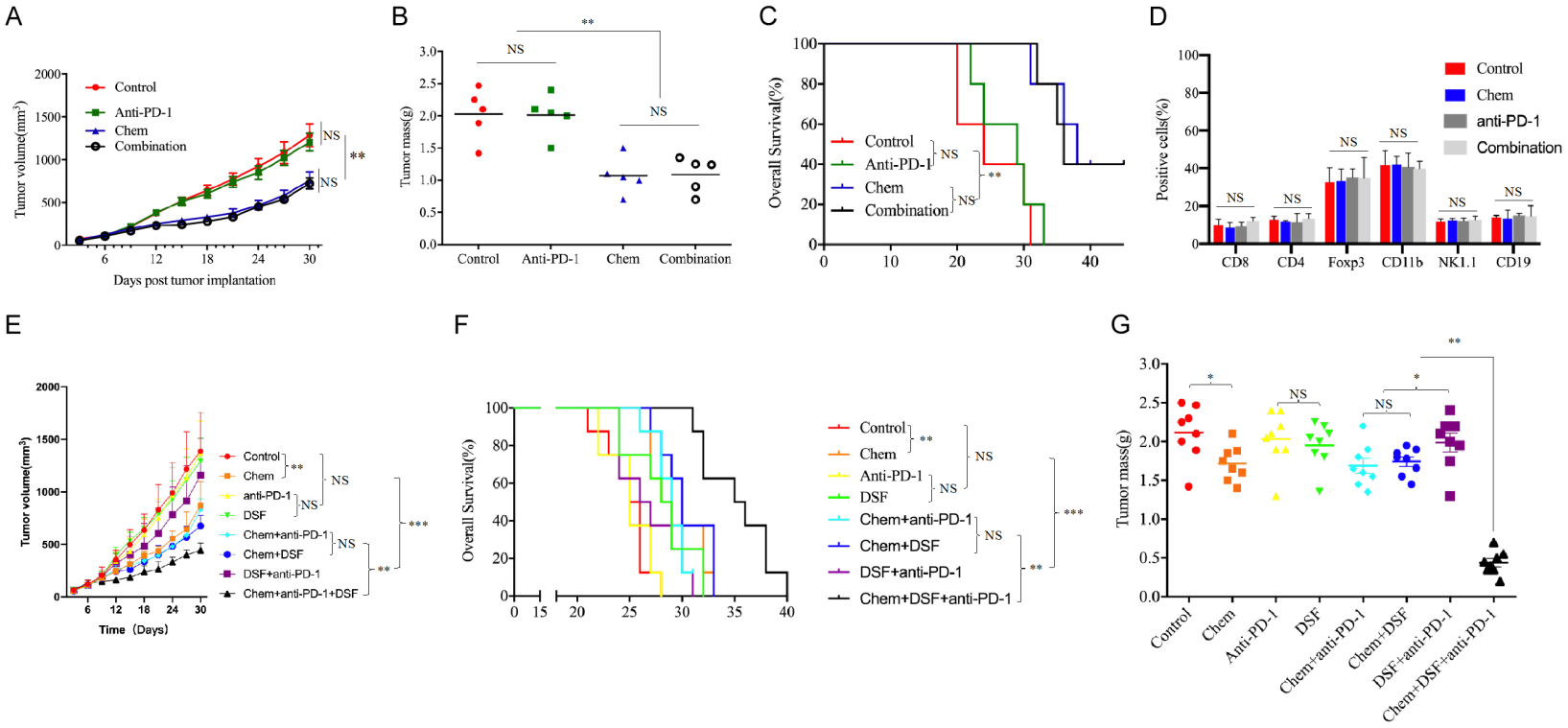

Figure 1. DSF combined with immunochemotherapy elevates antitumor efficacy. Tumor volume (A) and tumor mass (B) of the PAN02 cell-derived tumors (n = 8 per group). (C) The survival of mice bearing PAN02 tumors following treatment with chemotherapy and/or anti-PD-1 antibody (n = 8 per group). (D) The composition of immune cells in tumor tissues analyzed by flow cytometry. (E-G) Tumor volume, tumor mass, and the survival of mice bearing PAN02 tumors treated by DSF (50 mg/kg) and/or immunochemotherapy (**P < 0.01, ***P < 0.001, NS indicates no significance).

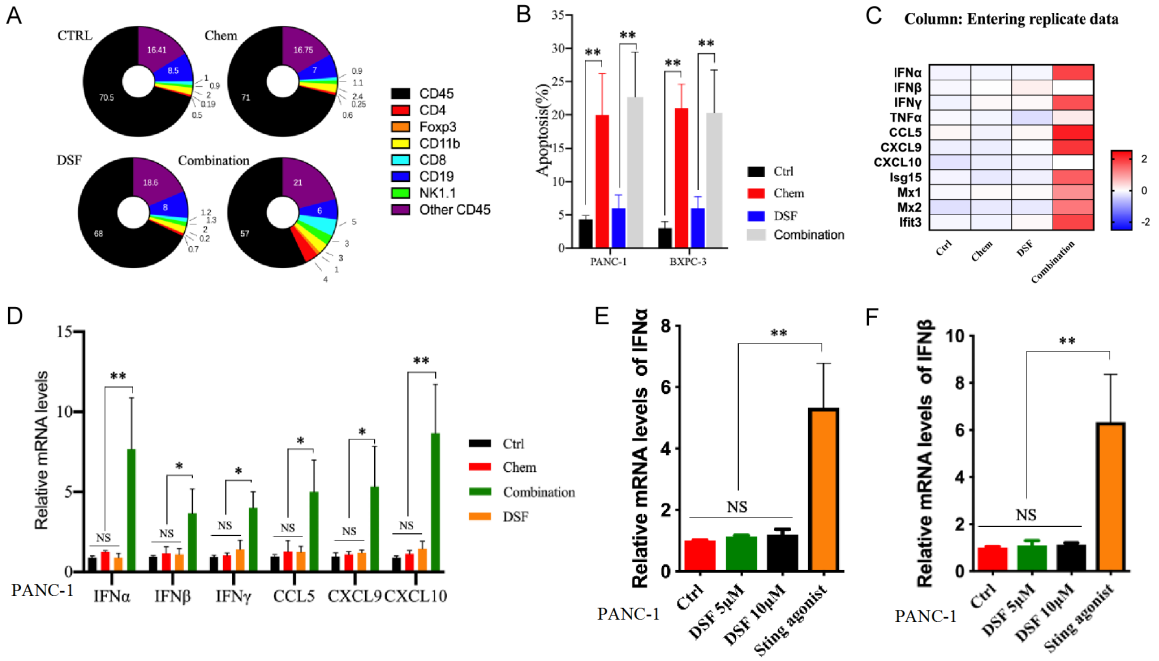

Figure 2. DSF combined with immunochemotherapy remodels the tumor immune microenvironment. A. The composition of immune cells in PAN02 cell-derived tumor tissues following treatment with DSF and/or immunochemotherapy was analyzed by flow cytometry. B. The apoptosis of PANC-1 and BXPC-3 cells induced by treatment with DSF (10 μM) and/or immunochemotherapy. C. Heatmap of the expression of cytokines in the treated PAN02-derived tumor tissues analyzed by the RNA-seq. D. The mRNA levels of cytokines in the treated PAN02-derived tumor tissues evaluated by qRT-PCR analysis. E and F. The mRNA levels of IFNα and IFNβ in PDAC cells treated by 5 and 10 μM DSF or 100 nM STING agonist (diABZI) for 4 hours as determined by qRT-PCR (**P < 0.01, ***P < 0.001, NS indicates no significance).

DSF activated cGAS-STING pathway through inhibiting PARP1

To further reveal the mechanisms by which DSF regulates cGAS-STING pathway, we investigated whether Poly (ADP-ribose) polymerase 1 (PARP1) was affected by the treatment of DSF and chemotherapy, as ours and other previous studies have shown that DSF/Cu^{2+} inhibits PARP1 activity [10, 23]. Additionally, PARP inhibitor treatment induces the antitumor immune response via the cGAS-STING pathway [24]. In this study, we observed that PARP1 expression was upregulated in a dose-dependent manner in PANC-1 and BXPC-3 cells treated by chemotherapy drug (**Figure 4A** and **4B**), while PARP1 expression was decreased upon DSF treatment. However, the combination of chemotherapy and DSF resulted in reduced expression of PARP1 (Supplementary Figure 1B) in PANC-1 cells. Subsequently, we examined the overall PARylation in PDAC cells treated by chemotherapy and found that chemotherapy could enhance the activity of PARP1 in a dose-dependent manner (**Figure 4C**). Furthermore, we determined whether the elevated PARP1 activity triggered by chemotherapy could be reversed by 10 μM DSF treatment. As expected, we found that DSF could inhibit the overall PARylation in cells, suggesting that DSF could reverse the activity of PARP1 induced by chemotherapy (**Figure 4D**). Furthermore, we also constructed PARP1-knockout PANC-1 cells and observed that PARP1 knockout could abolish the upregulated expression of IFNα and IFNβ induced by combined DSF and chemotherapy treatment (**Figure 4E**). Moreover, we also proved that GAS inhibition did not impact PARP1 expression (**Figure 4F**). Importantly, the immunohistochemistry staining of the subcutaneous tumor tissues demonstrated that the number of CD8 T cells was significantly elevated by the combination of DSF and chemotherapy treatment (**Figure 4G**). Together, these results revealed that DSF activated cGAS-STING pathway through PARP1 inhibition.

Discussion

Although DSF has shown anticancer activity against a broad spectrum of malignancies, the effect of DSF on antitumor immunity in PDAC

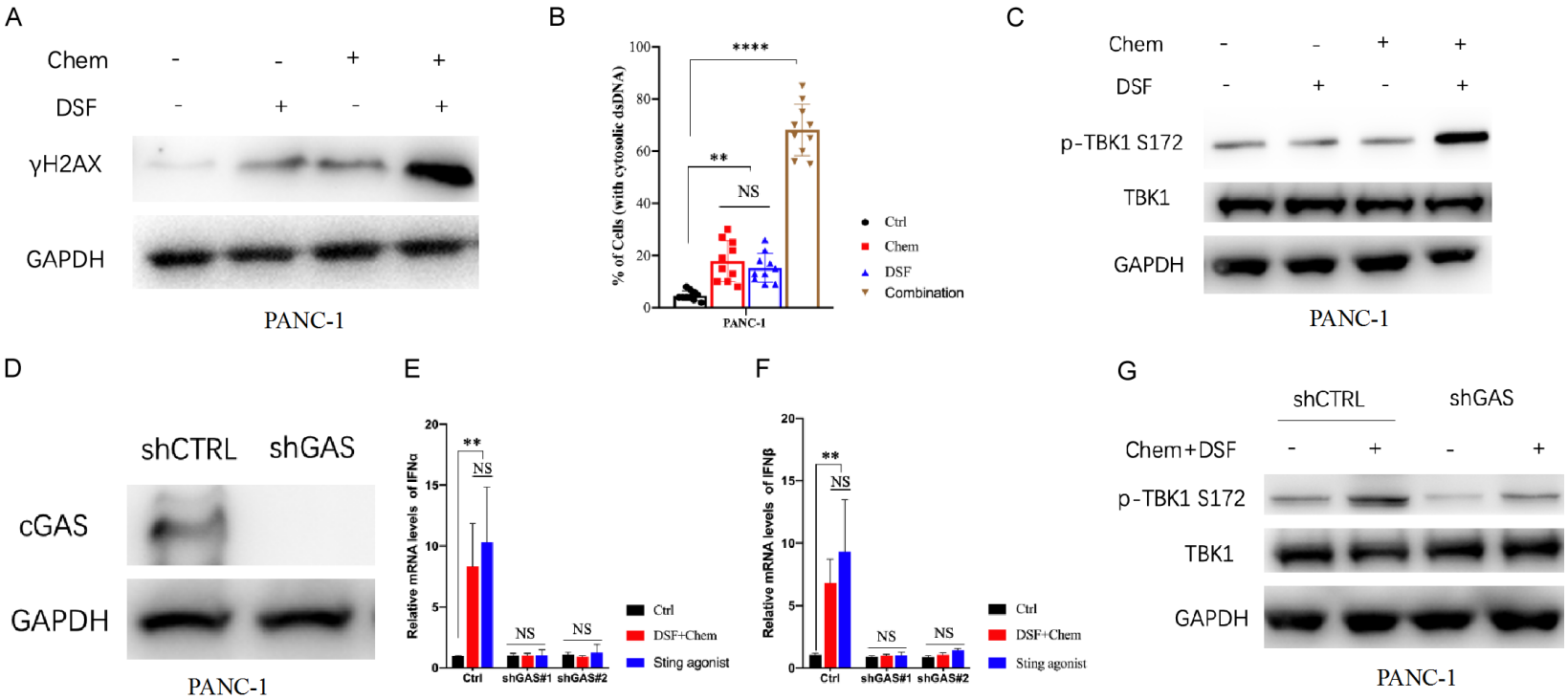

Figure 3. DSF activated antitumor immune response via the cGAS-STING pathway. A. The protein levels of γH2AX in PANC-1 cells treated by chemotherapy and/or DSF (10 μM) were detected by western blot. B. Quantification of the staining results of cytosolic dsDNA in PANC-1 cells. C. S172 phosphorylation of TBK1 in PANC-1 cells treated by chemotherapy and/or DSF (10 μM). D. cGAS protein expression was detected by western blot in PANC-1 cells transfected with shNC or shRNA targeting cGAS. E and F. The mRNA level of IFNα and IFNβ in PANC-1 cells transfected with shNC or shcGAS after being treated with DSF plus chemotherapy or STING agonist (diABZI) was determined by qRT-PCR. G. S172 phosphorylation of TBK1 and total TBK1 protein levels were detected in PANC-1 cells with or without chemotherapy plus DSF treatment ($^{**}P < 0.01$, $^{***}P < 0.001$, NS indicates no significance).

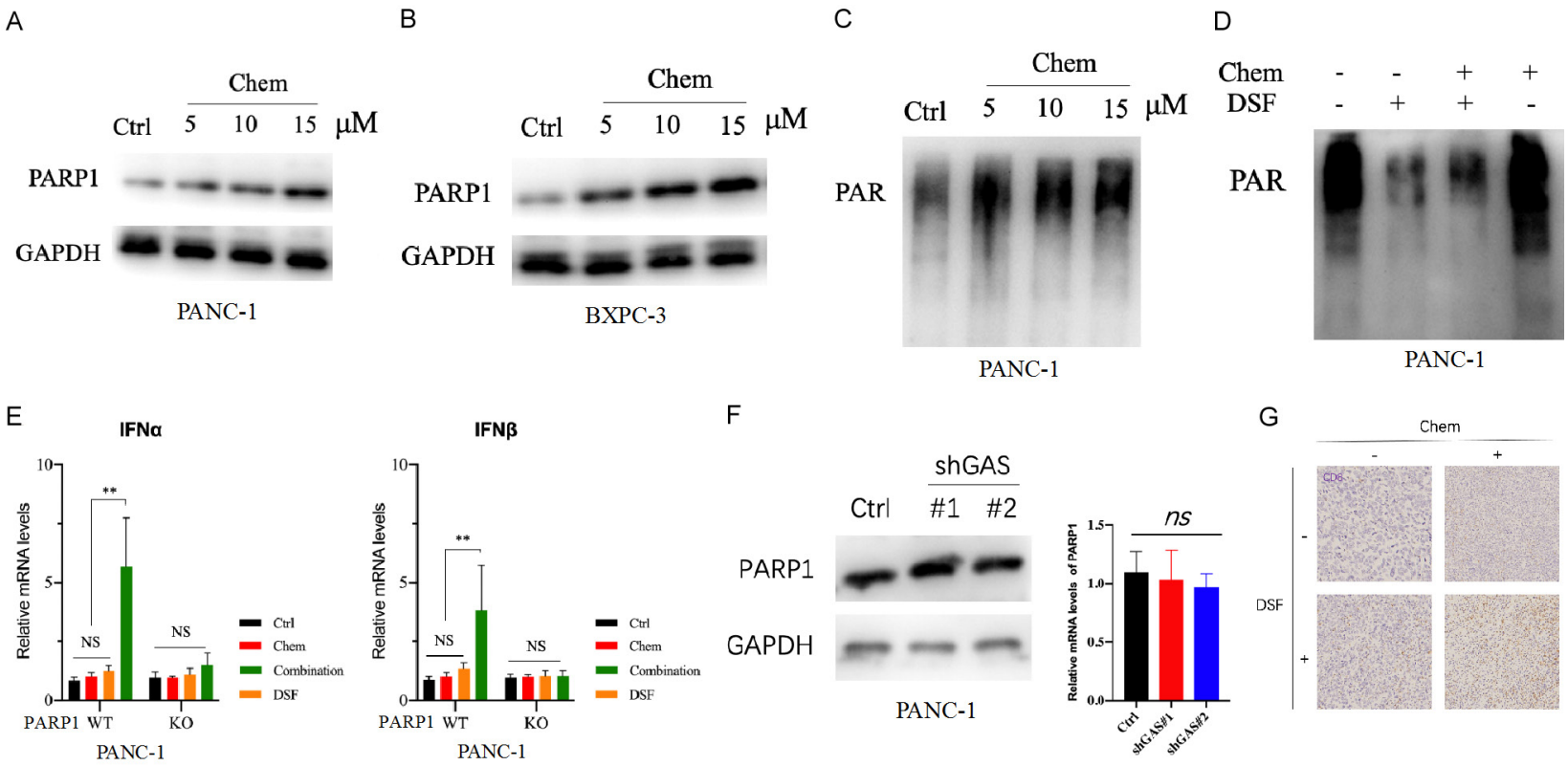

Figure 4. DSF upregulated cGAS-STING pathway through inhibiting PARP1 activity. The protein levels of PARP1 in PANC-1 (A) and BXPC-3 (B) cells treated with different doses of DSF were determined by western blot. (C) Total PARylation in PANC-1 cells treated with different doses of DSF was examined to evaluate the activity of PARP1. (D) Total PARylation in PANC-1 cells treated by DSF or chemotherapy was detected by western blot. (E) The mRNA levels of IFNα and IFNβ in wild type or PARP1 knockout PANC-1 cells treated by DSF or chemotherapy were determined by qRT-PCR. (F) PARP1 protein levels in PANC-1 cells transfected with shNC or shRNA targeting cGAS. (G) Immunohistochemistry staining of the subcutaneous tumor tissues treated with DSF or chemotherapy (**P < 0.01, ***P < 0.001, NS indicates no significance).

remains elusive. In this study, we for the first time demonstrated the tumor growth-suppressing effect of DSF when used in combination with immunochemotherapy in PDAC. By examining the alteration of immune cells and cytokines in the tumor microenvironment, we further revealed the activation of CD8 T cells and the upregulation of a series of cytokines caused by DSF plus chemoimmunotherapy treatment. Mechanistically, we determined that the combination of DSF and chemoimmunotherapy restored the activation of cGAS-STING pathway through inhibiting PARP1 activation.

To date, the first-line chemotherapy regimens for patients with advanced or metastatic pancreatic cancer are gemcitabine plus nanoparticle albumin-bound paclitaxel or the combinations of 5-fuorouracil/leucovorin with irinotecan and oxaliplatin (FOLFIRINOX) [25]. However, patients with PDAC develop chemotherapy resistance due to a complicated interplay between the genetic and epigenetic alterations, as well as a highly hypovascular, hypoxic and desmoplastic tumor microenvironment [3]. Immunotherapy, the fastest growing branch in oncology, has already revolutionized the treatment of few solid cancers; however, immune checkpoint inhibitor monotherapy, such as anti-PD-L1 or PD-1, only shows minimal response in PDAC [26]. Although immunotherapy and neoadjuvant chemotherapy are theoretically applicable, the actual effects still need to be investigated [26]. In our *in vivo* experiments using mouse tumor model, we also found immunochemotherapy had limited efficacy in suppressing the growth of PDCA in mice. However, DSF combined with immunochemotherapy showed a significant antitumor effect as the tumor size was significantly decreased and the survival of mice was prolonged. These findings supported our previous study, in which we also demonstrated that DSF/Cu^{2+} and anti-PD1 therapy could improve the antitumor effect in HCC [10].

Furthermore, in this study, we demonstrated that DSF combined with immunochemotherapy activated and recruited CD8 T cells through upregulating cytokine production during antitumor process. However, unlike chemotherapy, DSF had no significant cytotoxic effect. In addition, despite the upregulation of cytokines such as IFNα and IFNβ, which suggested the possible activation of STING signaling pathway, we found DSF could not directly upregulate IFNα and IFNβ expression in PDAC cells compared with STING agonist. On the other hand, increasing evidence has shown that chemotherapy and radiation therapy cause the formation of cytosolic dsDNA and micronuclei, which can activate the cGAS-STING signaling pathway as well as inflammatory responses in tumors [21, 22]. In this study, we also observed that chemotherapy could promote DNA double strand breaks. Intriguingly, DSF treatment could significantly magnify the chemotherapy-induced DNA double strand breaks. Furthermore, cGAS-STING signaling pathway activation also showed the same effect. Nevertheless, the mechanisms by which combined DSF and chemotherapy significantly activates cGAS-STING signaling pathway require further investigation.

PARP1 is an enzyme involved in modulating DNA damage response (DDR). Through sensing the genotoxic stress, PARP1 is recruited to DNA strand breaks and is activated to synthesize negatively charged Poly-ADP-ribose (PAR) polymers. One of the functions of these PAR chains is to serve as a platform to recruit the DDR machinery to repair and resolve these DNA breaks [27, 28]. Hence, PARP inhibitors (PARPi) have been extensively evaluated as monotherapy or combination therapy in clinic for the treatment of multiple malignancies [29]. As an inhibitor of PARP1, DSF can induce apoptosis by suppressing the PARP1 activity in tumor cells [10, 23]. In our study, we found that the expression and the activation of PARP1 was elevated in a dose-dependent manner in PDAC cells treated by chemotherapy, which was reversed markedly by DSF. In support with these findings, our results using PARP1 knockout cells also indicated that PARP1 was indispensable in IFNα and IFNβ upregulation induced by DSF plus chemotherapy.

In summary, our study revealed a promising combination therapy and further identified the regulatory mechanism by which DSF overcomes the PDAC resistance to chemotherapy via inhibiting PARP1 activation and thus activating cGAS-STING signaling pathway to promote the antitumor efficacy of the immune system. Our findings provide strong evidence to highlight the therapeutic strategy of DSF in combination with immunotherapy for the treatment of PDAC patients.

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Disclosure of conflict of interest

None.

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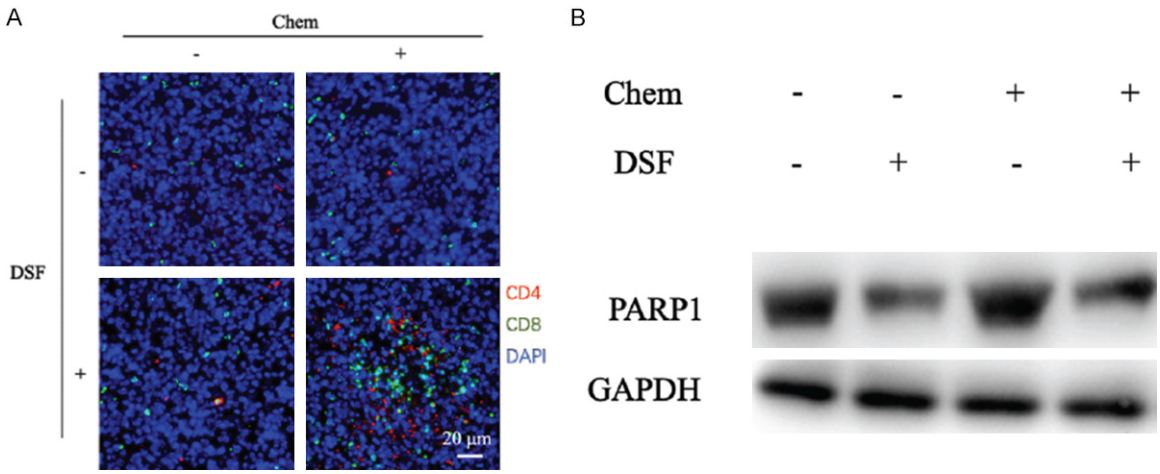

Supplementary Figure 1. A. Immunofluorescence staining for CD4, CD8 protein expression patterns in PAN02 tumors. Scale bar, 20 μM. B. PARP1 level in PANC-1 cells in the presence of the gemcitabine or DSF.